The effect of 60 days 6° head-down-tilt bed rest on the metabolic physiology of young, healthy males

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Authors Declaration

I hereby declare that this research is entirely the result of my own investigation and that appropriate credit has been given where reference has been made to the work of others. This work has not been submitted for any academic award, or part thereof, at this or any other educational establishment.

Signed	Date
Kiera Ward	12th September 2022
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Table of Contents

List of Tables

List of Figures

List of Equations

List of Abbreviations

AIT	Aerobic interval training
ALT	Alanine transaminase
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AP	Alkaline phosphatase
Apo-B	Apolipoprotein-B
ASP	Acylation stimulating protein
AST	Aspartate aminotransferase
ATP	Adenosine Triphosphate
AUC	Area under the curve
AUCG	Area under the curve for glucose
AUCI	Area under the curve for insulin
BAT	Brown adipose tissue
BCD	Basic core data
BDC	Baseline data collection
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
CHOL	Total cholesterol
CRP	C-reactive protein
CSA	Cross sectional area
CTRL	Control group
DAG	Diacylglycerol
DEXA	Dual-energy x-ray absorptiometry
DIO	Diet-induced obesity
DNL	De novo lipogenesis
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ESA	European Space Agency
FFA	Free fatty acid
FGF-21	Fibroblast growth factor 21

FMD	Flow-mediated dilation
GDR	Glucose disposal rate
GGT	Gamma-glutamyl transferase
GIR	Glucose infusion rate
GLUT	Glucose transporter
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein cholesterol
HDT	Head-down-tilt bed rest
HFD	High-fat diet
HIIT	High-intensity interval training
HMW	High molecular weight
HOMA-β	Homeostatic model assessment of beta-cell function
HOMA-IR	Homeostatic model assessment of insulin resistance
HR_{max}	Maximal heart rate
Hs-CRP	High sensitivity C-reactive protein
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IHL	Intra-hepatic lipid
IL-6	Interleukin 6
IRS	Insulin receptor substrate
JUMP	Jumping countermeasure group
KO	Knock-out
LDL	Low-density lipoprotein cholesterol
LM	Lean mass
LPL	Lipoprotein lipase
MET	Metabolic equivalents
MetSyn	Metabolic Syndrome
MHC	Myosin heavy chain
MHO	Metabolically-healthy obesity
MICT	Moderate intensity continuous training
mTOR	Mammalian target of rapamycin
MUO	Metabolically-unhealthy obesity

MVC	Maximal voluntary contraction
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-esterified fatty acids
NGT	Normal glucose tolerance
NRT	Non-linear resistance training
OGIS	Oral glucose insulin sensitivity
OGTT	Oral glucose tolerance test
PCOS	Polycystic ovary syndrome
PEPCK	Phosphoenolpyruvate carboxykinase
PPAR-α	Peroxisome proliferator-activated receptor alpha
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PV	Plasma volume
Q	Cardiac output
QUICKI	Quantitative insulin-sensitivity check index
RBP4	Retinol binding protein 4
RJT	Reactive jump training
RM	Repetition maximum
RMR	Resting metabolic rate
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SREBP-1C	Sterol-regulatory element binding protein-1C
SV	Stroke volume
T2DM	Type 2 diabetes mellitus
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
US-CRP	Ultra-sensitive C-reactive protein
VAT	Visceral adipose tissue
VLDL	Very-low-density lipoprotein cholesterol
$\dot{V}O_{2max}$	Maximal oxygen capacity
$\dot{V}O_{2peak}$	Peak oxygen capacity
WAT	White adipose tissue

Glossary of Terms

Adipokine

Factors secreted by adipose tissue which act through a network of autocrine, paracrine and endocrine pathways to regulate metabolic homeostasis.

Biomarker

A biological molecule found in blood, other body fluids or tissues that is measured and evaluated as an indicator of normal or pathological processes.

Countermeasure

An action or process taken to offset or mitigate another.

Gluconeogenesis

Synthesis of glucose from non-carbohydrate precursors including lactate, glycerol and amino acids.

Glucose Intolerance

An impaired ability for glucose disposal.

Glucose Tolerance

Ability to dispose of a glucose load.

Glycogenolysis

Process by which glycogen is broken down into glucose to provide immediate energy to maintain blood glucose levels during fasting.

Hepatokine

Factors secreted by the liver that can influence metabolic processes by exerting autocrine, paracrine and endocrine actions.

Insulin Resistance

A multi-faceted disruption of the action of insulin in skeletal muscle, adipose tissue, vasculature, brain, and the liver, leading to hyperinsulinemia and reduced glucose disposal.

Lipogenesis

Metabolic process through which carbon precursors of Acetyl Co-A are synthesised into fatty acids.

Lipolysis

Hydrolysis of triglycerides into free fatty acids and glycerol.

Myokine

Factors secreted by skeletal muscle which regulate whole-body metabolism in an autocrine, paracrine, or endocrine manner.

$\dot{V}O_{2max}$

The highest rate of oxygen uptake and utilization by the body during intense, maximal exercise that no further increases in work rate bring on additional rises in oxygen uptake.

$\dot{V}O_{2peak}$

The highest value of oxygen uptake attained upon an incremental or other high-intensity exercise test, designed to bring the subject to the limit of tolerance.

Important Terms

BDC: Baseline data collection phase (15 days): BDC-15 to and including BDC-1.

HDT: Head-down-tilt bed rest phase (60 days): HDT+1 to and including HDT+60.

R: Recovery phase: In house phase (15 days): R+0 to and including R+14.

Abstract

Introduction. Six-degree head-down-tilt (HDT) bed rest is a valuable experimental model for examining the physiological adaptations of gravity deprivation (spaceflight, physical inactivity, sedentary behaviour, immobilisation and ageing) including muscle atrophy, a shift in myofiber type composition, reduced cardiovascular and functional capacity and metabolic dysfunction. Establishing an exercise prescription which conserves time but mitigates these deleterious physiological adaptations is of profound importance for life in space and life on Earth. The overall aim was to examine the changes in physical characteristics, metabolic characteristics and circulating novel biomarkers of insulin sensitivity and insulin resistance in healthy young males, pre- and post-60 days of 6° HDT bed rest, with and without reactive jump training (RJT), a novel low volume, high-intensity jump training protocol.

Methodology. A total of 23 male subjects (29 ± 6 years, 181 ± 6 cm, 77 ± 7 kg) were randomised to a control (CTRL, n = 11) or RJT (JUMP, n = 12) group and exposed to 60 days of 6° HDT bed rest. RJT was performed 6 days per week and on average, each session consisted of 48 countermovement jumps and 30 hops, performed with maximal effort at a load equal to 80 – 90% body weight in a sledge jump system (≤ 4 minutes total exercise time). Body composition, $\dot{V}O_{2peak}$, muscle strength were measured and an oral glucose tolerance test (OGTT) was performed to estimate insulin sensitivity pre- and post-HDT bed rest. Circulating lipids, fetuin-A, retinol binding protein-4 (RBP4), irisin, adropin, adiponectin, acylation stimulating protein (ASP), apelin, apolipoprotein-J (apo-J) and fibroblast growth factor-21 (FGF-21) were quantified in fasting serum. A subanalysis was performed *a posteriori* to investigate individual metabolic response post-HDT bed rest.

Results. Body weight, lean mass and $\dot{V}O_{2peak}$ decreased in both groups post-HDT bed rest, with greater reductions observed in CTRL ($p < 0.05$). Significant main effects of time were found for increases in triglycerides, LDL-cholesterol and fetuin-A and decreases were observed in HDL-cholesterol, whole-body insulin sensitivity (Matsuda index) and tissue-specific insulin sensitivity, irisin, adropin, adiponectin and FGF-21 post-HDT bed rest ($p < 0.05$). In the subgroup with decreased insulin sensitivity, fetuin-A, RBP4, fasting insulin and glucose increased and irisin, adropin, adiponectin, FGF-21 and liver and adipose tissue insulin sensitivity decreased post-HDT bed rest ($p < 0.05$). In the subgroup with increased insulin sensitivity, adiponectin and FGF-21 decreased and liver insulin sensitivity increased post-HDT bed rest ($p < 0.05$).

Discussion. RJT preserved muscle mass and function, but could not mitigate the decline in insulin sensitivity or induce favourable changes in circulating novel biomarkers following HDT bed rest. In the subgroup with decreased insulin sensitivity, blunted insulin action and impaired peripheral glucose uptake were identified post-HDT bed rest. In the opposing subgroup with increased insulin sensitivity, an improvement in liver insulin sensitivity was found post-HDT bed rest. Fetuin-A, RBP4, irisin and adropin are candidate biomarkers for examining changes in insulin sensitivity in response to intervention.

Conclusion. This study provides insights into the physiological adaptation to HDT bed rest, including whole-body and tissue-specific insulin sensitivity, and highlights the importance of future studies to explore individual responses to obtain personalised information for the optimisation of exercise prescription to maintain health in all conditions of gravity deprivation in space and on Earth.

Peer-Reviewed Publications

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Chapter 1. Introduction

1.1. Introduction

The physiological adaptations in space and during head-down-tilt (HDT) bed rest, the most integrated ground-based analogue of microgravity, have been well-described and include muscle atrophy, a shift in myofiber type composition (slow-twitch oxidative to fast-twitch glycolytic muscle fibers), bone loss, reduced cardiovascular and functional capacity and metabolic dysfunction, among others (Bergouignan et al., 2011; Narici and de Boer, 2011; Ade et al., 2015; Vico and Hargens, 2018; Furukawa et al., 2021). In addition to aerospace medicine research, HDT bed rest involving healthy individuals is a suitable model for investigating the physiological adaptations to physical inactivity and high levels of sedentary time (i.e. the reduced use of gravity), as well as confinement and isolation from normal daily life and social networks. These conditions have been common place in the current COVID-19 pandemic, as entire populations have been asked to self-isolate and live in home-confinement for extended periods of time, leading to a dramatic increase in physical inactivity and sedentary behaviour, as well as emotional and physical stress, with ramifications on overall health and well-being (Choukér and Stahn, 2020; King et al., 2020; Narici et al., 2021). Furthermore, the physiological adaptations in spaceflight and HDT bed rest resemble those that occur during the ageing process (for example, declines in maximal aerobic capacity, sarcopenia, dynapenia and increased chronic disease risk), particularly when combined with limited or a lack of ambulatory activity (i.e. the reduced use of gravity over decades as a function of age) that can lead to a viscous cycle of physiological deconditioning, functional decline and hospitalisation (Chodzko-Zajko et al., 2009; Goswami, 2017). Prolonged inactivity, extreme sedentariness and bed rest are also experienced by individuals who have been immobilised as a result of, for example, a stroke, coma or spinal cord injury (Konda et al., 2019). Thus, the potential exists for a convergence of knowledge of the physiological adaptations to microgravity and the reduced use of gravity, ageing, confinement and isolation, to provide novel perspectives and inform countermeasure and rehabilitation protocol development.

Exercise is a well-regarded countermeasure that has been implemented to preserve physiological function following spaceflight and HDT bed rest (Macaulay et al., 2016).

However, despite on-going investigations and refinement of exercise prescription, no exercise countermeasure to date has fully mitigated the deleterious physiological adaptations associated with microgravity and gravitational deprivation. As astronaut time is at a premium, it is important to understand the minimum amount of exercise that is required to induce a substantial positive influence on overall physiological health (Hackney et al., 2015). One type of time-efficient exercise training that has the potential to elicit an osteogenic and hypertrophic stimulus and exert protective effects on cardiovascular and metabolic health is jumping. Reactive jump training is a form of low volume, high-intensity interval training incorporating whole-body exercises with high power output and rates of force development. Therefore, it is essential to investigate the efficacy of this novel exercise protocol and training device, particularly using the standardised conditions of ground-based HDT bed rest. Such investigations also have key implications for exercise prescription in Earth-based contexts including physical inactivity, sedentary behaviour, ageing, and immobilisation due to injury or illness.

There is evidence that underlying mechanisms, such as insulin resistance, play an important role in the regulation of systemic metabolism in HDT bed rest and spaceflight (Gratas-Delamarche et al., 2014). Insulin resistance can be described as a pathological defect in insulin signalling pathways, resulting in inappropriate cellular response to insulin in insulin-dependent tissues such as the liver, skeletal muscle and adipose tissue (Bourebaba and Marycz, 2019). It is also associated with increased circulating concentrations and deposition of lipids, impaired oxidative capacity and metabolic inflexibility (lower fasting fat oxidation and/or an impaired ability to oxidize carbohydrate during feeding or insulin-stimulated conditions). These whole-body and cellular changes have been observed following HDT bed rest studies, even when energy balance is maintained (Bergouignan et al., 2006; Bergouignan et al., 2009; Bergouignan et al., 2011; Kenny et al., 2017; Rudwill et al., 2018), suggesting physical inactivity and high levels of sedentary time and the physiological and metabolic adaptations to these are drivers of insulin resistance under these conditions. The severity of insulin resistance varies considerably between individuals and between the key organs and tissues of metabolic importance (Unnikrishnan, 2004; Abdul-Ghani et al., 2007; Hansen et al., 2020). Therefore, investigating and understanding individual responses in insulin

sensitivity could provide personalised information on the pathophysiology of insulin resistance and individualised interventions to maintain optimal health.

A key strategy for monitoring metabolic homeostasis is communication between peripheral tissues *via* secreted proteins, which perform autocrine, paracrine, and endocrine actions. Disruption of proper protein production and target-tissue action underpin the development of metabolic dysfunction including insulin resistance (Priest and Tontonoz, 2019). The quantitative measurement of circulating protein biomarkers, defined as indicators of a biological state or condition (Siderowf, 2001), is a relatively easy and minimally-invasive means of examining the etiology of insulin resistance.

The liver, adipose tissue and skeletal muscle are involved in the regulation of metabolic homeostasis by producing and secreting proteins (collectively referred to as organokines) known as hepatokines, adipokines and myokines (Priest and Tontonoz, 2019; Chung and Choi, 2020). These pleiotropic molecules regulate glucose and lipid metabolism, inflammation, oxidative stress, endothelial dysfunction and fat distribution by exerting various autocrine, paracrine and endocrine actions (Chung and Choi, 2020). Organokines including adropin, irisin, adiponectin, fibroblast growth factor 21 (FGF-21), apolipoprotein-J/clusterin (apo-J), acylation stimulating protein (ASP) and apelin improve insulin sensitivity, while fetuin-A and retinol binding protein 4 (RBP4) impair insulin sensitivity. Accordingly, the balance between the secretions of these two different types of organokines will either promote or deter metabolic dysfunction. Physical inactivity and high levels of sedentary time impair the secretion of health-enhancing organokines and promote the secretion of organokines that contribute to the development of metabolic diseases. Contrastingly, exercise training enhances the secretion of organokines that induce favourable changes in local and systemic metabolism (Leal et al., 2018). Hence, investigation of these circulating organokines and thus the behaviour of key metabolic organs may improve our understanding of the complex relationship of these metabolic networks and inter-organ communication and provide insights into individual responses in insulin sensitivity and metabolic deregulation following HDT bed rest.

Adiponectin, an adipokine, is one of the most extensively researched biomarkers to date and investigation into the regulation and physiological effects has enhanced our understanding of systemic metabolic homeostasis. In this view, it is possible that quantification of circulating adiponectin may provide insights into the dysregulation of glucose and lipid metabolism and the associated disruption of inter-organ communication after physical inactivity and high levels of sedentary time. Adiponectin and FGF-21, a hepatokine and multifunctional metabolic regulator, have been studied previously following bed rest providing an opportunity to extend these findings and determine whether these organokines are candidate biomarkers to assess physiological responsiveness to HDT bed rest (Hamburg et al., 2007; Rudwill et al., 2018; Petrocelli et al., 2020). Their response to HDT bed rest may also assist in the interpretation of the novel biomarker response to prolonged inactivity and extreme levels of sedentary time. Fetuin-A, RBP4, adropin, irisin, apo-J, ASP and apelin play key roles in the regulation of metabolic health and may be very useful in helping us to understand the pathophysiology of insulin resistance, but these novel biomarkers have not been measured previously in response to HDT bed rest. Identifying biomarkers or a panel of biomarkers that can successfully track changes in metabolic physiology in response to HDT bed rest and countermeasures is a fundamental component in understanding and mitigating the physiological adaptation to physical inactivity and extreme levels of sedentary time.

1.2. Aims

The primary aim of this study is to examine changes in physical characteristics, metabolic characteristics and circulating concentrations of novel biomarkers of insulin sensitivity and insulin resistance in healthy young males, pre- and post-60 days of 6° HDT bed rest.

The secondary aim is to determine the impact of reactive jump training (RJT), a low volume, high-intensity jump training protocol, on the physical characteristics, metabolic characteristics and circulating concentrations of novel biomarkers of insulin sensitivity and insulin resistance in healthy young males, pre- and post-60 days of 6° HDT bed rest.

1.3. Objectives

1. To investigate changes in body weight, body composition, peak aerobic capacity and muscle strength pre- and post-60 days of 6° HDT bed rest in young healthy males, with and without RJT.
2. To examine changes in glucose tolerance, insulin sensitivity and lipid metabolism pre- and post-60 days of 6° HDT bed rest in young healthy males, with and without RJT.
3. To quantify changes in circulating fetuin-A, RBP4, irisin, adropin, adiponectin, ASP, apelin, apo-J and FGF-21 pre- and post-60 days of 6° HDT bed rest in young healthy males, with and without RJT.

Chapter 2. Literature Review

2.1. Introduction

Spaceflight, characterised by prolonged exposure to microgravity (μg), has a profound impact on human physiology. The physiological adaptations have been well-described and include muscle atrophy, increased bone resorption, altered neurovestibular function, cardiovascular deconditioning and metabolic dysregulation, among others (Demontis et al., 2017; Tanaka et al., 2017). As space flights become longer and more frequent, it is becoming increasingly important to investigate these deleterious multi-system effects, and measures to counter these changes, which are critical for astronaut health and well-being, as well as safety and mission success (Mahadevan et al., 2021). Performing physiological research in space is challenging and is subject to multiple confounding factors including operational constraints, limited experimental controls and opportunities for repetition and validation and a small number of subjects (Shelhamer et al., 2020). Consequently, space agencies have used ground-based analogues of human spaceflight to enable higher quality, well-controlled and safer research.

Six-degree HDT bed rest is considered as the most integrated, Earth-based experimental model that reproduces several of the physiological adaptations induced by spaceflight (Guinet et al., 2020; Pandiarajan and Hargens, 2020). As opposed to horizontal bed rest, the focus of HDT bed rest is to induce the head-ward fluid shift observed in microgravity by altering the gravity vector over the body from anterior to posterior (Pandiarajan and Hargens, 2020). This analogue is also used to develop and test countermeasures through the evaluation of devices and strategies to simultaneously protect multiple physiological systems (Hargens and Vico, 2016). Spaceflight and HDT bed rest research has provided valuable data for understanding human physiology, particularly for public health and in clinical and nursing contexts (Pavy-Le Traon et al., 2007). Vernikos (2017; 2021) coined the term “The Gravity Deprivation Syndrome (GDS)” to highlight that spaceflight, physical inactivity, sedentary behaviour, immobilisation induced by injury or illness and the limited mobility of ageing fall on a continuum of reduced gravity exposure (from microgravity to the reduction of gravity to the reduced use of the gravity vector over decades of ageing), which affects all physiological systems, acting through common pathways and mechanisms. These aforementioned situations induce an atrophic

condition, which is not merely visually evident as musculoskeletal loss but also metabolically, morphologically and functionally. The atrophic effect differs only in the rate in which it is induced, determined by the lifestyle use of the gravity vector. The extreme common result is sarcopenia and frailty, which are characteristic features of the ageing process, which limit mobility and independence and can accelerate the deterioration of cardiometabolic, musculoskeletal, cognitive and mental health and well-being (Vernikos, 2017; Kirwan et al., 2020; Vernikos, 2021). Therefore, HDT bed can be considered as an important and unique model of human physiology, to explore the physiological adaptations of the GDS and investigate the underlying mechanisms of these changes which can improve our understanding and treatment of same.

2.2. Head-Down-Tilt (HDT) Bed Rest

The history of bed rest predates spaceflight. Bed rest was first introduced in the 19th century as a medical treatment to minimise the physiological demands on the body and allow time for rest and healing to aid recovery (Pavy-Le Traon et al., 2007; Parry and Puthucheary, 2015). However, during World War II, doctors observed that soldiers who were made ambulatory quickly after injury or surgery recovered more rapidly than those who remained in bed rest (Pavy-Le Traon et al., 2007). Thereafter, there was a gradual reduction in the practice of using bed rest as a medical treatment. In conjunction with the beginning of human spaceflight in 1961, water immersion was used as a ground-based model to simulate microgravity exposure. This analogue requires submersion in water from the neck down and utilises the hydrostatic pressure of the water environment to counteract intravascular hydrostatic pressure gradients, which is impractical in terms of long-term immersion (Pavy-Le Traon et al., 2007; Pandiarajan and Hargens, 2020). Subsequently, six-degree HDT bed rest became the ‘gold-standard’ spaceflight analogue to investigate the physiological adaptations induced by weightlessness. The focus of HDT bed rest is to induce the head-ward fluid shift and body unloading that occurs in microgravity (Koppelmans et al., 2015). Currently, HDT bed rest is considered the most integrated analogue for Earth-based studies of spaceflight and the physiological responses to short-, medium- and long-duration HDT bed rest are investigated by the European Space Agency (ESA) and National Aeronautics and Space Administration (NASA).

2.3. Physiological Adaptations to Physical Inactivity, High Levels of Sedentary Time, Bed Rest and Spaceflight

HDT bed rest induces multi-system effects which parallel those observed with microgravity exposure. These changes are well-described and include muscle atrophy, bone loss, altered cardiovascular capacity and impaired functional capacity, among others. The following subsections summarise the physiological adaptations observed during HDT bed rest and spaceflight.

2.3.1. Fluid Shift

In an upright stance, gravity creates a hydrostatic gradient with a mean arterial pressure of 70mmHg at the head, 100mmHg at the heart and 200mmHg in the lower limbs and feet (Figure 1) (Demontis et al., 2017). In space, this hydrostatic force is lost and arterial pressure is equalised throughout the body, leading to a subsequent translocation of venous fluid (1-2 litres) toward the head (Moore and Thornton, 1987; Clément, 2011a; Noskov, 2013; Nelson et al., 2014; Baker et al., 2019; Gallo et al., 2020; Bailey et al., 2021). This shift of fluid towards the head distends the heart and stimulates the baroreceptors of the central vasculature, triggering the suppression of the renin-angiotensin-aldosterone mechanism and reduction of renal sympathetic nerve activity and antidiuretic hormone (ADH) secretion (Williams et al., 2009). These events trigger the increased release of atrial natriuretic peptide (ANP) leading to a reduction in plasma volume (Williams et al., 2009). In microgravity, the loss of plasma volume does not result from increased diuresis or natriuresis, but rather likely from decreased interstitial pressures and upper body vascular pressures which together induce transcapillary fluid movement into the upper body interstitium (Watenpaugh, 2001; Vernice et al., 2020). In the first 24 hours, plasma volume decreases by approximately 17%, coupled with a gradual loss of red cell mass, leading to a net blood volume loss of around 10% (Williams et al., 2009). The facial fullness and unique puffy appearance of the head, in addition to the reduced volume of fluid in the lower limbs, resulting from fluid redistribution is described anecdotally as the “puffy face-bird leg” syndrome (Williams et al., 2009). Astronauts typically suffer from sinus and nasal congestion, loss of smell and taste, headaches, increased pressure inside the eyes and “space motion sickness”, gradually solved over 48 to 72 hours (Clément, 2011a; Demontis et al., 2017).

Unlike spaceflight, HDT bed rest redistributes arterial pressure across the posterior of the body, rather than being focused in the head to feet direction (Pandiarajan and Hargens, 2020). This posture does not completely abolish gravitational force but the headward fluid shift and cardiovascular adaptations are akin to those during microgravity exposure. Although, dissimilar to microgravity, HDT bed rest does not induce a loss in tissue weight (Pandiarajan and Hargens, 2020).

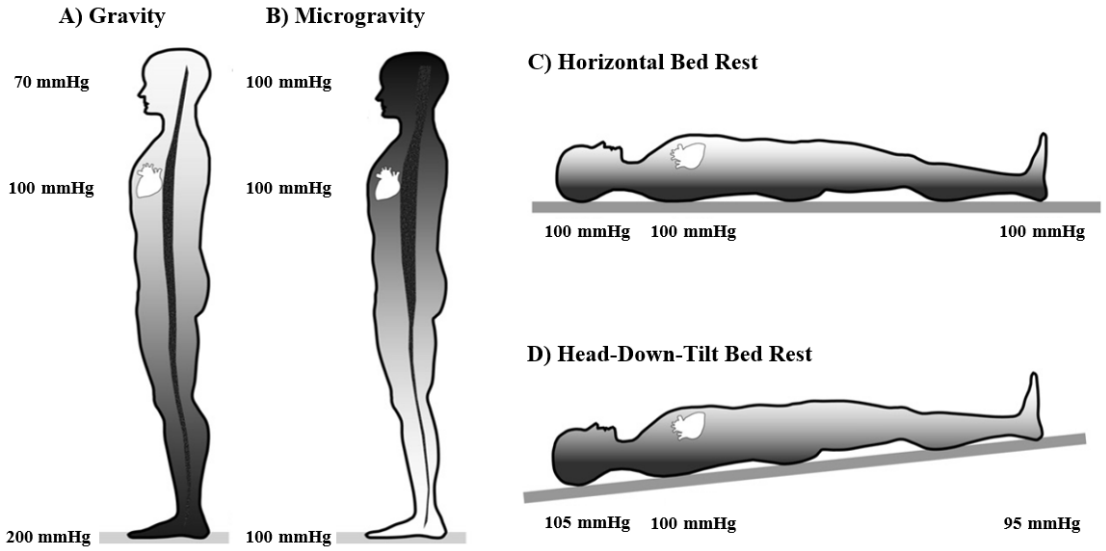

Figure 1. Hypothetical arterial blood pressures and fluid accumulation at the level of the head, heart and feet in a) gravity environment, b) microgravity, c) horizontal bed rest and d) head-down-tilt bed rest.

This diagram has been adapted from Hargens and Vico (2016).

2.3.2. Cardiovascular and $\dot{V}O_{2peak}$ Changes Associated with Bed Rest

There are multiple adaptive changes in the cardiovascular system in response to real or simulated weightlessness including, but not limited to, the reduction in plasma volume, red cell mass and stroke volume, decrease in left ventricular mass, altered vascular compliance, orthostatic intolerance, and a decrease in maximal or peak aerobic capacity (Ade et al., 2015; Rabineau et al., 2020). Maximal oxygen uptake ($\dot{V}O_{2max}$) can be defined as the highest rate at which oxygen can be taken up and utilised by the body during intense exercise and indicates the cardiopulmonary capacity of an individual (Sagiv et al., 2010). Peak oxygen uptake ($\dot{V}O_{2peak}$) is directly reflective of $\dot{V}O_{2max}$ and describes the highest value of oxygen consumption and utilisation during an incremental or other high-intensity exercise test, that is designed to bring the individual to the limit of tolerance (Cade et al.,

2018). $\dot{V}O_{2max}$ is an independent predictor of all-cause and cardiovascular disease mortality, and there is a compelling link between impaired cardiorespiratory fitness and components of the metabolic syndrome (MetSyn) (Lee et al., 2010; Myers et al., 2019).

The Fick equation defines $\dot{V}O_{2max}$ as the product of cardiac output (*Q*) multiplied by arteriovenous oxygen difference (a-vO_2 difference) (Sindall, 2020). *Q* is the product of heart rate (HR) and stroke volume (SV) and is measured in litres per minute. *Q* reflects the central factors that determine $\dot{V}O_{2max}$ which include the transport of oxygen from ambient air into the lungs, diffusion into the arterial blood supply and transport to the periphery through circulatory mechanisms (Sindall, 2020). Conversely, a-vO_2 difference reflects the peripheral factors that determine $\dot{V}O_{2max}$, relating to the processes within skeletal muscle that permit oxygen diffusion from arterial blood into the mitochondria to enable regeneration of adenosine triphosphate (ATP) and aerobic energy production. Taken together, $\dot{V}O_{2max}$ is a product of maximal oxygen supply and maximal oxygen extraction (Sindall, 2020).

The magnitude of change in $\dot{V}O_{2max}$ is dependent on the length of unloading and the training status of the individual, such that well-trained individuals show the fastest rates of decline (Ade et al., 2015; Ried-Larsen et al., 2017; Clark et al., 2020). Convertino (1997) proposed a linear decline in $\dot{V}O_{2max}$ of 0.9% per day over 30 days of bed rest. Although informative, this model of $\dot{V}O_{2max}$ decline was not appropriate for estimating the decrease in $\dot{V}O_{2max}$ in longer duration studies. Subsequently, Capelli et al. (2006) reported that while most of the decrease in $\dot{V}O_{2max}$ occurs in the first 14 days of HDT bed rest, the rate of decline becomes progressively smaller as the duration of HDT bed rest increases. The average daily rate of $\dot{V}O_{2max}$ decline was 0.99%, 0.39% and 0.35%, amounting to a reduction of 14%, 16% and 32% for 14, 42 and 90 days of HDT bed rest, respectively. These findings suggest that the $\dot{V}O_{2max}$ decrease with HDT bed rest tends toward an asymptote, rather than a linear decrease (Capelli et al., 2006). Similar reductions in $\dot{V}O_{2peak}$ have been reported in non-exercising subjects from other studies, including an 18% ($p < 0.01$) and 16% ($p < 0.001$) decline in $\dot{V}O_{2peak}$ in men and women, respectively, following 30 days HDT bed rest (Lee et al., 2007; Lee et al., 2009).

The main reasons for the initial decline (during the first 14 days) in $\dot{V}O_{2max}$ is related to the reduction in maximal oxygen delivery, as a result of decreases in *Q* and haemoglobin, in response to the HDT bed rest-induced cephalad fluid shift (Capelli et al., 2006). Conversely, the slow component (after 14 days) of the decrease in $\dot{V}O_{2max}$ is due to an impairment in intracellular oxidative metabolism, caused by a decrease in mitochondrial content and oxidative enzyme capacity, and a reduction in capillary volume leading to alterations in peripheral oxygen delivery and oxygen diffusion capacity (Capelli et al., 2006; Salvadego et al., 2011; Ade et al., 2015; Salvadego et al., 2016; Salvadego et al., 2018; Clark et al., 2020). Impaired vascular function and redistribution of maximal cardiac output are contributing factors to the decrease in oxygen diffusing capacity (Ade et al., 2015). These peripheral changes occur in concert with HDT-induced muscle atrophy, particularly a reduction in slow-twitch muscle fiber isoforms (Capelli et al., 2006; Clark et al., 2020). In accord with this hypothesis, and after 35 days HDT bed rest, the decrease in supine $\dot{V}O_{2max}$ was associated with reduced peripheral gas exchange (decreased mitochondrial volume), while a higher decline in upright $\dot{V}O_{2max}$ was related to a combination of decreased maximal oxygen delivery and impaired peripheral gas exchange (Bringard et al., 2010). The authors reported a 39% decrease in upright $\dot{V}O_{2max}$, which is larger than the decline reported in the abovementioned studies. This discrepancy was related to the timing of $\dot{V}O_{2max}$ assessment, which was one hour after re-ambulation compared to R+3 or R+4 (Lee et al., 2007; Lee et al., 2009), possibly allowing time for initial cardiovascular recovery.

A limited number of studies have investigated the effect of spaceflight exposure on changes in $\dot{V}O_{2max}$. Most recently, Ade et al. (2017) investigated changes in $\dot{V}O_{2max}$ in nine astronauts (4 women and 5 men, age, 49.5 ± 5.1 years) following long-duration spaceflight. The mean flight duration was 168.6 ± 19.2 days. $\dot{V}O_{2max}$, obtained by graded maximal cycle ergometry, decreased significantly post-flight (-16%, $p = 0.008$). A significant decrease in *Q* (-7%, $p = 0.05$), despite unchanged haemoglobin concentrations, resulted in a significant decrease in convective oxygen transport (QO_2, $p = 0.02$). Additionally, there was a significant decrease in oxygen diffusing capacity (DO_2, $p = 0.04$) and differences in arterial and venous oxygen content (CaO_2-CvO_2, $p = 0.007$). Maximal heart rate, ventilation and respiratory exchange ratio (RER) did not change post-flight. Linear regression analysis revealed that the change in $\dot{V}O_{2max}$ was significantly and

positively correlated with the change in DO_2 (r^2 = 0.47, p = 0.04). Taken together, exposure to spaceflight leads to a significant decrease in $\dot{V}O_{2max}$, relating, in part, to decrements along the oxygen transport pathway (Ade et al., 2017). Furthermore, the results of this study are congruent with the hypothesis that the decrease in $\dot{V}O_{2max}$ is non-linear, reaching an asymptote within a few weeks (Capelli et al., 2006). A similar reduction in $\dot{V}O_{2max}$ was reported by Moore et al. (2014), which used many of the same subjects in the previous publication. Following shorter duration spaceflight, Trappe et al. (2006) observed a 10% reduction in $\dot{V}O_{2peak}$ in four astronauts after a space shuttle mission (17 days), while Levine et al. (1996) reported a 22% reduction in $\dot{V}O_{2max}$ in six astronauts post-flight (9 or 14 days). Similar to HDT bed rest, the magnitude of decrease in $\dot{V}O_{2max}$ may be affected by the duration of the mission and timing of post-flight testing (Ade et al., 2017). Other factors contributing to the discrepancies between studies may include differences in gender and baseline fitness, daily living activities and individual variation (Trappe et al., 2006).

2.3.3. Muscle Physiology Deconditioning

Skeletal muscle is a highly plastic tissue which adapts to physiological stressors such as changes in contractile activity, mechanical load and nutritional state (Khodabukus, 2021). While physical activity and exercise can induce gains in muscle mass and function, immobilisation, bed rest and spaceflight can lead to skeletal muscle deconditioning (Arc-Chagnaud et al., 2020). Muscle deconditioning is a consequence of an imbalance in muscle protein homeostasis, which elicits a myriad of structural and functional alterations including the loss of muscle mass, myofiber atrophy and the loss of muscle strength and power. In addition, disused muscles undergo metabolic remodeling, affecting the myofiber typology and contractile properties (Arc-Chagnaud et al., 2020). These skeletal muscle adaptations are a liability for astronauts, who are required to perform physical work in space and move abruptly from microgravity to planetary gravity, and individuals recovering from illness or injury on Earth, that need to resume normal weight-bearing activities (Adams et al., 2003).

2.3.3.1. Structural Alterations in Muscle: Muscle Atrophy and Myosin Heavy Chain Type Changes

2.3.3.1.1. Muscle Atrophy

In simulated or actual microgravity, muscle atrophy is associated with a decline in muscle fiber size, with no change in fiber number (Clément, 2011c). Postural muscles, which are chronically contracted in gravity to support the weight of the body, are most susceptible to unloading compared to non-postural muscles (Qaisar et al., 2020). Additionally, substantial differences exist in the rate of atrophy among the postural muscles themselves, such that extensor muscles are more affected than flexor muscles (Clément, 2011c). A common observation is that the ankle plantar flexors (soleus and gastrocnemius) undergo the largest reduction in muscle volume (Narici and de Boer, 2011). On Earth, ankle plantarflexors balance the full weight of the body, whereas the hip extensors balance the weight of the trunk, upper extremities and head only. Therefore, ankle plantarflexors experience the largest change in load compared to previous loading history leading to the greatest atrophy (Berg et al., 2007). Muscle atrophy is a consequence of a disparity between protein synthesis and breakdown. Typically, muscle atrophy results from a predominant downregulation of protein synthesis, with no clear contribution from muscle protein breakdown (Wall et al., 2013). However, findings from a hindlimb unloading study suggest that muscle protein breakdown occurs at a higher rate in the soleus muscle (which is predominately made up of type I myofibers), suggesting that muscle protein breakdown is more apparent in slow oxidative postural muscles, consisting of primarily type I myofibers, compared to muscles of mixed fiber type such as the tibilias anterior and vastus lateralis and therefore experiences the greatest atrophy and may further contribute to disparities in different muscle groups in response to disuse (Baehr et al., 2017).

The response to unloading differs in human and mammalian muscle tissue. Adult rats have a 3 to 4 fold higher total protein turnover than adult humans, and protein synthetic rates are 2.5 fold greater. Thus, atrophy may occur at an accelerated and heightened rate in animals compared with humans (Phillips et al., 2009). In rodent models, soleus muscle mass has been reported to decrease by 37% after only 4 to 7 days of spaceflight (Fitts et al., 2000). In the same time frame in humans, soleus-gastrocnemius and quadriceps cross-sectional area (CSA) only decreased by 6% following 8 day spaceflight exposure

(LeBlanc et al., 1995). Following 5 days HDT bed rest, calf and thigh CSA decreased by approximately 2 – 3% (Mulder et al., 2015). Similarly, unilateral limb unloading (ULLS) caused a significant reduction in quadriceps CSA of 5.2% after 14 days and of 10% after 23 days, suggesting an approximate loss of 0.4% per day (de Boer et al., 2007).

Some authors speculate there is a continuous loss of muscle mass, such that the longer the flight duration, the greater the muscle atrophy experienced (LeBlanc et al., 2000). Conversely, others have argued that the loss of muscle mass is exponential with the duration of the flight and that a plateau is reached after a certain period of time. In accordance with this hypothesis, multiple studies have reported that the rates of muscle mass loss are heightened within in the first 30 days, at an approximate rate of ~0.6%/day, after which rates of loss begin to slow and a plateau is achieved, whilst others assert that the plateau is not attained for approximately 120 to 180 days (Fitts et al., 2000; di Prampero and Narici, 2003).

2.3.3.1.2. Myosin Heavy Chain (MHC) Phenotypes

Muscle atrophy is associated with intrinsic changes in muscle fibers and alterations in myosin heavy chain (MHC) phenotypes (Trappe et al., 2004). Such changes are associated with alterations in contractile properties and isolated fiber changes, which may reduce the force- and power-generating capacity (Baldwin and Haddad, 2001).

In mammals and rodents, slow-twitch muscles such as the soleus, predominately express the slow type I MHC isoform as well as a small proportion of the type IIa isoform. In humans, the soleus muscle exhibits an approximately equal distribution of type I and type IIa isoforms. In human fast-twitch muscles such as the vastus lateralis, there is commonly a mixed expression of type I, IIa and IIx MHC isoforms (Baldwin and Haddad, 2001). In addition, some muscles contain a mix of more than one phenotype (MHC I/IIa, IIa/IIx, I/IIa/IIx) which are known as hybrid fibers (Baldwin and Haddad, 2001; Bagley et al., 2012). Hybrid fibers are believed to represent the transition stage between one phenotype to another; for instance, MHC I will become I/IIa, before transitioning to IIa (Pette and Staron, 1997).

Rats exhibit preferential atrophy of type I fibers, over type II fibers, while in humans, the type II fibers are at least, or if not more so, inclined to atrophy after short duration space flight (Fitts et al., 2001). After 5 days space flight, changes in mean fiber CSA was 11% and 24% smaller in the type I and type II fibers, respectively. Furthermore, following 11 days of space shuttle flight, mean fiber CSA was 16 - 36% smaller with a relative effect of type IIb > IIa > I (Edgerton et al., 1995). However, it seems that following a period of long duration disuse, the atrophy in human muscle expresses the same pattern as rodents. This was evident in a study conducted by Trappe et al., (2004) in which single fiber diameter in the MHC I and MHC IIa fibers were 15 and 8% smaller ($p < 0.05$), respectively, following 84 days HDT bed rest.

2.3.3.1.3. Fiber Type Switch

Whilst examining MHC phenotype atrophy, it is essential to examine the microgravity-induced MHC phenotype switch. Following periods of unloading, rodent models display a clear shift from type I fibers (more oxidative) to type II fibers (more glycolytic) and an increase in hybrid fibers (Fitts et al., 2000; Baldwin and Haddad, 2001; Brooks and Myburgh, 2012). These changes are thought to be a consequence of changes in transcriptional processes associated with MHC expression (Bagley et al., 2012). This shift may result in decreased capillary density in both fiber types, reduced muscle performance, decreased muscle endurance and undesirable metabolic adaptations, such as impaired insulin sensitivity, myokine production and substrate utilisation (Bagley et al., 2012; Brooks and Myburgh, 2012).

In the previously mentioned 84 day HDT study by Trappe et al., (2004), there was an increase in hybrid fibers in the vastus lateralis, at the expense of the MHC I fibers which decreased by 29% and 19% ($p \leq 0.05$) in the control and exercise group, respectively. This finding compliments rodent-based research suggesting that anti-gravity muscles, such as the soleus, which is composed predominately of type I fibers, are most affected by weightlessness (Fitts et al., 2000). Further analysis on the results of this study highlighted that unloading had a larger than expected role in preserving muscle mass, power and function. Single muscle fiber power in the fibers of the vastus lateralis decreased in the type I fibers by 60% and by 25% in the type II fibers ($p < 0.05$) (Trappe

et al., 2004). In the years following, Trappe et al., (2009) examined changes in the gastrocnemius and soleus muscles of crew members returning from a six-month stay on the ISS. Similarly, muscle mass decreased (-13 ± 2%, $p < 0.05$) and a "microgravity-induced fiber type shift" occurred, causing a 12-17% ($p < 0.05$) shift in the MHC phenotype of the gastrocnemius and soleus. MHC I fibers decreased primarily with a redistribution among the faster phenotypes.

2.3.3.1.4. Nitrogen Concentrations

A biological indicator of a reduction in muscle mass following spaceflight or bed rest is the loss of nitrogen, an essential element of protein (Clément, 2011c). The examination of urinary nitrogen can be used as an indicator for muscle protein breakdown, and more specifically, the analysis of excreted 3-methylhistidine, creatine and sarcosine (Clément, 2011c). Multiple researchers have examined urinary nitrogen and found that a negative nitrogen balance exists in parallel to a reduction in protein synthesis and muscle mass (LeBlanc et al., 2000; Mulder et al., 2015).

2.3.3.2. Functional Alterations in the Muscle: Muscle Force, Strength and Power

2.3.3.2.1. Muscle Force and Strength

Many of the preceding structural alterations within the muscle and muscle fibers lead to and account for significant alterations in the function of the muscle. A disproportionate loss of muscle force, to that of muscle size, is often evident after a period of long-term bed rest indicating that atrophy alone cannot account solely for the inactivity-induced muscle weakness (Reeves et al., 2002; Pavy-Le Traon et al., 2007). Altered motor control, changes in contractile elements and properties of the muscles and impaired electromechanical signalling are implicated in functional alterations (Pavy-Le Traon et al., 2007).

In both human and animal studies, significant decrements in muscle strength have resulted from exposure to bed rest and other simulation models, in as little as 5 to 7 days (Mulder et al., 2015). A disproportionate loss of muscle force, to that of muscle size, is

often evident after long-duration bed rest indicating that atrophy alone cannot account solely for the hypo-activity induced muscle weakness (Reeves et al., 2002; Pavy-Le Traon et al., 2007). Voluntary force production is a function of neurological and skeletal muscle properties and composition and thus these mechanisms must be accountable for the loss of muscle strength. Kawakami et al. (2001) sought to delineate changes in the muscle of nine healthy males, as a consequence of HDT bed rest with respect to their size and composition and investigate alterations in neural activation. Following 20 days HDT, the control group exhibited a significant reduction in quadriceps mean PCSA of 8% ($p < 0.05$) and a decrease in mean knee extension force of 11% ($p < 0.05$). These results show that bed rest-induced a greater decrease in muscle strength than that of muscle size, advocating the existence of inadequate motor unit activation, which is known to be attenuated by bed rest and inactivity. In addition to this, subsequent linear regression suggested that the loss of force could have been related to changes in neural activation (Kawakami et al., 2001).

In addition to altered motor and neural control, changes in contractile elements and properties of the muscles, reduced intrinsic force of the single muscle fiber and impaired electro-mechanical signalling and force-generating cross-bridges have also been suggested to contribute to the loss in strength (de Boer et al., 2007; Pavy-Le Traon et al., 2007). Furthermore, Di Prampero et al., (2001) proposed that the ability to maintain tetanic forces, i.e. sustained muscle force, and decrease in the ratio between forces and muscle CSA may be due to a reduction in the efficiency of the electromechanical coupling, an insufficient neuromuscular response, a reduction in the fiber specific tension capacity and an increase in the amount of non-contractile tissue within the muscle (Di Prampero et al., 2001).

Similar to sarcopenia, disuse atrophy may result in a reduction in fascicle (bundle of muscle fibers surrounded by perimysium) length and pennation angle which is strongly associated with the loss of both in series and in-parallel sarcomeres (Reeves et al., 2002). Pennation angle refers to the angle of insertion of the fascicles into the deep aponeurosis. Decreased fascicle length has been pinpointed to obstruct shortening during contraction, with consequent negative implications for the force-length and force-velocity

relationships of the muscle (Reeves et al., 2002). Accordingly, reductions in knee extensor torque and strength and a shift in the torque-velocity relationship were reported following 23 days ULLS (de Boer et al., 2007). In a 90 day HDT bed rest study, fascicle length and pennation angle of the gastrocnemius muscle decreased by 10% ($p < 0.001$) and 13% ($p < 0.002$), respectively, in the non-exercising control group. These reductions caused a significant reduction in plantar flexor force (-55%, $p < 0.001$) (Reeves et al., 2002).

2.3.3.2.2. Muscle Power

Muscle power is a function of force and velocity. The loss of muscle mass and strength contribute to the reduction in muscle power reported following inactivity and high levels of sedentary time. Kortebein et al., (2007) reported that isotonic knee extensor strength was significantly reduced (-13.2 ± 4.1%, $p = 0.004$) following 10 days bed rest, coupled with a considerable decline in stair climbing power (-14 ± 4.1%, $p = 0.01$), in healthy older men and women. The same is true for explosive power events. For instance, a 24.1% reduction in jumping peak power was identified following 56 days bed rest ($p < 0.05$) (Buehring et al., 2011). Using a similar measure of peak jump power, a decrement of 27% was identified following 90 days of HDT bed rest ($p < 0.001$) (Rittweger et al., 2007).

Many authors have postulated that this reduction may be due to diminished motor unit recruitment patterns, electromechanical efficiency or predisposition to muscle damage (di Prampero and Narici, 2003; Narici and Maganaris, 2007). Ferretti et al (2001) investigated the relationship between the reduction of lower limb CSA and decline in instantaneous muscle power (Wp) during vertical jump performance following 42 days HDT bed rest. The researchers concluded that the reduction in CSA of the extensor muscles could explain the significant decrease in Wp after HDT bed rest, with impaired neural activation and fiber-specific tension accounting for the remaining variance. Single fiber specific tension and the mechanical properties of tendons can also impair the length-force relationship contributing to decreased whole muscle-specific force (Narici and Maganaris, 2007).

In addition to the above hypotheses, muscle damage, weakness and pain following re-ambulation have been highlighted as potential contributing factors for further deterioration in peak power measured following the transition back to the 1G environment or re-establishment of the upright posture following HDT bed rest (di Prampero and Narici, 2003). Buehring et al., (2011) emphasized that the timing of muscle function tests is extremely important in obtaining accurate measurements of power and muscle function, and inappropriate timing may affect the subsequent results and analysis.

2.3.4. Skeletal Alterations

Bone is a highly specialised supporting framework that undergoes constant and sequential remodeling through the action of osteoclasts, osteoblasts and osteocytes (Florencio-Silva et al., 2015). The balance between bone resorption and formation is influenced by a multitude of genetic, vascular, nutritional, hormonal and mechanical factors (Kini and Nandeesh, 2012). Mechanical loading is paramount for the maintenance of the dynamic skeleton. Physical activity on Earth permits muscular action to transmit tension to the bone, which is subsequently detected by the osteocyte network within the osseous fluid (Kini and Nandeesh, 2012). Opposingly, the reduction of gravitational force and mechanical loading that occurs during HDT bed rest and spaceflight results in reduced muscular activity leading to muscle atrophy and the decreased ability of muscle to rapidly produce high forces on bone (Lu et al., 1997; Robling, 2009; Avin et al., 2015; Novotny et al., 2015). This causes increased bone resorption, while bone formation is either unchanged or decreased (Kramer et al., 2012; Kini and Nandeesh, 2012). This adaptive process was proposed by Frost as the mechanostat theory, which suggests that the strain (i.e. deformation) of bone is maintained within a certain threshold and must be exceeded to drive bone formation and an increase in bone mass and strength (Frost, 1987).

Bone mineral density (BMD) is a composite measurement of bone mineral content (BMC) and CSA of bone (van der Schouw, 2009). As expected, the greater losses in BMD during bed rest and spaceflight occur in the lower limb skeletal sites. A recent review of 8 randomised controlled bed rest studies (duration 30 – 119 days) found that bed rest significantly decreased BMD at the hip (-5%) and the tibial epiphysis (-6%). These changes were accompanied with significant losses in quadriceps and calf muscles post-

bed rest (Konda et al., 2019). Further to this, some studies have attempted to investigate the loss that occurs in specific components of bone. Divergence exists in this regard, with some studies reporting a greater loss of trabecular bone (a highly porous form of bone tissue which surrounds pores filled with bone marrow) (Armbrecht et al., 2011; Belavy et al., 2011) compared to cortical bone (the dense outer surface of bone), whilst another study found larger reductions of cortical bone compared with trabecular bone in the tibia following bed rest (Rittweger et al., 2010).

In spaceflight, bone loss is about 1 – 2% per month (Clément, 2011c). Examination of ISS crewmembers on long-duration (4 – 6 months) space missions, identified a loss of BMD of 0.8 – 0.9% per month and 1.2 – 1.5% per month at the lumbar spine and hip, respectively (Lang et al., 2004). Using volumetric quantitative computed tomography (vQCT), compartmental assessment of the hip found that 90% of mineral loss came from cortical bone, which decreased by 1.6 – 1.7% per month. Trabecular volumetric BMD decreased at a rate of 2.2 – 2.7% per month, and while the loss of trabecular bone density was small in absolute terms, it was larger than the loss of integral and cortical bone and tended to be highest in the femoral neck (Lang et al., 2004). Currently, there is no indication that bone loss is abrogated with longer duration flights (Clément, 2011c). The uncoupling of bone resorption and formation can lead to an increase in calcium excretion and decrease in calcium absorption, causing a negative calcium balance (LeBlanc et al., 2007). Additionally, bone loss leads to an increase in fracture risk, which poses a problem from long-duration space missions, particularly when performing extravehicular activities. Similarly, the loss of BMD increases fragility and fracture risk in ageing and sedentary populations. In contrast to bed rest and spaceflight, bone loss is approximately 0.5 – 2.0% per year in osteoporosis (Clément, 2011c).

A consistent finding is the large variation in the loss of bone density between bone sites and between individuals (Rittweger et al., 2005; Smith et al., 2009; Williams et al., 2009). These observations are reported in spaceflight and bed rest studies. As physical activity, body composition and diet are strictly controlled in bed rest, it is possible that genetic variation may play an important role (Smith et al., 2009).

2.3.5. Conclusion of Physiological Adaptations

The physiological adaptations in space and during HDT bed rest, the pre-eminent ground-based analogue of microgravity, have been well-described and include muscle atrophy, bone loss and reduced cardiovascular and functional capacity, among others (Demontis et al., 2017; Tanaka et al., 2017). Research conducted during HDT bed rest and spaceflight provides valuable data for the general population, particularly in the contexts of physical inactivity, sedentary behaviour, ageing and immobilisation due to illness and injury, as these conditions induce similar physiological adaptations at a rate dependent on the use of the gravity vector (Vernikos, 2017; Vernikos, 2021). The HDT bed rest model also represents an essential opportunity to understand the health consequences of both microgravity and the reduced use of gravity and to test the effectiveness of countermeasures to simultaneously protect multiple physiological systems (Hargens and Vico, 2016) and these will be discussed later.

2.4. Metabolic Responses to Physical Inactivity, High Levels of Sedentary Time, Bed Rest and Spaceflight

2.4.1. Metabolism

This section begins with an overview of normal glucose and lipid metabolism in metabolically healthy individuals in order to subsequently understand the metabolic dysregulation that occurs in response to physical inactivity, high levels of sedentary time, HDT bed rest and spaceflight.

2.4.1.1. Normal Glucose Metabolism

The maintenance of normal glucose concentrations in the bloodstream is dependent upon precise regulation of glucose utilisation and endogenous glucose production or dietary glucose delivery (Giugliano et al., 2008). Plasma glucose is derived from three sources: post-prandial intestinal absorption, glycogenolysis (breakdown of glycogen) and gluconeogenesis (formation of glucose from sources such as lactate and amino acids). Glucose may be stored as glycogen, undergo glycolysis to pyruvate or be released into the circulation by the liver and kidneys to be used by other cells in the body (Giugliano et al., 2008). The process of maintaining plasma glucose at a constant level or within a small marginal range is known as glucose homeostasis. Glucose homeostasis is governed by pancreatic and gut-secreted hormones that exert effects on multiple target tissues including the brain, skeletal muscle, liver and adipose tissue. These glucoregulatory hormones include glucagon, insulin, amylin, glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), epinephrine, cortisol and growth hormone. The two key glucoregulatory hormones that control the bi-hormonal model of glucose homeostasis are glucagon and insulin (Figure 2) (Aronoff et al., 2004).

Glucagon is a potent hyperglycaemic and ketogenic hormone that is secreted from the pancreatic α-cells. In the fasted state, glucagon acts on the liver to increase hepatic glucose production through glycogenolysis and gluconeogenesis (Aronoff et al., 2004; Giugliano et al., 2008). Additionally, glucagon stimulates the release of free fatty acids (FFA) from the adipose tissue and subsequent ketogenesis in the liver (Nussey and Whitehead, 2001; Wilcox, 2005). Insulin is the pivotal glucoregulatory hormone, released from the pancreatic β-cells in response to elevated post-prandial blood glucose and amino

acids concentrations (Aronoff et al., 2004). The principal targets of the metabolic actions of insulin are the liver, adipose tissue and skeletal muscle (Nussey and Whitehead, 2001). In the liver, insulin promotes glycogen synthesis by upregulating glycogen synthetase and suppressing glycogen phosphorylase but has no effect on hepatic glucose uptake. Contrastingly, insulin permits glucose uptake in the skeletal muscle and adipose tissue by stimulating intracellular glucose transporter protein 4 (GLUT4) and increasing its cell-surface expression. Within the muscle, glucose is converted to glycogen. In adipose tissue, glucose is converted to fatty acids for storage as triglycerides. Insulin also plays a role in promoting the uptake of amino acids into skeletal muscle and the stimulation of protein synthesis. Simultaneously, insulin inhibits hepatic glycogen breakdown, lipolysis in the adipose tissue and the release of amino acids from the muscle (Nussey and Whitehead, 2001).

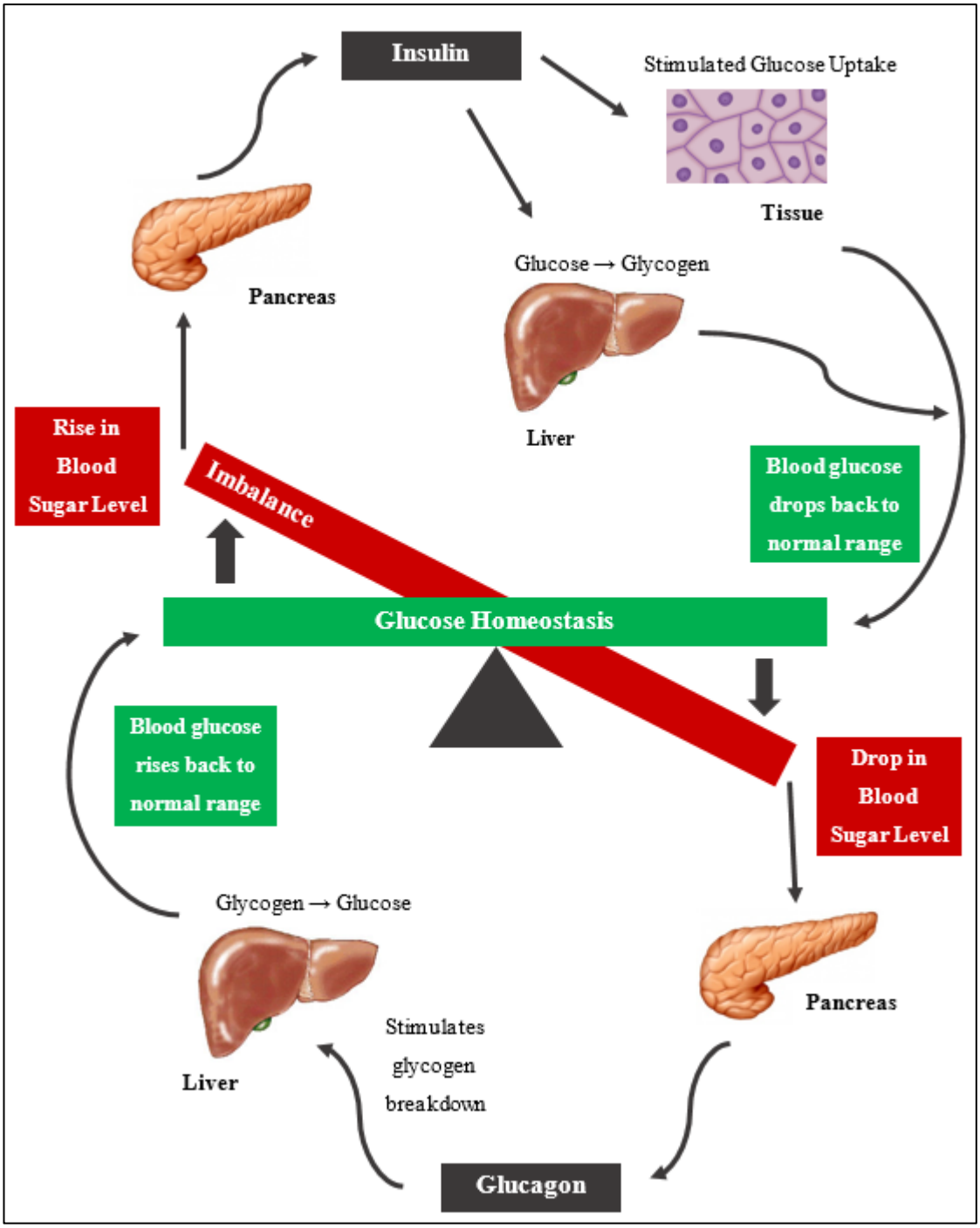

Figure 2. A schematic representation of the maintenance of glucose homeostasis by glucagon and insulin.

Adapted from Röder et al. (2016).

2.4.1.2. Insulin Signalling Cascade

The biological action of insulin depends upon the cascade of events subsequent to the interaction of insulin with its specific receptor (Figure 3). The insulin receptor is a glycosylated tetramer comprising two extracellular insulin binding α-subunits and two β-subunits that cross the cell membrane and express tyrosine kinase activity (Bugianesi et

al., 2005). Insulin binding permits the autophoshorylation of the receptor and tyrosine phosphorylation of the insulin receptor proteins (IRS-1 and IRS-2), which initiate a cascade of reactions leading to the binding of phosphoinositide 3 kinase (PI3K), activation of protein kinase B or Akt and translocation of intracellular GLUT4 to the cell membrane (Bugianesi et al., 2005; Wilcox, 2005; Mul et al., 2015). GLUT4 permits the transport of glucose along the concentration gradient from the extracellular space to the cytoplasm. During exercise, glucose transport is stimulated by pathways that are independent of PI3K and activated by 5' adenosine monophosphate–activated kinase (AMPK) (Bugianesi et al., 2005).

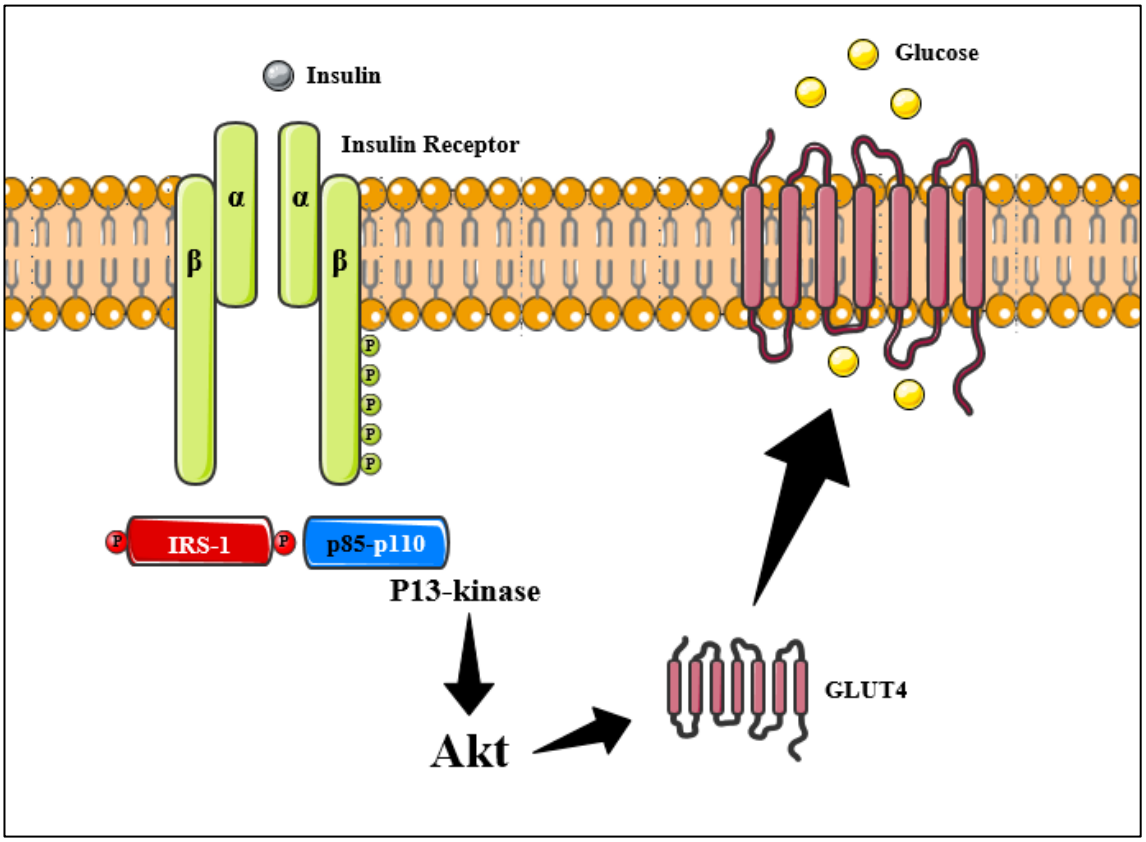

Figure 3. The insulin signalling cascade and the activation of GLUT4.

Adapted from Mul et al. (2015).

In addition to the PI3K-Akt pathway, insulin binding to the insulin receptor can cause a conformational change and phosphorylation of Shc (Src-homology collagen) proteins. Shc proteins activate the RAS-MAPK pathway (Boucher et al., 2014). Once phosphorylated Shc, recruits another protein known as growth factor receptor-bound

protein 2 (GRB2) which induces the activation of the RAS-RAF-MEK-ERK pathway. IRS-1 and IRS-2 proteins can also indirectly bind GRB2 and activate this pathway (Saad, 2018). The ERK pathway is involved in gene transcription, mitogenesis and proliferation, while the PI3K-Akt pathway is linked to the metabolic actions of insulin and nitric acid production (Saad, 2018; Draznin, 2020).

2.4.1.3. Normal Lipid Metabolism

Fat accumulation is regulated by a balance between the processes of fat synthesis (i.e. lipogenesis) and fat breakdown (i.e. lipolysis and fatty acid oxidation) (Figure 4) (Kersten, 2001; Song et al., 2018).

2.4.1.3.1. Lipogenesis

Lipogenesis is the process of triglyceride synthesis that occurs primarily in adipose tissue and to a lesser extent in the liver, muscle, heart and pancreas (Saponaro et al., 2015). The process is stimulated by a high fat or high carbohydrate diet and inhibited by polyunsaturated fatty acids and by fasting. These effects are partially regulated by hormones, which attenuate (leptin, growth hormone, glucagon, catecholamines) or stimulate (insulin) lipogenesis (Kersten, 2001; Saponaro et al., 2015). Triglyceride synthesis requires the activation of FFA to Acyl-CoA through the formation of monoacylglycerol (MAG) and diacylglycerol (DAG) by reacting with glycerol-3-phosphate (G3P). In adipose tissue, G3P can be formed by glycolysis or from non-carbohydrate sources (pyruvate, lactate or amino acids) catalysed by the enzyme phosphoenolpyruvate carboxykinase (PEPCK) in a process called glyceroneogenesis. In the liver, G3P can be synthesized by glycerol (Saponaro et al., 2015).

Triglycerides can be synthesized from either circulating FFA from the diet, peripheral lipolysis or *de novo* lipogenesis (DNL) (Saponaro et al., 2015). DNL occurs predominately in the liver but it can occur in the adipose tissue at lower rates. During glycolysis, citric acid is formed and this is converted to acetyl-CoA by ATP-citrate lipase (ACL) and subsequently to malonyl-CoA by acetyl-coA carboxylase (ACC). The multi-enzymatic complex fatty acid synthase (FAS) then converts malonyl-CoA to palmitate, the first fatty acid synthesized (Saponaro et al., 2015).

2.4.1.3.2. Lipolysis

Lipolysis is a catabolic pathway that involves the hydrolysis of triglycerides into FFA and glycerol which are released into circulation. This process requires the action of multiple lipases. Triglyceride hydrolysis into DAG is regulated by adipose triglyceride lipase (ATGL) and permits the release of one FFA. Following this, DAG is converted to MAG by the enzyme monoacylglycerol lipase (MGL) with the release of one fatty acid or is completely hydrolysed by hormone sensitive lipase (HSL) which permits the release of two FFAs and glycerol (Saponaro et al., 2015).

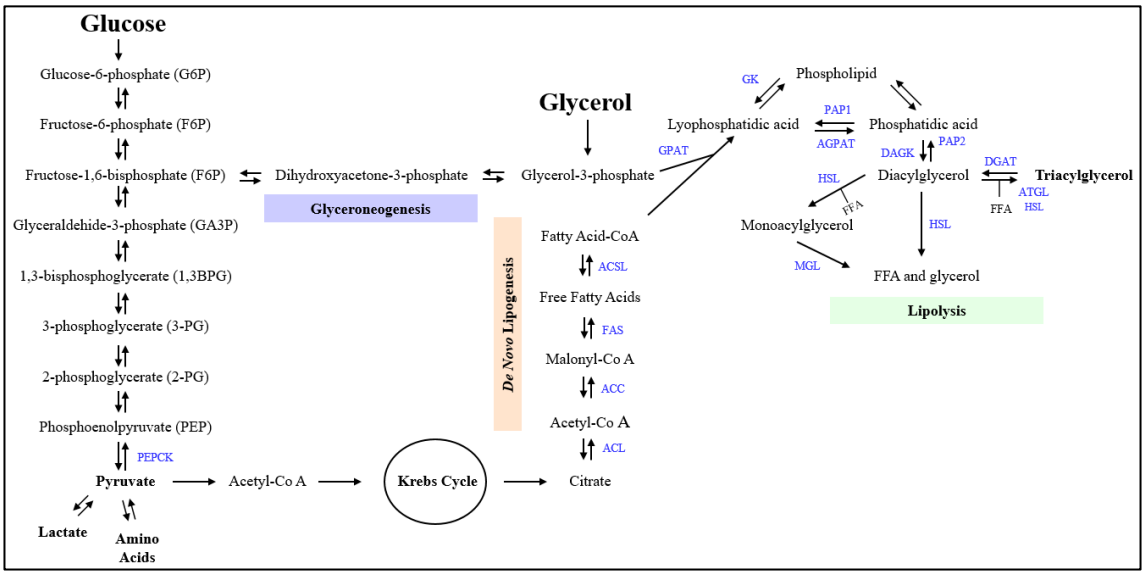

Figure 4. A schematic representation of the lipolytic and lipogenic pathways.

Adapted from Saponaro et al. (2015). Abbreviations: ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; ACSL, acyl-CoA synthetase; AGPAT, acyl CoA acylglycerol-3-phosphate acyltransferases; ATGL, adipose triglyceride lipase; DAGK, diacylglycerol kinase; DGAT, diacylglycerol acyltransferase; FAS, fatty acid synthase; GK, glucokinase; GPAT, glycerol-3-phosphate acyltransferase; HSL, hormone-sensitive lipase; MGL, monoacylglycerol lipase; PAP1, phosphohydrolase 1; PAP2, phosphohydrolase 2; PEPCK, phosphoenolpyruvate carboxykinase.

2.4.2. Insulin Resistance

Insulin resistance is one of the main hallmarks in the pathogenesis and etiology of type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD) (Rehman and Akash, 2016). Insulin resistance can be explained as a subnormal biological response to the action of insulin in skeletal muscle, adipose tissue, vasculature, brain and the liver, leading to compensatory hyperinsulinemia and reduced glucose disposal (Saad, 2018). However, as insulin has pleiotropic actions such as the modulation of lipid and protein metabolism, ion and amino acid transport, cell differentiation and proliferation, apoptosis

and nitric oxide synthesis, the whole array of functions must be considered in insulin resistance and not just the effect on glucose metabolism (Saad, 2018). Additionally, it is important to consider that the onset of insulin resistance is not uniform between the key target organs or between individuals (Unnikrishnan, 2004; Abdul-Ghani et al., 2007).

2.4.2.1. Insulin Signalling Disruption in Insulin Resistance

The regulation of insulin signalling may be described as a balance between positive regulation i.e. tyrosine phosphorylation of IRS proteins, and negative regulation, i.e. serine phosphorylation of IRS proteins (Saad, 2018). Serine phosphorylation of IRS proteins is detrimental to the normal conductance of insulin signalling and causes insulin resistance. Insulin and insulin-like growth factor 1 (IGF-1) permit tyrosine phosphorylation, while factors that cause insulin resistance such as increased tumor necrosis factor alpha (TNF-α), elevated FFAs and hyperinsulinemia can promote serine phosphorylation of the IRS-1 (Saad, 2018). These factors lead to the activation of the JNK (c-Jun N-terminal kinase) and IκB kinase (two isoforms- IKKε and IKKβ) pathways which results in the serine phosphorylation of IRS-1. IKKs will also indirectly activate the nuclear factor kappa B (NF-κB) pathway resulting in the elevated secretion of pro-inflammatory molecules which will contribute to insulin resistance. Activation of the JNK and IKK kinases highlights the convergence of the metabolic and inflammatory pathways and their contribution to insulin resistance. These kinases can also be upregulated in the innate immune response by toll-like receptor (TLR) in response to liposaccharides (LPS), peptidoglycan, double-stranded RNA and other microbial products (Saad, 2018).

2.4.3. Insulin Resistance in Peripheral Tissues

Impairment of normal insulin action can perturb metabolic homeostasis in insulin responsive tissues, with significant effects on the adipose tissue, liver and skeletal muscle (Suksangrat et al., 2019). In the context of glucose metabolism, insulin resistance can impair the suppression of hepatic glucose production, reduce glucose disposal in the skeletal muscle and adipose tissue and attenuate the suppression of lipolysis and very-low density lipoprotein (VLDL) production thereby increasing circulating non-esterified fatty acids (NEFA) and triglycerides, which can further exacerbate insulin resistance (Yki-Järvinen, 2010; Suksangrat et al., 2019).

2.4.3.1. Insulin Resistance in Adipose Tissue

Adipose tissue insulin resistance is the inability of insulin to stimulate glucose and lipid uptake and suppress lipolysis in adipocytes (Samuel and Shulman, 2016). However, compared to skeletal muscle, the contribution of adipocytes to insulin-stimulated glucose disposal *via* GLUT4 is quantitatively minor (~10% *vs.* 60-70%). In an insulin resistant state, the inability of insulin to suppress hormone sensitive lipase (HSL) and lipolysis in adipocytes upregulates circulating FFAs (Semenkovich, 2006; Ormazabal et al., 2018). An increased release of FFAs can interfere with insulin signalling and promote ectopic fat accumulation in the liver and skeletal muscle (Park and Seo, 2020). In addition to FFAs, adipocytes can secrete numerous cytokines, such as interleukin-6 (IL-6), TNF-α, plasminogen activator inhibitor 1 (PAI-1), angiotensinogen and leptin, exacerbating insulin resistance (Wilcox, 2005).

2.4.3.1.1. Insulin Resistance and Lipoprotein Changes

Elevated FFA delivery to the liver from adipose tissue and increased DNL through compensatory hyperinsulinemia, permits the post-translational stabilisation of apolipoprotein B-100 (ApoB) and enhances the formation and release of VLDL, resulting in hypertriglyceridemia (Adeli et al., 2001; Semenkovich, 2006; Ormazabal et al., 2018). Under normal conditions, insulin can inhibit VLDL synthesis and degrade ApoB through the activation of PI3K but these roles are attenuated in insulin resistance. Further to this, insulin resistance is known to downregulate lipoprotein lipase (LPL) activity, which impacts VLDL clearance from circulation (Ormazabal et al., 2018). Within the blood vessels, the triglycerides in VLDL are transferred to low-density lipoprotein cholesterol (LDL) and high-density lipoprotein cholesterol (HDL) by cholesterol ester transfer protein (CETP), producing triglyceride-rich particles. These triglyceride-rich particles are then hydrolysed by hepatic lipase forming small, dense LDL and HDL which are linked with a pro-atherogenic phenotype. The triglyceride-rich HDL are removed quickly from circulation by the kidneys, leaving fewer HDL particles to accept cholesterol from the vasculature to return to the liver (Adeli et al., 2001; Semenkovich, 2006; Jung and Choi, 2014; Ormazabal et al., 2018).

2.4.3.2. Insulin Resistance in Skeletal Muscle

Skeletal muscle insulin resistance is associated with decreased glucose uptake and glycogen synthesis and increased triglyceride accumulation. Impaired insulin-stimulated glucose disposal is traceable to defects in proximal insulin signalling, specifically the insulin receptor, IRS-1, PI3K and Akt activity, which attenuate insulin's ability to stimulate GLUT4 translocation and glycogen synthesis (Petersen and Shulman, 2018). In young, lean humans exhibiting skeletal muscle insulin resistance, ingested glucose that is not taken up by skeletal muscle is diverted to the liver, where it is used as a substrate for hepatic DNL, leading to increased triglyceride synthesis and circulating triglycerides, as well as reduced HDL concentrations (Petersen et al., 2007; Samuel and Shulman, 2016). Increased circulating FFA delivery from adipose tissue and expression of pro-inflammatory cytokines TNF-α and interleukin-1 beta (IL-1β) can enhance inflammatory signalling in myocytes, through the activation of protein kinase C (PKC), JNK and the nuclear factor kappa-light-chain-enhancer of activated B cells (IKK/NF-κB) pathways, leading to further disruption of insulin signalling (Wu and Ballantyne, 2017).

2.4.3.3. Insulin Resistance in the Liver

Hepatic insulin resistance can be explained as the impaired ability of insulin to suppress hepatic glucose production (Yki-Järvinen, 2010). The accumulation of toxic lipids such as DAGs and ceramides activate protein kinase Cε (PKCε) which binds and inactivates insulin receptor tyrosine kinase and reduces IRS-2 tyrosine phosphorylation. In downstream signalling, this causes reduced insulin activation of PI3K, RAC-β serine/threonine protein kinase (AKT2), glycogen synthase kinase-3 (GSK-3) and forkhead box protein O1 (FOXO1) leading to a decrease in hepatic glycogen synthesis and impaired suppression of hepatic gluconeogenesis (Savage et al., 2007; Yki-Järvinen, 2010). In addition, DNL is increased in hepatic insulin resistance and is regulated by mammalian target of rapamycin (mTOR) and sterol-regulatory element binding protein-1c (SREBP-1c) pathways (Armandi et al., 2021). Together, these changes result in hyperglycaemia, hyperlipidaemia and hepatic steatosis. Accordingly, the amount of intra-hepatic lipid (IHL) is strongly linked to liver and whole-body insulin resistance (Mu et al., 2018; Trouwborst et al., 2018).

2.4.4. Metabolic Regulation and Energy Balance in Bed Rest

In line with other ESA bed rest studies, dietary intake and physical activity levels are standardised and controlled prior to and throughout all study phases. For this study, and similar studies (Kenny et al., 2017; Rudwill et al., 2018; Kenny et al., 2020), subjects are maintained in energy balance. The aim of this unique approach is that fat accumulation does not occur as a consequence of reduced energy expenditure in HDT bed rest and thus fat accumulation is not a confounding factor on physiological or metabolic outcomes. Accordingly, decreases in body weight are reflective of muscle atrophy as fat mass is unchanged (Zahariev et al., 2005).

2.4.5. Sedentary Behaviour, Physical Inactivity, HDT Bed Rest and Metabolic Physiology

2.4.5.1. Distinction between Sedentary Behaviour and Physical Inactivity

Sedentary behaviour, light-intensity physical activity (LPA), moderate-intensity physical activity (MPA) and vigorous-intensity physical activity (VPA) are part of the same energy expenditure spectrum and movement continuum, but each are clearly distinctive (Figure 5) (Tremblay et al., 2010; van der Ploeg and Hillsdon, 2017). Sedentary behaviour can be described as "any waking behaviour characterized by an energy expenditure $\leq$ 1.5 metabolic equivalents (METs), while in a sitting, reclining or lying posture" (Tremblay et al., 2017; Bull et al., 2020). LPA is defined as "physical activity that is performed between 1.5 and 3 METs" and includes both static (e.g. standing) and ambulatory activities (e.g. slow walk). MPA refers to "physical activity that is performed between 3 and < 6 METs" (e.g. walking at $\leq$ 4mph on a level and firm surface). VPA describes "physical activity that is performed at $\geq$ 6 METs" (e.g. running, cycling and resistance training) (Bull et al., 2020).

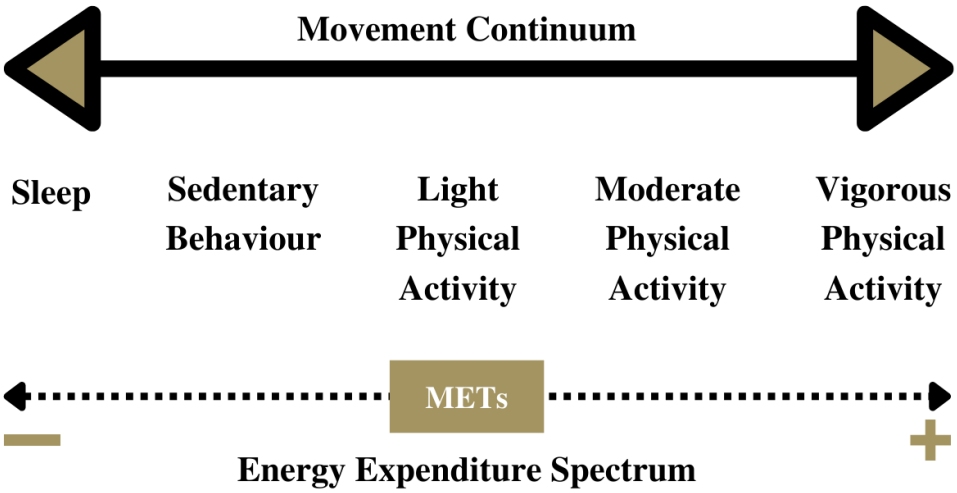

Figure 5. The movement continuum and energy expenditure spectrum between sedentary behaviour and physical activity.

Adapted from Tremblay et al. (2010).

According to the most recent physical activity guidelines, adults (aged 18 – 64 years) should undertake at least 150 – 300 minutes of moderate-intensity aerobic physical activity, or at least 75 – 150 minutes of vigorous-intensity aerobic physical activity; or an equal combination of both, throughout the week. In addition, adults are advised to do muscle strengthening activities on 2 or more days per week, at a moderate or greater intensity that involve all major muscle groups (Bull et al., 2020). Physical inactivity is, therefore, defined as "an insufficient physical activity level to meet the present physical activity recommendations" (Bull et al., 2020).

Overall, it is the balance between sedentary behaviour and physical activity that is important for health. For example, an individual can be physically active but highly sedentary. The opposite is also possible, for instance, an individual could be physically inactive but not sedentary. These situations have different ramifications for physical and metabolic health, but individuals who are physical inactive and highly sedentary are at the highest risk of chronic disease development (van der Ploeg and Hillsdon, 2017).

2.4.5.2. Sedentary Behaviour and Metabolic Physiology

Sedentary behaviour is common to every domain of modern daily life; transportation, occupational and leisure time activities. High volumes of sedentary time have a deleterious impact on cardiometabolic health. The significant reduction of energy expenditure is one of mechanisms by which sedentary behaviour induces deleterious physiological effects (Archer et al., 2017). When an individual is in a sitting, reclining or lying posture, the contractile activation of the musculoskeletal system is profoundly diminished. As the energy demand of skeletal muscle is dependent upon contractile activity, there is a substantial decline in the uptake of nutritive energy molecules (i.e. glucose, insulin and triglycerides) from blood by the skeletal muscles as a result of disuse. This will cause a rise in circulating concentrations of glucose, insulin and triglycerides. Increased concentrations of these metabolites in blood can stimulate pathological oxidative processes that lead to the production of reactive oxygen species (ROS) and oxidative damage to the intima, which underpin the development of atherosclerosis (Archer et al., 2017). Additionally, sedentary behaviour diminishes the metabolic functionality and oxidative capabilities of all muscle fibers, however, the specific impact on slow-twitch (oxidative) postural muscle fibers contributes to the reduced uptake and oxidation of circulating lipids and heightened oxidative damage. Reduced insulin sensitivity and suppressed activity of LPL in skeletal muscle can further exacerbate sedentarism-induced metabolic dysregulation and contribute to detrimental increases in visceral adiposity, increased inflammatory state and release of pro-inflammatory cytokines and injury of the endothelial cells of the vasculature. Ultimately, increased sedentary behaviour impairs the ability of skeletal muscle to remove glucose and lipids from the blood (Archer et al., 2017).

In adults, high amounts of sedentary behaviour are independently associated with all-cause, cardiovascular disease and cancer mortality and incidence of cardiovascular disease, cancer and T2DM (Bull et al., 2020). Of note, these associations are more pronounced at lower levels of moderate- and vigorous-intensity physical activity (MVPA) compared with higher levels, highlighting that higher levels of MVPA can attenuate the detrimental association between sedentary behaviour and poor health outcomes (Dempsey et al., 2020). However, based on evidence of a non-linear dose-response relationship, limiting the total time spent being sedentary and increasing LPA, increasing

MVPA, or a combination of both, can provide health benefits (Dempsey et al., 2020). Despite new recommendations to limit the amount of time spent sedentary from the WHO, there are currently no quantitative thresholds of sedentariness, to accurately distinguish sedentary individuals from non-sedentary individuals and insufficient evidence to determine whether specific health benefits vary by the type or patterns of sedentary behaviour or to determine the influence of frequency and duration of breaks in sedentary behaviour on overall physiological health, highlighting the importance of future research (Magnon et al., 2018; Bull et al., 2020). Additionally, experimental evidence on the independent effect of sedentary behaviour is lacking due to the challenges of isolating sedentary behaviour from those of physical inactivity (Le Roux et al., 2021).

2.4.5.3. Physical Inactivity and Metabolic Physiology

Physical inactivity is a public health risk of the modern lifestyle. Inactivity and a resultant low level of energy expenditure in skeletal muscle are key players in the complex phenomenon of inactivity-induced metabolic dysregulation (Thyfault and Krogh-Madsen, 2011). The transition to physical inactivity results in decreased peripheral insulin sensitivity and central adiposity, with the latter consequence occurring as a result of a chronic positive energy balance. Insulin resistance can impair the suppression of glucose production in the liver, reduce glucose disposal in the skeletal muscle and adipose tissue and attenuate the suppression of lipolysis and VLDL production thereby increasing circulating NEFA and triglycerides, which can further exacerbate insulin resistance (Yki-Järvinen, 2010; Suksangrat et al., 2019). Central and ectopic fat accumulation cause an increase in the release of reactive oxygen species and pro-inflammatory cytokines (e.g. TNF-α and IL-6) resulting in systemic oxidative stress, low-grade inflammation and promotion of insulin resistance (Gratas-Delamarche et al., 2014). Concomitantly, physical inactivity causes a decrease in cardiorespiratory fitness and muscle mass and strength and alters muscle fiber phenotype leading to an overall decline in overall physiological and metabolic function, as well as mobility and functional independence (Bowden Davies et al., 2019). In support of these mechanisms, overwhelming evidence from epidemiological studies shows that physical inactivity is a major risk factor for early mortality and common chronic diseases including obesity, insulin resistance, T2DM, the MetSyn and cardiovascular disease, among others (Booth et al., 2012; Le Roux et al., 2021).

Overall, sedentary behaviour is not physical inactivity by a different definition, as it has distinct health consequences compared to those from not exercising enough (van der Ploeg and Hillsdon, 2017). However, it is clear that individuals who are both physically inactive and highly sedentary are at the highest risk of poor health and require lifestyle advice and intervention. It is acknowledged that both of these activity behaviours occur in HDT bed rest and both have independent physiological consequences, therefore, it is very difficult to isolate the impact of sedentary behaviour or physical inactivity *per se* on the observed changes following HDT bed rest. More needs to be done in this regard in future HDT bed rest studies, possibly with the inclusion of ambulatory control groups (e.g. subjects who are physically active but highly sedentary or subjects who are physically inactive but not sedentary).

2.4.5.4. HDT Bed Rest and Metabolic Physiology

HDT bed rest induces physiological effects of combined physical inactivity and high levels of sedentary time that are associated with altered whole-body and tissue-specific functioning. Substantial reductions in muscle mass, muscle strength and cardiorespiratory fitness, as well as alterations in myofiber type composition, are associated with deleterious changes in physiological function following HDT bed rest. These changes are also implicated in HDT bed rest-induced metabolic dysfunction. Reduced insulin sensitivity (primarily in skeletal muscle), impaired oxidative capacity, increased circulating and deposition of lipids, and metabolic inflexibility (lower fasting fat oxidation and/or an impaired ability to oxidize carbohydrate during feeding or insulin-stimulated conditions) are eminent consequences of HDT bed rest, even in the absence of measurable changes in energy balance (Bergouignan et al., 2006; Bergouignan et al., 2009; Bergouignan et al., 2011; Kenny et al., 2017; Rudwill et al., 2018; Le Roux et al., 2021). These changes can increase the flux of dietary fatty acids to the liver leading to ectopic fat storage which has further implications for insulin sensitivity. Fat accumulation in the liver can drive DNL and increase the synthesis of VLDL, further exacerbating hyperlipidaemia and ectopic fat accumulation. Consequently, the steatotic liver will become insulin resistant leading to the worsening of hyperinsulinemia (Bergouignan et al., 2006; Le Roux et al., 2021). Similar metabolic alterations are associated with the etiology of multiple chronic diseases including obesity, T2DM, NAFLD and the MetSyn. These observations therefore support a key role of the combined effect of chronic

inactivity and extreme sedentariness in the onset and progression of metabolic dysfunction following HDT bed rest (Le Roux et al., 2021).

2.4.6. Ageing and Metabolism

Physiological function begins to gradually decline throughout adult life. Ageing is a natural biological process, characterised by structural and functional deterioration in most physiological systems, which makes the individual fragile and vulnerable to disease (Demontis et al., 2017; van den Beld et al., 2018). Metabolic dysfunction is a hallmark of ageing, underpinned by insulin resistance and changes in body composition (Barzilai et al., 2012). Ageing is associated with the loss of bone and muscle mass and strength, coupled with an increase in fat mass (van den Beld et al., 2018). Age-related increases in fat mass, as well as the redistribution of subcutaneous to intra-abdominal visceral depots and other ectopic sites including in the bone marrow, skeletal muscle and liver, contribute to impaired fatty acid handling and adipose tissue inflammation and dysfunction (Sepe et al., 2011). These impairments lead to a further reduction in adipogenesis, more lipotoxicity and activation of cellular stress pathways which, in turn, perpetuate inflammatory responses of pre-adipocytes and immune cells, leading to a vicious cycle and overall systemic dysfunction (Sepe et al., 2011). The physiological adaptations observed following spaceflight and HDT bed rest are similar to those that occur over decades of ageing (declines in cardiorespiratory fitness, sarcopenia, dynapenia and metabolic dysfunction), compounded by physical inactivity and high levels sedentary time (or reduced use of gravity over decades as a function of age) in older populations (Vernikos, 2017; Vernikos, 2021). Uncorrected, these changes can not only hasten ageing but are commonly associated with chronic diseases such as T2DM, cardiovascular disease, dementia, osteoporosis and arthritis (Vernikos, 2017; Sathya and Pandima Devi, 2020). Thus, the data collected from HDT bed rest will provide important insights into age-related declines in metabolic homeostasis and provide an opportunity for expanding knowledge in both space and ageing physiology.

2.4.7. Inter-Organ Communication to Regulate Metabolism

Systemic metabolism is governed by a complex series of pathways that regulate energy and nutrient intake and utilisation. Numerous organ systems within the human body must

communicate and work in concert to absorb, store and utilise chemical energy for the maintenance of metabolic homeostasis (Priest and Tontonoz, 2019). Dysregulation of these pathways will lead to pronounced metabolic dysregulation and pathologies such as obesity, T2DM, fatty liver and the MetSyn. The liver, adipose tissue and skeletal muscle are involved in the maintenance of metabolic homeostasis by producing and secreting proteins (collectively referred to as organokines) known as hepatokines, adipokines and myokines, respectively (Priest and Tontonoz, 2019; Chung and Choi, 2020). Organokines are pleiotropic molecules that regulate inflammation, glucose and lipid metabolism, oxidative stress, endothelial dysfunction and fat distribution via autocrine, paracrine and endocrine actions. They can also act on distant receptors independent of traditional metabolic functioning (Chung and Choi, 2020). Organokines have previously been classified into two categories; pro-inflammatory and anti-inflammatory (Ouchi et al., 2011). The balance between the secretions of these different types of organokines will either promote or deter metabolic dysfunction. Accordingly, physical inactivity can suppress the secretion of health enhancing organokines and promote the secretion of organokines that contribute to development of metabolic disease. Conversely, physical activity promotes the secretion of organokines that induce favourable changes in local and systemic metabolism (Leal et al., 2018). Therefore, understanding the production and target-tissue action of these secreted proteins is fundamental in the challenge to oppose metabolic dysregulation and onset of diseases such as obesity, insulin resistance, T2DM and NAFLD.

The following sections (2.4.8 to 2.4.10 inclusive) provide a comprehensive review of the current literature on a specifically identified and selected panel of adipokines (adiponectin, ASP, apelin), myokines (irisin) and hepatokines (fetuin-A, RBP4, apo-J), FGF-21 and adropin) that are known to influence insulin sensitivity. This in-depth review will provide details on the metabolic function of each individual biomarker and discuss its regulation in metabolic disease, exercise training, bed rest, physical inactivity, sedentary behaviour and ageing. Of note, emerging evidence has shown that fetuin-A, RBP4, adropin, irisin, apo-J, ASP and apelin play key roles in tissue-specific and whole-body insulin sensitivity but have not been measured previously in response to HDT bed rest. Adiponectin and FGF-21 have been examined in past bed rest studies and the findings of these studies, as well as the implications for metabolic health, will be

discussed. It is acknowledged that a large volume of research is presented in the sections below and so for the reader's convenience, and to assist in the interpretation of this research, a summary section is provided to synthesise the presented literature at the end of each individual biomarker review.

2.4.8. Adipokines

2.4.8.1. Adiponectin

Adiponectin is a 30 kDa multimetric peptide, that is expressed and secreted predominately by adipocytes, and is crucial for energy homeostasis (Coles, 2016). Adiponectin circulates in three forms with different molecular weights; low-molecular weight (LMW) homotrimer, moderate molecular weight (MMW) hexamer, and high molecular weight (HMW) multimer (Achari and Jain, 2017; Khoramipour et al., 2021). A smaller form of adiponectin that consists of a globular domain can also be found in circulation but is often in negligible amounts. In circulation, adiponectin levels are high, accounting for up to 0.01% of total plasma protein (Nigro et al., 2014).

2.4.8.1.1. Metabolic Actions of Adiponectin

Adiponectin is one of the most extensively studied biomarkers to date. There is a large number of published studies in humans, animals, isolated tissues and cultured cells that have reported that adiponectin exerts anti-diabetic, anti-inflammatory and anti-atherosclerotic effects, all of which have been comprehensively reviewed (Stern et al., 2016; Achari and Jain, 2017; Khoramipour et al., 2021). This makes adiponectin potentially a very useful biomarker to study in addition to novel biomarkers of insulin resistance, as it may assist in the interpretation of changes in novel biomarkers in response to intervention.

2.4.8.1.1.1. Adiponectin Functions in Metabolic Organs

The pleiotropic actions of adiponectin result in different effects in peripheral organs (Figure 6). The liver and skeletal muscle are major targets for adiponectin. In the liver, adiponectin decreases hepatic lipogenesis and increases β-oxidation through the activation of AMPK (Stern et al., 2016). Independent of AMPK, adiponectin inhibits

gluconeogenesis, thereby decreasing glucose output, and reduces ceramide accumulation in the liver by enhancing ceramidase activity (Stern et al., 2016). In skeletal muscle, adiponectin promotes insulin sensitivity and increases fatty acid oxidation and glucose uptake (Khoramipour et al., 2021). Adiponectin acts primarily on skeletal muscle to augment influx and combustion of FFA, thereby reducing muscle triglyceride content. As a consequence of decreased serum FFA and triglyceride levels, hepatic triglyceride content is decreased. Reduced triglyceride content can improve insulin signalling transduction and therefore improve insulin sensitivity (Yamauchi et al., 2001). In addition to the liver and skeletal muscle, adiponectin can act in an autocrine/paracrine manner to improve insulin sensitivity in adipose tissue (Fang and Judd, 2018). Adiponectin promotes triglyceride storage in subcutaneous adipose tissue thereby reducing excessive lipid deposition in the liver and skeletal muscle and permitting systemic improvements in insulin sensitivity and inflammation and preservation of β-cell mass in mice (Kim et al., 2007). Adiponectin has been shown to downregulate cytokine secretion and shift macrophage polarisation from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype (Dietze-Schroeder et al., 2005; Ohashi et al., 2010). Additionally, adiponectin can increase GLUT4-mediated glucose uptake in adipose tissue (Stern et al., 2016).

Overall, adiponectin can enhance whole-body insulin sensitivity through direct actions in the liver, skeletal muscle and adipose tissue. In addition, adiponectin can promote glucose-stimulated insulin secretion, prevent β-cell apoptosis and promote survival, as well as increase endothelial nitric oxide synthase (eNOS) activity and reduce oxidative stress in endothelial cells (Fang and Judd, 2018).

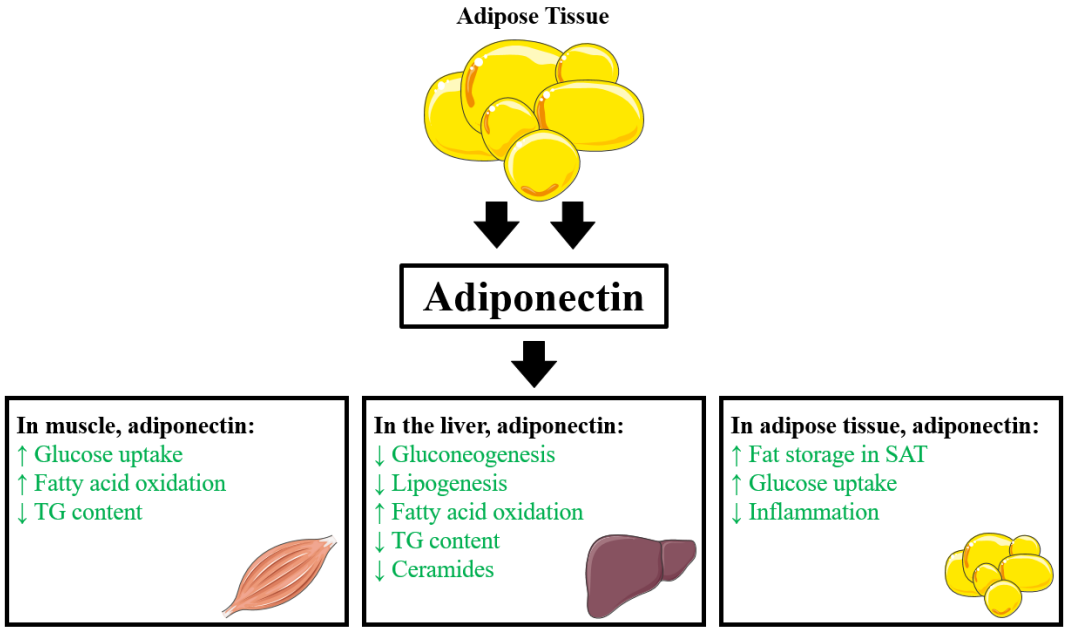

Figure 6. The metabolic actions of adiponectin under normal physiological conditions characterised by normal weight and a favourable metabolic profile.

Abbreviations: SAT, subcutaneous adipose tissue; TG, triglycerides.

2.4.8.1.2. Adiponectin in Metabolic Disease

The secretion of adiponectin from adipocytes is reduced in metabolic dysfunction, leading to decreased circulating levels of adiponectin (Andrade-Oliveira et al., 2015). Several hormones associated with obesity and insulin resistance including insulin, catecholamines, TNF-α as well as other pro-inflammatory cytokines (e.g. IL-6) downregulate the expression of adiponectin, thereby contributing to reduced circulating concentrations (Stefan and Stumvoll, 2002; Andrade-Oliveira et al., 2015). Accordingly, lower circulating concentrations of adiponectin have been found in individuals with obesity, insulin resistance, atherosclerosis, T2DM and the MetSyn (Arita et al., 1999; Hotta et al., 2000; Baratta et al., 2004; Goropashnaya et al., 2009; von Frankenberg et al., 2014; Ma et al., 2020).

An examination of the relationship between adipose tissue depots, fat cell size and secretion of adiponectin in obesity found that despite an obesity-induced reduction in adiponectin secretion, subcutaneous adipose tissue contributes more greatly to serum adiponectin than visceral adipose tissue (Meyer et al., 2013). Therefore, because of its

larger mass and greater secretion rate, even slight reductions in adiponectin production in subcutaneous adipose tissue have the potential to considerably lower circulating levels of adiponectin in obesity. There was evidence of reduced secretion of adiponectin from visceral adipose tissue from obese subjects, but as this adipose tissue depot only makes up 20% of total body fat, it is unlikely to account for more than 20% of the reduction in serum adiponectin suggesting other unknown factors may be associated with lower adiponectin levels in visceral obesity (Meyer et al., 2013).

In obese men with T2DM ($n = 48$, age 44 ± 9 years, BMI 31 ± 3 kg/m^2), there was a significant inverse relationship between circulating adiponectin and insulin resistance ($r = -0.59$, $p < 0.001$) indicating that lower concentrations of adiponectin are associated with higher insulin resistance (Izadi et al., 2011). Moreover, there was a significant inverse correlation between circulating adiponectin and fasting blood glucose ($r = -0.67$, $p < 0.001$) highlighting that a reduction in adiponectin is associated with high fasting glucose levels, which is a key determinant of T2DM in individuals with obesity (Izadi et al., 2011).

A meta-analysis including data from 13 prospective studies, 14598 participants and 2623 cases of incident diabetes, reported that higher circulating adiponectin levels are associated with a lower risk of T2DM (Li et al., 2009). This inverse association was consistent for multiple ethnicities and did not differ based on adiponectin assay, method of diabetes ascertainment, study size, follow up, BMI or the proportion of men and women (Li et al., 2009). In line with this, Yamamoto et al. (2014) examined the association between serum adiponectin and T2DM risk in 4591 subjects (aged 25–73 years, 4124 men and 467 women) who underwent a baseline health screening and a 3 year follow up. After 3 years, 214 diabetic cases were newly diagnosed. Of these 214 subjects, 87% had prediabetes at baseline. Between-group comparisons showed that subjects who developed T2DM were significantly older, had a higher family history of T2DM, BMI, visceral fat area, waist circumference, fasting glucose, glycated haemoglobin (HbA1c), fasting insulin and homeostatic model assessment of insulin resistance (HOMA-IR) but lower adiponectin levels compared to subjects without T2DM ($p < 0.05$). Baseline serum adiponectin was significantly and inversely associated with the 3-year incidence of

T2DM. This association persisted even after adjustment for risk factors of T2DM (age, sex, BMI, family history, smoking, drinking, physical activity) and measures of abdominal obesity (visceral fat area). However, when adjusted for HOMA-IR and HbA1c this association was attenuated and became non-significant. The findings of this study show that lower levels of circulating adiponectin are associated with higher risk of T2DM, independent of common risk factors and overall and intra-abdominal fat deposition (Yamamoto et al., 2014).

In a two-stage study using data from two separate cohorts (n = 172 in cohort 1 and n = 422 in cohort 2), von Frankenberg et al (2014) demonstrated that circulating concentrations of total adiponectin and HMW adiponectin were lower in the presence of the MetSyn ($p < 0.01$) and circulating concentrations of total and HMW adiponectin decrease with an increasing number of MetSyn components ($p < 0.01$). This association was independent of age, sex, smoking status, alcohol consumption, physical inactivity, waist circumference and insulin resistance in both studies. Circulating concentrations of adiponectin appear to be, in part, determined by HDL levels, triglycerides and abdominal obesity (von Frankenberg et al., 2014).

Overall, the paradoxical decrease in circulating adiponectin plays a central role in the development of obesity, insulin resistance, atherosclerosis, T2DM and the MetSyn in humans.

2.4.8.1.3. Adiponectin and Exercise Training

The effect of acute and chronic exercise on circulating concentrations of adiponectin have been studied extensively in healthy and clinical populations and the finding of these studies are discussed below.

2.4.8.1.3.1. Adiponectin and Acute Exercise

Studies investigating the impact of acute exercise training have reported conflicting results. Saunders et al. (2012) reported that acute aerobic exercise, irrespective of

intensity (low or high; 50% and 75% of $\dot{V}O_{2peak}$, respectively), markedly increased circulating adiponectin in inactive, abdominally obese men (low, n = 18, age 38.4 ± 11.2 years, BMI 33.0 ± 3.8 kg/m^2; high, n = 20, age 39.0 ± 7.6 years, BMI 34.0 ± 3.6 kg/m^2) immediately after and 30 minutes post-exercise. The exercise-induced increase in circulating adiponectin was not associated with changes in any markers of cardiometabolic risk including triglycerides, LDL, HDL, insulin or glucose (data not shown, $p > 0.05$) (Saunders et al., 2012). Contrastingly, Jamurtas et al. (2006) reported that acute aerobic exercise (cycling at an intensity relative to 65% $\dot{V}O_{2max}$ for 45 minutes) did not alter circulating adiponectin levels in healthy, overweight young males (n = 9, mean age 31.6 years, mean BMI 28.9 kg/m^2). In addition, circulating resistin and cortisol levels did not change following acute exercise. Insulin levels and HOMA-IR were significantly decreased immediately post-exercise ($p < 0.05$), but glucose concentrations did not change after exercise ($p > 0.05$). Examination of metabolic variables 24 and 48 hours post-exercise found no significant changes in adiponectin, resistin, cortisol, glucose, insulin or HOMA-IR at these time points ($p > 0.05$). There were no significant correlations between adiponectin and any of the measured metabolic variables pre- or post-exercise (Jamurtas et al., 2006).

In line with these findings, a single bout of moderate intensity aerobic exercise (cycling at 60% $\dot{V}O_{2peak}$ for 60 – 120 minutes, with the variable duration intended to bring about a wide range in exercise-induced changes in insulin sensitivity) did not change total or HMW adiponectin levels in healthy men and women ($p > 0.05$; n = 27, mean age 29 years, mean BMI 24.7 kg/m^2) (Magkos et al., 2010). Despite this, plasma glucose was significantly lower (~5%, $p = 0.001$) and insulin sensitivity, estimated using the HOMA2-IS score, was 20% higher ($p = 0.006$) after exercise compared to baseline levels. Plasma insulin did not change ($p = 0.083$). The exercise-induced improvement in insulin sensitivity was not related to changes in total adiponectin, HMW adiponectin or the ratio of HMW-to-total adiponectin ($p > 0.05$). Together, these studies suggests that the improvement in insulin sensitivity following a single bout of exercise is not mediated by changes in plasma adiponectin (Magkos et al., 2010).

Ferguson et al. (2004) examined the effect of a single exercise session (cycling at an intensity of 65% $\dot{V}O_{2max}$ for 60 minutes) on circulating adiponectin levels and compared exercise-induced metabolic changes in healthy males (n = 8, mean age 27.5 years, mean BMI 22.1 kg/m^2) and females (n = 8, mean age 27.8 years, mean BMI 26.3 kg/m^2), matched for $\dot{V}O_{2max}$. At baseline, male subjects had significantly higher body weight but lower %fat and BMI ($p < 0.01$). Resting adiponectin concentrations did not correlate significantly with %fat, $\dot{V}O_{2max}$, or fasting insulin ($p > 0.05$). Following acute exercise, glucose concentrations increased significantly in males and female subjects ($p < 0.05$), however, when adjusted for PV these changes were no longer significant ($p > 0.05$). Adiponectin levels did not change following exercise ($p > 0.05$). Exercise-induced changes in plasma adiponectin showed considerable inter-individual differences, with subjects displaying an increase, decrease or no change in circulating adiponectin. TNF-α increased significantly in male and female subjects post-exercise (24.8% and 11.9% respectively, $p < 0.05$), and no gender differences was identified. There was no significant main effect of time for plasma insulin, however there was a significant main effect of group ($p = 0.04$), with female subjects exhibiting a greater decline in plasma insulin following exercise compared to male subjects. The results of this study suggest that plasma adiponectin levels do not change with acute exercise in healthy male and female subjects. However, this study provides evidence of inter-individual variation in circulating adiponectin following a single cycling session. Furthermore, the authors proposed that the increase in plasma TNF-α during exercise may downregulate adiponectin levels, as TNF-α is known to negatively regulate adiponectin production (Ferguson et al., 2004).

In addition to aerobic exercise, the response of adiponectin to acute resistance training has also been examined. Varady et al. (2010) explored whether a single resistance training session could influence circulating adiponectin levels in sedentary and trained men. Male subjects (n = 43) were split into four separate groups based on their previous exercise history; sedentary (n = 10), weight-trained (n = 10), runners (n = 12) or weight-trained and runners (n = 11). Subjects in each group were young (26 to 28 years old), lean (%fat 17-22% and mean BMI 25 kg/m^2) healthy males. Acute resistance exercise involved a progressive leg press weight training session consisting of low weight (~30 – 40% of the 1-repetition maximum (1RM) for both of the weight-trained groups, and two to three

plates for other groups) for 2 sets of 8 – 12 repetitions each and then near-maximal exertion for 4 sets of 8 – 12 repetitions each, with 2 minutes rest between sets. There were no significant between-group differences in age, anthropometric measurements (body weight, height, BMI, waist circumference, %fat or fat-free mass), metabolic parameters (circulating adiponectin, total cholesterol, LDL, HDL, triglycerides, C-reactive protein (CRP)) or systolic and diastolic blood pressure at baseline ($p > 0.05$). Acute resistance exercise significantly increased circulating adiponectin concentrations in the weight-trained group and in the weight-trained and runner group (30 ± 7% and 37 ± 9%, respectively, $p < 0.05$). Adiponectin levels did not change in the sedentary or runner group post-exercise ($p > 0.05$). Total cholesterol, LDL, HDL, triglycerides, CRP or systolic and diastolic blood pressure did not change following exercise ($p > 0.05$). In the weight trained group and the weight-trained and runner group, the increase in adiponectin was associated with higher levels of HDL cholesterol after acute resistance exercise ($r = 0.71$, $p = 0.001$). The relationship between circulating adiponectin and brachial flow-mediated dilation (FMD) was also explored as part of this study. Reduced FMD is a hallmark of coronary artery disease. At baseline, brachial artery FMD was similar among the 4 experimental groups. Brachial FMD decreased significantly in the sedentary group, whereas brachial FMD increased significantly in the weight-trained, runners and weight-trained and runners after acute resistance training ($p < 0.05$). Exercise-induced improvements in FMD were associated with increases in adiponectin ($r = 0.61$, $p = 0.01$) in the weight-trained and weight-trained and runner groups only. The evidence from this study shows that acute resistance training can increase adiponectin concentrations in previously weight-trained men, and increased adiponectin levels are associated with enhanced endothelial function and elevated HDL concentrations suggestive of cardio-protective effects (Varady et al., 2010).

At present, studies investigating the effect of acute exercise have reported an increase or no change in circulating adiponectin in healthy, normal weight individuals and individuals with overweight/obesity. The mechanisms underlying the acute exercise-induced changes in adiponectin are unclear and therefore further studies are needed to understand alterations in adiponectin and insulin sensitivity following different modalities of acute exercise in healthy and clinical populations.

2.4.8.1.3.2. Adiponectin and Chronic Exercise

Research collected in systematic reviews and meta-analyses has shown that exercise training, particularly aerobic exercise, can significantly increase circulating adiponectin in individuals with prediabetes and T2DM (Becic et al., 2018) and overweight and obesity (Yu et al., 2017). In addition, elevated concentrations of adiponectin have been reported in healthy individuals following aerobic exercise training.

Lim et al. (2008) examined the effect of a 10-week long aerobic training intervention (3 sessions per week of cycling exercise at an intensity of 60 – 80% of $\dot{V}O_{2max}$ for 60 minutes) on circulating concentrations of adiponectin and changes in glucose metabolism in young (n = 36, age 22.4 ± 2.8 years, BMI 21.4 ± 2.9 kg/m^2) and middle-aged women (n = 38, age 59.8 ± 5.9 years, BMI 25.1 ± 2.9 kg/m^2). At baseline, older subjects had significantly higher BMI, waist circumference, blood pressure, fasting insulin, total cholesterol, triglycerides, LDL, HOMA-IR and adiponectin and significantly lower HDL, and $\dot{V}O_{2max}$ compared to younger subjects ($p < 0.05$). None of the subjects had diabetes, however three subjects in the younger age group and three subjects in the older age group had impaired fasting glucose (IFG). Two subjects in the older age group were also found to have impaired glucose tolerance (IGT). After 10 weeks of aerobic exercise, both age groups significantly reduced fasting glucose, area under the curve for glucose (AUCG), fasting insulin, total cholesterol, LDL, HOMA-IR and significantly increased $\dot{V}O_{2max,}$ adiponectin and HDL concentrations ($p < 0.05$). However, body weight, BMI, waist circumference, blood pressure and triglycerides significantly decreased in older subjects only after exercise training ($p < 0.05$). In both age groups, the increase in adiponectin correlated with decreases in fasting insulin and HOMA-IR and the increase in $\dot{V}O_{2max}$ (data not shown). In older subjects, the increase in adiponectin correlated with decreases in fasting glucose and obesity indices such as body weight, BMI and waist circumference (data not shown).

Following the primary analysis, all subjects were divided into two groups based on their individual response of adiponectin to the aerobic exercise intervention. Adiponectin responders were defined as subjects which displayed an increase in adiponectin after aerobic training. Adiponectin increased in 24 of the 36 younger subjects (67%) and 29 of

the 38 older subjects (76%). After exercise, adiponectin concentrations were increased by 56.6% in the younger responder group and by 73.0% in the older responder group ($p < 0.05$). Measurements relating to obesity and insulin resistance including body weight, BMI and HOMA-IR were decreased to a greater extent in the adiponectin responders compared with the non-responders ($p < 0.05$). The increase in adiponectin was negatively correlated with the decreases in fasting insulin, HOMA-IR and AUCG and positively correlated with the increase in $\dot{V}O_{2max}$ in the older adiponectin responder's only (data not shown). Regression analysis found that the increase in $\dot{V}O_{2max}$ and decrease in HOMA-IR were independently associated with the increase in adiponectin after the exercise intervention ($p = 0.009$ and $p = 0.043$, respectively). Overall, the results of this study show that 10 weeks of aerobic training significantly increased circulating adiponectin in both younger and older women, and the higher levels of adiponectin were independently associated with improvements in aerobic fitness and insulin resistance (Lim et al., 2008).

Contrastingly, some studies have reported non-significant changes in circulating adiponectin despite improved insulin sensitivity after an exercise intervention and these findings are discussed below. The effect of 12 weeks aerobic training and resistance training on changes in serum adiponectin and insulin resistance were compared in 24 overweight/obese men (Ahmadizad et al., 2007). Subjects were randomly assigned to 3 groups: aerobic training group (n = 8, age 41.3 ± 5.1 years, BMI 27.9 ± 2.2 kg/m^2), resistance training group (n = 8, age 40.9 ± 3.2 years, BMI 28.3 ± 2.3 kg/m^2) and the control group (n = 8, age 38.6 ± 3.2 years, BMI 29.4 ± 4.6 kg/m^2). Aerobic training consisted of 3 sessions per week of running at an intensity of 75 – 85% maximal heart rate (HR_{max}) for 20 – 30 minutes per day. Resistance training involved 3 sessions per week of circuit training, in which subjects performed 11 exercises for 4 sets of 12 repetitions, at an intensity of 50 – 60% 1RM and total duration of 50 – 60 minutes per session. Prior to the intervention, there was no significant differences in age, body weight, BMI, %fat, waist to hip ratio and $\dot{V}O_{2max}$ between the three groups. Baseline metabolic parameters including HOMA-IR and serum adiponectin were also similar among the three groups. Following 12 weeks of training, there was a significant increase in $\dot{V}O_{2max}$ in the aerobic training and resistance training groups ($p < 0.05$), but no change in the control group. Body weight, BMI and waist to hip ratio did not change in any group post-intervention. However, there was a significant reduction in %fat in the aerobic training

and resistance training groups ($p < 0.05$). Fasting glucose did not change in any group, however, fasting insulin and HOMA-IR decreased significantly following aerobic and resistance training ($p < 0.05$) but remained unchanged in the control group. When the change in HOMA-IR was compared between the three groups, there was a significant difference between the changes in the exercising groups compared to the control group ($p < 0.05$). However, the reduction in HOMA-IR was not significantly different in the aerobic and resistance training groups. Serum adiponectin did not change in response to aerobic or resistance training. Additionally, there was no significant differences identified for the changes in each of the three groups. At baseline, serum adiponectin correlated inversely with BMI ($r = -0.74$, $p < 0.001$), %fat ($r = -0.64$, $p < 0.001$), waist to hip ratio ($r = -0.59$, $p < 0.002$) and insulin resistance ($r = -0.41$, $p < 0.016$). However, these associations were not apparent when changes in response to training were assessed. In summary, aerobic and resistance training improved insulin resistance in healthy, overweight/obese men but this improvement was not accompanied by elevations in circulating adiponectin or reductions in body composition measurements (Ahmadizad et al., 2007).

Croymans et al. (2013) investigated the impact of 12 weeks of resistance training (3 times per week for 1 hour, with training overload determined by linear periodization) on changes in circulating adiponectin and OGTT derived markers of insulin sensitivity in 28 sedentary, young men (median age 22 years and BMI 30.9 kg/m^2). Following the intervention, BMI, lean body mass and resting energy expenditure significantly increased, whereas trunk fat mass, total fat mass and %fat significantly decreased ($p < 0.05$). However, there was no significant change in body weight or waist circumference. Relative 1RM chest, leg, and row strength significantly increased as well as the overall relative strength score ($p < 0.0001$). AUCG and area under the curve for insulin (AUCI) significantly decreased after resistance training ($p = 0.004$ and $p = 0.025$, respectively). The reductions were significant at OGTT time points 60 and 90 minutes for glucose and 90 and 120 minutes for insulin (all $p < 0.001$). Muscle insulin sensitivity and the disposition index (an index of β-cell function) significantly increased following resistance training (both $p = 0.003$). Conversely, hepatic insulin resistance, the insulinogenic index (early insulin response), adiponectin, leptin, amylin did not change. Examination of changes in muscle protein content identified a significant median percent increase in

hexokinase 2 (HK2), GLUT4 and AKT2 (all $p < 0.05$) following the intervention, but no change in IRS-1 or glycogen synthase kinase 3β (GSK3β) ($p > 0.05$). Together, these results show that resistance training improves OGTT-derived muscle insulin sensitivity and β-cell function, but not hepatic insulin resistance in young, overweight/obese men. Additionally, there was evidence of increased skeletal muscle content of proteins involved in insulin signalling toward increased glucose transport. The enhancement of muscle insulin sensitivity occurred in concert with improvements in lean body mass and reductions in total and trunk fat mass following resistance training, despite the absence of changes in body weight and circulating adiponectin (Croymans et al., 2013). Unfortunately, adipose tissue insulin resistance was not estimated in this study and therefore is not available for interpretation. There are similarities between the findings of this study and the previously reviewed study by Ahmadizad et al. (2007) such that there was no change in circulating adiponectin in response to exercise training that did not alter body weight or body mass, despite an improvement in insulin action. Therefore, it is possible that adiponectin may exert beneficial effects on insulin sensitivity with weight loss but not with exercise training, a hypothesis supported by other studies (Hulver et al., 2002).

Nikserecht et al. (2014) compared the effects of 12 weeks of non-linear resistance training (NRT, 3 sessions per week for 40 – 65 minutes per session with flexible periodisation) and aerobic interval training (AIT, 3 sessions per week of 4 x 4 minute interval runs at 80 – 90% HR_{max}, interspersed with 3 minutes recovery at 55 – 65% HR_{max}) and 4 weeks detraining on changes in circulating adiponectin, inflammatory markers and physical characteristics. Obese men, matched for age, %fat and $\dot{V}O_{2max}$, were randomly assigned to NRT (n = 12, age 40.4 ± 5.2 years), AIT (n = 10, age 39.6 ± 3.7 years) or the control group (n = 11, age 38.9 ± 4.1 years). At baseline, all anthropometric measurements were moderately and inversely correlated with adiponectin levels (all r = >-0.38, data not shown). Following 12 weeks of training, significant differences in body mass, %fat, waist circumference, waist-to-hip ratio and $\dot{V}O_{2max}$ were found by ANOVA. Body mass decreased significantly after AIT (3.3%), compared with the NRT ($p = 0.01$) and control ($p = 0.001$) groups, and %fat was reduced following AIT (7.8%) and NRT (7.4%), compared with the control group (both $p = 0.001$). After 4 weeks of detraining, body mass and %fat did not return to baseline values. In comparison to the control group, AIT and

NRT causes a significant reduction in waist circumference after the intervention ($p = 0.001$ and $p = 0.005$, respectively) and returned to baseline values after 4 weeks of detraining in the NRT group, but not the AIT group. There was a significant decrease in waist-to-hip ratio in the AIT group following 12 weeks of training compared to the control group ($p = 0.02$), but this measurement returned to baseline after the detraining period. $\dot{V}O_{2max}$ increased significantly in the AIT and NRT groups compared with the control following the intervention (both, $p < 0.01$) and the increase was significantly greater following AIT compared with NRT ($p = 0.004$). Detraining led to a significant decrease in $\dot{V}O_{2max}$ in both training groups (both $p < 0.001$), however, $\dot{V}O_{2max}$ was still higher than the control in the AIT group ($p = 0.003$) but had returned to baseline levels in the NRT group ($p = 0.72$).

After 12 weeks of training, there were significant time effects (both $p = 0.001$) and time*intervention interaction effects ($p = 0.002$ and $p = 0.023$) detected for adiponectin and insulin, respectively. Compared to the control group, there was a significant increase in circulating adiponectin in the AIT group (23.7%, $p = 0.03$) but no significant change found in the NRT ($p = 0.029$) after 12 weeks. Adiponectin concentrations decreased significantly in response to detraining in both exercise groups (both $p < 0.001$). Fasting insulin decreased significantly in the AIT and NRT groups after the intervention when compared with the control group (both $p \leq 0.05$), but returned to baseline levels in both groups following detraining. A significant main effect of time ($p = 0.001$) and interaction effect ($p = 0.042$) was found for soluble intercellular cell adhesion molecule 1 (sICAM-1). There was a significant decrease in sICAM-1 in the AIT (49%) and NRT (56%) after 12 weeks compared with the control group ($p = 0.04$ and $p = 0.01$, respectively). After detraining, sICAM-1 had returned to baseline levels. A significant main effect of time ($p = 0.001$) and interaction effect ($p = 0.008$) was further identified for IL-6. Exercise training did not significant alter IL-6 levels in the AIT or NRT group. However, there was a significant increase in IL-6 following the detraining period the AIT and NRT groups (both $p = 0.01$) in comparison to the control group. There were no significant main effect of time or intervention or an interaction effect for CRP or TNF-α found. The findings of this study show that 12 weeks of NRT and AIT significantly improved body composition, $\dot{V}O_{2max}$ and was effective in reducing fasting insulin and sICAM-1 in healthy, obese men. Inflammatory markers TNF-α, CRP and IL-6 remained unchanged

and circulating adiponectin increased significantly after AIT only. Body mass was also significantly decreased following AIT. After 4 weeks of detraining, $\dot{V}O_{2max}$ and adiponectin significantly decreased whereas IL-6 concentrations significantly increased in both exercise groups. Body mass and %fat remained lower than baseline values after the detraining period but other anthropometric measurements, fasting insulin and sICAM-1 returned to pre-intervention values. The authors proposed that the increase in adiponectin could be explained partly by the reduction in body mass and/or the larger increase in $\dot{V}O_{2max}$ in the AIT group after the exercise intervention (Nikseresht et al., 2014).

Investigations on the effect of chronic exercise training on changes in circulating adiponectin have reported discrepant findings, with studies reporting either no change or an increase in adiponectin following an exercise intervention in individuals with normal weight or overweight/obesity. In studies reporting significant increases in adiponectin following exercise training, favourable changes in body weight, insulin resistance and $\dot{V}O_{2max}$ have been reported.

2.4.8.1.4. Adiponectin and Bed Rest, Physical Inactivity and Ageing

Changes in circulating adiponectin have been investigated in response to short- and medium-duration bed rest but conflicting results have been reported. Rudwill et al. (2018) examined the effect of 21 days of strict HDT bed rest, with and without whey protein supplementation, on circulating adiponectin, fasting metabolic variables and body composition in 10 healthy, young males (mean age 31 years, mean BMI 24 kg/m^2). HDT bed rest significantly decreased body mass through a reduction in fat-free mass, particularly leg lean mass (time effects, $p < 0.05$). Fat mass remained stable indicating the maintenance of energy balance. Triglycerides, NEFA, leptin, LDL: HDL, AUCG, AUCI, quantitative insulin-sensitivity check index (QUICKI) and liver insulin sensitivity did not change. However, there was a significant main effect of time for reductions in muscle insulin sensitivity, total adiponectin and HMW adiponectin after HDT bed rest (all, $p < 0.001$). The significant reduction of both total and HMW adiponectin, despite fat mass remaining constant, is indicative of decreased insulin sensitivity after 21 days of bed rest (Rudwill et al., 2018).

In other studies, the effect of horizontal bed rest on changes in adiponectin have been explored. Petrocelli et al. (2020) characterised the response of circulating adiponectin, FGF-21 and ceramides to 5 days of controlled bed rest in younger (7 males, 6 females; age 23.4 ± 3.2 years, BMI 22.1 ± 3.4 kg/m^2) and older adults (11 males, 9 females; age 67.8 ± 5.5 years, BMI 24.9 ± 2.7 kg/m^2). In contrast to HDT bed rest, this model was designed to mimic an inpatient hospital stay. Older adults had higher fat mass, fasting glucose, adiponectin, FGF-21, ceramides and total ceramides at baseline ($p < 0.05$). Following 5 days of bed rest, lean mass and leg lean mass decreased significantly in the older adults only (age*interaction, both $p < 0.01$). There was a significant main effect of time for the decrease in insulin sensitivity (Matsuda), adiponectin and FGF-21 following bed rest ($p < 0.01$). In older adults, total ceramide levels decreased significantly after 5 days of bed rest ($p = 0.005$). Elevated ratios of ceramides are associated with increased cardiovascular risk. Ceramide ratios increased significantly in older adults after 5 days of bed rest ($p < 0.05$). The ratios of C16:0/C24:0 and C24:1/C24:0 were significantly higher in older adults compared to younger adults on day 5 ($p < 0.05$). LDL, HDL, triglycerides, total cholesterol, fasting glucose, fasting insulin or HOMA-IR did not change post-bed rest. In older adults, the percentage increases in C16:0/C24:0 and C20:0/C24:0 correlated inversely with the percentage decrease in adiponectin following bed rest ($r = -0.400$, $p = 0.009$ and $r = -0.403$, $p = 0.007$, respectively). When all subjects were combined, the percentage decrease in adiponectin correlated positively with the percentage decrease in FGF-21 ($r = 0.300$, $p = 0.014$). Overall, the findings of this study show that 5 days bed rest reduced insulin sensitivity concomitant with reductions in adiponectin and FGF-21 in younger and older adults. The elevation of ceramide ratios was associated with reductions in adiponectin in older adults after bed rest. Adiponectin has been shown to lower ceramide levels through the activation of ceramidase and therefore a reduction in adiponectin may impair this function and lead to the exacerbation of insulin resistance (Petrocelli et al., 2020).

In another 5 day bed rest study, changes in adiponectin and metabolic parameters were assessed in 20 healthy individuals (14 men and 6 women, mean weight 70.4 ± 11.4kg) (Hamburg et al., 2007). Bed rest significantly increased fasting glucose, fasting insulin, HOMA-IR, AUCG, AUCI, total cholesterol, LDL, systolic blood pressure and decreased insulin sensitivity (Gutt index) ($p < 0.05$). Body weight, heart rate, HDL, diastolic blood

pressure, IL-6, CRP, tumor necrosis factor receptor II (TNF receptor-II) and adiponectin did not change after bed rest. These results show that short-duration bed rest induced insulin resistance in healthy male and female subjects, but was no associated with changes in inflammatory markers or adiponectin levels (Hamburg et al., 2007).

Ageing is associated with several hormonal and metabolic alterations which exacerbate metabolic dysfunction. Adamczak et al. (2005) examined plasma adiponectin concentrations in apparently healthy men and women divided into 3 groups: younger than 50 years, between 50 – 70 years, and older than 70 years of age and reported that plasma adiponectin did not change with ageing in females, however there was a significant increase in plasma adiponectin in elderly males over 70 years of age. The authors postulated that the increase in circulating adiponectin in elderly males may be a defence mechanism against atherosclerosis and glucose intolerance, which are common features of ageing. In line with this, a review by Arai and colleagues (2019) proposed that in individuals with catabolic states, such as chronic heart failure or sarcopenia, adiponectin is upregulated as a compensatory mechanism to attenuate inflammation and oxidative stress. When this compensation fails and a state of adiponectin resistance ensues, high adiponectin levels predict high mortality in the advanced stage of disease or ageing. Conversely, in centenarians, higher circulating adiponectin concentrations are associated with a favourable metabolic phenotype including elevated HDL concentrations and enhanced insulin sensitivity, suggesting positive effects of this adipokine on enhancing longevity. However, as most studies on centenarians are based on a cross-sectional design, it is not yet known if high adiponectin concentrations are a cause or consequence of long life (Arai et al., 2019). Further longitudinal research is required to obtain a further understanding of the role of adiponectin in ageing and longevity.

2.4.8.1.5. Summary of the Comprehensive Review on Adiponectin

Adiponectin is an adipokine with insulin-sensitising, anti-inflammatory and atherosclerotic-protective properties. Adiponectin is an abundant serum protein in humans and plays a key role in metabolic homeostasis through communication between adipose tissue and other metabolic organs including the liver and skeletal muscle. In the liver, adiponectin reduces gluconeogenesis and lipogenesis and enhances fatty acid

oxidation. In skeletal muscle, adiponectin stimulates glucose uptake and fatty acid oxidation. Adiponectin promotes local effects in adipose tissue including fat storage in subcutaneous adipose tissue, increases glucose uptake and attenuates cytokine secretion and inflammation. Additionally, adiponectin enhances glucose-stimulated insulin secretion and β-cell survival and increases eNOS activity and decreases oxidative stress. Adiponectin levels are paradoxically decreased in states of metabolic dysfunction including obesity, insulin resistance, T2DM and the MetSyn. Unexpectedly, elevated concentrations of adiponectin have been proposed to indicate a compensatory response to attenuate metabolic distress in catabolic states or the presence of adiponectin resistance which can predict all-cause mortality in advance stages of disease or ageing. Conflicting findings have been reported in studies investigating the effect of acute and chronic exercise on changes in circulating adiponectin. Following acute exercise, circulating adiponectin increases or does not change in individuals with normal weight or overweight/obesity. However, the mechanisms underlying the acute exercise-induced changes are currently unknown. Studies investigating chronic exercise have reported either no change or an increase in circulating adiponectin after an exercise intervention in individuals with and without overweight/obesity. In studies reporting a significant increase in adiponectin following exercise training, favourable changes in body weight, insulin resistance and $\dot{V}O_{2max}$ have been reported. A significant decrease in circulating adiponectin has been shown previously in response to 21 days of HDT bed rest, supporting a reduction in insulin sensitivity which has been repeatedly observed following short- and long-duration bed rest (Bergouignan et al., 2011; Kenny et al., 2017).

2.4.8.2. Apelin

Apelin was first identified in 1998 as a ligand of the orphan G protein-coupled receptor APJ (Tatemoto et al., 1998). The apelin gene encodes a 77-amino acid prepropeptide. After cleavage, the proprotein of 55-amino acid residues generates multiple active forms/fragments including apelin-36, apelin-17 and apelin-13 (Castan-Laurell et al., 2011). Apelin-13 can also be post-translationally altered by the transformation of glutamine to form the pyroglutamated apelin-13 ([Pyr1]-apelin-13) (Castan-Laurell et al., 2012). These peptides all have distinct activities but the shorter peptides (C terminal structure with fewer than 13 residues) coordinate APJ binding and the biological actions of apelin (Tatemoto et al., 1998).

2.4.8.2.1. Metabolic Actions of Apelin

Apelin is produced mainly by adipocytes, and therefore can be classified as an adipokine (Wysocka et al., 2018). The beneficial effect of apelin on energy metabolism has been studied predominately in murine models (Figure 7). Investigations using obese and insulin-resistant mice have shown that apelin increases fatty acid oxidation and mitochondrial mass (biogenesis) in skeletal muscle, through the activation of AMPK (Castan-Laurell et al., 2012). AMPK activation can also contribute to enhanced glucose uptake in skeletal muscle (Castan-Laurell et al., 2012) and human adipose tissue (Attané et al., 2011). In mice, apelin has been shown to inhibit lipolysis (Yue et al., 2011), however this function was not supported in human adipose tissue or isolated adipocytes (Attané et al., 2011). The ability of apelin to improve glucose utilisation, fat oxidation, mitochondrial mass and function suggests that apelin promotes insulin sensitivity and protects against insulin resistance. However, more human experimental data is required to support or refute these functions and the physiological mechanisms involved.

In addition to its main role as an adipokine, there is evidence to show that apelin is acutely released in response to exercise and acts in an autocrine fashion to induce skeletal muscle hypertrophy (Besse-Patin et al., 2014; Vinel et al., 2018). Apelin production decreases with age and is associated with age-related loss of muscle mass in mice and humans (Vinel et al., 2018; Barbalho et al., 2020; de Oliveira dos Santos et al., 2021). The exact mechanism(s) responsible for the reduction in apelin with ageing are unknown but mechanical contraction, inflammation and senescence (a cell fate characterised by growth arrest and other phenotypic alterations, including the development of a pro-inflammatory secretome, that is implicated as a cause of a myriad of age-related diseases) have been proposed as possible contributing factors (Vinel et al., 2018). Interestingly, supplementation or overexpression of apelin in aged mice enhanced muscle capacities (mitochondrial biogenesis and function) and myofiber hypertrophy (Vinel et al., 2018). These beneficial autocrine effects of apelin in skeletal muscle may also contribute to enhanced insulin sensitivity.

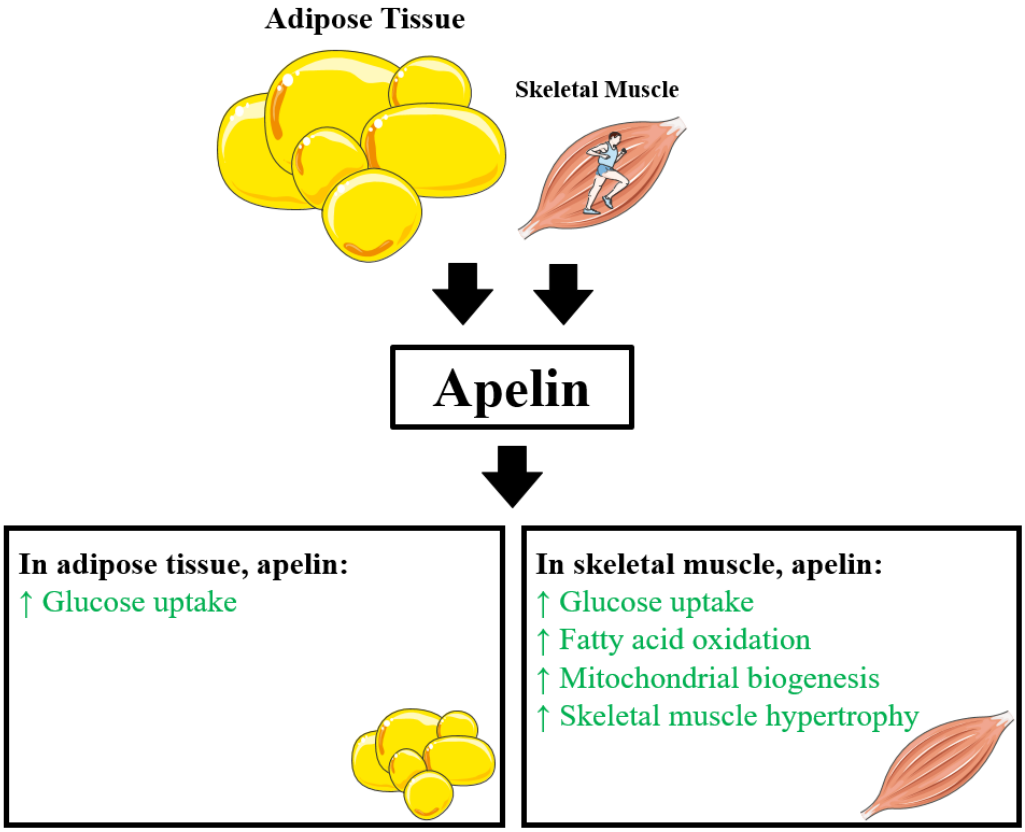

Figure 7. The secretion and positive metabolic actions of apelin under normal physiological conditions characterised by normal weight and a favourable metabolic profile.

2.4.8.2.2. Apelin in Metabolic Disease

Many studies have reported elevated concentrations of apelin in obesity, T2DM, NAFLD and MetSyn (Boucher et al., 2005; Castan-Laurell et al., 2008; Aktas et al., 2011; Krist et al., 2013; Karbek et al., 2014). Fasting and two-hour post-glucose load apelin concentrations were significantly higher in subjects with IGT and T2DM, compared to those with normal glucose tolerance (NGT) (Li et al., 2006). Further to this, increased concentrations of apelin were found to be independently associated with incident diabetes in men, but not in women (Ma et al., 2014). The authors proposed that this gender difference may be related to the influence of sex hormones on apelin secretion, but future studies are required to confirm this. The increased circulating concentrations of apelin in metabolic dysfunction would suggest either a possible resistance to apelin in these populations or that increased circulating concentrations of apelin are a compensatory mechanism intended to decrease insulin resistance (Sheibani et al., 2012; Bertrand et al., 2015).

The apelin resistance, or compensatory apelin hypersecretion observed in metabolic dysfunction, mirrors the insulin resistance, or compensatory insulin hypersecretion that is evident in metabolic dysfunction, highlighting a regulatory relationship between both biomarkers. Further investigation of the literature in murine and human studies reveals a direct regulation of apelin secretion by insulin (Boucher et al., 2005; Sheibani et al., 2012; Bertrand et al., 2015; Nam et al., 2020). Boucher et al. (2005) stated that the exact origin of this overproduction of apelin remains to be elucidated, but the striking up-regulation of apelin by insulin in adipocytes suggests an adipocyte origin of increased apelin in obesity. TNF-α is also shown to have a direct regulatory effect on apelin expression, suggesting inflammation-induced regulation of apelin. This is confirmed in both murine and human adipocytes (Daviaud et al., 2006). Since inflammation is present before the onset of insulin resistance, inflammatory cytokines including TNF-α may cause the initial rise in apelin concentrations, which is further exacerbated when insulin concentrations rise with insulin resistance. When insulin sensitivity improves and circulating concentrations of insulin decrease, circulating concentrations of apelin also decrease (Bertrand et al., 2015). Therefore, consistent with the putative role of this adipokine, there is accumulating evidence in humans that apelin functions to improve insulin sensitivity.

2.4.8.2.3. Apelin and Exercise Training

To date, the literature has predominately focused on examining exercise-induced changes in circulating apelin in individuals with obesity and T2DM, and the findings from acute and chronic exercise studies are discussed below.

2.4.8.2.3.1. Apelin and Acute Exercise

Son et al. (2019) recruited 34 women (21 lean and 13 overweight/obese) and 26 men (8 lean and 18 overweight/obese), age 30 – 59 years with a BMI between 18 – 30 kg/m^2 to examine the effect of a single bout of exhaustive exercise on a treadmill ($\dot{V}O_{2max}$ test) on apelin secretion. The acute bout of exhaustive exercise induced a significant increase in circulating apelin immediately after exercise in overweight/obese men ($p < 0.001$). This change was absent in lean men ($p > 0.05$) and there was a significant difference in apelin concentrations between overweight/obese men and lean men immediately post-exercise ($p < 0.05$). There were no significant differences in post-exercise apelin concentrations

found in women with or without overweight/obesity ($p > 0.05$). The authors proposed that these gender differences may be related to the compensatory upregulation associated with obesity, higher muscle mass and strength in men as well as hormonal differences. When all subjects were pooled, significant post-exercise correlations between plasma apelin (0 min) and lean mass, HOMA-IR, insulin secretion (HOMA-%B), lactate dehydrogenase, grip strength and isokinetic knee flexion and extension (all variables, $p < 0.05$). These correlations were not found at baseline and may suggest that apelin can be stimulated by muscle contraction and increases with post-exercise hyperglycaemia following acute exercise (Son et al., 2019). Further research is required to establish the effect of acute exercise, using different modalities (e.g. resistance training or high-intensity interval training (HIIT), on circulating apelin and its association with insulin sensitivity in individuals with metabolic dysfunction.

2.4.8.2.3.2. Apelin and Chronic Exercise

Conflicting results have been reported following aerobic training interventions in individuals with obesity and T2DM. Sheibani et al. (2012) investigated the effect of 8 weeks of aerobic training on plasma apelin concentrations in obese women (n = 20, age 38.6 ± 2.5 years, BMI 32.2 ± 1.4 kg/m^2). The training intervention consisted of 50 minutes of aerobic exercise (modality not defined) at an increasing intensity of 50 to 70% HR_{max}, 3 times per week for 8 weeks. Aerobic training significantly decreased plasma apelin concentrations in obese women ($p < 0.05$). Additionally, significant decreases in body weight, fat mass, fasting insulin and TNF-α were noticeable ($p < 0.05$). These results suggest that exercise-induced improvements in body composition and reductions in inflammation and insulin resistance may reduce apelin secretion in obesity (Sheibani et al., 2012).

In a more recent study, the impact of 8 weeks of endurance exercise (4 sessions per week of treadmill running at 65 – 70% $\dot{V}O_{2max}$ to burn 600 kilocalories) on changes in circulating apelin and the association with metabolic parameters was examined in young, normoglycaemic obese men (n = 28, age 25.04 ± 3.01 years, BMI 28.41 ± 2.37 kg/m^2) (Nam et al., 2020). Following the exercise intervention, body weight, BMI, %fat, %trunk fat, waist circumference, fasting insulin, HOMA-IR, homeostatic model assessment of beta-cell function (HOMA-β), LDL, systolic blood pressure, leptin and total adiponectin

were significantly decreased, whereas QUICKI and the percentage of HMW adiponectin were significantly increased ($p < 0.05$). Circulating apelin was significantly reduced after aerobic training ($p = 0.019$). No significant post-intervention changes were found for fasting glucose, AUCG, triglycerides, total cholesterol, HDL or diastolic blood pressure ($p > 0.05$). The exercise-induced reduction in serum apelin correlated positively with the reduction in waist circumference ($\beta = 0.419$, $p = 0.027$). Additionally, the reduction in serum apelin correlated positively with reductions in fasting insulin ($\beta = 0.607$, $p = 0.001$), HOMA-IR ($\beta = 0.440$, $p = 0.019$), HOMA-β ($\beta = 0.538$, $p = 0.003$) and correlated negatively with the increase in QUICKI ($\beta = -0.491$, $p = 0.008$). The associations with markers of glucose homeostasis measures remained significant after adjustment for changes in anthropometric measurements and conventional adipokines. These findings show that a reduction in serum apelin is associated with a lower insulin demand and improved insulin sensitivity in response to 8 weeks of aerobic training in normoglycaemic obese and propose apelin as an exercise-induced biomarker of insulin sensitivity (Nam et al., 2020).

Contrastingly, Besse-Patin et al. (2014) reported no significant change in circulating apelin in response to 8 weeks of aerobic training (5 days per week of cycling and running at a heart rate corresponding to 35 – 85% of $\dot{V}O_{2max}$ for 45 to 60 minutes per session) in sedentary, obese males (n = 11, age 35.4 ± 1.5 years, BMI 32.6 ± 2.3 kg/m^2). The exercise intervention significantly increased $\dot{V}O_{2max}$ ($p = 0.022$), reduced fat mass ($p = 0.033$) and tended to increase fat free mass ($p = 0.075$). However, no significant post-intervention changes were observed for body weight, fasting glucose, fasting insulin, AUCG, AUCI or the Matsuda index. Analysis of the skeletal muscle transcriptome identified a significant upregulation of *APLN* mRNA, the transcript that encodes apelin, following aerobic training ($n = 9$, $p < 0.01$). The increase in *APLN* mRNA correlated significantly and positively with the increase in insulin sensitivity ($r = 0.81$, $p = 0.008$) and negatively with the decrease in fasting insulin ($r = -0.70$, $p = 0.006$). Conversely, there was no change in *APLN* expression in adipose tissue (data not shown). These findings suggest that apelin secreted by skeletal muscle may contribute to exercise-induced improvements in insulin sensitivity in an autocrine and/or paracrine manner in obese subjects (Besse-Patin et al., 2014).

Krist et al. (2013) compared the effects of 12 weeks of aerobic training on changes in serum apelin concentrations in 3 experimental groups; subjects with NGT (n = 20, 9 males and 11 females, age 32.8 ± 2.5 years, BMI 24.3 ± 0.3 kg/m^2), IGT (n = 20, 9 males and 11 females, age 56.0 ± 3.6 years, BMI 29.8 ± 0.9 kg/m^2) and T2DM (n = 20, 11 males and 9 females, age 53.1 ± 1.5 years, BMI 31 ± 1 kg/m^2). The exercise intervention consisted of 3 sessions per week of running/cycling and swimming for a total of 60 minutes per session, at an intensity relative to an individual's submaximal heart rate measured during a $\dot{V}O_{2max}$ test. At baseline, subjects in the IGT and T2DM were older and had significantly higher BMI, fat mass percentage, fasting insulin, FFA and serum apelin and lower whole-body glucose uptake compared with the NGT group ($p < 0.05$). Fasting glucose was significantly higher in the T2DM group compared to the NGT group prior to the intervention ($p < 0.05$).

Aerobic training led to a significant improvement in $\dot{V}O_{2max}$ in all subject groups (data not shown). BMI and fasting glucose did not change in any group after exercise training ($p > 0.05$). Fat mass percentage and fasting insulin decreased significantly in the IGT and T2DM groups following aerobic training ($p < 0.05$). FFA decreased significantly in the NGT and IGT groups after the intervention ($p < 0.05$). Whole-body glucose uptake increased significantly in all groups after 8 weeks of training ($p < 0.05$). Further to this, significant improvements in insulin sensitivity (determined by the glucose infusion rate (GIR) during the steady state of an euglycemic-hyperinsulinemic clamp), HbA1c% and circulating high-sensitivity CRP (hsCRP) were observed in the IGT and T2DM group following the exercise intervention (data not shown). Aerobic training significantly reduced apelin concentrations by 20% in the IGT and T2DM groups and 10% in the NGT group ($p < 0.05$). Multivariate linear regression found that 25% of the reduction in serum apelin following exercise training could be explained by improvements in GIR, independent of changes in BMI ($p < 0.01$). The findings of this study imply that 12 weeks of aerobic training can reduce apelin concentrations and may contribute to the improvement in insulin sensitivity in individuals with varying degrees of glucose tolerance, even in the absence of significant weight loss (Krist et al., 2013).

Consistent with the results reported by Nikserecht et al. (2014) discussed in section 2.4.8.1.3.2, Nikserecht et al. (2016) reported that 12 weeks of non-linear resistance training (NRT) and aerobic interval training (AIT) significantly improved waist circumference, %fat, $\dot{V}O_{2peak}$, maximal strength and measures of glucose and lipid metabolism in obese men. After 4 weeks of detraining, body composition measurements, glucose and LDL concentrations remained lower than baseline values. Findings from the more recent publication show that circulating apelin increased significantly in the NRT and AIT groups, compared to within-group baseline values, and remained elevated after 4 weeks of detraining ($p < 0.05$).

In summary, divergent changes in circulating apelin have been reported following chronic exercise training in individuals with obesity and T2DM. However, in most training studies reporting significant improvements in body composition and insulin sensitivity, a significant reduction in circulating apelin concentrations has been reported. Discrepancies may be partly explained by the metabolic health status of subjects, and differences in the frequency, intensity, time and type of exercise being performed. Additional work is required to further elucidate the effect of chronic exercise training on circulating apelin and associated changes in insulin sensitivity in individuals with metabolic disease.

2.4.8.2.3.3. Apelin and Acute and Chronic Exercise in Healthy, Normal Weight Populations

In healthy, normal weight populations, the effect of acute and chronic exercise training on circulating apelin concentrations have also been investigated (Fujie et al., 2014; Kon et al., 2019; Waller et al., 2019). Plasma apelin concentrations were not significantly altered immediately following a $\dot{V}O_{2max}$ test, despite significant elevations in glucose and insulin post-exercise (both, $p < 0.05$) in apparently healthy males and females ($n = 12$; 7 males and 5 females, age 22.75 ± 2.96 years, BMI 23.37 ± 3.55 kg/m^2) (Waller et al., 2019). Contrastingly, acute sprint interval training (4 repetitions of 30 seconds maximal cycling bouts with resistance set to 7.5% of the subjects body weight, interspersed with 4 minutes of recovery) significantly increased apelin concentrations immediately post-exercise in healthy, normal weight men ($p < 0.05$; $n = 8$, mean age 20.3 years, mean BMI 23.2 kg/m^2). However, the elevation in apelin concentrations was not maintained 15, 30 or 120 minutes after the exercise session (Kon et al., 2019). A significant increase in

plasma apelin was reported following 8 weeks of aerobic training (3 sessions per week of cycling at an intensity of 60 – 70% for 45 minutes) in healthy, normal weight middle-aged and older adults (age 66.4 ± 2.1 years, BMI 24.7 ± 1.0 kg/m^2) (Fujie et al., 2014). Further research is needed to examine the effect of different types of exercise training (acute and chronic high-intensity interval training, aerobic training and resistance training) on apelin secretion and the association with insulin sensitivity in healthy, normal weight populations.

2.4.8.2.4. Apelin and Bed Rest, Physical Inactivity and Ageing

To our knowledge, the impact of HDT bed rest on circulating apelin has not be studied previously. Interestingly, many of the detrimental changes that occur in HDT bed rest such as the loss in lower extremity muscle mass and strength, parallel the normal ageing process, but occur at an accelerated rate (Kehler et al., 2019). The synthesis of apelin by skeletal muscle decreases with age and is associated with age-related loss of muscle mass in mice and humans (Vinel et al., 2018). In the study by Vinel et al. 2018, a cohort of elderly people was split into two experimental groups and included 27 subjects without muscle weakness (8 males and 19 females, mean age 75 years), and 33 subjects with sarcopenia (10 males and 24 females, mean age 76.5 years). Comparison of the groups identified significantly lower BMI (mean 23.2 kg/m^2 *vs.* 27.5 kg/m^2, $p = 0.002$), lean mass (mean 35.28 kg *vs.* 46.35 kg, $p < 0.0001$), appendicular skeletal mass (sum of lean tissue in the arms and legs) divided by height squared (mean 5.983 *vs.* 7.721, $p < 0.0001$) and Newman's index (appendicular lean mass divided by height and total fat mass: mean -1.885 *vs.* 2.200, $p < 0.0001$) in the sarcopenia group compared to the non-sarcopenia group, respectively. No significant between-group differences were identified for glucose, insulin, leptin, nerve growth factor, IL-8, IL-6, TNF-α, monocyte chemoattractant protein or hepatocyte growth factor ($p > 0.05$). Circulating apelin concentrations were significantly lower in individuals with sarcopenia, compared to those without sarcopenia ($p < 0.0001$), and were associated with sarcopenia, independent of body weight and fat mass. Consistent with these observations, in a mouse model of ageing (at 3, 12 and 24 months of age), plasma apelin levels were lower in an age-dependent manner and were associated with a specific loss of skeletal muscle apelin and its receptor (*Apln* and *Aplnr)* mRNA expression. Lower age-associated apelin expression was also confirmed in isolated muscle fibers from these mice, as well as cultured human

differentiated myocytes from 3 young and 3 older healthy donors. These results highlight a close relationship between ageing and skeletal muscle apelin (Vinel et al., 2018).

The loss of muscle mass can directly (through the storage of energy in atrophying muscle as fat) and indirectly (low total energy expenditure) increase fat storage (Hunter et al., 2019). Age-related increases in adiposity and accumulation of senescent cells exacerbate the release of pro-inflammatory cytokines and interfere with insulin signalling, causing insulin resistance (Barzilai and Ferrucci, 2012). In this situation, circulating apelin concentrations would be expected to increase (as a result of increased secretion from adipose tissue). In the study by Vinel et al. (2018) outlined above, individuals with sarcopenia had a normal-overweight BMI (21.51 – 26.17 kg/m^2) and were metabolically healthy. No significant between-group differences in markers of metabolic function were identified, however BMI was significantly greater in the individuals without sarcopenia (24.30 – 29.41 kg/m^2). As elevations of circulating apelin have been reported in obesity, this difference may partly explain the lower concentrations of apelin in the individuals with sarcopenia in this study. Additional research should be carried out to further explore the effect of ageing and sarcopenia on apelin secretion in humans.

2.4.8.2.5. Summary of the Comprehensive Review on Apelin

Apelin is secreted predominately by adipocytes and has insulin-sensitising effects through its ability to increase glucose utilisation in skeletal muscle and adipose tissue and enhance fatty acid oxidation and mitochondrial biogenesis in skeletal muscle. Additionally, there is evidence that apelin is a contraction-induced myokine that acts in an autocrine fashion to stimulate myofiber hypertrophy and enhance muscle function. In adipocytes, apelin appears to be directly regulated by insulin and TNF-α and numerous studies have reported increased concentrations of apelin in obesity, T2DM, NAFLD and the MetSyn. Accordingly, elevated concentrations of apelin have been proposed to reflect a possible resistance to apelin in states of metabolic dysfunction or a compensatory mechanism intended to attenuate insulin resistance. At present, there is a paucity of research examining the effect of acute exercise on apelin secretion. However, multiple studies investigating the impact of chronic aerobic and resistance training have reported significant reductions in circulating apelin concomitant with improvements in body

composition and insulin sensitivity in individuals with obesity and T2DM. The synthesis of apelin by skeletal muscle has been proposed to decrease with age and is associated with the age-related loss of muscle mass. However, changes in apelin secretion in response to ageing and sarcopenia in humans requires further investigation. HDT bed rest induces muscle atrophy, a shift in muscle fiber types towards a fast-twitch glycolytic phenotype, a reduction in mitochondrial oxidative capacity and insulin resistance, even when energy balance is maintained (Bergouignan et al., 2011).

2.4.8.3. ASP

Acylation stimulating protein (ASP, or C3adesArg) is an adipokine that plays a role in the regulation of lipid metabolism (Scantlebury-Manning, 2012). ASP is produced *via* the activation of the complement alternative pathway through the interaction of complement factors C3, factor B and adipsin, which are expressed and produced by adipocytes (Cianflone et al., 2003; Scantlebury-Manning, 2012; Saleh et al., 2019). ASP secretion and the responsiveness of the adipocyte to ASP has been shown to increase with adipocyte differentiation (Cianflone and Maslowska, 1995).

2.4.8.4.1. Metabolic Actions of ASP

In metabolically healthy individuals, ASP stimulates intracellular triglyceride synthesis in adipose tissue through three different mechanisms (Figure 8) (Saleh et al., 2019). Firstly, ASP can activate intracellular transport of glucose by increasing the translocation of glucose transporters to the cell surface. Subsequently, intracellular glucose forms glycerol, providing the backbone for FFA esterification. Secondly, ASP can stimulate diglyceride acyltransferase (DGAT) thereby enhancing fatty acid esterification. Enhanced fatty acid trapping by ASP mitigates feedback inhibition by fatty acids on LPL, thereby increasing fatty acid uptake further. Thirdly, ASP can inhibit the activity of hormone sensitive lipase (HSL) and intracellular lipolysis (Saleh et al., 2019). The lipogenic effects of ASP were found to be independent and additive of insulin (Germinario et al., 1993) and are permitted through an orphan G protein-coupled receptor C5L2 (Kalant et al., 2005). ASP was also shown to increase glucose uptake through the increased translocation of glucose transporters (GLUT1, GLUT3 and GLUT4) in rat L6

myotubes, suggesting that skeletal muscle is also responsive to ASP (Tao et al., 1997). However, these findings have not been confirmed in human skeletal muscle cells.

2.4.8.3.2. ASP in Metabolic Disease

Circulating ASP has been shown to increase in obesity, T2DM, NAFLD, cardiovascular disease and MetSyn (Maslowska et al., 1999; Koistinen et al., 2001; Faraj et al., 2003; Yesilova et al., 2005; Celik et al., 2013; Coelho et al., 2013; Mishra et al., 2017). However obesity is not an essential feature of elevated ASP as circulating ASP is increased in patients with T2DM and polycystic ovary syndrome (PCOS), independent of obesity (Yang et al., 2006; Wu et al., 2009). FFA and triglycerides were independent predictors of fasting ASP, accounting for 28% and 17% of the variance in a cohort of lean and obese Caucasians and Pima Indians. Body fat accounted for an additional 5% variance (Weyer and Pratley, 1999).

The action of ASP is a function of the concentrations of ASP as well as the expression and sensitivity of the ASP receptor (CL52) in target tissues to ASP (Oktenli et al., 2007). In humans, high levels of fasting ASP were associated with delayed postprandial triglyceride and NEFA clearance following an oral fat load (Cianflone et al., 2004). This paradox is similar to increased fasting insulin in subjects with glucose intolerance. Accordingly, higher ASP concentrations represent an ASP resistant state, whereas low ASP concentrations indicate increased sensitivity of the adipose tissue to ASP (Cianflone et al., 2004).

Defective ASP signalling impairs “fat trapping”, which results in the large escape of FFA into circulation. Increased flux of FFA to the liver results in hepatic apolipoprotein-B (apo-B) and VLDL production. To prevent excessive triglyceride deposition in the liver, part of VLDL is used by the muscle, but the excess flux to the adipose tissue results in storage and increased ASP secretion (Sniderman et al., 1998; Faraj et al., 2003). Enhanced triglyceride storage in adipose tissue increases adipocyte size causing macrophage recruitment and pro-inflammatory processes. Furthermore, chronically elevated ASP levels can lead to decreased insulin sensitivity through chronic activation of signalling

pathways (Fisette and Cianflone, 2010). Overall, elevations in circulating ASP reflect a deleterious state of ASP resistance that is related to adipose tissue dysfunction and which subsequently leads to adipose tissue inflammation, insulin resistance and dyslipidaemia (Paglialunga et al., 2008).

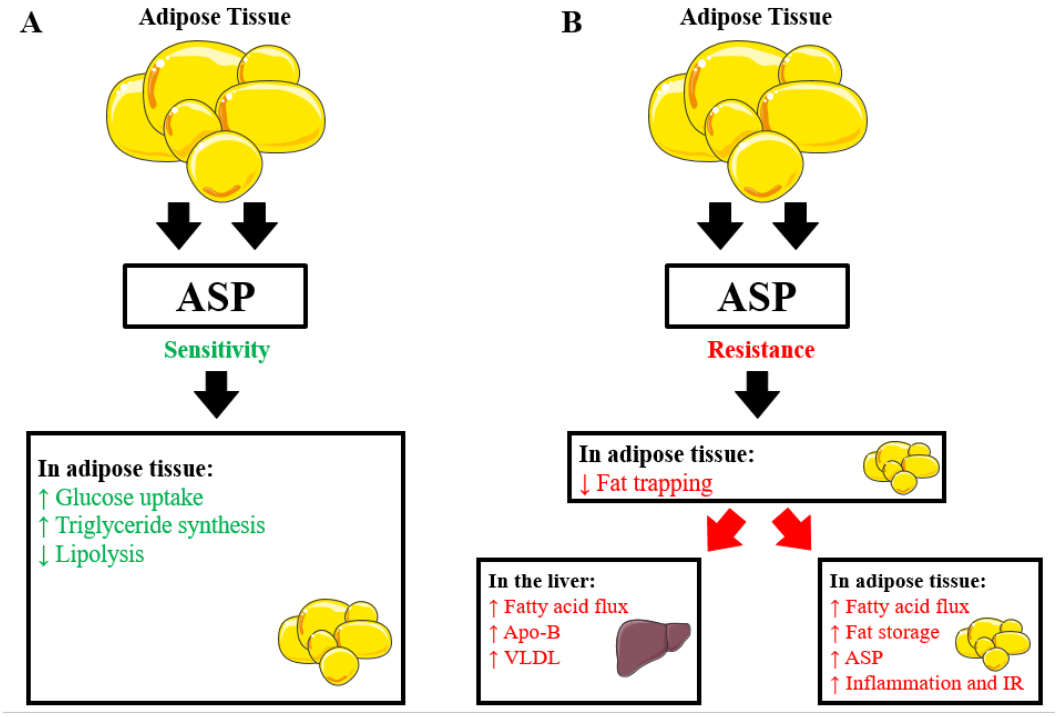

Figure 8. A) Summary of positive metabolic actions of ASP under normal physiological conditions characterised by normal weight, a favourable metabolic profile and ASP sensitivity. B) Negative metabolic actions of ASP in metabolic dysfunction associated with ASP resistance.

Abbreviations: Apo-B, apolipoprotein, B; VLDL, very-low-density lipoprotein cholesterol; IR insulin resistance.

2.4.8.3.3. ASP and Exercise Training

To date, there is only one study investigating the effect of exercise training on circulating concentrations of ASP. Schrauwen et al. (2005) investigated the effects of acute exercise (3-hour cycling test at 40% of maximal power output), before and after a short-term aerobic training intervention in 8 young, untrained healthy men (age 23.5 ± 3.4 years, BMI 22.4 ± 2.7 kg/m^2). The training intervention lasted 12 days and consisted of either 2 hours of endurance exercise (40 – 55% maximal power output) or 45 minutes of intermittent exercise training (alternating 3 minute intervals of 70% and 35% maximal power output), alternating on a daily basis. The intervention resulted in a significant

decrease (25%) in fasting ASP levels ($p < 0.001$). During exercise, there was a significant time *vs.* training effect identified for changes in ASP ($p < 0.05$). Prior to the intervention, ASP levels decreased significantly during the 3-hour exercise bout (0 *vs.* 180 minutes, $p < 0.05$). After training, ASP levels tended to increase slightly during exercise, but did not reach statistical significance (0 *vs.* 180 minutes, $p = 0.09$). The time until half of the glucose had disappeared was used as an indicator of insulin sensitivity but this measure did not change post-intervention ($p = 0.12$). Despite this, baseline ASP levels correlated significantly with glucose half time before ($r = 0.86$, $p < 0.01$), and after training ($r = 0.82$, $p < 0.05$). No significant post-intervention changes were identified for fasting FFA, glycerol, glucose or triglycerides. Plasma FFA and plasma glycerol were significantly increased in response to acute exercise, before and after training ($p < 0.05$), however the area under FFA concentrations and glycerol concentrations versus time curve were significantly lower after exercise training ($p < 0.05$). Plasma glucose and triglyceride levels decreased significantly in response to acute exercise, before and after training ($p < 0.05$), but the area under the curve was not significantly different.

This study showed that short-term endurance training significantly reduced fasting ASP concentrations in young, healthy men, and may represent improved ASP sensitivity in adipose tissue. The positive correlation between ASP and glucose half time highlights that lower levels of ASP were associated with higher insulin sensitivity, which could be related to ASP's ability to enhance glucose uptake in adipose tissue. Furthermore, the increase in ASP during acute exercise post-intervention, which coincided with an attenuated increase in FFA and glycerol, may be a physiological response to improve fatty acid re-esterification or reduce lipolysis (Schrauwen et al., 2005). Therefore, it seems that endurance training can improve ASP sensitivity in healthy men, however, future studies on exercise training, using different modalities and in healthy and clinical populations, are required to prove or confute these exercise-induced adaptations.

2.4.8.3.4. ASP and Bed Rest, Physical Inactivity and Ageing

To our knowledge, the effect of bed rest, physical inactivity and ageing on circulating concentrations of ASP has not been investigated previously. HDT bed rest serves as a useful model for examining the deleterious consequences of physical inactivity and accelerated ageing on metabolic physiology. Previous HDT bed rest studies (21 to 90

days in duration) have reported significant increases in NEFA (fasting), triglycerides (fasting and post-prandial), insulin resistance and lipogenesis, as well as significantly reduced adipose tissue lipolysis, even when energy balance was maintained (Bergouignan et al., 2006; Bergouignan et al., 2009; Rudwill et al., 2018). These findings suggest that extreme physical inactivity may induce ASP resistance and indicates a need to investigate the changes in ASP in response to HDT bed rest.

2.4.8.3.5. Summary of the Comprehensive Review on ASP

ASP is an adipokine that increases triglyceride synthesis in adipose tissue. This effect is achieved by promoting glucose uptake, enhancing FFA esterification and inhibiting intracellular lipolysis. Similar to insulin resistance, a state of ASP resistance has been proposed in a myriad of metabolic diseases, including obesity and T2DM. Ineffective ASP signalling reduces fatty acid trapping in adipose tissue. Increased flux of FFA to the liver upregulates apo-B and VLDL production, whereas increased fatty acid flux back to adipose tissue results in fat storage and increased ASP secretion. Chronic elevations in ASP augment adipose tissue inflammation, insulin resistance and dyslipidaemia. There is little published data on the effect of exercise training on ASP, however, it appears that short-term endurance training can decrease fasting ASP and is associated with improvements in insulin sensitivity. Conversely, insulin resistance and increased circulating and deposition of lipids are deleterious consequences of HDT bed rest.

2.4.9. Myokines

2.4.9.1. Irisin

Irisin is a polypeptide hormone that is secreted from skeletal muscle in response to exercise. Physical activity promotes the activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is responsible for the synthesis of fibronectin type III domain-containing protein 5 (FNDC5). Transmembrane FNDC5 is then cleaved and released in to circulation as irisin (Boström et al., 2012; Gamas et al., 2015), and is homologous between mice and humans (Arhire et al., 2019). Adipose tissue is also a source of irisin, however, in humans, the expression of FNDC5 is 200 times lower in adipose tissue compared with skeletal muscle (Moreno-Navarrete et al., 2013; Perakakis et al., 2017).

2.4.9.1.1. Metabolic Actions of Irisin

Irisin has multi-spectrum functions on the metabolic organs including the adipose tissue, skeletal muscle, liver and the pancreas (Figure 9). Irisin can promote energy expenditure through the browning of white adipose tissue (Boström et al., 2012; Arhire et al., 2019). This relevance of this function has been questioned in humans as adipose tissue (subcutaneous and omental) is predominately made up of white depots, with a low expression of brite-specific markers (Elsen et al., 2014), however, *in vitro* analysis of human adipocytes showed at transcriptional, translational and metabolite level that irisin was capable of inducing browning and controlling energy homeostasis (Huh et al., 2014a). Additionally, irisin has been shown to promote lipolysis *in vitro* and *in vivo* and this may be an important mechanism for reducing lipid accumulation (Xiong et al., 2015; Perakakis et al., 2017). In another study, irisin had no significant effect on lipolysis *in vitro* (Wang et al., 2015). Differences in cell culture and treatment, including concentrations of irisin used and length of treatment time, may explain these discrepancies and highlight that further research is warranted.

In muscle, irisin can activate the AMPK pathway by reducing intracellular ATP levels, or by increasing reactive oxygen species (ROS) or intracellular calcium levels (Huh et al., 2014b; Lee et al., 2015; Perakakis et al., 2017). Irisin has been proposed to reduce gluconeogenesis and increase glycogenesis in the liver (Liu et al., 2015b). Additionally, in human and mouse hepatocytes, irisin attenuated palmitic-acid induced lipogenesis and lipid accumulation, as well as oxidative stress and inflammation (Park et al., 2015). It has also been reported that irisin can reduce endoplasmic reticulum stress and contribute to β-islet cell mass survival and function (Arhire et al., 2019). Taken together, the effect of irisin on the main metabolic organs favours the maintenance of normoglycaemia and normolipidaemia and therefore irisin can be considered as a biomarker which enhances insulin sensitivity (Perakakis et al., 2017).

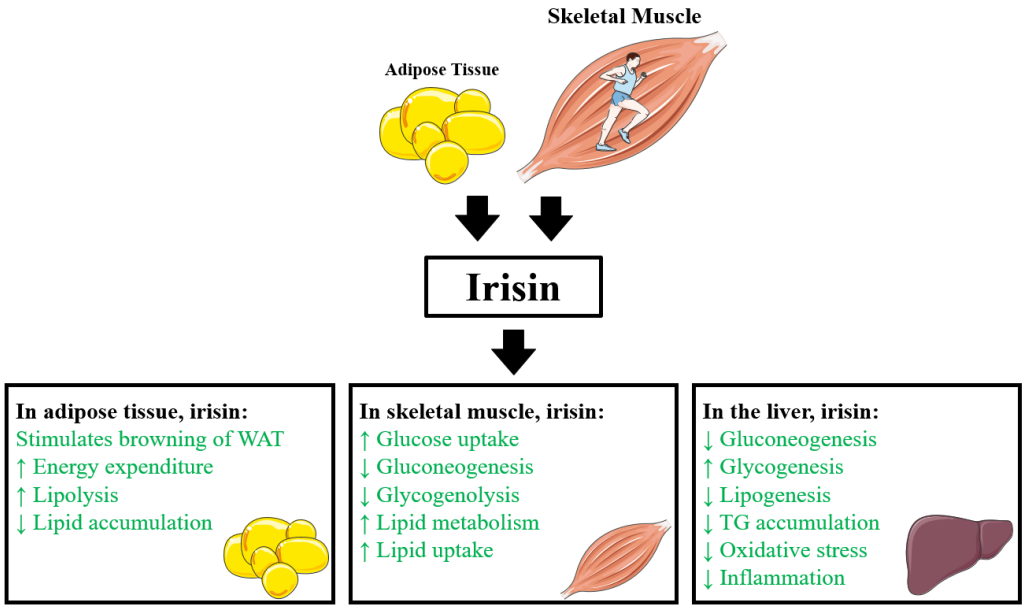

Figure 9. The secretion and positive metabolic functions of irisin under normal physiological conditions, characterised by normal weight and a favourable metabolic profile.

Abbreviations: TG, triglycerides; WAT, white adipose tissue.

2.4.9.1.2. Irisin in Metabolic Disease

The therapeutic potential of irisin has prompted many researchers to investigate the changes in circulating irisin in obesity and T2DM in humans. Associations between irisin levels, BMI and fat mass have been reported in multiple studies and these are discussed below. Stengel and co-authors (2013) investigated irisin levels over a broad spectrum of body weight, including subjects with anorexia nervosa, normal weight and morbid obesity (Stengel et al., 2013). Circulating irisin levels were 14% lower in subjects with anorexia nervosa compared to those with normal weight ($p > 0.05$) and concentrations in the morbidly obese subjects with a BMI > 40 kg/m^2 were higher compared to those with anorexia nervosa ($p < 0.05$) or normal weight ($p > 0.05$). Irisin levels positively and significantly correlated with BMI, body weight, fat mass and fat free mass (Stengel et al., 2013). Similar results were reported following a study on irisin in 145 female subjects split into anorexia nervosa, normal weight and obese groups (Pardo et al., 2014). Irisin concentrations were significantly higher in obese subjects ($p < 0.05$), and normal weight had a slight but not significant increase in irisin compared to subjects with anorexia nervosa ($p > 0.05$). Irisin also correlated significantly and positively with BMI, body weight, fat mass and fat free mass (Pardo et al., 2014). Other studies have supported an

increase in circulating irisin levels with obesity (Shoukry et al., 2016; Sahin-Efe et al., 2018), while some have not and even reported negative correlations between irisin and measures of adiposity (Moreno-Navarrete et al., 2013; Hou et al., 2015). Given the beneficial role of irisin in metabolism, the elevated concentrations of irisin that are evident in obesity may reflect (i) an increased secretion of irisin from the increased muscle and adipose tissue mass that is present in obesity, or (ii) a hypersecretion of irisin in an attempt to compensate for irisin resistance in this population. The latter response would be in line with hypersecretion of insulin in response to insulin resistance present in this population (Park et al., 2013; Hwang et al., 2016; Shoukry et al., 2016).

Lower concentrations of circulating irisin have been reported in prediabetes, newly-diagnosed and long-term T2DM (Choi et al., 2013; Liu et al., 2013; Hwang et al., 2016; Shoukry et al., 2016). A systematic review and meta-analyses of 17 cross-sectional and 6 case-control studies involving 1745 T2DM patients and 1339 non-T2DM controls, found that circulating irisin was significantly lower in patients with T2DM (Du et al., 2016). Three studies examined the effect of prediabetes (IFG) and/or IGT) on circulating concentrations of irisin. Assyov et al. (2016) found circulating irisin levels differed significantly between subjects with NGT, prediabetes and T2DM ($p < 0.05$) (Assyov et al., 2016). Duran et al. (2015) reported that irisin levels were significantly lower in subjects with both IFG and IGT and T2DM, compared to individuals with NGT ($p < 0.05$ and $p < 0.01$, respectively). However, irisin concentrations were comparable between subjects with NGT and subjects with isolated IFG or IGT (Duran et al., 2015). These studies would suggest that irisin levels decrease progressively with worsening of glucose tolerance. In contrast, Yucel et al. (2013) failed to detect any significant differences in irisin concentrations between patients with different stages of glucose intolerance (NGT, IFG, IGT, IFG/IGT and T2DM) (Yücel et al., 2018). However, it must be pointed out that the sample size in each group was lower in the third study. In subjects with NGT, multiple linear regression identified that irisin was associated with HOMA-β ($\beta = 1.872$, $p = 0.025$) and fasting glucose ($\beta = 1.012$, $p < 0.001$), after adjustment for 2 hour glucose, HbA1c, insulin and HOMA-IR, suggesting irisin may play a role in β-cell function (Yang et al., 2014). *In vitro* and *in vivo* studies on mice have shown that irisin promotes the expression of betatrophin, a novel liver and adipose tissue secreted protein, which promotes β-cell proliferation and improves glucose tolerance (Zhang et al., 2014). Therefore, irisin may

be important for regulating β-cell function and insulin secretion in healthy individuals and this function may be impaired in T2DM. Additionally, lower concentrations of irisin have also been related to compromised expression and secretion of irisin from skeletal muscle in T2DM (Shoukry et al., 2016).

2.4.9.1.3. Irisin and Exercise Training

In 2012, Bostrom and colleagues observed for the first time that irisin is released in response to exercise in humans. However, since then, research studies investigating the effects of acute and chronic exercise training on circulating irisin have yielded conflicting results.

2.4.9.1.3.1. Irisin and Acute Exercise

Acute sprinting exercise (2 – 3 sets of 80 meter sprints) significantly increased circulating irisin 30 minutes post-exercise, consistent with decreased muscle ATP content and increased markers of glycolytic and lipolytic metabolism in young healthy males (age 20.5 ± 1.5 years, BMI 21.9 ± 1.6 kg/m^2) (Huh et al., 2012).

The effect of a single session of HIIT (5 x 4 minutes walking on a treadmill at 3km/hr alternating with 4 x 4 minutes running at 90% HR_{max} for a total of 36 minutes), continuous moderate-intensity exercise (walking/running on a treadmill at 65% HR_{max} for 36 minutes) and resistance training (3 sets of 8 – 12 repetitions at 75-80% 1RM for six exercises targeting all major muscle groups for 45 minutes) on circulating irisin levels was investigated in healthy overweight males (age 41.1 ± 6.7 years, BMI 28.1 ± 4.2 kg/m^2) and males with the MetSyn (age 44.5 ± 8.5 years, BMI 30.1 ± 3.7 kg/m^2) (Huh et al., 2015). Circulating irisin was significantly different over time ($p < 0.001$) and there was a significant interaction between exercise session and time ($p < 0.001$). In contrast, neither a two-factor (time*group) or three-factor (time*group*session) ANOVA with repeated measures over time identified any significant differences, meaning that exercise-induced irisin changes were similar between healthy individuals and those with the MetSyn. Comparison of the exercise sessions in terms of percentage change found significant increases in irisin immediately after each session ($p < 0.05$). However, resistance training was found to be most effective in increasing irisin, in comparison to

the other two trials, and changes in lactate and creatine kinase were closely related to the changes in irisin (Huh et al., 2015).

Another comparison study investigated the effects of resistance training (3 – 4 sets of 12 repetitions of 8 exercise targeting all the major muscle groups for 60 minutes), endurance training (60 minutes pedalling at 65% $\dot{V}O_{2peak}$), and combined resistance and endurance training (30 minutes of endurance training preceded by 30 minutes of resistance training) in healthy males (mean ± SE: age; 23 ± 1 years, height; 172 ± 2 cm, weight; 70 ± 2 kg) (Tsuchiya et al., 2015). Plasma irisin concentrations were measured immediately (0) and then 0.5, 1, 2, 3, 4 and 6 hours after exercise. The endurance and combined endurance and resistance training protocols did not induce significant changes in irisin during the 6 hour period post-exercise. In the resistance training group, no significant post-exercise difference in irisin was found immediately after exercise, but there was a significant increase in irisin after one hour ($p < 0.05$). One-hour irisin was significantly higher in the resistance training group compared to the other two groups ($p < 0.05$). AUCI during the 6 hours following exercise was also significantly higher following resistance training compared to combined resistance and endurance training ($p < 0.05$). Multiple linear regression analysis found that the AUCI was significantly associated with AUCG ($r = 0.37$, $p < 0.05$), lactate ($r = 0.45$, $p < 0.05$) and serum glycerol ($r = 0.45$, $p < 0.05$) (Tsuchiya et al., 2015). In contrast to the abovementioned studies, a single resistance training session (5 sets of 10 repetitions of bilateral knee extensions with a load of 10RM) did not change circulating irisin levels post-exercise (immediately, 15 minutes- and 30 minutes-post) in men (age 34 ± 7 years, BMI 26 ± 2 kg/m^2) (Pekkala et al., 2013). These inconsistent results may be due to the type of resistance exercises used, which only targeted lower-limb muscles only.

Several other studies have investigated the effect of acute aerobic exercise training on circulating irisin. Kramer et al. (2013) demonstrated that irisin increased significantly in the middle (54 minutes) of a 90 minute submaximal endurance treadmill session, performed at 60% $\dot{V}O_{2max}$, in healthy males (age 22.7 ± 1.6 years, BMI 24.3 ± 2.9 kg/m^2) and females (age 23.8 ± 4.7 years, BMI 25.2 ± 5.0 kg/m^2). Post-hoc analysis revealed that irisin declined between the middle of the trial to the end of the trial and 20 minutes post-

trial (significant difference between 54 minutes and following 20 minutes recovery in men, $p = 0.021$, not significant in females $p > 0.05$) (Kraemer et al., 2014).

Another study examined the effect of acute exercise of different workloads and intensities on circulating irisin levels in healthy men and women (n = 35, age 23.0 ± 3.3 years, BMI 22.4 ± 2.5 kg/m^2) (Daskalopoulou et al., 2014). The assessment included one maximal workload session (incremental treadmill protocol to volitional exhaustion), one relative workload (10 minute bout of treadmill exercise at 70% $\dot{V}O_{2max}$) and one absolute workload (10 minutes of exercise on a cycle ergometer at 75 watts) session. A dose-response in irisin levels was identified, with the greatest and lowest increases observed 3 minutes after the maximal and absolute workloads, respectively ($p < 0.001$). The post-pre difference in irisin was significantly different between maximum and relative workloads, and maximum and absolute workloads ($p = 0.004$), but not significant between relative and absolute workloads. This paper also provided the results of a pilot trial involving one maximal workload session (same protocol as above) and reported that irisin levels increased by 35% 3 minutes post-exercise (no significance level reported) and following this, irisin levels tended to decrease towards baseline and remain relatively constant in the following 24 hours in 4 healthy individuals (2 men and 2 women, age 22.5 ± 7 years) (Daskalopoulou et al., 2014).

Together, the findings of the studies investigating the effects of acute exercise on circulating irisin suggest that the exercise-induced increase in irisin appears to be dependent on exercise intensity and duration and changes in muscle metabolism. Sampling time during and post-exercise appears to have an impact on the results and must be considered when comparing studies. Despite the limited number of studies with sampling times beyond one hour of exercise, it seems that irisin increases following acute exercise but tends to return to baseline relatively quickly.

2.4.9.1.3.2. Irisin and Chronic Exercise

Resistance training interventions of various durations (8 – 26 weeks), intensities (60 – 100% of 1RM or 20RM), volumes (≥ 8 exercises, 2 – 5 sets of 10 – 20 repetitions) and with a frequency of 3 – 6 days per week, did not observe any significant changes in

circulating irisin levels in healthy young and middle-aged males and females (Hecksteden et al., 2013; Bang et al., 2014; Scharhag-Rosenberger et al., 2014). Conversely, significant increases in circulating irisin were reported concomitant to improvements in muscle strength in ageing humans (mean age 74.45 years, mean BMI 24.88 kg/m^2) following 12 weeks of strength training (2 days a week of elastic band training), despite body composition remaining unchanged (Kim et al., 2015). These observations were also confirmed in experiments on ageing mice (19 month old male C57BL/6 mice) performing ladder exercises 3 days per week for a period of 12 weeks (Kim et al., 2015). Interestingly in mice, irisin protein level in skeletal muscle was significantly increased, particularly in the soleus muscle, which may be an important source of irisin induced by resistance exercise.

Another study examined the effect of 8 weeks of aerobic exercise (5 days/week of 60 minutes of treadmill/cycling exercise at 65 – 80% HR_{max}) versus resistance exercise (4 days/week of 60 minute upper and lower body sessions, with 3 sets of 10 – 12 repetitions at 65 – 80% 1RM) on circulating irisin levels in overweight and obese individuals (Aerobic, age 25.7 ± 4.1 years and BMI 26.4 ± 2.4 kg/m^2; Resistance, age 26.4 ± 2.9 years, BMI 27.0 ± 3.4 kg/m^2) (Kim et al., 2016). Following the intervention, there was significant favourable changes in body composition (decreased body weight, BMI, fat mass and %fat and increased skeletal mass per kg of body weight) in both groups but no significant changes in markers of glucose or lipid metabolism. $\dot{V}O_{2max}$ increased significantly in both training groups, while hand-grip strength and leg extensor peak torque significantly increased in the resistance training group only. Circulating irisin significantly increased in the resistance training group only ($p < 0.001$). When subjects from the training groups and control group (age 25.8 ± 5.5 years, BMI 26.5 ± 2.0 kg/m^2) were pooled, the change in circulating irisin concentrations were independently correlated with changes in skeletal muscle mass ($r = 0.432$, $p = 0.022$) and fat mass ($r = -0.407$, $p = 0.031$) (Kim et al., 2016). Comparison of 21 weeks of endurance training (2 days per week of cycling at different intensities relative to the aerobic and anabolic threshold) versus combined endurance and resistance training (2 days per week of each type of training, resistance training involved 3 x 7 week training blocks for targeting muscle strength endurance, muscle hypertrophy and maximising strength) found that serum irisin, as well as skeletal muscle PCG-1α and FNDC5, did not change in untrained middle-

aged men (endurance n = 9, age 57 ± 7 years and BMI 24 ± 2 kg/m^2; combination n = 9, age 62 ± 5 years and BMI 25 ± 3 kg/m^2) (Pekkala et al., 2013).

The impact of an aerobic training on circulating irisin in humans was first conducted in 2012. Bostrom and colleagues investigated the effect of 10 weeks of supervised endurance training (cycling at 65% of $\dot{V}O_{2max}$ for 20 to 35 minutes, 4 to 5 times per week) and reported a two-fold increase in circulating irisin in eight, non-diabetic men (Boström et al., 2012). A significant increase in circulating irisin in response to endurance exercise has also been reported in middle-aged/older (age 67 ± 8 years, BMI 23.7 ± 3.9 kg/m^2) males and females underwent an 8 week endurance training programme involving cycling at 60 – 70% $\dot{V}O_{2max}$ for 45 minutes, 3 days per week) (Miyamoto-Mikami et al., 2015). In contrast to these two studies, no significant change in circulating irisin levels was reported following 26 weeks of aerobic exercise training in healthy males and females (3 days per week of walking/running at 60% heart rate reserve for 45 minutes) (Hecksteden et al., 2013). Furthermore, 8 weeks of cycling/running of increasing intensity (up to 85% $\dot{V}O_{2max}$ for 45 to 60 minutes) failed to alter circulating irisin levels in obese males (Besse-Patin et al., 2014).

HIIT using the Tabata protocol (4 sets of 4 minute stages consisting of 8 exercises) for a period of 8 weeks, significantly increased serum irisin (30%) in sedentary men (age 32.4 ± 6.6 years, BMI 25.8 ± 2.9 kg/m^2) (Murawska-Cialowicz et al., 2020). Body weight, fat mass, %fat were significantly decreased, while lean mass and $\dot{V}O_{2max}$ significantly increased post-intervention. However, no significant correlations between the change in serum irisin and anthropometric variables or aerobic capacity was observed. Unfortunately, no additional metabolic parameters were measured in this study and therefore this data is not available for interpretation (Murawska-Cialowicz et al., 2020). In a study investigating the effect of sprint interval training (9 sessions of 4 to 8 30-second bouts of ‘‘all-out’’ maximal efforts separated by 4 minutes of recovery), plasma irisin and skeletal muscle protein content of FNDC5 were unchanged in young males and females (age 24 ± 1 years, BMI 26.8 ± 1.1 kg/m^2) (Scalzo et al., 2014). However, when gender differences were explored, there was a gender*training interaction for plasma irisin ($p = 0.012$). Sprint interval training significantly decreased plasma irisin in males only ($p =$

0.045), but the post-exercise concentrations of irisin were significantly higher in females ($p = 0.001$) (Scalzo et al., 2014). In contrast, 8 weeks of sprint interval training (3 days per week, including 2 to 3 sets of 2 80 meter sprints with 20 minutes between sets) did not change circulating irisin levels in young, moderately trained men (age 20.5 ± 1.5 years, BMI 21.9 ± 1.6 kg/m^2) (Huh et al., 2012).

Whole-body vibration training (2 sessions per week for 6 weeks) consisting of seven isometric exercises and a duration of 11 to 18.5 minutes (vibration frequency of 16 – 21 Hz and amplitude of 2.5 – 5 mm), failed to alter circulating irisin levels in healthy, untrained females (age 24.3 ± 2.6 years, BMI 20.4 ± 1.8 kg/m^2) (Huh et al., 2014c). However, a whole-body vibration protocol targeting muscle contractions of the flexor and extensors of the legs and trunk significantly increased serum PCG1-α and irisin levels in older patients with chronic obstructive pulmonary disease (age 66.4 ± 9.93, BMI 27.88 ± 7.87 kg/m^2) (Greulich et al., 2014).

In summary, studies investigating the effects of chronic exercise training using different modalities have reported inconsistent results, with many failing to observe a change in circulating irisin. In studies reporting significant increases in irisin following exercise interventions, favourable changes in body composition and increases in muscle strength have been reported. Unfortunately, metabolic data was not available in these studies but it would be an important consideration for future research.

2.4.9.1.4. Irisin and Bed Rest, Physical Inactivity and Ageing

To the best of our knowledge, changes in circulating irisin have not been examined in response to bed rest or physical inactivity. As circulating concentrations of irisin are increased by contractile activity, it is conceivable that irisin levels will decrease following exposure to long-duration bed rest (Arhire et al., 2019). HDT bed rest is used as a model of microgravity, inactivity and accelerated ageing in humans and induces a myriad of physiological alterations including bone loss, muscle atrophy, a shift in muscle fiber type toward fast-twitch glycolytic type, impaired lipid trafficking, metabolic inflexibility and insulin resistance (Bergouignan et al., 2011; Vico and Hargens, 2018). It is now well-

established that crosstalk exists between muscle and bone and alterations in the secretion of myokines, such as irisin, contribute to changes in bone formation and resorption (Khayrullin et al., 2020). Muscle atrophy can induce bone loss *via* increased bone resorption, but reductions in irisin may also suppress osteogenesis, the process of bone formation (Bettis et al., 2018). Furthermore, circulating levels of irisin are negatively correlated with age and lower concentrations of irisin are associated with pre-sarcopenia and sarcopenia (Chang et al., 2017). Given the beneficial roles of irisin on glucose homeostasis and insulin sensitivity, it is possible that alterations in circulating irisin play a role in the metabolic deregulation that is evident following HDT bed rest. Therefore, research examining changes in irisin levels and the associations with markers of metabolic function following inactivity is warranted.

2.4.9.1.5. Summary of the Comprehensive Review on Irisin

Circulating irisin originates primarily from exercising skeletal muscle, but is also secreted in small amounts by adipose tissue. Irisin has a broad range of functions which enhance insulin sensitivity and improve glucose and lipid metabolism in adipose tissue, skeletal muscle and the liver. Levels of irisin are increased in obesity and decreased in T2DM. Elevated concentrations of irisin in obesity may represent enhanced secretion of irisin from increased muscle and adipose tissue mass that is present in obesity, or hypersecretion of irisin that is intended to compensate for irisin resistance in individuals with obesity. Conversely, irisin concentrations have been shown to decrease progressively with worsening of glucose tolerance and lower concentrations of circulating irisin may be related to impaired expression and secretion of irisin in skeletal muscle in individuals with T2DM. Multiple studies have investigated the effect of acute and chronic exercise training on circulating concentrations of irisin. Acute exercise increases irisin secretion but circulating levels quickly return to baseline. While some research studies on chronic exercise training have reported no significant changes in irisin, others have found increases in circulating irisin consistent with favourable changes in body composition and increases in muscle strength. Exposure to microgravity, physical inactivity and sedentary behaviour are associated with physiological adaptations that result in muscle atrophy, bone loss and metabolic derangements including reduced insulin sensitivity. However, research to date has not yet determined the effect of these conditions on irisin.

2.4.10. Hepatokines

2.4.10.1. Fetuin-A

Fetuin-A, which is encoded by the alpha-2-HS-glycoprotein (*AHSG*) gene, is a glycoprotein synthesized and secreted predominately by the liver in humans (Bourebaba and Marycz, 2019). Recent reports have suggested that fetuin-A is also an adipokine, however, the expression of fetuin-A in adipose tissue remains unclear depending on the type of cells and species being examined (Jialal and Pahwa, 2019; Khadir et al., 2020). Further to this, fetuin-A mRNA expression is proposed to be at least 400 times higher in the liver than in adipose tissue in humans (Ren et al., 2021). In circulation, fetuin-A levels normally range between 0.2 to 0.8 g/L (Ochieng et al., 2018).

2.4.10.1.1. Metabolic Actions of Fetuin-A

Fetuin-A and insulin are the only two proteins that interact directly with the extracellular portion of the insulin receptor (Icer and Yildiran, 2021). Insulin binds to the α-subunit of the insulin receptor which initiates autophoshorylation of the intracellular portion of the β-subunits resulting in the recruitment and phosphorylation of IRS proteins and downstream signalling to increase glucose uptake (Bugianesi et al., 2005; Ochieng et al., 2018). Fetuin-A binds to the tandem fibronectin type 3 domains present on the extracellular portion of the transmembrane β-subunit of the insulin receptor and attenuates tyrosine kinase signalling thereby reducing glucose uptake (Figure 10) (Goustin et al., 2013; Ochieng et al., 2018). Therefore, fetuin-A antagonises the signalling actions initiated by activation of the insulin receptor, without interfering with insulin binding. In addition to the effects on insulin signalling, fetuin-A suppresses the production of insulin-sensitising hormone adiponectin in adipocytes, indirectly leading to a decrease in insulin sensitivity (Hennige et al., 2008). Fetuin-A is also implicated in lipid-induced insulin resistance by acting as an intermediary between palmitate and toll-like receptor 4 (TLR4) in adipocytes leading to a pro-inflammatory response and insulin resistance (Pal et al., 2012).

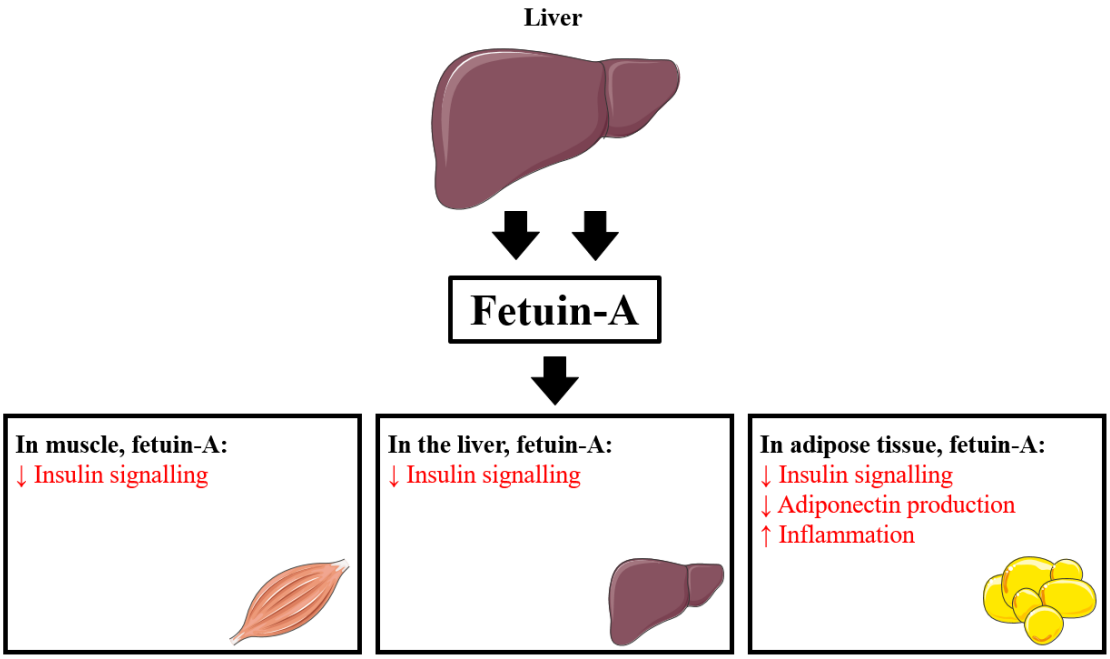

Figure 10. Secretion and negative metabolic actions of fetuin-A in metabolic derangements associated with elevated concentrations of fetuin-A.

2.4.10.1.2. Fetuin-A in Metabolic Disease

Multiple studies have reported elevated concentrations of fetuin-A in individuals with obesity, IGT, T2DM, NAFLD and PCOS (Stefan et al., 2006; Yilmaz et al., 2010b; Ou et al., 2011; Yin et al., 2014; Liu et al., 2020). In a systematic review and meta-analysis, higher concentrations of fetuin-A were linked to an increased risk of T2DM. Specifically, one standard deviation increment in circulating fetuin-A was associated with a 23% greater risk of developing T2DM (Guo et al., 2018).

Khadir et al. (2018) assessed the circulating levels of fetuin-A between individuals with T2DM (n = 118, 64 males and 54 females, age 52 ± 9 years, BMI 31.43 ± 4.50 kg/m^2) and without T2DM (n = 166, 67 males and 99 females, age 40 ± 12 years, BMI 29.19 ± 5.83 kg/m^2). Individuals with T2DM were older and had significantly higher BMI, %fat, waist circumference, systolic blood pressure, fasting blood glucose, HbA1c%, triglycerides and lower HDL and $\dot{V}O_{2max}$ in comparison to individuals without T2DM ($p < 0.05$). However, no significant between-group differences were found for total cholesterol, LDL, fasting insulin, C-peptide, hsCRP or fetuin-A. In all subjects, fetuin-A

correlated positively with fasting glucose ($r = 0.176$, $p < 0.01$), HbA1c% ($r = 0.151$, $p < 0.05$) and triglycerides ($r = 0.126$, $p < 0.05$) and negatively with HDL ($r = -0.131$, $p < 0.05$). Multiple stepwise linear regression showed that fasting glucose, HbA1c% and HDL were independently associated with circulating fetuin-A ($p < 0.05$). Following the primary analysis, individuals with T2DM were split into two subgroups based on glycaemic control (HbA1c% <7 good metabolic control, n = 44 and HbA1c% ≥ 7 poor metabolic control, n = 72). The group with poor metabolic control had significantly higher BMI, waist circumference, blood pressure, fasting blood glucose, and hsCRP ($p < 0.05$) but not fetuin-A ($p = 0.065$) compared with the group with good metabolic control. Individuals without T2DM were then subdivided into two subgroups as metabolically unhealthy obese (MUO, n = 97) and metabolically healthy obese (MHO, n = 64) based on the Adult Treatment Panel-III (NCEP-ATPIII guideline) criteria for MetSyn components (NCEP, 2002). Individuals in the MUO group were older, and had significantly higher BMI, %fat, waist circumference, hip circumference, blood pressure, triglycerides, fasting glucose, HbA1c%, fasting insulin and lower HDL in comparison to individuals in the MHO group ($p < 0.05$). Interestingly, fetuin-A levels were significantly elevated in the MUO group compared to the MHO group ($p = 0.026$), highlighting a link between circulating fetuin-A and components of the MetSyn (Khadir et al., 2018).

In accordance with these results, Chung et al. (2018) reported that fetuin-A levels were significantly increased in individuals with the MetSyn (metabolically abnormal) compared with individuals without the MetSyn (metabolically healthy), with and without obesity ($p = 0.016$ and $p = 0.001$, respectively). Serum concentrations of fetuin-A were strongly and independently associated with the MetSyn and its components, including central obesity, high blood pressure, high blood glucose and triglycerides, in community-dwelling Chinese adults (Xu et al., 2011).

Peter et al. (2018) examined the relationship between fetuin-A, liver fat and glucose homeostasis in two human cohorts. The first cohort consisted of 55 men and women (20 women and 35 men, age 62 ± 11 years, BMI 25.2 ± 3.9 kg/m^2) with available liver biopsies. Hepatic mRNA expression of fetuin-A was not associated with age or gender, but correlated positively with BMI ($r = 0.26$, $p = 0.05$), hepatic triglyceride content ($r =$

0.35, p = 0.009) and HOMA-IR (r = 0.29, p = 0.03). The latter two correlations remained significant after adjustment for BMI (p = 0.03 and p = 0.012, respectively). The second cohort included 220 subjects (130 women and 90 men, age 46 ± 11 years, BMI 29.5 ± 4.8 kg/m^2) who were at an increased risk of T2DM. Plasma fetuin-A correlated inversely with age (r = -0.29, p < 0.0001) but not gender (p = 0.32). Circulating fetuin-A correlated positively with liver fat (r = 0.22, p = 0.001), fasting insulin (r = 0.22, p = 0.001), HOMA-IR (r = 0.22, p = 0.002), AUCG (r = 0.21, p = 0.001) and negatively with insulin sensitivity during the OGTT (r = -0.25, p = 0.0003) and the clamp (r = -0.26, p = 0.0008, n = 167) and insulin clearance (r = -0.21, p = 0.003) after adjustment for age and gender. The positive correlation between fetuin-A and AUCG was attenuated after additional adjustment for insulin sensitivity (p = 0.11). Together, this data indicates that fetuin-A expression may be upregulated in the presence of insulin resistance and elevated liver fat (Peter et al., 2018). These findings are supported by a cross-sectional study reporting that high fetuin-A levels were independently associated with increased liver fat (Stefan et al., 2006). Further to this, analysis of longitudinal data following a lifestyle intervention (dietary counselling and increased physical activity) found that the changes in fetuin-A paralleled the changes in liver fat (Stefan et al., 2006).

In a recent study, the association between fetuin-A and the presence and severity of NAFLD was evaluated in 97 young adults (65% female, age 35.7 ± 12.4 years, BMI 22.7 ± 3.0 kg/m^2) (Filardi et al., 2021). Of the 97 subjects recruited, 65 were first degree relatives of patients with T2DM, whereas 32 had no family history of T2DM. None of the subjects displayed any OGTT alterations or presence of the MetSyn. There was no significant differences in serum fetuin-A levels between genders or between subjects with and without a family history of T2DM (p = 0.83). An inverse association between circulating fetuin-A and age was identified using linear regression (β = -0.12, p = 0.003). Fetuin-A levels positively correlated with BMI (β = 0.5, p = 0.048), waist circumference (β = 0.3, p = 0.027) and triglycerides (β = 0.1, p = 0.001) and uric acid (β = 1.7, p = 0.018) after adjustment for age and gender. The association with uric acid remained significant after adjustment for BMI and NAFLD (β = 1.8, p = 0.014). In multivariate analysis including age, BMI, waist circumference, triglycerides and uric acid, age (β = -0.2, p = 0.002) and triglycerides (β = 0.04, p = 0.02) were found to be independent predictors of circulating fetuin-A. The overall prevalence of steatosis in the study population was 66%,

categorised as 50.5% mild steatosis and 15.5% moderate to severe steatosis. Circulating fetuin-A did not differ between subjects with and without steatosis. In univariate logistic regression, the odds of having moderate to severe steatosis increased with age (odds ratio (OR) 1.1, p = 0.0001), BMI (OR 2.8, p = 0.001) and male sex (OR 3.4, p = 0.034) as well as increased fetuin-A, albeit non-significantly (OR 1.07, p = 0.095). However, in multivariate logistic regression, fetuin-A (OR 1.22, p = 0.036), age (OR 1.17, p = 0.01) and BMI (OR 2.75, p = 0.011) were independent predictors of moderate to severe steatosis. Specifically, for every unit increase in fetuin-A, the risk of moderate to severe steatosis increased by 22%. The findings of this study show that, in a sample of largely metabolically healthy young adults, fetuin-A is associated with moderate to severe NAFLD independent of confounders (Filardi et al., 2021).

Collectively, the evidence presented above shows that circulating fetuin-A is elevated in obesity, T2DM, NAFLD and the MetSyn and may be considered as a biological and predictive marker of metabolic disease.

2.4.10.1.3. Fetuin-A and Exercise Training

A number of studies have examined the effects of acute and chronic exercise on changes in circulating fetuin-A in healthy and clinical populations and the findings of these investigations are discussed below.

2.4.10.1.3.1. Fetuin-A and Acute Exercise

A single bout of moderate-intensity aerobic exercise (treadmill exercise performed at an intensity of 60% $\dot{V}O_{2peak}$ for 60 minutes) did not alter circulating fetuin-A in healthy normal weight (n = 11, age 36 ± 15 years, median BMI 23.4 kg/m^2) and overweight/obese men (n = 11, age 45 ± 14 years, median BMI 29.2 kg/m^2). The authors proposed that a higher exercise intensity or repeated bouts of exercise may be required to alter circulating levels of fetuin-A (Sargeant et al., 2018). In a more recent study, a single bout of moderate-intensity exercise (treadmill walking at an intensity 60 – 70% $\dot{V}O_{2max}$ to expend 500 kilocalories) on serum fetuin-A and insulin sensitivity was examined in 11 individuals with normal weight (age 43.3 ± 10.7 years, BMI 23.9 ± 2.0 kg/m^2) and 31

individuals with obesity (age 43.3 ± 9.2 years, BMI 36.0 ± 6.1 kg/m^2) (Ren et al., 2021). Serum fetuin-A was increased immediately following exercise ($p < 0.05$) and returned to pre-exercise levels 24 hours after the acute exercise bout in individuals with obesity, but did not change in individuals with normal weight ($p > 0.05$). The findings of this study show that a single bout of moderate-intensity aerobic exercise increased serum fetuin-A immediately post-exercise in individuals with obesity, but not in individuals with normal weight, and this change in fetuin-A was restored to baseline after 24 hours.

Together, the results of these two studies investigating the effect of acute exercise on circulating fetuin-A suggest that a single bout of moderate-intensity aerobic exercise does not alter circulating fetuin-A in individuals with normal weight, but transiently increases fetuin-A in individuals with obesity immediately post-exercise. It must be pointed out that these studies were conducted with small samples sizes and circulating protein concentrations were corrected for exercise-induced changes in PV in the former study only. Acute exercise can induce a significant reduction in PV and without correction, the absolute concentration of fetuin-A could artificially rise. Additional work is needed to explore the effect of different intensities and modalities of acute exercise, including HIIT and resistance training, on changes in circulating fetuin-A in healthy and clinical populations, to better understand the impact of a single bout of exercise on this hepatokine and the interaction with other metabolic parameters.

2.4.10.1.3.2. Fetuin-A and Chronic Exercise

Numerous published studies have investigated the effect of chronic exercise training on circulating fetuin-A in individuals with obesity, T2DM and NAFLD. These studies have examined the impact of aerobic, resistance and HIIT, as well as concurrent training, and the findings of these studies are discussed below.

Malin et al. (2013) investigated the effect of 7 days of aerobic exercise on circulating fetuin-A in middle-aged obese men and women with NAFLD (6 men and 7 women, age 50.5 ± 3.4 years, BMI 33.3 ± 0.9 kg/m^2). Subjects performed 60 minutes of aerobic treadmill walking and cycle ergometry at an intensity of 85% HR_{max} for a period of 7

days. Body weight, fat mass, hepatic triglyceride content and $\dot{V}O_{2max}$ when scaled to body weight or fat-free mass were unaltered after the exercise intervention. However, resting fat oxidation was significantly increased ($p = 0.04$). Aerobic training induced a significant reduction in fasting and 2 hour glucose and insulin levels, as well as a significant decrease in AUCG and AUCI ($p < 0.05$). Consistent with these changes, HOMA-IR and the whole-body insulin resistance index decreased by 19% ($p = 0.03$) and 29% ($p = 0.001$), respectively, after exercise training. Fasting NEFA and adipose tissue insulin resistance remained unchanged after the intervention. Exercise significantly reduced circulating fetuin-A by 11% ($p < 0.02$) and the reduction in fetuin-A correlated positively with exercise-induced reductions in insulin resistance ($r = 0.62$, $p < 0.04$) and AUCG ($r = 0.58$, $p < 0.05$). In addition, baseline fasting glucose correlated negatively with decreased circulating fetuin-A ($r = -0.65$, $p < 0.05$) and there was a trend for a negative correlation between baseline two-hour glucose and decreased circulating fetuin-A ($r = -0.52$, $p = 0.06$). Overall, short-term aerobic training did not alter body weight or $\dot{V}O_{2max}$ but induced a significant reduction in fetuin-A which correlated with improved insulin sensitivity (Malin et al., 2013).

In a subsequent study by Malin et al. (2014), the impact of 12 weeks of aerobic exercise training on circulating fetuin-A and the association with skeletal muscle and hepatic insulin resistance was investigated in 20 older obese men and women (9 men and 11 women, age 66.4 ± 0.9 years, BMI 34.1 ± 1.1 kg/m^2). The exercise intervention consisted of 60 minutes of treadmill walking at an intensity of 85% HR_{max}, 5 days per week for 12 weeks. Prior to and following the intervention, fasting and 2 hour blood samples were obtained after a 75g glucose load and a euglycemic-hyperinsulinemic clamp was performed. There was a significant decrease in body weight, BMI, %fat, systolic blood pressure, fasting and two-hour glucose and insulin, total cholesterol, LDL, triglycerides, leptin, hsCRP and a significant increase in $\dot{V}O_{2max}$ and metabolic flexibility post-intervention ($p < 0.05$). HDL, NEFA, HMW adiponectin, and TNF-α did not change. Skeletal muscle, hepatic and adipose tissue insulin resistance decreased significantly by 45%, 37% and 26%, respectively, after exercise training ($p < 0.05$). Aerobic training led to an 8% decrease in circulating fetuin-A ($p < 0.05$). The exercise-induced reduction in fetuin-A correlated positively with the exercise-induced reduction in hepatic insulin resistance ($r = 0.46$, $p < 0.01$) and increase in HMW adiponectin ($r = -0.57$, $p < 0.01$).

Furthermore, the lower concentration of fetuin-A correlated with higher metabolic flexibility at the 12 week time point ($r = -0.70$, $p < 0.01$). Fetuin-A remained a significant predictor of the reduction in hepatic insulin resistance and metabolic flexibility after the intervention, independent of changes in hsCRP, leptin, TNF-α or HMW adiponectin ($p = 0.03$). However, the independent association between elevated HMW adiponectin and reduced fetuin-A was diminished after adjustment for changes in hsCRP, leptin and TNF-α ($p = 0.07$). Together, the results of this study suggest that aerobic exercise training lowers circulating fetuin-A concomitant with improvements in hepatic insulin resistance, metabolic flexibility and systemic inflammation in older, obese individuals (Malin et al., 2014).

In contrast to the aforementioned studies, Mori et al. (2008) reported no significant change in circulating fetuin-A following 3 months of aerobic training (5 sessions per week of cycling exercise performed at an intensity relative to heart rate at the anaerobic threshold for 40 minutes per session) in individuals with T2DM ($n = 8$, 5 men and 3 women; age 62 ± 18 years, BMI 27.4 ± 4.2 kg/m^2). Aerobic exercise significantly decreased fasting glucose and HbA1c% and increased HDL ($p < 0.05$) but did not change BMI, systolic blood pressure, HOMA-IR, total cholesterol and triglyceride levels. Similarly, Schultes et al. (2010) reported no significant change in fetuin-A following 6 weeks of aerobic exercise training (3 sessions per week of exercise on a treadmill or bike at an intensity of 60% $\dot{V}O_{2peak}$ for 60 minutes per session) in pre-menopausal, obese women (mean age 43.2 years, mean BMI 36.5 kg/m^2). Following the 6 week intervention, there was a significant reduction in waist circumference, %fat, triglycerides and AST and a significant increase in resting energy expenditure ($p < 0.05$). However, fasting glucose, fasting insulin, HOMA-IR and GGT did not change. The findings of these two studies suggest that aerobic training does not modulate circulating fetuin-A in the absence of favourable changes in insulin resistance.

Winn et al. (2018) examined whether 4 weeks of HIIT could produce greater reductions in fetuin-A, intrahepatic lipid content (IHL) and NAFLD risk factors compared with energy-matched moderate-intensity continuous exercise training (MICT) in obese adults with hepatic steatosis. Recruited subjects were screened for hepatic steatosis and then

split into two exercise groups; MICT (n = 8, age 46 ± 9 years, BMI 40.3 ± 5.2 kg/m^2) and HIIT (n = 8, age 41 ± 14 years, BMI 33.8 ± 4.1 kg/m^2). Additionally, a group of age-matched, overweight or obese reference subjects agreed to participate as a non-exercise control (n = 5, age 51 ± 13 years, BMI 30.3 ± 3.7 kg/m^2). Exercise training was performed on a treadmill, 4 days per week for 4 weeks, for a duration equivalent to the consumption of ~80 L/O_2 (~400kcal) per session. The MICT group exercised at 55% $\dot{V}O_{2peak}$ and the HIIT group completed 4 minute intervals at 80% $\dot{V}O_{2peak}$ separated by 3 minutes of active recovery at 50% $\dot{V}O_{2peak}$. At baseline, body mass, BMI, waist circumference, fat mass, total abdominal fat, visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) were significantly higher in the MICT group compared with the HIIT and control groups ($p < 0.05$). There were no baseline between-group differences in fat-free mass or $\dot{V}O_{2peak}$ ($p > 0.05$), while time to exhaustion was significantly higher in the HIIT group compared to the MICT group ($p < 0.05$).

Following 4 weeks of exercise training, body mass, fat mass, fat free mass, %fat, total abdominal fat, VAT and SAT were not changed by either exercise intervention ($p > 0.05$). Exercise training for 4 weeks did not alter absolute or relative $\dot{V}O_{2peak}$ ($p > 0.05$). However, MICT and HIIT significantly increased exercise time to exhaustion and significantly decreased heart rate at the same absolute submaximal workload ($p < 0.05$). Pre-intervention IHL content was higher in subjects in the MICT and HIIT groups compared with control subjects (both $p < 0.01$), with no difference between the exercise groups. Despite significant differences in body mass between the MICT and HIIT groups, IHL content was tightly matched at baseline. Exercise training significantly reduced IHL content in the MICT and HIIT groups compared with the control group (group*time, $p = 0.02$) and the change in IHL was not significantly different between the exercising groups. Examination of inter-individual differences identified that 81% of subjects had >5% exercise-induced reduction in IHL, while 3 subjects did not show alterations in IHL or increased IHL following the exercise intervention. Additionally, 18% of subjects demonstrated resolution of fatty liver with IHL content <5.5% and 50% of subjects had <10% IHL levels after 4 weeks of exercise training, with no apparent differences in these responses between the training groups.

At baseline, fasting glucose, fasting insulin and HOMA-IR were significantly lower, while the Solomon index of insulin sensitivity was significantly higher in the HIIT group compared to the MICT group ($p < 0.05$). There were no further differences in indices of insulin resistance/sensitivity, circulating lipids, fetuin-A or markers of liver function and lipid peroxidation between the MICT and HIIT group at baseline. After 4 weeks of training, fasting insulin was significantly reduced in the MICT group, but not in the HIIT group (group*time, $p < 0.05$). In addition, the exercise-induced decrease in fasting glucose, HOMA-IR and adipose IR were greater in the MICT group compared to the HIIT group (group*time, $p < 0.05$). However, when baseline differences in body mass and adiposity were controlled, these significant time*intervention interactions were attenuated. There was a significant main effect of time for the increase in the Soloman index of insulin sensitivity and QUICKI (both, $p < 0.05$). Fasting NEFA, triglycerides, total cholesterol, HDL and LDL were unchanged after exercise training. Moreover, circulating fetuin-A, AST, ALT, cytokeratin 18 (CK18) and thiobarbituric acid reactive substances (TBARS) did not change after exercise training in the MICT or HIIT group. In summary, this study shows that energy-matched MICT and HIIT induces considerable reductions in IHL content in obese adults, without significant alterations in body weight and body composition, markers of hepatic function and circulating fetuin-A (Winn et al., 2018).

In another comparison study, the effects of 8 weeks of resistance training and aerobic training (3 days/week) on changes in circulating fetuin-A and physical and metabolic characteristics was performed in men with T2DM (Keihanian et al., 2019). Recruited participants were split into 3 groups; resistance training group (n = 12, age 52.4 ± 1.8 years, BMI 31.2 ± 1.2 kg/m^2), aerobic training group (n = 11, age 52.4 ± 1.5 years, BMI 32.4 ± 3.3 kg/m^2) and control group (n = 11, age 53.0 ± 1.1 years, BMI 32.6 ± 2.9 kg/m^2). Results from this study show that 8 weeks of aerobic and resistance training elicited favourable improvements on muscle strength, $\dot{V}O_{2max}$, markers of glucose and lipid metabolism and circulating fetuin-A in obese men with T2DM. Moreover, resistance training induced superior effects on circulating fetuin-A, fasting glucose and HbA1c suggesting in this study that this type of training may be more beneficial in enhancing insulin sensitivity (Keihanian et al., 2019).

In addition to comparing the effects of aerobic, resistance and HIIT, the effects of concurrent exercise training on circulating fetuin-A has been examined in multiple studies. Lee et al. (2017) studied the effect on 12 weeks of combined strength and interval training in 13 normal weight, normoglycaemic men (age 49.8 ± 2.1 years, BMI 23.5 ± 0.5 kg/m^2) and 13 overweight, dysglycemic men (age 52.5 ± 1.6 years, BMI 29.0 ± 0.7 kg/m^2). The exercise intervention consisted of 2 whole-body strength training sessions and 2 spinning bike interval sessions each week, for a total of 60 minutes per session. The key findings of this study show that 12 weeks of concurrent training can induce favourable changes in fetuin-A, liver fat and insulin sensitivity in normal weight, normoglycaemic men and overweight, dysglycemic men. Furthermore, lower concentrations of fetuin-A and less interaction with FFA may decrease TLR4-dependent insulin resistance in adipose tissue resulting in improved insulin sensitivity after exercise training. Additionally, changes in the composition of FFA following exercise may also influence the ability of fetuin-A to induce TLR4 signalling (Lee et al., 2017). In contrast, 3 months of combined aerobic and resistance training (45 minute of aerobic exercise of walking/running with a progressive increase in intensity to 60 – 75% age predicted HR_{max} (~300kcal) followed by 20 minutes of muscle strength training (~100kcal, no details provided), 5 times per week) did not induce a significant change in circulating fetuin-A in obese Korean women (age 45.3 ± 9.5 years, BMI 27.6 ± 2.4 kg/m^2) (Yang et al., 2011). BMI, waist circumference, systolic and diastolic blood pressure, triglycerides and arterial stiffness were significantly decreased ($p < 0.05$) but fasting glucose, HDL, LDL or HOMA-IR did not change following the intervention ($p > 0.05$) (Yang et al., 2011).

In summary, previous studies investigating the effect of chronic exercise training on circulating fetuin-A in individuals with metabolic dysfunction have reported conflicting findings, with exercise having no effect or causing a reduction in fetuin-A. In studies reporting a significant decrease in fetuin-A following an exercise intervention, improvements in body composition, liver fat and insulin sensitivity have been reported. According to a meta-analysis, aerobic and resistance training performed at a moderate to vigorous intensity, with a volume of 60 minutes per session and a minimum frequency of 4 – 7 sessions per week can markedly reduce circulating fetuin-A in dysglycemic and overweight/obese adults (Ramírez-Vélez et al., 2019).

2.4.10.1.4. Fetuin-A and Bed Rest, Physical Inactivity and Ageing

To our knowledge, circulating concentrations of fetuin-A have not been examined in response to bed rest or physical inactivity. However, it was previously shown that fetuin-A levels are significantly higher in low-active compared with highly-active men, and fetuin-A was inversely related to $\dot{V}O_{2max}$ (Jenkins et al., 2011). HDT bed rest represents an experimental model allowing for the investigation of physiological adaptations that occur with inactivity and microgravity, as well as accelerated ageing. Multiple age-related hormonal and metabolic changes contribute to the decline in physiological function that is associated with the pathophysiology of chronic diseases (Pataky et al., 2021). Unexpectedly, in multiple studies, an inverse association between circulating fetuin-A and age has been reported (Stefan et al., 2006; Ou et al., 2011; Sun et al., 2013; Filardi et al., 2021). The mechanism of the age-related decline in fetuin-A is unknown but it was recently hypothesized that a progressive fall in liver synthesis activity with increasing age may contribute to this reduction (Filardi et al., 2021).

According to epidemiological research, older adults with low levels of circulating fetuin-A are at a reduced risk of T2DM. Older individuals (70 – 79 years) in the highest tertile of fetuin-A (> 0.97 g/L) had a 2-fold higher risk of incident diabetes compared with older individuals in the lowest tertile of fetuin-A (≤ 0.76 g/L), in models adjusted for age, sex, race, demographics, waist circumference, body weight, physical activity, blood pressure, fasting glucose, HDL, triglycerides, and CRP levels (Hazards Ratio (HR) = 2.41 (95% confidence interval (CI) 1.28 – 4.53), $p < 0.01$). This association was not altered by adipocytokine levels (plasminogen activator inhibitor-1 (PAI-1), adiponectin, leptin, TNF-α or IL-6), but was moderately attenuated with adjustment for visceral adiposity (HR = 1.72, (95% CI 0.98 – 3.05), $p = 0.06$) (Ix et al., 2008). In a subsequent observational cohort study, it was reported that each standard deviation increment (0.42 g/L) in fetuin-A was associated with a 5% increase in visceral adipose tissue in older, community-living adults (Ix et al., 2009). Therefore, it appears that fetuin-A levels decrease with healthy ageing and maintenance of insulin sensitivity, but in the presence of metabolic dysfunction, fetuin-A levels will increase considerably.

2.4.10.1.5. Summary of the Comprehensive Review on Fetuin-A

Fetuin-A is a multi-faceted glycoprotein, synthesized and secreted by the liver, which is implicated in the causation of metabolic diseases. Fetuin-A promotes both insulin resistance and inflammation. Fetuin-A acts as an endogenous inhibitor of insulin receptor tyrosine kinase in the liver, adipose tissue and skeletal muscle. In addition to its direct effects on the insulin receptor, fetuin-A promotes insulin resistance by suppressing the production of adiponectin and triggering a pro-inflammatory state in adipose tissue. Elevated concentrations of fetuin-A have been reported in individuals with obesity, prediabetes, T2DM, NAFLD and the MetSyn. Contrastingly, fetuin-A levels have been shown to decrease with ageing, and this may be related to a progressive fall in liver synthesis with increasing age, when insulin sensitivity is maintained. At present, there is limited published data on the effect of acute exercise on circulating fetuin-A. However, fetuin-A is responsive to chronic exercise training and multiple studies have reported a decrease in circulating fetuin-A concomitant with improvements in body composition, liver fat and insulin sensitivity. To the best of our knowledge, changes in fetuin-A have not been studied previously following HDT bed rest.

2.4.10.2. RBP4

Retinol binding protein 4 (RBP4) is a member of the lipocalin family and the predominant transport protein of the hydrophobic molecule retinol, or vitamin A, in circulation (Steinhoff et al., 2021). Approximately 90% of circulating RBP4 is bound to retinol (holo-RBP4) and the remaining 10% is present in an unbound form (apo-RBP4) (Eckel, 2018). RBP4 is produced mainly in the liver but adipose tissue, and in particular mature adipocytes, are also an important source of this protein (approximately 20 – 40% of the liver contribution) (Bergmann and Sypniewska, 2013). Despite this, controversy still exists on whether RBP4 should be considered primarily as a hepatokine or adipokine. Since 2005, the existence of a mechanistic link between reduced expression of GLUT4 in adipocytes, increased circulating RBP4 and insulin resistance (discussed in detail below) prompted many researchers to consider RBP4 solely as an adipokine (Yang et al., 2005; Graham et al., 2006). In contrast, other studies have identified RBP4 as a hepatokine and reported that circulating RBP4 is derived exclusively from the liver (Ma et al., 2016; Thompson et al., 2017). It was recently hypothesized that RBP4 produced by extrahepatic tissues may reach circulation in certain disease states and conditions

(Steinhoff et al., 2021). Future studies are needed to further clarify the relative contributions of RBP4 from the liver, adipose tissue and other tissues to circulating RBP4 levels in mice and humans.

2.4.10.2.1. Metabolic Actions of RBP4

RBP4 is involved in the pathogenesis of insulin resistance and multiple research groups have examined the metabolic actions of RBP4 in inter-organ communication using various cell and mice models. Adipose tissue-specific knockout of insulin-regulated GLUT4 in mice (Adipose-GLUT4$^{-/-}$) causes insulin resistance, whereas overexpression of human GLUT4 (Adipose-GLUT4-Tg) in mice enhances glucose tolerance and insulin sensitivity (Yang et al., 2005). The reciprocal regulation of RBP4 in these genetic manipulations, as well as evidence of elevated circulating concentrations of RBP4 in multiple animal models of insulin resistance and in humans with obesity and T2DM, highlighted a causative role of increased RBP4 levels in insulin resistance (Yang et al., 2005). Additional experiments in RBP4 deficient mice (RBP4$^{-/-}$ mice) and mice overexpressing human RBP4 (RBP4-Tg mice) showed that increased RBP4 can impair insulin signalling in muscle and induce hepatic expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) concomitant to increased basal glucose production and impaired insulin-mediated suppression of glucose production in the liver (Yang et al., 2005).

In accordance with these findings, Ost et al. (2007) reported that RBP4 may be released by adipocytes in T2DM and act locally to inhibit IRS-1 phosphorylation. RBP4 has been shown to induce detrimental effects on insulin sensitivity and hepatic lipid content through increased inflammation in adipose tissue (Norseen et al., 2012; Moraes-Vieira et al., 2014; Lee et al., 2016; Moraes-Vieira et al., 2016; Moraes-Vieira et al., 2020). Experiments in adipocyte-specific RBP4 transgenic (adi-hRBP4) mice demonstrate increased mobilisation of FFA from adipocytes to the liver leading to enhanced hepatic FFA uptake and accumulation, even when fed a standard chow diet and before an elevation in RBP4 was detected in circulation. When exposed to a high-fat diet (HFD, 60% kcal from fat) for 24 days, the metabolic phenotype of adi-hRBP4 mice worsened and was characterised by reduced glucose clearance and increased fat mass, hepatic fat

accumulation, hepatic gluconeogenesis and adipose tissue inflammation (Lee et al., 2016).

Overall, research studies examining the mechanistic actions of RBP4 have reported that increased concentrations of RBP4 can impair insulin signalling in skeletal muscle and adipose tissue, perpetuate lipolysis and inflammation in adipose tissue and upregulate glucose production and lipid accumulation in the liver (Figure 11).

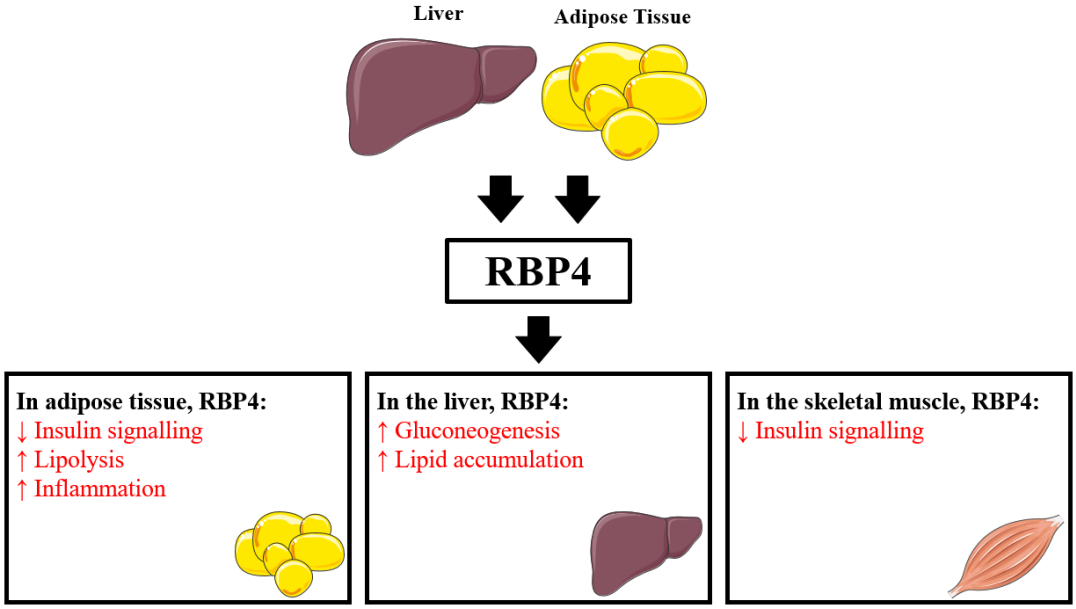

Figure 11. Secretion and negative metabolic actions of RBP4 in metabolic derangements associated with elevated concentrations of RBP4.

2.4.10.2.2. RBP4 in Metabolic Disease

Several studies have shown that circulating RBP4 levels are higher among individuals with obesity and morbid obesity compared with healthy weight controls and have shown associations between RBP4 and BMI (Graham et al., 2006; Haider et al., 2007). Conversely, other studies have reported that RBP4 concentrations do not differ with obesity status and are unrelated to BMI, fat mass and %fat (Cho et al., 2006; Korek et al., 2018; Wessel et al., 2019). Therefore, it has been suggested that circulating RBP4 may not be related to obesity itself, but the location of adipose tissue (Rychter et al., 2020).

The expression of RBP4 appears to be higher in visceral adipose tissue compared with subcutaneous adipose tissue and RBP4 has been considered as the best overall marker of intra-abdominal adipose tissue mass and predictor of insulin resistance (Klöting et al., 2007). In line with this, Jia et al. (2007) found that serum RBP4 levels were higher in men and women with visceral obesity compared to those without visceral obesity ($p < 0.01$), after age had been controlled. However, there was no difference in serum RBP4 levels when subjects were split into groups based on body mass index (lean, BMI < 25 kg/m^2; overweight BMI > 25 kg/m^2; obese BMI > 30kg/m^2) (Jia et al., 2007). Additionally, a significant positive correlation was identified between %trunk fat and circulating RBP4 ($r = 0.36$, $p = 0.001$) in 92 non-diabetic subjects (59 males and 33 females, age 49.4 ± 2.02 years, BMI 25.47 ± 0.29kg/m^2). However, circulating RBP4 did not correlate with absolute trunk mass, total body fat or %fat (Gavi et al., 2007).

Despite inconsistencies in obesity, elevated concentrations of RBP4 have been reported in insulin resistance, prediabetes, T2DM, NAFLD and the MetSyn (Cho et al., 2006; Jia et al., 2007; Norseen et al., 2012; El-Mesallamy et al., 2013; Chen et al., 2017; Li et al., 2018; Wessel et al., 2019; Huang et al., 2020). In individuals with NGT, fasting plasma glucose was an independent determinant of circulating RBP4 (Cho et al., 2006). In the same study, subjects with NGT, IGT and T2DM were pooled ($n = 154$) and divided into quartiles based on increasing levels of RBP4. The proportion of IGT and T2DM was higher with increasing RBP4 concentrations ($p < 0.001$ for trend). Additionally, the proportion of males and measurements of waist circumference, fasting and post-challenge glucose levels, HbA1c, triglyceride levels and HOMA-IR increased in parallel with increasing RBP4 ($p < 0.05$ for trends). Multivariate linear regression identified that sex and fasting glucose were independently associated with circulating RBP4 concentrations (Cho et al., 2006). Interestingly, circulating RBP4 have been found to be sexually dimorphic in other studies which report higher concentrations of RBP4 in men compared to women (Gavi et al., 2007; Jia et al., 2007; Qi et al., 2007; Chen et al., 2017; Wang et al., 2019). However, some studies have found no sex differences in circulating RBP4 (Klöting et al., 2007; Wessel et al., 2019). Wang et al. (2019) proposed that estrogen may have a regulatory role on RBP4 and may explain the interaction between RBP4 and sex influencing T2DM risk. Future studies are needed to examine RBP4 levels in males and

females, with and without chronic diseases, to determine whether differences in sex hormones can affect circulating RBP4.

El-Mesallamy et al. (2012) reported that serum RBP4 was significantly elevated individuals with T2DM, with and without obesity, compared to healthy, normal-weight controls ($p < 0.001$). This difference remained significant after adjustment for age, gender and BMI. There was no significant difference in serum RBP4 between non-obese or obese subjects with T2DM. Individuals with T2DM were then classified into controlled (HbA1c% < 7.5%) or uncontrolled (HbA1c% > 7.5%) diabetes. Serum RBP4 was significantly higher in individuals with uncontrolled diabetes, compared to those with controlled diabetes, even after adjustment for age, gender and BMI. In all subjects, there was a significant negative correlation between RBP4 and HOMA-β ($r = -0.67$, $p = 0.01$). Multiple regression analysis found a significant association of RBP4 with HbA1c% ($\beta = 0.422$, $p = 0.006$), HOMA-IR ($\beta = 0.237$, $p = 0.015$), and lipocalin-2 (LCN-2) ($\beta = 0.359$, $p = 0.002$), independent of age, sex, duration of diabetes, BMI, fasting glucose, insulin, HOMA-β, lipid profile and the ratio of insulin-like growth factor 1 to its binding protein-3 (IGFBP-3) (IGF-1/IGFBP-3 molar ratio). These results propose that elevated RBP4 may adversely affect glucose-insulin homeostasis (El-Mesallamy et al., 2013).

In support of this, a more recent study found that serum RBP4 was significantly and inversely correlated with β-cell function estimated by Stumvoll first-phase and second-phase insulin secretion indexes, but not HOMA-β, calculated during an oral glucose tolerance test (OGTT) in 291 subjects across a spectrum of glycaemia (Huang et al., 2020). This negative association was further confirmed using an oral minimal model test which assessed direct and model-derived measures of β-cell function in 74 individuals with NGT, IFG, IGT or T2DM, and these associations persisted after adjustment for potential confounders (age, gender, smoking, drinking, physical activity and family history of T2DM. This data suggests that RBP4 may negatively regulate β-cell function, but experimental evidence to confirm this association is warranted (Huang et al., 2020).

In contrast, Wessel et al. (2019) reported no significant differences between circulating RBP4 levels in individuals with ($n = 41$, age 60 ± 10 years, BMI $29.0 \pm 4.9 kg/m^2$) and without T2DM ($n = 37$, age 52 ± 9 years, BMI 25.5 ± 4.1 kg/m^2). Individuals with T2DM

were older and had significantly increased prevalence of the MetSyn, systolic and diastolic blood pressure, BMI, waist circumference, fasting glucose, HbA1c, HOMA-IR and triglycerides and significantly lower HDL concentrations compared to individuals without T2DM ($p < 0.05$). However, no significant between-group differences were noted for gender, total cholesterol, non-HDL, LDL, apo-B, apolipoprotein-A1 (apo-A1), RBP4, retinol or the ratio of RBP4/retinol. When subjects were pooled and then subdivided based on the presence ($n = 36$) or absence of the MetSyn ($n = 42$), serum RBP4 concentrations were significantly higher in individuals with the MetSyn ($p = 0.041$). The association of RBP4 with the presence of the MetSyn remained close to significance after adjustment for age and sex ($\beta = 0.224$, $p = 0.051$). In age- and sex-adjusted multivariable linear regression analysis, RBP4 was found to be independently associated with elevated triglycerides ($\beta = 0.348$, $p = 0.013$), but not with other individual components of the MetSyn ($p > 0.40$ for each, data not shown). Other studies have also reported a relationship between increased circulating RBP4 and components of the MetSyn. Strong positive correlations between circulating RBP4 and triglyceride concentrations were observed in subjects with and without obesity (obese, $r = 0.74$, $p = 0.03$; lean, $r = 0.62$, $p = 0.02$) (Korek et al., 2018). Plasma levels of RBP4 were also shown to increase gradually with an increasing number of components of the MetSyn in an ageing population, and particularly with elevated triglycerides, central obesity and high blood pressure (Qi et al., 2007).

In a subsequent analysis by Wessel et al. (2019), lipoprotein subfractions were quantified in individuals with ($n = 36$) and without T2DM ($n = 27$). Subjects with T2DM had significantly more large-sized VLDL particles, LDL particles, small-size LDL particles and HDL particles, but less large and medium-sized HDL particles ($p < 0.05$). Plasma RBP4 correlated positively with total cholesterol, non-HDL, LDL, triglycerides and (apo-B in subjects with and without T2DM, when groups were analysed together and separately ($p < 0.05$). RBP4 was found to positively correlate with VLDL particles, large and medium VLDL, LDL particles, small LDL and small HDL and inversely with large HDL ($p < 0.05$). These univariate correlations were similar when individuals with and without T2DM were also analysed separately. In multivariable linear regression analysis adjusted for age, sex, diabetes status and the use of glucose lowering and antihypertensive medications, RBP4 was independently and positively related to large VLDL particles (β

= 0.494, $p < 0.001$) and small LDL particles ($\beta = 0.511$, $p < 0.001$) and independently and inversely associated with large HDL particles ($\beta = -0.416$, $p = 0.011$). These findings show that higher concentrations of RBP4 may be involved in the pro-atherogenic plasma lipoprotein profile (Wessel et al., 2019).

Mounting evidence therefore indicates that elevated concentrations of RBP4 are associated with insulin resistance and metabolic risk factors such as elevated triglycerides and central obesity, which underlie clinical diseases such as obesity, T2DM, NAFLD, the MetSyn and cardiovascular disease.

2.4.10.2.3. RBP4 and Exercise Training

Changes in basal concentrations of RBP4 have been examined in multiple aerobic and resistance training interventions in healthy subjects and individuals with obesity, prediabetes and T2DM.

The effect of 4 weeks of aerobic exercise training (3 sessions per week of cycling and running for 60 minutes) on changes in RBP4, anthropometrics, aerobic fitness, and metabolic parameters was investigated in men and women with varying degrees of glucose tolerance (Graham et al., 2006). Based on the results of an OGTT, randomly selected participants were split according to having NGT (n = 20, 9 males and 11 females, age 33 ± 11 years, BMI 24.3 ± 1.5 years), IGT (n = 20, 9 males and 11 females, age 56 ± 12 years, BMI 29.8 ± 3.9 kg/m^2) and T2DM (n = 20, 11 males and 9 females, age 53 ± 7 years, BMI 30.7 ± 3.2 kg/m^2). None of the subjects had been previously diagnosed with IGT or T2DM and were therefore not taking any diabetic medication and those with NGT had no family history of T2DM. At baseline, subjects with T2DM had significant higher fasting glucose and lower fasting insulin compared to subjects with IGT ($p < 0.05$), which is indicative of diminished β-cell compensation for insulin resistance. Compared to subjects with NGT, subjects with IGT and T2DM had significantly lower glucose disposal rate (GDR), indicating insulin resistance ($p < 0.05$). Additionally, subjects with IGT and T2DM had significantly higher baseline BMI, waist to hip ratio, fasting insulin, fasting glucose, HbA1c, blood pressure and lower adiponectin levels compared to

subjects with NGT ($p < 0.05$). Baseline levels of plasma leptin, CRP, IL-6 and serum FFA were higher, and plasma adiponectin was lower, in subjects with IGT and T2DM compared to subjects with NGT (data not shown). Despite similar degrees of insulin resistance, subjects with T2DM had higher baseline levels of plasma CRP, IL-6 and serum FFA compared to subjects with IGT (data not shown). Prior to the intervention, concentrations of serum RBP4 were significantly higher in subjects with IGT and T2DM in comparison with subjects with NGT ($p < 0.05$) and there was no effect of gender on serum RBP4 levels. An inverse correlation between baseline levels of RBP4 and GDR was found in all subjects ($r = -0.78$, $p < 0.001$), and when subjects were separated based on gender (males, $r = - 0.77$, $p < 0.001$; females, $r = -0.79$, $p < 0.001$), even after the combined contributions of age and BMI had been controlled for by multivariate regression analysis. Further to this, baseline serum RBP4 correlated positively with waist to hip ratio, BMI, fasting glucose, fasting insulin, HbA1c and systolic blood pressure and inversely with HDL concentrations in all subjects ($p < 0.05$).

Examination of the change in whole-body insulin sensitivity demonstrated a variable response, with some subjects exhibiting little or no improvement in insulin sensitivity after 4 weeks of aerobic exercise. However, as changes in RBP4 correlated inversely with changes in GDR ($r = -0.83$, $p < 0.01$), post-hoc analysis was performed to examine changes in RBP4 and insulin resistance parameters when subjects were separated into 3 groups according to the magnitude of change in GDR post-intervention (lowest, middle and upper tertiles). Subjects in the lowest tertile had a marginal increase in insulin sensitivity ($p = 0.005$), whereas subjects in the middle and upper tertiles had a greater mean increase in GDR from baseline suggesting improved insulin sensitivity following aerobic exercise ($p < 0.001$ for both). In the highest tertile, circulating RBP4 decreased from pre-intervention levels in all subjects, except for one subject with RBP4 in the normal range at baseline ($p < 0.001$). Contrastingly, circulating RBP4 did not change or increased in all but one subject in the lowest tertile ($p = 0.002$). In the middle tertile, circulating RBP4 decreased in 11 subjects, albeit only marginally in some, and increased in 2 subjects ($p > 0.05$). Data from the middle and upper tertiles was subsequently pooled and exercise-induced changes in anthropometrics and metabolic parameters were assessed in subjects with either marginal or improved insulin sensitivity. Following the aerobic exercise intervention, significant reductions in BMI, % fat, waist to hip ratio and

fasting insulin and significant increases in HDL and $\dot{V}O_{2max}$ were evident in both groups ($p < 0.05$). However, significant decreases in fasting glucose and glucose levels during the OGTT were only found in individuals with improved insulin sensitivity post-exercise ($p < 0.05$). The results of this study suggest that RBP4 decreased in subjects with improved insulin sensitivity after aerobic exercise training and proposes RBP4 as a potential biomarker of individual responsiveness in insulin sensitivity following exercise training (Graham et al., 2006).

Lim and colleagues (2008) investigated whether 10 weeks of aerobic training (3 sessions per week of cycling exercise at an intensity of 60 – 80% of $\dot{V}O_{2max}$ for 60 minutes) could alter circulating RBP4, transthyretin (TTR, a protein that stabilises RBP4 in circulation) and adiponectin and if these changes would impact whole-body glucose metabolism in young (n = 36, age 22.4 ± 2.8 years, BMI 21.4 ± 2.9 kg/m^2) and middle aged women (n = 38, age 59.8 ± 5.9 years, BMI 25.1 ± 2.9 kg/m^2). At baseline, the older age group had significantly higher BMI, waist circumference, blood pressure, fasting insulin, total cholesterol, triglycerides, LDL, HOMA-IR, adiponectin and RBP4 levels and significantly lower HDL and $\dot{V}O_{2max}$ compared to the younger group ($p < 0.05$). None of the recruited subjects had diabetes, however three subjects in the younger age group and three subjects in the older age group had IFG as determined by the OGTT. Two subjects in the older age group were also found to have IGT. Following the exercise intervention, both age groups significantly decreased fasting glucose, AUCG, fasting insulin, total cholesterol, LDL, HOMA-IR and significantly increased $\dot{V}O_{2max,}$ adiponectin and HDL ($p < 0.05$). However, reductions in body weight, BMI, waist circumference, blood pressure, triglycerides and RBP4 were only evident in the older age group following exercise training ($p < 0.05$). In both age groups, changes in RBP4 correlated with changes in markers of insulin resistance, waist circumference, triglycerides and HOMA-IR (data not shown). In the older age group, there were significant negative correlations between the exercise-induced reduction in RBP4 and increase in adiponectin ($r = -0.517$, $p < 0.01$) and increase in $\dot{V}O_{2max}$ ($r = -0.547$, $p < 0.01$).

Following the primary analysis, all subjects were divided into two groups based on their individual response of RBP4 to aerobic exercise training. RBP4 responders were defined

as subjects which exhibited a decrease in RBP4 after the aerobic training intervention. RBP4 concentrations decreased in 18 of the 36 younger subjects (50%) and 29 of the 38 older subjects (76%). Specifically, RBP4 levels were reduced by 11.4% in the younger responders and 28.6% in the older responders ($p < 0.05$). TTR concentrations were also significantly decreased in RBP4 responders of both younger and older subjects ($p < 0.05$). A significant correlation between the reductions in RBP4 and TTR was found in the RBP4 responders only ($r = 0.565$, $p < 0.05$). Indices relating to obesity and insulin resistance such as body weight, BMI and HOMA-IR were decreased to a greater extent in the RBP4 responders compared to non-responders (data not shown). Correlational analysis found a positive correlation between the decrease in waist circumference and decrease in RBP4 in younger and older RBP4 responders (data not shown). In the older RBP4 responders only, the decrease in RBP4 was positively correlated with decreases in triglycerides, HOMA-IR and AUCG (data not shown). Additionally, there was borderline significance for correlations between the decrease in RBP4 and decreases in fasting plasma glucose and fasting plasma insulin (data not shown). Regression analysis determined that the decrease in HOMA-IR was significantly associated with the decrease in RBP4 after adjustment for age, and changes in BMI, $\dot{V}O_{2max}$, adiponectin and HOMA-IR ($p = 0.002$). Overall, the results of this suggest that the insulin-sensitising effects of aerobic exercise are more apparent in older subjects who are less fit and relatively insulin resistant compared with younger subjects. Interestingly, the older subjects with higher RBP4 concentrations at baseline, exhibited the greater decrease in RBP4 following the exercise intervention, with no difference in physical activity between the groups. These findings imply that changes in RBP4 following exercise training may be related to RBP4 concentrations at baseline (Lim et al., 2008).

The effect of 12 weeks aerobic exercise training (3 days per week of 45 – 60 minutes of aerobic exercise at 50 – 70% age-predicted HR_{max} and 15 – 25 minutes of recreational and cool-down activities) on circulating RBP4, adipokine concentrations and metabolic risk factors was investigated in 29 obese men (age 48 ± 2 years, BMI 29.6 ± 0.7 kg/m^2) (Numao et al., 2012). Body mass, BMI, fat mass, visceral fat mass, subcutaneous fat mass, blood pressure, total cholesterol, LDL, triglycerides, HbA1c, leptin and IL-6 decreased significantly, while $\dot{V}O_{2peak}$ and QUICKI increased significantly, after the exercise intervention ($p < 0.05$). Lean mass and circulating adiponectin did not change. There was

a significant reduction in circulating RBP4 following 12 weeks of aerobic exercise ($p < 0.05$). Stepwise multiple linear regression found that the exercise-induced decrease in RBP4 was related to the improvement in triglyceride concentrations ($\beta = 0.46$, $p = 0.012$), and this association was independent of age and the change in BMI, $\dot{V}O_{2peak}$, adiponectin, leptin, hsCRP, IL-6 and abdominal fat area. Overall, this study reports that aerobic training can improve body composition, insulin sensitivity and markers of lipid metabolism and reduce circulating RBP4 in obese men. Furthermore, reductions in RBP4 may be associated with exercise-induced improvements in triglyceride concentrations (Numao et al., 2012).

In comparison to the research presented above, Choi et al. (2019) reported no significant change in circulating RBP4 in response to 3 months of concurrent aerobic and resistance exercise training (5 times per week of 45 minutes of aerobic exercise at an intensity of 60 – 75% HR_{max} (300 kcal/day) and 20 minutes of a muscle strength programme (100kcal/day)) in obese women (n = 30, age 46.7 ± 7.5 years, BMI 28.4 ± 2.7 kg/m^2). Body weight, BMI, waist circumference, fasting glucose and total cholesterol decreased significantly and circulating IL-6 increased significantly after 3 months of training. However, no significant post-intervention differences were identified for blood pressure, triglycerides, HDL, LDL, AST, ALT, insulin or HOMA-IR. Taken together, these results show that circulating RBP4 was not altered following 3 months of concurrent aerobic and resistance training in obese women. The lack of change in RBP4 may be due to the absence of change in insulin sensitivity after the intervention (Choi et al., 2009).

The effect of concurrent training on changes in circulating RBP4 was also investigated in late middle-aged men with T2DM (Annibalini et al., 2017). Changes in anthropometric, metabolic parameters and concentrations of circulating adipokines, cytokines, chemokines and hsCRP were measured in the control (n = 8, age 60 ± 6.8 years, BMI 29.0 ± 3.8 kg/m^2) and exercise group (n = 8, age 57 ± 9.1 years, BMI 28.3 ± 1.5 kg/m^2) following a period of 16 weeks. The exercise programme consisted of 2 to 3 weekly sessions of combined aerobic and resistance training. Aerobic exercise involved walking on a treadmill with a progressive increase in intensity (45 – 60% of heart rate reserve) and duration (30 to 60 minutes) throughout the exercise intervention. Strength training

was gradually increased from 2 sets of 20 repetitions to 4 sets of 12 repetitions of exercises and was performed on the horizontal leg press, lat pull-down, lat machine and chest press, with loads ranging from 40 to 60% of the 1RM. There were no significant between-group differences in any physical or metabolic variables at baseline ($p > 0.05$). In comparison to the control group, the exercise group significantly reduced body weight, BMI, waist and hip circumferences, body fat, systolic and diastolic blood pressure and increased fat-free mass, $\dot{V}O_{2max}$ and muscular fitness after 16 weeks of concurrent exercise training ($p < 0.05$). The reduction in total cholesterol was significantly greater in the exercise group compared to the control ($p = 0.049$), whereas post-exercise changes in LDL, HDL, blood glucose, HbA1c and creatininemia were not significantly different between the two groups after the intervention ($p > 0.05$). Compared to the control group, RBP4, leptin, IL-6, monocyte chemoattractant protein-1 (MCP-1) and TNF-α decreased, and IGF-1 was increased, to a greater extent in the exercise group ($p < 0.05$). Changes in adiponectin, hsCRP and insulin-like growth factor-binding protein 3 (IGFBP-3) were not significantly different between the two experimental groups. Analysis of the plasma proteome from individuals with T2DM in the exercise group found that the level of RBP4 and TTR decreased after concurrent exercise training. Overall, this study found that concurrent exercise training reduced RBP4 in individuals with T2DM, and elicited significant beneficial effects on body composition, aerobic and muscular fitness, blood pressure, and circulating levels of various adipokines and cytokines. The authors proposed that the training-induced reduction in fat mass and favourable changes in inflammatory markers were associated with the reduction in RBP4 in individuals with T2DM (Annibalini et al., 2017).

A comparison of the effect of 12 weeks of circuit resistance training and walking exercise, performed 3 times per week for 60 minutes at an intensity of 60% heart rate reserve, on changes in glycaemic control and circulating RBP4 levels was examined in 15 post-menopausal women with T2DM (Kang et al., 2009). Subjects in the walking group (n = 7, mean age 52.5 years, mean BMI 23.6 kg/m^2) walked along a track and subjects in circuit resistance training group (n = 8, mean age 50.4 years, mean BMI 22.0 kg/m^2) performed stair climbing, stationary cycling and resistance exercises (3 sets of 12 repetitions of lat pull downs, abdominal exercises, leg curls and leg extensions). Body weight, BMI, %fat, HbA1c were significantly decreased and muscle mass and $\dot{V}O_{2max}$

were significantly increased in the circuit training group only ($p < 0.05$). Post-intervention measurements of body weight and % fat were significantly higher and $\dot{V}O_{2max}$ was significantly lower in the walking group compared to the circuit training group ($p < 0.05$). Conversely, fasting insulin and C-peptide decreased significantly in the walking group only post-intervention ($p < 0.05$). Circulating adiponectin and MCP-1 levels increased significantly in both exercise groups following the intervention, whereas RBP4 decreased significantly in the circuit training group only ($p < 0.05$). In summary, circuit training was superior to walking exercise in eliciting improvements in body composition, aerobic fitness and circulating RBP4 suggesting enhanced insulin sensitivity in normal-weight women with T2DM (Kang et al., 2009).

Collectively, the evidence from research studies investigating the effect of aerobic training and resistance training, as well as concurrent training, on changes in circulating RBP4 concentrations has shown that exercise interventions, with a minimum of 4 weeks duration, can significantly decrease RBP4 levels in concert with improvements in fat mass, insulin resistance and aerobic fitness. Some researchers have proposed that the exercise-induced change in RBP4 may be dependent upon the level of RBP4 before exercise training or the change in insulin sensitivity following exercise training. Others have highlighted that individuals with normal glycaemic control and insulin sensitivity may not exhibit substantial changes in RBP4 following an exercise intervention. In future investigations, the impact of HIIT on circulating concentrations of RBP4 in various human populations (healthy and clinical) and the association with changes in insulin sensitivity needs to be examined. Additionally, the effect of acute exercise training using different exercise modalities needs to be explored and compared to changes reported from chronic exercise training in healthy subjects and individuals with obesity and T2DM.

2.4.10.2.4. RBP4 and Bed Rest, Physical Inactivity and Ageing

To our knowledge, the impact of HDT bed rest and physical inactivity on circulating RBP4 has not previously been studied. HDT bed rest can provide unique insights into the physiology of gravitational unloading and disuse which induces age-related phenotypic changes that are associated with the aetiology of many chronic diseases. As age is an independent risk factor for insulin resistance, few research studies have evaluated the

association between circulating RBP4 and age. Cho et al. (2006)found that plasma RBP4 concentrations were significantly higher in women over 50 years of age, compared to women under 50 years of age ($p < 0.001$), with varying degrees of glucose homeostasis. However, no such differences were noticeable for men when subdivided by age (Cho et al., 2006). In another study, the physical and metabolic characteristics of elderly subjects (n = 46, 29 males and 17 females; age 67.41 ± 0.92 years) and young subjects (n = 46, 30 males and 16 females; age 31.39 ± 1.11 years) were compared (Gavi et al., 2007). Both groups had similar BMI (25.99 ± 0.40 kg/m^2 *vs.* 24.95 ± 0.40 kg/m^2; $p = 0.07$) but elderly subjects had reduced insulin sensitivity compared to the young subjects when measured using the hyperinsulinemic euglycemic clamp (8.60 ± 0.43 mg/kg/LBM/min *vs.* 10.32 ± 0.48 mg/kg/LBM/min; $p = 0.009$). Circulating RBP4 levels were significantly higher in the elderly subjects compared with the young subjects ($p < 0.001$). Serum RBP4 correlated significantly and positively with age ($r = 0.38$, $p < 0.001$) and multivariate regression analysis found that the relationship between RBP4 and age was independent of body fat distribution and insulin sensitivity (Gavi et al., 2007). This data suggests that RBP4 is increased in older compared with younger individuals, but does not imply that RBP4 directly causes insulin resistance in ageing.

2.4.10.2.5. Summary of the Comprehensive Review on RBP4

RBP4 is produced by the liver and adipose tissue and transports retinol/vitamin A *via* the circulation to peripheral tissues. In addition, RBP4 has been implicated in the pathogenesis of insulin resistance. Experimental studies in cells and mice have shown that elevations in RBP4 can block insulin signalling in the skeletal muscle and adipose tissue, upregulate lipolysis and inflammation in adipose tissue and promote gluconeogenesis and lipid accumulation in the liver. In humans, elevated concentrations of RBP4 are reported in visceral obesity, insulin resistance, prediabetes, T2DM, NAFLD and the MetSyn. Furthermore, RBP4 has been proposed to negatively regulate β-cell function, and is commonly associated with elevated triglycerides suggesting a role in lipid metabolism. Results from aerobic and resistance training interventions, as well as concurrent training interventions, have shown reductions in circulating RBP4 concomitant to improvements in fat mass, aerobic capacity and insulin resistance. Interestingly, it has been proposed that exercise-induced changes in RBP4 may be dependent on baseline levels of RBP4 and the change in insulin sensitivity following the

intervention. Thus, RBP4 may be a potential biomarker of inter-individual variability in insulin sensitivity following exercise training.

2.4.10.3. Adropin

Adropin, first identified in 2008, is a protein hormone which plays a role in the regulation of energy homeostasis by controlling glucose and fatty acid metabolism (Kumar et al., 2008; Kolben et al., 2020). Adropin is encoded by the energy homeostasis-associated (ENHO) gene, which is expressed predominantly in the brain and liver (Jasaszwili et al., 2020). This review highlights the metabolic effects of adropin, with particular focus on the beneficial role of adropin in insulin sensitivity.

2.4.10.3.1. Metabolic Actions of Adropin

The metabolic actions of adropin have been investigated in multiple cell and animal models (Figure 12). In mice, liver *Enho* expression and plasma adropin concentrations are regulated by fasting and dietary macronutrients. Adropin decreases with fasting and increases with short-term refeeding, particularly of a HFD (Kumar et al., 2008; Ganesh Kumar et al., 2012). Conversely, chronic exposure to a HFD reduces liver *Enho* expression suggesting deregulation with obesity (Kumar et al., 2008).

Transgenic overexpression or systemic treatment of adropin improves glucose homeostasis consistent with reduced obesity and independently of changes in obesity (Kumar et al., 2008). Conversely, adropin knock-out (KO) mice are insulin-resistant and this impairment is largely due to the impaired suppression of hepatic glucose production by insulin (Ganesh Kumar et al., 2012). In response to a HFD, adropin deficiency has a negative impact on metabolic homeostasis, resulting in increased fat mass, insulin resistance and elevated lipid and triglyceride content in the liver (Ganesh Kumar et al., 2012).

Studies on mice have also been used to investigate the mechanisms by which adropin regulates metabolic status and sensitizes insulin action in peripheral tissues. The role of

adropin in regulating whole-body substrate preference and substrate oxidation in skeletal muscle was examined in adropin knockout (AdrKO) and transgenic overexpression (AdrTG) mouse models fed with regular laboratory chow (Gao et al., 2014). To examine whether adropin regulates fuel preference in the fed state, the research group investigated fuel selection in AdrKO mice in the fed state and AdrTG mice in fasted state. The rationale for using these models is based on AdrKO mice being unable to increase adropin production in response to feeding, and fasting AdrTG mice have plasma adropin levels comparable to those of fed mice. The results of this study found that adropin promotes carbohydrate oxidation over fat oxidation, and skeletal muscle is the key target organ for mediating these effects. Similar results were observed in fasting mice after synthetic adropin treatment, whereas opposite effects were found in fasting wild-type mice and AdrKO mice which displayed preferential oxidation of fat. These findings highlight the role of adropin in modulating substrate utilisation in fasting and feeding cycles (Gao et al., 2014). A subsequent study revealed that adropin treatment in diet-induced obese mice improved glucose tolerance, insulin action, mitochondrial function and metabolic flexibility, highlighting that skeletal muscle is a key organ mediating the effects of adropin (Gao et al., 2015). In addition to skeletal muscle, adropin treatment mediates multiple intracellular signalling pathways in the liver to suppress fasting hyperglycaemia as a result of diet-induced obesity in mice (Gao et al., 2019). In the same study, *in vitro* analysis in mouse hepatocytes confirmed a direct effect of adropin on hepatic glucose production (Gao et al., 2019). Taken together, adropin has a regulatory role in substrate oxidation and can sensitize insulin-stimulated intracellular pathways in the liver and skeletal muscle. Further investigations are warranted to determine the metabolic effect of adropin in adipose tissue.

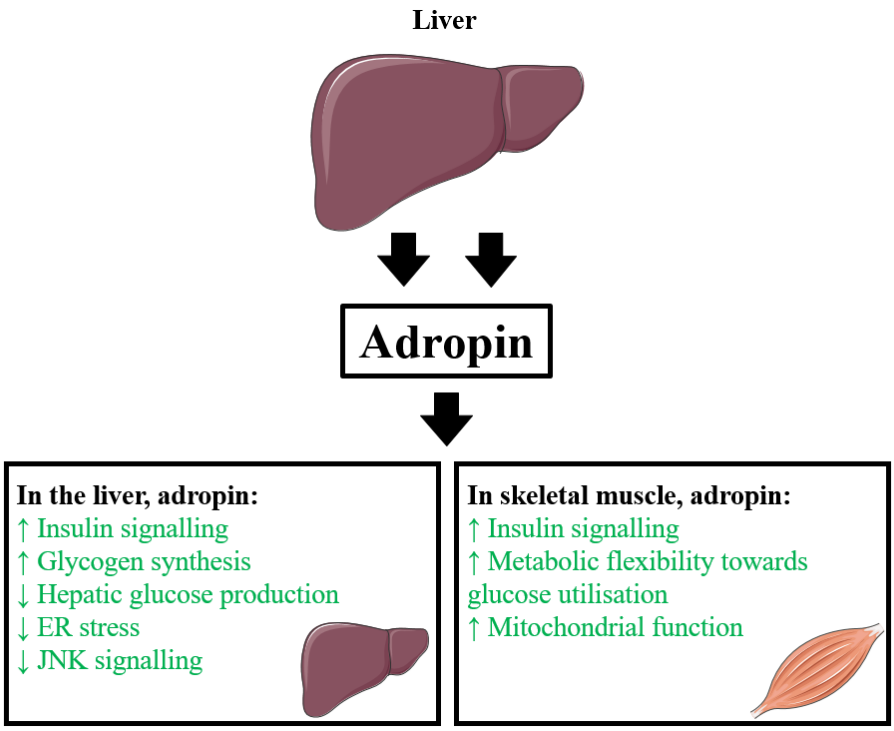

Figure 12. Metabolic actions of adropin.

Abbreviations: ER, endoplasmic reticulum and JNK, c-Jun N-terminal protein kinase.

2.4.10.3.2. Adropin in Metabolic Disease

In humans, lower circulating concentrations of adropin have been reported in obesity, insulin resistance, T2DM, hepatosteatosis and the MetSyn (Butler et al., 2012; Yosaee et al., 2017; Zang et al., 2018; Kutlu et al., 2019; Li et al., 2020). In women with PCOS, decreased adropin concentrations were associated with increased levels of TNF-α (Kume et al., 2016).

The association between circulating adropin, insulin resistance and NAFLD was examined in 51 patients diagnosed with grade 2 – 3 hepatosteatosis (26 males and 25 females, age 37.9 ± 9.96 years, BMI 29.2 ± 5.2 kg/m^2) and 30 healthy controls (14 males and 16 females, age 34.8 ± 9.5 years, BMI 27.8 ± 4.9 kg/m^2) (Kutlu et al., 2019). There were no significant between-group differences in BMI or gender or fasting concentrations of total cholesterol, HDL, LDL, creatinine, AST, ALT and GGT. Serum concentrations of adropin were significantly lower in subjects with NAFLD, compared with control subjects ($p < 0.001$). Subjects with NAFLD had significantly higher fasting glucose,

insulin, HOMA-IR and triglyceride levels compared to the control group ($p < 0.05$). Correlational analysis identified significant negative correlations between adropin levels and HOMA-IR ($r = -0.241$, $p = 0.030$), insulin ($r = -0.233$, $p = 0.036$), GGT ($r = -0.271$, $p = 0.014$), total cholesterol ($r = -0.229$, $p = 0.040$) and triglyceride levels ($r = -0.302$, $p = 0.006$) in the NAFLD group. All of the study subjects were then subdivided based on the presence (n = 35, 17 males and 18 females, age 38.1 ± 10.6 years, BMI 29.4 ± 5.3 kg/m^2) or absence (n = 46, 23 males and females, age 35.7 ± 9.2 years, BMI 28.1 ± 4.8 kg/m^2) of insulin resistance based on HOMA-IR score (HOMA-IR > 2.7 or < 2.7, respectively). There were no significant between-group differences in BMI or gender or fasting concentrations of total cholesterol, HDL, LDL, triglycerides, urea, creatinine, ALT or alkaline phosphatase (AP). Serum concentrations of adropin were significantly lower in subjects with insulin resistance ($p < 0.01$). Subjects with insulin resistance had significantly higher fasting glucose, insulin, AST and GGT ($p < 0.05$). The results of this study provide further evidence that adropin is decreased in metabolic disturbances including insulin resistance and NAFLD and in subject groups with similar BMI (Kutlu et al., 2019).

Zang et al. (2018) evaluated the relationship between adropin levels and metabolic parameters in T2DM. Participants were primarily split into a control group (n = 60, 32 males and 28 females, age 44.85 ± 9.92 years, BMI 23.47 ± 2.45 kg/m^2) and a T2DM group (n = 116, 68 males and 48 females, age 48.24 ± 11.48 years, BMI 25.21 ± 3.63 kg/m^2). There were no significant between-group differences in gender, age, current smoker, alcohol drinking or total cholesterol. Individuals with T2DM had significantly higher BMI, blood pressure, triglycerides, LDL, hsCRP, fasting glucose, fasting insulin, HbA1c and HOMA2-IR ($p < 0.05$) and significantly lower levels of HDL ($p < 0.001$). Serum adropin was significantly decreased in individuals with T2DM compared to the control group ($p < 0.001$). When all subjects were separated according to tertiles of adropin (low, middle, high), subjects in the lowest tertile had significantly higher BMI, triglycerides, fasting insulin and HOMA2-IR compared to the middle and highest tertile ($p < 0.05$). Subjects in the highest tertile had the lowest fasting glucose, HbA1c and highest HDL ($p < 0.05$). The presence of T2DM was 79.7% in the lower tertile, 70.7% in the middle tertile and 47.5% in the upper tertile. The percentage of overweight/obesity decreased as the concentrations of adropin increased ($p < 0.001$). When all subjects were

split according to BMI (normal weight BMI 18.5 – 25 kg/m^2, and overweight/obese BMI ≥ 25 kg/m^2), adropin concentrations in the control group were significantly lower in overweight/obese subjects compared to the normal weight subjects ($p < 0.05$). In subjects with T2DM, serum adropin was significantly lower in the overweight/obese subjects compared to normal weight subjects, and adropin concentrations were significantly lower in subjects with T2DM compared to subjects in the control group at each level of BMI ($p < 0.05$). Correlational analysis including all study subjects (n = 176) identified that circulating adropin was negatively associated with BMI, hsCRP, triglycerides, fasting glucose, fasting insulin, HOMA2-IR and HbA1c, and positively correlated with HDL ($p < 0.01$). Apart from hsCRP, all correlations remained significant after adjustment for BMI. Multiple linear regression identified HOMA2-IR and HbA1c as independent predictors of serum adropin. The results of this study show that circulating levels of adropin are decreased in T2DM, especially in those with overweight/obesity, and are associated with markers of glucose and lipid metabolism and insulin resistance (Zang et al., 2018).

The research presented here suggests that lower concentrations of adropin are found in metabolic diseases characterised by insulin resistance, lipid and lipoprotein abnormalities and obesity.

2.4.10.3.3. Adropin and Exercise Training

There is currently limited published research on the effect of exercise training on circulating adropin and these studies have predominately focused on the association between circulating adropin and endothelial function. Fujie et al. (2015) investigated the effects of 8 weeks of aerobic exercise training (cycling at 60 – 70% $\dot{V}O_{2peak}$ for 45 minutes, 3 days per week) on serum adropin and its association with plasma nitrite/nitrate (NOx) level. Healthy middle-aged and older males and females were split into an exercise group (age 66.9 ± 1.2 years; BMI, 24.1 ± 0.7 kg/m^2) and control group (age 68.2 ± 1.8 years, BMI 22.0 ± 1.2 kg/m^2). No significant between-group differences in $\dot{V}O_{2peak}$, carotid β-stiffness, plasma nitrite/nitrate (NOx) and serum adropin were reported at baseline. Significant interactions of group and time were identified for the changes in $\dot{V}O_{2peak}$, carotid β-stiffness, plasma NOx and serum adropin following the intervention (p

< 0.05). In the training group, carotid β-stiffness significantly decreased and $\dot{V}O_{2peak}$, plasma NOx and serum adropin significantly increased. No significant differences between groups or time were identified for age, height, body weight, BMI, heart rate, systolic and diastolic blood pressure, total cholesterol, HDL, triglycerides or blood glucose. In the exercise group, training-induced increases in circulating adropin were negatively correlated with decreased carotid β-stiffness ($r = -0.399$, $p < 0.05$) and positively correlated with increased plasma NOx ($r = 0.623$, $p < 0.001$) and $\dot{V}O_{2peak}$ ($r = 0.420$, $p < 0.05$). These correlations also remained significant after adjustment for age, sex, body weight, heart rate, blood pressure, lipid profiles and fasting glucose. The exercise-induced increase in plasma NOx was negatively correlated with decrease in carotid β-stiffness ($r = -0.680$, $p < 0.01$), and this correlation persisted after adjustment. Multiple linear regression identified that plasma NOx concentration was an independent factor associated with serum adropin concentrations ($\beta = 0.587$, $p < 0.05$). These findings suggest that the elevation of serum adropin in response to aerobic exercise may be related to a reduction in arterial stiffness, mediated by increased nitric oxide bioavailability (Fujie et al., 2015).

The same training protocol was employed in a subsequent study investigating the effects of 8 weeks of aerobic exercise on the relationship between circulating adropin, arterial stiffness and adiposity in 13 obese adults (4 males and 9 females, age 64.7 ± 1.7 years) (Fujie et al., 2017). In line with the previous study, there was a significant increase in serum adropin, plasma NOx concentrations and $\dot{V}O_{2peak}$, as well as a significant decrease in carotid β-stiffness following the intervention ($p < 0.05$). Additionally, there was a significant reduction in abdominal visceral fat area ($p = 0.019$) and %fat ($p = 0.036$). No significant changes in body weight, height, heart rate, systolic or diastolic blood pressure, total cholesterol, HDL, triglycerides and blood glucose were found following the exercise intervention. The increase in serum adropin correlated negatively with the reduction in carotid β-stiffness ($r = -0.573$, $p = 0.041$), and this correlation persisted after adjustment for sex. The increase in serum adropin also correlated negatively with the reduction in abdominal visceral fat area ($r = -0.585$, $p = 0.036$), even after adjustment for sex. Positive correlations were identified between the increase in serum adropin and the increase in plasma NOx ($r = 0.671$, $p = 0.012$) and $\dot{V}O_{2peak}$ ($r = 0.726$, $p = 0.005$) and these remained significant after adjustment for sex. A negative association between the exercise-induced

increase in plasma NOx and the decrease in carotid β-stiffness was also reported ($r = -0.654$, $p < 0.05$), and this persisted after adjusted for sex. In line with the findings of the first study, exercise-induced increases in circulating adropin were associated with the reduction of arterial stiffness, mediated by nitric oxide production. Additionally, elevations in serum adropin were associated with the decrease in adiposity in obese adults following the aerobic training intervention (Fujie et al., 2017).

The association between circulating adropin and changes in body composition, lipid metabolism and vascular endothelial function was examined before and after 12 weeks of aerobic exercise training (3-5 times per week of jogging or inclusion in sports such as badminton, table tennis, aerobics and cycling performed at 60 – 80% HR_{max} for 90 minutes) in obese adolescents (n = 45, 36 males and 9 females, age 17.9 ± 0.8 years, BMI 31.4 ± 2.7 kg/m^2) (Zhang et al., 2017). Following the intervention, obese adolescents significantly decreased body weight, BMI, waist circumference, waist to hip ratio and fat mass ($p < 0.001$). Systolic blood pressure was also significant decreased ($p < 0.001$), but diastolic blood pressure was not different after exercise training. Total cholesterol and LDL significant decreased ($p < 0.001$), whereas HDL significantly increased ($p < 0.001$) following the intervention. No significant changes in triglycerides were found. Fasting glucose, fasting insulin and HOMA-IR were all significantly decreased after exercise training ($p < 0.001$). $\dot{V}O_{2peak}$ significantly increased post-intervention ($p < 0.01$). Serum adropin levels significantly increased following the exercise intervention ($p < 0.001$). This increase occurred irrespective of post-intervention weight gain or weight loss suggesting the change was independent of the change in body weight. Vascular reactive hyperemia index (RHI), a non-invasive measure of endothelial function, significantly increased post-intervention ($p < 0.001$), and was also independent of changes in body weight. Multiple linear regression analysis identified that the change in serum adropin ($t = 3.261$, $p < 0.01$) was an independent factor of the change in RHI in obese adolescents. This study provides further evidence that the increase in serum adropin may improve endothelial function in obese adolescents following aerobic exercise training (Zhang et al., 2017).

In contrast to the studies above, one study reported a significant decrease in circulating adropin following exercise training in young, lean males (Ozbay et al., 2020). The acute and chronic effects of aerobic exercise training performed indoors (21 – 25°C) and outdoors (-5 – 5°C) on adropin and markers of lipid metabolism were investigated in 32 healthy male subjects (indoor n = 16, age 22.62 ± 1.58 years, %fat 9.16 ± 1.91%, outdoor n = 16, age 21.25 ± 2.40, %fat 9.26 ± 2.07%). In each group, participants performed 40 minutes of running at 65 – 70% HR_{max} or 50 – 55% $\dot{V}O_{2max,}$ 4 days per week for 18 weeks. Blood samples were taken before, immediately after the first training session and 24 hours after the 18 week training period, following a 3 to 4 hour fast. Unfortunately, glucose and insulin concentrations were not measured in this study and therefore this data is not available for interpretation. No significant differences in body mass, %fat or metabolic parameters were found at baseline. In both groups, body mass did not change but there was a significant decrease in %fat following the intervention ($p < 0.05$). HDL was unchanged after the first exercise session but significantly increased after 18 weeks of training in both groups ($p < 0.05$). The elevation in HDL in the outdoor group was significantly higher than the indoor group ($p < 0.05$). LDL and total cholesterol remained unchanged after the first training session and the exercise intervention. Serum adropin did not change after the first exercise session in both groups, however, circulating concentrations were significantly decreased following 18 weeks of outdoor aerobic training ($p < 0.05$), but unchanged following indoor aerobic training (Ozbay et al., 2020).

Collectively, the studies investigating the effect of exercise training on changes in circulating adropin suggest that aerobic exercise training of up to 12 weeks duration can significantly increase adropin levels concomitant with improvements in endothelial function and adiposity in healthy middle aged and older adults and obese populations. Adropin plays a key role in insulin sensitivity, however, there remains a paucity of evidence on exercise-induced changes in adropin and measures of insulin sensitivity (e.g. fasting insulin, HOMA-IR). Further research is required to investigate the effects of exercise training on circulating adropin and if these changes are associated with improvements in insulin sensitivity. Additionally, there is limited evidence on the effect of exercise training in healthy individuals and the research presented here shows a significant decrease in circulating adropin following 18 weeks of aerobic exercise in a young, lean population. Therefore, the effects of exercise training on circulating adropin

may be dependent upon the duration of the exercise intervention as well as the physical and metabolic characteristics of the population being studied. Future research is needed to investigate the time course of changes in adropin with exercise training, as well as the effects of different types of exercise such as resistance training and HIIT on adropin levels and changes in insulin sensitivity in healthy and clinical populations.

2.4.10.3.4. Adropin and Bed Rest, Physical Inactivity and Ageing

To our knowledge, the impact of HDT bed rest or inactivity on circulating adropin has not been investigated previously. HDT bed rest can be used to study physiological adaptations of microgravity, physical inactivity and extreme sedentariness, as well as accelerated human ageing. Ageing is associated with the deterioration of metabolic homeostasis and endocrine function, which can promote insulin resistance (Redman and Ravussin, 2009). In a pooled sample of 85 female (age 21 – 67 years, BMI 19.4 – 71.5 kg/m^2) and 45 male (age 18 – 70 years, BMI 19.1 – 62.6 kg/m^2) subjects from various clinical studies, adropin levels decreased with age, with a significant negative correlation identified between plasma adropin and age ($r = -0.251$, $p = 0.004$), that remained significant after adjustment for BMI ($r = -0.195$, $p = 0.027$) (Butler et al., 2012). When circulating adropin concentrations were analysed in groups categorised as 30 years and under (n = 34), 30 – 40 years (n = 39), 40 – 50 years (n = 30) and over 50 years (n = 27), adropin levels were significantly higher in those 30 years and under compared with those 40 – 50 years and over 50 years ($p < 0.05$). These results suggest that adropin decreases with ageing and may contribute to the dysregulation of metabolism that occurs with the ageing process (Butler et al., 2012).

2.4.10.3.5. Summary of the Comprehensive Review on Adropin

Adropin is produced in the liver and the brain and is present in circulation. Evidence suggests that adropin can contribute to the regulation of fat mass and glucose and lipid metabolism and has beneficial effects on the cardiovascular system by reducing arterial stiffness and improving endothelial function. Adropin can improve insulin action in the liver and muscle and suppress hepatic glucose production contributing to improved insulin sensitivity, particularly in animal models of obesity. In humans, lower circulating concentrations of adropin have been reported in obesity, insulin resistance and

hepatosteatosis and adropin levels are proposed to decrease with ageing. There is clear evidence for a crucial role of adropin in insulin sensitivity but metabolic data is scarce and only a few studies have investigated exercise-induced changes in circulating adropin and measurements of insulin sensitivity. Collectively, the available research shows that aerobic exercise training increases circulating adropin concomitant with improvements in adiposity, insulin resistance and endothelial function in middle aged and older adults and obese populations. However, further research should be undertaken to examine the effect of different types of exercise interventions (frequency, intensity, time and type) on circulating adropin and the association with insulin sensitivity in various human populations (healthy and clinical).

2.4.10.4. Apo-J/Clusterin

Apolipoprotein-J (Apo-J), also known as clusterin, is a secreted multi-functional protein that circulates as a soluble protein, as a component of HDL or bound to LDL or VLDL in a small portion (Hoofnagle et al., 2010; Seo et al., 2018). Apo-J has been functionally implicated in a multitude of physiological processes, however, this literature review will focus solely on the role of apo-J in insulin resistance and metabolic dysfunction. Some research studies refer to this protein as apo-J, while others use clusterin, therefore the term specified by each study will be used in this review.

2.4.10.4.1. Metabolic Actions of Apo-J/Clusterin

A recent study has reported apo-J as a circulating hepatokine that affects insulin signalling in skeletal muscle (Seo et al., 2020). In liver-specific apo-J deficient (L-Apo-J$^{-/-}$) mice, there was a lack of serum apo-J and levels of apo-J were very low or absent in numerous metabolic organs including adipose tissue and skeletal muscle. Further to this, increased circulating concentrations and apo-J content in metabolic organs was noticeable following injection of adenovirus apo-J to L-Apo-J$^{-/-}$ or global apo-J deficient mice (Apo-J$^{-/-}$) mice, demonstrating that liver-derived apo-J can accumulate in metabolic tissues, thereby playing a key role in inter-organ communication (Seo et al., 2020). L-Apo-J$^{-/-}$ mice were found to exhibit insulin resistance in the fasting state, indicated by fasting hyperglycaemia and the inability to normalise glucose levels in the presence of elevated insulin levels. This resistance was independent of adiposity. On further investigation, it was found that insulin-stimulated insulin receptor phosphorylation was reduced by ~50 –

65% in muscle, adipose tissue and the liver in L-Apo-J$^{-/-}$ mice. Additionally, phosphorylation of downstream components of insulin signalling (IRS-1/2, Akt, Akt substrate of 160 kDa (AS160) and GSK) in skeletal muscle and adipose tissue were also impaired in L-Apo-J$^{-/-}$ mice. This data suggests that insulin resistance caused by hepatic apo-J deficiency is due to reduced insulin signalling in insulin-target tissues at the level of the insulin receptor. Similar results were found following a HFD.

The effect of apo-J on insulin signalling in skeletal muscle is mediated through low-density lipoprotein receptor-related protein-2 (LRP2). Apo-J binding to LRP2 amplifies insulin action, specifically driving insulin receptor internalisation, which is crucial for downstream signalling and glucose uptake. Interestingly, both liver apo-J deficient (L-Apo-J$^{-/-}$) mice and muscle LRP2 deficient (M-LRP2$^{-/-}$) mice, which had high circulating apo-J, are insulin resistant. The authors proposed that impaired insulin signalling in skeletal muscle of the L-Apo-J$^{-/-}$ mice may be due to insufficient LRP2 signalling caused by a lack of its ligand apo-J, which inhibits insulin action on glucose uptake. Alternatively, in M-LRP2$^{-/-}$ mice, insulin resistance may occur as apo-J cannot act in skeletal muscle because of the lack of a cell surface receptor, thereby attenuating insulin's ability to activate signalling. Under these conditions, apo-J concentrations would remain elevated as they cannot be utilised by muscle. It is also possible that the apo-J/LRP2 axis affects insulin action independent of the insulin receptor, through the modulation of PI3K signalling (Seo et al., 2020).

Some contrasting findings to those presented above were reported in a publication by Bradley et al. (2019). In this study, the authors demonstrated that apo-J was secreted from adipocytes and in response to palmitate. However, Seo and authors (2020) reported that there was lack of detailed experimental methods, particularly on the antibody used and composition of the media, and highlighted that apo-J was only measured in the media and not in the adipocytes. Therefore, future experiments to confirm or refute these findings *in vivo* are warranted. On the basis of the findings in apo-J deficient mice, it is possible that apo-J in adipose tissue could be produced by the liver (Seo et al., 2020).

Cultured HepG2 cells, human adipocytes and human skeletal muscle cells were treated with insulin, with or without recombinant clusterin (Bradley et al., 2019). This investigation confirmed that clusterin binds to LRP2 to inhibit insulin signalling, however in contrast to the abovementioned study, clusterin treatment decreased insulin-induced Akt phosphorylation in HepG2 cells, but it had no effect on insulin action in skeletal muscle cells or adipocytes. Additionally, clusterin treatment increased genes representing enzymatic steps in hepatic gluconeogenesis and decreased expression of key regulators in lipogenesis and cholesterol efflux. The results of this study suggest that *in vitro* clusterin treatment impairs hepatic insulin sensitivity, promotes hyperglycaemia and increases cardiovascular disease risk (Bradley et al., 2019).

The role of clusterin in insulin resistance has also been investigated in wild-type and clusterin KO mice, following a HFD (Kwon et al., 2014). A HFD induced insulin resistance and enhanced clusterin expression concomitant to increased oxidative stress and inflammation in mice. Clusterin deficiency did not affect lipid levels and therefore alterations in lipid metabolism did not contribute to insulin resistance in clusterin KO mice fed a HFD. Higher insulin levels in the clusterin KO mice may be due to increased insulin secretion, which is supported by increased fasting C-peptide and islet size, which may increase to compensate for peripheral insulin resistance in these mice following a HFD. Elevated apolipoprotein-A1 (apo-A1) in the liver of clusterin KO mice may also represent a compensatory mechanism to decrease clusterin, as both proteins are known to interact. Overall, the results of this analysis suggest that clusterin may have a protective effect on HFD-induced insulin resistance inflammation and oxidative stress (Kwon et al., 2014).

Together these findings suggest that apo-J impacts insulin signalling and alters markers of oxidative stress and inflammation (Figure 13). Experimental studies have focused on the effects of apo-J in the liver and skeletal muscle, and therefore future studies are needed to examine the mechanisms of action of apo-J in adipose tissue. As hypothesized by Seo and authors (2020), a certain level of apo-J may be required in circulation to achieve glucose homeostasis, such that if apo-J is beyond a certain threshold or there is a lack of apo-J, apo-J could desensitize insulin action on glucose uptake.

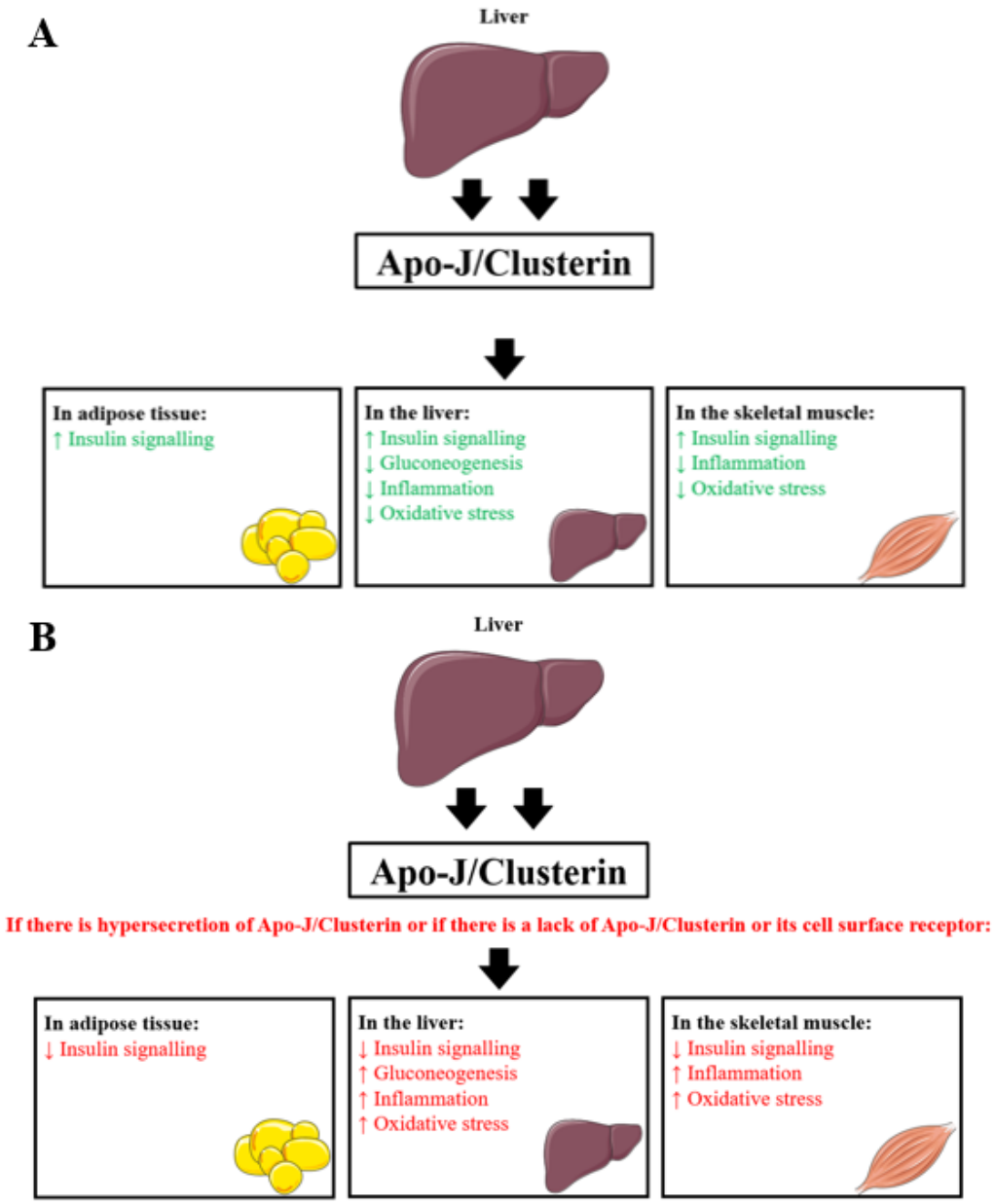

Figure 13. (A) Positive metabolic actions of Apo-J/Clusterin under normal physiological conditions characterised by normal weight and a favourable metabolic profile (B) Negative metabolic actions of Apo-J/Clusterin in metabolic dysfunction associated with the hypersecretion of Apo-J/Clusterin or a deficiency of Apo-J/Clusterin or lack of its cell surface receptors.

2.4.10.4.2. Apo-J/Clusterin in Metabolic Disease

Elevations in circulating apo-J/clusterin has been associated with metabolically dysregulated states including overweight/obesity, insulin resistance, T2DM and the MetSyn (Oberbach et al., 2011; Won et al., 2014; Seo et al., 2018; Bradley et al., 2019). In a cross-sectional study including 186 healthy male subjects (age 18.47 ± 0.15 years,

BMI 23.07 ± 3.87 kg/m^2), Spearman's correlations demonstrated a significant positive correlation between circulating clusterin levels and total cholesterol ($r = 0.21$, $p = 0.003$) and LDL ($r = 0.19$, $p = 0.008$) (Aronis et al., 2011). However, no other associations of clusterin with BMI, fat mass, fat free mass, total body water, basal metabolic rate, systolic and diastolic blood pressure, triglycerides, glucose and HDL could be found. Circulating clusterin levels were also not significantly correlated with leptin, resistin or total adiponectin. When subjects were divided into two groups based on LDL levels, subjects with LDL >130 mg/dl (or 3.37 mmol/L) had statistically higher circulating clusterin compared to those with LDL <130 mg/dl (86.41 ± 12.53 ng/ml *vs.* 78.19 ± 12.52 ng/ml, $p = 0.002$). This was the first study to highlight a potential link between clusterin and LDL and total cholesterol in healthy individuals (Aronis et al., 2011).

The relationship between circulating clusterin, adiposity, inflammation and the MetSyn were examined in a study of Korean adults (Won et al., 2014). Lean, overweight or obesity was defined as a BMI of 18.5 – 22.9, 23.0 – 24.9, or ≥ 25 kg/m^2, respectively, in accordance with the definitions for Asian adults proposed by WHO (World Health Organization. Regional Office for the Western, 2000). In total, 204 subjects were recruited and were split into a lean group (n = 38, 17 males and 21 females, age 37 ± 11 years and BMI 21.2 ± 1.5 kg/m^2) and overweight/obese group (n = 166, 94 males and 72 females, age 40 ± 10 years and BMI 26.0 ± 2.3 kg/m^2). Overweight/obese subjects had significantly higher waist circumference, waist to hip ratio, systolic and diastolic blood pressure compared to lean subjects ($p < 0.05$). Fasting insulin, triglycerides, hsCRP, uric acid, ferritin, leptin and RBP4 were significantly higher, whereas fasting HDL was significantly lower in overweight/obese subjects compared to lean subjects ($p < 0.05$). When all subjects were analysed together, plasma clusterin levels were significantly higher in men compared to women ($p < 0.05$). When separated by BMI, fasting plasma clusterin was significantly higher in overweight/obese subjects compared to lean subjects ($p < 0.001$). Age-adjusted clusterin levels were significantly increased in overweight and obese women compared to lean women ($p < 0.05$). In men, age-adjusted clusterin levels tended to be higher in overweight subjects, but were significantly higher in obese men compared with lean men and overweight men ($p < 0.05$). Smokers (n = 10 in lean, n = 48 in overweight/obese) had increased circulating clusterin compared to non-smokers too ($p < 0.001$).

Pearson's correlation analysis identified significant positive correlations between plasma clusterin and BMI, waist circumference, waist to hip ratio, total cholesterol: HDL ratio, triglyceride: HDL ratio, hsCRP, uric acid, ferritin and RBP4. When correlations were performed separately in men and women, clusterin levels in men were significantly and positively correlated with hsCRP ($r = 0.23$, $p = 0.016$) and BMI ($r = 0.21$, $p = 0.031$) and clusterin levels in women were significantly and positively correlated with BMI ($r = 0.21$, $p = 0.040$) and plasma leptin ($r = 0.21$, $p = 0.049$), and marginally with hsCRP ($r = 0.20$, $p = 0.053$). Using stepwise multiple linear regression, sex, hsCRP and BMI were independent predictors of plasma clusterin levels in all subjects. This study also identified that plasma clusterin levels were significantly higher in subjects with the MetSyn (n = 53, 38 men and 15 women), compared to those without the MetSyn (n = 151, 73 men and 78 women). Sex-stratified analysis also showed a trend towards an increase in circulating clusterin in those with the MetSyn, however this did not reach statistical significance. Additionally, there was an upward trend of plasma clusterin for increasing components of the MetSyn ($p < 0.05$). Taken together, these findings suggest that increases in plasma clusterin may be related to the pro-inflammatory state that is associated with increased adiposity in obesity. The mechanisms responsible for the difference in clusterin levels in males and females is yet to be identified (Won et al., 2014).

A comparison of serum concentrations of apo-J was conducted between non-T2DM (n = 27, 24 males and 3 females, age 54.2 ± 2.3 years, BMI 29.6 ± 1.0 kg/m^2) and T2DM (n = 26, 18 males and 8 females, age 57.4 ± 1.5 years, BMI 39.6 ± 1.1 kg/m^2) subjects (Seo et al., 2018). Subjects with T2DM had significantly higher BMI, fasting glucose, fasting insulin, HOMA-IR compared with subjects without T2DM, while neither sex, age, HOMA-β and blood pressure measurement differed significantly between the groups. Circulating apo-J was measured using western blotting analysis and ELISA and there was a strong correlation between the results of both methods ($r = 0.754$, $p < 0.0001$). Results of both types of analysis found a ~50% increase in serum levels of apo-J in T2DM subjects compared with non-T2DM subjects Further to this, when non-T2DM were classified according to level of HOMA-IR, subjects in the higher tertiles of HOMA-IR, had the highest apo-J concentrations ($p = 0.038$ for trend). In all subjects, circulating apo-J was significantly and positively correlated with fasting glucose, fasting insulin, HOMA-IR and BMI ($p < 0.05$). Multiple regression analysis found that circulating apo-J was a

significant independent association factor for fasting insulin and HOMA-IR, after adjustment for age, sex and BMI. When the subjects were separated according to T2DM status, these associations were maintained in the non-T2DM only. These results show that circulating apo-J is closely related to the magnitude of insulin resistance, independent of obesity, especially in non-T2DM subjects (Seo et al., 2018). Additionally, in a separate regression analysis model adjusted for age, sex and BMI, a positive correlation between circulating apo-J and HOMA-β was identified in non-T2DM subjects, but not in T2DM subjects. This result suggests that apo-J may be involved in the regulation of pancreatic β-cell function. The authors noted that β-cell dysfunction in a pathogenic mechanism of disease that worsens with disease progression and therefore it is possible that diabetic subjects included in this study have variable levels of β-cell dysfunction. Thus, the mixed effects of insulin resistance and insulin secretion on circulating apo-J may confound the correlations in the T2DM cohort (Seo et al., 2018).

2.4.10.4.3. Apo-J/Clusterin and Exercise Training

To our knowledge, there are currently only two studies examining the effect of exercise on circulating concentrations of apo-J. Oberach et al. (2011) examined the differences in serum clusterin between 5 lean (age 23.7 ± 1.99 years, BMI 23.8 ± 1.77 kg/m^2) and 5 obese (age 24.1 ± 3.11 years, BMI 37.3 ± 7.21 kg/m^2) males before, post- and 24 hours post-acute exercise training (a single session of resistance circuit training performed at 80% of individual maximal power for 60 minutes). Clusterin was significantly different between lean and obese subjects at all time-points ($p < 0.05$), and the results highlighted an opposing pattern of change between lean and obese individuals. Clusterin was significantly lower at baseline in lean subjects, but post-exercise clusterin was significantly higher in lean subjects. After 24 hours, clusterin was significantly lower in lean subjects with similar levels to baseline. Unfortunately, pre to post-exercise within-group changes were not presented and therefore cannot be interpreted (Oberbach et al., 2011).

The impact of combined aerobic and resistance training on changes in circulating apo-J was investigated in post-menopausal women with T2DM (Jeon et al., 2020). The mean duration of T2DM was 9.5 ± 7.9 years. Changes in anthropometrics, metabolic

characteristics and apo-J were examined in the control (n = 14, age 61.1 ± 7.0 years, BMI 25.3 ± 2.6 kg/m^2) and exercise group (n = 21, age 62.1 ± 7.3 years, BMI 24.5 ± 2.7 years) following a period of 12 weeks. The combined exercise training programme consisted of 3 aerobic training sessions (Folk dancing at an RPE of 11 to 12 for weeks 1 – 4 and 13 to 14 for weeks 5 – 12 for 20 minutes per session), and 3 resistance training sessions (Rubber band exercises of 9 movements to work the upper and lower body muscles for 30 minutes per session) every week. No significant between-group differences were identified at baseline.

After the exercise intervention, body weight, %fat and fat mass were significantly decreased in the exercise group only ($p < 0.05$). Weight-adjusted appendicular skeletal muscle mass (ASM/wt.) was significantly increased in the exercise group ($p = 0.005$), but not in the control group ($p = 0.820$). There were no significant differences in HbA1c, android fat mass or ASM/wt. in either group after 12 weeks ($p > 0.05$). HDL increased in the exercise group ($p = 0.026$), but did not change in the control group ($p = 0.573$). HOMA-IR was lower than baseline in the exercise group after the intervention, but this did not reach statistical significance ($p = 0.054$). At baseline, circulating apo-J in all subjects was significantly correlated with HDL ($r = 0.302$, $p = 0.048$) and ASM/wt. ($r = -0.332$, $p = 0.019$). Baseline circulating apo-J was positively correlated to HbA1c and HOMA-IR, albeit not significantly ($r = 0.286$, $p = 0.079$ and $r = 0.297$, $p = 0.067$, respectively). At week 8 and week 12, apo-J was significantly lower in the exercise group compared to the control group ($p = 0.019$ at week 8 and $p = 0.007$ at week 12 by repeated measures ANOVA). In the exercise group, concentrations of apo-J decreased from baseline by 26.3% and 19.4% at weeks 8 and 12, respectively, and was significantly different between the two groups at these time points ($p < 0.05$ and $p < 0.01$, respectively). Decreased concentrations of apo-J after 12 weeks of training was significantly correlated with changes in ASM/wt. ($r = -0.412$, $p = 0.016$) and HOMA-IR ($r = -0.352$, $p = 0.041$) in unadjusted analysis. After adjustment for age, the change in apo-J remained significantly correlated with ASM/wt. only ($r = -0.408$, $p = 0.024$). Overall, combined exercise training significantly reduced circulating apo-J concentrations, and the change was associated with improvements in muscle mass and an improvement in insulin sensitivity in patients with T2DM (Jeon et al., 2020).

2.4.10.4.4. Apo-J/Clusterin and Bed Rest, Physical Inactivity and Ageing

To our knowledge, the impact of bed rest and physical inactivity on circulating concentrations of apo-J has not been studied. There are limited studies concerning changes in circulating apo-J during ageing and of the studies available, most of the research is focused on apo-J as a biomarker of Alzheimer's disease. However, there is data to show that circulating clusterin levels increase in humans during the ageing process (Trougakos et al., 2002; Baralla et al., 2015). It has been proposed that the increase of clusterin during ageing and in age-related diseases such as T2DM and cardiovascular disease relates to increased oxidative stress which can promote proteotoxic stress, genomic instability, cellular growth arrest and cellular death (Trougakos and Gonos, 2006).

2.4.10.4.5. Summary of the Comprehensive Review on Apo-J/Clusterin

According to the literature, apo-J plays a key role in inter-organ communication and has been implicated in multiple metabolic and cardiovascular abnormalities. Under normal physiological conditions, characterised by normal weight and a favourable metabolic profile, apo-J can improve insulin signalling and reduce markers of oxidative stress and inflammation. Contrastingly, in conditions where there is hypersecretion of apo-J or a deficiency of apo-J or its cell surface receptors, apo-J can impair insulin signalling and increase markers of oxidative stress and inflammation. In line with this, higher concentrations of apo-J are found in metabolic diseases, such as obesity and T2DM, which are associated with increased adiposity, inflammation and insulin resistance. Despite this, the exact mechanistic and tissue-specific actions of apo-J remain elusive and further investigation is required to delineate the role of apo-J in whole-body and tissue-specific insulin sensitivity. Few studies have investigated the effects of exercise training on changes in circulating apo-J, however, the available evidence shows that apo-J decreases with chronic exercise training and is related to improvements in muscle mass and insulin sensitivity in individuals with T2DM. HDT bed rest represents a unique model of extreme inactivity and accelerated ageing that impairs whole-body and peripheral insulin sensitivity (Bergouignan et al., 2011; Kenny et al., 2017; Rudwill et al., 2018). Investigating the changes in apo-J in response to HDT bed rest, with or without reactive jump training, may provide valuable insights into the function of this biomarker in insulin resistance and metabolic dysfunction.

2.4.10.5. FGF-21

Fibroblast growth factor 21 (FGF-21) is a hormone which acts as a multifunctional metabolic regulator (Staiger et al., 2017). FGF-21 is abundantly expressed in the liver but it also expressed in the pancreas, white adipose tissue (WAT), brown adipose tissue (BAT) and stressed skeletal muscle (Inagaki, 2015). However, FGF-21 is commonly referred to as a hepatokine as circulating FGF-21 is derived mainly from the liver.

2.4.10.5.1. Metabolic Actions of FGF-21

Since its discovery in 2000, the metabolic actions of FGF-21 have been extensively studied in mice. FGF-21 functions physiologically to maintain energy homeostasis by improving insulin sensitivity and glycolipid metabolism and reducing hepatic lipid accumulation (Inagaki, 2015). At a molecular level, FGF-21 binds to β-klotho and fibroblast growth factor receptor (FGFR) and activates the dimerization and autophoshorylation of the FGFR, permitting its biological activity (Li et al., 2013b; Liu et al., 2015a).

2.4.10.5.1.1. Metabolic Functions of FGF-21 in the Liver

The liver is a major site of the production and actions of FGF-21. The hepatic expression and secretion of FGF-21 into circulation is upregulated in response to fasting and a ketogenic diet and rapidly suppressed by refeeding in mice and is regulated by peroxisome proliferator-activated receptor alpha (PPARα) (Badman et al., 2007). In response to fasting, NEFA are released from adipocytes into circulation and are taken up by hepatocytes. NEFA binds to and activates PPARα and stimulates the expression of FGF-21 in the liver (Mai et al., 2009; Murata et al., 2011). FGF-21 released from hepatocytes in response to fasting/starvation contributes to the stimulation of gluconeogenesis, hepatic fatty acid oxidation, ketogenesis and growth hormone resistance (Owen et al., 2015). The coordination of fatty acid oxidation and gluconeogenesis in the liver is mediated by PGC-1α (Potthoff et al., 2009).

In humans, starvation induced by 7 days of fasting significantly increased serum FGF-21 levels ($p < 0.05$), whereas shorter-term fasting (2 days) did not alter circulating

concentrations of FGF-21 (Gälman et al., 2008). The induction of ketogenesis independent of alterations in FGF-21 indicate that the metabolic actions of FGF-21 in humans may differ from that in mice (Gälman et al., 2008). As the metabolic rate of mice is approximately 10-fold higher than that of humans, energy stores are consumed at a much higher rate during fasting (Xie and Leung, 2017). In line with this, it been proposed that a 7 day fast in humans should be comparable to a fast of approximately 24 hours in mice (Xie and Leung, 2017). It is also important to note that humans exhibit a wide inter-individual variation in circulating FGF-21 concentrations (Gälman et al., 2008).

2.4.10.5.1.2. Metabolic Functions of FGF-21 in Adipose Tissue

Adipose tissue is another major target of the metabolic actions of FGF-21, with some variation between the regulation and effects in WAT and BAT. In WAT, FGF-21 is regulated by peroxisome proliferator-activated receptor gamma (PPARγ), while in BAT, it is regulated by activating transcription factor 4 (AFT2) (Hondares et al., 2011; Dutchak et al., 2012; Inagaki, 2015). In WAT, FGF-21 modulates lipolysis and enhances glucose uptake and mitochondrial oxidative capacity (Dolegowska et al., 2019). In addition, FGF-21 influences the regulation of thermogenesis in BAT and browning of WAT (Dolegowska et al., 2019). Furthermore, FGF-21 is a potent stimulator of adiponectin secretion and FGF-21 critically depends on adiponectin to elicit favourable glycaemic and insulin-sensitising effects in mice (Holland et al., 2013).

In both 3T3-L1 adipocytes and primary human adipocytes, FGF-21 stimulates glucose uptake in an insulin-independent manner (Kharitonenkov et al., 2005). However, upon co-treatment, FGF-21 can augment insulin activity thereby increasing glucose uptake further. Unlike insulin, FGF-21 has no effect on GLUT4, but acts through the upregulation of cellular GLUT1 (Kharitonenkov et al., 2005). In addition to glucose uptake, FGF-21 is involved in the regulation of lipolysis. As mentioned in the previous subsection, adipose tissue lipolysis is stimulated in response to fasting/starvation, allowing for the release NEFA, which in turn induces hepatic FGF-21 production *via* PPARα. Subsequently, FGF-21 inhibits lipolysis in adipocytes. This regulatory process forms a negative feedback loop in the control of lipolysis by FGF-21 (Mai et al., 2009; Murata et al., 2011). This feedback regulation helps to maintain the balance of lipid

distribution between the adipose tissue and the liver and potential lipotoxicity caused by a sustained elevation of FFAs (Ge et al., 2012). The suppressive effect of FGF-21 on lipolysis in the fasted state was confirmed in FGF-21-KO mice. This study also showed an opposing effect on lipolysis in the fed state, and concluded that FGF-21 regulates lipolysis in response to the metabolic state (Hotta et al., 2009).

2.4.10.5.1.3. Metabolic Functions of FGF-21 in Other Tissues

In skeletal muscle, the expression of FGF-21 appears to be regulated by PI3K-Akt signalling and by ATF4 under the conditions of mitochondrial dysfunction (Izumiya et al., 2008; Kim et al., 2013a; Inagaki, 2015). Two stimuli activating Akt in skeletal muscle are insulin and muscle contraction (Tanimura et al., 2016). Muscle-derived FGF-21 may act in a paracrine or autocrine manner to improve muscle metabolism. FGF-21 was shown to mediate glucose uptake in primary myotubes and in mouse myoblast C2C12 cells, however, there is currently no *in vivo* evidence to support this function in skeletal muscle (Mashili et al., 2011; Fisher and Maratos-Flier, 2016). Additionally, skeletal muscle is not typically considered as a target tissue for FGF-21 because of a low expression of β-klotho (Mashili et al., 2011; Fisher and Maratos-Flier, 2016). Conversely, FGF-21 expression and protein levels are high in the pancreas, however the metabolic function of FGF-21 in this tissue is still obscure (Fisher and Maratos-Flier, 2016). It appears that FGF-21 has a protective role in reducing inflammation and fibrosis, as well as injury, in acute pancreatitis (Algul, 2009). FGF-21 enhanced insulin content and glucose-induced insulin secretion in diabetic islets and protected rat islets and insulin-producing INS cells from glucolipotoxicity and cytokine-induced apoptosis (Wente et al., 2006). These functions suggest that FGF-21 may contribute to β-cell function and survival and elicit beneficial effects on glucose homeostasis in diabetic animals.

Collectively these studies show that FGF-21 is an endocrine factor secreted by the liver in response to the fasting state, which increases hepatic fatty acid oxidation, ketogenesis and gluconeogenesis. In addition, FGF-21 is an autocrine factor produced by adipose tissue which increases glucose uptake and modulates lipolysis according to the fasted or fed state (Figure 14). While favourable metabolic actions of FGF-21 in skeletal muscle

and the pancreas have been proposed, these physiological functions require further investigation.

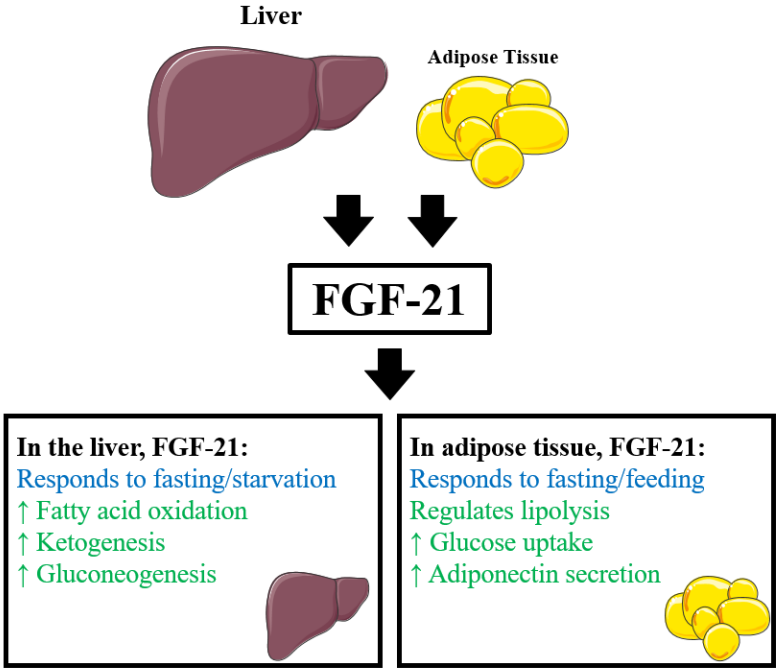

Figure 14. The metabolic actions of FGF-21 under different conditions of metabolic stress.

2.4.10.5.2. FGF-21 in Metabolic Disease

Circulating concentrations of FGF-21 are upregulated in individuals with overweight/obesity (Zhang et al., 2008), T2DM (Chen et al., 2008; Hong et al., 2019), and NAFLD (Yilmaz et al., 2010a; Yan et al., 2011; He et al., 2017). These findings are unexpected and indicate the presence of FGF-21 resistance or a compensatory response to the underlying metabolic stress in these conditions leading to increased secretion of FGF-21.

Fasting serum FGF-21 levels increased progressively from healthy subjects to individuals with IGT and T2DM ($p < 0.05$ for trend) (Lin et al., 2012). High serum FGF-21 levels were found to be an independent risk factor for the MetSyn (Zhang et al., 2008). Furthermore, high circulating concentrations of FGF-21 were found to be a predictor of NAFLD, independent of higher BMI, insulin resistance and the presence of the MetSyn

(Li et al., 2013a). Age-, gender- and body fat-matched individuals with morbid obesity (BMI ≥ 40 kg/m^2) were recruited and based on the insulin sensitivity index (Matsuda), were classified into a metabolic-healthy obese group (insulin sensitive) and a metabolically-unhealthy obese group (insulin resistant) (Berti et al., 2015). After adjustment for the confounders of gender, age, and BMI by multiple linear regression modelling, markedly higher concentrations of circulating FGF-21 were found in individuals stratified as metabolically-unhealthy obese ($p = 0.023$). The findings show that FGF-21 may contribute to metabolically detrimental effects and propose an adiposity-independent role in insulin resistance in humans (Berti et al., 2015).

FGF-21 concentrations were quantified in a cohort of 190 subjects undergoing a routine health examination to check for the presence of coronary artery disease (Hong et al., 2019). According to glucose tolerance, participants were split into 3 groups classified as having NGT (n = 48, 19 males and 29 females, age 57.8 ± 7.5 years, BMI 24.1 ± 2.8 kg/m^2), pre-diabetes (n = 53, 38 males and 15 females, age 57.6 ± 10.4 years, BMI 25.4 ± 2.9 kg/m^2) and T2DM (n = 89, 62 males and 27 females, age 56.4 ± 9.3 years, BMI 25.5 ± 2.6 kg/m^2). Analysis of between-group differences found no significant group differences in age or blood pressure, but gender was different among the three groups. BMI, fasting plasma glucose, 2 hour glucose, HbA1c%, fasting insulin, C-peptide and HOMA-IR were significantly higher in individuals with prediabetes and T2DM compared to those with NGT ($p < 0.05$). Fasting triglycerides were significantly higher and HDL was significantly lower in the individuals with T2DM, compared to individuals with NGT ($p < 0.05$). The Matsuda index was significantly lower in individuals with prediabetes and T2DM in comparison to individuals with NGT ($p < 0.05$). In the whole cohort, plasma FGF-21 correlated positively with triglycerides ($r = 0.292$, $p = 0.001$), HOMA-IR ($r = 0.246$, $p = 0.04$), BMI ($r = 0.212$, $p = 0.08$), LDL ($r = 0.182$, $p = 0.049$), and negatively with the Matsuda index ($r = -0.240$, $p = 0.016$). Plasma FGF-21 concentrations were significantly elevated in individuals with T2DM compared with individuals with prediabetes and NGT ($p = 0.041$). In a second cohort, plasma FGF-21 concentrations were also found to be significantly higher in individuals with T2DM (n = 23, 16 males and 7 females, age 68.1 ± 8.6 years, BMI 24.5 ± 4.0 kg/m^2) compared with individuals with NGT ($p = 0.009$, n = 17, 15 males and 2 females, age 65.4 ± 12.4 years, BMI 23.6 ± 2.2 kg/m^2). The results of this study show that circulating FGF-21 concentrations are elevated

in T2DM and plasma FGF-21 levels are positively associated with triglycerides, BMI and insulin resistance (Hong et al., 2019).

Another study investigating the relationship between FGF-21 and insulin sensitivity reported that plasma FGF-21 was increased in insulin resistant states (obesity, IFG/IGT and T2DM) and circulating levels of FGF-21 correlated inversely with whole-body (primarily reflecting muscle) insulin sensitivity ($r = -0.420$, $p = 0.007$) and positively with hepatic insulin resistance ($r = 0.344$, $p = 0.034$) and adipose tissue insulin resistance ($r = 0.318$, $p = 0.045$). These findings imply that FGF-21 may play a role in the pathogenesis of whole-body and peripheral insulin resistance in T2DM (Chavez et al., 2009).

Panahi et al. (2016) examined serum FGF-21 concentrations in individuals with well-controlled T2DM (n = 49, age 52.57 ± 11.09 years, BMI, 27.72 ±3.78 kg/m^2), poorly-controlled T2DM (n = 66, age 56.15 ± 11.26 years, BMI 26.98 ± 4.39kg/m^2) and healthy, non-T2DM controls (n = 26, age 51.77 ± 9.78 years, BMI 26.84 ± 3.76 kg/m^2). No between-group differences were identified for age, gender, BMI, blood pressure, triglycerides and HDL. There was significant between-group differences, in at least 2 groups, in fasting glucose, HbA1c%, creatinine and LDL ($p < 0.05$). Fasting glucose was significantly higher in individuals with poorly-controlled T2DM, compared with those with well-controlled T2DM and healthy controls, and was significantly increased in well-controlled T2DM compared with healthy controls ($p < 0.05$). Mean HbA1c% and creatinine levels were significantly greater in individuals with poorly-controlled T2DM in comparison to those with well-controlled diabetes ($p < 0.05$). LDL concentrations were significantly lower in healthy controls, compared with both T2DM subgroups ($p < 0.05$). When patients with T2DM were pooled, circulating FGF-21 was significantly higher in individuals with T2DM compared to those without diabetes ($p < 0.001$). Further analysis identified that FGF-21 levels were significantly higher in individuals with poorly-controlled T2DM compared with individuals with well-controlled T2DM ($p = 0.002$) and healthy controls ($p = 0.007$). However, no significant difference in circulating FGF-21 was observed between individuals with well-controlled T2DM and healthy controls ($p = 0.55$). Therefore, elevations in serum FGF-21 may be an indicator of patients with poorly-controlled diabetes (Panahi et al., 2016).

The paradoxical increase of circulating FGF-21 in metabolic dysfunction may reflect a state of FGF-21 resistance or a compensatory upregulation of FGF-21 as a defensive response to counteract insulin resistance (Zhang et al., 2008). The latter scenario is reminiscent of hyperinsulinemia and hyperleptinemia, which are both states of compensatory secretion to offset insulin and leptin resistance.

2.4.10.5.3. FGF-21 and Exercise Training

To date, several studies have investigated the effects of acute and chronic exercise on changes in circulating FGF-21 in healthy and clinical populations and the findings of these studies are discussed below.

2.4.10.5.3.1. FGF-21 and Acute Exercise

Cuevas-Ramos et al. (2012) examined the effect of an incremental treadmill exercise test on serum FGF-21 levels in young, sedentary healthy women (n = 60, age 24.0 ± 3.7 years, BMI 21.4 ± 7.0 kg/m^2). Metabolic parameters including glucose, insulin, FFA, epinephrine, leptin, adiponectin and FGF-21 were measured at baseline and 1 and 4 hours post-exercise. After 1 hour, no significant differences in glucose and insulin levels were identified ($p > 0.05$). After 4 hours, glucose concentrations were significantly reduced to a level below baseline ($p = 0.021$), and insulin concentrations increased significantly compared to baseline levels ($p = 0.0001$). Additionally, significant increases in epinephrine and leptin were documented after 1 and 4 hours ($p < 0.05$). Acute exercise did not alter circulating FGF-21, FFA or adiponectin. Based on these findings, it is possible that the elevated insulin response to acute exercise mitigated the expression and secretion of FGF-21 by inhibiting lipolysis (Cuevas-Ramos et al., 2012).

In contrast to the findings presented above, multiple research studies have reported significant increases in FGF-21 following acute exercise concomitant to increases in lipolytic markers. Kim et al. (2013) assessed the impact of acute exercise on serum FGF-21 levels in 13 healthy non-athletic males (mean age 22.1 years, mean BMI 21.4 kg/m^2). The trial consisted of a 30-minute treadmill run at 50% $\dot{V}O_{2max}$. Of the 13 subjects that completed the first trial, 8 subjects completed a second 30-minute treadmill run at 80%

$\dot{V}O_{2max}$, 3 days later. Blood samples were taken before, immediately after and 1hour post-exercise. FGF-21 concentrations were significantly higher 1 hour post-exercise following acute exercise at 50% and 80% $\dot{V}O_{2max}$ ($p < 0.001$ for both). However, there were no significant changes in FGF-21 immediately post-exercise compared to baseline levels for either trial. Further analysis found that FGF-21 levels 1 hour after high-intensity exercise (80% $\dot{V}O_{2max}$) were significantly higher when compared to circulating FGF-21 1 hour post mild intensity exercise (50% $\dot{V}O_{2max}$). This finding implies that the elevation in FGF-21 after acute exercise may be intensity dependent. In addition, serum FFA and β-hydroxybutyrate level (ketone body) were significantly elevated immediately and 1 hour post high-intensity exercise, suggesting an increase in exercise-induced lipolysis. Serum insulin concentrations were significant lower than baseline levels after 1 hour of recovery from high-intensity exercise ($p < 0.05$). Serum glucose increased immediately following high-intensity exercise ($p < 0.001$), but this elevation was not maintained 1 hour into recovery. Overall, these results show that a single bout of acute exercise increases circulating FGF-21 and enhances lipolysis in healthy male subjects (Kim et al., 2013b). These findings were supported in a subsequent study which reported that acute exercise (60 minute of cycling at 75% $\dot{V}O_{2max}$) significantly increased serum concentrations of FGF-21 in healthy men (n = 19, age 23.7 ± 2.3 years, BMI 22.4 ± 2.0 kg/m^2), consistent with an increase in lipolytic parameters (Tanimura et al., 2016).

In addition to FFA, changes in glucagon and the glucagon-to-insulin ratio have been proposed as important factors in the regulation of FGF-21 in response to acute exercise, particularly in aerobic exercise (Morville et al., 2018). Hansen et al. (2016) evaluated acute exercise-induced secretion of FGF-21 in young, healthy males (n = 6, age 22.7 ± 0.2 years, BMI 21.7 ± 0.4 kg/m^2) who completed two fasted exercise trials, which involved cycling for 2 hours at 60% $\dot{V}O_{2max}$, with and without a pancreatic clamp to block the increase in the glucagon-to-insulin ratio. The trials were preceded by 30 minutes of supine rest and baseline sample analysis, and then blood samples were collected every 30 minutes for 3 hours and then once hourly for an additional 4 hours. At the end of exercise, subjects rested in a supine position for 5 hours. Acute exercise induced a significant increase in the glucagon-to-insulin ratio (10 fold, $p = 0.002$), which peaks at the end of exercise and this change was abolished with the pancreatic clamp ($p = 0.21$). A two-way ANOVA found a significant effect of time ($p = 0.008$), group ($p = 0.001$) and time*group

interaction ($p = 0.008$) for the glucagon-to-insulin ratio. During exercise, blood glucose initially decreased and then remained suppressed throughout the trial ($p = 0.0003$). Conversely with the pancreatic clamp, glucose was continuously infused to maintain euglycaemia and a two-way ANOVA showed a significant effect of time ($p = 0.004$), group ($p < 0.0001$) and time*group interaction ($p < 0.05$). Exogenous glucose administration increased considerably during exercise, evidenced by a significant increase in glucose infusion rate ($p < 0.0001$). During the exercise trial, FFA increased significantly from baseline to the end of exercise and remained elevated during the recovery period ($p < 0.05$). Contrastingly, the FFA response to exercise was blunted with the pancreatic clamp and levels remained at baseline throughout the trial ($p = 0.55$). There was a significant effect of time, group and time*group interaction found by two-way ANOVA ($p < 0.05$). Plasma FGF-21 levels remained at baseline levels for the first hour of exercise, before increasing at 60 minutes, and reaching its peak at 120 minutes (8 fold increase, $p < 0.0001$). However, plasma FGF-21 levels returned to baseline within 2 hours of recovery. In contrast, plasma FGF-21 tended to decrease and was significantly decreased 3 to 5 hours after acute exercise ($p = 0.003$). When assessed using a two-way ANOVA, there was a significant effect of time ($p < 0.0001$), and time*group interaction ($p = 0.007$) and borderline significance for the effect of group ($p = 0.057$).

In a subsequent investigation reported in the same publication, the effect of acute exercise on circulating FGF-21 was examined in individuals with T2DM ($n = 7$) and healthy controls with NGT ($n = 8$). Subject groups were matched for age, BMI, and $\dot{V}O_{2max}$, and individuals with T2DM paused their medication for one week prior to the exercise trial. In the fasted state, subjects performed 60 minutes of cycling at an intensity of 50% of $\dot{V}O_{2max}$. Blood samples were collected prior to the exercise trial and then every 30 minutes for 2 hours and then once hourly for the final 2 hours. After the cessation of exercise, subjects rested for 180 minutes in a supine position. The glucagon-to-insulin ratio was higher in the NGT group compared to the T2DM group (effect of group, $p < 0.0001$). In the NGT group, the glucagon-to-insulin ratio increased initially with acute exercise with borderline significance (effect of time, $p = 0.056$), but remained unchanged in the T2DM group ($p = 0.47$). FFA were significantly higher in the T2DM group compared to the NGT group (effect of group, $p = 0.049$). In both groups, FFA decreased at the start of the exercise trial and then increased and remained elevated throughout the 3-hour recovery

period (one-way ANOVA for both groups, $p < 0.001$). Circulating FGF-21 was significantly greater in the T2DM group compared to the NGT group (effect of group, $p = 0.0004$). Acute exercise had no effect on circulating FGF-21 in the NGT group, when analysed in absolute concentrations. However, when expressed relative to baseline, there was a 1.5 fold increase in response to exercise (one-way ANOVA, $p = 0.008$), with the largest elevations 30 and 60 minutes after the cessation of exercise. In the T2DM group, plasma FGF-21 levels remained unchanged during and following acute exercise when analysed in absolute concentrations or fold changes from baseline (one-way ANOVA, $p = 1.00$ and $p = 0.92$, respectively). Analysis of the fold changes of circulating FGF-21 in the NGT group and T2DM revealed a borderline significant effect of group using a two-way ANOVA ($p = 0.056$). Taken together, the findings of this study suggest that glucagon and insulin, as well as FFA, are important regulators of FGF-21 secretion during acute exercise. Furthermore, exercise-induced secretion of FGF-21 is impaired in individuals with T2DM compared to healthy individuals (Hansen et al., 2016).

Overall, it can be concluded that acute exercise increases circulating FGF-21 in healthy individuals in response to elevations in the glucagon-to-insulin ratio and FFA. From the evidence presented here, it appears that FGF-21 levels return to baseline after 2 hours of recovery. In individuals with T2DM, a dysregulated exercise-induced FGF-21 response was reported but further studies are required to support or refute this finding. Sampling time during and post-exercise appears to have an impact on the results and must be considered when comparing studies.

2.4.10.5.3.2. FGF-21 and Chronic Exercise

Research on the influence of chronic exercise training on circulating FGF-21 concentrations has reported inconsistent results. Studies using different modalities of exercise have reported decreases (Taniguchi et al., 2016; Shabkhiz et al., 2020), increases (Cuevas-Ramos et al., 2012) and no changes (Besse-Patin et al., 2014; Kruse et al., 2017) in circulating FGF-21 in individuals with and without metabolic dysfunction. As exercise training improves insulin sensitivity and reduces intrahepatic lipid accumulation (Brouwers et al., 2016), it is conceivable that circulating concentrations of FGF-21 will decrease following a training intervention.

The metabolic effects of resistance training on circulating FGF-21 and insulin sensitivity were examined in elderly men with (n = 20, age 72.45 ± 6.00 years, BMI 26.15 ± 3.53 kg/m^2) and without T2DM (n = 24, age 72.08 ± 5.33 years, BMI 27.47 ± 4.02 kg/m^2) (Shabkhiz et al., 2020). Subjects were randomly assigned to either the resistance training group (n = 12 non-T2DM, n = 10 T2DM) or the control group (n = 12 non-T2DM, n = 10 T2DM) for a period of 12 weeks. The resistance training protocol was performed 3 times per week and consisted of 3 sets of 10 repetitions of 8 exercises targeting the whole-body (leg press, leg extension, seated leg curl, seated calf raise, bench press, compound row, triceps press and bicep curl), performed at 70% 1RM. At baseline, men with T2DM had significantly lower 1RM leg press strength ($p = 0.015$) and fasting insulin ($p = 0.001$) and higher fasting glucose ($p = 0.001$) and serum FGF-21 ($p = 0.002$) compared with non-T2DM men. However, there were no significant differences in age, body weight, BMI, or HOMA-IR between non-T2DM and T2DM men prior to the intervention.

Following the resistance training intervention, leg press 1RM was significantly increased in T2DM and non-T2DM men ($p = 0.01$ and $p = 0.001$, respectively). The increase in leg press strength was similar between the groups (interaction of training*diabetes status, $p = 0.44$). Conversely, leg press 1RM did not change in either of the control groups ($p > 0.05$). Body weight and BMI did not change in the resistance training groups of T2DM and non-T2DM men. In the control group, body weight and BMI increased in T2DM ($p < 0.05$), but not in non-T2DM men. Resistance training significantly reduced fasting glucose in non-T2DM and T2DM ($p = 0.001$ for both groups), but only induced a significant reduction in fasting insulin and HOMA-IR in non-T2DM men ($p < 0.05$). A significant interaction effect of fasting glucose ($p = 0.012$) highlighted that the decrease in fasting glucose was more pronounced after resistance training in T2DM men, compared to non-T2DM men. No significant changes in fasting glucose, fasting insulin or HOMA-IR were noticeable in the control group. Resistance training elicited a significant decrease in serum FGF-21 in T2DM and non-T2DM men ($p = 0.008$ and $p = 0.002$, respectively). FGF-21 levels did not change in the control groups. In elderly men with T2DM, the reduction in FGF-21 correlated negatively with the increase in leg press strength ($r = -0.59$, $p = 0.006$) and positively with the decrease in fasting glucose ($r = 0.64$, $p = 0.002$). In elderly non-T2DM men, the reduction in FGF-21 correlated negatively with the increase in leg press strength ($r = -0.52$, $p = 0.009$) and positively with the decreases in

fasting insulin (r = 0.61, p = 0.002) and HOMA-IR (r = 0.62, p = 0.001). Taken together, these results show that chronic resistance training induced a significant decrease in FGF-21 in elderly T2DM and non-T2DM men and this reduction was associated with improvements in muscle strength and insulin sensitivity (Shabkhiz et al., 2020).

Tanguchi et al. (2016) evaluated whether endurance training could modulate hepatic fat content and circulating FGF-21 levels in elderly Japanese men (n = 33, age 69.6 ± 4.2 years, BMI 23.1 ± 2.6 kg/m^2). Subjects were randomly assigned to an exercise group or control group and were then changed in a cross-over design. Subjects in the exercise group undertook a 5 week training intervention consisting of 3 sessions per week of cycling exercise with progressive intensity and duration (60 – 75% $\dot{V}O_{2max}$ for 30 – 45 minutes). Absolute changes in each variable were compared between the exercise and control periods using a 2 x 2 cross-over ANOVA for the factors, treatment and sequence. Aerobic training significantly increased $\dot{V}O_{2max}$ and the absolute change was higher in the exercise period compared to the control period ($p < 0.001$). Contrastingly, aerobic training significantly reduced hepatic fat content and serum FGF-21, and the absolute changes were greater in the exercise period compared to the control period ($p = 0.021$ and $p = 0.026$, respectively). There was no significant interaction between treatment and sequence for $\dot{V}O_{2max,}$ intrahepatic fat or FGF-21 levels, implying no carryover effects with the sequence of the experiment. HbA1c% was significantly different between the exercise and control period, however a significant interaction between treatment and sequence was detected indicating that the carryover effect influenced HbA1c% ($p = 0.013$). No significant changes in body weight, BMI, %fat, visceral fat, subcutaneous fat, AST, ALT, γ-glutamyl transpeptidase (γ-GTP), triglycerides, FFA, fasting glucose, fasting insulin or HOMA-IR were detected. Correlation analysis identified that the decrease in serum FGF-21 was positively correlated with the decrease in hepatic fat ($r = 0.366$, $p = 0.006$) and this relationship was maintained even after adjustment for age, baseline serum FGF-21 and changes in $\dot{V}O_{2max}$ and visceral fat area ($r = 0.321$, $p = 0.03$). The reduction in serum FGF-21 was not significantly correlated with any anthropometric or metabolic parameters. Overall, endurance exercise training reduced hepatic fat content in spite of the absence of weight loss in elderly men, and the decrease in FGF-21 was positively associated with the reduction in intrahepatic fat (Taniguchi et al., 2016).

Contrastingly, 10 weeks of aerobic training (cycling on a stationary bike for 20 – 35 minutes at ~65% $\dot{V}O_{2peak}$, 4 – 5 days per week) did not alter circulating FGF-21 levels 12 overweight/obese men with T2DM (age 53.2 ± 1.6 years, BMI 33.2 ± 0.8 kg/m^2) or 12 overweight/obese glucose tolerant men (age 53.0 ± 1.4 years, BMI 33.5 ± 0.8 kg/m^2), despite significant exercise-induced improvements in insulin-stimulated GDR (Kruse et al., 2017). Moreover, exercise training did not alter the response of serum FGF-21 to insulin administration, implying that the ability of insulin to increase FGF-21 levels is dissociated from changes in insulin action on glucose uptake in peripheral tissues (Kruse et al., 2017). Consistent with these findings, an 8 week aerobic training intervention (5 days per week of cycling and running at a target heart rate corresponding to 35 – 85% of $\dot{V}O_{2max}$ for 45 to 60 minutes per session) did not alter circulating or skeletal muscle gene expression of FGF-21 in sedentary obese males (age 35.4 ± 1.5 years, BMI 32.6 ± 2.3 kg/m^2) (Besse-Patin et al., 2014). Exercise training significantly increased $\dot{V}O_{2max}$ ($p = 0.022$), decreased fat mass ($p = 0.033$) and tended to increase fat free mass ($p = 0.075$). However, no significant changes were reported for body weight, fasting glucose, fasting insulin or the Matsuda index of insulin sensitivity (Besse-Patin et al., 2014).

Cuevas-Ramos et al. (2012) investigated the impact of a 2 week intensive exercise programme on serum FGF-21 levels, in a group of young sedentary healthy women (n = 60, age 24.0 ± 3.7 years, BMI 21.4 ± 7.0 kg/m^2). Subjects were required to perform a treadmill exercise test following the Bruce protocol, a total of 9 times during the 14 day period. In brief, the test commences at a treadmill speed of 2.7 km and an incline of 10% gradient for 3 minutes. The speed and inclination are then subsequently increased every 3 minutes in a simultaneous way until volitional exhaustion. The mean duration of the exercise tests was 14.2 ± 1.4 minutes per session. The majority of subjects reached stage 4 of the Bruce protocol, with a mean metabolic equivalent (METs) consumption of 12.2 ± 2.4. No significant changes in anthropometric measurements (BMI, waist circumference, hip circumference, waist to hip ratio, %fat, and fat free mass) or circulating concentrations of total cholesterol, HDL, LDL, ALT, uric acid, creatinine, leptin or adiponectin were identified following 2 weeks of exercise training. As expected, there was a significant increase in daily physical activity after the intervention ($p < 0.0001$). Circulating AST, GGT and triglycerides were significantly decreased after 2 weeks of exercise ($p < 0.05$). Serum FGF-21 and plasma FFA concentrations increased

significantly following exercise training ($p < 0.0001$ and $p < 0.001$, respectively). Plasma epinephrine was also significantly increased after the intervention ($p < 0.0001$).

The delta (Δ, final – baseline levels) of log serum FGF-21 positively correlated with Δepinephrine ($r = 0.53$, $p < 0.001$), METs ($r = 0.40$, $p < 0.0001$) and ΔFFA levels ($r = 0.35$, $p < 0.0001$), and these correlations were maintained after adjustment for BMI. The mean resting heart rate ($r = 0.43$, $p < 0.0001$) and HR_{max} ($r = 0.54$, $p < 0.0001$) correlated positively with the Δlog serum FGF-21. The ΔFFA correlated positively with Δepinephrine ($r = 0.40$, $p < 0.01$), mean HR_{max} ($r = 0.22$, $p < 0.05$), METs ($r = 0.25$, $p < 0.05$), mean maximum diastolic ($r = 0.25$, $p = 0.025$) and systolic blood pressure ($r = 0.026$, $p = 0.03$). The mean duration of the exercise test correlated negatively with ΔFFA ($r = -0.34$, $p = 0.007$). Stepwise linear regression showed that baseline fasting glucose, ΔFFA, Δepinephrine, mean HR_{max} and MET's were factors independently associated with the increase in FGF-21 after exercise ($F = 3.48$, $r^2 = 0.54$, $p < 0.0001$). Additionally, fasting insulin, mean duration per exercise test, Δepinephrine and Δlog FGF-21 were independently related to the increment in FFA following the intervention ($F = 3.4$, $r^2 = 0.44$, $p = 0.001$). The authors concluded that the increase in circulating FGF-21 in response to exercise training could be explained by the exercise-induced adrenergic and lipolytic response in healthy, young women (Cuevas-Ramos et al., 2012).

Exercise training studies examining the changes in FGF-21 and the association with insulin sensitivity have reported discrepant findings. The studies reviewed above have considerable variability in study design (exercise frequency, intensity, time and type) and participant characteristics and have utilised relatively small sample sizes. Therefore, further studies are required to establish the impact of different modalities of exercise training, particularly aerobic, resistance and HIIT, on circulating FGF-21 and changes in whole-body and peripheral insulin sensitivity in healthy and clinical populations. However, there is some evidence to show that exercise-induced reductions in FGF-21 are associated with improvements in insulin sensitivity and intrahepatic lipid accumulation in older men with and without T2DM.

2.4.10.5.4. FGF-21 and Bed Rest, Physical Inactivity and Ageing

In a recent study, the effect of 5 days of controlled bed rest on circulating FGF-21, adiponectin and ceramides levels in healthy younger and older men and women was examined (Petrocelli et al., 2020). Changes in body composition and metabolic parameters were measured in younger (7 males, 6 females; age 23.4 ± 3.2 years, BMI 22.1 ± 3.4 kg/m^2) and older adults (11 males, 9 females; age 67.8 ± 5.5 years, BMI 24.9 ± 2.7 kg/m^2) before and after bed rest. At baseline, older adults had higher fat mass, fasting glucose, adiponectin, FGF-21, ceramides and total ceramides compared to the younger adults ($p < 0.05$). After 5 days of bed rest, lean mass and leg lean mass decreased significantly in the older adults only (age*interaction, both $p < 0.01$). There was a significant main effect of time for the decrease in insulin sensitivity (Matsuda), adiponectin and FGF-21 following bed rest ($p < 0.01$). Older adults displayed a significant reduction in total ceramide levels following 5 days of bed rest ($p = 0.005$). Increased ratios of ceramides are associated with increased cardiovascular risk. Ceramide ratios significantly increased after bed rest in older adults ($p < 0.05$). The ratios of C16:0/C24:0 and C24:1/C24:0 were significantly higher in older adults compared to younger adults on day 5 ($p < 0.05$). There were no significant changes in LDL, HDL, triglycerides, total cholesterol, fasting glucose, fasting insulin or HOMA-IR post-bed rest. The percentage increases in C16:0/C24:0 and C18:0/C24:0 inversely correlated with the percentage decrease in FGF-21 after bed rest in older adults ($r = -0.475$, $p = 0.003$ and $r = -0.483$, $p = 0.002$, respectively). In all subjects combined, the percentage change in FGF-21 correlated with the percentage change in adiponectin ($r = 0.300$, $p = 0.014$). The findings of this study show that 5 days of bed rest reduced insulin sensitivity and circulating FGF-21 and adiponectin in older and younger adults. The reduction in FGF-21 is unexpected as FGF-21 is commonly elevated in states of insulin resistance. The increase in ceramide ratios was associated with decreases in FGF-21 and adiponectin in older adults (Petrocelli et al., 2020). Further research is required to determine the specific roles of FGF-21 and adiponectin in bed rest-induced deterioration of cardiovascular function and insulin sensitivity.

Ageing is a risk factor linked to the deterioration of energy and glucose homeostasis. An age-related increase in circulating FGF-21 was observed in healthy individuals ranging from 5 to 80 years of age, independently of body composition (Hanks et al., 2015). In line

with these findings, serum FGF-21 levels were significantly increased in elderly individuals (n = 28, 14 males and 14 females; age 80.8 ± 1.1 years, BMI 27.4 ± 0.6 kg/m^2) compared with younger healthy individuals (n = 35, 23 males and 12 females; age 38.1 ± 0.6 years, BMI 24.4 ± 0.6 kg/m^2) (Villarroya et al., 2018). Despite the absence of T2DM, elderly individuals had elevated glycaemia and insulinemia compared to younger individuals ($p < 0.05$). Circulating FGF-21 levels were positively correlated with fasting glucose ($r = 0.197$, $p = 0.048$), fasting insulin ($r = 0.224$, $p = 0.038$) and HOMA-IR ($r = 0.427$, $p = 0.005$). Analysis of subcutaneous adipose tissue biopsies from elderly (n = 13) and younger (n = 10) adults, as well as adipose explants from aged and young mice, showed that elevations in circulating FGF-21 in healthy ageing are not associated with impaired responsiveness of aged adipose tissue to FGF-21, a finding that contrasts with observations in metabolic disease (Villarroya et al., 2018).

2.4.10.5.5. Summary of the Comprehensive Review on FGF-21

FGF-21 is a pleiotropic regulator that elicits physiological effects on glucose and lipid metabolism thereby contributing to energy balance. Much of what is currently known about the favourable metabolic actions of FGF-21 has been derived from animal studies. These actions occur predominately in the liver and adipose tissue, where the expression of FGF-21 is mainly under the control of PPARα and PPARγ, respectively. In the liver, FGF-21 mediates the metabolic adaptation to fasting/starvation and stimulates fatty acid oxidation, ketogenesis and gluconeogenesis. In adipose tissue, FGF-21 enhances glucose uptake and regulates lipolysis according to the metabolic state. These changes can result in improved insulin sensitivity. Unexpectedly, circulating FGF-21 levels are elevated in metabolic diseases characterised by insulin resistance including obesity, T2DM, NAFLD and the MetSyn. The elevated concentrations of FGF-21 may indicate the presence of FGF-21 resistance or a compensatory response to overcome the metabolic distress in these conditions leading to the upregulation of FGF-21. Several studies have also reported an age-related increase in circulating FGF-21. Acute exercise training increases circulating FGF-21 in response to elevations in the glucagon: insulin ratio and FFA in healthy individuals. However, acute exercise-induced secretion of FGF-21 appears to be blunted in individuals with T2DM. Divergent results have been reported from studies investigating the effect of chronic exercise training on circulating FGF-21 and therefore further investigation is required. To our knowledge, there is currently only one study that

has examined changes in circulating FGF-21 in response to bed rest. This study reported a paradoxical decrease in FGF-21 following 5 days of horizontal bed rest in younger and older adults, in concert with reduced insulin sensitivity and circulating adiponectin. The mechanisms underlying this change have not yet been determined and further investigation into the effects of severe physical inactivity on FGF-21 and insulin sensitivity is warranted.

2.4.11. Individual Variability

Up to now, the majority of researchers have reported a group of individual's responses to an intervention as means and standard deviations with a focus on the main effects (Royal et al., 2021). However, emerging evidence from human studies has confirmed that the magnitude of physiological response to even a standardised intervention differs between individuals (Hecksteden et al., 2015; Ross et al., 2019). Therefore, this analytical approach does not accurately reflect the true range of individual responses leading to potentially inappropriate inferences. The issue of individual variability to intervention is one of the most important in exercise and space physiology, particularly in the context of "personalised medicine", and explains the growing interest in this topic in recent years. Yet, standard approaches for the quantification and prediction of the inter-individual response or "subject-by-training interaction" remain to be established. Furthermore, biostatisticians in the fields of exercise and space sciences have highlighted concerns regarding the experimental and statistical rigor required to analyse individual response heterogeneity (Bonafiglia et al., 2019). Particular challenges in this field include the consideration of sources of variability, as well as the definitions and classification of "responders" and "non-responders" (Hecksteden et al., 2018). Furthermore, the sample size has to be sufficiently large, up to four times larger than that required to estimate the mean effect, or individual differences cannot be tested (Hopkins, 2018; Tobita et al., 2022). Substantial individual variability in physiological variables has been reported recently following bed rest, despite highly standardised and controlled experimental conditions (Fernandez-Gonzalo et al., 2021; Böcker et al., 2022). This has important implications for the optimisation of personalised interventions to maintain health following physical inactivity, extreme sedentarism and spaceflight. However, it is clear that many limitations need to be considered when quantifying individual variability in the context of bed rest. To overcome these limitations, several research groups have used

more conservative statistical approaches and the findings of these studies are presented below. It is clear that the investigation of individual variability in physiological responses to bed rest is pivotal, but best practice in this regard is still under development.

2.4.11.1. Sources of Variation

When a measurement is taken, the observed value is influenced by both the individual's true value and random measurement error, also known as the typical error of measurement (TE_M). TE_M can result from a combination of the technical error introduced by equipment and/or tester reliability and the random day-to-day variability in biological factors that affect an individual's mental and/or physical state during testing (for example, behavioural and lifestyle factors including diet, physical activity, sleep) (Bonafiglia et al., 2019; Böcker et al., 2022). Both technical error and day-to-day variability will introduce "noise" to any measurement, but it is expected that this interference will be normally distributed around the true observed value. In a training intervention, the individuals observed change will encompass the true change from pre- to post-intervention and the TE_M relative to both pre- and post-intervention observed values (Bonafiglia et al., 2019). Biological variability, specifically chronic changes in behaviour and/or environmental factors external to the intervention, can also influence an individual's observed change. This source of variation is termed as within-subject variability and the impact of this is expected to increase with longer duration interventions (Atkinson and Batterham, 2015; Bonafiglia et al., 2019). Additionally, there is a familial/genetic component which contributes to physiological response variability (Ross et al., 2019).

2.4.11.2. Isolating Individual Differences

The standard deviation of observed pre- to post-changes in the intervention group provides a basic estimate of gross response variability. However, this standard deviation comprises both the subject-by-training interaction and random variation, leading to a possible overestimation of response (Hecksteden et al., 2018). From a methodological point of view, repeating the intervention (after a sufficient washout period) is the most viable solution for delineating the individual response from confounding factors. However, this is rarely employed as it is logistically challenging and relies on the assumption that the intervention leads to reversible adaptations only. More practical

alternatives for the separation of random error from the training response are: 1) control group designs; 2) external reliability; 3) use of repeated measurements during the intervention (Hecksteden et al., 2018).

Comparing the variability of pre-post changes in the intervention group and a control group, e.g. parallel-design randomised controlled training study (RCT), by determining the standard deviation of individual responses (SD_{IR}) has been proposed as the most feasible approach to isolate and quantify the magnitude of individual responses due to the intervention *per se* (Atkinson and Batterham, 2015; Hopkins, 2015; Hecksteden et al., 2018; Hopkins, 2018). SD_{IR} is calculated using the following formula: $SD_{IR} = \sqrt{(SD_{INT})^2 - (SD_{CON})^2}$, where SD_{INT} and SD_{CON} represent the standard deviation of observed response to the intervention or control, respectively. Once calculated, the typical error (TE) of SD_{IR} and the 90% confidence intervals (CI) can be calculated using the following equations: $TE\ (SD_{IR})^2 = \sqrt{[2\ (SD_{INT}^4 / df_{INT} + SD_{CON}^4 / df_{CON})}$ with df = n - 1 for respective group, and 90% CI: $[(SD_{IR})^2 - 1.65 * TE\ (SD_{IR})^2] < (SD_{IR})^2 < [(SD_{IR})^2 + 1.65 * TE\ (SD_{IR})^2]$ (Hecksteden et al., 2018). It is important to note that the calculation of confidence intervals for SD_{IR} assumes a normal distribution of sampling variance. When SD_{INT} and SD_{CON} are similar, it can be concluded that there is negligible inter-individual variability in the response to the intervention. However, when the SD_{INT} is substantially greater than the SD_{CON}, an individual response is inferred. This would justify further investigation into the potential moderators and mediators, using more advanced statistical modelling (Williamson et al., 2017; Fernandez-Gonzalo et al., 2021). In some instances, the variance in individual responses and/or confidence intervals may be negative, implying greater variability in response in the control group *versus* the intervention group. This could be due to imprecision in the estimation of individual response as a result of small sample sizes or a large amount of "noise" and/or the intervention "homogenising" outcome measurements thereby reducing the standard deviation changes relative to the control group (Atkinson and Batterham, 2015).

In the absence of a control group, reliability characteristics of the physiological outcome can be used to estimate the contribution of random measurement error or TE_M to observed response variability. It may be estimated using data from repeated measurements from

several subjects (i.e. a "reliability trial"). Alternatively, external reliability can be used and in this instance, TE may be estimated by multiplying the published coefficient of variation with the mean baseline level in the present trial (Hecksteden et al., 2018). External data should be obtained from a research study with similar subjects, measurement timing and protocols (Atkinson and Batterham, 2015; Hopkins, 2018).

Repeated measurements permit the calculation of the slope of the personal regression line of measured variables overtime (Hecksteden et al., 2018). The uncertainty in the response estimate can be quantified on an individual basis as the scatter of measured values around the regression line (TE of B). Subsequently, SD_{IR} may be estimated by calculating the standard deviation of B. In contrast to pre-post designs with single measurements only, repeated measurements allow for the calculation of the subject-by-training interaction using linear mixed models. Further to this, personal estimates of central tendency (i.e. individual slope or overall change) and variation (TE of B or standard deviation of segmental changes) enable the calculation of frequentist indicators (e.g. effect sizes) at the fully individualised level (Hecksteden et al., 2018).

2.4.11.3. Limitations on the Interpretation of SD_{IR}

The inherent assumptions of a parallel-design RCT study imply that subjects in the intervention and control groups differ only by the treatment they receive (e.g. exercise and no exercise, respectively) and the TE_M and within-subject variability are the same in both the intervention and control groups (Hopkins, 2018; Bonafiglia et al., 2019). If the latter assumption is violated, then the SD_{IR} must be interpreted with caution. Non-optimal RCT designs (e.g. different equipment, experiments and/or testing schedules), and insufficient participant adherence and compliance (e.g. < 90% attendance to training) may invalidate the assumption that TE_M and within-subject variability are equal between the intervention and control groups. However, these can be avoided or reduced by rigorously controlled and standardised RCT's. Inherent limitations (e.g. inability to blind participants) causes risk to the within-subject variability assumption as it may introduce performance or preference bias and needs to be considered in future studies (Bonafiglia et al., 2019).

2.4.11.4. Responders and Non-Responders

The definitions of "responders" and "non-responders" varies considerably. Categorisation of individuals as "responders" and "non-responders" according to an observed change in a physiological outcome requires a specified response threshold and a method to deal with the limited accuracy of the individual response estimate (Hecksteden et al., 2015; Hecksteden et al., 2018). The determination of a response or non-response is also dependent on subject characteristics as well as the length and type of a training intervention and criterion outcome, as the timeline of adaptations can differ considerably (Hecksteden et al., 2015; Hecksteden et al., 2018). A multitude of response thresholds have been proposed including (1) zero change as a fixed threshold value; (2) the upper limit of observed differences that may be expected as a result of random variation; (3) quantiles of observed changes due to the stressor or intervention and (4) lower limit of a clinically or practically meaningful difference (Hecksteden et al., 2015; Hecksteden et al., 2018).

Defining a non-response as a difference from pre- to post-intervention as zero or less (for outcomes that typically increase with an intervention) is relatively straightforward. However, this approach does not take into account the limited accuracy of observed training effects (Hecksteden et al., 2015). Alternatively, the borderline between trivial and substantial effects has been suggested as a preferable criterion, using a cut-off as the minimum clinically important difference (MCID) or smallest worthwhile difference, typically 0.2 of the between-subject SD at baseline (Atkinson and Batterham, 2015; Hecksteden et al., 2018). For health-related indicators, the criteria may be associated with a small substantial change in risk of morbidity or mortality, a hazard ratio of 10/9 (Hopkins et al., 2009; Hopkins, 2018). From a statistical perspective, the coefficient of variation for the physiological outcome delineates differences that may be expected as a result of random variation from true changes. Despite a more conservative approach, there is uncertainty in the classification of an individual because of random variation in observed values and within-subject variability in physiological response, which may occur regardless of the chosen cut-off value. This uncertainty may be quantified using linear mixed modelling, which provides specification of the confidence limits for each individual's response (Hecksteden et al., 2015). A combination of this statistical technique with prefixed limits of practical relevance, detailed by Hopkins (2000), is the

most informative option to determine response or non-response in a single observed training effect. However, it must be noted that the underlying assumption of negligible within-subject variability in the efficacy of an intervention is untested. The use of quantiles allows for a suitable way to contrast balanced subgroups with marked differences in physiological responses, but it fails to provide insights into the distribution and variability of such responses because a predefined percentage of subjects will be classified as non-responders (Hecksteden et al., 2015).

2.4.11.5. Individual Variability in Bed Rest Studies

Until recently, individual variability in physiological responses to bed rest (horizontal or HDT) had not been investigated, despite providing a highly standardised and controlled experimental testbed. However, the sample size in bed rest studies is typically small due to staff and economic constraints (Fernandez-Gonzalo et al., 2021). To overcome this limitation, researchers have compiled data from numerous studies thereby increasing statistical power to address individual variability. Fernandez-Gonzalo et al., (2021) examined the individual responses in skeletal muscle outcomes following bed rest, through the compilation of data from three different bed rest studies conducted in Planica (21 day PlanHab; 10 day FemHab and LunHab). Within each study, subjects (n = 35) participated in three cross-over campaigns: normoxic (NBR) and hypoxic bed rest (HBR) and hypoxic ambulation (HAMB, which acted as the control). The findings of this study indicated that knee extensor torque, thigh muscle area and calf muscle area were most significantly affected by bed rest. Furthermore, individual variability in knee extensor torque and calf muscle area were classified as clinically relevant (i.e. range of the overall typical overall effect was greater than 5%). Specifically, if a random individual underwent a period of bed rest in similar conditions than those in Planica, a loss of 0 to 18% and 2 to 12% could be expected for knee extensor torque and calf muscle area, respectively. Examination of potential moderators of individual variability found that baseline conditioning, but not deviations in tailored dietary intake, was an influencing factor on the loss of muscle mass and strength, such that individuals who are bigger and/or stronger (in terms of muscle mass) will lose the most muscle mass and strength during bed rest. However, it was highlighted that these individuals have bigger safety margins to overcome the physiological adaptation to bed rest. Further to this, examination of repeatability suggested that the magnitude of muscle atrophy for a specific individual

after bed rest could be predicted fairly accurately by the extent of muscle mass lost in a previous unloading intervention (Fernandez-Gonzalo et al., 2021). The findings of this study have important implications for the health management and personalised training recommendations for healthy bed rest subjects and astronauts, as well as individuals who are hospitalised due to illness or injury.

In a more recent publication, Böcker et al. (2022) analysed individual responses from peripheral quantitative computer tomography (pQCT) data of the lower leg of 76 subjects from 8 different bed rest studies (AGBRESA, BBR, LTBR, MEP, NUC, PlanHab, RSL and Valdoltra). Specifically, between-subject variability, within-subject variability and measurement uncertainty were quantified using a defined statistical framework. The data was predominately from control subjects only, except for calculations of measurement uncertainty. The results of this study showed that measurement uncertainty using pQCT was small, albeit higher for muscle than bone sites, and therefore cannot explain the musculoskeletal adaptation to bed rest. There was evidence of substantial between-subject variability for bone loss and muscle atrophy (even paradoxical responses) and this was greater in muscle compared to bone measures. However, adjusted between-subject deviation (i.e. the uncertainty of individual response relative to the averaged change per week for each study group) was higher for bone than muscle. With regards to within-subject variability, there appeared to be little variability in the bed rest response within an individual's calf musculature, while there was large variability in the tibia, even after dividing epiphyseal measurement sites into compact and trabecular bone. The authors proposed that exercise participation and dietary habits prior to the bed rest studies may have contributed to the between-subject variation, in addition to genetic and epigenetic pre-disposition. Additionally, differences in the characteristics and metabolic processes of bone and muscle may explain the presence of within-subject variation (Böcker et al., 2022).

Together, the results of these recent investigations highlight substantial individual variation in physiological adaptation under controlled conditions, and highlight that some individuals (and astronauts) may be more resilient than others to maintain a sufficient physiological status, following reduced gravitational unloading. Baseline training status

appears to be a key moderator of individual variability in physiological responsiveness following bed rest, in addition to changes in pre-study dietary intake and genetic and epigenetic factors. It is important to highlight that these few studies have focused on the individual variability in muscle and bone, but not metabolic characteristics, in response to inactivity and extreme sedentariness. Further, the findings of these studies provide impetus for a more personalised approach to countermeasure design and prescription to maintain optimal health (Böcker et al., 2022).

2.4.11.6. Conclusion of Individual Variability

It can be concluded that understanding individual variability is of general interest. The quantification of individual response heterogeneity to intervention is complex and dependent on good trial design supported by appropriate statistical analysis and sufficient sample size. Both exercise training and bed rest studies have demonstrated the importance of duplicate baseline (repeated) measurements, unifying standard operating procedures, monitoring physical activity and dietary habits prior to the study and inclusion of a control group. Future research studies need to acknowledge and implement these recommendations to further optimise the estimation of individual response to intervention. It is recommended that bed rest studies should include an ambulatory control group to determine whether bed rest only or bed rest with an intervention modulated the individual variation in physiological response (Fernandez-Gonzalo et al., 2021). Investigation of individual variability in metabolic responses to bed rest, using compiled data and appropriate statistical modelling akin to the most recent bed rest publications (Fernandez-Gonzalo et al., 2021; Böcker et al., 2022), is required to explore the mechanisms that influence individual responsiveness and guide personalised countermeasure prescription. However, such analysis is beyond the scope of this PhD. This research would offer new tools to ameliorate the physiological adaptations to physical inactivity, extreme sedentarism and spaceflight.

2.5. Exercise as a Countermeasure

A countermeasure refers to the use of procedures, whether physical, chemical, biological, or psychological to sustain physiological functioning and mission performance (Clément, 2011b). The aim of a countermeasure is to prevent or mitigate the undesirable

physiological adaptations associated with spaceflight to maintain astronaut health and safety. Optimising countermeasures is a priority for space agencies to permit long-duration spaceflight.

2.5.1. Exercise in Space

Exercise is a well-regarded countermeasure used during spaceflight to combat the deleterious physiological adaptations of microgravity exposure (Macaulay et al., 2016). However, in-flight exercise data is often classified and protected medical information and is only published on the basis of obtaining data sharing agreements. Additionally, the evaluation of in-flight exercise data is difficult due to issues relating to instrumentation malfunction, downlink interruption, software bugs and operator error and therefore, it is often challenging to document in detail. Despite this, it is clear that exercise prescription during spaceflight has focused predominately on concurrent resistance and aerobic training. Numerous studies have shown that resistance training can stimulate muscle hypertrophy and produce an osteogenic stimulus to preserve musculoskeletal mass and function, elicit improvements in neuromuscular function and strength and insulin sensitivity (increased lean mass, glucose disposal and resting metabolic rate) (Strasser and Schobersberger, 2011; Fyfe et al., 2021) and aerobic training can improve $\dot{V}O_{2max}$, through enhancements in cardiac output and muscle perfusion, increase the proportion of slow-twitch muscle fibers and mitochondria and induce favourable changes in body composition and insulin sensitivity (Hellsten and Nyberg, 2015; Bird and Hawley, 2017). Hence, these two different types of exercise training form the basis of exercise training in spaceflight since they have the potential to counteract the negative physiological adaptations that occur in astronauts in space.

Early exercise hardware on the International Space Station (ISS) consisted of a treadmill with a vibration isolation system (TVIS), which was limited to low running velocities (~11.3km/hr peak velocity) and an interim resistive exercise device (iRED), which was restricted to low loads (136kg peak load). Therefore, the default exercise prescription for astronauts was curtailed to low-intensity, high-volume exercise training, which failed to protect against decrements in musculoskeletal mass, strength and function and cardiorespiratory fitness (Lang et al., 2004; Gopalakrishnan et al., 2010; English et al.,

2015; Moore et al., 2015). To facilitate higher intensity aerobic and resistance training, the ISS exercise hardware was replaced in 2009/2010 with a second generation treadmill (T2) permitting increased running velocities (~19.3 km/hr peak velocity) and the advanced resistive exercise device (ARED) with enhanced loading capabilities (~272kg peak load). A cycle ergometer with a vibration isolation system (CEVIS) is also available on board the ISS. Similarly, the T2 and ARED are mounted on a vibration isolation system to protect the structural integrity of the ISS by minimising the transfer of force generated during exercise to the modular space station (English et al., 2020).

Exercise prescription in space is provided by Astronaut Strength, Conditioning, and Rehabilitation specialists (ASCRs) based on individual fitness assessments (including $\dot{V}O_{2peak}$ tests performed 60-90 days before launch and ground-based resistance training sessions) and extensive experience working with other astronauts in microgravity. The current time allocation for exercise training in space is 2.5 hours per day, 6 days per week. Astronauts typically perform ~ 60 – 75 minutes of resistance training and ~ 30 – 45 minutes of aerobic training using the ARED, T2 and CEVIS (Scott et al., 2019; English et al., 2020). This equates to a weekly exercise volume total of approximately 9 hours to 12 hours. The remaining allocated time is taken up by equipment setup, hardware inspections, and transitions between exercises, data logging, stowage and cleaning up.

In line with the growing body of literature demonstrating that high-intensity, low volume exercise training can elicit positive musculoskeletal and cardiovascular adaptations, which may be superior to that of low-intensity, high volume exercise training (Gibala and McGee, 2008; Shiraev and Barclay, 2012; MacInnis and Gibala, 2017), the effectiveness of a new evidence-based exercise prescription for ISS crewmembers has been examined and published. According to the review by Hackney et al., (2015), this new exercise prescription was influenced by detraining studies reporting that exercise intensity is crucial for maintaining cardiorespiratory fitness, exercise studies confirming the efficacy of different interval durations for improving cardiorespiratory fitness and findings from HDT bed rest and limb immobilisation studies showing that lower frequency (~3 days per week) of resistance training is sufficient to maintain muscle mass and strength. English et al., (2020) compared physiological outcomes after long-duration spaceflight (~6

months) in ISS crewmembers performing either (i) low-intensity, high volume exercise training (control or CON group; n = 17 (14 males and 3 females), 46 ± 6 years, 176 ± 6 cm, 80.6 ± 10.5 kg; resistance training and aerobic training, 6 days/week with a total weekly volume of 9 – 10 hours) or (ii) high-intensity, low volume exercise training (SPRINT group; n = 9 (8 males and 1 female), 48 ± 7 years, 178 ± 5 cm and 77.7 ± 12.0 kg; resistance training, continuous aerobic training and aerobic interval training, 3 days/week each with a total weekly volume of 6 hours).

Exercise prescription for the CON group was consistent with the standardised programme for ISS astronauts provided above. Aerobic training consisted of interval or continuous steady state exercise at an intensity of 70 – 100% pre-$\dot{V}O_{2peak}$ on the CEVIS and 70 – 100% pre-HR_{max} on T2 with external loading of 60 – 80% body weight. Resistance training followed a 9 day periodised programme with linear progression of loads and undulating volume in two 12 week mesocycles and incorporated exercises for the lower body, upper body and stability musculature. Exercise prescription for the SPRINT group consisted of high-intensity interval aerobic exercise (one type of following intervals per session: 8 repetitions of 30 second intervals at 100% pre-$\dot{V}O_{2peak}$; 6 repetitions of 2 minute intervals at 70 – 100% pre-$\dot{V}O_{2peak}$; or 4 repetitions of 4 minute intervals at 90% pre-$\dot{V}O_{2peak}$), continuous aerobic exercise (30 minutes at 75% pre-$\dot{V}O_{2peak}$) and high-intensity resistance training (an undulating periodised programme in a single 24 week mesocycle, performed 4 – 6 hours prior to continuous aerobic exercise and involving exercises for the lower body, upper body and stability musculature). For a full detailed explanation of the training protocol, the author directs the reader to the supplementary information provided by English et al., (2020).

The results of this study showed significant decreases in muscle strength and endurance, BMD, functional muscle performance (cone agility test) and $\dot{V}O_{2peak}$ post-flight and these changes were mostly independent of the exercise protocol performed. Significant group*time interaction effects were only detected for flexibility (sit and reach) and two measures of muscle strength and endurance immediately post-flight, which were attenuated in SPRINT compared to CON. Leg lean mass did not change, but there was a significant decrease in leg fat mass irrespective of the exercise protocol performed. Taken

together, both exercise protocols elicited similar physiological outcomes following long-duration spaceflight and provided substantially better physiologic protection when compared to historic exercise programmes, but the SPRINT protocol required at least 33% (~3 hours) less time than the current standard training protocol for astronauts on the ISS (English et al., 2020). Therefore, in the interest of conserving time while optimally preserving the physiological health of astronauts, the SPRINT protocol or a similar high-intensity, low volume form of exercise training should be utilised in future spaceflight exercise prescriptions.

2.5.2. Exercise in HDT Bed Rest

Due to the challenges of collecting in-flight exercise data, HDT bed rest is widely used as an experimental model to evaluate the effectiveness of exercise countermeasures. The impact of resistance training, combined resistance and aerobic training and resistive vibration exercise has been examined in various horizontal and HDT bed rest studies spanning from 21 – 90 days. It is important to note that comparisons between the findings of HDT bed rest studies can be challenging due to differences in the total number of subjects included in a specific analysis conducted by each contributing research team and the lack of a centralised database of published papers from specific HDT bed rest studies.

Resistance flywheel exercise, performed for 35 minutes every 3 days during 90 days HDT bed rest (involving a progressive warm-up, 4 sets of 7 repetitions of concentric/eccentric squats and 4 sets of 14 repetitions of a calf press with 2 minutes rest between sets and 5 minutes rest between exercises), attenuated the loss of muscle mass, knee extensor strength and alterations in myofiber size and phenotype but did not prevent the loss of plantarflexor strength or deleterious changes in glucose and lipid metabolism in young healthy males following long-duration HDT bed rest (Gallagher et al., 2005; Bergouignan et al., 2006; Belavy et al., 2017).

A combined protocol of resistance training (3 days per week of maximal concentric and eccentric actions in the supine squat and calf press for 35 minutes) and aerobic training (2 days per week on a vertical treadmill in a lower-body negative-pressure (LBNP;

application of ambient pressure lower than normal atmospheric pressure to the lower quadrant of the body to induce foot-ward fluid shift) chamber at any intensity ranging between 40 and 80% pre-bed rest $\dot{V}O_{2peak}$ for 50 minutes) performed during 60 days HDT bed rest preserved myofiber size and phenotype, $\dot{V}O_{2peak}$ and increased calf press strength, but could not attenuate the loss of isometric knee extensor strength in young, healthy females (Trappe et al., 2007; Salanova et al., 2008; Schneider et al., 2009). Additionally, this combined protocol mitigated the shift in substrate utilisation in the fasting and fed states and the rise in postprandial insulin following HDT bed rest. However, the exercise group were in a negative energy balance after HDT bed rest limiting the ability to dissociate the metabolic impact of negative energy balance from that due to exercise (Bergouignan et al., 2009). The efficacy of aerobic training with added LBNP (6 days per week of supine treadmill exercise at 40 – 80% pre–bed rest $\dot{V}O_{2peak}$ against LBNP at an intensity of 1.0 to 1.2 times body weight for 40 minutes, followed by 5 minutes of resting LBNP) to maintain upright exercise capacity was also confirmed in a 30 day HDT bed rest study in identical male twins (Lee et al., 2007).

In addition to resistance training, the efficacy of whole-body vibration during resistive exercise (commonly termed resistive vibration exercise or RVE) to mitigate the physiological adaptations of HDT bed rest has been examined. The premise of vibration training is the utilisation of a machine-generated force at the feet to elicit a training stimulus (Gruber et al., 2019). The vibratory inputs stimulate additional muscle activity during contraction, *via* the muscle spindle system, permitting greater force generation and a stimulus for muscle maintenance and/or hypertrophy (Belavý et al., 2009). Typically, RVE is done with vibration frequencies of 20 – 40 Hz and with peak-to-peak amplitudes up to 12mm (Gruber et al., 2019). Following a 21 day HDT bed rest study, RVE (5 sessions in total of a leg press protocol involving bilateral squats, single heel raises and bilateral heel raises performed on a vibration platform – 8 mm peak-to-peak amplitude and 25 Hz frequency) proved to be an efficient strategy for maintaining insulin sensitivity and mitochondrial respiration, but not lean mass or $\dot{V}O_{2peak}$, in healthy young males (Kenny et al., 2017; Kenny et al., 2020). In a longer duration study, RVE (11 sessions per week of 6 minutes duration, involving 4 resistive exercises (squats, heel raises, toe raises, and kicking exercises) on a vibration platform – 3.5 – 4 mm amplitude and 19 – 26 Hz frequency) was effective in attenuating the atrophic response, preserving myofiber

phenotype and size and muscle strength of the leg extensors and preventing bone loss in healthy young males after 56 days horizontal bed rest (Blottner et al., 2006; Mulder et al., 2006; Belavý et al., 2009; Rittweger et al., 2010). Additionally, in a follow-up study, it was demonstrated that vibration *per se* can contribute to bone, but not muscle, maintenance (Mulder et al., 2009; Belavý et al., 2010; Belavý et al., 2011).

To summarise, it is clear that exercise training can elicit some positive benefits for astronauts in space, but in spite of on-going investigations and refinement of exercise prescription in space and HDT bed rest, no exercise protocol to date has fully mitigated the deleterious physiological adaptations associated with microgravity and gravitational deprivation. In future space exploration missions, it is possible that greater restrictions will be placed on exercise time as well as space for exercise hardware and therefore, it is critical to establish a time-efficient mode of training with limited equipment usage that has a substantial positive influence on an astronaut's overall physiological health. Such investigations also have important implications for exercise prescription in Earth-based contexts including physical inactivity, sedentary behaviour, ageing, and immobilisation due to injury or illness.

2.5.3. High-Intensity, Plyometric Based Training – A Novel Training Mode

2.5.3.1. Mechanical Loading and the Impact on Bone Remodelling

The principal of using an exercise capable of inducing a higher strain rate is based on the mechanostat theory of bone remodelling and the knowledge that muscle contraction is important in the maintenance of the skeletal system (Rittweger, 2007; Kramer et al., 2012; Tyrovola and Odont, 2015). The mechanostat model, originally proposed by H.M. Frost suggests there is an existence of a homeostatic regulatory mechanism within bone tissue for sensing exposure to mechanical loading and subsequently alternating bone structure, size and strength to meet the required biomechanical demands (Frost, 1987; Frost, 1996). If the strain elicited is within the normal range (e.g. 1000 – 2000 micro-strains), bone mass and strength is maintained. If the strain rate is too low, bone degradation and resorption will occur (Kramer et al., 2012). If bone is exposed to high strain rates, bone formation will occur leading to an increase in bone strength. On Earth, movements such as walking and jumping create enough force to stimulate the deformation of bone. The

opposite is true for inactivity and spaceflight, in which muscular loading is dramatically reduced (Kramer et al., 2012). This reduction in mechanical loading will have an adverse effect on bone through the acceleration of bone resorption, the process in which old bone is removed from the skeleton (Kini and Nandeesh, 2012).

Osteoclasts (large bone cells) facilitate bone resorption by dissolving the mineral matrix and removing the osteoid matrix of the bones (Figure 15). Macrophages assist in the completion of the process and the release of growth factors contained in the matrix including transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF) and insulin-like growth factor 1 and 2 (IGF-1 and IGF-2) (Kini and Nandeesh, 2012). Alternatively, if an exercise countermeasure facilitated muscle contraction and sufficient mechanical loading to be detected by the osteocyte network, the subsequent phases of the bone remodelling process to produce new bone could be initiated. Following bone resorption, remodelling occurs during the reversal phase in which bone resorption is overcome by bone formation. Once osteoclasts have resorbed bone, they retract from the bone surface, leaving the cavities filled with monocytes, osteocytes and preosteoblasts to activate bone formation. The osteoblasts then attach to the lining of the bone and the growth factors within the cavities are released to act as chemotactics and proliferate. A cementing substance is then secreted, upon which new bones attach, and bone morphogenic proteins are stimulated to allow for differentiation and the formation of an osteoid matrix which will fill the perforated areas of the bone. Bone formation continues until the osteoblasts transform into lining cells that cover the new bone surface and the process ends with mineralisation of the bone. Mechanical loading can further increase bone strength by influencing collagen alignment as new bone is being formed (Kini and Nandeesh, 2012).

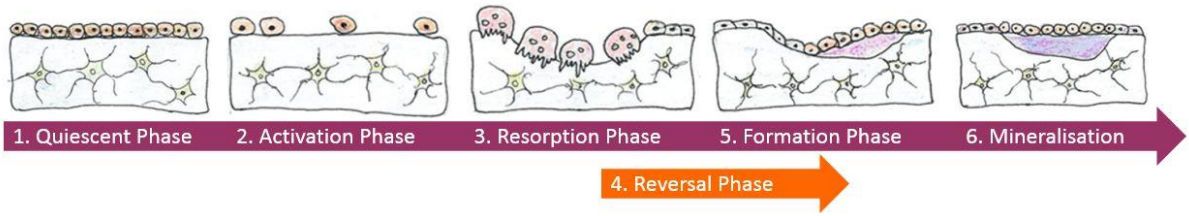

Figure 15. Schematic diagram of the process of bone remodelling.

Adapted from Kini and Nandeesh (2012).

2.5.3.2. Mechanical Loading and the Regulation of Muscle Mass and Strength

Mechanical loading has a similar effect of muscle mass and strength in addition to bone. Reductions in muscle mass and strength occur as a result of reduced mechanical loading causing protein breakdown rates to exceed those of protein synthesis (Fanzani et al., 2012). Conversely, high load, resistance-based exercise training permits the growth of skeletal muscles through an increase in protein contractile content (Hoppeler et al., 2011).

Multiple different molecular pathways control the formation and degradation of muscle mass. The key component and principal integrator of the signalling pathways involved in hypertrophy is mTOR (Hoppeler et al., 2011). mTOR is a serine-threonine protein kinase, that is imperative for cell survival and proliferation, apoptosis, autophagy and is a key factor in the regulation of muscle protein synthesis (Miyazaki and Esser, 2009). The three main activators of mTOR are insulin and growth hormone dependent growth factors which act through the insulin receptor (IGF-R)-Akt pathway, mechanical loading through protein kinase B (Akt)-dependent and Akt-independent pathways in a PI3K independent manner and nutritional influences, particularly in the presence of leucine and other essential amino acids that can be sensed through mTOR (Hoppeler et al., 2011). mTOR facilitates protein synthesis, through the activation of translation and elongation, by phosphorylating its effector kinase p70S6K. The activity of mTOR is inhibited by two important regulators AMPK and myostatin. In states of low cellular energy status, mTOR activity is decreased through the activation of AMPK and AMPK-dependent phosphorylation of tuberous sclerosis complex 2 (TSC2). Myostatin, a potent molecule in muscle wasting, can depress the activity of mTOR (Hoppeler et al., 2011).

mTOR exists in two multiprotein complexes which differ in structural and functional capacity: mTORC1 and mTORC2. mTORC1 is a protein complex consisting of five core units; regulatory-associated protein of mTOR named Raptor, mammalian LST8 (mLST8), proline-rich Akt substrate 40 kDa (PRAS40) and Deptor, the DEP-domain-containing mTOR-interacting protein (Hoppeler et al., 2011; Laplante and Sabatini, 2009). mTORC1 has a ribosomal protein S6 kinase β-1 (p70S6K1) as a primary phosphorylation target and augments translation through the inhibition of the eukaryotic translation initiation factor 4E-binding protein (4E-BP1) (Hoppeler et al., 2011). The mTORC1 pathway integrates the inputs of growth factors and hormonal status, amino acid availability, energy status and mechanical stress (Bond, 2016). In contrast to mTORC1, mTORC2 regulates insulin-stimulated glucose uptake *via* a feedback mechanism on Akt, but it is not involved in muscle contraction signalling (Hoppeler et al., 2011).

The exercise-induced increase in protein accretion is a consequence of mTOR associated signalling. However, muscle growth has been shown to be limited to 20% of the initial muscle fiber size. Increases in size beyond this are supported by DNA recruitment through the recruitment of satellite cells, which function in proliferation and differentiation to maintain the nucleus/cytoplasm ratio (Hoppeler et al., 2011). Resistance-based, intense muscular contractions have been shown to increase anabolic muscle signalling through the activation of the mTOR pathway, thus it is likely that jump training would be sufficient in eliciting the necessary stimulus to activate the molecular pathway to stimulate muscle hypertrophy (Figure 16).

The signalling network that is involved with the loss of muscle atrophy through inactivity involves protein kinase B (Akt). Akt plays a role in the phosphorylation of forkhead box O (FOXO) transcription factors, specifically blocking FOXO3 from entering the nucleus and upregulating the transcription of muscle atrophy F-Box (MAFbx/Atrogin-1) and muscle RING finger- 1 (MuRF1) (Fanzani et al., 2012). These two ligases are responsible for protein degradation which is controlled by the ubiquitin-proteasomal pathways. This pathway can be influenced by classic hormones including insulin, IGF-1 and TNF-a, such that reductions in insulin and release of pro-inflammatory cytokines lead to increased

ubiquitin expression and increased ubiquitinated proteins such as MAFbx. Another molecule associated with muscle atrophy is NF-κB. It is often linked with increased nitric oxide concentrations and can directly bind to MuRF1 and instigate a proteasome-dependent loss of muscle mass (Fanzani et al., 2012).

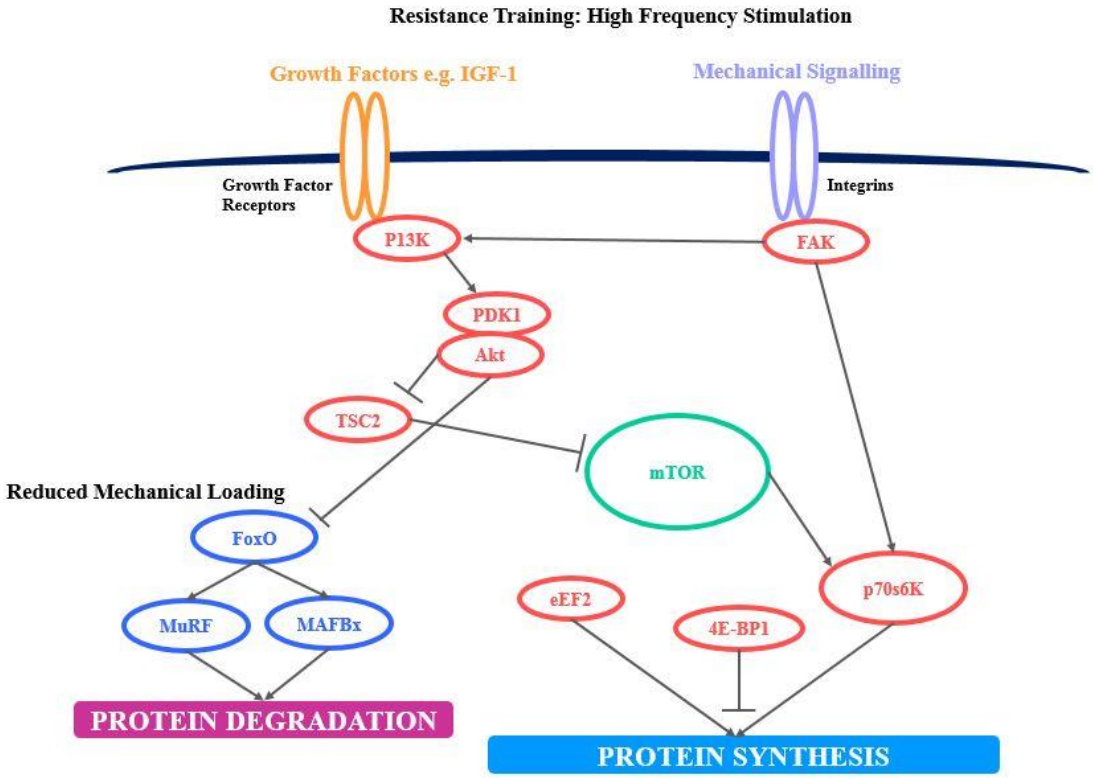

Figure 16. The molecular pathways by which skeletal muscle growth is stimulated as a result of high load, resistance based training and muscle atrophy ensues from reduced mechanical loading.

Adapted from Hoppeler et al., (2011). This diagram depicts two of the major influencers in the activation of mTORC – growth factors and mechanical loading. Phosphoinositide-3-kinase (PI3k) activation phosphorylates and stimulates protein kinase B (Akt) and its movement to the plasma membrane. Akt activates mTOR. Insulin-like growth factors can activate the mTORC1pathway through the receptor tyrosine kinase (TRK)-Akt-PKB signalling pathway. Akt phosphorylates tuberous sclerosis complex 2 (TSC2), leading to the activation of mTORC1, permitting protein synthesis *via* insulin signalling. Focal adhesion kinase (FAK) is also involved in the mechno-regulated signalling process of hypertrophy, and can activate ribosomal protein S6 kinase β-1 (p70S6K) in an Akt-independent way. Muscle degradation is activated through the phosphorylation of forkhead box O (FOXO) transcription factors, which upregulate the transcription of muscle atrophy F-Box (MAFbx/Atrogin-1) and muscle RING finger-1 (MuRF1).

From the evidence presented here it is inconceivable that reductions in mechanical loading through physical inactivity, sedentary behaviour and microgravity exposure will cause an imbalance in anabolic versus catabolic factors leading to the loss of protein content of the muscle and insufficient tension on be relied on to the skeleton system leading to increased bone degradation over formation (Kini and Nandeesh, 2012). Therefore, exercise countermeasures pertaining high forces, which can be relayed in short

periods of time to allow for high rates of force development (RFD), are essential to generate high strain rates to maintain, and possibly increase, musculoskeletal geometry and strength (Kramer et al., 2012). The loss of musculoskeletal structure is higher in the leg muscles and bones, in contrast to the trunk and arms, which usually bear the force of gravitational loading with movement on Earth and thus most countermeasure are designed to specifically enhance lower limb musculoskeletal activity (Kramer et al., 2012).

2.5.3.3. Reactive Jumps as a Novel Training Mode

A known, natural movement that produces high ground reaction forces (GRF) and RFD is reactive jumps. The movement of reactive jumps requires the performance of explosive movements encompassing short ground contact times and rapid upward accelerations. The movement requires high leg stiffness to store the elastic energy produced in the eccentric phase, and its release during the subsequent concentric phase, a mechanism known as the stretch-shortening cycle (Gollhofer and Kyröläinen, 1991). Reactive jumps have been shown to elicit markedly higher GRF and RFD when compared with other exercise modes including running and walking (Ebben et al., 2010). The reactive jumps require the individual to be in a “tip-toe” position, as seen in Figure 17. Due to the mechanical advantage of the ankle joint (4cm: 12cm = 1: 3), the calf muscles are required to produce a force more than three times larger than the GRF when the heel is lifted off the ground and propelled during the jumping movement (Rittweger, 2007). Thus, the kinetic characteristics of reactive jumps allow for the establishment of force and loading required to produce an osteogenic stimulus (Ebben et al., 2010).

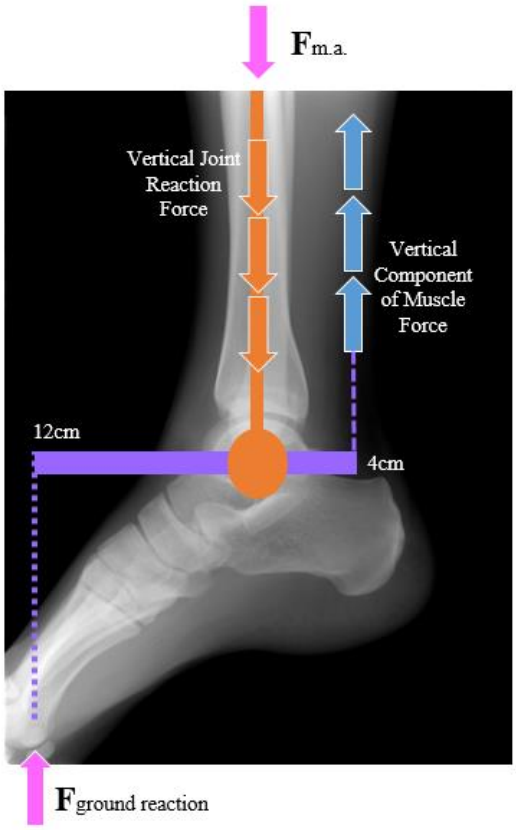

Figure 17. The biomechanical forces exerted during reactive jumps.

Adapted from Rittweger (2007).

The ability of jump training specifically to produce this bone formation stimulus has been tested previously in both humans and animals. Hinton et al., (2015) conducted a 12-month randomised trial to determine whether resistance training or jump training would increase whole-body, total hip and lumbar spine BMD and markers of bone formation and resorption in middle aged, healthy men. Results of this study found that both resistance training and jump training significantly increased whole-body and lumbar spine BMD, but only resistance training increased hip BMD ($p < 0.05$). Bone formation markers were increased, whilst bone resorption markers decreased advocating that both types of training had an anti-resorptive and anabolic effect on bone (Hinton et al., 2015). Similarly, but in a much shorter time frame, jump training was effective in stimulating bone formation in young, healthy females. The bone formation markers, bone alkaline phosphatase (BAP) and C-terminal telopeptides of type I collagen (CTX), increased significantly following fifteen sessions of high-intensity, low volume jumps over a three week period ($p \leq 0.05$) (Reiger and Yingling, 2016). This view was supported by another research study whereby

jump exercise applied during three-week hind limb unloading in tail-suspended rats proved to be effective in inhibiting bone loss and trabecular bone architectural deterioration (Ju et al., 2013). The performance of reactive jumps requires precise judgement of muscular activity before ground contact is achieved. Hence, this type of exercise may be additionally advantageous to the maintenance of sensorimotor and neuromuscular function, which is noticeably deteriorated following HDT bed rest or spaceflight (Kramer et al., 2012).

Another advantage for the use of a high-intensity and resistance-based countermeasure is the ability of the two combined exercise modalities to increase lean mass, reduce regional and total fat mass, improve $\dot{V}O_{2max}$, increase muscle strength and bone mineral content and reduce risk factors associated with metabolic diseases such as reducing systolic blood pressure and improving glycaemic control and insulin sensitivity (Winett and Carpinelli, 2001; Castaneda et al., 2002; Gibala and McGee, 2008; Shiraev and Barclay, 2012). Increases in muscle mass permit an increase in daily energy expenditure, through an augmented RMR which may facilitate fat loss over time, and enhance glycaemic control by increasing the storage space available for glucose (Castaneda et al., 2002). From the comprehensive review on novel adipokines, myokines and hepatokines provided above, it is clear that exercise training promotes the secretion of health-enhancing organokines and deters the secretion of organokines that promote metabolic dysfunction, thereby inducing favourable effects on local and systemic metabolism and function. Therefore, if reactive jump training can adequately preserve muscle mass and strength, cardiorespiratory fitness and body composition, it is possible that this low volume, high-intensity countermeasure may help to maintain regular inter-organ crosstalk and tissue-specific and systemic metabolic health and function following HDT bed rest.

Overall, plyometric training induces high-loading force capable of stimulating signalling pathways involved in bone formation and hypertrophy, particularly in the leg extensors that experience the highest rate of atrophy following HDT bed rest. Additionally, reactive jump training is a form of high-intensity interval training, with short rest intervals, which may confer protective effects on the cardiovascular system and overall metabolic health. If this type of training is proven to be effective in preventing the deleterious physiological

and metabolic effects of HDT bed rest, it will be a very time-efficient countermeasure that would be highly compliant with space operations, as the current allocated time for exercise training is 2.5 hours per day, 6 days per week (Gruber et al., 2019; English et al., 2020). In addition to spaceflight, reactive jump training may be a quick and effective type of exercise training which could be adapted for inactive, sedentary and ageing populations.

2.6. Conclusion of Literature Review

HDT bed rest simulates the effects of microgravity and is used to study the physiological adaptations to spaceflight (Gao and Chilibeck, 2020). These include muscle atrophy, bone loss, impaired cardiovascular and functional capacity and metabolic dysregulation, among others (Bergouignan et al., 2011; Hart and Zernicke, 2020). These documented changes are similar, albeit less severe, than those observed in physically inactive, sedentary and/or ageing cohorts and in response to immobilisation due to injury or illness, due to reductions in gravitational loading (Vernikos, 2017; Vernikos, 2021). There is evidence that underlying mechanisms, such as insulin resistance, play an important role in the regulation of whole-body metabolic changes. Insulin resistance can be described as a pathological defect in insulin signalling pathways, resulting in inappropriate cellular response to insulin in tissues such as the liver, skeletal muscle and adipose tissue and is a well-known consequence of HDT bed rest, even when energy balance is maintained (Bergouignan et al., 2011; Kenny et al., 2017; Bourebaba and Marycz, 2019). The severity of insulin resistance varies considerably between individuals and between the tissues of metabolic importance (Abdul-Ghani et al., 2007; Hansen et al., 2020). A key strategy for monitoring metabolic homeostasis is communication between the liver, skeletal muscle and adipose tissue *via* secreted proteins known as hepatokines, myokines and adipokines, respectively, which perform autocrine, paracrine and endocrine actions. Disruption of protein secretion and target-tissue underpin the development of metabolic dysregulation, including insulin resistance (Priest and Tontonoz, 2019). Investigation of the roles of circulating novel biomarkers of insulin sensitivity and insulin resistance in in regulating whole-body and tissue-specific insulin sensitivity may fill important knowledge gaps regarding individual responses in insulin sensitivity and potentially assist with personalised prescription of interventions to maintain health following HDT bed rest and other conditions of gravity deprivation.

Chapter 3. Methodology

3.1. Overview of Full Study Design

This research was conducted as part of the "Reactive jumps in a sledge jump system as a countermeasure during long-term bed rest" (RSL) study funded by the European Space Agency (ESA), which ran as two separate bed rest campaigns, commencing in August 2015 and January 2016, respectively. This parallel-design randomised controlled training study was conducted at the ":envihab" facility at the German Aerospace Centre (Deutsches Zentrum für Luft- und Raumfahrt, DLR). In brief, the study was split into 3 phases: a 15-day baseline data collection phase (BDC-15 to BDC-1), 60 days of strict 6° head-down-tilt bed rest (HDT1 to HDT60), followed by a post-testing phase (R+0 to R+14), with a total duration of 90 days (Figure 18). The study protocols were approved by the ethics committee of the North Rhine Medical Association (Ärztekammer Nordrhein) in Düsseldorf, Germany, as well as the Federal Office for Radiation Protection (Bundesamt für Strahlenschutz). All subjects gave their written informed consent before commencing the study in accordance with the Declaration of Helsinki. This study was registered retrospectively with the German Clinical Trial Registry (#DRKS00012946, 18th September 2017).

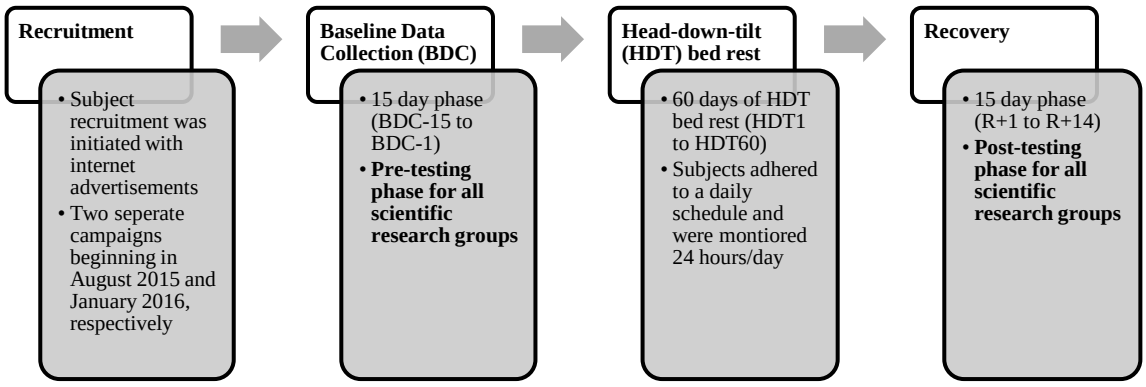

Figure 18. Schematic representation of each of the phases included in the full RSL bed rest study.

The text in bold highlights the involvement of the metabolic research team (TUS and DCU) in the full RSL bed rest study.

The following subsections (3.1.1 to 3.1.9) will describe the experimental procedures of this HDT bed rest study, including the process of participant recruitment, subjects, bed rest routine, dietary intake, exercise countermeasure and training protocol, physiotherapy and reconditioning. Following this, Section 3.2 will explain the procedures of basic core data collection that are relevant for the interpretation of the results of the PhD study. Finally, Section 3.3 will outline the experimental procedures performed specifically for this PhD study including the OGTT and subsequent laboratory and statistical analysis.

3.1.1. Participant Recruitment

The basic inclusion criteria for participation in this bed rest study were as follows: physically and mentally healthy males, aged between 20 and 45 years old with a body mass index (BMI) of 20 – 26 kg/m^2, a non-smoker, taking no medication, a non-competitive athlete and no history of bone fractures. Following the first information session, volunteers who satisfied the basic inclusion criteria and successfully passed the primary psychological test were invited to the :envihab to undergo a full medical examination with strictly defined exclusion criteria. These included: chronic hypertension, diabetes, obesity, arthritis, hyperlipidaemia, hepatic disease (A or C), or a disorder of calcium or bone metabolism. The presence of heritable blood clotting disorders (AT III, S-Akt, Lupus-PTT, ferritin, Factor V Leiden, Factor IV, and Factor II) was screened for and volunteers with positive test results were excluded from participation. Volunteers deemed medically eligible underwent the next stage of psychological screening, involving questionnaires and interviews. As part of this recruitment process, questionnaires and interviews were also conducted to determine the presence of food intolerances and allergies (e.g. volunteers who had coeliac disease or who were vegetarian) and to identify volunteers with a lot of dislikes to food items that would be used in the study, which led to exclusion from participation. The recruitment process ended with a dual-energy X-ray absorptiometry (DEXA) scan to determine bone mineral density of the femur and lumbar vertebra column. Of 494 original volunteers, 121 attended the information session; 60 were medically screened; 45 completed the psychological interview, and 31 underwent the screening DEXA scan.

3.1.2. Subjects

Following the recruitment process, 27 volunteers were eligible to participate in the study and of these, 24 were enrolled (Figure 19). One subject discontinued the study on BDC-4 of the second campaign due to medical reasons that were unrelated to the study and could not be replaced. On HDT1, subjects were selected in pairs and each subject was randomly assigned to either the control group (CTRL) or the jump countermeasure group (JUMP) using a dice roll (Table 1). Two subjects that completed the study (one CTRL, one JUMP) were re-ambulated on HDT49 and HDT50 respectively, due to medical reasons and completed the recovery process with scheduled measurements except for VO_{2peak}. Each subject was given a specific anonymous ID for the duration of the study. Subjects received financial reward for participating in the study (a total of €15,000 for the whole study, including follow-up measurements).

Table 1. Group characteristics at baseline, separately for the CTRL and the JUMP group.

	CTRL	**JUMP**
N	11	12
Age (years)	28 ± 6	30 ± 7
Height (cm)	181 ± 5	181 ± 7
BMI (kg/m²)	23.33 ± 2.03	23.75 ± 1.80

Data are presented as mean ± standard deviation (SD). Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; N, number; BMI, body mass index.

Figure 19. Subjects from the second phase of the ESA RSL bed rest study.

Image credits: DLR/envihab.

3.1.3. Bed Rest Routine and Safety Measures

For the duration of the bed rest study, each subject was accommodated in a single-person room equipped with a television, telephone and laptop with wireless internet access. Room temperature was kept stable between 20 – 23°C and humidity was regulated between 30 – 50%. For the intervention phase (beginning at 9.00am on HDT1), subjects remained in the 6° HDT position 24 hours/day (Figure 20), with the exception for experiments that needed to be performed horizontally. All essential daily activities (such as eating, showering, and bowel movements) and leisure activities (reading and watching television) were carried out in the HDT position. Each subject was required to have one shoulder in contact with the bed at all times and was not allowed to elevate their head 30° above horizontal (in respect to the mattress). Each subject was allowed to have one pillow, but this could be changed for a thicker or thinner pillow. Therefore, each subject had a different tilt angle, but this concerned the position of the head only. Horizontal displacements were permitted, but static and dynamic muscle contractions were strictly prohibited. To monitor compliance, subjects were supervised around the clock by DLR staff and CCTV. During the non-bed rest phases (BDC and R), subjects were free to walk around the designated facility, but they did not engage in any physical activity aside from the familiarisation sessions with the exercise protocol.

To ensure subject safety, health and well-being, 24 hours/day medical and paramedical care was available. General health indicators including blood pressure and heart rate were assessed daily (Intellivue MMS X2, Philips, Best, The Netherlands) in the fasted state, during scheduled wake-up at 6.30am. An independent medical doctor monitored the subjects' health status during daily ward rounds and periodically reviewed safety parameters from the blood and urine samples. The safety parameters included hemoglobin (g/dl), hematocrit (%), red blood cells (RBC, $10^6/mm^3$), RBC volume (μm^3), mean corpuscular hemoglobin (MCH, pg), MCH concentration (MCHC, g/dl), platelets ($10^3/mm^3$), urea (mg/dl), creatinine (mg/dl), total protein (g/dl), bilirubin (mg/dl), glucose (mg/dl), C-reactive protein (mg/dl), alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), alkaline phosphatase (AP, U/L), gamma-glutamyl transferase (GGT, U/L), creatine kinase (CK, U/L), lactate dehydrogenase (LDH, U/L), prothrombine time (PT, %), activated partial thromboplastin time (APTT, seconds), fibrinogen (mg/dl), ferritin (µg/l), and vitamin D (µg). The total volume of blood drawn throughout the whole study (2 year time period) was 767.1ml. With respect to the size of the available monovettes, this amount could have been 30 – 40ml higher. Due to the duration of the study, no more than 370 ml of blood could be obtained in any two month period. Subjects received psychological support from an independent psychologist during weekly individual support sessions. Subjects were asked to keep a diary throughout the bed rest study to report any critical events or experiences. The daily schedule of the subjects was determined by the various principal investigators due to the large volume of tests being performed (~ 90 individual experiments). Lights out were at 11.00pm daily.

Figure 20. A subject at the :envihab facility in the HDT bed rest position.

Image credits: DLR/envihab.

3.1.4. Diet

All subjects received a strictly controlled and individualised diet for the entire duration of the study, which was set to maintain energy balance. To individually tailor the diet, resting metabolic rate (RMR) was measured on BDC-14 by means of indirect calorimetry (metalyzer 3B, CORTEX Biophysik, GmBH) and estimated using the Weir equation, assuming urinary nitrogen to be 8.64g/day (Weir, 1949). The energy content of the diet during BDC and R was set to 1.6 multiplied (*) by the subject's RMR. During HDT bed rest, the energy content equalled 1.3 * RMR for the CTRL group and 1.33 * RMR for the JUMP group to account for the higher energy requirements of the exercise training sessions. To ensure validity of the diet, daily measurements of body weight, 24-hour urine pools, as well as periodic assessments of body composition by DEXA were implemented throughout the study. In the event of systemic changes in total fat mass that exceeded 1kg, the total energy intake was adjusted, while keeping the relative contributions of the macronutrients the same.

In terms of total energy intake, and macronutrient content of the diet, protein intake was set at 1.2g per kg of body weight per day (kg/BW/day), fibre 30g/day and fat was kept

constant at 35% of total energy intake a day. Thus, the relative intake levels for all of the macronutrients were as follows: carbohydrates 49%, fats 35%, protein 14% and fibre 2%. Water intake was set at 50ml/kgBW/day and intake of caffeine or alcohol was strictly prohibited. The average daily energy and water intake during all study phases is summarised in Table 2.

Table 2. Average daily energy and water intake during all study phases (BDC, HDT, and R) in both experimental groups (CTRL, JUMP).

	BDC	**HDT**		**R**
		CTRL	**JUMP**	
Energy (kcal/d)	2688 ± 267	2080 ± 222	2066 ± 129	2692 ± 239
Protein (g/d)	92 ± 8	92 ± 9	93 ± 8	92 ± 8
Protein (g/kgBW/d)	1.21 ± 0.01	1.21 ± 0.01	1.20 ± 0.02	1.21 ± 0.01
Protein (%TEE/d)	14 ± 1	18 ± 2	19 ± 1	14 ± 1
Fat (g/d)	102 ± 10	79 ± 8	78 ± 5	102 ± 9
Fat (%TEE/d)	35 ± 0	35 ± 0	35 ± 0	35 ± 0
Carbohydrates (g/d)	332 ± 39	236 ± 33	231 ± 17	334 ± 34
Carbohydrates (%TEE/d)	51 ± 1	46 ± 2	46 ± 1	51 ± 1
Fiber (g/d)	31 ± 1	33 ± 2	32 ± 1	32 ± 1
Fluid (g/d)	3820 ± 338	3830 ± 390	3851 ± 324	4239 ± 441
Fluid (ml/kgBW/d)	50.0 ± 0.3	50.5 ± 0.7	50.0 ± 0.1	56.0 ± 6.0

Estimated nutritional intake is presented as mean ± standard deviation. Abbreviations: BDC, baseline data collection phase; HDT, head-down-tilt bed rest phase; R, recovery phase; CTRL, control group; JUMP, jumping countermeasure group; kcal, kilocalories; g, grams; /d, per day; kgBW, per kilograms of body weight; TEE, total energy expenditure; ml, millilitres.

To compensate for sweat and energy loss during physically demanding experiments, additional fluid and energy intake was given in the form of water and diluted apple juice. During recovery (R+0 to R+5), there was no upper limit for water consumption to compensate for the reambulation-induced fluid shift. To account for the lack of sunlight exposure, all subjects were supplemented with vitamin D (1000 IU/day) throughout the study. The intake of all vitamins and elements was controlled and each subject received at a minimum the recommended Dietary Reference Intakes (http://ods.od.nih.gov).

The DLR internal research team were responsible for nutrition in the RSL bed rest study. The menus were prepared daily and designed using PRODI (Kluthe Prodi 6.3 ® expert), a comprehensive organisational software, permitting effective calculation of recipes, daily schedules, meal plans and nutrition logs that can easily be compared with intake recommendations. The CTRL and JUMP groups got the same meal on the same study day. All meals were prepared in the metabolic kitchen in the :envihab facility and all foods were weighed to ± 0.5 g using laboratory scales. Subjects were encouraged to consume all the food provided. However, if they consumed less than provided, the leftovers were weighed and considered in subsequent dietary analysis.

3.1.5. Exercise Countermeasure

The exercise countermeasure chosen for this HDT bed rest study consisted of a constructed system that permitted the performance of reactive jumps independent of gravitational forces and thus allowing the system to be used in the horizontal position on Earth. The sledge jump system (Figure 21) was designed by Novotec Medical GmbH (Pforzheim, Germany), on behalf of ESA (AO/1-5251/06/NL/IA; ESA Technology Research Program, contract #20767; ESA Life Sciences Program TEC-MMG/2006/82) and aimed to target the human musculoskeletal and neuromuscular systems. The system comprised a lightweight frame on wheels, which had a sledge attached to the rails on both sides of the frame. The sledge was attached to the rails by straps that permitted some movement perpendicular to the movement direction and a small degree of rotation. The subject is strapped into the device *via* two straps around the shoulders that attached to a small neck and back support. The force plate is located at the bottom of the sledge (Figure 22). The force that pulls the sledge towards the force plate is generated by four low-pressure cylinders, which could each produce forces of up to 450 newton's (N). Ground reaction forces were measured and the position of the sledge was logged by an incremental encoder. Above the force plate was a feedback monitor, which provided live feedback (e.g. peak force) to the subjects following every single jump.

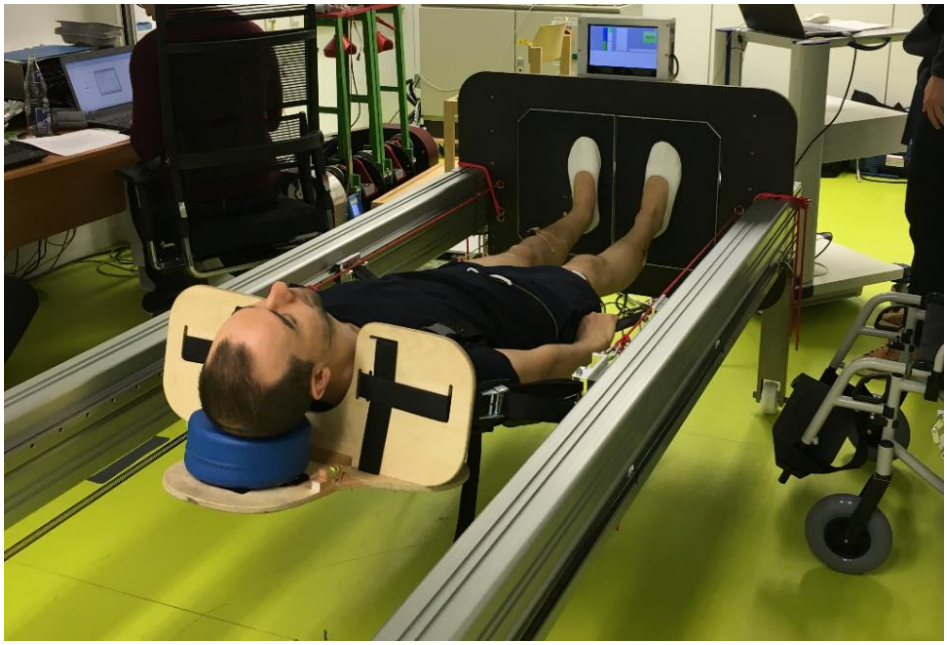

Figure 21. A subject strapped into the sledge jump system ready to perform reactive jump training.

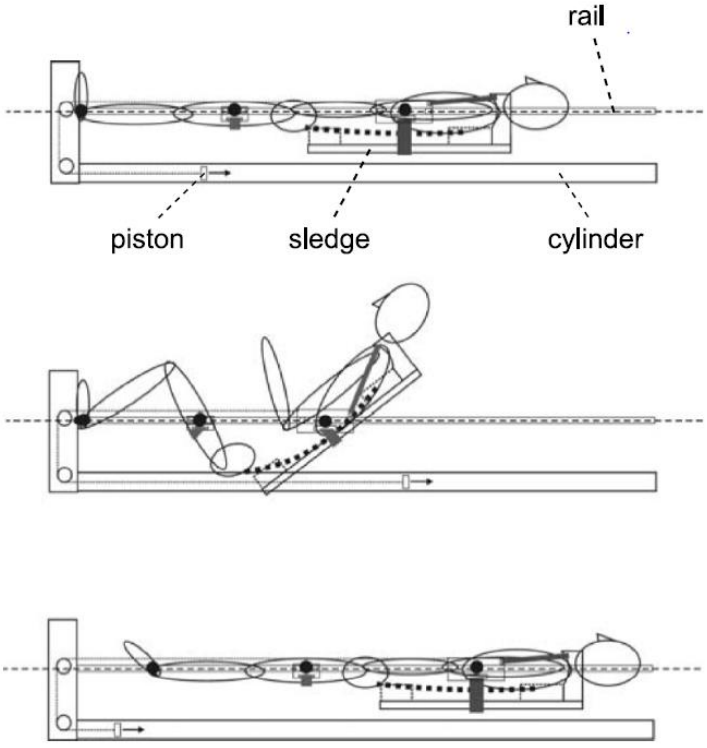

Figure 22. The longitudinal section of the sledge jump system as illustrated by Kramer et al. (2017b).

3.1.6. Familiarisation Training

During the BDC period, all subjects completed a total of 9 familiarisation sessions to ensure the correct jumping technique in the sledge jump system. Each familiarisation session included a warm up (mobilisation of the ankle joints, six heel raises, six deep static squats, three submaximal countermovement jumps (CMJs) and one series of submaximal hops) followed by 6 CMJs and 4 sets of 10 hops. During these sessions, the force in the sledge jump system was gradually increased from a resistance of 50% of the subject's body weight up to 100% during the final session. Subjects were instructed through the correct jumping technique and received verbal feedback on their movements following each set on jumps or hops.

3.1.7. Exercise Training

A total of 48 sessions of reactive jump training (RJT) were completed throughout the 60 day HDT bed rest period. Each session had a different training plan (6 x session A, 15 x session B, 14 x session C and 13 x session D) and the details of these can be found below in Table 3. In brief, each session consisted of a warm-up (detailed above in section 3.1.6) and a varying number of CMJs and repetitive hops and three maximal CMJs at 80% of the subject's body weight. Body weight was measured every morning and the force was adjusted accordingly. For the CMJs subjects were asked to "drop quickly into a squat and then burst into a jump as high as possible". For the hops, subjects were instructed to "jump as stiff and as high as possible whilst flexing the ankle, knee and hip joints; do not let the heels hit the force plate on landing, keep the contact time as brief as possible and jump constantly for the set". All sessions were supervised and recorded, and subjects received on-going verbal encouragement as well as live visual feedback through the feedback monitor. Similar to the familiarisation sessions, the ground reaction forces and sledge positioning were recorded for each jump.

Table 3. Details of the different types of exercise sessions completed by the JUMP group during the intervention.

Training Type	Session A	Session B	Session C	Session D
Amount of CMJs	2 sets, 12 reps	2 sets, 12 reps	2 sets, 15 reps	4 sets, 15 reps
Amount of Hops	3 sets, 10 reps	4 sets, 10 reps	2 sets, 15 reps 2 sets, 10 reps	1 set, 12 reps
Load	85% BW	90% BW	80% BW	80% BW
Breaks between sets (secs)	60	90	30	60
Training duration (mins:secs)	17:00	14:30	09:30	08:30
Duration excluding breaks (mins:secs)	03:00	04:00	04:00	01:30

Abbreviations: BW, body weight; CMJ, countermovement jumps; mins, minutes; reps, repetitions; secs, seconds.

3.1.8. Physiotherapy

In campaign 1, physiotherapy was provided to all subjects weekly from HDT6 and from week 3, physiotherapy was increased to twice weekly. In campaign 2, additional physiotherapy sessions were given between HDT2 and HDT5 to alleviate back pain and were subsequently given twice a week thereafter. The main aim of the physiotherapy sessions was passive movements of the joints in the lower extremity. Subjects also received a light massage with little pressure placed on the back to release muscle tension. Traction was used, at the level of the cervical spine, to relieve the spinal discs. If the patient was experiencing any other discomfort e.g. tension headaches, the physiotherapist sought to these appropriately.

3.1.9. Reconditioning

During the BDC period, subjects were familiarised with the exercises they would be completing during the reconditioning sessions. Following HDT bed rest, each subject was scheduled for 6 reconditioning sessions during the recovery phase following re-

ambulation (R+2 to R+14). Due to foot soreness, one subject was not able to complete one of the sessions (1%). The reconditioning programme involved active stretching, fast footwork with an "agility ladder" and exercises using a Bosu ball (Bosu, Ashland, Ohio, USA). The overall aim of the reconditioning sessions was to increase the subject's range of motion, muscle strength, contraction speed and coordination and to improve core stability and body posture. The exercises began slow and easy and then progressed in speed and difficulty according to the individual's status of recovery.

3.2. Basic Core Data Collection

Basic core data (BCD) is a set of standardised experiments that are performed in every HDT bed rest study sponsored by the ESA, to enable optimal countermeasure comparisons between various HDT bed rest studies. BCD includes the measurements of body weight, body composition, $\dot{V}O_{2peak}$ and muscle strength. These assessments were conducted by the internal research team at the DLR and the data was shared amongst all research groups involved in the study, based on data sharing agreements signed by principal investigators before the commencement of the HDT bed rest study. We were kindly invited to observe these procedures when we were present for pre- and post-HDT bed rest testing at the :envihab facility.

3.2.1. Body Weight and Body Composition

Daily measurements of body weight were taken following the first urine void of the day (DVM 5703, Sartorius, Göttingen, Germany). Body composition was examined using the whole-body scan feature on the Prodigy Full Pro (GE Healthcare GmbH, Solingen, Germany; Figure 23). The manufacturer's enCORE software (version 16.10.151) was used to generate automated reports of total lean mass, fat mass and bone mineral content. A total of 2 scans were completed during BDC, 4 during HDT bed rest and 2 during recovery to track the maintenance of energy balance.

Figure 23. A subject undergoing a whole-body DEXA scan in the :envihab facility.

Image credits: DLR/envihab.

3.2.2. Peak Oxygen Uptake Capacity

Peak oxygen uptake ($\dot{V}O_{2peak}$) was measured using an electrically-braked cycle ergometer (Lode, Groningen, The Netherlands), on BDC-8 and R+1. The test began with the subject completing 5 minutes of seated rest, before being instructed to start pedalling. The warm up consisted of 3 minutes cycling at 50W, followed by one minute stages in which the load was increased by 25W per stage until volitional exhaustion with strong verbal encouragement. Continuous breath-by-breath systemic oxygen uptake and carbon dioxide efflux was measured using the Innocor system (Innovision, Odense, Denmark) and heart rate was monitored using a 12-lead electrocardiogram (Padsy, Medset Medizintechnik, Germany). If the peak respiratory exchange ratio (RER) did not exceed 1.10, the trial was deemed not exhaustive and not considered for further analyses. This lead to the exclusion of the data from 1 subject. Two subjects (one CTRL, one JUMP) were re-ambulated on HDT49 and HDT50, respectively, due to medical reasons and did not take part in the post-HDT bed test $\dot{V}O_{2peak}$ test. Therefore, only 20 subjects (n = 10 CTRL, n = 10 JUMP) were included in the analysis of $\dot{V}O_{2peak}$ pre and post-HDT bed rest.

3.2.3. Muscle Strength

Maximal voluntary isometric torque was measured during the four following movements using the Isomed 2000 dynamometer (D&R Ferstl GmbH, Hemau, Germany): knee extension, knee flexion, plantarflexion and dorsiflexion. Subjects were familiarised with

the equipment and test procedures six days prior to the first measurements (BDC-7) and then measured once directly before (BDC-1) and after HDT bed rest (R+0). Knee extension and knee flexion torque were examined in a seated position with a hip angle of 90°, knee angle of 90° and ankle angle of 0°. Plantarflexion and dorsiflexion torque was assessed in a supine position with hip, knee and ankle angles of 0°. A warm up, consisting of 5 minutes of cycling at 75 watts (W) and 5 isokinetic submaximal contractions, was conducted prior to the test. For each of the 4 movements, the subjects performed 6 repetitions of 5 seconds (3 repetitions separated with 1 minute of recovery, followed by a 2 minute break and a second block of 3 repetitions).

3.3. Experimental Overview of this PhD Study

This section will focus on the experimental procedures of this PhD study (Figure 24). This includes the OGTT, which was performed specifically for our metabolic research team (a small group of researchers from the Technological University of the Shannon and Dublin City University) to quantify glucose tolerance, insulin sensitivity and the lipid profile prior to and following 60 days HDT bed rest. Due to standardisation and training purposes, the internal research team at the DLR performed the cannulation, blood draws, and centrifugation and aliquoting procedures during the OGTT. Our metabolic research team was responsible for collecting and transporting the individual subjects from the bed rest module to the human physiology laboratory before and after the OGTT during pre- and post-HDT bed rest testing. After the cessation of the RSL study, the samples were transported from Germany to Ireland for laboratory analysis. This section also provides a timeline of training, identification and analysis of circulating concentrations of biomarkers of insulin sensitivity and insulin resistance pre- and post-HDT bed rest. In addition, the process of correcting measured parameters for hemoconcentration and statistical analysis are provided in this section.

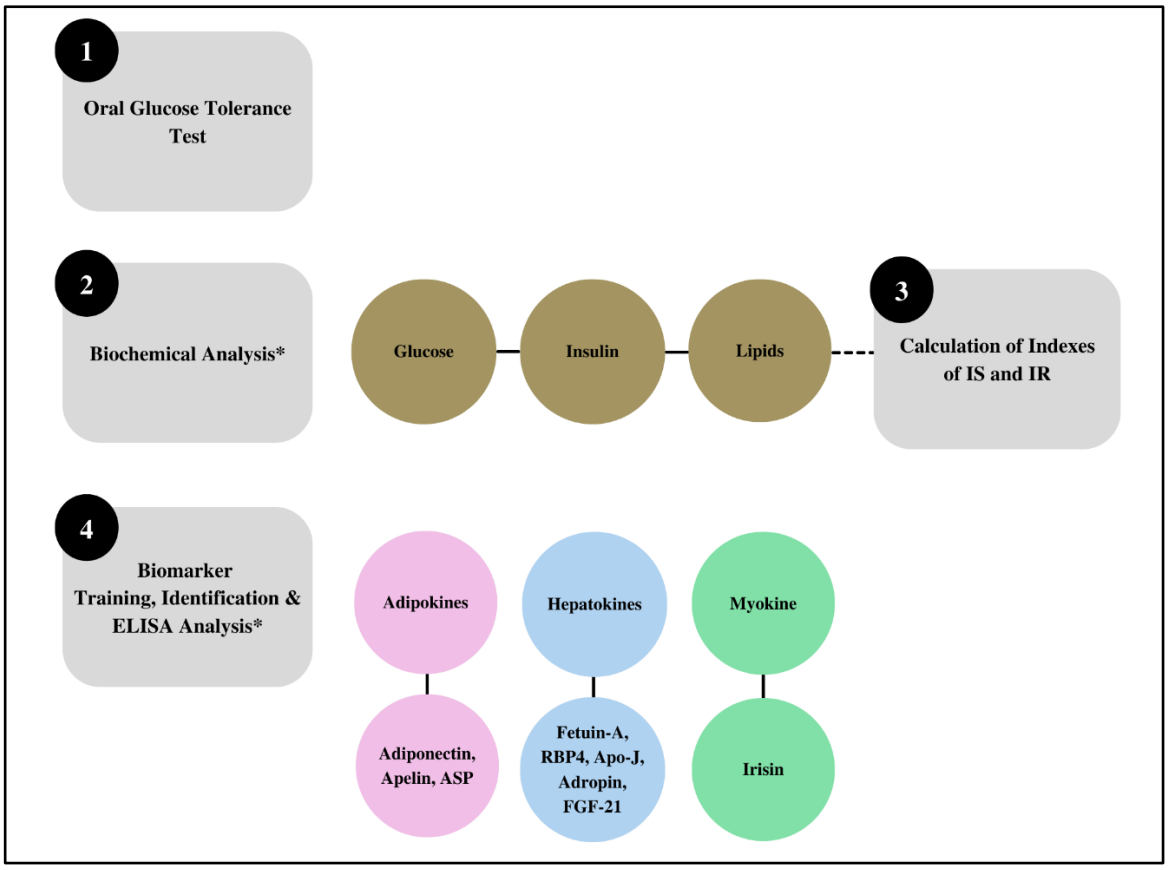

Figure 24. Experimental overview of this PhD study.

Abbreviations: IS, insulin sensitivity; ASP, acylation stimulating protein; RBP4, retinol binding protein 4; Apo-J, apolipoprotein-J; FGF-21, fibroblast growth factor 21. *Final concentrations of measured parameters, quantified using biochemical or ELISA analysis, were corrected for hemoconcentration.

3.3.1. OGTT

An OGTT was performed after a 12-hour overnight fast, in the morning of BDC-5 and HDT59. For standardisation purposes, no physically demanding assessments were performed in the 24-hour period prior to the OGTT (Appendix I) and subjects remained supine during the OGTT. A catheter was placed in the antecubital vein and blood samples were drawn before and at 30-minute intervals (30, 60, 90, 120 minutes) after the ingestion of a 75g glucose equivalent (ACCU Chek® Dextro OGT, Roche Diagnostics Deutschland GmbH, Mannheim) dissolved in 300ml water. Following sample extraction, serum was left to coagulate at room temperature for 30 minutes before centrifugation, whilst tubes containing fluoride and EDTA were centrifuged immediately (3000 rpm or 184 x *g*, 4°C, 10 minutes. Varifuge 3.0R, Heraeus Sepatech). Serum and plasma were then aliquoted and labelled and stored at -80°C until analysis.

3.3.2. Biochemical Analysis

Concentrations of glucose, NEFA, total cholesterol (CHOL), LDL, HDL and triglycerides (TG) were all measured using colorimetric assay kits on the Randox RX Daytona™ (Crumlin, United Kingdom). Serum insulin was quantified using an immunoassay method on the Cobas® 8000 modular analyser (module e602, Roche Diagnostics, North America).

3.3.3. Indexes of Insulin Sensitivity and Insulin Resistance

AUCG and AUCI were calculated according to the trapezoidal rule for 30 minutes and 120 minutes. Indexes of insulin sensitivity and insulin sensitivity including HOMA-IR, HOMA-β, QUICKI, Matsuda index, insulinogenic index, disposition index, oral glucose insulin sensitivity (OGIS) index, Gutt index, liver insulin sensitivity, muscle insulin sensitivity, adipose tissue insulin resistance and hepatic insulin resistance were calculated using previously reported equations (Matthews et al., 1985; Matsuda and DeFronzo, 1999; Gutt et al., 2000; Katz et al., 2000; Mari et al., 2001; Bergman et al., 2002; Abdul-Ghani et al., 2007; Utzschneider et al., 2009; Lomonaco et al., 2012). In the equations below, $glucose_0$ represents fasting glucose and $glucose_{30}$ represents the concentration of glucose 30 minutes after the ingestion of the oral glucose load. The same acronyms are used for insulin and NEFA.

Equation 1: $AUCG_{30}$

$\mathbf{AUCG_{30}} = (0.5*(Glucose_0 + Glucose_{30})*30)$

Glucose is expressed in mmol/L.

Equation 2: $AUCG_{120}$

$\mathbf{AUCG_{120}} = (0.5*(Glucose_0 + Glucose_{30})*30) + (0.5*(Glucose_{30} + Glucose_{60})*30) + (0.5*(Glucose_{60} + Glucose_{90})*30) + (0.5*(Glucose_{90} + Glucose_{120})*30)$.

Glucose is expressed in mmol/L.

Equation 3: $AUCI_{30}$

$AUCI_{30}$ = $(0.5*(Insulin_0 + Insulin_{30})*30)$

Insulin is expressed in pmol/L.

Equation 4: $AUCI_{120}$

$AUCI_{120}$ = $(0.5*(Insulin_0 + Insulin_{30})*30) + (0.5*(Insulin_{30} + Insulin_{60})*30) + (0.5*(Insulin_{60} + Insulin_{90})*30) + (0.5*(Insulin_{90} + Insulin_{120})*30)$.

Insulin is expressed in pmol/L.

Equation 5: HOMA-IR

HOMA-IR = $Insulin_0 * Glucose_0 / 22.5$

Insulin is expressed in µU/mL. Glucose is expressed in mmol/L.

Equation 6: HOMA-β

HOMA-β = $20 * insulin_0 / glucose_0 – 3.5$

Insulin is expressed in µU/mL. Glucose is expressed in mmol/L.

Equation 7: QUICKI

QUICKI = $1 / \log(insulin_0) + \log(glucose_0)$

Insulin is expressed in µU/mL. Glucose is expressed in mg/dL.

Equation 8: Matsuda Index

Matsuda = $10{,}000 / \sqrt{glucose_0 * insulin_0 * glucose_{mean} * insulin_{mean}}$

$Glucose_{mean} = 15 * glucose_0 + 30 * glucose_{30} + 30 * glucose_{60} + 30 * glucose_{90} + 15 * glucose_{120}$ / 120 minutes.

$Insulin_{mean} = 15 * insulin_0 + 30 * insulin_{30} + 30 * insulin_{60} + 30 * insulin_{90} + 15 * insulin_{120}$ / 120 minutes.

Insulin is expressed in µU/mL. Glucose is expressed in mg/dL.

Equation 9: Insulinogenic Index

Insulinogenic Index = $(Insulin_{30} - Insulin_0) / (Glucose_{30} - Glucose_0)$

Insulin is expressed in µU/mL. Glucose is expressed in mg/dL.

Equation 10: Disposition Index

Disposition Index = Matsuda Index * Insulinogenic Index

Insulin is expressed in µU/mL. Glucose is expressed in mg/dL.

Equation 11: OGIS Index

OGIS Index = $f(Glucose_0, Glucose_{90}, Glucose_{120}, Insulin_0, Insulin_{90}, Insulin_{120}, D_0)$.

D_0 is the oral glucose dose (g/m^2 body surface area). Insulin is expressed in pmol/L. Glucose is expressed in mmol/L. The function *f* is complex but is easily programmed using a spreadsheet downloadable from http://webmet.pd.cnr.it/ogis/index.php.

Equation 12: Gutt Index

Gutt Index = $75000 + (glucose_0 - glucose_{120}) * 0.19 * body\ weight / 120 * glucose_{mean} * \log(insulin_{mean})$

Body weight is measured in kg. $Glucose_0$ and $glucose_{120}$ are expressed is mg/dL. $Glucose_{mean}$ is the average value of $glucose_0$ and $glucose_{120}$ expressed is mmol/L. $Insulin_{mean}$ is the average value of $insulin_0$ and $insulin_{120}$ expressed in µU/mL.

Equation 13: Liver Insulin Sensitivity

Liver Insulin Sensitivity = $100 * (k / glucose_0 * insulin_0)$, where $k = 22.5 * 18$

Insulin is expressed in µU/mL. Glucose is expressed in mg/dL.

Equation 14: Muscle Insulin Sensitivity

Muscle Insulin Sensitivity = $dG / dt / insulin_{mean}$

dG/dt represents the rate of decline in plasma glucose concentrations and is calculated as the slope of the least square fit to the decline in plasma glucose from its peak to its nadir. $Insulin_{mean}$ refers to the average insulin concentration during the OGTT (0, 30, 60, 90 and 120 minutes). Insulin is expressed in µU/mL. Glucose is expressed in mg/dL.

Equation 15: Adipose Tissue Insulin Resistance

Adipose Insulin Resistance = $NEFA_0 * insulin_0$

NEFA is measured in mmol/L. Insulin is expressed in µU/mL.

Equation 16: Hepatic Insulin Resistance

Hepatic Insulin Resistance = $(Glucose_0 + Glucose_{30} / 2) * 0.5) * (Insulin_0 + Insulin_{30} / 2) * 0.5)$

Insulin is expressed in pmol/L. Glucose is expressed in mmol/L.

3.3.4. Timeline of Biomarker Training, Identification and Analysis

A schematic representation of the timeline of biomarker training, identification and analysis performed for this PhD study can be found in Figure 25. Each stage of this process is explained in more detail in the subsections to follow.

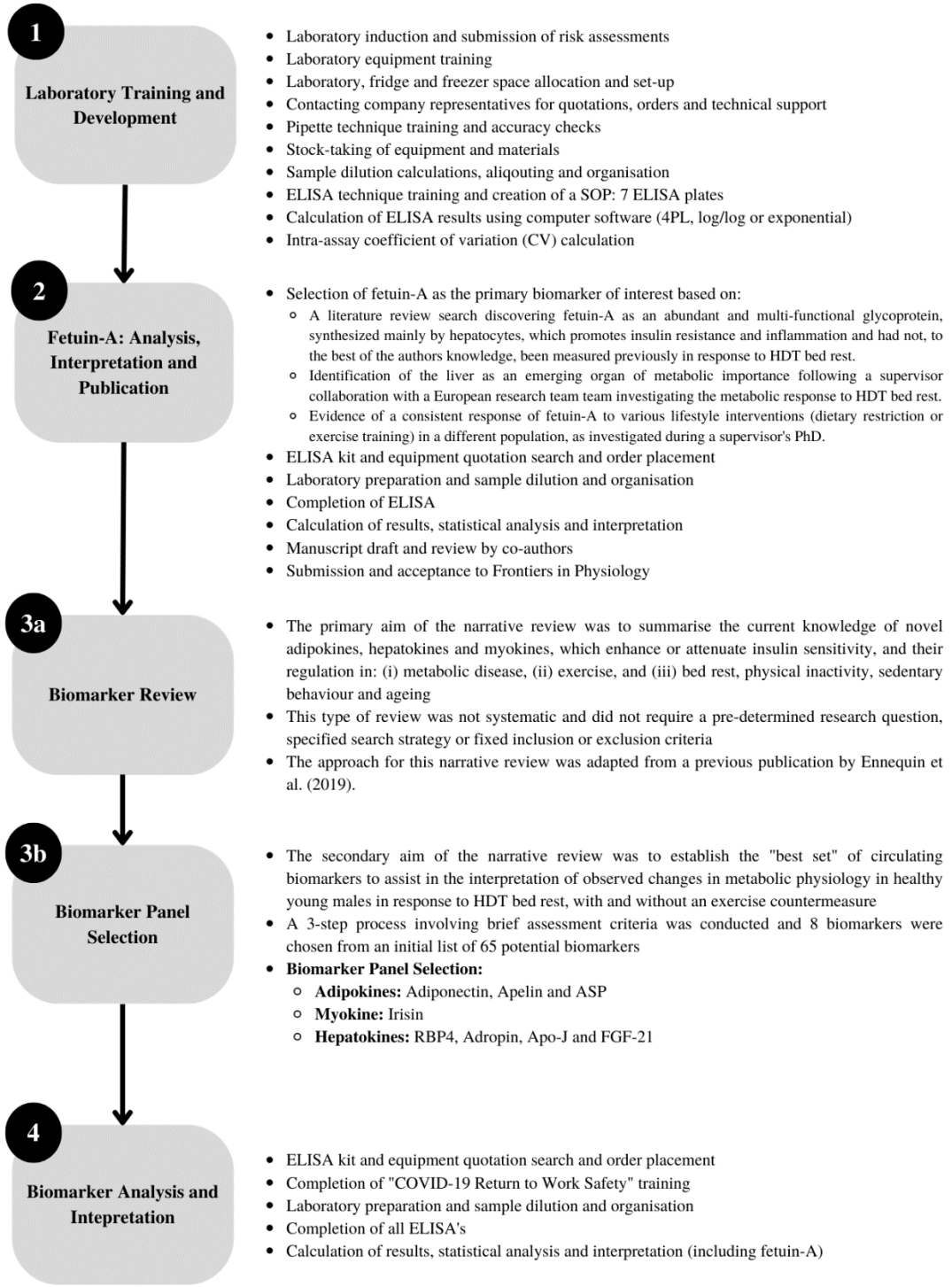

Figure 25. A schematic representing the timeline of biomarker training, identification and analysis for this PhD study.

3.3.4.1. Laboratory training and development

Laboratory training and development is critical for effective research. All research students in the Bioscience Research Institute (BRI) at TUS are required to undergo laboratory induction training, which covers the basic competencies and regulatory requirements for those working in the laboratories (e.g. personal protective equipment (PPE), proper waste handling and disposal, decontamination and spill clean-up procedures). Additionally, each research student is required to submit a biological and chemical risk assessment form detailing general information on their laboratory research, processes involving the use of hazardous chemical and biological agents, potential experimental outcomes, potential exposure to hazards or biological agents, storage and waste disposal arrangements, PPE, training, supervision, risk control measures, emergency procedures and a declaration signed by the student, principal supervisor and facility director. These mandatory procedures were completed by the researcher in the first year of the PhD. Following this, training on frequently used equipment in the laboratories (e.g. autoclave training) was completed and laboratory, fridge and -80° freezer space was designated and set up appropriately. Laboratory assistance and other forms of training were provided by fellow research students and facility directors creating a positive and productive learning environment.

A critical technique for this PhD study was sample pipetting. Pipette training and competence monitoring are essential components for ensuring the accuracy and precision of laboratory results. Pipette training, using single and multi-channel pipettes, was completed by the researcher at the start of the PhD. In addition, pipetting accuracy checks were completed before sample aliquoting and each enzyme-linked immunosorbent assay (ELISA), by weighing pipetted water on an analytical balance to ensure sufficient pipetting precision as well as the maintenance and calibration of the pipettes. Sample aliquoting involves taking a small amount of serum from a larger serum sample and placing it into a separate tube so it can be tested (e.g. taking 100µL from an 800µL sample). This process prevents repeated freeze-thaw cycles and preserves the stability of analytes. In advance of the biomarker analyses in this PhD study, one pre- and post-HDT bed rest fasting serum sample (500µL) for each subject was divided into 10 sample aliquots (50µL). These aliquots were labelled and stored appropriately and recorded on a reference sheet.

ELISA is a plate-based assay technique designed for detecting and quantifying substances, including antibodies, antigens, proteins, glycoproteins and hormones, in a variety of samples (e.g. serum, plasma). The detection of these substances is accomplished by complexing antibodies and antigens to produce a measurable result (Alhajj and Farhana, 2022). An important element of laboratory training and development involved contacting numerous ELISA and laboratory equipment companies and communicating with sales advisors and technical support advisors to obtain information on, and become familiar with, ELISA kit formats, protocols, best practices and techniques and troubleshooting guides. Establishing these connections provided invaluable support throughout all stages of this PhD, particularly when obtaining quotations, processing orders and answering technical queries (e.g. confirmation of specific protein type being analysed, sample dilution calculation and application of curve fitting). The two different types of ELISA that were most suitable for the proposed analysis in this PhD were the sandwich ELISA (two specific antibodies are used to "sandwich" the antigen) and competitive ELISA (a conjugated antigen is used to compete for binding with the antigen present in the sample, particularly useful for determining concentrations of small molecules or hormones). These types of ELISA kits have similar step-by-step protocols and are fully validated, pre-coated and ready-to-run. An example ELISA procedure can be found in Appendix III.

To optimise accuracy, precision and reproducibility in the quantification of circulating biomarkers of insulin sensitivity and insulin resistance, while conserving the sample volume (≤ 1ml for each subject for pre- and post-HDT bed rest) from the RSL bed rest study for the main analyses, 7 practice ELISA's were conducted using samples obtained from previous lifestyle intervention studies led by the researchers PhD supervisor. These ELISA kits quantified circulating concentrations of fetuin-A (hepatokine), follistatin (hepatokine), myostatin (myokine) and fatty acid binding protein 4 (FABP4, adipokine) in human sera. These ELISA kits had different sample dilution and activation procedures, standards and controls, reagent preparations, incubation conditions, aspirations, optical density wavelength measurements and sample calculation procedures. The kits were specifically chosen for the acquisition and perfection of these various skills and techniques. Each ELISA took approximately 6 – 8 hours to run and each standard, control and sample was assayed in duplicate. Performing these practice ELISA's assisted with

the optimisation of a biomarker research workflow and creation of a standard operating procedure (SOP) for the researcher. The SOP contained a list of all equipment required for an ELISA and detailed the procedures to be conducted on the day prior to the ELISA (laboratory and materials set-up, sample dilution calculations and identification of required sample volume, sample aliquoting and organisation, pipetting accuracy check, equipment booking, cleaning, autoclaving and drying and printing of protocols and plate layout sheets) and on the day of the ELISA (use of PPE, defrosting samples, ensuring reagents are at room temperature and equipment is powered on prior to commencement of the ELISA's, completing the full ELISA procedure, setting up the plate reader protocol, saving the results to an external memory key and ensuring laboratory clean up and correct waste handling and disposal is complete). This SOP was also used as a reference for routine stock checks of equipment and materials throughout the entirety of the PhD study.

In addition to the laboratory skills necessary for each ELISA, the skills involved in calculating and evaluating ELISA data are of paramount importance. The duplicate readings for each standard, control and sample are averaged and the average zero standard optical density (O.D.) is subtracted from each value (the latter calculation is not required in a competitive ELISA). After this, a standard curve is created by reducing the data using computer software (online curve fitting (mycurvefit.com, myassays.com, elisaanalysis.com), PRISM and Microsoft Excel) capable of generating a four parameter logistic (4PL), log/log or exponential curve fit. If samples are diluted, the concentration obtained from the standard curve is multiplied by the dilution factor used. Using the data from the 7 practice ELISA's, the researcher became proficient using various computer software to calculate the concentration of circulating biomarkers in the unknown samples and intra-assay coefficient of variation (CV; reproducibility between wells within an assay plate). The %CV is calculated by dividing the standard deviation of the duplicate results by the duplicate mean and multiplying the result by 100. The average of the individual CVs is then reported as the intra-assay CV. On average, intra-assay CV should be less than 10% (Calvi et al., 2017) and this was attained prior to quantifying the circulating biomarkers of insulin sensitivity and insulin resistance in the actual samples collected prior to and following the RSL bed rest study.

3.3.4.2. Fetuin-A: Analysis, Interpretation and Publication

Fetuin-A was chosen as the first circulating biomarker to be quantified in the fasting serum samples obtained before and after 60 days of HDT bed rest. The reasons for this decision are threefold. Firstly, a narrative review was conducted to provide a comprehensive, critical and objective overview of the current research on fetuin-A. The purpose of this review was to gain an understanding of the metabolic actions of fetuin-A, its regulation in metabolic disease and acute and chronic exercise and to establish whether fetuin-A has been measured in response to HDT bed rest, physical inactivity, sedentary behaviour and ageing. To avoid repetition, the written review on fetuin-A can be found in section 2.4.10.1, starting on page 79 of this PhD thesis. In brief, fetuin-A is an abundant and multi-functional glycoprotein synthesized mainly by hepatocytes, which promotes insulin resistance and inflammation and had not, to the best of the author's knowledge at the time of writing, been studied previously in HDT bed rest.

Secondly, following a supervisor collaboration with a European research team investigating the metabolic response to HDT bed rest, the liver was identified as an emerging organ of metabolic importance in both normal physiology and metabolic dysfunction. The liver is a key regulator of systemic energy balance by sensing nutrient availability and altering metabolite and energy production for various tissues (Kim et al., 2021). The liver communicates with these tissues (e.g. adipose tissue, skeletal muscle and central nervous system) in part by producing and secreting hepatokines, such as fetuin-A, which play a fundamental role in the inter-organ communication network. Dysregulation of liver function and these related pathways may result in metabolic dysfunction and the development of insulin resistance, which is a well-known consequence of HDT bed rest (Kenny et al., 2017; Kenny et al., 2020). Therefore, it was of great interest to focus primarily on the response of circulating hepatokines to HDT bed rest as this may provide insights into changes in liver metabolism and systemic metabolic homeostasis following prolonged inactivity and high levels of sedentary behaviour.

Thirdly, an investigation of the effectiveness of different lifestyle interventions (dietary restriction or various exercise interventions including aerobic training, resistance training and eccentric training) on circulating biomarkers of insulin sensitivity and insulin

resistance in a different population conducted during a supervisor's PhD, highlighted a large variation in biomarker responses, except for fetuin-A, which showed a consistent circulating response. Taken together, it was clear that fetuin-A plays a fundamental role in insulin signalling and energy metabolism and the identification and evaluation of this circulating hepatokine, which is modulated under various physiological statuses but exhibits minor variation in circulating response, may provide novel insights into metabolic disturbances following HDT bed rest.

As part of laboratory training and development, fetuin-A was one of the circulating biomarkers quantified using the ELISA technique and therefore, the researcher was familiar with order processing, laboratory preparation and the experimental protocol. Briefly, circulating concentrations of fetuin-A were quantified in fasting serum samples collected before and after HDT bed rest using a sandwich ELISA, according to the manufacturer's instructions (Appendix III). Information on the specific ELISA kit used for this analysis, as well as the linear range, dilution factor, and intra-assay CV, can be found below in Table 4.

Table 4. ELISA kit used to quantify circulating concentrations of fetuin-A in fasting serum.

Biomarker	Kit Manufacturer	Linear Range	Dilution Used	Intra-Assay CV
Fetuin-A	R&D Systems	7.8 - 500 ng/mL	1:4000	11.24%

Abbreviations: CV, coefficient of variation.

Following the ELISA procedure, the duplicate readings for each standard, control and samples were averaged and the average zero standard O.D. was subtracted from each value. A standard curve was created by reducing the data using computer software (online curve fitting (mycurvefit.com, myassays.com, elisaanalysis.com) and PRISM) capable of generating a 4PL curve fit. To account for the sample dilution, the concentrations obtained from the standard curve were then multiplied by the dilution factor of 4000. After this, concentrations of fetuin-A following HDT bed rest were corrected for changes in hemoconcentration (please see Section 3.3.5 on page 191 for explanation). The final results were statistically analysed and the overall findings were interpreted by the

researcher. A draft manuscript was subsequently prepared and sent to all co-authors for review before submission. Our research paper titled “Fetuin-A as a Potential Biomarker of Metabolic Variability Following 60 Days of Bed Rest” was published in the Environmental, Aviation and Space Physiology section of the journal Frontiers in Physiology in October 2020 (Appendix IV).

3.3.4.3. Biomarker Review and Panel Selection

A narrative review was conducted with the aim of summarising the current knowledge of novel adipokines, hepatokines and myokines which enhance or attenuate insulin sensitivity and their regulation in: (i) metabolic disease, (ii) acute and chronic exercise, and (iii) bed rest, physical inactivity, sedentary behaviour and ageing. The secondary aim of this narrative review was to establish a panel of biomarkers to assist in the interpretation of changes in metabolic physiology in response to HDT bed rest, with and without an exercise countermeasure. This type of review was not systematic and did not require a pre-determined research question, specified search strategy or fixed inclusion or exclusion criteria (Demiris et al., 2019; Furley and Goldschmied, 2021). However, to maintain consistency, a 4-step process was followed: 1) Literature search; 2) Identification of search terms; 3) Review of abstracts and articles; 4) Summary and synthesis of research findings (Demiris et al., 2019). The current approach was also adapted from the published review by Ennequin et al. (2019) which summarised the current literature on hepatokines and their regulation by acute and chronic exercise in the context of metabolic disorders.

Searches for original articles, reviews, meta-analyses, systematic reviews and abstracts focusing on novel adipokines, hepatokines and myokines known to enhance or attenuate insulin sensitivity or insulin resistance and/or have been studied in response to exercise, bed rest, physical inactivity, sedentary behaviour and ageing, up until September 2020 were performed in PubMed and Google Scholar. The search terms used were “hepatokine”, “myokine”, “adipokine”, “organokine”, “exerkine”, “biomarker”, “proteins”, “hormones”, “secreted factors”, “adipose tissue”, “skeletal muscle”, “liver”, “insulin sensitivity”, “insulin resistance”, “inter-organ crosstalk”, “inter-organ communication”, “bed rest”, “inactivity”, “sedentary behaviour” and “ageing”. The

commands “AND” and “OR” were included to direct the search. All published literature identified in the search were in the English language.

After the search was complete, a total of 65 biomarkers were identified (n = 27 adipokines, n = 19 hepatokines and n = 19 myokines). Using the published articles and abstracts found in the literature search, information on each individual biomarker including the full name, aliases, secretory organ, year of first appearance in the literature, role in enhancing or attenuating insulin sensitivity, main physiological functions, measurement in bed rest, availability of a validated ELISA kit to quantify serum concentrations and the number of PubMed search results was obtained. The findings were summarised and can be found in Appendix VI.

Prior to formulating the written review, the research team (TUS and DCU) conducted a step-by-step process (detailed below) to determine whether quantifying changes in any of these organokines could help to understand the observed changes in physical and metabolic characteristics and estimates of insulin sensitivity and insulin resistance in healthy young males after HDT bed rest, as discussed in our publication (Appendix IV). As part of this decision-making process, the research team had to consider the total volume of sample available for analyses (≤ 1ml pre and post-HDT bed rest for each subject), as well as the time and cost of conducting the analysis. One validated, pre-coated and ready-to-run ELISA kit costs approximately €500 – 800 and takes approximately 6 – 8 hours to run. Two ELISA kits were required per biomarker to accommodate the quantification of the pre- and post-HDT bed rest samples for each study participant. Therefore, it was imperative to identify and select a small panel of circulating biomarkers that could be quantified by ELISA and could provide valuable insights into observed changes in systemic and tissue-specific metabolic health following prolonged HDT bed rest, with and without an exercise countermeasure.

To do this, a 3-step process was conducted (Figure 26). In phase 1, the information collected on each of the 65 individual biomarkers was assessed for (i) novelty and (ii) evidence of a specific role in insulin sensitivity or glucose or lipid metabolism. Following

Phase 1, three biomarkers were selected for analysis (apelin, irisin and RBP4) and 34 biomarkers were removed from the list of potential analytes due to reasons, not limited to, roles in obesity, inflammation, adipose tissue browning, immunity or mitochondrial function, being a paralog protein or growth factor, or a results of insufficient published evidence or specific mechanistic actions only. In Phase 2, the remaining 28 biomarkers were further examined to establish whether their physiological role(s) could potentially explain the currently observed changes in physical and metabolic characteristics in healthy young males following HDT bed rest (Appendix VII). After Phase 2, two biomarkers were selected for analysis (adiponectin and ASP) and 10 biomarkers were removed from the list of potential analytes. Phase 3 involved a similar but secondary review of the physiological role(s) of the remaining 17 biomarkers to determine if they could further assist in the interpretation of the observed physical and metabolic adaptations in healthy young males after HDT bed rest (Appendix VIII). Following Phase 3, three biomarkers were selected for analysis (adropin, apo-J and FGF-21) and 14 biomarkers were removed from the list of potential analytes. The reasons for removal in Phase 2 and Phase 3 included, but was not limited to, further links with inflammation, immunity or obesity, roles in apoptosis, angiogenesis, muscle hypertrophy, liver fibrosis, β-cell function, structural or role similarities to other proteins, poorly understood underlying mechanisms or lack of ability to examine them in more detail (e.g. hepatic AMPK expression), low level of detection in human serum (particularly in young healthy males) and a lack of ELISA specificity. Overall, an initial list of 65 potential biomarkers was reduced to a panel of 8 biomarkers following this selection process (Appendix V).

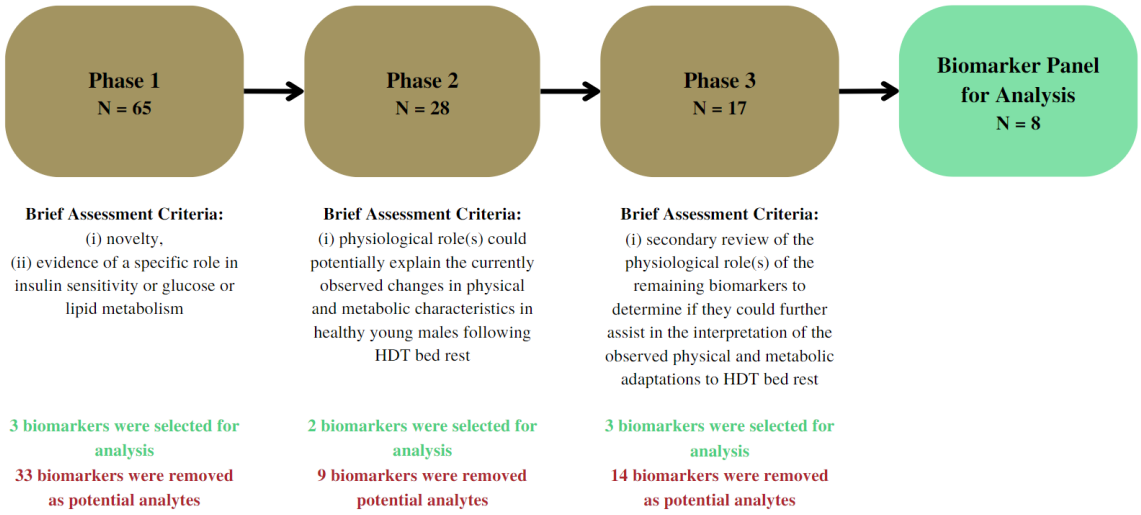

Figure 26. An overview of the 3-step selection process to determine the "best set" of circulating biomarkers to assist in the interpretation of the observed changes in physical and metabolic characteristics and estimates of insulin sensitivity and insulin resistance in healthy young males following HDT bed rest.

The final biomarkers chosen for analysis in this HDT bed rest study were RBP4, irisin, adropin, adiponectin, apo-J, FGF-21, ASP and apelin. To gain an in-depth understanding of the chosen biomarkers, the written review focused solely on the current literature relating to this biomarker panel (as well as fetuin-A) and their regulation by (i) metabolic disease, (ii) acute and chronic exercise, and (iii) bed rest, physical inactivity, sedentary behaviour and ageing. The comprehensive narrative review can be found in Chapter 2, starting on page 36.

3.3.4.4. Biomarker Panel: Analysis and Interpretation

Quotations and technical information on ELISA kits and laboratory equipment was obtained from a total 18 companies and orders were placed in a total of 7 companies. Due to COVID-19 pandemic and implications of Brexit, the lead-time and delivery of orders were significantly affected. Additionally, the government guidelines to enforce periodic closures of academic institutions caused disruption to all research students, especially in regard to receiving deliveries and accessing laboratories and offices. In accordance with government advice (at that specific time), research students were required to obtain access approval from facility directors and complete "COVID-19 Return to Work Safety" training. In addition, research students were required to pre-book laboratory space and communal equipment, complete pre-screening COVID-19 questionnaires and log

attendance. Efficient communication between research students and facility directors and company representatives and organisation was pivotal during this time to ensure minimal disruption and continuation of laboratory work.

When laboratory analysis was permitted, circulating concentrations of adiponectin, adropin, apelin, apo-J, ASP, FGF-21, irisin and RBP4 were quantified in fasting serum samples collected before and after HDT bed rest using the ELISA technique, according to the manufacturer's instructions. More specifically, a sandwich ELISA was used to quantify circulating concentrations of adiponectin, adropin, apo-J, ASP and FGF-21, whereas a competitive ELISA was used to quantify circulating concentrations of irisin, RBP4 and apelin. Information on the specific ELISA kits used for this analysis, as well as the linear ranges, dilution factors, and intra-assay CV, can be found below in Table 5.

Table 5. ELISA kits used to quantify circulating concentrations of the biomarker panel in human serum.

Biomarker	Kit Manufacturer	Linear Range	Dilution Used	Intra-Assay CV
Adiponectin	Biovendor	5 – 100 ng/ml	1:300	4.48%
Adropin	Novus Biologicals	12.5 – 800 pg/ml	1:20	13.61%
Apelin	Phoenix	0.09 – 1.44 ng/ml	1:5	5.15%
Apo-J	R&D Systems	3.12 – 200 ng/ml	1:2000	6.45%
ASP	MyBioSource	3.12 – 100 nmol/L	None	8.03%
FGF-21	R&D Systems	31.3 – 2000 pg/ml	None	6.92%
Irisin	Biovendor	0.001 – 5 µg/ml	1:10	8.57%
RBP4	Biovendor	0.001 – 5 µg/ml	1:100	8.93%

Abbreviations: CV, coefficient of variation; Apo-J, apolipoprotein-J; ASP, acylation stimulating protein; FGF-21, fibroblast growth factor 21; RBP4, retinol binding protein 4.

Following each ELISA procedure, the duplicate readings for each standard, control and samples were averaged. The average zero standard O.D. was subtracted from each value obtained using a sandwich ELISA only. A standard curve was created by reducing the data using computer software (online curve fitting (mycurvefit.com, myassays.com, elisaanalysis.com), PRISM and Microsoft Excel) capable of generating a 4PL, log/log or

exponential curve fit. To account for the sample dilutions, the concentrations of adiponectin, adropin, apelin, apo-J, irisin and RBP4 obtained from the standard curve were multiplied by their respective dilution factors (Table 5). After this, the concentrations of all biomarkers following HDT bed rest were corrected for changes in hemoconcentration (section 3.3.5., page 191). The final results were statistically analysed and the overall findings were interpreted by the researcher.

3.3.5. Correction for Changes in Hemoconcentration

As the 6° HDT angle caused the fluid shift and consequent loss of plasma volume (PV), the concentrations of biochemical parameters following HDT bed rest were corrected for changes in hemoconcentration. The change in PV (Δ%PV) following HDT bed rest was calculated as follows: $\Delta\%PV = 100 * ((Hb_{pre}/Hb_{post}) * (100 - Htc_{post}) / (100 - Htc_{pre}) - 1)$, where haemoglobin (Hb) is given in g/dL and haematocrit (Htc) is expressed as a percentage (%). To correct measured parameters for changes in PV, the following calculation was used: $[parameter]_c = [parameter]_u * (1 + \Delta PV(\%) / 100)$, where the c and u indices represent corrected and uncorrected concentrations, respectively (Dill and Costill, 1974; Alis et al., 2015).

3.3.6. Statistical Analysis

Statistical analysis was performed in SPSS 26.0 (IBM Corp., Armonk, NY) considering a two-sided 0.05 significance level. Data is presented as mean ± standard deviation (SD) for normally distributed values or as median (interquartile range, IQR) for non-normally distributed values. Normality of distribution was evaluated specific to the statistical procedure being conducted, using either the Shapiro-Wilk test or by inspecting residuals on a Q-Q Plot. Differences in baseline characteristics between the two experimental groups were assessed using independent samples t-tests, the Welch t-test, or the non-parametric Mann-Whitney U test. Physical and metabolic changes in response to bed rest were analysed using a mixed between-within factorial analysis of variance (or mixed ANOVA) using time as the within-group factor (pre, post) and group as the between-group factor (CTRL, JUMP). For the mixed ANOVA, data violating the assumption of normality was transformed to normality using either a square root transformation (moderately positively skewed), log transformation (strongly positively skewed) or

inverse transformation (extremely positively skewed). If a significant two-way interaction was identified, univariate analysis was conducted to determine if there was a significant simple main effect of group (difference in the dependent variable between groups at each category of time) and a mixed ANOVA with split groups was used to investigate if there was a significant simple main effect of time (difference in the dependent variable between time points for each category of group). Data on the simple main effects are mean ± standard error, unless otherwise stated. Conversely, if the two-way interaction was not significant, the main effects for the between-group and within-group factors were interpreted and reported.

Following this, subjects from both groups were pooled for further analyses (n = 23). The justification for the pooling of subjects from the CTRL and JUMP groups and for not performing adjustments in subsequent regression analysis was three-fold. Firstly, a similar statistical analysis approach was used during a supervisor collaboration with a European research team investigating the effect of 21 days HDT bed rest, with and without whey protein supplementation, on metabolic flexibility in healthy young males and published by Rudwill et al., (2018). Secondly, the pre- to post-HDT bed rest changes in metabolic characteristics exhibited main effects of time only. Thirdly, both the CTRL and JUMP group were physically inactive and exposed to high levels of sedentary time for 60 days HDT bed rest, based on the estimation of MVPA and energy cost of reactive jump training (2.5 minutes of MVPA per day in the JUMP group) (Le Roux et al., 2021).

Associations between the significant pre- to post-HDT bed rest changes (Δ) in indexes and biomarkers of insulin sensitivity and insulin resistance and all other measured physical and metabolic characteristics were examined using Pearson's correlations. All correlation coefficients with a significance value $p < 0.05$, $p < 0.100$ and $p < 0.200$ were selected for further assessment of validity. Subsequently, predictors of the significant Δindexes and Δbiomarkers of insulin sensitivity and insulin resistance were examined using multiple regression. Both stepwise and backwards regression methods were used to determine the best overall model, which met the specific assumptions of multiple regression (Pallant, 2011; Laerd-Statistics, 2015), and to ensure that each independent variable made a significant contribution to the prediction of the dependent variable. The

justification for this approach was based on having a large amount of predictor variables and a small number of subjects. In a small sample size ($n \leq 30$), some moderate correlations do not reach statistical significance at the traditional $p < 0.05$ level (Pallant, 2011). Additionally, a cut-off value of $p < 0.200$ to select independent variables for regression analysis has been used previously and reported within the literature (Van Middelkoop et al., 2008; O'Connor et al., 2019). Another reason for the inclusion of independent variables with correlation coefficients with a significance value $p < 0.05$, $p < 0.100$ and $p < 0.200$ is that multiple regression examines independent variables together based on how they predict or how they are associated with the dependent variable and therefore it is worthwhile to include a broader range.

Furthermore, the justification for not making any alpha adjustments is based on the consistent use of individual testing in this PhD study. Individual testing permits researchers to test individual null hypotheses that do not compromise joint null hypotheses. In this instance, a single test result is used to make a decision about a single null hypothesis, therefore providing only one opportunity to make a type I error about that null hypothesis. Thus, the alpha level of the test does not need to be lowered (Rubin, 2021). This logic applies even when multiple instances of individual testing take place side-by-side within the same study. Each decision to reject each individual null hypothesis depends on no more than one significance test so none of the individual tests constitute a "family" with respect to any single hypothesis. Hence, it is not necessary to adjust alpha levels on the basis of any family-based error rate (Rubin, 2021).

3.3.7. Subgroup Analysis

The quantification of inter-individual variability in insulin sensitivity is complex and requires appropriate statistical analysis and sample size and is therefore beyond the scope of this PhD study. However, in an attempt to further understand the different individual metabolic responses post-HDT bed rest, the subject data from the two groups was pooled and divided into two subgroups based on subjects who decreased (↓ Matsuda, n = 17, CTRL n = 7, JUMP n = 10) or increased (↑ Matsuda, n = 6, CTRL n = 4 and JUMP n = 2) insulin sensitivity post-HDT bed rest. Normality of distribution was evaluated specific to the statistical procedure being conducted. Differences in baseline characteristics

between the two subgroups were examined using independent samples t-tests or the Welch t-test. Paired sample t-tests were used to examine the significance of pre- to post-HDT bed rest changes in physical and metabolic characteristics, firstly in the subgroup with decreased insulin sensitivity following HDT bed rest and secondly, in the subgroup with increased insulin sensitivity following HDT bed rest. Subsequently, Pearson's correlations were performed to investigate the pre- to post-HDT bed rest changes (Δ) in metabolic physiology that contributed to the significant decrease in insulin sensitivity and significant changes in circulating biomarkers in the subgroup which became less insulin-sensitive post-HDT bed rest. Following this, Pearson's correlations were performed to investigate the pre- to post-HDT bed rest changes (Δ) in metabolic physiology that contributed to the significant increase in insulin sensitivity and significant changes in circulating biomarkers in the subgroup which became more insulin-sensitive post-HDT bed rest. All correlation coefficients with a significance value $p < 0.05$, $p < 0.100$ and $p < 0.200$ were retained for further assessment of validity. Predictors of the significant Δindexes and Δbiomarkers of insulin sensitivity and insulin resistance in both subgroups were explored using multiple regression. Both stepwise and backwards regression methods were used to determine the best overall model, which met the specific assumptions of multiple regression (Pallant, 2011; Laerd-Statistics, 2015), and to ensure that each independent variable made a significant contribution to the prediction of the dependent variable. Statistical analysis was performed in SPSS 26.0 (IBM Corp., Armonk, NY) considering a two-sided 0.05 significance level.

Chapter 4. Results

4.1. Physical Characteristics: Body Weight, Body Composition, $\dot{V}O_{2peak}$ and Muscle Strength

Subject's baseline characteristics are displayed in Table 6. Anthropometric measurements were obtained on BDC-3 and HDT60 (Table 7). No significant between-group differences in age, height and measures of body weight and body composition were identified at baseline. There was a significant interaction between time and group on body weight ($F_{1, 21} = 5.620$, $p = 0.027$, partial $\eta^2 = 0.211$), lean mass ($F_{1, 21} = 11.837$, $p = 0.002$, partial $\eta^2 = 0.360$) and fat mass ($F_{1, 21} = 6.534$, $p = 0.018$, partial $\eta^2 = 0.237$). Following 60 days of HDT bed rest, body weight decreased significantly in both groups, with a larger reduction observed in the CTRL group (M = -3.63 kg, SE = 0.54 kg, $p < 0.001$) compared to the JUMP group (M = -2.23 kg, SE = 0.28 kg, $p < 0.001$). Similarly, HDT bed rest significantly reduced lean mass in both groups, with a higher decline noticeable in the CTRL group (M = -3.91 kg, SE = 0.70 kg, $p < 0.001$) in comparison to the JUMP group (M = -1.34 kg, SE = 0.32 kg, $p = 0.002$). Fat mass decreased significantly in the JUMP group (M = -0.87 kg, SE = 0.23 kg, $p = 0.003$) but did not change in the CTRL group (M = 0.10 kg, SE = 0.31 kg, $p = 0.757$) following HDT bed rest. Bone mineral content did not change after HDT bed rest.

The main effect of time showed a significant reduction in trunk total mass ($F_{1, 21} = 11.135$, $p = 0.003$, partial $\eta^2 = 0.346$) and trunk lean mass ($F_{1, 21} = 7.191$, $p = 0.014$, partial $\eta^2 = 0.255$) following HDT bed rest. There was a significant interaction between time and intervention on body weight on trunk fat mass ($F_{1, 21} = 9.772$, $p = 0.005$, partial $\eta^2 = 0.318$). Trunk fat mass decreased significantly in the JUMP group (M = -0.53 kg, SE = 0.14 kg, $p = 0.002$) but did not change significantly in the CTRL group (M = 0.23 kg, SE = 0.21 kg, $p = 0.298$) after HDT bed rest. There was also a significant interaction between time and group on leg total mass ($F_{1, 21} = 59.243$, $p < 0.001$, partial $\eta^2 = 0.738$) and leg lean mass ($F_{1, 21} = 49.777$, $p < 0.001$, partial $\eta^2 = 0.703$). Leg total mass decreased significantly post-HDT bed rest with a greater loss identified in the CTRL group (M = -3.13 kg, SE = 0.21 kg, $p < 0.001$) compared to the JUMP group (M = -1.28 kg, SE = 0.13 kg, $p < 0.001$). Additionally, there was a trend for a between-group difference in leg total mass following HDT bed rest, which was greater in the JUMP group (M = 2.23 kg, SE =

1.08 kg, p = 0.052). Similarly, leg lean mass was significantly decreased following HDT bed rest, with a larger reduction evident in the CTRL group (M = -3.10 kg, SE = 0.23 kg, p < 0.001) in comparison to the JUMP group (M = -1.03 kg, SE = 0.18 kg, p < 0.001). In addition, there was a significant between-group difference in leg lean mass post-HDT bed rest, which was significantly higher in the JUMP group (M = 1.79 kg, SE = 0.74 kg, p = 0.024). Leg fat mass, arm total mass and arm lean mass did not change. The main effect of time revealed a significant reduction in arm fat mass after HDT bed rest ($F_{1, 21}$ = 11.238, p = 0.003, partial η^2 = 0.349).

Table 6. Baseline descriptive characteristics of the subjects in the RSL bed rest study.

	CTRL	**JUMP**
N	11	12
Age (years)	28 ± 6	30 ± 7
Height (cm)	181 ± 5	181 ± 7
BMI (kg/m²)	23.33 ± 2.03	23.75 ± 1.80

Data are presented as mean ± standard deviation (SD). Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; N, number; BMI, body mass index.

Table 7. The effect of 60 days HDT bed rest on measures of anthropometry.

Measurement	CTRL (n = 11)		JUMP (n = 12)		Statistics		
	Pre	Post	Pre	Post	Time	Group	T*G
BW (kg)	76.10 ± 8.06	72.47 ± 6.76*	77.85 ± 6.55	75.63 ± 6.39*	**<0.001**	0.405	**0.027**
LM (kg)	56.94 ± 6.57	53.03 ± 5.11*	56.41 ± 5.18	55.08 ± 4.29*	**<0.001**	0.731	**0.002**
FM (kg)	16.91 ± 3.95	17.00 ± 3.41	19.21 ± 6.42	18.34 ± 6.18*	0.055	0.412	**0.018**
BMC (kg)	3.14 ± 0.36	3.14 ± 0.37	3.00 ± 0.32	3.00 ± 0.32	0.605	0.355	0.800
Trunk TM (kg)	35.79 ± 4.34	34.97 ± 3.15	37.14 ± 3.50	36.20 ± 3.60	**0.003**	0.403	0.823
Trunk LM (kg)	26.85 ± 3.39	25.80 ± 2.15	26.66 ± 2.91	26.27 ± 2.37	**0.014**	0.900	0.241
Trunk FM (kg)	8.05 ± 2.36	8.28 ± 2.01	9.61 ± 3.84	9.07 ± 3.95*	0.222	0.388	**0.005**
Leg TM (kg)	26.94 ± 2.83	23.81 ± 2.58*	27.32 ± 2.67	26.04 ± 2.59*	**<0.001**	0.251	**<0.001**
Leg LM (kg)	19.61 ± 2.36	16.54 ± 2.13*	19.36 ± 1.37	18.33 ± 1.36*#	**<0.001**	0.320	**<0.001**
Leg FM (kg)	6.11 ± 1.26	6.07 ± 1.18	6.79 ± 2.35	6.53 ± 2.07	0.117	0.454	0.249
Arm TM (kg)	9.26 ± 1.09	9.25 ± 1.05	9.34 ± 1.11	9.32 ± 0.96	0.800	0.860	0.941
Arm LM (kg)	7.00 ± 0.93	7.10 ± 0.89	7.01 ± 1.15	7.07 ± 1.00	0.248	0.981	0.787
Arm FM (kg)	1.82 ± 0.45	1.71 ± 0.35	1.90 ± 0.49	1.81 ± 0.45	**0.003**	0.609	0.784

Data are presented as mean ± standard deviation (SD). Anthropometric measurements were taken on BDC-3 and HDT60. Significant p-values ($p \leq 0.05$) are indicated in bold. When a significant interaction effect was found, an asterisk (*) represents a significant difference from pre in each group. A hashtag (#) represents a significant difference between groups post-HDT bed rest. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Group, main effect of group; T*G, time*group interaction effect; BW, body weight; LM, lean mass; FM, fat mass; BMC, bone mineral content; TM, total mass.

$\dot{V}O_{2peak}$ was measured on BDC-8 and R+1 (Table 8). No significant between-group differences in $\dot{V}O_{2peak}$ were found at baseline. There was a significant interaction between time and group on absolute $\dot{V}O_{2peak}$ ($F_{1, 18} = 17.564$, $p = 0.001$, partial $\eta^2 = 0.494$) and relative $\dot{V}O_{2peak}$, when normalised for changes in body weight ($F_{1, 18} = 20.931$, $p < 0.001$, partial $\eta^2 = 0.538$) and lean mass ($F_{1, 18} = 20.653$, $p < 0.001$, partial $\eta^2 = 0.534$). Absolute $\dot{V}O_{2peak}$ decreased significantly in both groups after HDT bed rest, with a greater loss

identified in the CTRL group (M = -1.28 L/min, SE = 0.17 L/min, p < 0.001) compared to the JUMP group (M = -0.33 L/min, SE = 0.15 L/min, p = 0.049). $\dot{V}O_{2peak}$, when normalised for changes in body weight, decreased significantly in the CTRL group (M = -14.86 ml/kg/min, SE = 1.84 ml/kg/min, p < 0.001) but did not change in the JUMP group (M = -2.85 ml/kg/min. SE = 1.87 ml/kg/min p = 0.161) following HDT bed rest. Similarly, $\dot{V}O_{2peak}$, when normalised for changes in lean mass, decreased significantly in the CTRL group (M = 18.82 ml/kgLM/min, SE = 2.26 ml/kgLM/min, p < 0.001) but did not change in the JUMP group (M = -4.58 ml/kgLM/min, SE = 2.17 ml/kgLM/min, p = 0.063) after HDT bed rest.

Table 8. The effect of 60 days HDT bed rest on measures of cardiorespiratory capacity.

Measurement	CTRL (n = 10)		JUMP (n = 10)		Statistics		
	Pre	Post	Pre	Post	Time	Group	T*G
$\dot{V}O_{2peak}$ (L/min)	3.85 ± 0.68	2.57 ± 0.48*	3.32 ± 0.76	2.99 ± 0.53*	**<0.001**	0.848	**0.001**
$\dot{V}O_{2peak}$ (ml/kg/min)	50.11 ± 7.31	35.25 ± 7.05*	42.65 ± 8.72	39.79 ± 6.58	**<0.001**	0.639	**<0.001**
$\dot{V}O_{2peak}$ (ml/kgLM/min)	67.55 ± 8.42	48.74 ± 9.35*	58.72 ± 10.95	54.14 ± 8.03	**<0.001**	0.660	**<0.001**

Data are presented as mean ± SD. $\dot{V}O_{2peak}$ was measured on BDC-8 and R+1. Significant p-values ($p \leq 0.05$) are indicated in bold. When a significant interaction effect was found, an asterisk (*) represents a significant difference from pre in each group. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Group, main effect of group; T*G, time*group interaction effect; $\dot{V}O_{2peak}$, peak aerobic capacity.

Measurements of muscle strength were recorded on BDC-1 and R+0 (Table 9). No significant between-group differences in muscle strength were identified at baseline. There was a significant interaction between time and group on isometric maximal voluntary contraction (MVC) torque during knee extension ($F_{1, 21}$ = 38.490, p < 0.001, partial η^2 = 0.647) and plantarflexion ($F_{1, 21}$ = 33.240, p < 0.001, partial η^2 = 0.613). Maximal leg strength during knee extension decreased significantly in the CTRL group (M = -126.49 Nm, SE = 16.69 Nm, p < 0.001) but did not change in the JUMP group (M = -10.86 Nm, SE = 9.23 Nm, p = 0.264) following HDT bed rest. Additionally, maximal leg strength during knee extension was significantly greater in the JUMP group post-HDT bed rest (M = 112.43 Nm, SE = 19.34 Nm, p < 0.001). Maximal leg strength during

plantarflexion decreased significantly in both groups after HDT bed rest, with a larger reduction found in the CTRL group (M = -97.08 Nm, SE = 12.34 Nm, $p < 0.001$) in comparison to the JUMP group (M = -18.50 Nm, SE = 6.53 Nm, $p = 0.016$). In addition, maximal leg strength during plantarflexion was significantly higher in the JUMP group post-HDT bed rest (M = 71.70 Nm, SE = 15.22 Nm, $p < 0.001$). The main effect of time showed a significant reduction in isometric MVC torque during knee flexion ($F_{1, 21} = 16.661$, $p = 0.001$, partial $\eta^2 = 0.442$) and dorsiflexion ($F_{1, 21} = 15.576$, $p = 0.001$, partial $\eta^2 = 0.426$) after HDT bed rest.

Table 9. The effect of 60 days HDT bed rest on measurements of muscle strength.

Measurement	CTRL (n = 11)		JUMP (n = 12)		Statistics		
	Pre	Post	Pre	Post	Time	Group	T*G
Knee Extension (Nm)	297.67.± 68.58	171.18 ± 34.06*	294.47 ± 66.88	283.61 ± 55.16#	**<0.001**	**0.023**	**<0.001**
Knee Flexion (Nm)	125.89 ± 18.38	105.10 ± 14.03	129.31 ± 19.17	120.85 ± 17.75	**0.001**	0.147	0.100
Plantarflexion (Nm)	235.10 ± 41.15	138.02 ± 29.01*	228.22 ± 46.88	209.72 ± 42.09*#	**<0.001**	**0.049**	**<0.001**
Dorsiflexion† (Nm)	39.00 (34.55)	37.69 (27.97)	45.16 (24.87)	39.90 (25.59)	**0.001**	0.661	0.711

Data are presented as mean ± SD or median (IQR). Maximal voluntary isometric strength was measured on BDC-1 and R+0. Significant p-values ($p \leq 0.05$) are indicated in bold. A dagger (†) denotes that data was transformed for the two-way mixed ANOVA. When a significant interaction effect was found, an asterisk (*) represents a significant difference from pre in each group. A hashtag (#) represents a significant difference between groups post-HDT bed rest. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Group, main effect of group; T*G, time*group interaction effect.

4.2. Metabolic Characteristics: Glucose Tolerance, Insulin Sensitivity and Lipid Metabolism

Changes in the glucose and insulin response to the OGTT following 60 days HDT bed rest are presented in Table 10 and Figure 27. No significant between-group differences in measurements of glucose tolerance and insulin sensitivity were found at baseline. Metabolic characteristics were corrected for changes in hemoconcentration following HDT bed rest (mean ΔPV% CTRL 0.88%, JUMP -2.13%). The main effect of time

showed a significant increase in $glucose_{60}$ ($F_{1, 21} = 19.409$, $p < 0.001$, partial $\eta^2 = 0.480$) and $glucose_{120}$ ($F_{1, 21} = 7.995$, $p = 0.010$, partial $\eta^2 = 0.276$) after HDT bed rest. $Glucose_0$, $glucose_{30}$, and $glucose_{90}$ did not change. The main effect of time revealed a significant increase in $AUCG_{120}$ ($F_{1, 21} = 9.535$, $p = 0.006$), but not $AUCG_{30}$, post-HDT bed rest. The main effect of time showed a significant increase in $insulin_0$ following HDT bed rest ($F_{1, 21} = 7.650$, $p = 0.012$, partial $\eta^2 = 0.267$). Additionally, the main effect of group showed a significant difference in $insulin_0$ between groups ($F_{1, 21} = 5.004$, $p = 0.036$, partial $\eta^2 = 0.192$). $Insulin_{30}$, $insulin_{60}$, $insulin_{90}$, $insuin_{120}$ and $AUCI_{30}$ did not change post-HDT bed rest. Despite this, the main effect of time showed a significant increase in $AUCI_{120}$ ($F_{1, 21} = 5.361$, $p = 0.031$, Partial $\eta^2 = 0.203$) following HDT bed rest.

Table 10. The effect of 60 days HDT bed rest on glucose tolerance and insulin sensitivity.

Measurement	CTRL (n = 11)		JUMP (n = 12)		Statistics		
	Pre	Post	Pre	Post	Time	Group	T*G
$Glucose_0$ (mmol/L)	5.18 ± 0.40	5.24 ± 0.51	5.32 ± 0.64	5.47 ± 0.59	0.447	0.311	0.732
$Glucose_{30}$ (mmol/L)	8.77 ± 1.25	9.28 ± 0.99	8.62 ± 1.53	9.08 ± 1.22	0.114	0.691	0.933
$Glucose_{60}$ (mmol/L)	6.77 ± 1.67	8.49 ± 1.33	7.90 ± 2.06	8.99 ± 1.87	**<0.001**	0.233	0.332
$Glucose_{90}$ (mmol/L)	7.67 ± 1.59	8.11 ± 1.96	7.90 ± 2.52	8.62 ± 1.69	0.126	0.628	0.704
$Glucose_{120}$ (mmol/L)	6.95 ± 1.01	8.51 ± 1.80	7.36 ± 1.82	7.98 ± 1.98	**0.010**	0.919	0.234
$AUCG_{30}$ (mmol/L*min)	209.17 ± 22.30	217.80 ± 20.08	208.99 ± 27.29	218.30 ± 25.83	0.133	0.985	0.953
$AUCG_{120}$ (mmol/L*min)	878.09 ± 128.83	982.98 ± 140.28	922.54 ± 187.30	1002.46 ± 154.12	**0.006**	0.583	0.681
$Insulin_0$ (pmol/L)	45.00 ± 13.09	51.48 ± 16.49	55.55 ± 14.92	67.22 ± 19.14	**0.012**	**0.036**	0.437
$Insulin_{30}$ (pmol/L)	466.73 ± 237.48	539.37 ± 165.34	556.61 ± 289.34	456.43 ± 157.69	0.771	0.965	0.077
$Insulin_{60}$ (pmol/L)	418.55 ± 220.00	490.29 ± 217.96	454.56 ± 220.72	536.05 ± 190.38	0.060	0.614	0.901
$Insulin_{90}$ (pmol/L)	454.79 ± 272.03	500.08 ± 238.30	514.60 ± 228.79	561.16 ± 249.70	0.154	0.546	0.984
$Insulin_{120}$† (pmol/L)	335.50 (678.30)	512.51 (1201.60)	480.20 (842.40)	491.88 (913.33)	0.054	0.587	0.264
$AUCI_{30}$ (pmol/L*min)	7676.03 ± 3684.34	8862.74 ± 2636.68	9182.33 ± 4426.95	7856.15 ± 2567.66	0.922	0.843	0.089
$AUCI_{120}$ (pmol/L*min)	46084.83 ± 20023.15	54110.26 ± 16896.34	53632.08 ± 17872.47	55010.45 ± 15743.61	**0.031**	0.558	0.117

Data are presented as mean ± SD or median (IQR). Metabolic characteristics were measured on BDC-5 and HDT59. Significant p-values ($p \leq 0.05$) are indicated in bold. A dagger (†) denotes that data was transformed for the two-way mixed ANOVA. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Group, main effect of group; T*G, time*group interaction effect; $Glucose_0$, fasting glucose; $Glucose_{120}$, glucose concentrations 120 minutes after the glucose load; AUCG, area under the curve for glucose; $Insulin_0$, fasting insulin; $Insulin_{120}$, insulin concentrations 120 minutes after the glucose load, AUCI, area under the curve for insulin.

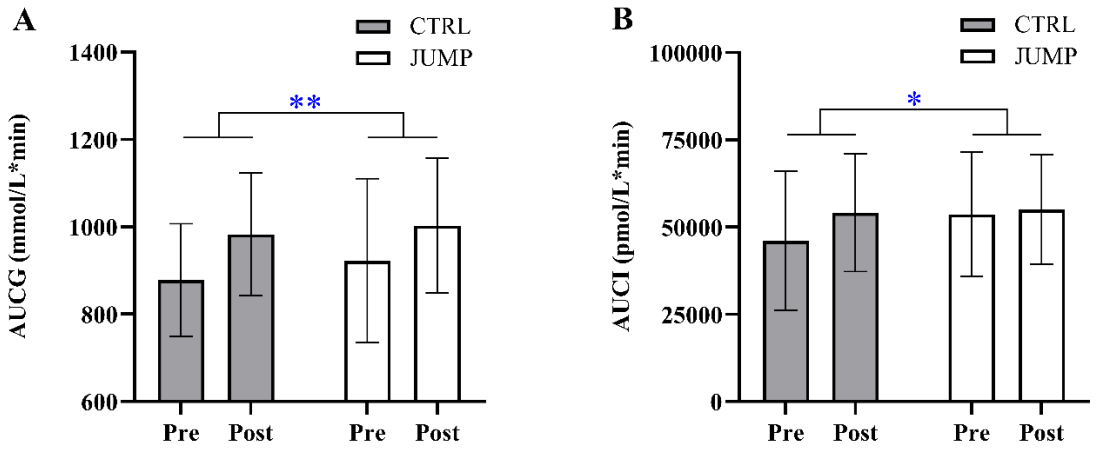

Figure 27. The effect of 60 days HDT bed rest on the glucose and insulin response to the OGTT measured on BDC-5 (pre) and HDT59 (post).

Data are presented as mean ± SD. Pre- to post-HDT bed rest changes in the 120 minute area under the curve totals for glucose and insulin are shown in (A) and (B), respectively. A significant main effect of time indicated as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Changes in measures of the lipid profile and lipid ratios after 60 days HDT bed rest are shown below in Table 11. Analysis of between-group differences at baseline found a significant difference in TG: HDL only, with a higher mean ratio identified in the JUMP group prior to HDT bed rest (p = 0.040). The main effect of time showed a significant increase in triglycerides ($F_{1, 21}$ = 7.337, p = 0.013, partial η^2 = 0.259) and LDL ($F_{1, 21}$ = 10.470, p = 0.004, partial η^2 = 0.333) following HDT bed rest. Additionally, the main effect of time found a significant decrease in HDL ($F_{1, 21}$ =18.968, p < 0.001, partial η^2 = 0.475) after HDT bed rest. NEFA and total cholesterol did not change. The main effect of time identified a significant increase in LDL: HDL ($F_{1, 21}$ = 87.786, p < 0.001, partial η^2 = 0.807) and CHOL: HDL ($F_{1, 21}$ = 86.298, p < 0.001, partial η^2 = 0.804) post-HDT bed rest. The main effect of time showed a significant increase in TG: HDL following HDT bed rest ($F_{1, 21}$ = 45.506, p < 0.001, partial η^2 = 0.684). In addition, the main effect of group showed a significant difference in TG: HDL between groups ($F_{1, 21}$ = 4.359, p = 0.049, partial η^2 = 0.172).

Table 11. The effect of 60 days HDT bed rest on measures of the lipid profile and lipid ratios.

Measurement	CTRL (n = 11)		JUMP (n = 12)		Statistics		
	Pre	Post	Pre	Post	Time	Group	T*G
NEFA (mmol/L)	0.40 ± 0.13	0.41 ± 0.16	0.41 ± 0.14	0.52 ± 0.17	0.124	0.232	0.215
TG (mmol/L)	0.87 ± 0.26	1.00 ± 0.26	1.16 ± 0.45	1.21 ± 0.41	**0.013**	0.102	0.245
CHOL (mmol/L)	4.09 ± 0.72	4.27 ± 0.86	4.11 ± 0.57	4.06 ± 0.57	0.619	0.710	0.381
HDL (mmol/L)	1.16 ± 0.17	0.99 ± 0.19	1.08 ± 0.27	0.91 ± 0.17	**<0.001**	0.304	0.955
LDL (mmol/L)	2.76 ± 0.71	3.10 ± 0.71	2.73 ± 0.51	2.99 ± 0.56	**0.004**	0.780	0.647
LDL: HDL (mmol/L)	2.42 ± 0.72	3.19 ± 0.78	2.62 ± 0.64	3.33 ± 0.62	**<0.001**	0.546	0.704
CHOL: HDL (mmol/L)	3.57 ± 0.69	4.36 ± 0.74	3.91 ± 0.64	4.52 ± 0.66	**<0.001**	0.370	0.249
TG: HDL (mmol/L)	0.77 ± 0.27	1.04 ± 0.27	1.16 ± 0.54‡	1.40 ± 0.57	**<0.001**	**0.049**	0.711

Data are presented as mean ± SD. Metabolic characteristics were measured on BDC-5 and HDT59 Significant p-values ($p \leq 0.05$) are indicated in bold. A double dagger (‡) represents a significant difference between groups pre-HDT bed rest. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Group, main effect of group; T*G, time*group interaction effect; NEFA, non-esterified fatty acids; TG, triglycerides; CHOL, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

Changes in estimates of insulin sensitivity and insulin resistance after 60 days of HDT bed rest are displayed in Table 12. No significant between-group differences in indexes of insulin sensitivity and insulin resistance were identified at baseline. The main effect of time revealed a significant decrease in the Matsuda index ($F_{1, 21} = 11.735$, $p = 0.003$, partial $\eta^2 = 0.358$), disposition index ($F_{1, 21} = 8.845$, $p = 0.007$, partial $\eta^2 = 0.296$), Gutt index ($F_{1, 21} = 4.681$, $p = 0.042$, partial $\eta^2 = 0.182$) and muscle insulin sensitivity ($F_{1, 21} = 4.560$, $p = 0.045$, partial $\eta^2 = 0.178$) following HDT bed rest. The main effect of time showed a significant increase in HOMA-IR post-HDT bed rest ($F_{1, 21} = 6.613$, $p = 0.018$, partial $\eta^2 = 0.240$). In addition, the main effect of group revealed a significant difference in HOMA-IR between groups ($F_{1, 21} = 5.129$, $p = 0.034$, partial $\eta^2 = 0.196$). The main effect of time identified a significant decrease in QUICKI after HDT bed rest ($F_{1, 21} = 5.796$, $p = 0.025$, partial $\eta^2 = 0.216$). Additionally, the main effect of group showed a significant difference in QUICKI between groups ($F_{1, 21} = 5.591$, $p = 0.028$, partial $\eta^2 =$

0.210). Similarly, the main effect of time highlighted a significant decrease in liver insulin sensitivity following HDT bed rest ($F_{1, 21} = 6.222$, $p = 0.021$, partial $\eta^2 = 0.229$) and the main effect of group revealed a significant difference in liver insulin sensitivity between groups ($F_{1, 21} = 5.842$, $p = 0.025$, partial $\eta^2 = 0.218$). The main effect of time showed a significant increase in adipose tissue insulin resistance after HDT bed rest ($F_{1, 21} = 8.327$, $p = 0.009$, partial $\eta^2 = 0.284$). Further to this, the main effect of group found a significant difference in adipose tissue insulin resistance between groups ($F_{1, 21} = 4.526$, $p = 0.045$, partial $\eta^2 = 0.177$). HOMA-β, insulinogenic index, OGIS index and hepatic insulin resistance did not change.

Table 12. The effect of 60 days HDT bed rest on estimates of insulin sensitivity and insulin resistance.

Measurement	CTRL (n = 11)		JUMP (n = 12)		Statistics		
	Pre	Post	Pre	Post	Time	Group	T*G
HOMA-IR	1.51 ± 0.51	1.74 ± 0.67	1.88 ± 0.50	2.34 ± 0.72	**0.018**	**0.034**	0.409
HOMA-β†	73.83 (77.34)	83.89 (193.29)	96.99 (97.54)	99.20 (128.94)	0.135	0.300	0.881
QUICKI	0.36 ± 0.02	0.36 ± 0.02	0.35 ± 0.01	0.34 ± 0.01	**0.025**	**0.028**	0.324
Matsuda†	5.10 (6.37)	4.18 (3.47)	3.75 (5.50)	3.24 (3.48)	**0.003**	0.125	0.720
Ins. Index	1.06 ± 0.71	0.99 ± 0.34	1.33 ± 0.61	0.91 ± 0.45	0.052	0.630	0.154
Disp. Index†	4.67 (11.06)	3.74 (5.11)	5.48 (16.97)	2.66 (6.78)	**0.007**	0.803	0.171
OGIS Index	386.14 ± 49.57	361.85 ± 42.42	369.69 ± 44.86	354.97 ± 43.24	0.075	0.464	0.651
Gutt Index	74.63 ± 14.25	61.63 ± 16.89	71.66 ± 28.11	62.87 ± 19.40	**0.042**	0.902	0.680
Liver IS	0.74 ± 0.29	0.64 ± 0.20	0.57 ± 0.18	0.46 ± 0.11	**0.021**	**0.025**	0.886
Muscle IS	0.03 ± 0.04	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	**0.045**	0.372	0.302
Hepatic IR	446.07 ± 208.71	537.08 ± 166.87	547.95 ± 308.17	473.85 ± 158.66	0.875	0.799	0.136
Adipose IR†	2.57 (3.14)	2.30 (6.53)	3.52 (6.97)	5.07 (5.59)	**0.009**	**0.045**	0.211

Data are presented as mean ± SD or median (IQR). Metabolic characteristics were measured on BDC-5 and HDT59. Significant p-values ($p \leq 0.05$) are indicated in bold. A dagger (†) denotes that data was transformed for the two-way mixed ANOVA. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Group, main effect of group; T*G, time*group interaction effect; HOMA-IR, homeostatic model of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function; QUICKI, quantitative insulin-sensitivity check index; Ins. Index, insulinogenic index; Disp. Index, disposition index; OGIS Index, oral glucose insulin sensitivity index; IS, insulin sensitivity; IR, insulin resistance.

4.3. Biomarkers of Insulin Sensitivity and Insulin Resistance

Changes in circulating biomarkers of insulin sensitivity and insulin resistance are displayed in Table 13 and Figure 28. There were no significant between-group differences in circulating fetuin-A, RBP4, irisin, adiponectin, ASP, adropin, apelin or FGF-21 at

baseline. There was a significant interaction between time and group on circulating Apo-J ($F_{1, 21} = 5.716$, $p = 0.026$, partial $\eta^2 = 0.214$). Apo-J was significantly higher in the JUMP group at baseline (M = 53.25 μg/ml, SE = 22.08 μg/ml, $p = 0.025$) but there was no significant difference in apo-J between groups post-intervention (M = 10.14 μg/ml, SE = 20.74 μg/ml, $p = 0.680$). Apo-J did not change in the CTRL group ($p = 0.082$) or JUMP group ($p = 0.170$) following HDT bed rest. A significant main effect of time showed a significant increase in fetuin-A ($F_{1, 21} = 13.990$, $p = 0.001$, partial $\eta^2 = 0.400$) and a significant decrease in irisin ($F_{1, 21} = 7.751$, $p = 0.011$, partial $\eta^2 = 0.270$), adiponectin ($F_{1, 21} = 28.428$, $p < 0.001$, partial $\eta^2 = 0.575$), adropin ($F_{1, 21} = 14.967$, $p = 0.001$, partial $\eta^2 = 0.416$) and FGF-21 ($F_{1, 21} = 32.790$, $p < 0.001$, partial $\eta^2 = 0.633$) post-HDT bed rest. RBP4, ASP and apelin did not change after HDT bed rest.

Table 13. The effect of 60 days HDT bed rest on circulating biomarkers of insulin sensitivity and insulin resistance.

Measurement	CTRL		JUMP		Statistics		
	Pre	Post	Pre	Post	Time	Group	T*G
Fetuin-A† (mg/ml)	0.34 (0.59)	0.47 (0.40)	0.40 (0.47)	0.51 (0.68)	**0.001**	0.609	0.769
RBP4 (µg/ml)	37.85 ± 8.13	39.33 ± 13.05	38.82 ± 15.17	43.04 ± 10.13	0.256	0.597	0.582
Irisin (µg/ml)	6.85 ± 2.47	5.18 ± 2.10	6.95 ± 2.96	5.79 ± 2.76	**0.011**	0.712	0.623
Apo-J (µg/ml)	176.68 ± 55.12	210.38 ± 60.55	229.93 ± 50.80‡	200.24 ± 37.17	0.777	0.175	**0.026**
Adiponectin (µg/ml)	8.06 ± 2.05	6.58 ± 1.90	8.00 ± 2.44	5.28 ± 1.52	**<0.001**	0.366	0.131
Adropin (ng/ml)	3.67 ± 1.44	2.47 ± 1.79	2.56 ± 1.22	1.82 ± 1.30	**0.001**	0.123	0.378
Apelin (ng/ml)	0.89 ± 0.23	0.88 ± 0.30	0.82 ± 0.18	0.75 ± 0.23	0.447	0.276	0.629
ASP (nmol/l)	6.97 ± 2.09	7.06 ± 2.53	6.13 ± 2.64	7.62 ± 3.88	0.232	0.922	0.288
FGF-21 (pg/ml)	133.27 ± 92.50	73.63 ± 36.43	175.67 ± 77.35	95.92 ± 37.60	**<0.001**	0.227	0.419

Data are presented as mean ± SD or median (IQR). Metabolic characteristics were measured on BDC-5 and HDT59. Significant p-values ($p \leq 0.05$) are indicated in bold. A dagger (†) denotes that data was transformed for the two-way mixed ANOVA. A double dagger (‡) represents a significant difference between experimental groups pre-HDT bed rest. The results for fetuin-A, RBP4, irisin, apo-J, adiponectin and adropin include n = 11 in CTRL and n = 12 JUMP. For apelin, n = 9 CTRL and n = 11 JUMP. For ASP, n = 7 CTRL and n = 7 JUMP. For FGF-21, n = 10 CTRL and n = 11 JUMP. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Group, main effect of group; T*G, time*group interaction effect.

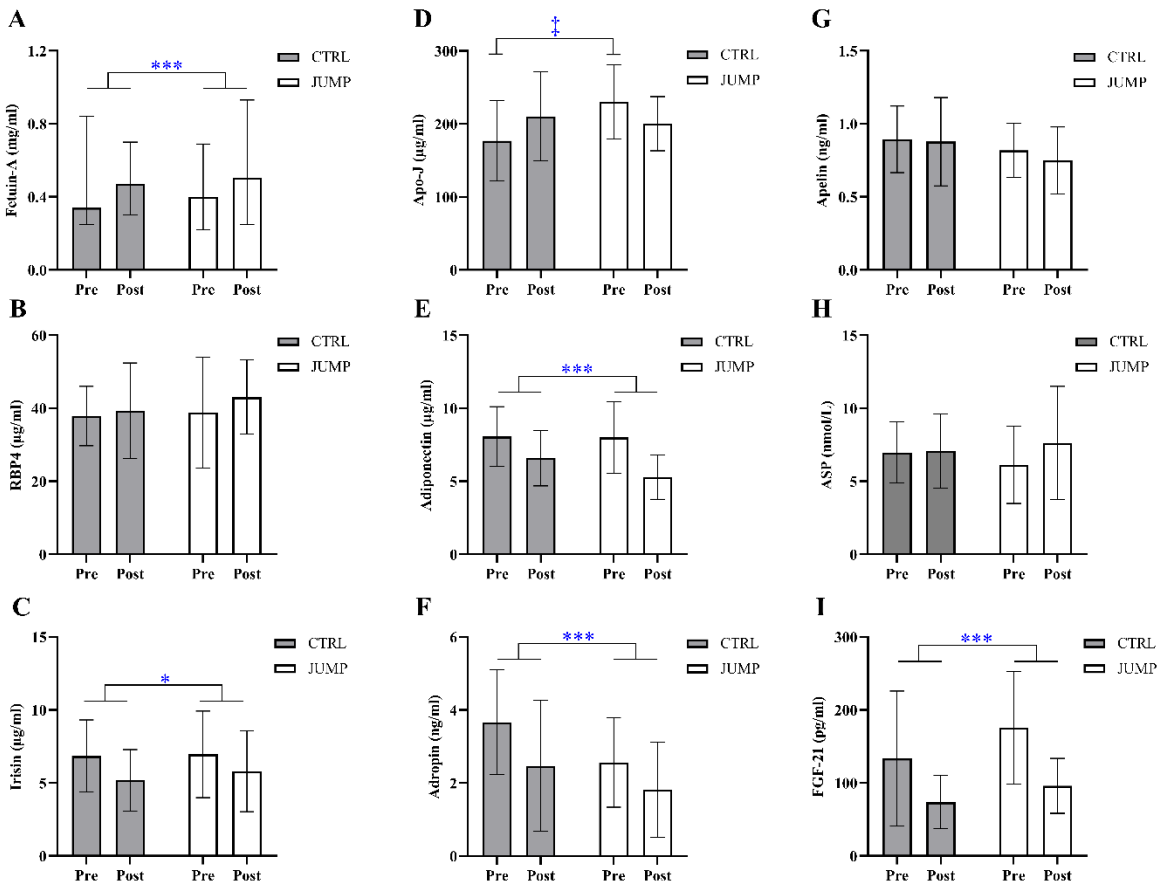

Figure 28. The effect of 60 days HDT bed rest on biomarkers of insulin sensitivity and insulin resistance measured on BDC-5 (pre) and HDT59 (post).

Data are presented as mean ± SD or median (IQR). Metabolic characteristics were measured on BDC-5 and HDT59. The results for fetuin-A, RBP4, irisin, apo-J, adiponectin and adropin (Figure 3A-F) include n = 11 in CTRL and n = 12 JUMP. For apelin (Figure 3G), n = 9 CTRL and n = 11 JUMP. For ASP (Figure 3H), n = 7 CTRL and n = 7 JUMP. For FGF-21 (Figure 3I), n = 10 CTRL and n = 11 JUMP. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group. A significant main effect of time indicated as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. A double dagger (‡) represents a significant difference between experimental groups pre-HDT bed rest.

4.4. Correlation and Regression Analysis

4.4.1. Indexes of Insulin Sensitivity and Insulin Resistance

The ΔMatsuda index was significantly and negatively correlated with the Δtriglycerides, Δtotal cholesterol and ΔLDL in all subjects post-HDT bed rest (Table 14). Additionally, the ΔMatsuda index was significantly and positively correlated with Δknee flexion in all subjects after HDT bed rest. Multiple regression analysis determined that the Δtriglycerides significantly explained 35.80% (adjusted r^2 = 32.80%) of the variance in the Matsuda index in all subjects following HDT bed rest ($F_{1, 21}$ = 11.725, p = 0.003. Table 15).

Table 14. Correlational analysis for ΔMatsuda index in all subjects following HDT bed rest.

	ΔMatsuda (*r*)
ΔTG	**-0.599****
ΔCHOL	**-0.528****
ΔLDL	**-0.554****
ΔRBP4	-0.369ǂ
ΔKnee flexion	**0.463***
ΔDorsiflexion	0.307§

Note. §p ≤ 0.200, ǂp ≤ 0.100, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: TG, triglycerides; CHOL, total cholesterol; LDL, low-density lipoprotein cholesterol; RBP4, retinol binding protein 4.

Table 15. Multiple regression analysis to predict the ΔMatsuda index in all subjects following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	-0.536	0.288	-1.134	0.062				
ΔTG	-5.489	1.603	-8.823	-2.155	-0.599	-0.599	0.359	**0.003**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: TG, triglycerides; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The Δliver insulin sensitivity correlated significantly and negatively with the Δtriglycerides in all subjects after HDT bed rest (Table 16). Multiple regression analysis revealed that the Δtriglycerides significantly accounted for 19.90% (adjusted r^2 = 16.10%) of the Δliver insulin sensitivity in all subjects following HDT bed rest ($F_{1, 21}$ = 5.215, p = 0.033. Table 17).

Table 16. Correlational analysis for Δliver insulin sensitivity in all subjects following HDT bed rest.

	ΔLiver IS (*r*)
ΔTG	**-0.446***
ΔLDL	-0.365ǂ
ΔFM	0.298§
ΔTrunk FM	0.392ǂ
ΔArm LM	-0.360ǂ
ΔRBP4	-0.399ǂ
ΔKnee flexion	0.325§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: Liver IS, liver insulin sensitivity; TG, triglycerides; LDL, low-density lipoprotein cholesterol; FM, fat mass; LM, lean mass; RBP4, retinol binding protein 4.

Table 17. Multiple regression analysis to predict the Δliver insulin sensitivity in all subjects following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	-0.06	0.046	-0.155	0.035				
ΔTG	-0.581	0.255	-1.111	-0.052	-0.446	-0.446	0.199	**0.033**

Note. Model = "Stepwise" method. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: TG, triglycerides; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The ΔQUICKI was significantly and positively correlated with the Δtrunk fat mass and Δfat mass and negatively correlated with ΔRBP4 in all subjects after HDT bed rest (Table 18). Multiple regression analysis found that the Δtrunk fat mass, ΔRBP4 and Δtriglycerides together significantly predicted 56.10% (adjusted r^2 = 49.10%) of the ΔQUICKI in all subjects following HDT bed rest ($F_{3, 19}$ = 8.078, p = 0.001. Table 19).

Table 18. Correlational analysis for ΔQUICKI in all subjects following HDT bed rest.

	ΔQUICKI (*r*)
ΔTG	-0.384ǂ
ΔLDL	-0.305§
ΔFM	**0.493***
ΔTrunk FM	**0.570****
ΔArm LM	-0.339§
ΔRBP4	**-0.486***
ΔDorsiflexion	0.386ǂ

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: QUICKI, quantitative insulin-sensitivity check index; TG, triglycerides; LDL, low-density lipoprotein cholesterol; FM, fat mass; LM, lean mass; RBP4, retinol binding protein 4.

Table 19. Multiple regression analysis to predict the ΔQUICKI in all subjects following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	-0.002	0.003	-0.009	0.004				
ΔTrunk FM	0.013	0.004	0.004	0.021	0.462	0.448	0.201	**0.008**
ΔRBP4	-0.001	0.000	-0.001	0.000	-0.353	-0.343	0.118	**0.036**
ΔTG	-0.038	0.018	-0.075	-0.001	-0.325	-0.324	0.105	**0.047**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: FM, fat mass; RBP4, retinol binding protein 4; TG, triglycerides; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In all subjects, the ΔHOMA-IR was significantly and negatively correlated with Δfat mass and Δtrunk fat mass, and significantly and positively correlated with the ΔRBP4, following HDT bed rest (Table 20). Multiple regression analysis revealed that the Δtrunk fat mass significantly accounted for 22.70% (adjusted r^2 = 19.00%) of the ΔHOMA-IR in all subjects post-HDT bed rest ($F_{1, 21}$ = 6.171, p = 0.021. Table 21).

Table 20. Correlational analysis for ΔHOMA-IR in all subjects following HDT bed rest.

	ΔHOMA-IR (*r*)
ΔTG	0.321§
ΔFM	**-0.454***
ΔTrunk FM	**-0.477***
ΔArm LM	0.283§
ΔRBP4	**0.454***
ΔDorsiflexion	-0.386ǂ

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: HOMA-IR, homeostatic model assessment of insulin resistance; TG, triglycerides; FM, fat mass; LM, lean mass; RBP4, retinol binding protein-4.

Table 21. Multiple regression analysis to predict the ΔHOMA-IR in all subjects following HDT bed rest.

Model 1	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	0.278	0.125	0.018	0.538				
ΔTrunk FM	-0.446	0.180	-0.819	-0.073	-0.477	-0.477	0.228	**0.021**

Note. Model = "Stepwise" method. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: FM, fat mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In all subjects, the Δdisposition index was significantly and positively correlated with Δtrunk total mass and Δtrunk lean mass following HDT bed rest (Table 22). In addition, the Δdisposition index was significantly and negatively correlated with ΔASP in all subjects' post-HDT bed rest. Multiple regression analysis revealed that the Δtrunk lean

mass was the best single predictor of Δdisposition index, accounting for 18.90% (adjusted r^2 = 15.00%) of the variance in all subjects after HDT bed rest ($F_{1, 21}$ = 4.896, p = 0.038. Table 23).

Table 22. Correlational analysis for Δdisposition index in all subjects following HDT bed rest.

	ΔDisp. Index (*r*)
ΔNEFA	-0.371ǂ
ΔTrunk TM	**0.608****
ΔTrunk LM	**0.435***
ΔRBP4	-0.285§
ΔAdiponectin	0.374ǂ
ΔASP	**-0.667****
ΔKnee flexion	0.279§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: Disp. Index, disposition index; NEFA, non-esterified fatty acids; TM, total mass; LM, lean mass; RBP4, retinol binding protein 4; ASP, acylation stimulating protein.

Table 23. Multiple regression analysis to predict the Δdisposition index in all subjects following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	-1.225	0.825	-2.940	0.490				
ΔTrunk LM	1.256	0.568	0.076	2.437	0.435	0.435	0.189	**0.038**

Note. Model = "Stepwise" method. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: LM, lean mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The ΔGutt index was significantly and negatively correlated with Δtotal cholesterol and ΔLDL in all subjects after HDT bed rest (Table 24). The ΔGutt index was also significantly and positively correlated with $\Delta\dot{V}O_{2peak}$, when normalised for changes in body weight, in all subjects' post-HDT bed rest. Multiple regression analysis identified

that the Δtotal cholesterol significantly explained 36.70% (adjusted r^2 = 33.70%) of the variance in the Gutt index in all subjects following HDT bed rest ($F_{1, 21}$ = 12.194, p = 0.002. Table 25).

Table 24. Correlational analysis for ΔGutt index in all subjects following HDT bed rest.

	ΔGutt Index (*r*)
ΔNEFA	-0.298§
ΔCHOL	**-0.606****
ΔLDL	**-0.507***
$\Delta\dot{V}O_{2peak}$ (ml/kg/min)	**0.473***
$\Delta\dot{V}O_{2peak}$ (L/min)	0.418ǂ
$\Delta\dot{V}O_{2peak}$ (ml/kgLM/min)	0.418ǂ
ΔIrisin	-0.336§
ΔDorsiflexion	0.367ǂ

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: NEFA, non-esterified fatty acids; CHOL, total cholesterol; LDL, low-density lipoprotein cholesterol; $\dot{V}O_{2peak}$, peak oxygen uptake.

Table 25. Multiple regression analysis to predict the ΔGutt index in all subjects following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	-9.405	4.039	-17.805	-1.004				
ΔCHOL	-23.885	6.840	-38.109	-9.660	-0.606	-0.606	0.367	**0.002**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: CHOL, total cholesterol; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The Δadipose tissue insulin resistance correlated significantly and positively to the ΔLDL in all subjects post-HDT bed rest (Table 26). Multiple regression analysis found that 18.50% (adjusted r^2 = 14.60%) of the Δadipose tissue insulin resistance could be

significantly predicted by the ΔLDL in all subjects after HDT bed rest ($F_{1, 21} = 4.773$, $p = 0.040$. Table 27).

Table 26. Correlational analysis for Δadipose tissue insulin resistance in all subjects following HDT bed rest.

	ΔAdipose IR (*r*)
ΔCHOL	0.298§
ΔLDL	**0.430***
ΔFM	-0.391ǂ
ΔTrunk FM	-0.387ǂ
ΔRBP4	0.398ǂ

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: Adipose IR, adipose tissue insulin resistance; CHOL, total cholesterol; LDL, low-density lipoprotein cholesterol; FM, fat mass; RBP4, retinol binding protein 4.

Table 27. Multiple regression analysis to predict the Δadipose tissue insulin resistance in all subjects following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	0.522	0.450	-0.413	1.457				
ΔLDL	1.864	0.853	0.090	3.639	0.430	0.430	0.185	**0.040**

Note. Model = "Stepwise" method. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: LDL, low-density lipoprotein cholesterol; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

Correlation and multiple regression analysis could not successfully determine the variables associated with, and those that contributed to, the Δmuscle insulin sensitivity in all subjects following HDT bed rest.

4.4.2. Biomarkers of Insulin Sensitivity and Insulin Resistance

In all subjects, the Δadropin correlated significantly and positively with the Δlean mass, Δtrunk lean mass and Δabsolute $\dot{V}O_{2peak}$ following HDT bed rest (Table 28). Multiple regression analysis found that the Δlean mass significantly accounted for 22.00% (adjusted r^2 = 18.20%) of the variance in adropin in all subjects post-HDT bed rest ($F_{1, 21}$ = 5.909, p = 0.024. Table 29).

Table 28. Correlational analysis for Δadropin in all subjects following HDT bed rest.

	Δ Adropin (*r*)
ΔBMI	0.307§
ΔBW	0.324§
ΔBM	0.301§
ΔLM	**0.469***
ΔTrunk LM	**0.460***
ΔLeg LM	0.346§
$\Delta\dot{V}O_{2peak}$ (ml/kg/min)	0.433ǂ
$\Delta\dot{V}O_{2peak}$ (L/min)	**0.449***
$\Delta\dot{V}O_{2peak}$ (ml/kgLM/min)	0.395ǂ
ΔPlantarflexion	0.293§
ΔHOMA-β	-0.344§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: BMI, body mass index; BW, body weight; BM, body mass; LM, lean mass; $\dot{V}O_{2peak}$, peak oxygen uptake; HOMA-β, homeostatic model assessment of beta-cell function.

Table 29. Multiple regression analysis to predict the Δadropin in all subjects following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-0.303	0.352	-1.035	0.429				
ΔLM	0.256	0.105	0.037	0.475	0.469	0.469	0.220	**0.024**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: LM, lean mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In all subjects, the Δirisin was not significantly associated with changes in any other measured variable following HDT bed rest (Table 30). Despite this, multiple regression analysis found that a combination of the ΔHOMA-β and Δfetuin-A significantly explained 31.40% (adjusted $r^2 = 24.50\%$) of the Δirisin in all subjects following HDT bed rest ($F_{2, 20} = 4.575$, $p = 0.023$. Table 31).

Table 30. Correlational analysis for Δirisin in all subjects following HDT bed rest.

	ΔIrisin (*r*)
ΔFetuin-A	-0.294§
ΔFGF-21	0.325§
ΔArm TM	0.324§
ΔHDL	0.349§
ΔHOMA-β	-0.376ǂ

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: FGF-21, fibroblast growth factor 21; TM, total mass; HDL, high-density lipoprotein cholesterol; HOMA-β, homeostatic model assessment of beta-cell function.

Table 31. Multiple regression analysis to predict the Δirisin in all subjects following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-0.293	0.581	-1.505	0.920				
ΔHOMA-β	-0.028	0.011	-0.052	-0.005	-0.497	-0.477	0.228	**0.018**
ΔFetuin-A	-6.015	2.681	-11.608	-0.423	-0.433	-0.416	0.173	**0.036**

Note. Model = "Backward" method. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: HOMA-β, homeostatic model assessment of beta-cell function; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The Δadiponectin was significantly and positively correlated with the Δleg fat mass in all subjects after HDT bed rest (Table 32). Additionally, there was a significant and negative correlation between the Δadiponectin and ΔRBP4 and ΔASP in all subjects following HDT bed rest. Multiple regression analysis determined that 44.90% (adjusted r^2 = 39.40%) of the variance in adiponectin could be explained by the ΔRBP4 and Δleg fat mass in all subjects post-HDT bed rest ($F_{2, 20} = 8.154$, $p = 0.003$. Table 33).

Table 32. Correlational analysis for Δadiponectin in all subjects following HDT bed rest.

	ΔAdiponectin (*r*)
ΔRBP4	**-0.463***
ΔASP	**-0.722****
ΔFM	0.328§
ΔTrunk TM	0.297§
ΔLeg FM	**0.486***
ΔIns. Index	0.402ǂ
ΔDisp. Index	0.374ǂ

Note. §$p \leq 0.200$, ǂ$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Abbreviations: RBP4, retinol binding protein 4; ASP, acylation stimulating protein; FM, fat mass; TM, total mass; Ins. Index, insulinogenic index; Disp. Index; disposition index.

Table 33. Multiple regression analysis to predict the Δadiponectin in all subjects following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-1.566	0.345	-2.285	-0.847				
ΔLFM	2.149	0.736	0.613	3.684	0.484	0.484	0.234	**0.008**
ΔRBP4	-0.078	0.028	-0.136	-0.019	-0.462	-0.462	0.213	**0.011**

Note. Model = "Stepwise" method. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: LFM, leg fat mass; RBP4, retinol binding protein 4; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In all subjects, the ΔFGF-21 was not significantly associated with changes in any other physiological measurement following HDT bed rest (Table 34). Multiple regression analysis revealed that the Δarm total mass could explain 15.60% (adjusted $r^2 = 11.20\%$) of the ΔFGF-21 in all subjects following HDT bed rest ($F_{1, 19} = 3.517$, $p = 0.076$. Table 35).

Table 34. Correlational analysis for ΔFGF-21 in all subjects following HDT bed rest.

	ΔFGF-21 (*r*)
ΔFetuin-A	-0.304§
ΔIrisin	0.325§
ΔTrunk LM	0.305§
ΔArm TM	0.395ǂ
ΔNEFA	-0.302§

Note. §$p \leq 0.200$, ǂ$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Abbreviations: LM, lean mass; TM, total mass; NEFA, non-esterified fatty acids.

Table 35. Multiple regression analysis to predict the ΔFGF-21 in all subjects following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-68.965	11.385	-92.794	-45.135				
ΔArm TM	77.372	41.258	-8.982	163.725	0.395	0.395	0.156	0.076

Note. Model = "Backward" method. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: TM, total mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In all subjects, the Δfetuin-A was significantly and negatively correlated with the Δarm total mass following HDT bed rest (Table 36). Multiple regression analysis revealed that the Δarm total mass significantly accounted for 22.30% (adjusted r^2 = 18.60%) of the variance in fetuin-A in all subjects after HDT bed rest ($F_{1, 21} = 6.033$, $p = 0.023$. Table 37).

Table 36. Correlational analysis for Δfetuin-A in all subjects following HDT bed rest.

	ΔFetuin-A (*r*)
ΔIrisin	-0.294§
ΔFGF-21	-0.304§
ΔArm TM	**-0.472***
ΔNEFA	0.406ǂ

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: FGF-21, fibroblast growth factor 21; TM, total mass; NEFA, non-esterified fatty acids.

Table 37. Multiple regression analysis to predict the Δfetuin-A in all subjects following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	0.111	0.032	0.044	0.179				
ΔArm TM	-0.288	0.117	-0.532	-0.044	-0.472	-0.472	0.223	**0.023**

Note. Model = "Stepwise" method. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: TM, total mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

Chapter 5. Results

5.1. A Subanalysis Exploring Individual Metabolic Response Post-HDT Bed Rest

It is clear that the investigation of individual variability in metabolic responses to HDT bed rest is pivotal, particularly to explore the mechanisms that influence individual responsiveness and optimise personalised interventions to maintain health following prolonged physical inactivity, extreme sedentarism and spaceflight. However, the quantification of individual response heterogeneity to intervention is complex and requires appropriate statistical analysis and sufficient sample size, which is beyond the scope of this PhD (Fernandez-Gonzalo et al., 2021; Böcker et al., 2022). Despite this, and in an attempt to further explore the observed individual metabolic response post-HDT bed rest, the subject data from the two groups (CTRL, JUMP) was pooled and then divided based on subjects who decreased (↓ Matsuda index, n = 17, CTRL n = 7, JUMP n = 10) or increased (↑ Matsuda index, n = 6, CTRL n = 4 and JUMP n = 2) insulin sensitivity after HDT bed rest. The Matsuda index is a composite measure of whole-body insulin sensitivity that encompasses both hepatic and peripheral tissues and is calculated using dynamic glucose and insulin values obtained during an OGTT (Matsuda and DeFronzo, 1999). This index has been validated to predict insulin sensitivity against the euglycaemic-hyperinsulinemic clamp in healthy, lean individuals (Trikudanathan et al., 2013) and has been examined previously in bed rest (Heer et al., 2014; Nielsen et al., 2016; Dirks et al., 2018; Petrocelli et al., 2020). The other surrogate indices of insulin sensitivity and insulin resistance used in this HDT bed rest study either primarily reflect hepatic insulin sensitivity, peripheral insulin sensitivity or insulin secretion or have not been studied previously in response to bed rest. For these reasons, the Matsuda index was used as the measure of whole-body insulin sensitivity and subjects were classified into two subgroups based on a decrease or an increase in whole-body insulin sensitivity post-HDT bed rest.

The purpose of this subanalysis was to, firstly, investigate and profile the physical and metabolic changes that occurred in individuals with decreased insulin sensitivity following HDT bed rest and to identify specific pre- to post-HDT bed rest changes in metabolic physiology that may have contributed to the significant deterioration of insulin

sensitivity noticeable in this subgroup, using correlation and multiple regression analysis. Secondly, to investigate and profile the physical and metabolic changes that occurred in individuals with increased insulin sensitivity following HDT bed rest and to identify the specific pre- to post-HDT bed rest changes in metabolic physiology that may have contributed to the significant improvement of insulin sensitivity noticeable in this subgroup, using correlation and multiple regression analysis. A detailed analysis of HDT bed rest-induced changes in physical characteristics, metabolic characteristics and biomarkers of insulin sensitivity and insulin resistance in the two subgroups can be found in sections 5.1.1, 5.1.2 and 5.1.3. The results of the correlation and regression analysis for the subgroup with decreased insulin sensitivity and the subgroup with increased insulin sensitivity are outlined in sections 5.1.4 and 5.1.5, respectively.

5.1.1. Physical Characteristics: Body Weight, Body Composition, $\dot{V}O_{2peak}$ and Muscle Strength

Baseline characteristics of the two subgroups are displayed below in Table 38. Changes in anthropometric measurements in the subgroups with decreased and increased insulin sensitivity following HDT bed rest are presented in Table 39. No significant between-subgroup differences in age, height and measures of body weight or body composition were identified at baseline. In the subgroup with decreased insulin sensitivity following HDT bed rest, the pre- to post-decreases in body weight, lean mass, trunk total mass, leg total mass, leg lean mass and arm fat mass were statistically significant. Fat mass, bone mineral content, trunk lean mass, trunk fat mass, leg fat mass, arm total mass and arm lean mass did not change in the subgroup with decreased insulin sensitivity after HDT bed rest. In the subgroup with increased insulin sensitivity post-HDT bed rest, the pre- to post-decreases in body weight, lean mass, trunk total mass, trunk lean mass, leg total mass and leg lean mass were statistically significant. Fat mass, bone mineral content, trunk fat mass, leg fat mass, arm total mass, arm lean mass and arm fat mass did not change in the subgroup with increased insulin sensitivity following HDT bed rest.

Table 38. Baseline descriptive characteristics when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity post-HDT bed rest.

Measurement	Decreased IS Subgroup	Increased IS Subgroup
N	17	6
Age (years)	29 ± 6	30 ± 7
Height (cm)	181 ± 6	180 ± 6
BMI (kg/m²)	23.37 ± 1.97	24.06 ± 1.67

Data are presented as mean ± standard deviation (SD). Abbreviations: IS, insulin sensitivity; N, number; BMI, body mass index.

Table 39. The effect of 60 days HDT bed rest on measures of anthropometry when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity post-HDT bed rest.

Measurement	Decreased IS Subgroup (n = 17)		p-value	Increased IS Subgroup (n = 6)		p-value
	Pre	Post		Pre	Post	
BW (kg)	76.56 ± 7.51	73.90 ± 7.02	**<0.001**	78.30 ± 6.68	74.73 ± 5.86	**0.003**
LM (kg)	55.83 ± 5.67	53.53 ± 4.60	**<0.001**	59.03 ± 5.83	55.72 ± 5.04	**0.020**
FM (kg)	18.51 ± 5.83	18.09 ± 5.36	0.143	16.97 ± 4.16	16.60 ± 3.93	0.254
BMC (kg)	3.03 ± 0.32	3.03 ± 0.33	0.955	3.19 ± 0.41	3.17 ± 0.40	0.455
Trunk TM (kg)	36.41 ± 4.25	35.64 ± 3.66	**0.033**	36.73 ± 2.95	35.53 ± 2.71	**0.016**
Trunk LM (kg)	26.38 ± 3.15	25.81 ± 2.22	0.111	27.81 ± 2.82	26.70 ± 2.33	**0.047**
Trunk FM (kg)	9.15 ± 3.56	8.96 ± 3.43	0.321	8.05 ± 2.17	7.94 ± 2.12	0.551
Leg TM (kg)	26.97 ± 2.72	24.93 ± 2.99	**<0.001**	27.60 ± 2.78	25.08 ± 2.30	**0.005**
Leg LM (kg)	19.22 ± 1.78	17.32 ± 1.95	**<0.001**	20.22 ± 2.09	17.91 ± 2.07	**0.017**
Leg FM (kg)	6.57 ± 2.04	6.43 ± 1.75	0.237	6.71 ± 1.55	5.97 ± 1.57	0.273
Arm TM (kg)	9.17 ± 1.10	9.18 ± 0.99	0.871	9.68 ± 1.01	9.59 ± 0.99	0.261
Arm LM (kg)	6.87 ± 1.01	6.97 ± 0.91	0.220	7.41 ± 1.06	7.42 ± 0.97	0.938
Arm FM (kg)	1.88 ± 0.47	1.78 ± 0.42	**0.018**	1.82 ± 0.48	1.72 ± 0.38	0.059

Data are presented as mean ± SD. Anthropometric measurements were taken on BDC-3 and HDT60. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: IS, insulin sensitivity; BMI, body mass index; BW, body weight; LM, lean mass; FM, fat mass; BMC, bone mineral content; TM, total mass.

Changes in absolute and relative $\dot{V}O_{2peak}$, when normalised for changes in body weight and lean mass, in the subgroups with decreased and increased insulin sensitivity following HDT bed rest are shown in Table 40. No significant between-subgroup differences in $\dot{V}O_{2peak}$ were found at baseline. In the subgroup with decreased insulin sensitivity following HDT bed rest, the pre- to post-decreases in absolute and relative $\dot{V}O_{2peak}$, when

normalised for changes in body weight and lean mass, were statistically significant. In the opposing subgroup with increased insulin sensitivity after HDT bed rest, absolute and relative $\dot{V}O_{2peak}$ did not change.

Table 40. The effect of 60 days HDT bed rest on measures of cardiorespiratory capacity when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity post-HDT bed rest.

Measurement	Decreased IS Subgroup (n = 15)		p-value	Increased IS Subgroup (n = 5)		p-value
	Pre	Post		Pre	Post	
$\dot{V}O_{2peak}$ (L/min)	3.61 ± 0.78	2.78 ± 0.53	**<0.001**	3.51 ± 0.75	2.78 ± 0.63	0.233
$\dot{V}O_{2peak}$ (ml/kg/min)	47.16 ± 9.26	37.79 ± 6.53	**<0.001**	44.02 ± 7.19	36.72 ± 9.19	0.293
$\dot{V}O_{2peak}$ (ml/kgLM/min)	64.31 ± 10.21	51.91 ± 7.74	**<0.001**	59.62 ± 11.90	50.04 ± 12.85	0.282

Data are presented as mean ± SD. $\dot{V}O_{2peak}$ was measured on BDC-8 and R+1. Significant p-values ($p \leq 0.05$) are indicated in bold. $\dot{V}O_{2peak}$ measurements were available in 20 subjects only (n = 15 decreased IS subgroup, n = 5 increased IS subgroup) due to the absence of post-HDT bed rest data and failure to meet the test criteria. Abbreviations: IS, insulin sensitivity; LM, lean mass; $\dot{V}O_{2peak,}$ peak aerobic capacity.

Changes in muscle strength in the subgroups with decreased and increased insulin sensitivity post-HDT bed rest are displayed in Table 41. No significant between-subgroup differences in muscle strength were identified at baseline. In the subgroup with decreased insulin sensitivity post-HDT bed rest, the pre- to post-decreases in maximal leg strength during knee extension, knee flexion, plantarflexion and dorsiflexion were statistically significant. In the subgroup with increased insulin sensitivity following HDT bed rest, maximal leg strength during plantarflexion decreased significantly. Despite this, changes in muscle strength during knee extension, knee flexion and dorsiflexion were not statistically significant in subjects who became more insulin-sensitive after HDT bed rest.

Table 41. The effect of 60 days HDT bed rest on measurements of muscle strength when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity post-HDT bed rest.

Measurement	Decreased IS Subgroup (n = 17)		p-value	Increased IS Subgroup (n = 6)		p-value
	Pre	Post		Pre	Post	
Knee Extension (Nm)	288.06 ± 63.12	231.18 ± 76.29	**0.001**	318.49 ± 75.42	226.04 ± 69.81	0.084
Knee Flexion (Nm)	127.94 ± 19.59	112.44 ± 19.87	**0.003**	126.93 ± 16.39	115.79 ± 9.90	0.191
Plantarflexion (Nm)	231.89 ± 44.12	182.83 ± 54.38	**<0.001**	230.44 ± 45.22	154.44 ± 36.11	**0.044**
Dorsiflexion (Nm)	43.56 ± 6.33	39.78 ± 5.83	**<0.001**	44.79 ± 13.40	43.14 ± 10.27	0.386

Data are presented as mean ± SD. Maximal voluntary isometric strength was measured on BDC-1 and R+0. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: IS, insulin sensitivity.

5.1.2. Metabolic Characteristics: Glucose Tolerance, Insulin Sensitivity and Lipid Metabolism

Changes in the glucose and insulin response to the OGTT for the subgroups with decreased and increased insulin sensitivity following HDT bed rest are presented in Table 42. Analysis of between-subgroup differences at baseline found a statistically significant difference in $insulin_{30}$ only, with higher mean values in subjects with increased insulin sensitivity following HDT bed rest ($p = 0.050$). In subjects who became less insulin-sensitive after HDT bed rest, the pre- to post-increases in $glucose_0$, $glucose_{60}$, $glucose_{90}$, $glucose_{120}$, $AUCG_{30}$, $AUCG_{120}$, $insulin_0$ and $AUCI_{120}$ were statistically significant. $Glucose_{30}$, $insulin_{30}$, $insulin_{60}$, $insulin_{90}$, $insulin_{120}$ and $AUCI_{30}$ did not change in the subgroup with decreased insulin sensitivity post-HDT bed rest. In subjects who became more insulin-sensitive following HDT bed rest, the pre- to post-decreases in $AUCG_{30}$, $insulin_{30}$ and $AUCI_{30}$ were statistically significant. $Glucose_0$, $glucose_{30}$, $glucose_{60}$, $glucose_{90}$, $glucose_{120}$, $AUCG_{120}$, $insulin_0$, $insulin_{60}$, $insulin_{90}$, $insulin_{120}$ and $AUCI_{120}$ did not change in the subgroup with increased insulin sensitivity post-HDT bed rest.

Table 42. The effect of 60 days HDT bed rest on glucose tolerance and insulin sensitivity when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity post-HDT bed rest.

Measurement	Decreased IS Subgroup (n = 17)		p-value	Increased IS Subgroup (n = 6)		p-value
	Pre	Post		Pre	Post	
$Glucose_0$ (mmol/L)	5.15 ± 0.39	5.50 ± 0.51	**0.011**	5.52 ± 0.80	4.96 ± 0.47	0.058
$Glucose_{30}$ (mmol/L)	8.56 ± 1.47	9.31 ± 1.09	0.060	9.05 ± 1.08	8.80 ± 1.14	0.183
$Glucose_{60}$ (mmol/L)	7.61 ± 2.09	9.13 ± 1.48	**0.001**	6.64 ± 1.27	7.69 ± 1.65	0.126
$Glucose_{90}$ (mmol/L)	7.88 ± 2.37	8.88 ± 1.62	**0.029**	7.53 ± 0.99	6.94 ± 1.57	0.240
$Glucose_{120}$ (mmol/L)	7.17 ± 1.65	8.61 ± 1.70	**0.005**	7.15 ± 0.89	7.17 ± 2.11	0.978
$AUCG_{30}$ (mmol/L*min)	205.73 ± 24.00	222.21 ± 21.81	**0.023**	218.55 ± 25.40	206.30 ± 22.99	**0.039**
$AUCG_{120}$ (mmol/L*min)	906.41 ± 178.31	1031.41 ± 122.54	**0.002**	886.73 ± 102.75	884.73 ± 157.82	0.957
$Insulin_0$ (pmol/L)	49.23 ± 15.91	62.78 ± 20.65	**0.003**	54.11 ± 11.35	50.94 ± 12.06	0.279
$Insulin_{30}$ (pmol/L)	450.31 ± 244.17‡	470.47 ± 152.35	0.750	693.00 ± 251.45	568.91 ± 185.44	**0.046**
$Insulin_{60}$ (pmol/L)	449.39 ± 223.39	513.30 ± 181.50	0.167	403.20 ± 209.50	516.63 ± 267.81	0.201
$Insulin_{90}$ (pmol/L)	499.65 ± 275.99	569.01 ± 265.60	0.084	447.30 ± 145.26	426.94 ± 106.70	0.622
$Insulin_{120}$ (pmol/L)	428.44 ± 279.31	542.63 ± 309.92	0.062	359.35 ± 68.99	357.95 ± 134.68	0.984
$AUCI_{30}$ (pmol/L*min)	7493.21 ± 3761.01	7998.69 ± 2478.20	0.594	11206.63 ± 3920.85	9297.70 ± 2902.07	**0.041**
$AUCI_{120}$ (pmol/L*min)	49145.69 ± 20498.88	55664.21 ± 16667.60	**0.027**	52506.88 ± 14672.98	51507.78 ± 14590.59	0.477

Data are presented as mean ± SD. Metabolic characteristics were measured on BDC-5 and HDT59. Significant p-values ($p \leq 0.05$) are indicated in bold. A double dagger (‡) represents a significant difference between subgroups at baseline. Abbreviations: IS, insulin sensitivity; Glucose0, fasting glucose; Glucose120, glucose concentrations 120 minutes after the glucose load; AUCG, area under the curve for glucose; Insulin0, fasting insulin; Insulin120, insulin concentrations 120 minutes after the glucose load, AUCI, area under the curve for insulin.

Changes in measures of the lipid profile and lipid ratios for the subgroups with decreased and increased insulin sensitivity post-HDT bed rest are displayed in Table 43. No significant between-subgroup differences in lipids and lipid ratios were found at baseline. In the subgroup with decreased insulin sensitivity post-HDT bed rest, the pre- to post-decrease in HDL and pre- to post-increases in triglycerides, LDL, LDL: HDL, CHOL: HDL and TG: HDL were statistically significant. NEFA and total cholesterol did not change in subjects who became less insulin-sensitive following HDT bed rest. In the subgroup with increased insulin sensitivity after HDT bed rest, the pre- to post-decrease in HDL and pre- to post-increases in LDL: HDL, CHOL: HDL and TG: HDL were statistically significant. NEFA, triglycerides, total cholesterol and LDL did not change in subjects who became more insulin-sensitive post-HDT bed rest.

Table 43. The effect of 60 days HDT bed rest on measures of the lipid profile and lipid ratios when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity post-HDT bed rest.

Measurement	Decreased IS Subgroup (n = 17)		p-value	Increased IS Subgroup (n = 6)		p-value
	Pre	Post		Pre	Post	
NEFA (mmol/L)	0.42 ± 0.14	0.50 ± 0.19	0.137	0.35 ± 0.09	0.38 ± 0.07	0.667
TG (mmol/L)	1.02 ± 0.40	1.14 ± 0.38	**0.009**	1.03 ± 0.39	1.03 ± 0.30	0.969
CHOL (mmol/L)	4.14 ± 0.51	4.23 ± 0.66	0.519	4.00 ± 0.95	3.96 ± 0.90	0.887
LDL (mmol/L)	2.75 ± 0.40	3.09 ± 0.53	**0.003**	2.73 ± 1.03	2.92 ± 0.89	0.440
HDL (mmol/L)	1.13 ± 0.26	0.98 ± 0.19	**0.007**	1.09 ± 0.14	0.87 ± 0.10	**0.003**
LDL: HDL (mmol/L)	2.52 ± 0.57	3.23 ± 0.59	**<0.001**	2.54 ± 0.97	3.38 ± 0.99	**0.001**
CHOL: HDL (mmol/L)	3.76 ± 0.59	4.39 ± 0.57	**<0.001**	3.71 ± 0.94	4.59 ± 1.02	**0.002**
TG: HDL (mmol/L)	0.97 ± 0.47	1.23 ± 0.50	**<0.001**	0.99 ± 0.50	1.22 ± 0.45	**0.001**

Data are presented as mean ± SD. Metabolic characteristics were measured on BDC-5 and HDT59. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: NEFA, non-esterified fatty acids; TG, triglycerides; CHOL, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.

Changes in estimates of insulin sensitivity and insulin resistance when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity following HDT bed rest are shown in Table 44. Additionally, changes in the Matsuda index in the subgroup with decreased insulin sensitivity post-HDT bed rest and the subgroup with increased insulin sensitivity post-HDT bed rest are displayed in Figure 29. Examination of between-subgroup differences at baseline found a statistically significant difference in liver insulin sensitivity only, with higher mean values in subjects who became less insulin sensitive following HDT bed rest ($p = 0.048$). In subjects who became less insulin-sensitive post-HDT bed rest, the pre- to post-decreases in the Matsuda index, QUICKI, liver insulin sensitivity, disposition index, OGIS index and Gutt index and the pre- to post- increases in HOMA-IR and adipose tissue insulin resistance were statistically significant. HOMA-β, insulinogenic index, muscle insulin sensitivity and hepatic insulin resistance did not change in the subgroup with decreased insulin sensitivity after HDT bed rest. In subjects who became more insulin-sensitive following HDT bed rest, the pre- to post-increases in the Matsuda index, QUICKI and liver insulin sensitivity and the pre- to post-decreases in HOMA-IR and hepatic insulin resistance were statistically significant. HOMA-β, insulinogenic index, disposition index, OGIS index, Gutt index, muscle insulin sensitivity and adipose tissue insulin resistance did not change in the subgroup with increased insulin sensitivity post-HDT bed rest.

Table 44. The effect of 60 days HDT bed rest on estimates of insulin sensitivity and insulin resistance when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity post-HDT bed rest.

Measurement	Decreased IS Subgroup (n = 17)		p-value	Increased IS Subgroup (n = 6)		p-value
	Pre	Post		Pre	Post	
HOMA-IR	1.64 ± 0.59	2.22 ± 0.79	**0.001**	1.88 ± 0.27	1.60 ± 0.31	**0.015**
HOMA-β	87.35 ± 27.39	95.73 ± 40.77	0.400	87.84 ± 37.84	120.00 ± 74.06	0.145
QUICKI	0.36 ± 0.02	0.34 ± 0.02	**0.001**	0.35 ± 0.01	0.36 ± 0.01	**0.012**
Matsuda	5.16 ± 2.28	3.64 ± 1.20	**<0.001**	4.09 ± 0.65	4.51 ± 0.59	**0.011**
Ins. Index	1.07 ± 0.58	0.88 ± 0.30	0.202	1.56 ± 0.80	1.16 ± 0.58	0.107
Disp. Index	5.59 ± 4.48	3.06 ± 1.05	**0.025**	6.05 ± 2.37	5.11 ± 2.26	0.241
OGIS	382.45 ± 48.07	345.98 ± 33.42	**0.001**	363.70 ± 44.12	393.06 ± 47.19	0.174
Gutt Index	75.00 ± 25.25	57.91 ± 14.18	**0.005**	67.64 ± 8.14	74.64 ± 22.56	0.458
Liver IS	0.70 ± 0.28‡	0.51 ± 0.19	**0.001**	0.54 ± 0.08	0.64 ± 0.10	**0.012**
Muscle IS	0.02 ± 0.03	0.01 ± 0.01	0.141	0.02 ± 0.01	0.01 ± 0.00	0.058
Hepatic IR	440.28 ± 267.28	498.55 ± 178.71	0.405	666.24 ± 184.62	519.79 ± 114.30	**0.030**
Adipose IR	3.12 ± 1.84	4.58 ± 2.26	**0.008**	2.78 ± 1.05	2.79 ± 0.97	0.991

Data are presented as mean ± SD. Metabolic characteristics were measured on BDC-5 and HDT59. Significant p-values ($p \leq 0.05$) are indicated in bold. A double dagger (‡) represents a significant difference between subgroups at baseline. Abbreviations: HOMA-IR, homeostatic model of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function; QUICKI, quantitative insulin-sensitivity check index; Ins. Index, insulinogenic index; Disp. Index, disposition index; OGIS Index, oral glucose insulin sensitivity index; IS, insulin sensitivity; IR, insulin resistance.

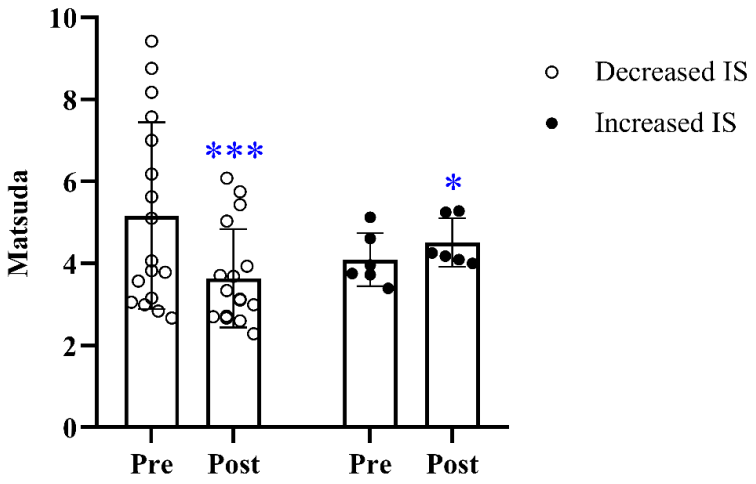

Figure 29. Changes in the Matsuda index in the subgroup with decreased insulin sensitivity post-HDT bed rest and the subgroup with increased insulin sensitivity post-HDT bed rest.

Abbreviations: IS, insulin sensitivity; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

5.1.3. Biomarkers of Insulin Sensitivity and Insulin Resistance

Changes in circulating biomarkers of insulin sensitivity and insulin resistance when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity post-HDT bed rest are displayed in Table 45 and Figure 30. No significant between-subgroup differences in biomarkers of insulin sensitivity and insulin resistance were noticeable at baseline. In the subgroup with decreased insulin sensitivity post-HDT bed rest, the pre- to post-increases in fetuin-A and RBP4 and pre- to post-decreases in adiponectin, adropin, irisin and FGF-21 were statistically significant. Apo-J, apelin and ASP did not change in subjects who became less insulin-sensitive after HDT bed rest. In the subgroup with increased insulin sensitivity following HDT bed rest, the pre- to post-decreases in adiponectin and FGF-21 were statistically significant. Fetuin-A, RBP4, irisin, apo-J, adropin, apelin and ASP did not change in subjects who became more insulin-sensitive post-HDT bed rest.

Table 45. The effect of 60 days HDT bed rest on circulating biomarkers of insulin sensitivity and insulin resistance when subjects were divided into two subgroups based on an increase or decrease in insulin sensitivity post-HDT bed rest.

Measurement	Decreased IS Subgroup		p-value	Increased IS Subgroup		p-value
	Pre	Post		Pre	Post	
Fetuin-A (mg/ml)	0.40 ± 0.16	0.51 ± 0.20	**0.019**	0.36 ± 0.07	0.49 ± 0.16	0.141
RBP4 (µg/ml)	38.33 ± 11.74	43.33 ± 12.05	**0.047**	38.41 ± 14.09	35.44 ± 7.91	0.655
Irisin (µg/ml)	7.27 ± 2.58	5.99 ± 2.52	**0.028**	5.86 ± 2.91	4.12 ± 1.59	0.228
Apo-J (µg/ml)	203.21 ± 60.76	201.83 ± 55.10	0.939	208.02 ± 56.35	214.33 ± 25.17	0.834
Adiponectin (µg/ml)	8.48 ± 2.30	6.23 ± 1.97	**<0.001**	6.76 ± 1.38	4.98 ± 0.59	**0.048**
Adropin (ng/ml)	3.23 ± 1.44	2.24 ± 1.38	**0.003**	2.70 ± 1.38	1.82 ± 2.10	0.193
Apelin (ng/ml)	0.87 ± 0.17	0.84 ± 0.29	0.650	0.81 ± 0.30	0.72 ± 0.12	0.452
ASP (nmol/l)	6.53 ± 2.46	7.53 ± 3.30	0.221	6.62 ± 2.26	6.65 ± 3.06	0.980
FGF-21 (pg/ml)	154.95 ± 89.30	85.97 ± 39.72	**0.001**	156.80 ± 83.17	83.66 ± 36.27	**0.014**

Data are presented as mean ± SD. Significant p-values ($p \leq 0.05$) are indicated in bold. Metabolic characteristics were measured on BDC-5 and HDT59. Abbreviations: IS, insulin sensitivity; RBP4, retinol binding protein 4; apo-J, apolipoprotein-J; ASP, acylation stimulating protein; FGF-21, fibroblast growth factor 21. The results for fetuin-A, RBP4, irisin, apo-J, adiponectin and adropin include n = 17 in the decreased IS subgroup and n = 6 in the increased IS subgroup. For apelin, n = 15 in the decreased IS subgroup and n = 5 in the increased IS subgroup. For ASP, n = 11 in the decreased IS subgroup and n = 3 in the increased IS subgroup. For FGF-21, n = 15 in the decreased IS subgroup and n = 6 in the increased IS subgroup.

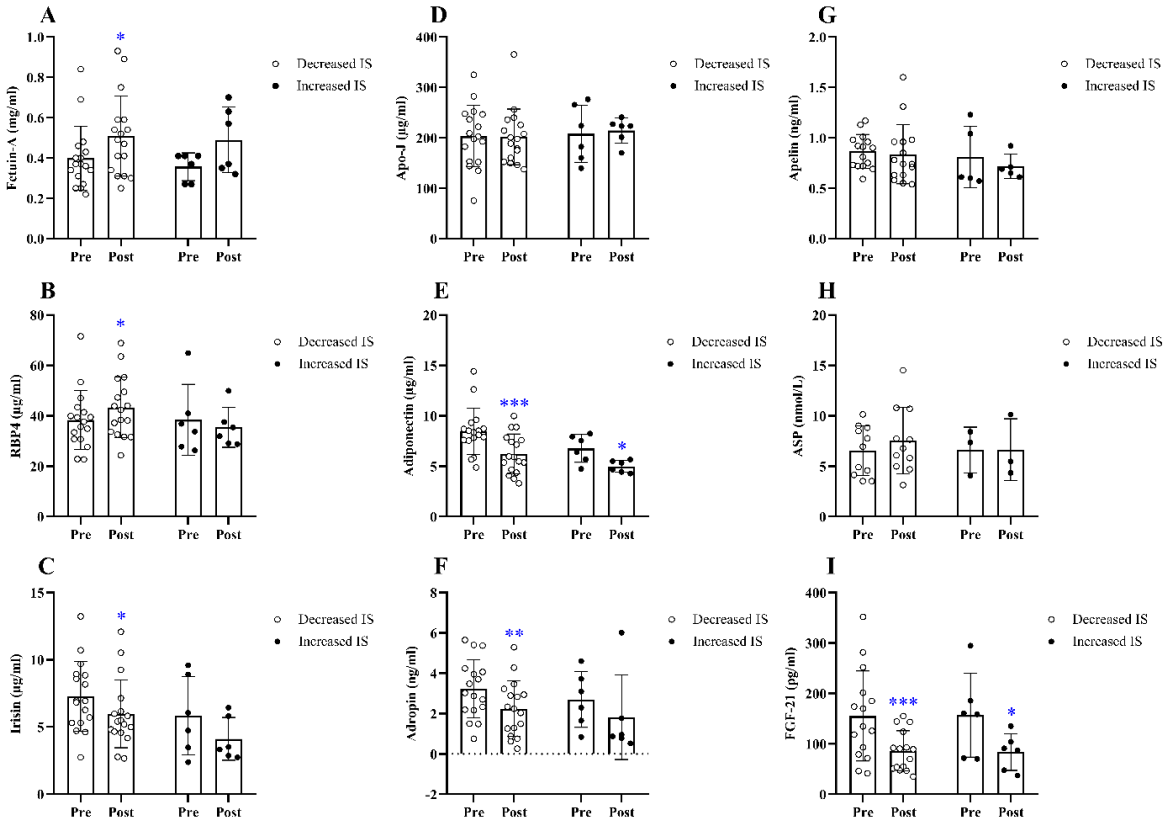

Figure 30. Changes in circulating biomarkers of insulin sensitivity and insulin resistance when subjects were divided into two subgroups based on a decrease or an increase in insulin sensitivity post-HDT bed rest.

Data are presented as mean ± SD. Metabolic characteristics were measured on BDC-5 and HDT59. Pre- to post-HDT bed rest changes in fetuin-A, RBP4, irisin, apo-J, adiponectin, adropin, apelin, ASP and FGF-21 are shown in Figure 2A-I, respectively. Abbreviations: IS, insulin sensitivity; RBP4, retinol binding protein 4; apo-J, apolipoprotein-J; ASP, acylation stimulating protein; FGF-21, fibroblast growth factor 21. The results for fetuin-A, RBP4, irisin, apo-J, adiponectin and adropin include n = 17 in the decreased IS subgroup and n = 6 in the increased IS subgroup. For apelin, n = 15 in the decreased IS subgroup and n = 5 in the increased IS subgroup. For ASP, n = 11 in the decreased IS subgroup and n = 3 in the increased IS subgroup. For FGF-21, n = 15 in the decreased IS subgroup and n = 6 in the increased IS subgroup. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

5.1.4. Correlation and Regression Analysis for the Subgroup with Decreased Insulin Sensitivity

5.1.4.1. Indexes of Insulin Sensitivity and Insulin Resistance

In the subgroup with decreased insulin sensitivity following HDT bed rest, the ΔMatsuda index was significantly and negatively correlated with Δtriglycerides, Δtotal cholesterol and ΔLDL (Table 46). Additionally, in this subgroup, there were significant and positive correlations between the ΔMatsuda index and Δknee extension and Δknee flexion post-HDT bed rest. Multiple regression analysis determined that a combination of the ΔLDL and Δknee extension significantly predicted 57.90% (adjusted r^2 = 51.90%) of the ΔMatsuda index in subjects who became less insulin-sensitive after HDT bed rest ($F_{2, 14}$ = 9.637, p = 0.002. Table 47).

Table 46. Correlational analysis for ΔMatsuda index in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔMatsuda Index (*r*)
ΔTG	**-0.558***
ΔCHOL	**-0.641****
ΔLDL	**-0.662****
ΔKnee extension	**0.611****
ΔKnee flexion	**0.591***

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: TG, triglycerides; CHOL, total cholesterol; LDL, low-density lipoprotein cholesterol.

Table 47. Multiple regression analysis to predict the ΔMatsuda index in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-0.419	0.344	-1.158	0.319				
ΔLDL	-1.672	0.639	-3.042	-0.302	-0.496	-0.454	0.206	**0.020**
ΔKE	0.009	0.004	0.000	0.019	0.410	0.375	0.141	**0.048**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: LDL, low-density lipoprotein cholesterol; KE, knee extension; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The ΔQUICKI was significantly and positively correlated with Δfat mass, Δtrunk fat mass and Δapelin in the subgroup with reduced insulin sensitivity after HDT bed rest (Table 48). Further to this, ΔQUICKI was significantly and negatively correlated with ΔRBP4 in this subgroup post-HDT bed rest. Multiple regression analysis determined that a combination of the Δtrunk fat mass, Δknee flexion and Δapelin could significantly predict 76.10% (adjusted $r^2 = 69.60\%$) of the variance in QUICKI in subjects showing a decline in insulin sensitivity following HDT bed rest ($F_{3, 11} = 11.666$, $p = 0.001$. Table 49).

Table 48. Correlational analysis for ΔQUICKI in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔQUICKI (*r*)
ΔFM	**0.603****
ΔTrunk FM	**0.684****
ΔArm LM	-0.341§
ΔLDL	-0.336§
ΔRBP4	**-0.506***
ΔApelin	**0.570***
ΔKnee flexion	0.368§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: QUICKI, quantitative insulin sensitivity check index; FM, fat mass; LM, lean mass; LDL, low-density lipoprotein cholesterol; RBP4, retinol binding protein 4.

Table 49. Multiple regression analysis to predict the ΔQUICKI in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	-0.007	0.003	-0.015	0.000				
ΔTFM	0.012	0.003	0.004	0.019	0.544	0.504	0.254	**0.006**
ΔKF	0.000	0.000	0.000	0.001	0.426	0.424	0.180	**0.015**
ΔApelin	0.028	0.011	0.004	0.053	0.407	0.375	0.141	**0.027**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: TFM, trunk fat mass; KF, knee flexion; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In the subgroup exhibiting a decline in insulin sensitivity following HDT bed rest, the Δliver insulin sensitivity correlated significantly and positively with Δknee extension (Table 50). Multiple regression analysis identified that the Δknee extension and Δtrunk fat mass significantly accounted for 65.40% (adjusted r^2 = 60.40%) of the variance in

liver insulin sensitivity in subjects with decreased insulin sensitivity post-HDT bed rest ($F_{2, 14}$ = 13.203, p = 0.001. Table 51).

Table 50. Correlational analysis for Δliver insulin sensitivity in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔLiver IS (*r*)
ΔFM	0.366§
ΔTrunk FM	0.445ǂ
ΔArm LM	-0.360§
ΔTG	-0.384§
ΔLDL	-0.478ǂ
ΔRBP4	-0.398§
ΔApelin	0.464ǂ
ΔKnee extension	**0.495***
ΔKnee flexion	0.425ǂ
ΔDorsiflexion	0.404§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: IS, insulin sensitivity; FM, fat mass; LM, lean mass; TG, triglycerides; LDL, low-density lipoprotein cholesterol; RBP4, retinol binding protein 4.

Table 51. Multiple regression analysis to predict the Δliver insulin sensitivity in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	-0.029	0.043	-0.123	0.064				
ΔKE	0.002	0.001	0.001	0.003	0.713	0.675	0.456	**0.001**
ΔTFM	0.163	0.040	0.077	0.249	0.675	0.639	0.408	**0.001**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: KE, knee extension; TFM, trunk fat mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The Δdisposition index was significantly and positively correlated with the Δtrunk total mass, Δtrunk lean mass, Δbody weight, Δbody mass and ΔASP in the subgroup which decreased insulin sensitivity after HDT bed rest (Table 52). Multiple regression analysis found that the Δbody mass was the best single predictor of Δdisposition index, accounting for 23.60% (adjusted r^2 = 18.50%) of the variance in subjects who became less insulin-sensitive following HDT bed rest ($F_{1, 15}$ = 4.641, p = 0.048. Table 53).

Table 52. Correlational analysis for Δdisposition index in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔDisp. Index (*r*)
ΔBMI	0.447ǂ
ΔBW	**0.487***
ΔBM	**0.486***
ΔTrunk TM	**0.725*****
ΔTrunk LM	**0.537***
ΔNEFA	-0.376§
ΔRBP4	-0.449ǂ
ΔAdiponectin	0.366§
ΔASP	**-0.650***
ΔKnee flexion	0.339§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: Disp. Index, disposition index; BMI, body mass index; BW, body weight; BM, body mass; FM, fat mass; TM, total mass; LM, lean mass; NEFA, non-esterified fatty acids; RBP4, retinol binding protein 4; ASP, acylation stimulating protein.

Table 53. Multiple regression analysis to predict the Δdisposition index in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	0.838	1.813	-3.026	4.703				
ΔBody Mass	1.235	0.573	0.013	2.457	0.486	0.486	0.236	**0.048**

Note. Model = "Stepwise" method. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The ΔGutt index was significantly and negatively correlated with the Δtotal cholesterol in subjects showing a decline in insulin sensitivity following HDT bed rest (Table 54). Multiple regression analysis showed that the Δtotal cholesterol significantly explained 38.90% (adjusted r^2 = 34.80%) of the variance in the Gutt index in the subgroup with decreased insulin sensitivity following HDT bed rest ($F_{1, 15} = 9.557$, $p = 0.007$. Table 55).

Table 54. Correlational analysis for ΔGutt index in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔGutt Index (*r*)
$\Delta\dot{V}O_{2peak}$ (ml/kg/min)	0.444ǂ
$\Delta\dot{V}O_{2peak}$ (L/min)	0.391§
$\Delta\dot{V}O_{2peak}$ (ml/kgLM/min)	0.361§
ΔCHOL	**-0.624****
ΔLDL	-0.382§

Note. §$p \leq 0.200$, ǂ$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Abbreviations: $\dot{V}O_{2peak,}$ peak oxygen uptake; CHOL, total cholesterol; LDL, low-density lipoprotein cholesterol.

Table 55. Multiple regression analysis to predict the ΔGutt index in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-14.933	4.291	-24.078	-5.787				
ΔCHOL	-22.928	7.417	-38.737	-7.120	-0.624	-0.624	0.389	**0.007**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: CHOL, total cholesterol; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In the subgroup showing a decrease in insulin sensitivity post-HDT bed rest, the ΔOGIS index correlated significantly and negatively with the Δtotal cholesterol and positively with Δdorsiflexion (Table 56). Multiple regression analysis found that the Δtotal cholesterol significantly explained 34.30% (adjusted $r^2 = 29.90\%$) of the variance in the OGIS index in this subgroup following HDT bed rest ($F_{1, 15} = 7.824$, $p = 0.014$, Table 57).

Table 56. Correlational analysis for ΔOGIS index in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔOGIS Index (*r*)
ΔCHOL	**-0.585***
ΔLDL	-0.410§
ΔKnee flexion	0.392§
ΔDorsiflexion	**0.493***

Note. §$p \leq 0.200$, ŧ$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Abbreviations: OGIS, oral glucose insulin sensitivity; CHOL, total cholesterol; LDL, low-density lipoprotein cholesterol.

Table 57. Multiple regression analysis to predict the ΔOGIS index in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-32.890	7.864	-49.653	-16.127				
ΔCHOL	-38.024	13.594	-67.000	-9.049	-0.585	-0.585	0.342	**0.014**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: CHOL, total cholesterol; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The ΔHOMA-IR was significantly and negatively correlated with Δfat mass, Δtrunk fat mass and Δapelin in the subgroup with decreased insulin sensitivity following HDT bed rest (Table 58). Additionally, there was a significant and positive correlation between ΔHOMA-IR and ΔRBP4 in this subgroup after HDT bed rest. Multiple regression analysis identified that the Δtrunk fat mass was the best significant predictor, accounting for 29.80% (r^2 = 25.10%) of the variance in HOMA-IR in subjects who became less insulin-sensitive post-HDT bed rest ($F_{1, 15} = 6.362$, $p = 0.023$. Table 59).

Table 58. Correlational analysis for ΔHOMA-IR in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔHOMA-IR (*r*)
ΔFM	**-0.544***
ΔTrunk FM	**-0.546***
ΔLeg FM	-0.378§
ΔRBP4	**0.493***
ΔApelin	**-0.579***

Note. §$p \leq 0.200$, ǂ$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Abbreviations: HOMA-IR, homeostatic model assessment of insulin resistance; FM, fat mass; RBP4, retinol binding protein 4.

Table 59. Multiple regression analysis to predict the ΔHOMA-IR in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	0.498	0.129	0.222	0.774				
ΔTFM	-0.420	0.167	-0.775	-0.065	-0.546	-0.546	0.298	**0.023**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: TFM, trunk fat mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The Δadipose tissue insulin resistance was significantly and positively correlated with ΔRBP4 in the subgroup with decreased insulin sensitivity following HDT bed rest (Table 60). Multiple regression analysis found that the ΔRBP4 significantly accounted for 32.30% (adjusted $r^2 = 27.70\%$) of the Δadipose tissue insulin resistance in this subgroup after HDT bed rest ($F_{1, 15} = 7.142$, $p = 0.017$. Table 61).

Table 60. Correlational analysis for Δadipose tissue insulin resistance in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔAdipose IR (*r*)
ΔFM	-0.358§
ΔTrunk FM	-0.346§
ΔLDL	0.465§
ΔRBP4	**0.568***
ΔIrisin	-0.392§
ΔKnee flexion	-0.330§

Note. §$p \leq 0.200$, ǂ$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Abbreviations: Adipose IR, adipose tissue insulin resistance; FM, fat mass; LDL, low-density lipoprotein cholesterol; RBP4, retinol binding protein 4.

Table 61. Multiple regression analysis to predict the Δadipose tissue insulin resistance in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	0.880	0.462	-0.105	1.866				
ΔRBP4	0.117	0.044	0.024	0.210	0.568	0.568	0.323	**0.017**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: RBP4, retinol binding protein 4; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

5.1.4.2. Biomarkers of Insulin Sensitivity and Insulin Resistance

In the subgroup with decreased insulin sensitivity following HDT bed rest, the Δfetuin-A was significantly and negatively correlated with the Δirisin (Table 62). Multiple regression analysis revealed that the Δirisin significantly predicted 24.80% (adjusted r^2 = 19.80%) of the variance in fetuin-A in this subgroup post-HDT bed rest ($F_{1, 15} = 4.959$, $p = 0.042$. Table 63).

Table 62. Correlational analysis for Δfetuin-A in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔFetuin-A (*r*)
ΔIrisin	**-0.498***
ΔNEFA	0.367§
ΔFGF-21	-0.385§

Note. §$p \leq 0.200$, ǂ$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Abbreviations: NEFA, non-esterified fatty acid; FGF-21, fibroblast growth factor 21.

Table 63. Multiple regression analysis to predict the Δfetuin-A in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	0.059	0.044	-0.034	0.152				
ΔIrisin	-0.039	0.018	-0.077	-0.002	-0.498	-0.498	0.248	**0.042**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In the subgroup with decreased insulin sensitivity following HDT bed rest, the ΔRBP4 was significantly and positively correlated with ΔASP, ΔNEFA, $\Delta glucose_0$, $\Delta glucose_{30}$, $\Delta AUCG_{30}$, ΔHOMA-IR and Δadipose tissue insulin resistance (Table 64). In addition, the ΔRBP4 was significantly and negatively correlated with Δadiponectin and ΔQUICKI in this subgroup post-HDT bed rest. Multiple regression analysis revealed that the Δadiponectin, Δhepatic insulin resistance and Δtrunk fat mass together significantly explained 68.90% (adjusted r^2 = 61.70%) of the variance in ΔRBP4 in the subgroup exhibiting a decline in insulin sensitivity after HDT bed rest ($F_{3, 13}$ = 9.582, p = 0.001. Table 65).

Table 64. Correlational analysis for ΔRBP4 in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔRBP4 (*r*)
ΔAdiponectin	**-0.517***
ΔASP	**0.780****
ΔApelin	-0.377§
ΔTrunk TM	-0.370§
ΔTrunk FM	-0.350§
ΔNEFA	**0.509***
ΔLDL	0.413ǂ
$\Delta Glucose_0$	**0.492***
$\Delta Glucose_{30}$	**0.572***
$\Delta Insulin_0$	0.336§
$\Delta Insulin_{30}$	0.329§
$\Delta AUCG_{30}$	**0.623****
$\Delta AUCI_{30}$	0.351§
ΔHOMA-IR	**0.493***
ΔQUICKI	**-0.506***
ΔDisp. Index	-0.449ǂ
ΔLiver IS	-0.398§
ΔHepatic IR	0.406§
ΔAdipose IR	**0.568***

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: RBP4, retinol binding protein 4; ASP, acylation stimulating protein; FM, fat mass; TM, total mass; NEFA, non-esterified fatty acid; LDL, low-density lipoprotein cholesterol; $Glucose_0$, fasting glucose; $Glucose_{30}$, glucose concentrations 30 minutes after the glucose load; $Insulin_0$, fasting insulin; $Insulin_{30}$, insulin concentrations 30 minutes after the glucose load; $AUCG_{30}$, area under the curve for glucose for 30 minutes; $AUCI_{30}$, area under the curve for insulin for 30 minutes; HOMA-IR, homeostatic model of insulin resistance; QUICKI, quantitative insulin-sensitivity check index; Disp. Index, disposition index; IS, insulin sensitivity; IR, insulin resistance.

Table 65. Multiple regression analysis to predict the ΔRBP4 in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-2.489	2.230	-7.306	2.328				
ΔAdiponectin	-2.230	0.743	-3.836	-0.624	-0.480	-0.464	0.215	**0.010**
ΔTFM	-6.061	2.137	-10.678	-1.445	-0.490	-0.439	0.193	**0.014**
ΔHepatic IR	0.023	0.006	0.010	0.035	0.662	0.608	0.370	**0.002**

Note. Model = "Backward" method. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: TFM, trunk fat mass. IR, insulin resistance; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The Δadiponectin was significantly and negatively correlated with ΔASP and ΔRBP4 in the subgroup with decreased insulin sensitivity after HDT bed rest (Table 66). In addition, there was a significant and positive correlation between Δadiponectin and Δleg fat mass and Δtrunk total mass in this subgroup post-HDT bed rest. Multiple regression analysis found that a combination of the ΔRBP4, Δleg fat mass and Δtrunk lean mass significantly predicted 63.40% (adjusted r^2 = 55.00%) of the Δadiponectin in subjects showing a reduction in insulin sensitivity following HDT bed rest ($F_{3, 13}$ = 7.509, p = 0.004. Table 67). However, the unique contribution of ΔRBP4 to the prediction of Δadiponectin did not reach statistical significance (p = 0.053).

Table 66. Correlational analysis for Δadiponectin in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔAdiponectin (*r*)
ΔRBP4	**-0.517***
ΔASP	**-0.681***
ΔFGF-21	0.365§
ΔFM	0.373§
ΔTrunk TM	**0.486***
ΔTrunk LM	0.341§
ΔLeg FM	**0.494***
ΔIns. Index	0.404§
ΔDisp. Index	0.366§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: RBP4, retinol binding protein 4; ASP, acylation stimulating protein; FGF-21, fibroblast growth factor 21; FM, fat mass; TM, total mass; LM, lean mass; Ins. Index; insulinogenic index; Disp. Index, disposition index.

Table 67. Multiple regression analysis to predict the Δadiponectin in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	-1.103	0.417	-2.005	-0.202				
ΔRBP4	-0.079	0.037	-0.160	0.001	-0.368	-0.356	0.127	0.053
ΔLFM	2.587	0.793	0.874	4.300	0.584	0.547	0.299	**0.006**
ΔTLM	0.679	0.269	0.098	1.261	0.454	0.423	0.179	**0.025**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: RBP4, retinol binding protein 4; LFM, leg fat mass; TLM, trunk lean mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The Δadropin was significantly and positively correlated with Δlean mass and Δtrunk lean mass in subjects who became less insulin-sensitive following HDT bed rest (Table 68). Multiple regression analysis found that the Δlean mass significantly accounted for 32.00% (adjusted r^2 = 27.40%) of the Δadropin in the subgroup with decreased insulin sensitivity after HDT bed rest ($F_{1, 15}$ = 7.054, p = 0.018. Table 69).

Table 68. Correlational analysis for Δadropin in subjects with decreased insulin sensitivity following HDT bed rest.

	Δ Adropin (*r*)
ΔFGF-21	0.368§
ΔBMI	0.369§
ΔBW	0.390§
ΔBM	0.375§
ΔLM	**0.566***
ΔTrunk LM	**0.520***
ΔLeg LM	0.411§
$\Delta\dot{V}O_{2peak}$ (ml/kg/min)	0.483ǂ
$\Delta\dot{V}O_{2peak}$ (L/min)	0.486ǂ
$\Delta\dot{V}O_{2peak}$ (ml/kgLM/min)	0.381§
ΔPlantarflexion	0.397§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: FGF-21, fibroblast growth factor 21; BMI, body mass index; BW, body weight; BM, body mass; LM, lean mass; $\dot{V}O_{2peak}$, peak oxygen uptake.

Table 69. Multiple regression analysis to predict the Δadropin in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-0.281	0.357	-1.042	0.480				
ΔLM	0.307	0.116	0.061	0.553	0.566	0.566	0.320	**0.018**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: LM, lean mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance

In subjects who became less insulin-sensitive following HDT bed rest, the Δirisin was correlated significantly and negatively correlated with the Δfetuin-A, $\Delta insulin_{30}$ and $\Delta AUCI_{30}$ (Table 70). Additionally, there was a significant and positive correlation between Δirisin and ΔFGF-21 in this subgroup after HDT bed rest. Multiple regression analysis found that 84.10% (adjusted r^2 = 80.40%) of the Δirisin could be explained significantly by a combination of the Δfetuin-A, ΔLDL: HDL and $\Delta insulin_{30}$ in the subgroup with decreased insulin sensitivity after HDT bed rest ($F_{3, 13} = 22.930$, $p < 0.001$. Table 71).

Table 70. Correlational analysis for Δirisin in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔIrisin (*r*)
ΔFetuin-A	**-0.498***
ΔFGF-21	**0.519***
ΔNEFA	-0.348§
ΔHDL	0.370§
ΔLDL: HDL	-0.474ǂ
ΔCHOL: HDL	-0.414ǂ
$\Delta Insulin_{30}$	**-0.514***
$\Delta AUCI_{30}$	**-0.526***
ΔHepatic IR	-0.458ǂ
ΔAdipose IR	-0.392§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: FGF-21, fibroblast growth factor 21; NEFA, non-esterified fatty acid; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; CHOL, total cholesterol; $Insulin_{30}$, insulin concentrations 30 minutes after the glucose load; $AUCI_{30}$, area under the curve for insulin for 30 minutes; IR, insulin resistance.

Table 71. Multiple regression analysis to predict the Δirisin in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	1.971	0.542	0.801	3.142				
ΔLDL: HDL	-3.452	0.632	-4.818	-2.087	-0.613	-0.604	0.365	**<0.001**
$\Delta Insulin_{30}$	-0.005	0.001	-0.007	-0.003	-0.556	-0.550	0.303	**<0.001**
ΔFetuin-A	-6.737	1.418	-9.799	-3.674	-0.530	-0.525	0.276	**<0.001**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; $Insulin_{30}$, insulin concentrations 30 minutes after the glucose load; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The ΔFGF-21 was significantly and positively correlated to the Δirisin in subjects who became less insulin-sensitive following HDT bed rest (Table 72). Multiple regression analysis identified that the Δirisin significantly explained 26.90% (adjusted r^2 = 21.30%) of the variance in FGF-21 in this subgroup after HDT bed rest ($F_{1, 13}$ = 4.789, p = 0.048. Table 73).

Table 72. Correlational analysis for ΔFGF-21 in subjects with decreased insulin sensitivity following HDT bed rest.

	ΔFGF-21 (*r*)
ΔFetuin-A	-0.385§
ΔIrisin	**0.519***
ΔAdiponectin	0.365§
ΔAdropin	0.368§
ΔTrunk LM	0.376§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: FGF-21, fibroblast growth factor 21; LM, lean mass.

Table 73. Multiple regression analysis to predict the ΔFGF-21 in the subgroup with decreased insulin sensitivity following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	-50.907	15.919	-85.298	-16.515				
ΔIrisin	14.082	6.435	0.180	27.983	0.519	0.519	0.269	**0.048**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

5.1.5. Correlation and Regression Analysis for the Subgroup with Increased Insulin Sensitivity

5.1.5.1. Indexes of Insulin Sensitivity and Insulin Resistance

In the subgroup with increased insulin sensitivity after HDT bed rest, the ΔMatsuda index was significantly and negatively correlated with Δtotal cholesterol (Table 74). Multiple regression analysis could not successfully determine the factor(s) that predicted the ΔMatsuda index in subjects with increased insulin sensitivity following HDT bed rest.

Table 74. Correlational analysis for the ΔMatsuda index in subjects with increased insulin sensitivity following HDT bed rest.

	ΔMatsuda Index (*r*)
ΔCHOL	**-0.877***
ΔTG: HDL	0.789ǂ
ΔAdropin	-0.662§
ΔApelin	-0.855ǂ

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: CHOL, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol.

The ΔQUICKI was not significantly associated with changes in any other physiological measurement in subjects with increased insulin sensitivity following HDT bed rest (Table 75). Multiple regression analysis could not successfully determine the factor(s) which explained the ΔQUICKI in subjects who became more insulin-sensitive after HDT bed rest.

Table 75. Correlational analysis for the ΔQUICKI in subjects with increased insulin sensitivity following HDT bed rest.

	ΔQUICKI (*r*)
ΔTrunk LM	-0.751ǂ
ΔHDL	-0.674§
ΔApelin	-0.854ǂ
ΔKnee flexion	-0.711§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: QUICKI, quantitative insulin sensitivity check index; LM, lean mass; HDL, high-density lipoprotein cholesterol.

In subjects who became more insulin-sensitive following HDT bed rest, the Δliver insulin sensitivity was not significantly associated with changes in any other measured variable after HDT bed rest (Table 76). Multiple regression analysis found that the Δapelin predicted 65.60% (adjusted r^2 = 54.10%) of the variance in liver insulin sensitivity in the subgroup with increased insulin sensitivity following HDT bed rest ($F_{1, 3}$ = 5.716, p = 0.097. Table 77).

Table 76. Correlational analysis for Δliver insulin sensitivity in subjects with increased insulin sensitivity following HDT bed rest.

	ΔLiver IS (*r*)
ΔApelin	-0.810ǂ
ΔIrisin	-0.617§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: IS, insulin sensitivity.

Table 77. Multiple regression analysis to predict the ΔLiver insulin sensitivity in the subgroup with increased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	0.083	0.022	0.014	0.153				
ΔApelin	-0.212	0.089	-0.495	0.070	-0.810	-0.810	0.656	0.097

Note. Model = "Backward" method. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In the subgroup with increased insulin sensitivity following HDT bed rest, the ΔHOMA-IR was significantly and positively correlated with the Δapelin (Table 78). Multiple regression analysis revealed that the Δapelin significantly accounted for 80.30% (adjusted $r^2 = 73.70\%$) of the variance in HOMA-IR in subjects who became more insulin-sensitive post-HDT bed rest ($F_{1, 3} = 12.235$, $p = 0.040$. Table 79).

Table 78. Correlational analysis for ΔHOMA-IR in subjects with increased insulin sensitivity following HDT bed rest.

	ΔHOMA-IR (*r*)
ΔApelin	**.896***
ΔIrisin	0.759ǂ

Note. §$p \leq 0.200$, ǂ$p \leq 0.10$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. Abbreviations: HOMA-IR, homeostatic model of insulin resistance.

Table 79. Multiple regression analysis to predict the ΔHOMA-IR in the subgroup with increased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-0.219	0.048	-0.371	-0.068				
ΔApelin	0.679	0.194	0.061	1.298	0.896	0.896	0.803	**0.040**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

The Δhepatic insulin resistance was not significantly correlated with changes in any measured variable in subjects with increased insulin sensitivity following HDT bed rest (Table 80). Multiple regression analysis revealed that the Δleg fat mass and Δirisin together predicted 98.40% (adjusted $r^2 = 97.40\%$) of the Δhepatic insulin resistance in the subgroup with increased insulin sensitivity following HDT bed rest ($F_{2, 3} = 95.230$, $p = 0.002$. Table 81).

Table 80. Correlational analysis for the Δhepatic insulin resistance in subjects with increased insulin sensitivity following HDT bed rest.

	ΔHepatic IR (*r*)
ΔLeg FM	0.755ǂ
ΔIrisin	0.745ǂ
ΔAdiponectin	0.725§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: IR, insulin resistance; FM, fat mass.

Table 81. Multiple regression analysis to predict the Δhepatic insulin resistance in the subgroup with increased insulin sensitivity following HDT bed rest.

Model	B	SE	95% CI for B		β	SPC	Cont.	Sig.
			LB	UB				
(Constant)	-62.812	9.944	-94.459	-31.164				
ΔLFM	206.606	22.660	134.492	278.720	0.662	0.656	0.430	**0.003**
ΔIrisin	25.087	2.803	16.168	34.006	0.650	0.644	0.415	**0.003**

Note. Model = "Backward" method. Significant p-values ($p \leq 0.05$) are indicated in bold. Abbreviations: LFM, leg fat mass; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

5.1.5.2. Biomarkers of Insulin Sensitivity and Insulin Resistance

In the subgroup showing an improvement in insulin sensitivity post-HDT bed rest, the Δadiponectin was significantly and negatively correlated with $\Delta insulin_{60}$ (Table 82). In addition, the Δadiponectin was significantly and positively correlated with $\Delta insulin_{30}$ and $\Delta AUCI_{30}$ in this subgroup after HDT bed rest. Multiple regression analysis found that a combination of the $\Delta insulin_{60}$ and ΔTG: HDL could significantly account for 97.40% (adjusted r^2 = 95.60%) of the variance in adiponectin in this subgroup following HDT bed rest ($F_{2, 3}$ = 55.375, p = 0.004. Table 83).

Table 82. Correlational analysis for Δadiponectin in subjects with increased insulin sensitivity following HDT bed rest.

	ΔAdiponectin (*r*)
ΔIrisin	0.702§
ΔApo-J	-0.690§
ΔTG: HDL	-0.741ǂ
$\Delta Insulin_{30}$	**0.825***
$\Delta Insulin_{60}$	**-0.914***
$\Delta Insulin_{90}$	0.643§
$\Delta Insulin_{120}$	0.692§
$\Delta AUCI_{30}$	**0.844***
ΔMuscle IS	0.746ǂ
ΔAdipose IR	0.725§

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: Apo-J, apolipoprotein-J; TG, triglycerides; HDL, high-density lipoprotein cholesterol; $Insulin_{30}$, insulin concentrations 30 minutes after the glucose load; $AUCI_{30}$, area under the curve for insulin for 30 minutes; IS, insulin sensitivity; IR, insulin resistance.

Table 83. Multiple regression analysis to predict the Δadiponectin in the subgroup with increased insulin sensitivity following HDT bed rest.

Model	**B**	**SE**	**95% CI for B**		**β**	**SPC**	**Cont.**	**Sig.**
			LB	UB				
(Constant)	1.021	0.505	-0.587	2.629				
$\Delta Insulin_{60}$	-0.006	0.001	-0.009	-0.003	-0.728	-0.651	0.424	**0.006**
ΔTG: HDL	-9.127	2.305	-16.464	-1.790	-0.415	-0.371	0.138	**0.029**

Note. Model = "Stepwise" and "Backward" methods. Significant p-values (p ≤ 0.05) are indicated in bold. Abbreviations: $Insulin_{60}$, insulin concentrations 60 minutes after the glucose load; TG, triglycerides; HDL, high-density lipoprotein cholesterol; B, unstandardized coefficient; SE, standard error; CI, confidence interval; LB, lower bound; UB, upper bound; β, standardised coefficient; SPC, semi-partial correlation; Cont., estimated contribution of the independent variable to the total r square calculated by squaring the semi-partial coefficient; Sig, significance.

In subjects who became more insulin-sensitive after HDT bed rest, the ΔFGF-21 was significantly and positively correlated with $\Delta AUCG_{30}$ (Table 84). Multiple regression could not successfully identify the factor(s) that accounted for the ΔFGF-21 in the subgroup with increased insulin sensitivity following HDT bed rest.

Table 84. Correlational analysis for ΔFGF-21 in subjects with increased insulin sensitivity following HDT bed rest.

	ΔFGF-21 (*r*)
ΔRBP4	0.783ǂ
$\Delta Glucose_0$	0.719§
$\Delta AUCG_{30}$	**0.826***

Note. §p ≤ 0.200, ǂp ≤ 0.10, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Abbreviations: FGF-21, fibroblast growth factor 21; RBP4, retinol binding protein 4; $Glucose_0$, fasting glucose; $AUCG_{30}$; area under the curve for glucose for 30 minutes.

Chapter 6. Discussion

In this chapter, the key results presented in chapter 4 and chapter 5 will be interpreted and compared to previous literature to form a discussion.

The primary aim of this study was to examine changes in physical characteristics, metabolic characteristics and circulating concentrations of novel biomarkers of insulin sensitivity and insulin resistance in healthy young males, pre- and post-60 days of 6° HDT bed rest. The secondary aim was to determine the impact of reactive jump training (RJT), a low volume, high-intensity jump training protocol, on the physical characteristics, metabolic characteristics and circulating concentrations of novel biomarkers of insulin sensitivity and insulin resistance in healthy young males, pre- and post-60 days of 6° HDT bed rest. In line with the objectives, the changes in body weight, body composition, peak aerobic capacity and muscle strength will be discussed in section 6.1. The changes in glucose tolerance, insulin sensitivity and lipid metabolism will be discussed in section 6.2. The changes in circulating fetuin-A, RBP4, irisin, adropin, adiponectin, ASP, apelin, apo-J and FGF-21 will be discussed in section 6.3.

A subanalysis investigating individual metabolic response post-HDT bed rest was conducted *a posteriori*. The purpose of this subanalysis was to firstly, investigate and profile the physical and metabolic changes that occurred in individuals with following HDT bed rest and to identify specific pre- to post-HDT bed rest changes in metabolic physiology that may have contributed to the significant deterioration of insulin sensitivity noticeable in this subgroup, using correlation and multiple regression analysis. Secondly, to investigate and profile the physical and metabolic changes that occurred in individuals with increased insulin sensitivity following HDT bed rest and to identify the specific pre- to post-HDT bed rest changes in metabolic physiology that may have contributed to the significant improvement of insulin sensitivity noticeable in this subgroup, using correlation and multiple regression analysis. The results of this subanalysis will be discussed in section 6.4.

6.1. Physical Characteristics: Body Weight, Body Composition, $\dot{V}O_{2peak}$ and Muscle Strength

The physiological adaptations during spaceflight and HDT bed rest include muscle atrophy, a shift in myofiber phenotype (slow oxidative to fast glycolytic muscle fibers), decreased strength and power, reduced aerobic capacity and metabolic dysfunction (Bergouignan et al., 2011; Narici and de Boer, 2011; Ade et al., 2015; Vico and Hargens, 2018; Furukawa et al., 2021). Exercise countermeasures have been widely implemented during spaceflight and HDT bed rest to negate these physiological adaptations, however, at present, no countermeasure has fully alleviated the deleterious multi-system effects (Ploutz-Snyder, 2016; Kenny et al., 2017; Rudwill et al., 2018; Gao and Chilibeck, 2020). Currently, astronauts perform a high weekly training volume of 9 to 12 hours (~ 60 – 75 minutes of resistance training and ~ 30 – 45 minutes of aerobic training, 6 days per week), which considerably limits the time available to perform other in-flight tasks.

In the RSL bed rest study described in this PhD thesis, which was funded by ESA and hosted by the DLR, twelve research teams collaborated to investigate the physiological adaptations to HDT bed rest. The overall purpose of this study was to examine the efficacy of reactive jump training, encompassing plyometric movements with high rates of force development, to preserve musculoskeletal mass and strength was examined (Kramer et al., 2017b). It is estimated that subjects in JUMP group performed a total of 2.5 hours of MVPA (or 2.5 minutes of MVPA per day), without breaks, during 60 days HDT bed rest, which is the equivalent of the time astronauts currently spend exercising every day in space. In terms of the physical characteristics, the results of this PhD study show that this time-efficient countermeasure attenuated the loss of whole-body lean mass, leg lean mass, $\dot{V}O_{2peak}$ and muscle strength. These findings have also been published by the other research teams (Kramer et al., 2017b; Kramer et al., 2017a). Additionally, there is evidence that the short-duration, high-impact reactive jump training preserved myofiber size and phenotype (Blottner et al., 2019). Taken together, these results are similar to those reported following resistance exercise (7-9 minutes every 3 days) (Gallagher et al., 2005; Bergouignan et al., 2006; Belavy et al., 2017) and resistive vibration exercise (2 x 6 minutes daily) (Blottner et al., 2006; Mulder et al., 2006; Belavý

et al., 2009) and support a role for low volume, high-force producing jumping exercise in the preservation of physical capacity during long-duration HDT bed rest. Additionally, from an applied perspective, these findings support the further optimisation of low volume exercise prescription to achieve a similar, if not superior, physiological effect for astronauts in space, which would alleviate an already very full daily schedule.

Bed rest studies conducted by the ESA are strictly controlled to study physiological and metabolic outcomes (Kenny et al., 2020). In this study, and previous bed rest studies (Bergouignan et al., 2006; Kenny et al., 2017; Rudwill et al., 2018), the goal was to maintain energy balance throughout HDT bed rest to ensure that the change in fat mass was not confounding factor on metabolic perturbations. However, this does not exclude that the behaviour and distribution of fat in peripheral tissues may be altered in response to same. Fat mass was maintained in the CTRL group during HDT bed rest but decreased in the JUMP group (-0.9kg or -5%), possibly due to the challenges of estimating energy expenditure of the exercise protocol. Lean mass decreased by ~4kg (-7%) in the CTRL group, which is consistent with other HDT bed rest studies of similar duration (Bergouignan et al., 2009; Rudwill et al., 2015; Trim et al., 2021). Leg lean mass decreased by ~3kg (-16%) in the CTRL group, which supports the loss of muscle mass occurring predominately in the lower quadrant of the body as a result of reduced gravitational forces (Belavy et al., 2017). Lean mass and leg lean mass decreased by ~1kg in the JUMP group (-2% and -5%, respectively), and leg lean mass was higher in the JUMP group after HDT bed rest. The reduction in body weight in the CTRL group (~4kg or -5%) is therefore attributed to the decrease in lean mass, whereas the reduction in body weight in the JUMP group (~2kg or -3%) is due to the decrease in lean mass and fat mass, post-HDT bed rest. We observed no change in bone mineral content in either group following HDT bed rest. Skeletal muscle mass is regulated by a balance between protein synthesis and degradation (Naro et al., 2020). It is now clear that blunted or suppressed muscle protein synthesis is the main driver of muscle atrophy in response to chronic inactivity and high levels of sedentary time, associated with the decreased activation of the Akt-mTOR pathway (Gao et al., 2018). The efficacy of reactive jump training to attenuate muscle atrophy may be related to the provision of stretch-shortening stimuli and the ability to induce tissue strains when pull-down forces are eliminated by unloading or

microgravity, constituting a strong stimulus for muscle growth (Blottner et al., 2019; Gruber et al., 2019).

Oxidative, anti-gravity muscles such as the soleus are more prone to deconditioning as a result of microgravity or HDT bed rest resulting in reduced fiber CSA, slow-to-fast fiber-type transition and muscle weakness, compared to non-postural, mixed fast-slow muscles such as the vastus lateralis (Gao et al., 2018; Blottner et al., 2019). Results from our HDT bed rest study, published by another group (Blottner et al., 2019), show reactive jump training preserved myofiber size in the soleus and myofiber type composition in the soleus and vastus lateralis. There was no evidence of HDT bed rest-induced atrophy in the vastus lateralis in either group. Additionally, reactive jump training attenuated capillary rarefaction, but did not mitigate the loss of local capillary to fiber ratio (LCFR) per fiber perimeter (which reflects the number of capillaries supplying a fiber) following HDT bed rest. Skeletal muscle oxidative capacity or aerobic capacity/mitochondrial content of the muscle fibers in the vastus lateralis did not change in either group following 60 days HDT bed rest. These results have important implications for the preservation of physical capacity and metabolism following HDT bed rest with the performance of short-duration, high-impact reactive jump training (Blottner et al., 2019). The relevance of these findings for $\dot{V}O_{2peak}$ and muscle performance will be discussed in the following three paragraphs.

Reactive jump training was effective in mitigating the loss of $\dot{V}O_{2peak}$, while the CTRL group exhibited a decrease of approximately 30%, in line with the results from other long-duration HDT bed rest studies (Capelli et al., 2006; Schneider et al., 2009). It is clear that the magnitude of change in $\dot{V}O_{2max}$ is dependent on the duration of extreme physical inactivity and sedentarism as well as the initial training status of the individual subject, such that well-trained individuals at baseline commonly exhibit the fastest rates of decline (Ade et al., 2015; Ried-Larsen et al., 2017; Clark et al., 2020). However, large variation exists between bed rest studies due to differences in test protocols, timing of the test after re-ambulation and assessment criteria, highlighting that further harmonisation of $\dot{V}O_{2peak}$ procedures is required (Bringard et al., 2010; Ried-Larsen et al., 2017). Previous research has demonstrated that the rate of decline in $\dot{V}O_{2max}$ becomes progressively smaller as the

duration of HDT bed rest increases (14 < 42 < 90 days) and indicates that most of the $\dot{V}O_{2max}$ decrease occurs in the first 14 days of HDT bed rest (Capelli et al., 2006). It is suggested that the fast component (first 14 days) of the decrease in $\dot{V}O_{2max}$ is related to the reduction in maximal oxygen delivery, as a result of decreases in maximal cardiac output and haemoglobin, in response to the HDT bed rest-induced cephalad fluid shift. Conversely, the slow component (after 14 days) of the decrease in $\dot{V}O_{2max}$ is due to an impairment intracellular oxidative metabolism and a reduction in capillary volume leading to alterations in peripheral oxygen delivery and oxygen diffusion capacity associated, at least in part, with HDT bed rest-induced muscle atrophy, particularly a reduction in slow-twitch muscle fiber isoforms (Capelli et al., 2006; Salvadego et al., 2011; Salvadego et al., 2016; Salvadego et al., 2018; Clark et al., 2020). In agreement with the above hypotheses, and following 35 days HDT bed rest, decreases in supine $\dot{V}O_{2max}$ have been attributed to reduced peripheral gas exchange (decreased mitochondrial volume), whereas larger reductions in upright $\dot{V}O_{2max}$ are due to the combined effect of decreased maximal oxygen delivery and impaired peripheral gas exchange (Bringard et al., 2010). The 39% reduction in upright $\dot{V}O_{2max}$ in the aforementioned study is larger than the decrease reported in the present study, despite a longer bed rest duration, and this was attributed to the timing of the $\dot{V}O_{2max}$ test which occurred only 1 hour after re-ambulation, suggesting limited initial fluid balance recovery. $\dot{V}O_{2peak}$ was measured one day after re-ambulation in this study (R+1) allowing for a possible partial cardiovascular recovery.

The decrease in plasma volume is a well-known consequence of HDT bed rest that has been shown to contribute largely to the decrease in maximal cardiac output following HDT bed rest (Spaak et al., 2005). In this study, there was a significant main effect of time for the reduction in plasma volume following HDT bed rest, which decreased by 13%, advocating that reactive jump training did not maintain plasma volume in the JUMP group. Similarly, a recent publication reported a 15% reduction in plasma volume following 21 days HDT bed rest that could not be mitigated by resistive vibration exercise (Guinet et al., 2020). Conversely, an integrated countermeasure of treadmill exercise and lower body negative pressure mitigated the loss of plasma volume in the exercise group after 30 days HDT bed rest, highlighting that aerobic training is a superior modality for

preserving plasma volume during HDT bed rest (Lee et al., 2007). Additionally, the concentration of haemoglobin did not change following 60 days HDT bed rest (data not shown). Maximal cardiac output was not measured during the $\dot{V}O_{2peak}$ test, however, it appears that factors other than plasma volume and haemoglobin influenced post-HDT bed rest $\dot{V}O_{2peak}$. In addition to central limitations, impairments and decreases in vascular function, skeletal muscle mass, oxygen diffusing capacity and mitochondrial enzyme activity can contribute to the reduction in $\dot{V}O_{2peak}$ following extreme inactivity (Ferretti et al., 1997; Capelli et al., 2006; Ade et al., 2015). Accordingly, the efficacy of reactive jump training to preserve muscle mass, myofiber type composition and capillarisation (detailed above) may have contributed to the smaller decline in $\dot{V}O_{2peak}$ in the JUMP group.

The results of the maximal strength tests show that reactive jump training prevented the loss of knee extensor strength and attenuated the loss of plantar flexor strength following HDT bed rest. In the CTRL group, knee extensor and plantar flexor strength decreased by approximately 40%, which is similar to the declines reported after 60 and 90 days HDT bed rest (Alkner and Tesch, 2004; Gallagher et al., 2005; Trappe et al., 2007). In a recent 60 day HDT bed rest study, isometric maximal voluntary contraction of the quadriceps and triceps surae decreased by 32% and 21%, respectively, in the control group (Arc-Chagnaud et al., 2020). It is clear from this study and comparison studies that the decrease in muscle strength exceeds the loss of muscle mass in response to HDT bed rest. This may be explained, at least in part, by altered motor control, reduced electromechanical signalling and changes in intrinsic skeletal muscle properties (Alkner and Tesch, 2004; Pavy-Le Traon et al., 2007).

Reactive jump training incorporated high peak forces and high power output of the leg extensors in a whole-body movement that required precise activation and coordination of muscle groups. The isometric MVC tests showed no change in knee extensor strength and only a small decrease in plantar flexor strength (-8%) in the JUMP group after HDT bed rest. Resistance flywheel exercise maintained knee extensor strength but could not attenuate the loss in plantar flexor strength following 90 days HDT bed rest (Gallagher et

al., 2005). Conversely, the combination of flywheel and treadmill exercise increased isometric calf press strength but could not prevent the decrease in isometric knee extensor strength after 60 days HDT bed rest (Trappe et al., 2007). To date, the only other countermeasure that was equally effective in preserving leg extensor strength (reduction of 9% in isometric plantar flexor strength) was resistive exercise in combination with superimposed whole-body vibration during 56 days of HDT bed rest (Blottner et al., 2006; Mulder et al., 2006). These results highlight the effectiveness of plyometric and resistive vibration exercise in preserving the calf musculature despite a low volume of training, which is challenging due to its prominence in habitual loading in daily life and higher proportion of slow type myofibers (Alkner and Tesch, 2004; Blottner et al., 2019). Reactive jump training did not have any additional protective effect on muscle strength during knee flexion or dorsiflexion following HDT bed rest. However, it is well known that leg flexor strength is less affected by HDT bed rest than leg extensor strength due to the higher susceptibility of anti-gravity muscles to muscle atrophy (Lee et al., 2014b; Kramer et al., 2017a). Reactive jump training focuses particularly on the leg extensors and this may explain the absence of a significant training-induced effect on the leg flexors, which is consistent with other countermeasures (Lee et al., 2014b).

6.2. Metabolic Characteristics: Glucose Tolerance, Insulin Sensitivity and Lipid Metabolism

Following 60 days 6° HDT bed rest, there was evidence of reduced whole-body glucose tolerance and insulin sensitivity and an altered lipid profile, characterised by increased fasting triglycerides and LDL and decreased HDL. The metabolic response to HDT bed rest was not different in the CTRL and JUMP group. There was a significant increase in fasting insulin levels and in response to an OGTT, one- and two-hour glucose concentrations, as well as two-hour AUC values for glucose and insulin, were significantly higher. Fasting glucose, NEFA and total cholesterol did not change. These findings are in agreement with previous HDT bed rest studies (Rudwill et al., 2015; Bergouignan et al., 2009; Kenny et al., 2017) and support links between the decrease in muscle contraction and elevated triglycerides (Bergouignan et al., 2011), decreases in the amount and activity of key proteins associated with muscle glucose uptake (Biensø et al.,

2012), and altered mitochondrial function (Kenny et al., 2017). Similar metabolic alterations are associated with the etiology of multiple chronic diseases including obesity, T2DM, NAFLD, and the MetSyn. These observations therefore support a key role of physical inactivity and high levels of sedentary time in the onset and progression of metabolic dysfunction (Le Roux et al., 2021).

In addition to glucose and insulin measurements during the OGTT, we examined changes in multiple OGTT-derived indexes of insulin sensitivity and insulin resistance after HDT bed rest. We report a significant increase in HOMA-IR and significant decreases in the Matsuda index, disposition index, Gutt index and QUICKI following 60 days HDT bed rest, irrespective of group. However, the OGIS index, HOMA-β and insulinogenic index did not change. Furthermore, as the severity of insulin resistance has been found to vary between peripheral tissues after HDT bed rest (Abdul-Ghani et al., 2007; Rudwill et al., 2018), we used relevant indices reported in the literature to estimate changes in insulin sensitivity in the liver, adipose tissue and skeletal muscle. Liver and muscle insulin sensitivity significantly decreased, and adipose insulin resistance significantly increased post-HDT bed rest, regardless of group. Conversely, hepatic insulin resistance did not change. Heer and colleagues (2014) investigated the effect of 21 days HDT bed rest on the Matsuda index, HOMA-IR and hepatic insulin resistance in lean, healthy males. No significant changes in fasting glucose, fasting insulin, HOMA-IR and hepatic insulin resistance were reported. However, there was a significant increase in two-hour AUC for glucose and insulin, and a significant decrease in the Matsuda index. These results reflect a reduction in peripheral, but not hepatic, insulin sensitivity following HDT bed rest (Heer et al., 2014). Similarly, in another 21 day HDT bed rest study, muscle insulin sensitivity decreased significantly but no significant pre- to post-changes in fasting glucose, fasting insulin, QUICKI or liver insulin sensitivity were found (Rudwill et al., 2018).

Impaired insulin sensitivity is generally defined by impaired suppression of hepatic glucose production, enhanced lipolysis in adipose tissue and decreased glucose clearance in skeletal muscle (Roden et al., 2017). These changes may develop simultaneously but, as mentioned above, the severity of insulin resistance can differ among the tissues (Abdul-

Ghani et al., 2007). HOMA-IR and QUICKI primarily reflect changes in hepatic insulin sensitivity. As fasting glucose and NEFA did not change, the significant pre- to post-HDT bed rest increase in HOMA-IR and decreases in QUICKI, liver insulin sensitivity and adipose tissue insulin resistance are due to elevated fasting insulin concentrations. Therefore, we propose that the reduction in whole-body insulin sensitivity following 60 days HDT bed rest is associated mainly with the development of insulin resistance in skeletal muscle. This leads to the diversion of excess glucose and lipids to the liver, which can cause hepatic insulin resistance, and when combined with compensatory hyperinsulinemia, upregulates hepatic de novo lipogenesis, synthesis of triglycerides and secretion of VLDL resulting in elevated circulating triglycerides and reduced circulating HDL (Roden et al., 2017).

While reactive jump training had protective effects on muscle mass and function, it could not attenuate the dysregulation of glucose and lipid metabolism that typically occurs with HDT bed rest. Similar results were reported following flywheel exercise (Bergouignan et al., 2006) and the authors, in this case, postulated that insufficient energy expenditure during the resistance training sessions may have been the key factor. While reactive jump training is a form of high-intensity interval training consisting of 48 jumps and 30 hops, the overall workload may not be sufficient to improve insulin sensitivity. The physical inactivity and extreme sedentarism of HDT bed rest is the primary intervention and the exercise countermeasure in this case is designed to maintain rather than enhance physiological function. Combined resistance (3 days per week of maximal concentric and eccentric actions in the supine squat and calf press for 35 minutes) and aerobic (2 days per week on a vertical treadmill in a lower-body negative-pressure at intensities ranging between 40 and 80% pre-bed rest $\dot{V}O_{2peak}$ for 50 minutes) training preserved myofiber size and phenotype as well as counteracting the shift in substrate utilisation in the fasted and fed state and postprandial insulin concentrations following HDT bed rest (Salanova et al., 2008; Bergouignan et al., 2009). However, the exercise group was in negative energy balance as evidenced by a reduction in fat mass (-10%) in the exercise group. In contrast, resistive vibration exercise (5 training sessions of a leg press protocol involving bilateral squats, single heel raises and bilateral heel raises performed on a vibration platform – 8mm peak-to-peak amplitude and 25Hz frequency) maintained insulin

sensitivity following 21 days HDT bed rest but was not sufficient to offset the decrease in lean body mass (Kenny et al., 2017; Kenny et al., 2020). The maintenance of physiological health in microgravity requires countermeasures capable of mitigating atrophy as well as maintaining metabolic homeostasis. Therefore, a combination of reactive jump training and resistance vibration exercise may optimally preserve physiological functioning in a time-efficient manner. Further research is warranted to optimise an exercise protocol and determine the efficacy of this countermeasure in mitigating multi-organ deconditioning.

To explore the factors that may have contributed to the decline in insulin sensitivity in all subjects post-HDT bed rest, we performed correlation and multiple regression analysis using the pre- to post-HDT changes in estimates of insulin sensitivity and insulin resistance as dependent variables. The increase in circulating triglycerides accounted for 36% and 20% of the decrease in the Matsuda index and liver insulin sensitivity, respectively, in all subjects following HDT bed rest. These associations are in agreement with previous HDT bed rest studies which have reported elevated triglycerides concomitant with decreased insulin sensitivity following extreme inactivity (Bergouignan et al., 2011).

A combination of the decrease in trunk fat mass and increases in triglycerides and RBP4 accounted for 56% of the decrease in QUICKI in all subjects after HDT bed rest. The decrease in trunk fat mass predicted 23% of the increase in HOMA-IR in all subjects' post-HDT bed rest. Similarly, the decrease in trunk fat mass made the largest unique contribution to explaining the decrease in QUICKI, followed by RBP4 and triglycerides, respectively. This finding is unexpected as the literature consistently reports improvements in insulin sensitivity concomitant with reductions in fat mass, particularly trunk fat mass (Chait and den Hartigh, 2020). However, the decrease in trunk fat mass was minimal (-0.2kg), so it is possible that the behaviour of existing fat mass may have changed unfavourably in response to HDT bed rest. This is supported by the findings of a previous study showing reductions in circulating adiponectin and muscle insulin sensitivity when total fat mass was maintained in lean healthy males following HDT bed

rest (Rudwill et al., 2018). RBP4 is a hepatokine and adipokine that has a negative impact on insulin sensitivity by impairing insulin signalling in skeletal muscle and adipose tissue, increasing lipolysis and inflammation in adipose tissue and upregulating gluconeogenesis and lipid accumulation in the liver (Yang et al., 2005; Ost et al., 2007; Berry et al., 2011; Norseen et al., 2012; Lee et al., 2016). These findings may imply a negative change in the behaviour of adipose tissue and the liver in response to HDT bed rest.

The decrease in trunk lean mass explained 19% of the decrease in disposition index in all subjects after HDT bed rest, suggesting that muscle atrophy plays a role in the decline of the insulin sensitivity/secretion index. The increase in total cholesterol was the best predictor of the decrease in the Gutt index, accounting for 37% of the variance in all subjects after HDT bed rest. The increase in LDL predicted 19% of the increase in adipose tissue insulin resistance following HDT bed rest. Taken together, muscle atrophy and alterations in the behaviour of adipose tissue, liver and skeletal muscle underpin the metabolic dysregulation in all subjects after HDT bed rest.

6.3. Biomarkers of Insulin Sensitivity and Insulin Resistance

Investigation of secreted hepatokines, myokines and adipokines and their roles in inter-organ crosstalk has the potential to identify and understand the etiology of insulin resistance in the context of HDT bed rest (Priest and Tontonoz, 2019). In this study, we examined circulating adropin, irisin, adiponectin, FGF-21, fetuin-A, RBP4, apo-J, ASP and apelin pre- and post-60 days 6° HDT bed rest, with and without reactive jump training. These secreted biomarkers are known to enhance (adropin, irisin, adiponectin, FGF-21, apo-J, ASP and apelin) or attenuate (fetuin-A and RBP4) insulin sensitivity.

Adropin is a recently identified hepatokine which modulates metabolic homeostasis, demonstrating systemic effects on insulin sensitivity (Mushala and Scott, 2021). Adropin acts on the liver and skeletal muscle and improves insulin signalling and metabolic flexibility and reduces hepatic glucose production, endoplasmic reticulum stress and JNK activity (Gao et al., 2014; Gao et al., 2015; Gao et al., 2019). It has been proposed that

liver dysfunction decreases adropin expression and triggers insulin resistance (Mushala and Scott, 2021). Accordingly, lower circulating adropin levels have been reported in humans with obesity, insulin resistance, T2DM and hepatosteatosis (Butler et al., 2012; Zang et al., 2018; Kutlu et al., 2019). To our knowledge, the impact of bed rest on circulating adropin has not been investigated previously.

We report a significant decrease in circulating adropin after 60 days 6° HDT bed rest, irrespective of group. Adropin deficiency, examined in adropin-knockout mice, is associated with insulin resistance due to impairments in insulin-mediated suppression of hepatic glucose production and glucose disposal and is accompanied by dyslipidaemia (Ganesh Kumar et al., 2012). These deleterious effects on glucose and lipid metabolism are exacerbated in obesity (Ganesh Kumar et al., 2012). In this study, fasting glucose and hepatic insulin resistance did not change following HDT bed rest suggesting that hepatic glucose production remained unchanged. Conversely, there was a significant increase in fasting insulin and a significant decrease in whole-body insulin sensitivity after HDT bed rest, associated with a pronounced disturbance in peripheral insulin sensitivity, irrespective of group. These changes were accompanied with an increase in fasting triglycerides and LDL and decrease in HDL post-HDT bed rest, regardless of group. Additionally, lean mass decreased significantly following HDT bed rest, with a higher mean decline noticeable in the CTRL group compared to the JUMP group. Multiple regression analysis found that the decrease in lean mass predicted 22% of the decrease in adropin in all subjects after HDT bed rest. Skeletal muscle is a key target organ in mediating the favourable metabolic effects of adropin (Gao et al., 2014). The reduction in adropin secretion may have contributed to impaired insulin signalling leading to reduced peripheral glucose uptake and decreased clearance of triglycerides from circulation, which was compounded with the reduction in skeletal muscle mass. While we did not measure changes in metabolic flexibility in this study, previous research has reported that HDT bed rest-induced glucose intolerance is preceded by a metabolically inflexible state (Rudwill et al., 2018; Le Roux et al., 2021), which could also be impacted by lower concentrations of adropin.

Irisin is secreted primarily from skeletal muscle in response to exercise and elicits multiple beneficial metabolic effects in adipose tissue, skeletal muscle, liver and pancreas. Irisin maintains glucose and lipid metabolism contributing to improved insulin sensitivity, reduces endoplasmic reticulum stress and contributes to β-islet cell survival and function (Huh et al., 2014b; Liu et al., 2015b; Park et al., 2015; Perakakis et al., 2017; Arhire et al., 2019). Studies in the literature have reported that irisin levels decrease progressively with worsening of glucose tolerance and are decreased in T2DM (Choi et al., 2013; Liu et al., 2013; Assyov et al., 2016). Lower concentrations of irisin have been associated with compromised expression and secretion of irisin from skeletal muscle in T2DM (Shoukry et al., 2016). To our knowledge, changes in circulating irisin have not been measured in response to bed rest.

We report a significant decrease in circulating irisin, irrespective of group, after 60 days 6° HDT bed rest. This change occurred concomitant to significant elevations in fasting insulin, triglycerides and LDL and reductions in HDL and insulin sensitivity, regardless of group. In addition, HDT bed rest significantly decreased lean mass in both groups, with a higher mean decline identified in the CTRL group compared to the JUMP group. It is possible that the pronounced reduction in lean mass and contractile activity may have blunted the secretion of irisin in response to HDT bed rest leading to reduced glucose disposal. Further to this, lower concentrations of irisin may have reduced lipid metabolism and lipid uptake in skeletal muscle and elevated lipogenesis in the liver, thereby contributing to hyperlipidaemia following HDT bed rest. Multiple regression analysis revealed that increases in HOMA-β and fetuin-A predicted 31% of the decrease in circulating irisin in all subjects following HDT bed rest. HOMA-β was reported to be independently, but positively, associated with irisin in subjects with normal glucose tolerance, suggesting a role of irisin in pancreatic β-cell function (Yang et al., 2014). However, the mechanism underlying this association is unknown. The increase in HOMA-β may reflect increased insulin release from the pancreas, which is commonly noticeable in the early stages of T2DM development (Liu et al., 2017). Irisin and fetuin-A have pivotal roles in glucose homeostasis. Irisin augments insulin-induced PI3K/Akt signalling leading to increased glucose disposal (Yano et al., 2021). Conversely, fetuin-A inhibits the activity of the insulin receptors and PI3K/Akt signalling causing insulin

resistance (Pan et al., 2020). Therefore, this association may be related to the reduction in peripheral insulin sensitivity in response to HDT bed rest. However, additional experiments are needed to confirm this. Overall, the reduction in irisin appears to be implicated in the deterioration of the metabolic profile of young, healthy males after 60 days 6° HDT bed rest.

Adiponectin is an abundant protein hormone secreted by adipocytes, with multiple salutary insulin-sensitising, anti-inflammatory and anti-atherogenic effects (Achari and Jain, 2017). The liver and skeletal muscle are principal targets for adiponectin. Adiponectin stimulates fatty acid oxidation in both tissues, stimulates glucose uptake in skeletal muscle and attenuates gluconeogenesis and lipogenesis in the liver (Khoramipour et al., 2021). Locally in adipose tissue, adiponectin promotes glucose uptake and subcutaneous fat storage and reduces inflammation (Fang and Judd, 2018). Additionally, this adipokine can enhance glucose-stimulated insulin secretion, β-cell survival, eNOS activity and decrease oxidative stress (Fang and Judd, 2018). A primary physiological function of adiponectin is therefore to improve insulin sensitivity, with decreased circulating concentrations associated with insulin resistance and metabolic dysfunction (Andrade-Oliveira et al., 2015; Fang and Judd, 2018).

In this study, we observed a significant decrease in circulating adiponectin, regardless of group, after 60 days of 6° HDT bed rest. This finding is consistent with previous a 21 day HDT bed rest study and supports a reduction in insulin sensitivity after extreme inactivity (Rudwill et al., 2018). When all the subjects from the CTRL and JUMP groups are combined, the percentage decrease in adiponectin is 24%. This result is in line with the 23% decrease in circulating adiponectin reported recently in young, healthy males following 60 days HDT bed rest (Trim et al., 2021). The authors proposed that, given the role of adiponectin as an insulin-sensitising hormone, the reduction in circulating adiponectin may reflect a homeostatic re-adjustment to increased fasting insulin levels (Trim et al., 2021). In line with this, we report a significant increase in fasting insulin, in addition to an altered lipid profile and evidence of impaired peripheral insulin sensitivity, post-HDT bed rest. These changes occurred irrespective of group. It is possible that

reductions in circulating adiponectin may have negatively affected glucose uptake, lipid oxidation and lipogenesis contributing to the onset of insulin resistance during HDT bed rest, even in the absence of fat mass gain.

Multiple regression analysis revealed that the increase in RBP4 and decrease in leg fat mass explained 45% of the decrease in circulating adiponectin in all subjects following HDT bed rest. A significant independent, positive correlation between leg fat mass and circulating adiponectin has been reported recently (Bellissimo et al., 2021). Lower body distribution of adipose tissue contributes less to metabolic deregulation than visceral adipose tissue (Chait and den Hartigh, 2020). Thus, it is possible that the positive correlation between the decreases in adiponectin and leg fat mass is not indicative of adipose tissue dysfunction in lean individuals. RBP4 is a liver- and adipose tissue-derived protein that is implicated in the pathogenesis of insulin resistance through its ability to impair insulin signalling in skeletal muscle and adipose tissue, upregulate lipolysis and inflammation in adipose tissue and increase glucose production and lipid accumulation in the liver (Yang et al., 2005; Ost et al., 2007; Norseen et al., 2012; Lee et al., 2016). Multiple studies have reported an inverse relationship between circulating RBP4 and adiponectin in humans, including non-diabetic, non-obese adults with hypercholesterolemia (Jia et al., 2007; Shin et al., 2007; Christou et al., 2012). Decreased adiponectin and increased RBP4 concentrations are associated with reduced fatty acid oxidation and insulin signalling in the skeletal muscle and liver and increased gluconeogenesis in the liver (Esteve et al., 2009). Therefore, decrease in adiponectin and increase in RBP4 may underlie the reduction in insulin sensitivity in all subjects following HDT bed rest, particularly in the liver and skeletal muscle.

FGF-21 is a potent metabolic regulator, derived mainly from the liver. It is also expressed in the pancreas, adipose tissue and stressed skeletal muscle (Inagaki, 2015). FGF-21 increases fatty acid oxidation, ketogenesis and gluconeogenesis in the liver in response to the fasting state (Owen et al., 2015). In adipose tissue, FGF-21 increases glucose uptake, stimulates adiponectin secretion, modulates lipolysis according to the fasted or fed state, and regulates thermogenesis in brown adipose tissue (Holland et al., 2013; Dolegowska

et al., 2019). These physiological functions help to maintain energy homeostasis and improve insulin sensitivity. Contrary to expectations, circulating FGF-21 concentrations are increased in insulin resistance, reflecting FGF-21 resistance, and have been shown to correlate inversely with whole-body insulin sensitivity and positively with hepatic and adipose tissue insulin resistance (Chavez et al., 2009).

In this present study, we report a significant decrease in circulating FGF-21, irrespective of group, after 60 days of 6° HDT bed rest. This finding was unexpected given the increase in insulin resistance observed in the current study, but is in accord with a previous study showing a decrease in circulating FGF-21 after 5 days of horizontal bed rest, concomitant to decreased circulating adiponectin and reduced insulin sensitivity, measured using the Matsuda index (Petrocelli et al., 2020). The authors provided additional evidence of a positive correlation between the decrease in FGF-21 and decrease in adiponectin, suggesting a possible mechanistic link between these two biomarkers. While we did not observe a positive association between these biomarkers in this study, we did report a significant main effect of time for the decreases in FGF-21, adiponectin and the Matsuda index following 60 days HDT bed rest. Findings presented in the review article by Hui et al. (2016) suggest that the transcription and secretion of adiponectin is induced by FGF-21, partially dependent on PPAR-γ, and adiponectin is a downstream effector mediating the beneficial metabolic actions of FGF-21. The combined actions of FGF-21 and adiponectin, referred to as the "FGF-21-adiponectin axis", maintain systemic metabolic homeostasis (Hui et al., 2016). Therefore it is possible that the reduction in circulating FGF-21 and adiponectin and consequent dysregulation of this axis may contribute to the decrease in insulin sensitivity in response to HDT bed rest. However, this mechanism requires further investigation.

It is generally assumed that circulating FGF-21 is reflective of hepatic production, although changes in expression in skeletal muscle, adipose tissue and the pancreas may be, at least partly, responsible (Angelin et al., 2012). Also, the subjects in this study were lean, healthy males and it may be that compensatory hypersecretion of FGF-21 observed in the literature is specific to individuals with obesity and excessive ectopic fat

accumulation (Hong et al., 2019). When all subjects were combined, the decrease in FGF-21 was not significantly correlated with changes in any physical or metabolic variables after HDT bed rest and therefore, there were no appropriate regression models to explain the decrease in FGF-21 post-HDT bed rest. The lack of associations between FGF-21 and other physiological measures may be impacted by the relatively small sample size, which is generally low (8 – 12 participants per group) due to the logistics and cost associated with running HDT bed rest studies (Kenny et al., 2020), and the individual responses in circulating FGF-21 and insulin sensitivity reported in humans (Gälman et al., 2008; Hansen et al., 2020). Investigations into the regulation of FGF-21 and its association with insulin sensitivity after HDT bed rest are required.

Fetuin-A is a multifunctional glycoprotein that is synthesized mainly by hepatocytes and promotes insulin resistance and inflammation (Bourebaba and Marycz, 2019). Fetuin-A attenuates insulin signalling, suppresses the production of adiponectin and triggers lipid-induced inflammation (Hennige et al., 2008; Pal et al., 2012; Ochieng et al., 2018). Circulating levels of fetuin-A are upregulated in the presence of insulin resistance and elevated liver fat content (Peter et al., 2018). Accordingly, elevated concentrations of fetuin-A are reported in individuals with obesity, prediabetes, T2DM, and NAFLD (Stefan et al., 2006; Yilmaz et al., 2010b; Ou et al., 2011; Ismail et al., 2012). To our knowledge, changes in circulating fetuin-A have not been investigated in response to bed rest.

We found a significant increase in circulating fetuin-A concomitant to reduced whole-body insulin sensitivity, following 60 days of 6° HDT bed rest. These changes occurred irrespective of the group. Excessive release of FFAs and elevated glucose levels can stimulate the synthesis of fetuin-A from hepatocytes through the activation of the nuclear factor kappa B (NF-κB) and extracellular signal-regulated protein kinase (ERK1/2) signalling pathways (Bourebaba and Marycz, 2019). Fetuin-A then inhibits insulin receptor auto-phosphorylation and tyrosine kinase activity leading to decreased glucose uptake in peripheral tissues, triggering insulin resistance (Bourebaba and Marycz, 2019). Multiple regression analysis identified that the decrease in arm total mass as a sole

predictor of 22% of the increase in fetuin-A in all subjects following HDT bed rest, but there was a trend for a positive correlation between the increases in fetuin-A and NEFA post-HDT bed rest, suggesting a normal response of adipose tissue to the effects of insulin. In contrast to other studies demonstrating an increase in fetuin-A concomitant to metabolic dysfunction (Khadir et al., 2018; Peter et al., 2018), the change in fetuin-A was not significantly associated with any other markers of glucose and lipid metabolism or measurements of insulin sensitivity/resistance after HDT bed rest. Unfortunately, no measurements of liver fat were obtained as part of this study precluding our ability to explore the association between fetuin-A and hepatic fat accumulation in response to HDT bed rest. Similar to FGF-21, it is possible that these associations are masked by the small sample size, which is typical for HDT bed rest studies (Rudwill et al., 2015; Rudwill et al., 2018; Kenny et al., 2017), and individual variation in insulin sensitivity previously reported in the literature (Hansen et al., 2020). Therefore, further investigation into the potential role of fetuin-A in the regulation of whole-body insulin sensitivity following HDT bed rest is required.

RBP4 belongs to the lipocalin family of transport proteins and is synthesised by the liver and adipose tissue (Bergmann and Sypniewska, 2013). RBP4 contributes to the development of insulin resistance by impairing insulin signalling in skeletal muscle and adipose tissue, increasing lipolysis and inflammation in adipose tissue and upregulating gluconeogenesis and lipid accumulation in the liver (Yang et al., 2005; Ost et al., 2007; Norseen et al., 2012; Lee et al., 2016). Higher circulating levels of RBP4 have been documented in visceral obesity, insulin resistance, prediabetes, T2DM, NAFLD and the MetSyn (Cho et al., 2006; Jia et al., 2007; El-Mesallamy et al., 2013; Chen et al., 2017; Wessel et al., 2019). To our knowledge, changes in circulating RBP4 have not been examined in response to bed rest.

In this study, we show that circulating RBP4 did not change in response to 60 days of 6° HDT bed rest, regardless of group. Fasting glucose is reported to be an independent determinant of circulating RBP4 (Cho et al., 2006). We found no significant change in fasting glucose following HDT bed rest, which may reflect unchanged hepatic glucose

production, as RBP4 has been shown to increase the expression of PEPCK a key enzyme in gluconeogenesis in the liver. In addition to fasting glucose, RBP4 has been found to be independently associated with elevated triglycerides, large VLDL particles, small LDL particles and reduced large HDL particles, suggesting a link between higher RBP4 concentrations and a pro-atherogenic plasma lipoprotein profile (Qi et al., 2007; Wessel et al., 2019). We report a significant increase in circulating triglycerides and LDL and decrease in HDL, irrespective of group, after 60 days HDT bed rest. In addition, we found a significant main effect of time for the significant reduction in whole-body insulin sensitivity following HDT bed rest, associated with a pronounced decrease in peripheral insulin sensitivity. A significant negative correlation between increase in RBP4 and decrease in adiponectin in all subjects post-HDT bed rest was also identified. Therefore, RBP4 may interfere with the metabolism of triglyceride-rich apo-B-containing lipoproteins, fatty acid oxidation and insulin signalling in response to HDT bed rest. Previous research has proposed RBP4 as a potential biomarker of individual variability in insulin sensitivity, a phenomenon that is reported within the literature (Graham et al., 2006; Hansen et al., 2020). Further research is required to investigate individual responses in RBP4 and insulin sensitivity, as well as the regulation and proposed effects of RBP4, in response to HDT bed rest.

Apo-J/clusterin is a hepatokine which impacts metabolic homeostasis. In states characterised by a hypersecretion of apo-J (metabolic dysregulation in humans or muscle-specific deletion of LRP2 in mice) or a deficiency of apo-J and its receptors (liver-specific Apo-J deficient mice or clusterin knock-out mice), apo-J can impair insulin signalling and perpetuate inflammation and oxidation stress in the liver, skeletal muscle and adipose tissue (Seo et al., 2020). Elevated concentrations of apo-J have been reported in overweight/obesity, insulin resistance, T2DM and the MetSyn (Oberbach et al., 2011; Won et al., 2014; Seo et al., 2018; Bradley et al., 2019). To our knowledge, changes in circulating apo-J have not been studied in response to bed rest.

We report no significant change in circulating apo-J after 60 days of 6° HDT bed rest, irrespective of group. Previous research has shown that apo-J is tightly linked to glucose

metabolism and insulin sensitivity, with apo-J levels representing a significant independent association factor for fasting insulin and HOMA-IR in non-diabetic subjects and a combined group of non-diabetic and diabetic subjects (Seo et al., 2018). In the same study, there was evidence to suggest that apo-J may also have a role in the regulation of insulin secretion (Seo et al., 2018). In another study, clusterin was significantly and positively correlated with LDL and total cholesterol in young lean healthy males, but was not associated with any other metabolic variables or adipokines relating to energy homeostasis and metabolism (Aronis et al., 2011). Following 60 days HDT bed rest, we found a significant increase in fasting insulin, LDL and HOMA-IR and decrease in HDL, regardless of group. However, indices of insulin secretion and β-cell function (HOMA-β and insulinogenic index), as well as total cholesterol did not change. These findings imply that apo-J concentrations did not change despite the evidence of metabolic dysregulation following HDT bed rest.

Apo-J has also been shown to be independently predicted by sex, hsCRP and BMI suggesting that increases in plasma clusterin may be associated with a pro-inflammatory state induced by excess adiposity in obesity (Won et al., 2014). While our study is not focused on changes in apo-J in obesity, changes in adiposity and skeletal muscle mass can affect circulating apo-J in humans (Jeon et al., 2020). In this study, lean mass decreased significantly in both groups following HDT bed rest, with a larger reduction found in the CTRL group compared to the JUMP group. Conversely, fat mass was maintained in the CTRL group, but decreased significantly in the JUMP group after HDT bed rest. Therefore, it is possible that the absence of fat mass gain may have attenuated the increase in circulating apo-J in response to HDT bed rest. Additionally, individual responses in circulating apo-J has been observed within the literature, in individuals with and without metabolic dysregulation (Won et al., 2014; Seo et al., 2018; Jeon et al., 2020). Further research to explore and understand the specific role of apo-J in the context of insulin sensitivity following HDT bed rest is warranted.

ASP is an adipokine that stimulates intracellular triglyceride synthesis in adipose tissue. Specifically, ASP promotes glucose uptake, stimulates fatty acid esterification and

inhibits lipolysis (Saleh et al., 2019). Elevated circulating levels of ASP reflect an ASP resistant state, which occurs in obesity, T2DM, NAFLD and the MetSyn (Maslowska et al., 1999; Koistinen et al., 2001; Faraj et al., 2003; Cianflone et al., 2004; Yesilova et al., 2005; Celik et al., 2013; Coelho et al., 2013; Mishra et al., 2017). To our knowledge, changes in ASP have not been reported previously following bed rest.

In this study, we found no significant change in circulating ASP following 60 days of 6° HDT bed rest, irrespective of group. It is possible that ASP sensitivity in adipose tissue is maintained throughout HDT bed rest, permitting efficient fatty acid trapping and avoiding the large escape of FFAs into circulation. This would subsequently prevent enhanced ASP secretion and onset of adipose tissue inflammation, insulin resistance and dyslipidaemia. In this study, we report no significant changes in circulating NEFA after HDT bed rest, regardless of group. Additionally, fat mass was maintained in the CTRL group but decreased significantly in the JUMP group following HDT bed rest. Fasting FFAs were the largest independent predictor of fasting ASP concentrations in lean and obese populations (Weyer and Pratley, 1999). The circulating levels of ASP and NEFA, as well as the influence of these metabolites on insulin resistance and dyslipidaemia, may have been exacerbated by positive energy balance during HDT bed rest. Furthermore, considerable individual responses in circulating concentrations of ASP have been reported in healthy, normal weight individuals and those with metabolic dysfunction (Maslowska et al., 1999; Celik et al., 2013; Mishra et al., 2017). Future studies are needed to explore individual response in circulating ASP to intervention and elucidate whether ASP resistance occurs following HDT bed rest, in different states of energy balance.

Apelin is an insulin-sensitising adipokine, which increases glucose uptake in skeletal muscle and adipose tissue and enhances fatty acid oxidation and mitochondrial biogenesis in skeletal muscle (Castan-Laurell et al., 2012; Wysocka et al., 2018). In addition, there is evidence to suggest that apelin is an acutely exercise-induced myokine that acts in an autocrine manner to induce skeletal muscle hypertrophy and enhance muscle function (Besse-Patin et al., 2014; Vinel et al., 2018). Insulin and TNF-α upregulate apelin secretion from adipocytes, and increased concentrations of apelin are present in states of

metabolic dysfunction suggesting apelin resistance or compensatory apelin secretion (Daviaud et al., 2006; Bertrand et al., 2015). To our knowledge, circulating apelin concentrations have not been measured following bed rest.

Following 60 days of 6° HDT bed rest, we found no significant change in circulating apelin, irrespective of group. This is a somewhat unexpected finding based on the observation of a significant main effect of time for the increase in fasting insulin after HDT bed rest, which is a known regulator of apelin secretion (Boucher et al., 2005; Bertrand et al., 2015). Additionally, in spite of the absence of change in circulating apelin, HDT bed rest led to significant increases in circulating triglycerides and LDL and significant decreases in HDL and whole-body insulin sensitivity, particularly peripheral insulin sensitivity, regardless of group. Fat mass was sustained in the CTRL group but decreased significantly in the JUMP group following HDT bed rest. It is possible that adipose tissue dysfunction is blunted when energy balance is regulated in lean individuals and this may explain why circulating apelin did not change after HDT bed rest. Apelin has previously been considered as a biomarker of insulin sensitivity, such that as insulin sensitivity improves and circulating concentrations of insulin decrease, circulating apelin concentrations are reduced (Krist et al., 2013; Bertrand et al., 2015; Nam et al., 2020). Therefore, individual responses in insulin sensitivity reported in the literature (Hansen et al., 2020), may have a direct impact on circulating apelin levels following HDT bed rest. Further work to explore the individual variability in circulating apelin and insulin sensitivity after HDT bed rest are required.

A plethora of research has shown that different modalities of exercise training, including aerobic, resistance and high-intensity interval training, as well as concurrent training, can improve body composition, aerobic fitness, muscle strength, arterial stiffness, endothelial function, intrahepatic lipid content and insulin sensitivity in healthy and clinical populations (Schrauwen et al., 2005; Graham et al., 2006; Lim et al., 2008; Kim et al., 2016; Taniguchi et al., 2016; Zhang et al., 2017; Keihanian et al., 2019; Jeon et al., 2020; Murawska-Cialowicz et al., 2020; Nam et al., 2020; Shabkhiz et al., 2020). These changes are associated with favourable alterations in myokines, hepatokines and adipokines,

which interplay through inter-organ crosstalk and physiological adaptations to mediate the beneficial effects of exercise. In contrast, there is also documented evidence that circulating muscle-, liver- and adipose tissue-derived proteins do not change in response to different types of exercise training, despite favourable changes in the physical and metabolic characteristics of individuals, with and without metabolic dysregulation (Mori et al., 2008; Choi et al., 2009; Schultes et al., 2010; Croymans et al., 2013; Hecksteden et al., 2013; Besse-Patin et al., 2014; Kruse et al., 2017). These findings suggest that there is considerable individual variability in the physiological response to exercise training. The concentration and effect of circulating biomarkers can also change in response to the intensity, duration and frequency of exercise (Gonzalez-Gil and Elizondo-Montemayor, 2020).

In this study, we found that the circulating concentrations of adropin, irisin, adiponectin, FGF-21, fetuin-A, RBP4, apo-J, ASP and apelin were not significantly affected by reactive jump training performed during 60 days of 6° HDT bed rest. It is possible that the low volume of training (≤ 4 minutes total training time per session, total of 48 sessions) was not sufficient to induce favourable changes in these circulating biomarkers or to improve insulin sensitivity. As mentioned previously, the primary intervention in the current study is the extreme inactivity and high levels of sedentary time as a result of HDT bed rest and the exercise countermeasure in this case was designed to maintain leg muscle function and bone mass. Changes in adiponectin and FGF-21 have been reported following bed rest, however, to our knowledge the impact of an exercise countermeasure on the circulating levels of these biomarkers has not been studied previously in response to HDT bed rest. A countermeasure capable of attenuating multi-organ deterioration is required to sustain a favourable organokine profile following HDT bed rest. In line with the research presented above, a combination of reactive jump training and resistance vibration exercise may optimally preserve physical and metabolic homeostasis in a time-efficient manner. However, further research is required to optimise an exercise protocol and examine the efficacy of such countermeasure in maintaining overall physiological health after HDT bed rest.

Despite factors that influence biomarker secretion changing in response to HDT bed rest (e.g. reduction of muscle mass, $\dot{V}O_{2peak}$ and insulin sensitivity), the circulating concentrations of some biomarkers did not change. Furthermore, while reactive jump training attenuated the loss of lean mass and $\dot{V}O_{2peak}$, as well as muscle strength and myofiber size and phenotype, the exercise countermeasure did not elicit any favourable effects on insulin sensitivity or the concentration of circulating biomarkers after HDT bed rest. In agreement with the literature presented above, inter-individual variation is a well-recognised phenomenon in human research studies including HDT bed studies, with highly standardised experimental conditions (O'Donoghue et al., 2019; Scott et al., 2021). Investigation of the inter-individual variability in insulin sensitivity in response to HDT bed rest is the first critical step in understanding the existence of individual variation in metabolic homeostasis following extreme inactivity and high levels of sedentary time. While it was not feasible to do that specific analysis for the reasons mentioned in the literature review, it was important to attempt to understand the individual metabolic response post-HDT bed rest, which is discussed in detail in the next section.

6.4. Individual Metabolic Response

The issue of individual variability to intervention is one of the most important in human, exercise and space physiology, particularly in the context of "personalised medicine" to maintain optimal health, and explains the growing interest in this topic in recent years. However, many limitations need to be considered when quantifying individual variability in the context of bed rest (e.g. appropriate statistical analysis, inclusion an ambulatory control group and sufficient sample size) (Fernandez-Gonzalo et al., 2021; Böcker et al., 2022) and therefore this analysis is beyond the scope of this PhD study. Despite this, and in an attempt to further examine the observed individual metabolic response post-HDT bed rest, the subject data from the two groups (CTRL, JUMP) was pooled and then divided based on subjects who decreased (↓ Matsuda index, n = 17, CTRL n = 7, JUMP n = 10) or increased (↑ Matsuda index, n = 6, CTRL n = 4 and JUMP n = 2) insulin sensitivity after HDT bed rest. The Matsuda index is a composite measure of whole-body insulin sensitivity that incorporates both hepatic and peripheral tissues and is calculated using dynamic glucose and insulin values obtained during an OGTT (Matsuda and DeFronzo, 1999). This index has been validated to predict insulin sensitivity against the

euglycaemic-hyperinsulinemic clamp in healthy, lean individuals (Trikudanathan et al., 2013) and has been measured previously in bed rest (Heer et al., 2014; Nielsen et al., 2016; Dirks et al., 2018; Petrocelli et al., 2020). The other surrogate indices of insulin sensitivity used in this HDT bed rest study either primarily reflect hepatic insulin sensitivity, peripheral insulin sensitivity or insulin secretion or have not been studied previously in response to bed rest. For these reasons, the Matsuda index was used as the measure of whole-body insulin sensitivity and subjects were classified into two subgroups based on a decrease or an increase in whole-body insulin sensitivity post-HDT bed rest.

The purpose of this subanalysis was to, firstly, determine and profile the physical and metabolic changes that occurred in individuals with decreased insulin sensitivity following HDT bed rest and to ascertain specific pre- to post-HDT bed rest changes in metabolic physiology that may have contributed to the deterioration of insulin sensitivity noticeable in this subgroup, using correlation and multiple regression analysis. Secondly, to determine and profile the physical and metabolic changes that occurred in individuals with increased insulin sensitivity following HDT bed rest and to ascertain the specific pre- to post-HDT bed rest changes in metabolic physiology that may have contributed to the improvement of insulin sensitivity noticeable in this subgroup, using correlation and multiple regression analysis. From this analysis, we aim to determine a panel of biomarkers that provide important insights into whole-body and tissue-specific insulin sensitivity and are useful to track changes in physiological responsiveness to intervention.

6.4.1. Subgroup with Decreased Insulin Sensitivity

In individuals who became less insulin-sensitive after HDT bed rest, there was a significant reduction in body weight, lean mass, trunk total mass, leg total mass, leg lean mass, arm fat mass, muscle strength and $\dot{V}O_{2peak}$. Fat mass, trunk lean mass, trunk fat mass, leg fat mass, arm total mass, arm lean mass and bone mineral content did not change. Circulating triglycerides and LDL increased significantly and HDL decreased significantly in this subgroup post-HDT bed rest, while NEFA and total cholesterol did not change. There was a significant increase in fasting insulin and fasting glucose and in response to an OGTT, glucose concentrations after 60, 90 and 120 minutes, as well as 30-

and 120-minute AUC values for glucose and 120 minute AUC values for insulin, were significantly higher. Conversely, glucose concentrations after 30 minutes and insulin concentrations after 30, 60, 90 and 120 minutes did not change in this subgroup after HDT bed rest. In addition to the significant reduction in whole-body insulin sensitivity estimated using the Matsuda index, there was a significant decrease in QUICKI, liver insulin sensitivity, disposition index, OGIS index and Gutt index and a significant increase in HOMA-IR and adipose tissue insulin resistance following HDT bed rest. HOMA-β, insulinogenic index, and hepatic insulin resistance did not change. These results suggest blunted insulin action following HDT bed rest, reflected by a significant increase in fasting insulin and fasting glucose, as well as a marked impairment in peripheral glucose uptake evidenced by higher glycaemia in the last hour of the OGTT in individuals who became less insulin-sensitive following HDT bed rest. Furthermore, the increase in circulating triglycerides and decrease in HDL may represent resistance in the ability of insulin to suppress VLDL production in the subgroup with decreased insulin sensitivity after HDT bed rest.

To further explore the factors that may have contributed to the decline in whole-body insulin sensitivity in this subgroup after HDT bed rest, we performed correlation and multiple regression analysis using the pre- to post-HDT changes in estimates of insulin sensitivity and insulin resistance as dependent variables. Overall, the results of this analysis show that unloading-induced muscle atrophy, decrements in muscle strength, increased circulating lipids and alterations in the behaviour of tissues of metabolic importance contributed to the decrease in insulin sensitivity in this subgroup following HDT bed rest. To avoid repetition, similar findings, in terms of the significant pre- to post-HDT bed rest changes and the results of the correlation and multiple regression analysis to determine the factors that contributed to the significant deterioration of insulin sensitivity in all subjects' post-HDT bed rest, have been discussed earlier and can be found in section 6.2. Additionally, and specific to the subgroup which became less insulin-sensitive following HDT bed rest, there was a significant pre- to post-decrease in the OGIS index. Multiple regression analysis identified that the pre- to post-HDT bed rest increase in total cholesterol, albeit not significant, solely predicted the significant decrease in the OGIS index in this subgroup after HDT bed rest, accounting for 34% of

the variance. This finding further highlights a shift in cholesterol metabolism towards enhanced synthesis and decreased absorption concomitant to reduced insulin sensitivity (Gylling et al., 2010).

Inter-organ crosstalk is an important physiological mechanisms to maintain whole-body metabolic homeostasis. Tissues such as the liver, adipose tissue and skeletal muscle have well-established roles in insulin sensitivity (Priest and Tontonoz, 2019). Physical inactivity and high levels of sedentary time can suppress the secretion of health enhancing organokines and promote the secretion of organokines that contribute to development of metabolic disease. Conversely, exercise training promotes the secretion of organokines that induce favourable changes in local and systemic metabolism (Leal et al., 2018). Therefore, to further understand the individual response in insulin sensitivity and deterioration of metabolism in this subgroup following HDT bed rest, changes in the individual biomarker response and thus behaviour in organs and tissues of metabolic importance must be explored. As detailed in the literature review, circulating concentrations of fetuin-A, RBP4, apo-J, ASP, apelin and FGF-21 are elevated when insulin sensitivity is decreased, particularly in the presence of obesity. The increases in apo-J, ASP, apelin and FGF-21 indicate the presence of biomarker resistance or a compensatory response to underlying metabolic stress leading to enhanced secretion. Conversely, circulating concentrations of adiponectin, adropin and irisin are lower when insulin sensitivity is decreased.

In subjects with decreased insulin sensitivity after HDT bed rest, circulating fetuin-A increased significantly and adiponectin, adropin, irisin and FGF-21 decreased significantly. Conversely, apo-J, apelin and ASP concentrations did not change in this subgroup post-HDT bed rest. Interestingly, these findings are in accord with the pre- to post-HDT bed rest changes reported in the whole-group and therefore to avoid repetition, the interpretation of the change in each individual biomarker can be found in section 6.3. Additionally and specific to the subgroup which became less insulin-sensitive after HDT bed rest, the increase in circulating RBP4 was statistically significant. Therefore, with the exception of FGF-21, the increases in fetuin-A and RBP4 and decreases in adiponectin,

adropin and irisin were expected given the observed decline in insulin sensitivity in this subgroup following HDT bed rest. Hence this panel of biomarkers may be useful to track changes in systemic and tissue-specific metabolic health in response to HDT bed rest. Apo-J, ASP and apelin did not change following HDT bed rest and it is possible that in a lean population, and in the absence of fat mass gain during HDT bed rest, the compensatory hypersecretion of these biomarkers observed in overweight populations is not increased. Thus, these biomarkers might not be appropriate to track changes in whole-body and peripheral insulin sensitivity in a lean population.

As further examination of circulating biomarker concentrations may help us to understand the individual metabolic response following HDT bed rest, we conducted correlation and multiple regression analysis using the significant pre- to post-changes in circulating biomarkers as dependent variables to ascertain the factors that contributed to the changes in biomarker secretion, in the subgroup with decreased insulin sensitivity post-HDT bed rest. Interestingly in this subgroup, the results of the pre- to post-HDT bed rest changes and correlation and multiple regression analysis for adiponectin and adropin are similar to those observed in the whole-group and therefore to avoid repetition, please refer to Section 6.3 for the interpretation of these findings. Below, the variables predicting the significant increases in RBP4 and fetuin-A and the significant decreases in irisin and FGF-21 in the subgroup with decreased insulin sensitivity following HDT bed rest are discussed.

The increase in RBP4 in individuals with decreased insulin sensitivity after HDT bed rest was accounted for by multiple factors including the decreases in serum adiponectin and trunk fat mass and increase in hepatic insulin resistance. This group of factors explained 69% of the increase in RBP4. Interestingly, the increase in hepatic insulin resistance made the strongest unique contribution to explaining the elevation in RBP4. Moreover, the increase in circulating RBP4 correlated strongly and positively with increases in $glucose_0$, $glucose_{30}$, $AUCG_{30}$, HOMA-IR and negatively with the decrease in QUICKI following HDT bed rest. These correlations (with fasting and/or 30-minute indices of insulin sensitivity/resistance) may reflect a stronger effect of RBP4 on hepatic insulin sensitivity,

due to the absence of associations with indices of peripheral or whole-body insulin sensitivity, possibly relating to the stimulatory effect of RBP4 on gluconeogenesis, as well as the positive association with liver fat in humans (Yang et al., 2005; Stefan et al., 2007). In line with this, the negative relationship between the increase in RBP4 and decrease in adiponectin is associated with increased gluconeogenesis in the liver, as well as decreased fatty acid oxidation and insulin signalling in skeletal muscle and adipose tissue (Esteve et al., 2009). As mentioned above, the decrease in trunk fat mass (-0.2kg) was minimal and not significant in individuals showing a decline in insulin sensitivity after HDT bed rest. While the increase in RBP4 was significantly and positively correlated with the increases in NEFA and adipose tissue insulin resistance, it is possible that the proposed role of RBP4 in upregulating lipolysis in adipose tissue was diminished in the absence of fat mass gain.

The single best predictor of the increase in fetuin-A in this subgroup was the decrease in irisin, which accounted for 25% of the variance in this circulating hepatokine post-HDT bed rest. As mentioned earlier in this discussion, it is possible that the association between irisin and fetuin-A is related to the impairment in peripheral glucose uptake in individuals with decreased insulin sensitivity following HDT bed rest, as irisin is known to augment insulin signalling whereas fetuin-A can attenuate insulin signalling. A combination of the increases in fetuin-A, LDL: HDL ratio and $insulin_{30}$ predicted 84% of the decrease in circulating irisin in individuals who became less insulin-sensitive following HDT bed rest. Of these three variables, LDL: HDL made the strongest unique contribution to explaining the decrease in irisin. Evidence of an inverse association between circulating irisin and total- and LDL-cholesterol has been emerging (Huh et al., 2012; Duran et al., 2015; Oelmann et al., 2016) and is proposed to be related to the ability of irisin to suppress hepatic cholesterol production *via* a mechanism dependent on AMPK and sterol regulatory element-binding protein 2 (SREBP2) signalling (Tang et al., 2016). Therefore, it is possible that the increase in the LDL: HDL ratio may reflect attenuated suppression of cholesterol synthesis in the liver as a consequence of reduced irisin levels after HDT bed rest. However, this mechanism requires further investigation. Based on the findings of the two regression models presented above, there appears to be a regulatory role between circulating irisin and circulating fetuin-A. This relationship needs to be

examined further but it is possible that there is a cyclical relationship between both of these biomarkers which could impact insulin signalling. Irisin has been shown to promote β-cell survival and glucose-stimulated insulin secretion (Arhire et al., 2019). The negative association between the decrease in irisin and increase in $insulin_{30}$ may indicate that these roles are sustained in spite of a decrease in the circulating concentrations of this myokine following HDT bed rest.

The decrease in irisin could further explain 27% of the decrease in FGF-21 in individuals with reduced insulin sensitivity after HDT bed rest. Both hormones have been previously reported to enhance insulin sensitivity by preserving β-cell function, promoting glucose uptake in peripheral tissues and improving hepatic glucose and lipid metabolism (Xie and Leung, 2020). Additionally, irisin and FGF-21 have been shown to work in concert to enhance adipocyte browning and thermogenesis in humans (Lee et al., 2014a). Given the association with irisin, the decrease in FGF-21 in response to HDT bed rest may represent reduced expression of FGF-21 in skeletal muscle, possibly due to the lack of muscle contraction, leading to the attenuation of its insulin-sensitising effects.

Investigation of the alterations in biomarker secretion from organs and tissues of metabolic importance in individuals with decreased insulin sensitivity following HDT bed rest has provided important insights to help us to further understand the etiology of insulin resistance in the context of extreme physical inactivity and high levels of sedentary time. Multidirectional interactions between active metabolic organs including the liver, adipose tissue and skeletal muscle maintains whole-body insulin sensitivity, whereas dysregulation of these lines of communication contributes to the pathophysiology of insulin resistance (Priest and Tontonoz, 2019). Alterations in the behaviour of skeletal muscle, adipose tissue and the liver and their ability to regulate metabolism *via* inter-organ crosstalk has a significant impact on overall physiological health. We propose that extreme inactivity and sedentary time leads to muscle atrophy and changes in the behaviour of adipose tissue and the liver, causing a significant increase in fetuin-A and RBP4 and decrease in adiponectin, irisin, adropin and FGF-21, that contributes to the reduction in insulin sensitivity in lean individuals following HDT bed rest. With the

exception of FGF-21, the increases in fetuin-A and RBP4 and decreases in adiponectin, irisin and adropin were anticipated given the observation of decreased insulin sensitivity in this subgroup after HDT bed rest. Thus, this panel of circulating biomarkers may be useful to assess changes in whole-body and tissue-specific insulin sensitivity in lean individuals in response to HDT bed rest. Apo-J, apelin and ASP did not respond to decrements in insulin sensitivity with compensatory hypersecretion, suggesting that obesity may be a key determinant of elevations in these biomarkers in metabolic dysfunction. Therefore these biomarkers may not be appropriate to track changes in systemic metabolism in a lean population.

6.4.2. Subgroup with Increased Insulin Sensitivity

In individuals who became more insulin-sensitive after HDT bed rest, there was a significant reduction in body weight, lean mass, trunk total mass, trunk lean mass, leg total mass, leg lean mass, and muscle strength during plantarflexion. Fat mass, bone mineral content, trunk fat mass, leg fat mass, arm total mass, arm lean mass, arm fat mass did not change. Additionally, measurements of $\dot{V}O_{2peak}$ and muscle strength during knee extension, knee flexion and dorsiflexion did not change in this subgroup following HDT bed rest. HDL decreased significantly in individuals exhibiting an improvement in insulin sensitivity post-HDT bed rest, while circulating NEFA, triglycerides, total cholesterol and LDL did not change. There was a significant decrease in $insulin_{30}$ and 30-minute AUC values for glucose and insulin in this subgroup after HDT bed rest. However, glucose and insulin concentrations at fasting or 30, 60, 90 or 120 minutes after the ingestion of an oral glucose load did not change. In addition to the significant increase in whole-body insulin sensitivity estimated using the Matsuda index, there was a significant increase in QUICKI and liver insulin sensitivity and a significant decrease in HOMA-IR and hepatic insulin resistance following HDT bed rest. HOMA-β, insulinogenic index, disposition index, OGIS index, Gutt index, muscle insulin sensitivity and adipose tissue insulin resistance did not change. Taken together, the significant decreases in $insulin_{30}$, $AUCG_{30}$, $AUCI_{30}$, HOMA-IR and hepatic insulin resistance and significant increases in QUICKI and liver insulin sensitivity, are reflective of an increase in hepatic insulin sensitivity in this subgroup following HDT bed rest. Therefore, the improvement in whole-body insulin sensitivity may be an indirect effect of liver metabolism.

We acknowledge that the sample size in the subgroup with improved insulin sensitivity is small (n = 6, n = 4 CTRL and n = 2 JUMP), however, the observation that whole-body and liver insulin sensitivity improved in a subgroup of bed rest subjects is intriguing and supports the current emphasis on personalised medicine approaches to disease treatment. To determine the factors that may have contributed to the increase in insulin sensitivity in this subgroup post-HDT bed rest, we performed correlation and multiple regression analysis using the pre- to post-HDT changes in estimates of insulin sensitivity and insulin resistance as dependent variables. There are inherent challenges with statistical analysis using low sample sizes, however, it is important to investigate the possible mediators and alterations in metabolic physiology that contributed to the improvement in insulin sensitivity in this subgroup following HDT bed rest.

The best predictor of the decrease in HOMA-IR was the decrease in circulating apelin, which accounted for 80% of the variance in this subgroup following HDT bed rest. Additionally, the decrease in circulating apelin explained, albeit not significantly, 66% of the increase in liver insulin sensitivity after HDT bed rest. The increases in QUICKI and Matsuda index were also negatively, but not significantly, associated with the decrease in circulating apelin after HDT bed rest. Apelin, secreted predominately by adipocytes, enhances glucose utilisation and insulin sensitivity (Antushevich and Wojcik, 2018). Fluctuations in circulating apelin are attributed to changes in multiple interrelated factors including insulin, insulin resistance and inflammatory cytokines (Hamza et al., 2021). Reductions in circulating apelin are associated with an improvement in insulin sensitivity (Bertrand et al., 2015). Skeletal muscle and adipose tissue are the principal targets of apelin action; however, there is evidence to suggest that apelin can ameliorate the TNF-α-induced decrease in glycogen synthesis and inhibit lipid accumulation in the liver. Chu et al. (2013) reported that apelin reversed TNF-α-induced reduction of glycogen synthesis in HepG2 cells, mouse primary hepatocytes and liver tissue of C57BL/6J mice, by inhibiting reactive oxygen species (ROS) generation and improving the insulin signalling (JNK-IRS1-AKT-GSK) pathway. These actions were mediated through the APJ receptor, the only known receptor of apelin, as apelin is not expressed in the liver (Chu et al., 2013). More recently, Huang et al. (2017) provided evidence that apelin-APJ signalling can

protect against lipid accumulation in human and mouse hepatocytes. This anti-steatotic function is mediated through two signalling pathways involving AMPK activation and PPAR-α induction (Huang et al., 2017). Therefore, the improvement in liver and whole-body insulin sensitivity may be related to the anti-insulin resistance properties of apelin in the liver, skeletal muscle and adipose tissue. In this view, apelin may be a candidate biomarker for examining changes in whole-body and tissue-specific metabolic health in response to HDT bed rest or lifestyle interventions.

The increase in the Matsuda index was significantly and negatively associated with decrease in total cholesterol post-HDT bed rest. This finding corroborates previous literature reporting that insulin sensitivity is inversely associated with cholesterol synthesis, independent of obesity, such that cholesterol synthesis is lower and cholesterol absorption is higher in individuals with normoglycaemia (Gylling et al., 2010). As the liver is the main site of cholesterol biosynthesis (Luo et al., 2020), this finding further infers that the improvement in insulin sensitivity in this subgroup may be an indirect effect of liver metabolism. The reduction in hepatic insulin resistance was almost exclusively (98%), explained by a combination of the decrease in leg fat mass and decrease in circulating irisin. Adipose tissue in the lower body is proposed to exert protective effects on insulin sensitivity (Goossens, 2017). As the decrease in leg fat mass was minimal (-0.2kg) it is possible that the metabolic phenotype of this tissue was sustained following HDT bed rest. Furthermore, as the subjects in this study were lean healthy males, it is possible that irisin did not change in individuals with improved insulin sensitivity due to the absence of obesity and therefore, the positive metabolic effects of irisin in controlling glucose and lipid metabolism in the liver, skeletal muscle and adipose tissue were sustained.

As explored in the literature review, circulating concentrations of adiponectin, adropin and irisin are elevated concomitant with improvements in insulin sensitivity. On the other hand, circulating concentrations of fetuin-A, RBP4, apo-J, ASP, apelin and FGF-21 are reduced when insulin sensitivity is increased. The reductions in apo-J, ASP, apelin and

FGF-21 are indicative of enhanced biomarker sensitivity, thus requiring lower concentrations of these biomarkers to maintain normal physiological function.

We observed a significant decrease in circulating adiponectin and FGF-21 in subjects with improved insulin sensitivity after HDT bed rest. Contrastingly, fetuin-A, RBP4, irisin, apo-J, adropin, apelin and ASP did not change in this subgroup following HDT bed rest. The observation that fetuin-A, RBP4, irisin and adropin did not change is interesting as these four organokines changed unfavourably in response to a decrease in insulin sensitivity after HDT bed rest. As fetuin-A and RBP4 impair insulin signalling and irisin and adropin improve insulin signalling, it is possible that the improvement in insulin sensitivity may be related to the maintenance of normal insulin signalling in peripheral tissues. The reduction in FGF-21 is indicative of improved insulin sensitivity and therefore FGF-21 may be a useful biomarker to track individual responses in metabolic physiology due to inactivity or lifestyle interventions. To determine the factors that contributed to alterations in biomarker secretion in individuals showing an improvement in whole-body and liver insulin sensitivity after HDT bed rest, we conducted correlation and regression analysis with the pre- to post-HDT bed rest changes in circulating biomarkers as the dependent variables. We acknowledge the limitation and difficulties associated with a small sample size, however, unravelling the complex physiology and relations between organokines may enhance our understanding of the variation in insulin sensitivity following HDT bed rest.

There was a significant positive association between the reductions in FGF-21 and $AUCG_{30}$ in this subgroup after HDT bed rest. The liver is a major site of the production and actions of FGF-21. FGF-21 mediates the metabolic adaptation to fasting and increases fatty acid oxidation, ketogenesis and gluconeogenesis in the liver (Owen et al., 2015). Additionally, in adipose tissue, FGF-21 increases glucose uptake, enhances adiponectin production, modulates lipolysis according to the fasting or fed state, and regulates adipocyte browning and thermogenesis (Holland et al., 2013; Dolegowska et al., 2019). The observation of a positive association between reductions in FGF-21 and $AUCG_{30}$ may reflect improved regulation of glucose homeostasis in the liver through

improvements in hepatic insulin sensitivity in this subgroup after HDT bed rest. This is supported by previous experimental evidence showing that FGF-21 treatment *in vivo* and chronic administration of FGF-21 to obese and diabetic mice improves glucose metabolism and insulin sensitivity in the liver (Berglund et al., 2009; Liu et al., 2018).

A combination of the increases in TG: HDL ratio and insulin concentrations one-hour after the oral glucose load predicted 97% of the decrease in circulating adiponectin in individuals with improved insulin sensitivity after HDT bed rest. These findings are unexpected as adiponectin is proposed to increase, whereas the TG: HDL ratio and one-hour insulin concentrations are expected to decrease, as insulin sensitivity improves. Instead, it appears that the reduction in circulating adiponectin may reflect a homeostatic readjustment to disturbances in glucose and lipid metabolism in response to HDT bed rest (Trim et al., 2021). Further research is needed to explore changes in circulating adiponectin and individual metabolic response, to determine if the anti-atherosclerotic and insulin-sensitising effects of adiponectin contribute to the improvement in insulin sensitivity and to determine if this biomarker can be used to track individual changes in metabolic homeostasis.

In summary, our analysis identified six subjects (26%) that increased insulin sensitivity following HDT bed rest. One possibility is that the improvement in whole-body insulin sensitivity may be an indirect effect of liver metabolism. The amount of intra-hepatic lipid (IHL) is strongly linked to liver and whole-body insulin resistance (Mu et al., 2018; Trouwborst et al., 2018). Unfortunately, no measurements of liver fat were obtained as part of this study impeding our ability to explore the association between insulin sensitivity and hepatic fat accumulation in response to HDT bed rest. Despite this, we propose that apelin and FGF-21 play a role in the improvement of insulin sensitivity in this subgroup and identify that these biomarkers may be useful to track changes in metabolic physiology in response to intervention including HDT bed rest. It is possible that the small sample size in this subgroup is too small for definite conclusions, and therefore, we suggest that further studies are required to investigate the specific alterations

in systemic metabolism, with particular focus on inter-organ crosstalk, that contributed to the improvement insulin sensitivity observed in these subjects after HDT bed rest.

To conclude, investigating changes in metabolic physiology that occurred in a subgroup with decreased insulin sensitivity following HDT bed rest and a subgroup with increased insulin sensitivity following HDT bed rest has provided some important insights into individual responses in insulin sensitivity following prolonged inactivity and high levels of sedentary time (Figure 31). In the subgroup with decreased insulin sensitivity after HDT bed rest, there was evidence of blunted insulin action, reflected by significant increases in fasting insulin, glucose and triglycerides, as well as a significant decrease in HDL, and a considerable impairment in peripheral glucose uptake. Conversely, in the subgroup with increased insulin sensitivity after HDT bed rest, there was a significant improvement in liver insulin sensitivity, implying that the improvement in whole-body insulin sensitivity may be an indirect effect of liver metabolism. In consideration of all of the analysis, we propose that fetuin-A, RBP4, irisin and adropin may represent the best set of circulating biomarkers to provide insights into whole-body and tissue-specific insulin sensitivity and to track changes in physiological responsiveness to intervention in a lean population. Additionally, if confirmed in a larger study, apelin may also be a candidate biomarker for examining changes in systemic and tissue metabolism in response to intervention.

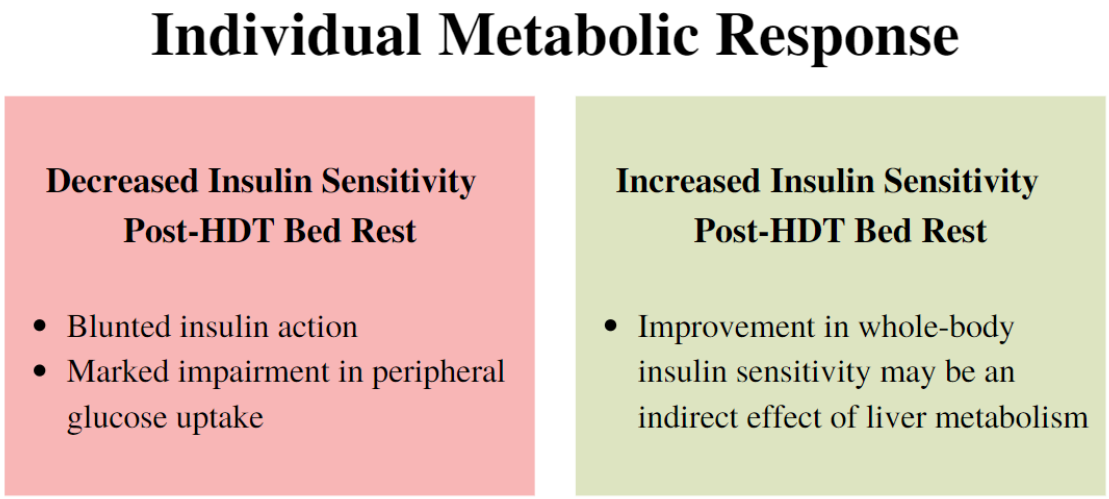

Figure 31. Key findings of the subgroup analysis investigating individual metabolic response.

Chapter 7. Thesis Summary, Conclusions and Future Recommendations

7.1. Main Findings

In the present study, we demonstrate that 60 days HDT bed rest impaired insulin action, reflected by a significant increase in fasting insulin, triglycerides and LDL and a significant decrease in HDL, and induced a pronounced reduction in peripheral glucose uptake, evidenced by higher glycaemia one- and two-hours after the glucose load, irrespective of group. Inter-organ communication plays a key role in regulating metabolic homeostasis. Secreted proteins from the liver, adipose tissue and skeletal muscle are major crosstalk molecules regulating tissue-specific insulin sensitivity (Romero and Eckel, 2021). Physical inactivity and high levels of sedentary time is known to suppress the secretion of health enhancing organokines and promote the secretion of organokines that contribute to development of metabolic disease. In line with this, we report a significant increase in circulating fetuin-A and a significant decrease in circulating adropin, irisin, adiponectin and FGF-21 following 60 days of HDT bed rest, irrespective of group. Despite factors that influence biomarker secretion changing in response to HDT bed rest (e.g. reduction of muscle mass, $\dot{V}O_{2peak}$ and insulin sensitivity), circulating RBP4, apo-J, ASP and apelin did not change in all subjects after 60 days of HDT bed rest. Overall, multiple regression analysis identified that muscle atrophy, increased circulating lipids and alterations in the behaviour of tissues of metabolic importance contributed the metabolic dysregulation evident in all subjects following HDT bed rest. Additionally, while reactive jump training had protective effects for muscle function, it could not prevent the dysregulation of glucose and lipid metabolism. It is possible that that the overall workload, consisting of 48 jumps and 30 hops per session, was not sufficient to improve insulin sensitivity or induce favourable changes in these circulating biomarkers.

The principal findings in the subgroup with decreased insulin sensitivity after HDT bed rest show that prolonged inactivity and extreme levels of sedentary time impaired insulin action, indicated by a significant increase in fasting insulin, glucose and triglycerides and a significant decrease in HDL, and induced a significant impairment in peripheral glucose uptake, evidenced by higher glycaemia in the last hour of the OGTT in individuals who

became less insulin-sensitive following HDT bed rest. Muscle atrophy, decrements in muscle strength, increased circulating lipids and alterations in the behaviour of active metabolic organs contributed to the deterioration in insulin sensitivity noticeable in this subgroup following HDT bed rest. In subjects with decreased insulin sensitivity post-HDT bed rest, circulating concentrations of RBP4 and fetuin-A increased significantly and adiponectin, adropin, irisin and FGF-21 decreased significantly. Overall, muscle atrophy and alterations in inter-organ communication contributed to changes in circulating biomarker concentrations and consequent metabolic dysregulation in subjects who became less insulin-sensitive after HDT bed rest Associations between RBP4 and fasting and/or 30-minute indices of insulin sensitivity/resistance (e.g. fasting glucose, glucose and AUC values for glucose 30 minutes after the glucose load, HOMA-IR, QUICKI and hepatic insulin resistance) may suggest a stronger effect of RBP4 on hepatic insulin sensitivity, due to the absence of associations with indices of peripheral or whole-body insulin sensitivity, possibly relating to the stimulatory effect of RBP4 on gluconeogenesis, as well as the positive association with liver fat in humans. To our knowledge, this is the first time that RBP4, fetuin-A, irisin, adropin, apo-J, ASP and apelin have been studied under conditions of HDT bed rest. Excluding FGF-21, the increases in fetuin-A and RBP4 and decreases in adiponectin, irisin and adropin were expected given the observed decrease insulin sensitivity in this subgroup after HDT bed rest. Thus, this panel of circulating biomarkers may be useful to examine changes in whole-body and tissue-specific insulin sensitivity in lean individuals in response to HDT bed rest. Apo-J, apelin and ASP did not respond to the decline in insulin sensitivity with compensatory hypersecretion, suggesting that obesity may be a key factor in the upregulation of these biomarkers in metabolic dysfunction. Thus, these biomarkers may not be appropriate to track changes in systemic metabolism in a lean population.

In the subgroup with increased insulin sensitivity following HDT bed rest, there was evidence of a substantial improvement in liver insulin sensitivity, indicated by significant decreases in insulin concentrations 30 minutes after the glucose load, 30-minute AUC values for glucose and insulin, HOMA-IR and hepatic insulin resistance and a significant increase in Matsuda index, QUICKI and liver insulin sensitivity. Therefore, we propose that the improvement in whole-body insulin sensitivity in this subgroup may be an

indirect effect of liver metabolism. We acknowledge that the sample size in the subgroup with improved insulin sensitivity is small (n = 6, n = 4 CTRL and n = 2 JUMP), however, the observation that whole-body and liver insulin sensitivity improved in a subgroup following HDT bed rest is interesting and supports the current emphasis on personalised medicine approaches to disease treatment. The amount of intra-hepatic lipid (IHL) is strongly linked to liver and whole-body insulin resistance (Mu et al., 2018; Trouwborst et al., 2018). Unfortunately, we did not obtain measurements of liver fat as part of this HDT bed rest study and therefore we cannot explore the association between insulin sensitivity and fat accumulation in the liver in response to prolonged inactivity and high levels of sedentary time. Despite this, we propose that the insulin-sensitising effects of apelin in the liver, skeletal muscle and adipose tissue may be related to the improvement in insulin sensitivity observed in this subgroup following HDT bed rest. The observation that fetuin-A, RBP4, irisin and adropin did not change in the subgroup with improved insulin sensitivity is noteworthy because these organokines changed unfavourably in response to a decline in insulin sensitivity after HDT bed rest. As fetuin-A and RBP4 impair insulin signalling and irisin and adropin improve insulin signalling, it is possible that the improvement in insulin sensitivity may be related to the maintenance of normal insulin signalling in peripheral tissues.

In conclusion, our subanalysis has provided important insights to help us to further understand the etiology of insulin resistance in the context of physical inactivity and high levels of sedentary time. It has allowed us to profile the physical and metabolic characteristics of subjects who decreased insulin sensitivity post-HDT bed rest and subjects who increased insulin sensitivity post-HDT bed rest and determine the specific changes in metabolic physiology which contributed to the deterioration or improvement in insulin sensitivity following HDT bed rest. Furthermore, when the findings of both subgroups are considered, we propose that fetuin-A, RBP4, irisin and adropin may represent the best set of circulating biomarkers to provide insights into whole-body and tissue-specific insulin sensitivity and to track changes in physiological responsiveness to intervention in a lean population. Additionally, if confirmed in a larger study, apelin may also be a candidate biomarker for examining changes in systemic and tissue metabolism in response to intervention.

7.2. Practical Applications

Firstly, the results of this PhD highlight a clear potential for the convergence of knowledge of the physiological adaptations of all situations of gravity deprivation (from microgravity to the reduced influence of gravity as a result of decreased movement and acceleration or the reduced use of gravity over decades as function of age). Spaceflight, physical inactivity, sedentary behaviour, immobilisation induced by COVID-19, illness or injury, and the limited mobility of ageing induce similar physiological adaptations, acting through common pathways and mechanism (Vernikos, 2017; Vernikos, 2021). These situations induce an atrophic condition which is not merely visually evident as musculoskeletal loss but also metabolically, morphologically and functionally, and only differs by the rate at which it is induced. There is strong evidence that the direction of physiological changes is overwhelming similar in all aforementioned conditions, including substantial reductions in muscle mass, strength and function, bone mass, neurovestibular function, cardiorespiratory fitness and insulin sensitivity, among others (Le Roux et al., 2021; Vernikos, 2021). Overall, the HDT bed rest model can be used to provide an understanding of the kinetics and magnitude of physiological adaptation.

Secondly, the findings of this PhD show that low volume, high-intensity reactive jump training elicited protective effects on muscle mass and function, but could not prevent the dysregulation of glucose and lipid metabolism. While future research is needed to optimise the exercise prescription of reactive jump training, this type of plyometric training would be highly compliant with current ISS operations, as the total training time (~17.5 of MVPA per week without breaks) is sufficiently lower than the current allocated training time of two and a half hours per day. This would be of particular advantage in future human exploration missions with restrictions on exercise time and minimal ground communication (Gruber et al., 2019). In addition to spaceflight, this type of high-intensity plyometric training, with short rest intervals, has the potential improve physiological health and function on Earth. From a practical perspective, it is unlikely that the sledge jump system is applicable for deconditioned and ageing populations, however, the training principles underpinning reactive jump training are important and may result in a pronounced decrease in sarcopenia and frailty and preservation of cardiometabolic health.

Thirdly, the findings of this PhD highlight that, while it is not normally a prime consideration during study design, the analysis of individual variability in physiological and metabolic responses in HDT bed rest has important and practical implications in many areas. For space agencies, the examination of inter-individual variability in physiological and metabolic responses may help to determine which astronauts are more resilient to microgravity exposure when compared to others (e.g. individuals who exhibit less muscle atrophy) and this may subsequently guide selection. For public health agencies, the examination of inter-individual variability has important implications for the health management and personalised exercise training recommendations of physical inactive, sedentary and ageing populations, or those who are immobilised due to hospitalisation (e.g. COVID-19), illness or injury. In line with this, the findings of this PhD show that the quantification of circulating hepatokines, adipokines and myokines is a relatively straight-forward and minimally-invasive means of exploring and understanding the complexity of metabolic crosstalk and insulin resistance, which is an eminent consequence of conditions of gravity deprivation. Furthermore, the formulation of a biomarker panel is likely to confer more power in terms of identifying the possible mediators of the variation of insulin sensitivity in these conditions. Further work is required to establish whether fetuin-A, RBP4, irisin and adropin represent the best set of circulating biomarkers to provide insights into whole-body and tissue-specific insulin sensitivity and to track changes in physiological responsiveness to intervention in different populations and under different conditions of gravity deprivation, however, they may assist in refining exercise prescription to maintain optimal health in microgravity, physical inactivity, sedentary behaviour, immobilisation and ageing.

7.3. Limitations

Despite the highly controlled nature of this HDT bed rest study, several limitations have to be acknowledged. Firstly, we acknowledge that the subgroup analysis investigating individual metabolic response post-HDT bed rest was conducted *a posteriori* and therefore, a power analysis was not conducted. In line with this, we understand that the quantification of individual response heterogeneity to intervention is dependent on sufficient sample size, inclusion of an ambulatory control group and appropriate statistical analysis (Fernandez-Gonzalo et al., 2021; Böcker et al., 2022) and thus, in light of the

study design being pre-determined, this analysis was beyond the scope of this PhD. Secondly, although it was not the objective of this study, we did not obtain any measurements of liver fat precluding our ability to fully explore the association between insulin sensitivity and hepatic lipid accumulation after HDT bed rest; this requires further investigation. Thirdly, an OGTT was used to estimate insulin sensitivity prior to and following HDT bed rest. While a euglycaemic-clamp is the gold standard measure, it was not possible in the current study. However, the OGTT sufficiently reflects changes in glucose tolerance and the measurement of whole-body insulin sensitivity using the OGTT has been validated previously in healthy non-overweight adults (Trikudanathan et al., 2013). Fourthly, in spite of examining the literature on the normal concentration ranges of all biomarkers in lean healthy subjects, consulting technical support and performing ELISA analysis with different dilution factors, pre- to post-HDT bed rest changes in circulating biomarkers could not be determined in some subjects (i.e. ASP, apelin). However, we have presented and analysed all the available data. Finally, the results of this study have been obtained from healthy lean adult males and similar investigations will need to be extended to other populations (e.g. women, elderly, metabolically unhealthy) to determine the specific links with disease etiology.

7.4. Future Recommendations

Disruption of protein production and target-tissue action underpin the development of metabolic dysfunction including insulin resistance. The quantitative measurement of circulating biomarkers is a relatively easy and minimally-invasive means of identifying and understanding the etiology of insulin resistance. The results of our subgroup analysis propose that the improvement in whole-body insulin sensitivity is an indirect effect of liver metabolism, while the deterioration of whole-body insulin sensitivity in the opposing subgroup is associated with impairments in the liver and skeletal muscle after HDT bed rest. Therefore, the liver remains an important area to investigate. As no direct measurements of liver fat have been performed in humans for ethical reasons, it is imperative that future studies investigate the effect of prolonged inactivity and high levels of sedentary time on additional circulating hepatokines to determine how changes in liver metabolism affect other organ systems and overall systemic metabolic homeostasis. This may help to further optimise a “best set” of circulating biomarkers which can provide

insights into tissue-specific and whole-body insulin sensitivity and track changes in physiological responsiveness to intervention. In line with this, it is acknowledged that changes in skeletal muscle mass and adipose tissue affect bone structure and function and this may impact the secretion and circulating concentrations of osteokines which can modulate insulin sensitivity. Therefore, future studies should investigate the effect of HDT bed rest on changes in circulating osteokines, bone mass, bone strength and the influence of these on whole-body metabolic homeostasis. Additionally, future researchers should perform repeated sampling, through the collection of fasting blood samples, throughout HDT bed rest to track changes in the circulating concentrations of biomarkers over time. This type of investigation may offer novel perspectives on the short-term, medium-term or long-term biomarker response and assist in the interpretation of changes in insulin sensitivity over time in HDT bed rest. This analysis could provide important personalised information for the optimisation of interventions to maintain health.

To our knowledge, this is the first study to report individual responses in insulin sensitivity following HDT bed rest. However, the development of insulin resistance is an eminent consequence of extreme inactivity and high levels of sedentary time. Thus, it would be interesting to determine whether individual variability in insulin sensitivity is a common phenomenon among HDT bed rest studies. The quantification of individual response heterogeneity to intervention is multifaceted and dependent on good trial design supported by suitable statistical analysis and sufficient sample size. HDT bed rest provides a highly controlled experimental testbed for the examination of inter-individual metabolic response to physical inactivity and extreme sedentarism. Additionally, in HDT bed rest studies conducted by the ESA, subjects are maintained in energy balance so fat accumulation is not a confounding factor on physiological or metabolic outcomes. Standardisation of HDT bed rest conditions between studies means that, in principle, results of different studies, in particular the control (i.e. bed rest only) groups could be compared and potentially combined (Scott et al., 2021). However, there is an undisputable necessity for the inclusion of an ambulatory control group in future studies (i.e. a “free-living control” group which undergo the same testing procedures) to determine whether bed rest only or bed rest with an intervention modulated the individual variation in physiological response. Compiled data and appropriate statistical modelling akin to the

most recent bed rest publications (Fernandez-Gonzalo et al., 2021; Böcker et al., 2022), is pivotal for exploring the mechanisms that influence individual responsiveness and guide personalised countermeasure prescription. This research would further our understanding of the pathogenesis of insulin resistance and offer new tools to ameliorate the physiological adaptations to physical inactivity, extreme sedentarism and spaceflight. The recommendation for future studies to include an ambulatory control group(s) may provide an opportunity to delineate and further explore the independent effects of physical inactivity and sedentary behaviour on health outcomes. Additionally, it is apparent that confinement and social isolation can markedly alter human physiology (Pagel and Choukèr, 2016), but the independent effect of these stressors on physiological adaptations to HDT bed rest needs to be further investigated and one viable opportunity is to use an ambulatory control group (e.g. to control for the influence of confinement, an ambulatory confinement group could be examined). Such proposed investigations would need to be tightly controlled.

Establishing an exercise protocol that conserves time but mitigates the deleterious physiological adaptations associated with microgravity and gravitational deprivation is of pivotal importance for life in space and life on Earth. While reactive jump training in the present study had protective effects on muscle mass and function, it could not prevent the dysregulation of glucose and lipid metabolism following 60 days HDT bed rest. Future research is required to investigate the efficacy of both a greater intensity and volume of reactive jump training or an exercise prescription of reactive jump training superimposed with vibration in mitigating the deleterious changes in physiology and metabolism known to occur after HDT bed rest. While it unlikely that the sledge jump system is applicable for the ageing population, from a practical perspective, the training principles underpinning this type of training may be useful and applicable in this population for preserving muscle mass, strength and function, bone mass and cardiorespiratory fitness. This has important implications for physiological health, functional independence, morbidity and mortality in this population.

Finally, and in agreement with Ekman et al., (2022), reconditioning and rehabilitation protocols following HDT bed rest have received very little attention and the specifics of these are not well reported within the literature. Astronauts are provided with a comprehensive and individualised exercise programme following spaceflight to optimise recovery. In the same way, defining an optimal recovery programme after HDT bed rest is warranted. Investigation into the recovery dynamics of the different physiological systems using either controlled but generic, or controlled and individualised, recovery programmes is of distinct interest and would help to determine the time and resources required for recovery. Additionally, this work could provide novel approaches that have practical applications in primary care to promote recovery in individuals who have been immobilised due to COVID-19, illness or injury.

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Appendices

Appendix I: Post-HDT Bed Rest Testing Schedule

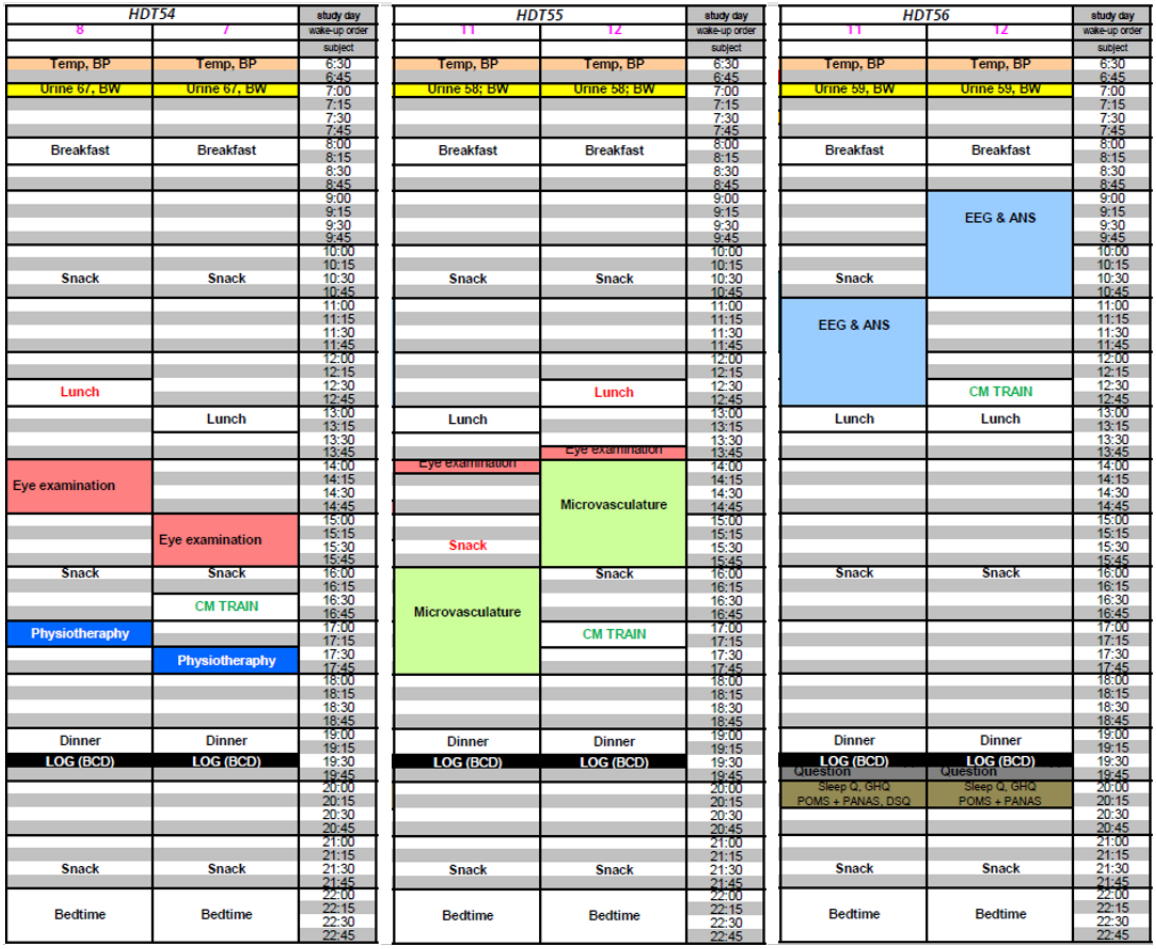


Figure 32. Testing schedule for two subjects on HDT54, HDT55 and HDT56.

For each study day, the column on the left represents the schedule for a subject in the CTRL group and the column on the right represents a schedule for a subject in the JUMP group. Abbreviations: ANS, Autonomic Nervous System; BCD, basic core data; BP, blood pressure; BW, body weight; CM, countermeasure, DSQ, device specific questionnaire; EEG, electroencephalogram; GHQ, general health questionnaire; HDT, head-down-tilt bed rest; LOG, log of critical incidents; PANAS, positive and negative affect schedule; POMS, profile of mood states; Temp, temperature.

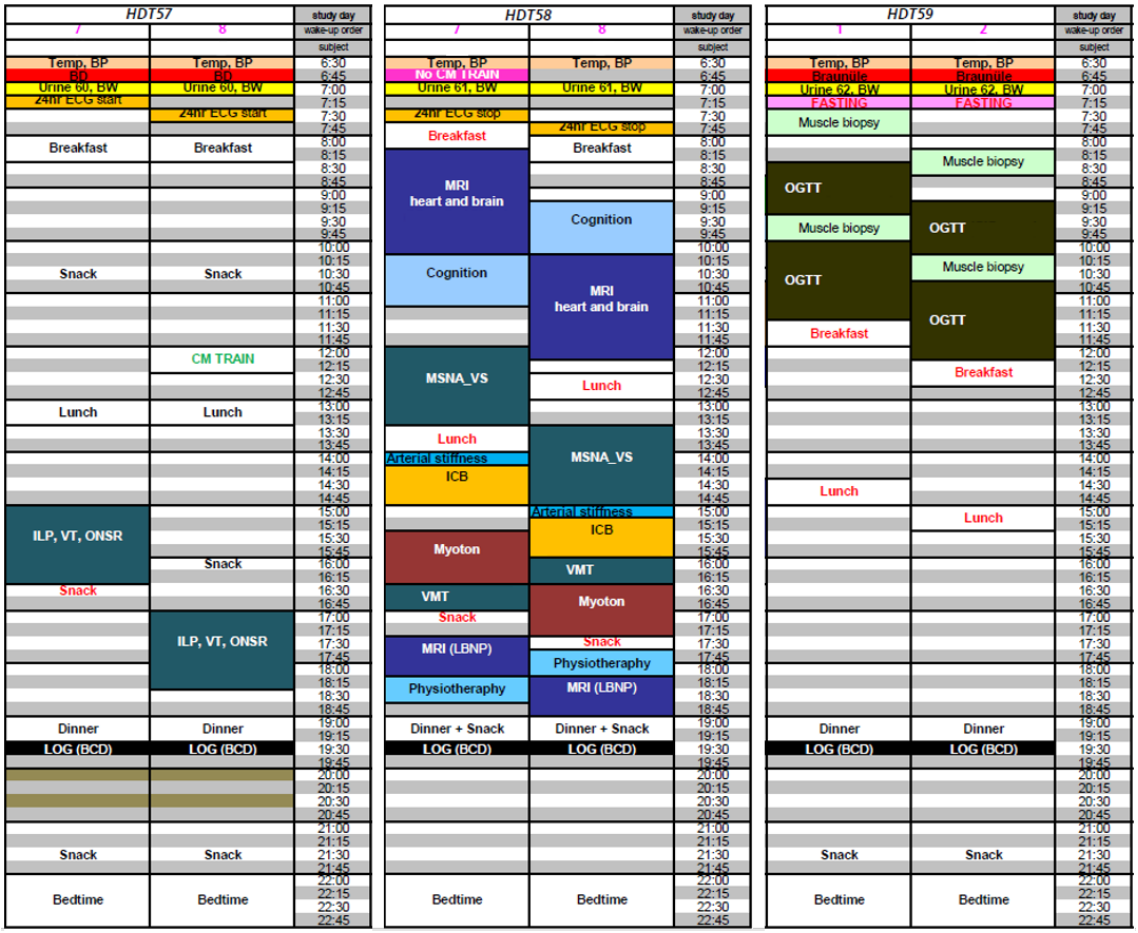


Figure 33. Testing schedule for two subjects on HDT57, HDT58 and HDT59.

For each study day, the column on the left represents the schedule for a subject in the CTRL group and the column on the right represents a schedule for a subject in the JUMP group. Abbreviations: BCD, basic core data; BD, blood draw; BP, blood pressure; BW, body weight; CM, countermeasure; ECG, electrocardiogram; HDT, head-down-tilt; ICB, imposed and controlled breathing; ILP, intra-labyrinthine pressure; LBNP, lower body negative pressure; LOG, log of critical incidents; MRI, magnetic resonance imagery; MSNA, muscle sympathetic nerve activity; OGTT, oral glucose tolerance test; Temp, temperature; VMT, visual manual testing; VS, vestibular stimulation; VT, vestibular testing.

Figure 34. Testing schedule for two subjects on HDT60, R+0 and R+1.

For each study day, the column on the left represents the schedule for a subject in the CTRL group and the column on the right represents a schedule for a subject in the JUMP group. Abbreviations: Baro, baroreflex evaluation; BCD, basic core data; BD, blood draw; BP, blood pressure; cVT, comprehensive vestibular testing; DEXA, dual-energy x-ray absorptiometry; DGI, dynamic gait index; ECG, electrocardiogram; HDT, head-down-tilt; ICB, imposed and controlled breathing; ILP, intra-labyrinthine pressure; LBNP, lower body negative pressure; LOG, log of critical incidents; MVC, maximal voluntary contraction; PG, posturography; pQCT, peripheral quantitative computed tomography; R, recovery; Temp, temperature; VMT, visual manual testing.

Appendix II: Insulin Equation Confirmation

A. Hepatic Insulin Resistance Equation

Question to Author: When calculating "Glucose0-30(AUC)", is it correct to use the AUCG formula i.e. (0.5*(Glucose0+Glucose30)/30) or should I add the concentrations of glucose at 0 and 30 time points together and multiply them against the same for the insulin? Can I also ask the units of measurements that you use for this equation? Is it mmol/L for glucose and pmol/L for insulin?

Answer from Author, Muhammad Abdul-Ghani, MD, Ph.D.: The formula is calculated with the total area under the plasma insulin and plasma glucose concentrations between 0-30 minutes (not the incremental). ((Glucose 0 + Glucose 30)/2) X 0.5) and similar formula for insulin and multiply the two numbers. We multiplied by 0.5 (half an hour) because we calculated them per hour, you also can multiply by 30 (calculate the area by minute), it is equally good. You should not worry about the absolute number. This highly depends on the insulin assay you have and the units you use for insulin and glucose measurement (uU/ml, pmol/l, mg/dl, mmol), as long as you use the same units before and after treatment, it should not make any difference, because you are comparing the change in the index before versus after treatment.

B. Matsuda Index Equation

Masafumi Matsuda, MD, Ph.D., Professor and Department of Endocrinology and Diabetes at Saitama Medical University confirmed that the formula to calculate the "Mastuda Index" of insulin sensitivity uses the trapezoid rule.

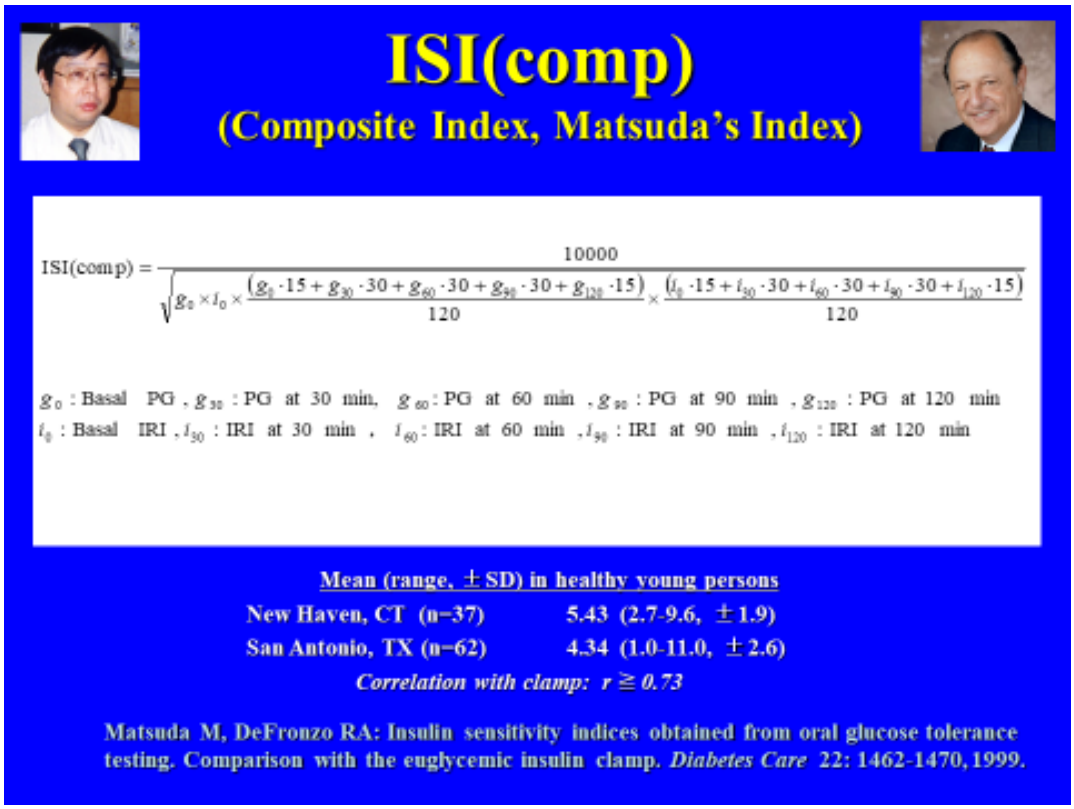


Figure 35. Confirmation of Matsuda Index Equation

Appendix III: Example of Manufacturer Instructions for the Quantification of Fetuin-A

Quantikine® ELISA

Human Fetuin A Immunoassay

Catalog Number DFTA00

For the quantitative determination of human Fetuin A concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Fetuin A Microplate	893822	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Fetuin A.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Fetuin A Standard	893824	Recombinant human Fetuin A in a buffered protein base with preservative; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store at ≤ -20 °C for up to 1 month. Avoid repeated freeze-thaw cycles.
Human Fetuin A Conjugate	893823	21 mL of a monoclonal antibody specific for human Fetuin A conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1S	895137	11 mL of a buffered protein base with preservative. *For cell culture supernate samples.*	
Assay Diluent RD1X	895121	11 mL of a buffered protein base with preservative. *For serum/plasma samples. May contain crystals. Warm to room temperature to dissolve.*	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Used diluted 1:10 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 50 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.
- Human Fetuin A Controls (optional; available from R&D Systems).

PRECAUTIONS

Fetuin A is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 4000-fold dilution. A suggested 4000-fold dilution can be achieved by adding 10 µL of sample to 990 µL of Calibrator Diluent RD5-26 (diluted 1:10).* Complete the 4000-fold dilution by adding 25 µL of the diluted sample to 975 µL Calibrator Diluent RD5-26 (diluted 1:10).

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of Fetuin A are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

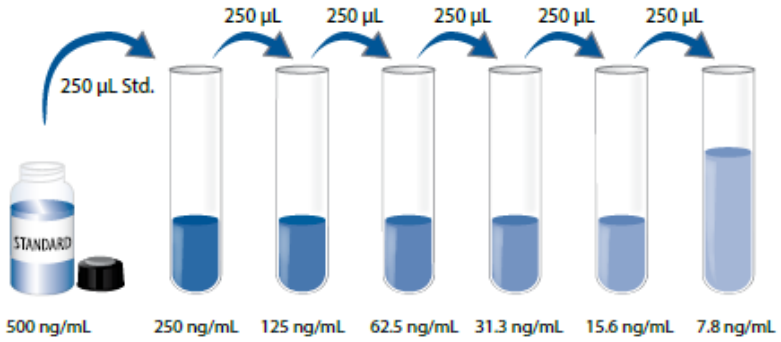
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (diluted 1:10) - Add 5 mL of Calibrator Diluent RD5-26 Concentrate into 45 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5-26 (diluted 1:10).

Human Fetuin A Standard - Refer to the vial label for recontitution volume. Reconstitute the Human Fetuin A Standard with Calibrator Diluent RD5-26 (diluted 1:10). This reconstitution produces a stock solution of 500 ng/mL. Allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 250 µL of Calibrator Diluent RD5-26 (diluted 1:10) into six tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human Fetuin A Standard (500 ng/mL) serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:10) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

Note: *High concentrations of Fetuin A are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. **For Culture Supernate Samples:** Add 100 µL of Assay Diluent RD1S to each well.
   For Serum/Plasma Samples: Add 100 µL of Assay Diluent RD1X to each well. *May contain crystals. Warm to room temperature to dissolve.*
4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human Fetuin A Conjugate to each well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

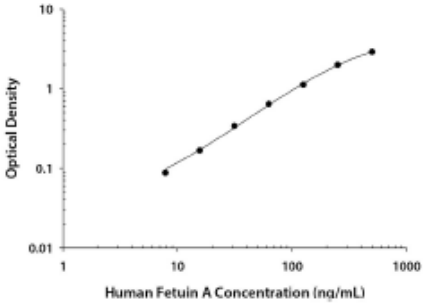
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human Fetuin A concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

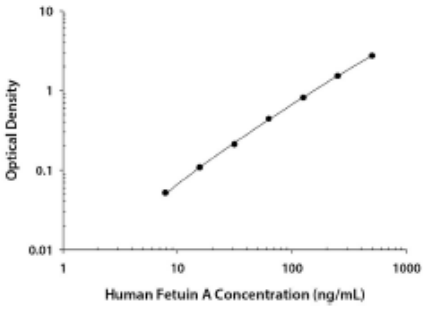
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.026 0.026	0.026	—
7.8	0.110 0.118	0.114	0.088
15.6	0.192 0.194	0.193	0.167
31.3	0.358 0.372	0.365	0.339
62.5	0.656 0.674	0.665	0.639
125	1.120 1.168	1.144	1.118
250	1.982 2.033	2.008	1.982
500	2.853 2.979	2.916	2.890

SERUM/PLASMA ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.033 0.045	0.039	—
7.8	0.089 0.092	0.091	0.052
15.6	0.145 0.148	0.147	0.108
31.3	0.241 0.258	0.250	0.211
62.5	0.460 0.500	0.480	0.441
125	0.830 0.873	0.852	0.813
250	1.538 1.586	1.562	1.523
500	2.741 2.786	2.764	2.725

Appendix IV: Publication

frontiers in Physiology

ORIGINAL RESEARCH
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Fetuin-A as a Potential Biomarker of Metabolic Variability Following 60 Days of Bed Rest

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Background: Fetuin-A is a hepatokine linked to the development of insulin resistance. The purpose of this study was to determine if 60 days head-down-tilt (HDT) bed rest increased circulating fetuin-A and if it was linked to whole body insulin sensitivity (IS). Additionally, we examined whether reactive jump training (RJT) could alleviate the metabolic changes associated with bed rest.

Methods: 23 young men (29 ± 6 years, 181 ± 6 cm, 77 ± 7 kg) were randomized to a control (CTRL, $n = 11$) or RJT group (JUMP, $n = 12$) and exposed to 60 days of bed rest. Before and after bed rest, body composition and $\dot{V}O_{2peak}$ were measured and an oral glucose tolerance test was performed to estimate IS. Circulating lipids and fetuin-A were measured in fasting serum.

Results: Body weight, lean mass, and $\dot{V}O_{2peak}$ decreased in both groups following bed rest, with greater reductions in CTRL ($p < 0.05$). There was a main effect of time, but not the RJT intervention, for the increase in fetuin-A, triglycerides (TG), area under the curve for glucose (AUCG) and insulin (AUCI), and the decrease in Matsuda and tissue-specific IS ($p < 0.05$). Fetuin-A increased in participants who became less insulin sensitive ($p = 0.019$). In this subgroup, liver IS and adipose IS decreased ($p < 0.05$), while muscle IS was unchanged. In a subgroup, where IS did not decrease, fetuin-A did not change. Liver IS increased ($p = 0.012$), while muscle and adipose tissue IS remained unchanged.

Conclusions: In this study, we report an increase in circulating fetuin-A following 60 days of bed rest, concomitant with reduced IS, which could not be mitigated by RJT. The amount of fetuin-A released from the liver may be an important determinant of changes in whole body IS. In this regard, it may also be a useful biomarker of individual variation due to inactivity or lifestyle interventions.

Keywords: bed rest, fetuin-A, hepatokine, insulin sensitivity, liver, metabolism

INTRODUCTION

Physical inactivity and exposure to microgravity induce aging-like phenotypic changes that are associated with the etiology of many chronic diseases (Bergouignan et al., 2011; Hart and Zernicke, 2020). The physiological changes in space and during head-down-tilt (HDT) bed rest, the Earth-based analogue of microgravity, have been well-described and include altered cardiovascular capacity, bone loss, muscle atrophy, impaired functional capacity, and metabolic dysregulation, among others (Bergouignan et al., 2011; Narici and de Boer, 2011; Ade et al., 2015; Vico and Hargens, 2018; Konda et al., 2019). There is evidence that underlying mechanisms, such as insulin resistance, play an important role in the regulation of whole body metabolic changes (Gratas-Delamarche et al., 2014). Insulin resistance is a multi-faceted disruption of the action of insulin in skeletal muscle, adipose tissue, vasculature, brain, and the liver, leading to hyperinsulinemia and reduced glucose disposal (Cox-York and Pereira, 2020). It is also associated with impaired oxidative capacity, increased circulating and deposition of lipids, and metabolic inflexibility. These whole body and cellular changes have been observed following bed rest studies, even when energy balance is maintained (Bergouignan et al., 2006, 2009, 2011; Kenny et al., 2017; Rudwill et al., 2018). The severity of insulin resistance has been found to vary considerably between individuals and between the key target organs (Unnikrishnan, 2004; Abdul-Ghani et al., 2007). Therefore, understanding the individual variability in insulin resistance could provide personalized information on disease etiology and individualized interventions to maintain health.

A key strategy for monitoring metabolic homeostasis is communication between peripheral tissues *via* secreted proteins, which perform autocrine, paracrine, and endocrine actions. Disruption of protein production and target-tissue action underpin the development of metabolic dysfunction including insulin resistance (Priest and Tontonoz, 2019). The quantitative measurement of circulating protein biomarkers is a relatively easy and minimally-invasive means of identifying and understanding the etiology of insulin resistance.

The liver is a major regulator of systemic glucose metabolism. Liver-derived proteins, known as hepatokines, are released into circulation and some of them are known to enhance or attenuate insulin sensitivity (Stefan and Häring, 2013; Iroz et al., 2015; Choi, 2016). Fetuin-A is a novel hepatokine, encoded by the alpha-2-HS-glycoprotein (AHSG) gene in humans and is a known regulator of metabolism (Stefan and Häring, 2013). In particular, an increase in fetuin-A is associated with the development of insulin resistance and the pathophysiology of type 2 diabetes mellitus (T2DM). Fetuin-A impairs the insulin signaling cascade by binding to the tandem fibronectin type 3 domains present on the extracellular portion of the transmembrane β-subunit of the insulin receptor, attenuating tyrosine kinase signaling and leading to reduced glucose uptake (Goustin et al., 2013; Ochieng et al., 2018). Additionally, fetuin-A has been proposed to inhibit the production of the insulin-sensitizing hormone adiponectin in adipocytes, indirectly leading to a decrease in insulin sensitivity (Hennige et al., 2008). Finally, fetuin-A is implicated in lipid-induced insulin resistance by acting as an intermediary between palmitate and toll-like receptor 4 (TLR4) leading to adipose tissue inflammation and insulin resistance (Pal et al., 2012).

Exercise training, using a wide-range of modalities, is known to promote glucose control and improve insulin sensitivity in healthy and clinical populations (Ennequin et al., 2019). One of the mechanisms that contributes to improved metabolic health is training-induced alterations in the production and secretion of pro-inflammatory and anti-inflammatory cytokines from tissues of metabolic importance. Numerous studies have reported that exercise training decreases the secretion of fetuin-A from the liver concomitant with improvements in whole body and liver insulin sensitivity in patients with metabolic disease (Malin et al., 2013, 2014; Lee et al., 2017; Ennequin et al., 2019).

The purpose of this study was to determine if 60 days of extreme physical inactivity increased circulating fetuin-A and if those changes correlated with whole body insulin sensitivity. We used the European Space Agency 60 day bed rest model, where subjects were maintained in energy balance, despite a decrease in physical activity. This unique approach means fat accumulation is not a confounding factor on the metabolic outcomes. In addition, we examined whether reactive jump training (RJT), a countermeasure used to maintain skeletal muscle mass, was able to attenuate the deterioration in metabolic health that occurs with prolonged inactivity.

MATERIALS AND METHODS

General Study Information

This research was conducted as part of the "Reactive jumps in a sledge jump system as a countermeasure during long-term bed rest" (RSL) study funded by the European Space Agency, which ran as two separate bed rest campaigns, commencing in August 2015 and January 2016, respectively. This parallel-design randomized controlled training study was conducted at the "envihab" facility at the German Aerospace Center (DLR). A detailed description of the subject recruitment procedures, experimental conditions, diet, countermeasure, and training protocol have been published previously (Kramer et al., 2017b).

In brief, the study was split into three phases: a 15-day baseline data collection phase (BDC-15 to BDC-1), 60 days of strict 6° head-down-tilt bed rest (HDT1 to HDT60), followed by a post-intervention testing phase (R + 0 to R + 14), with a total duration of 90 days. Subjects were randomly assigned to a control group (CTRL) or intervention group involving

Abbreviations: Adipose IR, Adipose tissue insulin resistance; AUCG, Area under the curve for glucose; AUCI, Area under the curve for insulin; BDC, Baseline data collection; BMI, Body mass index; CTRL, Control group; ELISA, Enzyme-linked immunosorbent assay; HDT, Head-down-tilt; IS, Insulin sensitivity; JUMP, Jump countermeasure group; Liver IS, Liver insulin sensitivity; Muscle IS, Muscle insulin sensitivity; OGTT, Oral glucose tolerance test; RJT, Reactive jump training; PV, Plasma volume; T2DM, Type 2 diabetes mellitus; VO2peak, Peak aerobic capacity.

reactive jump exercise (JUMP). For the duration of the bed rest period, subjects remained at the 6° HDT angle for 24 h/day.

The inclusion criteria have been described previously (Kramer et al., 2017b) but included men, aged 20–45 years, body mass index (BMI) 20–26 kg/m², non-smoker, no medication, no history of bone fractures, non-competitive athlete, and no medical conditions. Initially, 24 male volunteers were enrolled for the study, however one subject discontinued during baseline data collection due to medical reasons unrelated to the study. In addition, two subjects were ambulated after 49-days HDT (CTRL) and 50-days HDT (JUMP) due to medical reasons but all post-bed rest data were collected except for $\dot{V}O_{2peak}$.

On HDT1, participants were randomly assigned to either the control group (CTRL, n = 11, age 28 ± 6 years, BMI 23.3 ± 2 kg/m²) or the countermeasure group (JUMP, n = 12, age 30 ± 7 years, BMI 23.8 ± 2 kg/m²), which performed RJT 5–6 days per week in a horizontal sledge jump system. A total of 48 training sessions were completed during the HDT bed rest period. Each training session involved a varying amount of repetitive hops and countermovement jumps with an average load equal to or exceeding 80% of the individual's body weight. The maximal workload in one session excluding breaks did not exceed 4 min. During the entire study, the subjects received a strictly controlled and individualized diet, which was tailored to maintain energy balance. The study protocols were approved by the Ethics Committee of the North Rhine Medical Association (Ärztekammer Nordrhein) in Düsseldorf, Germany, as well as the Federal Office for Radiation Protection (Bundesamt für Strahlenschutz). All subjects gave their written informed consent before commencing the study in accordance with the Declaration of Helsinki. This study was registered with the German Clinical Trial Registry (#DRKS00012946, 18th September 2017).

Body Weight and Body Composition

Measurements of body weight and body composition were taken on numerous days before and after bed rest. This is a large scale bed rest study and data are reported by different research groups. The core data have been published elsewhere (Kramer et al., 2017b), however, this study has compared measurements of body weight and body composition recorded on BDC-3 and HDT60. Body weight was measured daily following the first urine void of the day (DVM 5703, Sartorius, Göttingen, Germany). Body composition was examined with dual-energy X-ray absorptiometry (DEXA), using the whole body scan feature on the Prodigy Full Pro (GE Healthcare GmbH, Solingen, Germany) and the manufacturer's enCORE software (version 16.10.151) was used to generate automated reports of total lean mass, fat mass, and bone mineral content.

Peak Oxygen Consumption Test

Peak aerobic capacity ($\dot{V}O_{2peak}$) was measured on BDC-8 and R + 1 using cycle ergometry (Lode, Groningen, The Netherlands) as previously described (Kramer et al., 2017a). In brief, after an initial 5 min of seated rest, subjects were instructed to start pedaling and to maintain a cadence of 75 revolutions per minute (rpm). The warm-up consisted of 3 min cycling at 50 W, followed by 1 min stages, in which the load was increased by 25 W per stage until volitional exhaustion, despite strong verbal encouragement. If the peak respiratory exchange ratio (RER) did not exceed 1.10, the trial was deemed not exhaustive and not considered for further analyses. Due to the absence of post-bed rest data, two subjects were removed from our analysis of $\dot{V}O_{2peak}$.

Oral Glucose Tolerance Test

An oral glucose tolerance test (OGTT) was performed after a 12-h overnight fast, in the morning of BDC-5 and HDT59. A catheter was placed in the antecubital vein and blood samples were drawn before and at 30 min intervals (30, 60, 90, and 120 min) after the ingestion of a 75 g glucose equivalent (ACCU Chek® Dextro OGT, Roche Diagnostics Deutschland GmbH, Mannheim) dissolved in 300 ml water. Following sample extraction, serum was left to coagulate at room temperature for 30 min before centrifugation, while vacutainers containing fluoride and EDTA were centrifuged immediately (184 × g, 4°C, 10 min). Serum and plasma were then aliquoted and stored at −80°C until analysis.

Plasma Volume Correction

As the 6° HDT angle induces a fluid shift and consequent loss of plasma volume (PV), the change in PV was calculated and the concentrations of biochemical parameters following HDT bed rest were corrected for changes in hemoconcentration (Dill and Costill, 1974; Alis et al., 2016). The change in PV (Δ%PV) was calculated as follows: $\Delta\%PV = 100^{*}((Hb_{pre}/Hb_{post})^{*}(100-Htc_{post})/(100-Htc_{pre})-1)$, where hemoglobin (Hb) is given in g/dL and hematocrit (Hct) is expressed as a percentage (%). To correct measured parameters for changes in PV, the following calculation was used: [parameter]c = [parameter] u*(1 + ΔPV(%)/100), where the c and u indices represent corrected and uncorrected concentrations, respectively.

Biochemical Analysis and Assays

Concentrations of glucose, non-esterified fatty acids (NEFA), total cholesterol, LDL-cholesterol (LDL), HDL-cholesterol (HDL), and triglycerides (TG) were measured using colorimetric assay kits on the Randox RX Daytona™ (Crumlin, United Kingdom). Serum insulin was quantified using an immunoassay method on the Cobas® 8000 modular analyser (module e602, Roche Diagnostics, North America). Area under the curve for glucose (AUCG) and insulin (AUCI) were calculated according to the trapezoidal rule. Indexes of insulin resistance and insulin sensitivity including the Matsuda index, liver insulin sensitivity (liver IS), muscle insulin sensitivity (muscle IS), and adipose tissue insulin resistance (adipose IR) were calculated using previously reported equations (**Supplementary Material**; Matsuda and DeFronzo, 1999; Abdul-Ghani et al., 2007; Lomonaco et al., 2012). Using fasting serum samples from the OGTT, concentrations of fetuin-A were assayed in duplicate using the human fetuin-A quantikine ELISA kit, according to the

manufacturer's instructions (Cat No: DFTA00. R&D Systems Inc. Minneapolis, United States). The intra-assay coefficient of variance (%CV) was 11%.

Statistical Analysis

All experimental data are presented as mean ± SD. Normality of distribution for each variable was evaluated using the Shapiro-Wilk test and data violating the assumption of normality was transformed. Differences in baseline characteristics between the two experimental groups were assessed using independent samples *t*-tests or the non-parametric Mann-Whitney U test. Physical and metabolic changes in response to bed rest were analyzed using a mixed between-within factorial analysis of variance using time as the within-group factor and experimental group as the between-group factor (CTRL and JUMP). When a statistically significant interaction was found, simple main effects are reported as mean (M) and standard error (SE). Statistical analysis was performed in SPSS 26.0 (IBM Corp., Armonk, NY, United States) considering a two-sided 0.05 significance level.

In-Depth Data Analysis

An association between insulin sensitivity and fetuin-A has been well-established within the literature (Ochieng et al., 2018; Bourebaba and Marycz, 2019). In the current study, pre- to post-differences in insulin sensitivity and fetuin-A exhibited significant effects of time only, so to further explore the relationship between both variables additional subanalysis was conducted. The participant data from the two experimental groups were pooled and then divided into two subgroups based on participants who improved (↑ Matsuda, $n = 6$, CTRL $n = 4$, and JUMP $n = 2$) or reduced (↓ Matsuda, $n = 17$, CTRL $n = 7$, and JUMP $n = 10$) insulin sensitivity post-HDT bed rest. Paired sample *t*-tests were used to assess the significance of pre- to post-bed rest changes in physical and metabolic parameters in both subgroups.

RESULTS

Physical Characteristics: Body Weight, Body Composition, and $\dot{V}O_{2peak}$

Anthropometric measurements were obtained on BDC-3 and HDT60 (**Table 1**). No significant between-group differences in physical characteristics were identified at baseline. Following 60 days of HDT bed rest, body weight decreased significantly in both groups, with a larger reduction observed in the CTRL group ($M = -3.63$ kg, $SE = 0.54$ kg, $p < 0.001$) compared to the JUMP group ($M = -2.23$ kg, $SE = 0.28$ kg, $p < 0.001$). Similarly, bed rest significantly reduced lean mass, with a higher decline noticeable in the CTRL group ($M = -3.91$ kg, $SE = 0.70$ kg, $p < 0.001$) in comparison to the JUMP group ($M = -1.34$ kg, $SE = 0.32$ kg, $p = 0.002$). Fat mass decreased significantly in the JUMP group ($M = -0.87$ kg, $SE = 0.23$ kg, $p = 0.003$) but did not change significantly in the CTRL group ($M = 0.10$ kg, $SE = 0.31$ kg, $p = 0.757$) following HDT bed rest. Bone mineral content did not change significantly. Absolute $\dot{V}O_{2peak}$, measured on BDC-8 and R + 1, decreased significantly in both groups after HDT bed rest, with a greater loss identified in the CTRL group ($M = -1.28$ L/min, $SE = 0.17$ L/min, $p < 0.001$) in comparison to the JUMP group ($M = -0.33$ L/min, $SE = 0.15$ L/min, $p = 0.049$). $\dot{V}O_{2peak}$, when normalized for changes in lean mass, decreased significantly in the CTRL group ($M = -18.82$ ml/kgLW/min, $SE = 2.26$ ml/kgLM/min, $p < 0.001$) but did not change in the JUMP group ($M = -4.58$, $SE = 2.17$, $p = 0.063$) after HDT bed rest.

Metabolic Characteristics: Glucose Tolerance, Insulin Sensitivity, Lipid Metabolism, and Fetuin-A

The changes in metabolic parameters are presented in **Figure 1** and **Table 2**. No significant between-group differences in metabolic characteristics were found at baseline. Metabolic characteristics were corrected for changes in hemoconcentration

TABLE 1 | Effects of 60 days HDT bed rest on measures of anthropometry and cardiorespiratory capacity.

Measurement	CTRL ($n = 11$)		JUMP ($n = 12$)		Statistics		
	Pre	Post	Pre	Post	Time	Int	T*Int
Age (years)	28 ± 6		30 ± 7				
Height (cm)	181 ± 5		181 ± 7				
BMI (kg/m²)	23.33 ± 2.03	22.22 ± 1.67*	23.75 ± 1.80	23.07 ± 1.81*	**<0.001**	0.410	**0.021**
BW (kg)	76.10 ± 8.06	72.47 ± 6.76*	77.85 ± 6.55	75.63 ± 6.39*	**<0.001**	0.405	**0.027**
LM (kg)	56.94 ± 6.57	53.03 ± 5.11*	56.41 ± 5.18	55.08 ± 4.29*	**<0.001**	0.731	**0.002**
FM (kg)	16.91 ± 3.95	17.00 ± 3.41	19.21 ± 6.42	18.34 ± 6.18*	0.055	0.412	**0.018**
BMC (kg)	3.14 ± 0.36	3.14 ± 0.37	3.00 ± 0.32	3.00 ± 0.32	0.605	0.355	0.800
Measurement	**CTRL ($n = 10$)**		**JUMP ($n = 10$)**		**Statistics**		
	Pre	**Post**	**Pre**	**Post**	**Time**	**Int**	**T*Int**
$\dot{V}O_{2peak}$ (L/min)	3.85 ± 0.68	2.57 ± 0.48*	3.32 ± 0.76	2.99 ± 0.53*	**<0.001**	0.848	**0.001**
$\dot{V}O_{2peak}$ (ml/kgLM/min)	67.55 ± 8.42	48.74 ± 9.35*	58.72 ± 10.95	54.14 ± 8.03	**<0.001**	0.660	**<0.001**

*Data are presented as mean ± standard deviation (SD). Significant $p < 0.05$ are indicated in bold. Anthropometric measurements were taken on BDC-3 and HDT60. $\dot{V}O_{2peak}$ was measured on BDC-8 and R + 1. When a significant interaction effect was found, an asterisk (*) denotes a significant difference from pre in each intervention group. CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Int, main effect of intervention; T*Int, time*intervention interaction effect; BMI, body mass index; BW, body weight; LM, lean mass; FM, fat mass; BMC, bone mineral content; $\dot{V}O_{2peak}$, peak aerobic capacity.*

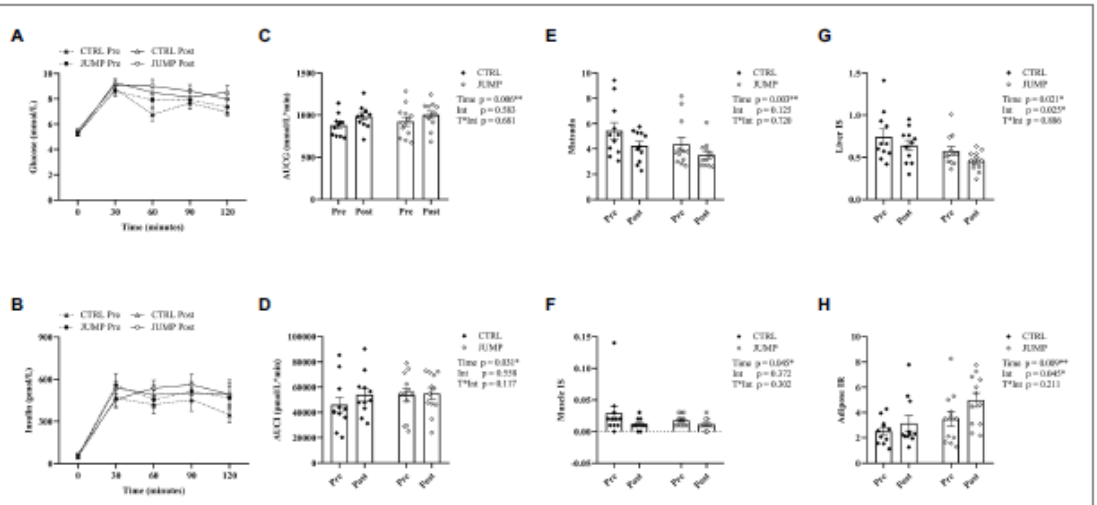

FIGURE 1 | The effects of 60 days of HDT bed rest on metabolic variables measured on BDC-5 (pre) and HDT59 (post). Data are presented as mean ± standard error of mean (SEM). The glucose and insulin response curve to the OGTT are displayed in **(A)** and **(B)**, respectively. The 120 min area under the curve totals for glucose are shown in **(C)** and for insulin in **(D)**. The pre to post changes in OGTT derived indexes of Matsuda, liver IS, muscle IS, and adipose IR are presented in **(E–H)**. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; AUCG, area under the curve for glucose for 120 min; AUCI, area under the curve for insulin for 120 min; IS, insulin sensitivity; IR, insulin resistance; Time, main effect of time; Int, main effect of intervention; T*Int, time*intervention interaction effect. $^{*}p \leq 0.05$ and $^{**}p \leq 0.010$.

TABLE 2 | The effects of 60 days HDT bed rest on metabolic characteristics.

Measurement	CTRL (n = 11)		JUMP (n = 12)		Statistics		
	Pre	Post	Pre	Post	Time	Int	T*Int
$Glucose_0$ (mmol/L)	5.18 ± 0.40	5.24 ± 0.51	5.32 ± 0.64	5.47 ± 0.59	0.447	0.311	0.732
$Glucose_{120}$ (mmol/L)	6.95 ± 1.01	8.51 ± 1.80	7.36 ± 1.82	7.98 ± 1.98	**0.010**	0.919	0.234
$Insulin_0$ (pmol/L)	45.00 ± 13.09	51.48 ± 16.49	55.55 ± 14.92	67.22 ± 19.14	**0.012**	**0.036**	0.437
$Insulin_{120}$† (pmol/L)	347.16 ± 184.12	496.38 ± 321.36	468.39 ± 281.21	492.68 ± 260.41	0.054	0.587	0.264
NEFA (mmol/L)	0.40 ± 0.13	0.41 ± 0.16	0.41 ± 0.14	0.52 ± 0.17	0.124	0.232	0.215
TG (mmol/L)	0.87 ± 0.26	1.00 ± 0.26	1.16 ± 0.45	1.21 ± 0.41	**0.013**	0.102	0.245
CHOL (mmol/L)	4.09 ± 0.72	4.27 ± 0.86	4.11 ± 0.57	4.06 ± 0.57	0.619	0.710	0.381
HDL (mmol/L)	1.16 ± 0.17	0.99 ± 0.19	1.08 ± 0.27	0.91 ± 0.17	**<0.001**	0.304	0.955
LDL (mmol/L)	2.76 ± 0.71	3.10 ± 0.71	2.73 ± 0.51	2.99 ± 0.56	**0.004**	0.780	0.647

*Data are presented as mean ± standard deviation (SD). Significant $p < 0.05$ are indicated in bold. †Denotes that data were transformed for statistical analysis. Metabolic characteristics were measured on BDC-5 and HDT59. CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Int, main effect of intervention; T*Int, time*intervention interaction effect; $Glucose_0$, fasting glucose; $Glucose_{120}$, glucose concentrations 120 min after the glucose load; $Insulin_0$, fasting insulin; $Insulin_{120}$, insulin concentrations 120 min after the glucose load; NEFA, non-esterified fatty acids; TG, triglycerides; CHOL, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.*

following HDT bed rest (mean ΔPV% CTRL 0.88%, JUMP -2.13%). NEFA, total cholesterol, fasting glucose ($glucose_0$), and 2-h insulin ($insulin_{120}$) did not change significantly following HDT bed rest. There was a significant effect of time, but not intervention, for the increase in 2-h glucose ($glucose_{120}$; $p = 0.010$), TG ($p = 0.013$), and LDL ($p = 0.004$) and decrease in HDL ($p < 0.001$) after HDT bed rest (**Table 2**). A significant effect of time and intervention was identified for fasting insulin ($insulin_0$), which increased significantly following HDT bed rest and was significantly higher overall in the JUMP group (**Table 2**). There was a significant effect of time, but not intervention, for the increase in AUCG and AUCI post-HDT bed rest (**Figures 1A–D**). A significant main effect of time only was also identified for the decrease in Matsuda and muscle IS following HDT bed rest (**Figures 1E–F**). The main effect of time and intervention were statistically significant for the change in liver IS and adipose IR following HDT bed rest (**Figures 1G–H**). Liver IS decreased significantly after HDT bed rest and was significantly higher overall in the CTRL group. In contrast, adipose IR increased significantly following HDT bed rest and was found to be significantly higher overall in the JUMP group. A significant main effect of time, but not intervention, was found for the increase in circulating fetuin-A (**Figure 2A**). Fetuin-A increased from

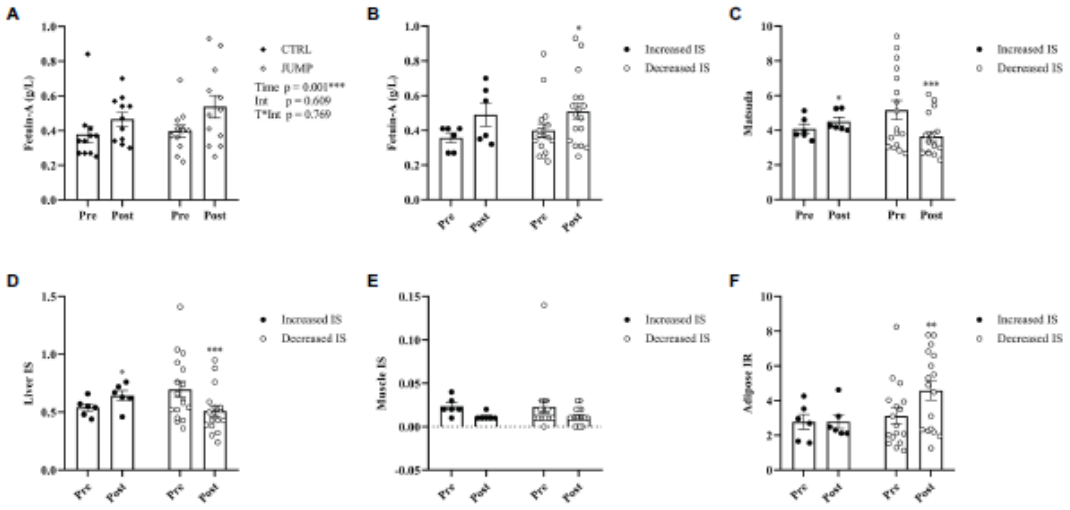

FIGURE 2 | (A) Circulating fetuin-A in the CTRL and JUMP groups before and after 60 days of HDT bed rest. **(B–F)** The effect of HDT bed rest on fetuin-A and OGTT derived indexes of insulin sensitivity and insulin resistance when subjects were divided into two subgroups based on an increase ($n = 6$) or decrease ($n = 17$) in insulin sensitivity (Matsuda) post-HDT bed rest. Data are presented as mean ± SEM. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; IS, insulin sensitivity; IR, insulin resistance; Time, main effect of time; Int, main effect of intervention; T*Int, time*intervention interaction effect. $^{*}p \leq 0.05$, $^{**}p \leq 0.010$, and $^{***}p \leq 0.001$.

0.38 ± 0.16 to 0.47 ± 0.13 g/L in the CTRL group and from 0.40 ± 0.12 to 0.54 ± 0.23 g/L in the JUMP group after HDT bed rest.

Subanalysis Exploring the Relationship Between Insulin Sensitivity and Fetuin-A

Changes in the physical and metabolic parameters in subgroups with decreased and increased insulin sensitivity following HDT bed rest are presented in **Figures 2B–F** and **Supplementary Tables 1** and **2**.

In the subgroup with improved insulin sensitivity following HDT bed rest, the pre- to post-increase in liver IS was statistically significant. Despite this, muscle IS and adipose IR did not change significantly after HDT bed rest. In subjects who became more insulin-sensitive following HDT bed rest, the change in fetuin-A was not statistically significant.

In the opposing subgroup, with reduced insulin sensitivity after HDT bed rest, the pre- to post-decrease in liver IS was statistically significant. Adipose IR increased significantly following HDT bed rest. The change in muscle IS was not statistically significant. Interestingly, in those who became less insulin-sensitive during HDT bed rest, circulating concentrations of fetuin-A significantly increased.

DISCUSSION

The main findings of the current study demonstrate that 60 days of HDT bed rest elicited a significant increase in fetuin-A concomitant with reduced insulin sensitivity, which could not be mitigated by RJT. As considerable individual differences have been found in the responsiveness to lifestyle interventions, we compared changes in metabolic variables in subgroups with decreased and increased insulin sensitivity following HDT bed rest. Our results suggest that fetuin-A may have a role in the regulation of peripheral insulin sensitivity during bed rest and physical inactivity. In addition, fetuin-A has potential as a biomarker to track individual changes in metabolic homeostasis.

Exercise and diet countermeasures have been widely implemented to abrogate deconditioning during bed rest. The RJT protocol combined plyometric movements with high rates of force development with the aim of preserving musculoskeletal mass and strength (Kramer et al., 2017a). The results, from previous publications, show that this time-efficient countermeasure attenuated the loss of whole body lean mass, leg lean mass, $\dot{V}O_{2peak}$ (Kramer et al., 2017a,b), and myofiber size and phenotype (Blottner et al., 2019). While RJT had protective effects for muscle function, it could not prevent the dysregulation of glucose and lipid metabolism. Similar results were reported following flywheel exercise (Bergouignan et al., 2006) and the authors, in this case, suggested that insufficient energy expenditure during the training sessions may be the key factor. While the RJT is a form of high intensity interval training consisting of 48 jumps and 30 hops, the overall workload may not be sufficient to improve insulin sensitivity. There was a decrease in fat mass (0.9 kg), indicating a negative energy balance in the JUMP group but this may be due to the challenges of estimating energy expenditure of the exercise protocol.

Another challenge for countermeasure design and implementation is the individual variation in response to lifestyle interventions (Bouchard and Rankinen, 2001; Solomon, 2018; O'Donoghue et al., 2019). While a specific exercise program may, on average, be effective there can be a broad range in the individual response. This is particularly important for long-term missions in microgravity, where a standard countermeasure program may not confer the same benefit to all the astronauts. It will be important to have individualized countermeasure programs that can be monitored and adjusted depending on the response. While changes in muscle mass can be observed with relative ease, monitoring changes in metabolism is more challenging in microgravity. The role of circulating biomarkers may serve as a simple and effective strategy to track metabolic changes on the health continuum and guide countermeasure recommendations. Previous research has reported that the beneficial and detrimental effects of physical activity and inactivity, respectively, are linked with changes in circulating biomarkers, which are key messengers for inter-organ communication (Pedersen and Febbraio, 2012; Ennequin et al., 2019; Mika et al., 2019).

Fetuin-A is a multifunctional glycoprotein that is predominately synthesized and secreted by hepatocytes (Bourebaba and Marycz, 2019) and associated with insulin action (Ochieng et al., 2018). To the best of our knowledge, fetuin-A has not been previously reported following bed rest and we report a significant increase in circulating fetuin-A following 60 days of HDT bed rest, irrespective of the experimental group. The regulation of hepatic fetuin-A secretion is incompletely understood (Haukeland et al., 2012). Previous research has reported that fetuin-A mRNA expression in the liver correlated positively with hepatic triglyceride content and homeostatic model of insulin resistance (HOMA-IR) and these associations remained significant after adjustment for BMI (Peter et al., 2018). These findings were supported by a cross-sectional study reporting higher fetuin-A in subjects with elevated liver fat content (Stefan et al., 2006). Analysis of longitudinal data following a lifestyle intervention found that the changes in fetuin-A paralleled the changes in liver fat (Stefan et al., 2006). Collectively, this evidence presents fetuin-A as a possible biological and predictive marker of metabolic disease.

Fetuin-A is also responsive to exercise training and a number of studies have reported a decrease in circulating levels with accompanying improvements in body composition, liver fat, and insulin sensitivity (Malin et al., 2013, 2014). Reductions in fetuin-A following aerobic exercise training have been attributable to decreases in waist circumference and body weight and increases in adiponectin, an insulin sensitizing hormone (Zhang et al., 2018). Additionally, favorable changes in fetuin-A, liver fat and insulin sensitivity have also been reported following long-term (12 weeks) aerobic and resistance exercise training (Lee et al., 2017). A recent meta-analysis reported that both aerobic and resistance training significantly reduced fetuin-A in dysglycemic and overweight/obese individuals when performed at a moderate or vigorous intensity, with a volume of 60 min per session and a minimum frequency of 4–7 sessions per week (Ramírez-Vélez et al., 2019).

In this study, we found that circulating concentrations of fetuin-A and insulin sensitivity were not significantly affected by RJT performed during HDT bed rest. This may be due to the type, intensity, and duration of the exercise protocol, which was primarily designed to preserve muscle mass and function. In addition, the physical inactivity of bed rest is the primary intervention and the exercise countermeasure in this case is designed to maintain rather than enhance physiological function. We observed a significant reduction in whole body insulin sensitivity, concomitant with an increase in circulating TG after 60 days HDT bed rest in energy-balanced conditions. These findings are in agreement with other bed rest studies (Bergouignan et al., 2009; Rudwill et al., 2015; Kenny et al., 2017), which support links between the decrease in muscle contraction and elevated TG (Bergouignan et al., 2011), decreases in the amount and activity of key proteins associated with muscle glucose uptake (Biensø et al., 2012), and altered mitochondrial function (Kenny et al., 2017). The observation that whole body and liver insulin sensitivity improved in a subgroup of bed rest participants is intriguing and supports the current emphasis on personalized medicine approaches to disease treatment. Interestingly, the improvement in insulin sensitivity was greater in the CTRL group than in the JUMP group. It has previously been highlighted that there is substantial inter-individual variability in physiological responses following lifestyle interventions (Bouchard and Rankinen, 2001; Solomon, 2018; O'Donoghue et al., 2019). In addition, the genes and pathways underlying the response to exercise training and physical inactivity differ (Booth et al., 2012; Pillon et al., 2020). Therefore, it is important to learn more about the possible mediators of the variation in insulin sensitivity following bed rest.

Our analysis identified six participants (26%) that improved insulin sensitivity following HDT bed rest. In addition, there were no significant changes in fetuin-A, fasting glucose, or TG in this subgroup. One possibility is that the improvement in whole body insulin sensitivity may be an indirect effect of liver metabolism. The amount of intra-hepatic lipid (IHL) is strongly linked to liver and whole body insulin resistance (Mu et al., 2018; Trouwborst et al., 2018). If liver insulin sensitivity does not decrease, it is possible that the release of fetuin-A would be attenuated and the negative effect on peripheral tissues would be mitigated (**Figure 3**). In support of this hypothesis, fetuin-A levels were found to be significantly higher in metabolically-unhealthy compared with metabolically-healthy obese subjects (Khadir et al., 2018). Fetuin-A knockout (KO) mice display enhanced insulin sensitivity, improved glucose tolerance, and lowered plasma lipid content (Mathews et al., 2002). When fed a high fat diet, fetuin-A KO mice remain insulin-sensitive, are resistant to weight gain and have less adiposity than wild-type controls. As there is inter-individual variation in IHL following lifestyle interventions (Winn et al., 2018), it is possible that fetuin-A could be a driver of peripheral insulin resistance and a biomarker to track the physiological responsiveness to bed rest and physical inactivity.

Despite the highly controlled nature of this bed rest study, there are some limitations to acknowledge. Firstly, the sample size for parallel-designed bed rest studies is generally low, in the order of 8–12 participants per group (Bergouignan et al., 2006, 2009; Rudwill et al., 2015, 2018; Kenny et al., 2017).

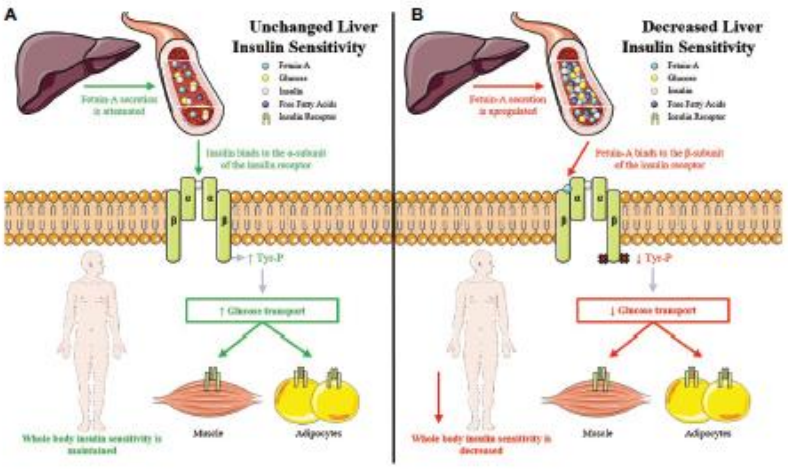

FIGURE 3 | The impact of liver insulin sensitivity and fetuin-A secretion on whole body insulin sensitivity. **(A)** Unchanged liver insulin sensitivity: if liver insulin sensitivity is unchanged then the release of fetuin-A from the liver is attenuated. This allows insulin to bind to the α-subunit of the insulin receptor, which promotes the auto-phosphorylation of the insulin receptor and subsequent tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins. These actions initiate a cascade of events leading to increased glucose uptake in peripheral tissues and an improvement in whole body insulin sensitivity. **(B)** Decreased liver insulin sensitivity: when liver insulin sensitivity is decreased, the release of fetuin-A from the liver is upregulated. Fetuin-A binds to the extracellular portion of the β-subunit of the insulin receptor. Fetuin-A inhibits insulin receptor auto-phosphorylation and tyrosine kinase activity leading to decreased glucose uptake in peripheral tissues and a reduction in whole body insulin sensitivity.

It is possible that the small sample size in the subgroup with improved insulin sensitivity is too small for a definite conclusion, and therefore, we suggest that further studies are required to investigate the potential role of fetuin-A in the regulation of whole body insulin sensitivity following HDT bed rest. Secondly, although it was not the objective of this study, we did not obtain any measurements of liver fat precluding our ability to fully explore the association between fetuin-A and hepatic insulin resistance in response to physical inactivity; this requires further investigation. Thirdly, an OGTT was used to estimate insulin sensitivity. While a euglycaemic-clamp is the gold standard measure, it was not possible in the current study. However, the OGTT sufficiently reflects changes in glucose tolerance and the measurement of whole body insulin sensitivity using the OGTT has been validated previously in healthy non-overweight adults (Trikudanathan et al., 2013). Finally, the results of this study have been obtained from healthy, lean adult males and similar investigations will need to be extended to other populations (e.g., women, elderly, and metabolically unhealthy) to determine the specific links with disease etiology.

In conclusion, we report that 60 days of HDT bed rest led to a significant increase in circulating fetuin-A and decreased insulin sensitivity, which could not be ameliorated by RJT. Exploring individual responses to lifestyle interventions is a growing area of interest in personalized medicine. While HDT bed rest reduced insulin sensitivity at the group level, there was considerable individual responses, including a subgroup for which insulin sensitivity improved. We propose that the regulation of insulin sensitivity is related to circulating fetuin-A, which is attenuated when liver metabolism is maintained. Collectively, our results show that fetuin-A is a candidate biomarker to assess the physiological responses to bed rest and physical inactivity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the North Rhine Medical Association (Ärztekammer Nordrhein) in Düsseldorf, Germany, as well as the Federal Office for Radiation Protection (Bundesamt für Strahlenschutz). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KW, DC, and DO'G had full access to the data used in this study and take responsibility for the integrity of the data and the accuracy of the data analysis. The study was designed by KW, DC, DO'G, EM, and PF-M. EM was the project scientist and PF-M leads the work package "biological samples" and "nutrition" for the ESA RSL study. KW, DC, and DO'G conducted

the biological sample analysis and were responsible for the formal analysis and interpretation of the data. KW, DC, and DO'G drafted the original manuscript and all authors were involved in the critical review for important intellectual content. Approval of the final manuscript was given by all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.573581/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix V: Summary of the Process of Biomarker Selection

Table 85. A summary of the 3-step process to determine the circulating biomarkers that could assist in the interpretation of observed changes in metabolic physiology in healthy young males following HDT bed rest.

	Phase 1	Phase 2	Phase 3
Acylation stimulating protein (ASP)	M	Y	
Adipolin	M	M	N
Adiponectin	M	Y	
Adipsin	M	N	
Adropin	M	M	Y
Angiopoietin like 3 (ANGPTL3)	M	M	N
Angiopoietin like 4 (ANGPTL4)	M	N	
Angiopoietin like 6 (ANGPTL6)	M	N	
Apelin	Y		
Apolipoprotein-J (Apo-J)/Clusterin	M	M	Y
Asprosin	N		
Betatrophin/ Angiopoietin like 8 (ANGPTL8)	M	M	N
Brain-derived neurotrophic factor (BDNF)	N		
Chemerin	N		
Chitinase 3-like 1 (CHI3L1)/YKL-40	M	M	N
Circulating Complement-C1q TNF-Related Protein-1 (CTRP1)	N		
C-X-C motif chemokine ligand 10 (CXCL10)	M	M	N
Decorin	N		
Dipeptidyl peptidase IV (DPP-4)	N		
Erythropoietin	N		
Fatty acid binding protein 4 (FABP4)	N		
Fetuin-B	M	N	
Fibroblast growth factor 21 (FGF-21)	M	M	Y
Follistatin	M	N	
Follistatin Like 1 (FSTL1)	M	M	N
Fractalkine	N		
Ghrelin	N		
Heat Shock Protein 72 (HSP72)	N		
Hepatocyte derived fibrinogen protein 1 (HFREP1)	M	M	N
Hepatocyte Growth Factor (HGF)	N		
Hepcidin	N		
Inhibin beta-E chain (Inhibin βE)	M	N	
Insulin-like growth factor binding protein 1 (IGFBP1)	N		
Interleukin 10 (IL-10)	N		
Interleukin 15 (IL-15)	N		

	Phase 1	Phase 2	Phase 3
Irisin	**Y**		
Leptin	**N**		
Leukaemia Inhibitory Factor (LIF)	**N**		
Leukocyte cell–derived chemotaxin 2 (LECT2)	**M**	**M**	**N**
Lipocalin-2 (LCN2)	**N**		
Meteorin-like protein (Metrnl)	**N**		
Musculin	**N**		
Myonectin	**M**	**M**	**N**
Myostatin	**M**	**N**	
Nefastin-1	**N**		
Neuregulin 4 (NRG4)	**N**		
Omentin	**N**		
Osteoglycin	**N**		
Plasminogen activator inhibitor-1 (PAI-1)	**M**	**M**	**N**
Preptin	**N**		
Progranulin	**N**		
Resistin	**M**	**M**	**N**
Retinol-binding protein 4 (RBP4)	**Y**		
Secreted frizzled-related protein 4 (SRFP4)	**N**		
Secreted frizzled-related protein 5 (SRFP5)	**M**	**N**	
Secreted Protein Acidic and Rich in Cysteine (SPARC)	**M**	**M**	**N**
Selenoprotein P (SEPP1)	**M**	**M**	**N**
Sex-hormone binding globulin (SHBG)	**N**		
Tissue inhibitor of metalloproteinase (TIMP)	**N**		
Vapsin	**N**		
Visfatin	**M**	**M**	**N**
WNT1-inducible-signaling pathway protein 1 (WISP-1)	**M**	**N**	
Zinc-alpha2-glycoprotein (ZAG)	**N**		
α-ketoglutaric acid	**N**		
β-aminoisobutyric acid (BAIBA)	**N**		

***Abbreviations**: Y, yes; M; maybe; N, no.*

Appendix VI: Phase 1 of the Biomarker Selection Process

Name	Other Name	Organ	Appeared	IR	Exercise	IS or IR?		Main Functions (Summary)	Previously measured in other bed rest studies?	ELISA Kit	PubMed Search 2020 (English Language)						
											Initial	Humans	IR	IS	Exercise	Inactivity	Bed Rest
Follistatin		Liver	1990	↑	↑ Acute		IS	Increases hypertrophy, decreases muscle breakdown, inhibits myostatin, promotes B-cell survival and proliferation.	No	R&D	2397	1201	33	9	53	1	0
FGF-21	OR "Fibroblast growth factor 21"	Primarily Liver	2005	↑	↑		IS	Increases glucose uptake, regulates lipolysis and increases adiponectin production	Yes	R&D	1812	914	213	104	67	0	1
Betatrophin/ ANGPTL8	"RIFL" OR "refeeding-induced fat and liver" OR "Lipasin" OR "angiopoietin-like protein 8" OR "ANGPTL8"	Liver and Adipose Tissue	2012	↑	↓	IR		Inhibits lipoprotein lipase (LPL) and regulates triglyceride (TG) levels (muscle and brown adipose tissue), and is associated with HDL. Decrease in exercise due to reduction in body weight and body fat.	No	BioVendor & Aviscera	297	180	62	9	4	1	0
ANGPTL3	OR "Angiopoietin-like protein 3"	Liver	1999	↑	↔	IR		Inhibits LPL and regulated TG levels (muscle and BAT), functionally dependent on ANGPTL8, increases lipolysis.	Yes	All 3 Companies	353	237	19	7	3	1	1
ANGPTL4	OR "Angiopoietin-like 4"	Liver	2000		↑	IR		Closely related to ANGPTL3. Involved in the regulation of TG and inhibition of LPL (white adipose tissue).	No	ThermoFisher	886	538	31	15	19	0	0
ANGPTL6	OR "Angiopoietin-like 6" OR "angiopoietin-related growth factor"	Liver		↑	↓		IS	Increases systemic energy expenditure and antagonises metabolic diseases. Improves lipid profile and insulin senstivity.	No	Novus, ELISA Genie, MSC, Abbexa	79	54	14	6	1	0	0
Apolipoprotein-J	OR "Apo-J" OR "Clusterin"	Hepatocytes	1990	↑	↓		IS	Found in HDL and LDL in plasma. ApoJ has a key role as a co-factor in coordinating insulin-mediated signalling events - absence or high levels impair insulin signalling.	No	R&D & BioVendor	2860	1761	12	5	3	0	0
LECT2	OR "Leukocyte Cell–Derived Chemotaxin 2"	Hepatocytes	1996	↑		IR		Increases in obesity and fatty liver and shown to have a link with obesity and skeletal muscle insulin resistance.	No	BioVendor	175	90	11	1	4	0	0
Selenoprotein P	OR "SEPP1"	Liver	1982	↑	↔	IR		Inhibits the insulin signalling pathway by inactivating AMP-activated protein kinase (AMPK).	No	Abbexa, ELISAGenie	764	449	39	10	8	0	0
Adropin	OR "Energy-homeostasis-associated protein"	Liver - but exp. In multiple tissues	2008	↓	↑		IS	Regulation of energy homeostasis and controls glucose and fatty acid metabolism. Decreases hepatic glucose production (HGP) and increases liver insulin sensitivity.	No	Novus Bio, ELISA Genie	167	98	31	11	6	0	0
RBP4	OR "Retional-binding protein 4"	Hepatocytes, Adipocytes and Macrophages	2001	↑	↓ ↔	IR		RBP4 impairs insulin action and exerts a pro-inflammatory response.	No	R&D	1302	890	423	123	25	1	1
HFREP1	OR "Hepassocin" OR "Hepatocyte derived fibrinogen protein 1"	Hepatocytes	2001	↑	N/A	IR		Involved in the development of insulin resistance through the ERK1/2 pathway. Structurally similar to ANGPTL proteins.	No	Abbexa, NovusBio, ThermoFisher	80	51	5	3	0	0	0
SHBG	OR "sex-hormone binding globulin"	Hepatocytes	2009	↓	↑	IR		Protein in the liver which transports the hormones testosterone, dihydrotestosterone and estradial in the blood. May have direct effects on HGP. Insulin inhibits SHBG so it may be a consequence rather than a cause of IR.	Yes	R&D	8413	7584	923	345	329	11	1
Inhibin βE	OR activin beta-E chain OR "inhibin beta E subunit" OR "INHBE"	Liver		↑	N/A	IR		Increased by hyperinsulinemia and decreases fat oxidation.	No	Abbexa, ELISAGenie	44	25	2	2	0	0	0
Fetuin-B		Hepatocytes	2000	↑	↓	IR		Decreases glucose uptake in myotubes and impaired insulin action in hepatocytes.	No	BioVendor and ThermoFisher	98	45	11	1	1	0	0
Insulin-like growth factor binding protein 1	OR "IGFBP1" OR "IGFBP-1"	Liver	1988	↓			IS	IGFBP1 increases as a result of hepatic IR	Yes	R&D	3949	2929	264	161	117	3	1
Heat Shock Protein 72	OR "HSP72"	Hepatosplanchnic tissue/stressed muscle	2002	↓	↑	IR		Involved with mitochondrial function and/or fatty acid oxidation. Possible that HSP72 increases to combat metabolic dysfunction then declines.	No	BioMatik, EnzoLS	2,341	979	21	10	128	2	1
Hepcidin		Liver	2000	↓	↑	IR		Hepcidin plays a role in iron metabolism and is involved in the development of T2DM.	No	R&D	4,116	2,539	43	8	80	3	0
Hepatocyte Growth Factor	OR "HGF"	Liver	1989	↑		IR		HGF has a role in the metabolic flux of glucose in insulin sensitive cells.	Yes - Elderly	Invitrogen, ELISAGenie	14,082	9,170	35	9	44	2	1

Figure 36. Hepatokines included in biomarker selection phase 1.

Name	Other Name	Organ	Appeared	IR	Exercise	IS or IR?		Main Functions (Summary)	Previously measured in other bed rest studies?	ELISA Kit	PubMed Search 2020 (English Language)						
											Initial	Humans	IR	IS	Exercise	Inactivity	Bed Rest
Myostatin	OR "GDF-8"	Myocytes	2004	↑	↓	IR		Decreases muscle mass development and muscle insulin sensitivity. Predominantely measued in muscle.	Yes	R&D	2877	1041	67	31	225	13	10
Myonectin	OR "CTRP15"	Skeletal Muscle	2011	↓	↑ [c]		IS	Regulates lipid metabolism by increasing fatty acid uptake and storage in the liver and muscle.	No	Aviscera	56	24	7	2	8	0	0
Irisin	OR "Fibronectin type III domain-containing protein 5" OR "FNDC5"	Skeletal muscle/adipose tissue	2012	[c]	↑		IS	Role in thermogenesis and increasing energy expenditure. Increases glucose uptake, improves hepatic glucose and lipid metabolism, increases β-cell survival.	No	BioVendor	1127	609	141	57	251	7 [1]	1
YKL-40	OR "Chitinase 3-like 1" OR "CHI3L1"	Skeletal muscle, Inflammatory cells	1989	↑	↑[acute, RT]	IR		Acute phase reactant which is regulated by pro-inflammatory cytokines - proposed to counteract TNFa-induced inflammation and play a role in lipid metabolism, angiogenesis and Phosphoinositide 3-kinases (P13K) and protein kinase B (Akt) signalling.	No	R&D	1536	1177	28	6	9	0	1
SPARC	OR "Secreted Protein Acidic and Rich in Cysteine" OR "Osteonectin"	Skeletal muscle/bone/ adipose tissue	1981	↑	↔	IR		SPARC ↑leptin, insulin and adipose tissue inflammation (it then increases subcutaneous adipose tissue fibrosis) thereby increasing lipids in circulation and promotes lipid storage in other organs thus contributing to insulin resistance. Related to obesity.	No	R&D	3792	2192	14	2	26	0	0
Fractalkine	OR "CX3CL1"	Skeletal muscle/adipose tissue	2009	↑	↔	IR		Obesity-induced increase in chemokine production may be a compensatory mechanism to sustain insulin secretion, however it may then lead to hyperinsulinemia and β-cell failure.	No	R&D	2232	1243	18	6	11	0	0
Meteorin-like protein	OR "Metrnl" OR "subfatin"	Skeletal muscle/white adipose tissue	2008	[c]	↑		IS	Circulating protein in muscle induced by exercise. Improves whole-body energy expenditure and glucose tolerance by enhancing browning of white adopose tissue. Improves insulin sensitivity and fatty acid oxidation (cells and mice).	No	Aviscera, ELISAGenie	53	26	6	1	2	0	0
IL-10	OR "Interleukin-10"	Macrophages, dendritic cells, T cells and B cells	1989	↓ [c]	[c]		IS	Induces anti-inflammatory effects and improves glucose tolerance.	Yes	R&D	63,165	33,543	205	56	557	13	8
CXCL10	"IP-10" OR "C-X-C motif chemokine ligand 10" OR "Interferon gamma-induced protein 10" OR "small-inducible cytokine B10"	Various cell types	1985	↑	↓	IR		Inflammatory cytokines that binds to CXCR3 to mediate immune responses.	Yes	R&D	8844	5575	39	3	25	2	1
Osteoglycin	"OGN" OR "osteoinductive factor" OR "mimecan"	Myoblastic cells	2012	[↓OB]	N/A		IS	In vivo, osteoglycin is shown to increase Akt phosphorylation - synergistic effect of insulin.	No	MSC, ELISA Genie, Abbexa	340	172	1	0	2	0	1
IL-15	OR "Interleukin-15"	Skeletal muscle and other cells	1994	↓ [c]	↑		IS	Related to obesity. Improves insulin sensitivity, lipogenesis, increases brown fat and fatty acid oxidation. Similar function to IL-2.	No	R&D	6056	3885	20	8	74	2	1
FSTL1	OR "Follistatin Like 1"	Skeletal muscle/adipose tissue	1993/2012	↑	↑ [acute]	IR		Belongs to the SPARC family and has a follistatin like domain. Lack of mechanistic data but FSTL-1 is implicated in glucose metabolism, insulin signalling inhibition and inflammation.	No	MSC, ELISA Genie and Aviscera	259	147	3	1	9	1	0
Decorin		Contracting human myotubes	1986	↑	↑ ↔		IS	Restructing of muscle during hypertrophy. Increases follistatin and decreases myostatin.	No	Thermofisher, Abcam	2811	1539	7	1	19	3	0
LIF	OR "Leukemia Inhibitory Factor"	Skeletal muscle	1988		↑in Muscle	IR		Belongs to the IL-6 cytokine superfamily. Promotes myocyte proliferation (multiplication and replication of myoblasts) but more research is required.	No	R&D	10609	4056	5	2	25	3	2
BDNF	OR "Brain-derived neurotrophic factor"	Growth factor	1982	↓ [c]	↑	IR		Released from the brain, and may be affected by high glucose levels. After exercise, there was an increase in BDNF and fat oxidation in vitro and ex vivo.	Yes (Brain Related)	R&D	24,674	8,718	82	26	554	13	2
Erythropoietin	OR "EPO"	Kidney (proposed as a myokine in 2009)	1985		↔↑		IS	EPO plays a role in the improvement of diet-induced obesity, inflammation and IR in animal and cell models.	Yes	Invitrogen, R&D	30,289	21,706	52	31	524	8	9
Musculin	OR "Osteocrin"									Abbexa, ELISA Genie	36	20	1	1	2	0	0
BAIBA	OR "β-aminoisobutyric acid"									None	X	X	X	X	X	X	X
a-ketoglutaric acid	"alpha-ketoglutaric acid" OR "2-Oxoglutaric acid" OR "2-ketoglutaric acid" OR "2-Oxopentanedioic acid"									None	4,592	870	5	2	17	1	0

Figure 37. Myokines included in biomarker selection phase 1.

Name	Other Name	Organ	Appeared	IR	Exercise	IS or IR?		Main Functions (Summary)	Previously measured in other bed rest studies?	ELISA Kit	PubMed Search 2020 (English Language)						
											Initial	Humans	IR	IS	Exercise	Inactivity	Bed Rest
FABP4	"adipocyte FABP" OR "A-FABP" OR "adipocyte fatty acid binding protein" OR "aP2"	Mature adipocytes and macrophages	1983, 1986	↑	↓	IR		FABP4 is involved in lipid signalling and may represent changes in fat transport.	No	R&D	7526	3189	231	76	13	1	0
Chemerin	OR "retinoic acid receptor responder protein 2" OR "tazarotene-induced gene 2 protein" OR "RAR-responsive protein TIG2"	VAT/SAT by mature adipocytes	1997	↑	↓	IR		Chemerin impairs glucose uptake, induces inflammation and atherosclerotic plaque development.	No	R&D	921	595	160	44	24	0	0
Omentin	OR "intelectin" OR "omentin-1" OR "intestinal lactoferrin receptor" OR "endothelial lectin HL-1" OR "galactofuranose-binding lectin"	Stromal vascular cells in VAT	2001/4	↓	↑		IS	Omentin regulates insulin sensitivity and exerts anti-inflammatory and anti-atherogenic effects.	No	BioVendor	629	426	131	43	15	0	0
Visfatin	OR "PBEF" OR "pre-B-cell colony-enhancing factor 1" OR "Nicotinamide phosphoribosyltransferase" OR "NAMPT"	Adipocytes and macrophages VAT/SAT	1993	↑	↓		IS	Visfatin exerts properties similar to those of insulin.	Yes	BioVendor	2627	1761	426	131	60	3	3
Apelin		Adipocytes	1998	↑			IS	Apelin stimulates glucose uptake and inhibits insulin secretion. Improves insulin sensitivity , oxidative capabilites and mitochondrial biogenesis.	No	NovusBio, ELISAGenie etc.	1659	854	105	35	29	0	0
WISP-1	OR "WNT1-inducible-signaling pathway protein 1" OR "CCN4"	Adipocytes (may be hard to detect in healthy individuals)	2007	↑ OB	N/A	IR		WISP-1 is a pro-inflammatory cytokine that impairs insulin signalling.	No	BioVendor	312	188	10	2	0	0	0
CTRP1	OR "Circulating Complement-C1q TNF-Related Protein-1"	Stromal vascular cells of AT	2004	↑	↑ [acute]		IS	CTRP1 is a paralog of adiponectin. Plausible role in accounting for adiponectin in its absence. Promotes glucose uptake and muscle fatty acid oxidation.	No	BioVendor	61	36	10	4	1	0	0
Adiponectin	Member of CTRP family	Adipose Tissue, Primarily WAT	1995	↓	↑		IS	Adiponectin improves peripheral IS, lipid metabolism and exerts anti-inflammatory and anti-atherogenic effects.	Yes	R&D	20,402	13,364	4713	1924	870	32 [3]	4
Asprosin		White adipose tissue	2016	↑	N/A	IR		Fasting-induced glucogenic hormone - increases glucose release from the liver. Asprosin increases in insulin resistance and promotes inflammation.	No	Abbexa, ELISAGenie	49	16	6	0	0	0	0
Adipsin	OR "Complement Factor D"	Adipocytes and Monocytes/Macrophages	1987	↓	↓ [acute]		IS	Improves pancreatic β-cell function by increasing first-phase insulin secretion - may be a compensatory mechanism in metabolic dysfunction.	No	R&D	795	482	35	8	4	1	0
Resistin		Adipocytes/Monocytes	2001	↑		IR		Produced during adipocyte differentiation and antagonises the action of insulin to reduces glucose uptake in peripheral tissues.	Yes	R&D	4163	2798	1090	312	138	7	1
Leptin		White Adipocytes	1994	↑	↓		IS	Regulates energy homeostasis and improves peripeheral insulin sensitivity and prevents lipid accumulation. Decreases hepatic glucose production (HGP).	Yes	R&D	35,936	19,555	3963	1336	1295	51	18
Vaspin	OR "visceral adipose tissue-derived serine protease inhibitor" OR "Serpin A12"	VAT and SAT	2005	↑	↓		IS	Vaspin has anti-inflammatory and insulin sensitizing effects.	No	BioVendor	414	308	135	57	8	1	0
LCN2	OR "lipocalin-2"	Adipocytes, macrophages and neutrophils	1994	↑	↓	IR		Plays a role in insulin resistance by modulating inflammation - this adopokine is from a large superfamily of proteins including RBP4.	Yes	R&D	3481	2483	66	15	26	0	1

Figure 38. Adipokines included in biomarker selection phase 1 (part A).

Name	Other Name	Organ	Appeared	IR	Exercise	IS or IR?		Main Functions (Summary)	Previously measured in other bed rest studies?	ELISA Kit	PubMed Search 2020 (English Language)						
											Initial	Humans	IR	IS	Exercise	Inactivity	Bed Rest
Adipolin	OR "CTRP12" OR "FAM132A" OR "C1qdc2"	Adipocytes, Exp in WAT	2011	↓	N/A		IS	Anti-inflammatory adipokine that has beneficial effects on glucose metabolism - activates Akt signalling and suppresses gluconeogenesis while increasing glucose uptake in adipocytes.	No	NovusBio	38	23	11	4	0	0	0
Preptin		Pancreatic beta cells	2001	↑	N/A		IS	Co-secreted with insulin and amylin. Preptin is secreted in response to glucose levels and modulates insulin secretion (cells and animals).	No	ELISAGenie, MSC	47	36	8	0	0	0	0
Ghrelin		65%–90% synthesized in the stomach	1999	↓	↑	IR		Increases glucagon and decreases glucose-induced insulin release. Increases HGP and decreases glucose uptake in skeletal muscle and adipose tissue.	Yes	NovusBio, Thermofisher	10531	5963	549	259	432	9	4
PAI-1	OR "plasminogen activator inhibitor-1 " OR "Serpin E1"	Visceral adipocytes, liver, endothelial cells	1963	↑		IR		PAI-1 is upregulated in states of hyperinsulinemia, elevated free fatty acids and hepatic steatosis.	Yes (Hemostatic Purposes)	R&D	14097	10562	807	247	295	10	3
ASP	OR "acylation stimulating protein"	Adipose Tissue	1993	↑	↓		IS	ASP activates TG synthesis through several mechanisms in adipose tissue (independent of insulin).	No	Abbexa	218	164	48	15	5	0	0
DDP4	OR "DDP IV" OR "Dipeptidyl peptidase IV"	Preadipocytes and Adipocytes	1966	↑	↓	IR		DPP4 has a role linking obesity and pathogenesis of T2DM by mediating inflammation and IR in AT and the liver.	Yes	BioVendor	10,222	6,450	347	122	143	3 [1]	0
SFRP4	OR "Secreted frizzled-related protein 4"	Adipose Tissue	1998	↑		IR		SFRP4 has a role in inflammation, defective insulin secretion and angiogenesis.	No	Abbexa, ELISAGenie, MSC	419	283	12	4	2	0	0
SFRP5	OR "Secreted frizzled-related protein 5"	Adipose Tissue		↓ [c]	↑		IS	SFRP5 reduces chronic inflammation and improves insulin sensitivity by blocking Wnt signalling.	No	ELISA Genie, MSC	283	193	26	10	1	0	0
Neuregulin 4	OR "NRG-4"	White/Brown Adipose Tissue	1999	[c]	N/A	IR		Nrg4 exerts an important role in balancing hepatic lipogenesis - ↑ in diet-induced obesity.	No	Abbexa, MSC, ELISA Genie	84	53	13	0	0	0	0
Zinc-alpha2-glycoprotein		SAT	1961	OB ↓	Acute RT ↑		IS	ZAG is an adipokine that acts as a lipid-mobilising factor in adipose tissue. ↓ZAG = accumulation of lipids and metabolic dysfunction. Linked with obesity.	No	BioVendor	175	121	19	12	1	0	0
Nefastin-1	OR "NUCB2"	AT and Muscle	2006	↑ nT2DM, ↓duration	↓		IS	Nesfatin has important effects on food intake and energy homeostasis	No	BioVendor	408	227	34	3	6	0	0
Progranulin	OR "PGRN" OR "Granulin Epithelin Precursor" OR "Proepithelin" OR "Acrogranin"	Adipocytes	1990	↑	↓T2DM	IR		PGRN is related to central obesity and is involved in the regulation of IR and liver dysfunction possibly through inflammation and oxidative/ER stress.	No	BioVendor	1,316	940	27	9	1	0	0
Tissue inhibitor of metalloproteinase	OR "TIMP-1"	Stromal Vascular Cells and Adipocytes.	1970s	↑OB	↔T2DM	IR		TIMP-1 appears to be related to nutritional-induced obesity (adipose tissue functions) and may have a role in CVD risk.	No	R&D	9,731	6,195	59	16	89	5	2

Figure 39. Adipokines included in biomarker selection phase 1 (part B).

Appendix VII: Phase 2 of the Biomarker Selection Process

Phase 2

SELECTION OF NOVEL BIOMARKERS FOR ANALYSIS

MYOKINE

Myonectin/ CTRP15

Maybe

Main Functions:

Myonectin expression is restricted to skeletal muscle and this myokine promotes fatty acid uptake in the adipose tissue and liver.

Reasons For:

- Myonectin is a myokine that plays a role in inter-tissue crosstalk to increase nutrient uptake in peripheral tissues – it enhances fatty acid uptake in hepatocytes and adipocytes.

Reasons Against:

- CTRP15 was found to be homologous with adiponectin in the Q region, and it is thus considered to have a similar roles.
- Myonectin is stimulated by two main factors; exercise and nutrients – the RSL intervention may not be sufficient to alter myonectin.
- Concentrations of NEFA were unchanged following 60 days HDTBR (main analysis and subanalysis groups).
- ST muscle fibers have a higher transcript level of myonectin – CTRL group were found to have ↓type I fibers and ↑hybrid fibers after HDTBR.

MYOKINE

Myostatin

No

Main Functions:

Decreases muscle mass development.

Predominantly measured in muscle.

Reasons For:

- Myostatin prevents muscle mass development by inhibiting myogenesis.
- Decreased muscle mass is a well-known consequence of HDTBR.
- HDT bed rest significantly reduced lean mass, with a higher decline noticeable in the CTRL group (M = -3.91 kg, SE = 0.70 kg, p < 0.001) in comparison to the JUMP group (M = -1.34 kg, SE = 0.32 kg, p = 0.002).
- It is possible that we could see differences in myostatin between the CTRL and JUMP groups following HDTBR, similar to Irimia et al. (2017).

Reasons Against:

- Analysing biomarkers related to changes in muscle mass are not the primary focus of this study.
- Myostatin is commonly measured in muscle and changes in response to HDTBR have been reported in the literature – it is not a novel biomarker.
- The evidence about the influence of myostatin on insulin action and IR is contradictory (also stated by Toloza et al. 2018).

MYOKINE

YKL-40/ CHI3L1

Maybe

Main Functions:

Acute phase reactant which is regulated by pro-inflammatory cytokines. YKL-40 is proposed to counteract TNF-α-induced inflammation and play a role in lipid metabolism, angiogenesis and P13K and Akt signalling.

Reasons For:

- The development of chronic low grade inflammation precedes insulin resistance (however this is more common in obesity!)
- YKL-40 is proposed to counteract inflammation and IR in human skeletal muscle.

Reasons Against:

- YKL-40 is increased in inflammatory conditions – it is induced by pro-inflammatory cytokine.
- YKL-40 is also secreted by endothelial cells and vascular smooth muscle cells and modulates vascular endothelial cell morphology indicating a role in angiogenesis – suggested as a marker of inflammation, angiogenesis and atherosclerosis (Rathcke et al. 2006).
- Unsure of whether YKL-40 is involved in the pathogenesis of T2DM or if it is a general marker of inflammation (Nielson et al. 2008).

MYOKINE

CXCL10/ IP-10

Maybe

Main Functions:

Inflammatory cytokine, induced by interferon-γ (IFN-γ) and liposaccharides, that binds to CXCR3 to mediate immune responses.

Reasons For:

- Supporting evidence: In 2015, Hoff and co-authors measured IP-10 in response to 60 days of HDTBR (BBR2). IP-10 increased during bed rest in the CTRL group but remained stable in the exercise groups (RE + RVE). The authors concluded that this change was indicative of pro-inflammatory conditions in the CTRL group following HDTBR and protective role of training on immunity.

Reasons Against:

- Investigating changes in immune responses are not the primary aim of this study.
- CXCL10 displays a multitude of effects in immunity, angiogenesis and organ-specific metastases of cancer. It is also a risk factor in auto-immune diseases e.g. type 1 diabetes (Ahmadi et al. 2012).

MYOKINE

SPARC

Maybe

Main Functions:

↑leptin, insulin and AT inflammation contribute to ↑ SPARC. SPARC increases SAT fibrosis (growth of AT) thereby ↑lipids in circulation and promotes lipid storage in other organs thus contributing to IR. Related to obesity!

Reasons For:

- SPARC inhibits adipogenesis and contributes to adipose-tissue fibrosis – fibrosis of SAT may restrict TG accumulation in this tissue leading to ↑TG in circulation (systemic hyperlipidaemia), which are diverted for storage in the liver and skeletal muscle (predisposes tissues to IR).
- There was a significant main effect of time for the increase in TG following HDTBR. Subgroup analysis found a significant pre to post-difference in TG in the decreased IS group (↑) only.

Reasons Against:

- SPARC is often found in humans and animals with obesity and insulin resistance – the subjects in the RSL were lean, healthy males!
- SPARC is a multifunctional protein with roles in angiogenesis, osteogenesis, would healing, tumorigenesis and hepatic and renal fibrosis – these roles are unrelated to our research.

MYOKINE

Follistatin Like 1

Maybe

Main Functions:

Follistatin Like-1 is an adipo-myokine that belongs to the SPARC family and has a follistatin like domain. FSTL-1 is implicated in glucose metabolism and inflammation (often in obesity). Underlying mechanisms are poorly understood.

Reasons For:

- In a recent study by Xu et al. (2020), serum levels of FSTL-1 was significantly ↑ in nT2DM (BMI 25.3kg/m2) and positively associated with markers of adiposity, HOMA-IR, lipid profile and glucose metabolism and negatively with adiponectin and HDL. HOMA-IR and FFA were independent factors associated with circulating FSTL-1. EHC and lipid infusion studies in healthy humans provided evidence that hyperinsulinemia and FFA contribute to ↓FSTL-1 → FSTL-1 is associated with IR.
- There was a significant main effect of time for the increase in AUCI and time and intervention for fasting insulin and HOMA-IR following HDTBR. No significant main effects for the change in NEFA.

Reasons Against:

- Mechanistic data is lacking and conflicting on the role of FSTL-1 in glucose metabolism and insulin signalling.
- FSTL-1 is implicated in apoptosis, inflammation and adipogenesis and therefore it functions in many processes related to obesity – proposed that FSTL-1 increases in OW/OB but decreases in morbidly OB.
- FSTL-1 has been shown to increase in response to acute exercise, before decreasing to baseline levels – we may not see a change with RJT!

ADIPOKINE

Visfatin

Maybe

Main Functions:

Visfatin is an adipokine that exerts endocrine, paracrine and autocrine effects. It has a physiological role in lowering plasma glucose by activating insulin signalling, which mimics the action of insulin.

Reasons For:

- Visfatin is a well-studied adipokine, that shares properties with insulin.
- Visfatin has been shown to enhance glucose uptake, inhibit hepatic glucose production, induce triglyceride accumulation and its synthesis from glucose.
- We would expect that with the metabolic dysregulation evident from the RSL study, that we would see a change in visfatin following HDTBR.

Reasons Against:

- Visfatin is not a novel adipokine, however there is a large body of literature that can be used to help explain our findings.
- Visfatin has been measured in response to bed rest previously.
  - Jurdana et al. (2015): Serum visfatin was lower in younger compared to older individuals at baseline. After 14 days BR, visfatin concentrations increased in the younger subjects but did not change in older adults. In all participants, serum visfatin positivelyy associated with IL-6.
  - Rudwill et al. (2013): Visfatin increased by 50% in response to 3 months HDTBR, but did not change with HDTBR + EXE. There was no change in visfatin in response to a 1 month detraining, while 10 days confinement led to a 55% increase in visfatin. All experiments were conducted in healthy, NW men.

ADIPOKINE

WISP-1

No

Main Functions:

WISP-1 is a pro-inflammatory cytokine that impairs insulin signalling. WISP-1 is increased in obesity, but levels do not differ between diabetics and non-diabetics – a possible marker of impaired AT homeostasis and function.

Reasons For:

- WISP-1 is a pro-inflammatory cytokine that has been shown to inhibit insulin-mediated Akt phosphorylation in cell models.
- Fetuin-A inhibits insulin receptor auto-phosphorylation and tyrosine kinase activity leading to decreased downstream insulin signalling – we would expect that this biomarker would be increased and opposing changes would be noticeable in the subgroups!

Reasons Against:

- In a sub-cohort of healthy individuals, WISP-1 was only detected in 13% of subjects (27/207, BMI 26.5kg/m2, age 56 years). In another sub-cohort of subjects with different levels of glucose intolerance, WISP-1 detection was 58% (BMI 31.6kg/m2, age 59 years). Therefore, levels may be more detectable in those with increased metabolic dysregulation. In another study, two subjects had WISP-1 levels under the limit of detection too.
- WISP-1 is an adipocyte derived target/regulator of the Wnt pathway – due to the complexity of this pathway, the exact mechanisms by which WISP-1 inhibits insulin action haven't been determined. These proteins are known to be altered in obesity too.

ADIPOKINE

Adiponectin

Yes

Main Functions:

Adiponectin improves peripheral IS, lipid metabolism and exerts anti-inflammatory and antiatherogenic effects.

Reasons For:

- Adiponectin is one of the most well-studied adipokines and plays a role in protecting against insulin resistance and cardiovascular disease.
- Interaction of adiponectin with its receptors (AdipoR1 and AdipoR2) results in the activation of multiple signalling pathways (IRS1/2, AMPK and p38 MAPK) to sensitize insulin action, improve glucose uptake and increase FA oxidation.
- Correlates with numerous other biomarkers and has been measured in bed rest before. Fetuin-A is proposed to inhibit the production of adiponectin.

Reasons Against:

- Adiponectin cannot be classified as a novel biomarker and has been measured in response to bed rest.
  - Jurdana et al. (2015): Basal adiponectin was significantly lower in younger compared to older adults. In response to 14 days BR, serum adiponectin increased in young and older subjects. Δadiponectin was negatively correlated with ΔTNF-α in young adults, and Δresistin in older adults.
  - Hojbjerre et al. (2011): After 10 days BR, FDR of patients with T2DM decreased plasma adiponectin [basal ad insulin-stimulated] while control subjects increased plasma adiponectin [insulin-stimulated only].

ADIPOKINE

Adipsin

No

Main Functions:

Improves pancreatic B-cell function by increasing first-phase insulin secretion - may be a compensatory mechanism in metabolic dysfunction.

Reasons For:

- First-phase insulin secretion occurs when the β-cell is exposed to rapid changing glucose levels. This leads to a prompt inhibition of EGP in the liver to restrain glucose levels.
- Following 60 days of bed rest, there was a significant main effect of time, and a significant main effect of intervention, for liver insulin sensitivity (higher overall in CTRL).
- Liver insulin sensitivity was decreased in the subgroup with decreased WBIS and increased in the subgroup with increased WBIS.

Reasons Against:

- Adipsin was the first adipokine described and therefore it cannot be considered as a novel biomarker.
- ASP is produced via the activation of the complement alternative pathway by the interaction of complement factors C3, Factor B, and adipsin.

ADIPOKINE

ASP

Yes

Main Functions:

ASP activates TG synthesis in adipose tissue (independent of insulin).

Reasons For:

- ASP regulates TG synthesis in the adipose tissue via 3 different mechanisms (Saleh et al. 2019).
  - (1) activating glucose uptake to provide glycerol backbone for TG,
  - (2) enhancing FA esterficiation by activating DGAT - key enzyme for TG synthesis,
  - (3) inhibits HSL and intracellular lipolysis.
- There was a significant main effect of time for the increase in TG following HDTBR. Subgroup analysis found a significant pre to post-difference in TG in the decreased IS group (↑) only.

Reasons Against:

- Metabolic disturbances may overcome the role of ASP, i.e. high ASP levels could represent ASP resistance.
- ASP is produced via the activation of the complement alternative pathway by the interaction of complement factors C3, Factor B, and adipsin.
- Elevated ASP in obesity could be related to increased fat mass – our subjects are lean, healthy males.

ADIPOKINE

Resistin

Maybe

Main Functions:

Produced during adipocyte differentiation and antagonises the action of insulin to reduces glucose uptake in peripheral tissues.

Reasons For:

- Resistin exerts pro-inflammatory effects and induces insulin resistance. Resistin exerts its potent metabolic actions on the liver, skeletal muscles, and adipocytes through multiple peripheral and central mechanisms (especially through NF-kB activation). Resistin is upregulated by CRP, IL-1, IL-12 and TNF-a (through the induction of NF-kB) or other pro-inflammatory stimuli. It can also enhance the Th1 pro-inflammatory immune response.. It is possible that the pro-inflammatory effects of resistin may be mediated by its binding to TLR4.
- If resistin is elevated during bed rest, this could signify an increase pro-inflammatory response to bed rest which may impair insulin signaling in peripheral tissues.

Reasons Against:

- Resistin is not a novel biomarker and has been measured in response to bed rest previously.
  - Jurdana et al. (2015): Resistin levels were lower in younger, compared to older subjects. Young subjects significantly increased resistin following 14 days BR, while there was no differences in resistin in the older subjects.

ADIPOKINE

PAI-1

Maybe

Main Functions:

PAI-1 is an endogenous inhibitor of fibrinolysis but it has also been implicated in metabolic dysfunction.

Reasons For:

- It has been proposed that although it might take insulin resistance/chronic hyperinsulinemia and hepatic steatosis to ↑PAI-1 concentrations, it is insulin resistance, with the compensatory ↑ in insulin leading to an ↑ in liver fat that is common amongst this relationship.
- In the fetuin-A paper, we proposed that the improvement in WBIS may be an indirect effect of liver metabolism. The amount of intra-hepatic lipid is strongly linked to liver and WBIR.

Reasons Against:

- Plasminogen activator inhibitor-1 (PAI-1) is an acute phase protein and the primary regulator of fibrinolysis – it is often implicated in liver fibrosis.
- Adipose tissue, liver and the endothelium are important sources of PAI-1 and its production can be stimulated by numerous different molecules e.g. insulin, proinsulin, IGF-1, TNF-α etc.

ADIPOKINE

Adipolin/ CTRP12

Maybe

Main Functions:

Anti-inflammatory adipokine that has beneficial effects on glucose metabolism - activates Akt signalling and suppresses gluconeogenesis while increasing glucose uptake in adipocytes.

Reasons For:

- Adipolin is an endocrine factor secreted by adipocytes that circulates in plasma and that preferentially acts on adipose tissue and liver to modulate whole body glucose metabolism. Low CTRP12 levels reflect insulin resistance.
- Weng et al. (2012): *In vivo* studies on genetic and diet-induced obese mice found that CTRP12 overexpression can improve insulin sensitivity by enhancing insulin signalling in the adipose tissue and liver. *In vitro* studies indicate that CTRP12 activates P13k-Akt signalling, independent on insulin, to suppress gluconeogenesis in hepatocytes and promote glucose uptake in adipocytes.
- Dysregulation of these mechanisms may support the increase in fetuin-A following prolonged HDTBR.

Reasons Against:

- Adipolin is part of the CTRP family, however it is considered as a distant relation of other CTRPs and adiponectin – it has limited sequence similarity to other CTRPs including adiponectin (21%).

ADIPOKINE

SFRP5

No

Main Functions:

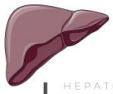
SFRP5 reduces chronic inflammation and improves insulin sensitivity by blocking Wnt signalling.

Reasons For:

- SFRP5 is expressed in white adipose tissue and has been found to associate with glucose metabolism and insulin sensitivity.
- WNT5A is a glycoprotein that increases inflammatory responses by activating the non-canonical WNT signalling pathway. SFRP5 connects to the frizzled receptors competing with WNT5A and inhibits the non-canonical WNT signalling. Therefore, ↑ the expression of WNT5A along with ↓ the expression of SFRP5 leads to an ↑ in inflammatory symptoms and IR (+ ↑ fat deposition).
  - Mediated by activating the JNK pathway and serine phosphorylation of insulin receptor substrate.

Reasons Against:

- As mentioned in articles relating to WISP-1, WNT signalling is a very complex pathway and is dysregulated in obesity.



HEPATOKINE

HFREP1

Maybe

Main Functions:

In cell and animal studies, HFREP1 plays a role in the development of IR through the ERK1/2 pathway.

Reasons For:

Wu et al. (2016) conducted human, animal and cell studies to identify to the role of HFREP1 in the development of IR. Plasma HFREP1 concentrations were significantly ↑ in participants with IFG, IGT and NDD and were independently associated with FPG, HOMA-IR, prediabetes and diabetes. In mice, HFREP1 impaired glucose utility and induced IR. HFREP1 impaired insulin signalling in both the liver and muscle. Analysis of HFREP1 in the cell model found that HREP1 blocked insulin signalling through an ERK1/2-dependent pathway in primary hepatocytes. Wu et al. (2013): HFREP1 could contribute to hepatic steatosis through this pathway also.

- Relatable to fetuin-A: High glucose concentrations can upregulate fetuin-A secretion through the ERK1/2 pathway.

Reasons Against:

- HFREP1 is structurally similar to ANGPTL proteins.

HEPATOKINE

Follistatin

No

Main Functions:

Increases muscle mass by inhibiting myostatin and activin A, promotes β-cell survival and proliferation.

Reasons For:

- Follistatin-induced muscle growth is regulated by its inhibitory action on myostatin and activin A, leading to diminished phosphorylation of Smad3 and upregulation of the Akt/mTOR signalling pathway.
- Prolonged HDTBR leads to a significant ↓ in muscle mass (and ↑ myostatin) and therefore it is expected that follistatin would ↓.
- As circulating follistatin is ↑ in states of energy deprivation i.e. conditions associated with ↑ gluconeogenesis – follistatin may act in a negative feedback loop to regulate gluconeogenesis. In addition, animal studies suggest that follistatin may ↓ hepatic lipid uptake and synthesis.

Reasons Against:

- In humans, the glucagon-to-insulin ratio is a key determinant of circulating follistatin levels – increased ratio in response to prolonged fasting and exercise. However, there was no change in follistatin found in response to acute glucose or insulin concentrations in healthy subjects (n=5).
- In healthy controls, follistatin did not correlated with any physical or metabolic characteristics. However in T2DM, follistatin correlated positively with FPG, HbA1c, CHOL and TG. Multivariable analysis found that 2 hour glucose and CHOL were independent predictors of follistatin in T2DM (Hansen et al. 2013).

HEPATOKINE

Adropin

Maybe

Main Functions:

Regulation of energy homeostasis and controls glucose and fatty acid metabolism. Decreases HGP and increases liver insulin sensitivity.

Reasons For:

- Adropin is involved in the regulation of metabolic homeostasis and is important for maintaining insulin sensitivity and preventing dyslipidaemia.
- Adropin regulates the expression of PPAR-γ, gene related to FA metabolism – regulates macrophage polarization and can reduce fat accumulation and inflammation in adipocytes.
- In a pooled sample (n=130), the association between plasma adropin and fasting TG persisted when age and BMI were controlled for (r = -0.305, p = 0.003) suggesting that adropin impacts TG metabolism (synthesis or clearance).

Reasons Against:

- Adropin is encoded by the Energy Homeostasis Associated gene.
- Liver Enho expression is regulated by energy status and dietary nutrient content, and is altered with obesity (Kumar et al. 2008).
  - Butler et al. (2012) reported no circadian, meal or fasting related changes suggested adropin is not regulated by acute changes.

HEPATOKINE

LECT2

Maybe

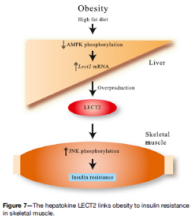


Figure 7—The hepatokine LECT2 links obesity to insulin resistance in skeletal muscle.

Main Functions:

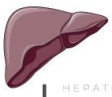
Associated with obesity and IR. Increases in obesity and fatty liver and shown to have a link with obesity and skeletal muscle IR.

Reasons For:

- LECT2 is a hepatokine that impairs insulin signalling in skeletal muscle, by activating JNK. In animal models, AMPK negatively regulates LECT2.
  - Commonly related to obesity, as well as IR.
- In a sample of 200 individuals (BMI 22.9kg/m2, HOMA-IR 1.4) significant positive correlations were found between serum LECT2 and BMI, WC, HOMA-IR, HbA1c, SBP, SeP (hepatokine that induces IR) and negative correlations with Matsuda index (Lan et al. 2014). In another study, HDL, HOMA-IR, BMI, FINS and TG were significant independent determinants of LECT2 (Zhang et al. 2017).
- AMPK is reduced by physical inactivity (Bergouignan et al. 2011).
  ↓AMPK → ↑LECT2 → ↑JNK → ↑ IR in skeletal muscle (possible effects on lipids too)

Reasons Against:

- Unsure of the effect of energy balance during bed rest on AMPK – we have no measurements of hepatic AMPK.



HEPATOKINE

ANGPTL3

Maybe

Main Functions:

ANGPTL3 regulates TG levels by inhibiting LPL and may induce deterioration of glucose metabolism by increasing lipolysis in AT. Functionality depends on ANGPTL8 (increased with both)

True hepatokine of the ANGPTL family.

Reasons For:

- ANGPTL3 is a hepatokine that modulates the metabolism of TG-rich lipoproteins by inhibiting the activity of lipoprotein lipase (LPL). ANGPTL3 also stimulates lipolysis of adipose tissue and FFA release into the circulation, which can also affect hepatic IR. Animal studies have also proposed that ANGPTL3 inhibits the activity of endothelial lipase, an endothelial lipase implicated in the catabolism of HDL particles.
- Measured in response to HDTBR: Rudwill et al. (2015).
  - CTRL group ANGPTL3 at HDT60 (+29%; P = 0.02)
  - Moderate changes might show that volunteers did not reach severe stages of NAFLD and ↑TG may be due to defects in fat uptake at the muscle level.

Reasons Against:

- In mice, the N-terminal region of ANGPTL3 has been identified to be essential for the regulation of TG, while the C-terminal region containing a fibrinogen-like domain is important for angiogenesis.
- In some studies, the level of ANGPTL3 in plasma does not seem to correlate with plasma TG concentrations, which may be due to the samples being collected in the fasted state – ANGPTL3 expression does not seem to be substantially affected by fasting/refeeding but its activity on LPL may be more pronounced in the fed or re-fed state.
  - However, other studies have reported correlations with the lipid profile.

HEPATOKINE

ANGPTL4

No

Main Functions:

Closely related to ANGPTL3. Involved in the regulation of TG and inhibition of LPL (WAT).

Reasons For:

- Alike ANGPTL3, ANGPTL4 inactivates LPL via similar but no completely identical mechanisms.
- ANGPTL4 is the central component of a fatty acid driven feedback mechanism that regulates plasma TG hydrolysis and subsequent tissue fatty acid uptake in response to changes in lipid availability and cellular fuel demand (i.e. during fasting, cold exposure and exercise). Expression of ANGPTL4 is sensitively regulated by the fatty acid-activated PPAR-γ – feedback mechanism to prevent lipotoxicity – and can be suppressed by AMPK.
- It is has also been reported that insulin suppresses ANGPTL4 expression and glucocorticoids increase ANGPTL4 expression.

Reasons Against:

- It has been reported as pro- and anti-angiogenic protein and proposed to play a role as gatekeeper regulating vascular integrity and angiogenesis in a context-dependent manner – role still under investigation.
- ANGPTL4 is the best-studied member of ANGPTL proteins family and it exhibits a widespread distribution of tissue expression.
  - ANGPTL4 is highly expressed in liver and adipose tissue and to a lesser extent in heart, skeletal muscle, intestine and several other tissues – it is not liver specific.

HEPATOKINE

Betatrophin/ ANGPTL8

Maybe

Main Functions:

Inhibits LPL and regulated TG levels (muscle and BAT), and is associated with HDL.

Reasons For:

- ANGPTL3 serves as a binding partner and activator of ANGPTL8/betatrophin, which inhibits LPL thereby regulating TG levels. ↓fasting and ↑post-prandial state – stress and insulin can increase ANGPTL8.
- Irisin has been proposed to promote the expression of betatrophin – PCG-1α through a ↑FNDC5 secretes irisin, which acts on WAT to stimulate UCP1. This promotes the expression of betatrophin and ↓IR. The opposite effect is proposed to happen in aging and IR.
  - However, betatrophin shown to increase, while irisin was significantly decreased in T2DM compared to NGT, matched for age, sex and BMI.

Reasons Against:

- Some studies have mentioned that some of contradictory findings on betatrophin may be due to the lack of specificity of the ELISA's (full-length or N-terminal only), duration of long term storage and freeze-thaw cycles.

HEPATOKINE

SEPP-1

Maybe

Main Functions:

Selenoprotein-P inhibits the insulin signalling pathway by inactivating AMPK.

Reasons For:

- Selenoprotein-P is the most abundant selenoprotein in plasma and is primarily produced in the liver. The expression of SELENOP is regulated by FoxOs and SREBP-1. SeP expression is regulated positively with glucose and saturated FAs and negatively with insulin and cytokine levels (IL-1b, TNF-a, IFN-γ, and TGF-β).
- SeP has been found to be independently associated with adiponectin and insulin resistance (others reported obesity rather than IR ↑SeP)
- It is believed that SeP causes insulin resistance in the liver, at least in part, by inactivating AMPK.
  - AMPK is reduced by physical inactivity (Bergouignan et al. 2011).

Reasons Against:

- Unsure of the effect of energy balance during bed rest on AMPK – we have no measurements of hepatic AMPK.
- Mechanism responsible for SeP-induced IR in skeletal muscle is not known.

HEPATOKINE

Fetuin-B

No

Main Functions:

Fetuin-B decreases glucose uptake in myotubes and impaired insulin action in hepatocytes.

Reasons For:

- Fetuin-B secretion from the liver is increased by steatosis and diminishes glucose lowering through insulin-independent mechanisms.

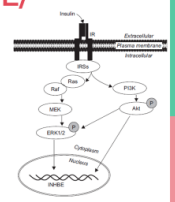
Reasons Against:

- Fetuin-B presents 22% homology with fetuin A.
  - Circulating and mRNA expression of these hepatokines has been shown to correlate, albeit weakly, with each other.
- Mechanism of action by which fetuin-B modulates glucose homeostasis is unknown.
  - Fetuin-B does not impact pro-inflammatory signalling, adipocyte lipolysis, fatty acid metabolism in myotubes and hepatocytes or AMPK signalling in the skeletal muscle and liver.
- Fetuin-B levels have been found to be higher in women than men.

HEPATOKINE

Inhibin-βE/ Activin E

No



Main Functions:

Inhibin-βE is a hepatokine that plays a role in glucose metabolism.

Reasons For:

- INHBE mRNA is upregulated in humans with insulin resistance and obesity. Furthermore, the upregulation of INHBE mRNA expression was also found in an animal model of obese insulin resistance (Sugiyama et al. 2018).
- In vitro studies have shown that insulin can stimulate the expression of INHBE suggesting that this hepatokine plays a role in glucose metabolism (Hashimoto et al. 2009. Ramalingam et al. 2016).

Reasons Against:

- Multiple reported functions of INHBE/Activin E are related to obesity.
  - Recent animal studies have reported that activin E can stimulate EE through brown and beige adipocyte activation leading to ↑ glucose tolerance and insulin sensitivity in mice with active E overexpression (Hashimoto et al. 2018. Sekiyama et al. 2019). FGF-21 was ↑ in the transgenic mice.
  - Preliminary in vivo siRNA study analysing whole-body metabolic function suggest the possibility that the hepatokine INHBE ↓fat utilization and ↑ fat mass in the mice model of obesity.
- There is limited research on Activin E and it is not well studied in humans – future studies are needed.

HEPATOKINE

ANGPTL6

No

Main Functions:

ANGPTL6 is a hepatokine that antagonizes obesity and insulin resistance by increasing energy expenditure.

Reasons For:

- ANGPTL6 has been shown to be increased in type 2 diabetes compared to non-diabetic controls (Ebert et al. 2009. Qaddoumi et al. 2020).
  - *For example, in a sample of 60 patients on chronic hemodialysis (CD; 32 diabetic and 28 nondiabetic subjects) and 60 controls (30 diabetic and 30 nondiabetic subjects) with a glomerular filtration rate (GFR) greater than 50 mL/min, fasting glucose positively predicted circulating AGF/ANGPLT6 independent of age, sex, GFR and BMI. AGF levels were sig. higher in T2DM and non-T2DM.*

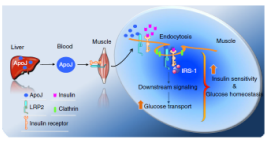
Reasons Against:

- ANGPTL6, also known as angiopoietin-related growth factor (AGF), is a hepatocyte-derived angiogenic factor that promotes angiogenesis.
- ANGPTL6 has been proposed as a modulator of energy metabolism and adiposity in animal studies. In vivo and in vitro models have shown that leptin regulates the hepatic expression of ANGPTL6, with serum levels dependent on the status of metabolic stress induced by HFD or exercise.
  - We have no measurements of serum leptin and our lean, healthy males were in energy balance throughout HDTBR.
- Upregulation of serum ANGPTL6 levels in humans with metabolic disease may be a compensatory increase or resistance, similar to that of insulin and leptin – however these scenarios have not been studied.

HEPATOKINE

Apo-J

Maybe



Main Functions:

Found in HDL LDL and VLDL. ApoJ has a key role as a co-factor in coordinating insulin-mediated signalling events - absence or high levels impair insulin signalling.

Reasons For:

- ApoJ acts as a hepatokine that plays a pivotal role in modulating muscle glucose metabolism through LRP2, highlighting an inter-organ communication network between liver and muscle.
  - This is either through a defect in the internalisation of the IR or ApoJ/LRP2 modulates P13K signalling – *studies in mice.*
- ApoJ has been found to negatively regulate gene expression of SREBP1c, a master regulator of the lipogenic pathway but the importance of hepatic ApoJ requires further investigation.
- ApoJ levels were a significant independent association factor for fasting insulin and HOMA-IR after adjusting for age, sex and BMI in a group of ND and T2DM.

Reasons Against:

- Seo and authors (2020) propose that a certain level of ApoJ in circulation is required to achieve glucose homeostasis, such that if ApoJ is beyond a certain threshold or lack of ApoJ, this hepatokine could desensitize insulin action.

HEPATOKINE

FGF-21

Maybe

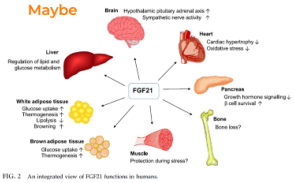


FIG. 2 An integrated view of FGF21 functions in humans.

Main Functions:

FGF-21 is an endocrine factor secreted by the liver which acts as a metabolic regulator.

Reasons For:

- Fibroblast growth factor 21 (FGF21) is a hormone that regulates important metabolic pathways. Considering the beneficial effects of FGF-21 in murine glucose and lipid metabolism, it is unexpected that levels of FGF-21 are increased in metabolic dysfunction. This has prompted the hypothesis of FGF-21 resistance, which is similar to insulin and leptin resistance.
- Elevated fasting FGF-21 levels in disease states characterised by increased liver fat.
  - Staiger et al. (2017): The strongest BMI-independent determinant of hepatic FGF-21 expression and circulating FGF-21 concentrations is liver fat content [review of 4 studies].

Reasons Against:

- The FGF21 function is complicated and well debated due to its different sites of production and action and different mechanisms are proposed in animals and humans.
- FFA and PPARα appear to be the most important regulators of FGF21 under physiological energy balance in humans. Fasting but not post-prandial FFAs are closely linked to human FGF-21 concentrations.
  - We saw no change in NEFA concentrations in response to HDTBR.

Appendix VIII: Phase 3 of the Biomarker Selection Process

Phase 3

SELECTION OF NOVEL BIOMARKERS FOR ANALYSIS

MYOKINE

Myonectin/ CTRP15

No – as we are analysing adiponectin.

Main Functions:

Myonectin expression is restricted to skeletal muscle and this myokine promotes fatty acid uptake in the adipose tissue and liver.

Reasons For:

- Myonectin is a myokine that plays a role in inter-tissue crosstalk to increase nutrient uptake in peripheral tissues – it enhances fatty acid uptake in hepatocytes and adipocytes.

Reasons Against:

- CTRP15 was found to be homologous with adiponectin in the Q region, and it is thus considered to have a similar roles.
- Myonectin is stimulated by two main factors; exercise and nutrients – the RSL intervention may not be sufficient to alter myonectin.
- Concentrations of NEFA were unchanged following 60 days HDTBR (main analysis and subanalysis groups).
- ST muscle fibers have a higher transcript level of myonectin – CTRL group were found to have ↓type I fibers and ↑hybrid fibers after HDTBR.

MYOKINE

YKL-40/ CHI3L1

No – inflammatory markers.

Main Functions:

Acute phase reactant which is regulated by pro-inflammatory cytokines (TNF-a, IL-6, IL-1B and IFN-γ).

Reasons For:

- The development of chronic low grade inflammation precedes insulin resistance (however this is more common in obesity!).
- ↑ glucose and FFA mediate the expression of inflammatory mediators through TLR dependent pathways (TLR2 and TLR4), indicating that the state of IR may be enabled by TLR signaling. Fetuin-A has been shown to be an endogenous ligand for TLR4. However, so far the receptors that mediate the effects CHI3L1 have not been defined, and only a potential link between TLR4-and CHI3L1-signalling pathways has been reported.
- In another study, YKL-40 was proposed to counteract inflammation and IR in human skeletal muscle.

Reasons Against:

- YKL-40 is also secreted by endothelial cells and vascular smooth muscle cells and modulates vascular endothelial cell morphology indicating a role in angiogenesis – suggested as a marker of inflammation, angiogenesis and atherosclerosis (Rathcke et al. 2006).
- Unsure of whether YKL-40 is involved in the pathogenesis of T2DM or if it is a general marker of inflammation (Nielson et al. 2008).

MYOKINE

CXCL10/ IP-10

No – inflammatory markers.

Main Functions:

IP-10 is an inflammatory cytokine, induced by interferon-γ (IFN-γ) and liposaccharides, that binds to CXCR3 to mediate immune responses.

Reasons For:

- Supporting evidence: In 2015, Hoff and co-authors measured IP-10 in response to 60 days of HDTBR (BBR2). IP-10 increased during bed rest in the CTRL group but remained stable in the exercise groups (RE + RVE). The authors concluded that this change was indicative of pro-inflammatory conditions in the CTRL group following HDTBR and protective role of training on immunity.
- IP-10 appears to be a marker of progressive liver injury and a sensitive marker for the need of a liver biopsy.
- **In vitro studies found** that insulin resistance encouraged IP-10 production during monocyte stimulation by TLR ligands, especially TLR2 ligands (could be related to IKK and JNK pathways).

Reasons Against:

- CXCL10 displays a multitude of effects in immunity, angiogenesis and organ-specific metastases of cancer. It is also a risk factor in auto-immune diseases e.g. type 1 diabetes (Ahmadi et al. 2012).

MYOKINE

SPARC

No

Main Functions:

↑leptin, insulin and AT inflammation contribute to ↑ SPARC. SPARC increases SAT fibrosis (growth of AT) thereby ↑lipids in circulation and promotes lipid storage in other organs thus contributing to IR. Related to obesity!

Reasons For:

- SPARC inhibits adipogenesis and contributes to adipose-tissue fibrosis – fibrosis of SAT may restrict TG accumulation in this tissue leading to ↑TG in circulation (systemic hyperlipidaemia), which are diverted for storage in the liver and skeletal muscle (predisposes tissues to IR).
- There was a significant main effect of time for the increase in TG following HDTBR. Subgroup analysis found a significant pre to post-difference in TG in the decreased IS group (↑) only.

Reasons Against:

- SPARC is often found in humans and animals with obesity and insulin resistance – the subjects in the RSL were lean, healthy males!
- SPARC is a multifunctional protein with roles in angiogenesis, osteogenesis, would healing, tumorigenesis and hepatic and renal fibrosis.

MYOKINE

Follistatin Like 1

No

Main Functions:

Follistatin Like-1 is an adipo-myokine that belongs to the SPARC family and has a follistatin like domain. FSTL-1 is implicated in glucose metabolism and inflammation (often in obesity). Underlying mechanisms are poorly understood.

Reasons For:

- In a recent study by Xu et al. (2020), serum levels of FSTL-1 was significantly ↑ in nT2DM (BMI 25.3kg/m2) and positively associated with markers of adiposity, HOMA-IR, lipid profile and glucose metabolism and negatively with adiponectin and HDL. HOMA-IR and FFA were independent factors associated with circulating FSTL-1. EHC and lipid infusion studies in healthy humans provided evidence that hyperinsulinemia and FFA contribute to ↑FSTL-1 → FSTL-1 is associated with IR.
- There was a significant main effect of time for the increase in AUCI and time and intervention for fasting insulin and HOMA-IR following HDTBR. No significant main effects for the change in NEFA.

Reasons Against:

- Mechanistic data is lacking and conflicting on the role of FSTL-1 in glucose metabolism and insulin signalling.
- FSTL-1 is implicated in apoptosis, inflammation and adipogenesis and therefore it functions in many processes related to obesity – proposed that FSTL-1 increases in OW/OB but decreases in morbidly OB.
- FSTL-1 has been shown to increase in response to acute exercise, before decreasing to baseline levels – we may not see a change with RJT!

ADIPOKINE

Visfatin

No

Main Functions:

Visfatin is an adipokine that exerts endocrine, paracrine and autocrine effects. It has a physiological role in lowering plasma glucose by activating insulin signalling, which mimics the action of insulin.

Reasons For:

- Visfatin is a well-studied adipokine, that shares properties with insulin.
- Experiments in cells and mice have shown that visfatin can bind and activate insulin signalling but does so at a different site to insulin. However, it follows the same cascade upon activation (phosphorylation of IR, IRS-1, IRS-2, binding of P13kinase to IRS-1 and IRS-2 and phosphorylation of Akt and MAPK).
- We would expect that with the metabolic dysregulation evident from the RSL study, that we would see a change in visfatin following HDTBR.

Reasons Against:

- Visfatin is not a novel adipokine, however there is a large body of literature that can be used to help explain our findings.
- Visfatin has been measured in response to bed rest previously.
  - Jurdana et al. (2015): Serum visfatin was lower in younger compared to older individuals at baseline. After 14 days BR, visfatin concentrations increased in the younger subjects but did not change in older adults. In all participants, serum visfatin positivelyy associated with IL-6.
  - Rudwill et al. (2013): Visfatin increased by 50% in response to 3 months HDTBR, but did not change with HDTBR + EXE. There was no change in visfatin in response to a 1 month detraining, while 10 days confinement led to a 55% increase in visfatin. All experiments were conducted in healthy, NW men.

ADIPOKINE

Resistin

No

Main Functions:

Produced during adipocyte differentiation and antagonises the action of insulin to reduces glucose uptake in peripheral tissues.

Reasons For:

- Resistin exerts pro-inflammatory effects and induces insulin resistance. Resistin exerts its potent metabolic actions on the liver, skeletal muscles, and adipocytes through multiple peripheral and central mechanisms (especially through NF-kB activation). Resistin is upregulated by CRP, IL-1, IL-12 and TNF-a (through the induction of NF-kB) or other pro-inflammatory stimuli. It can also enhance the Th1 pro-inflammatory immune response.. It is possible that the pro-inflammatory effects of resistin may be mediated by its binding to TLR4.
- If resistin is elevated during bed rest, this could signify an increase pro-inflammatory response to bed rest which may impair insulin signaling in peripheral tissues.

Reasons Against:

- Resistin is not a novel biomarker and has been measured in response to bed rest previously.
  - Jurdana et al. (2015): Resistin levels were lower in younger, compared to older subjects. Young subjects significantly increased resistin following 14 days BR, while there was no differences in resistin in the older subjects.

ADIPOKINE

PAI-1

No

Main Functions:

PAI-1 is an endogenous inhibitor of fibrinolysis but it has also been implicated in metabolic dysfunction.

Reasons For:

- It has been proposed that although it might take insulin resistance/chronic hyperinsulinemia and hepatic steatosis to ↑PAI-1 concentrations, it is insulin resistance, with the compensatory ↑ in insulin leading to an ↑ in liver fat that is common amongst this relationship.
- In the fetuin-A paper, we proposed that the improvement in WBIS may be an indirect effect of liver metabolism. The amount of intra-hepatic lipid is strongly linked to liver and WBIR.

Reasons Against:

- Plasminogen activator inhibitor-1 (PAI-1) is an acute phase protein and the primary regulator of fibrinolysis – it is often implicated in liver fibrosis.
- Adipose tissue, liver and the endothelium are important sources of PAI-1 and its production can be stimulated by numerous different molecules e.g. insulin, proinsulin, IGF-1, TNF-α etc.

ADIPOKINE

Adipolin/ CTRP12

No

Main Functions:

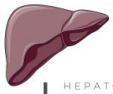
Anti-inflammatory adipokine that has beneficial effects on glucose metabolism - activates Akt signalling and suppresses gluconeogenesis while increasing glucose uptake in adipocytes.

Reasons For:

- Adipolin is an endocrine factor secreted by adipocytes that circulates in plasma and that preferentially acts on adipose tissue and liver to modulate whole body glucose metabolism. Low CTRP12 levels reflect insulin resistance.
- Weng et al. (2012): *In vivo* studies on genetic and diet-induced obese mice found that CTRP12 overexpression can improve insulin sensitivity by enhancing insulin signalling in the adipose tissue and liver. *In vitro* studies indicate that CTRP12 activates P13k-Akt signalling, independent on insulin, to suppress gluconeogenesis in hepatocytes and promote glucose uptake in adipocytes.
- Dysregulation of these mechanisms may support the increase in fetuin-A following prolonged HDTBR.

Reasons Against:

- Adipolin is part of the CTRP family, however it is considered as a distant relation of other CTRPs and adiponectin – it has limited sequence similarity to other CTRPs including adiponectin (21%).



HEPATOKINE

HFREP1

No

Main Functions:

In cell and animal studies, HFREP1 plays a role in the development of IR through the ERK1/2 pathway.

Reasons For:

Wu et al. (2016) conducted human, animal and cell studies to identify to the role of HFREP1 in the development of IR. Plasma HFREP1 concentrations were significantly ↑ in participants with IFG, IGT and NDD and were independently associated with FPG, HOMA-IR, prediabetes and diabetes. In mice, HFREP1 impaired glucose utility and induced IR. HFREP1 impaired insulin signalling in both the liver and muscle. Analysis of HFREP1 in the cell model found that HREP1 blocked insulin signalling through an ERK1/2-dependent pathway in primary hepatocytes. Wu et al. (2013): HFREP1 could contribute to hepatic steatosis through this pathway also.

- Relatable to fetuin-A: High glucose concentrations can upregulate fetuin-A secretion through the ERK1/2 pathway.

Reasons Against:

- HFREP1 is structurally similar to ANGPTL proteins.

HEPATOKINE

Adropin

Yes

Main Functions:

Regulation of energy homeostasis and controls glucose and fatty acid metabolism. Decreases HGP and increases liver insulin sensitivity.

Reasons For:

- Adropin is involved in the regulation of metabolic homeostasis and is important for maintaining insulin sensitivity and preventing dyslipidaemia.
- Adropin regulates the expression of PPAR-γ, gene related to FA metabolism – regulates macrophage polarization and can reduce fat accumulation and inflammation in adipocytes.
- In a pooled sample (n=130), the association between plasma adropin and fasting TG persisted when age and BMI were controlled for (r = -0.305, p = 0.003) suggesting that adropin impacts TG metabolism (synthesis or clearance).

Reasons Against:

- Adropin is encoded by the Energy Homeostasis Associated gene.
- Liver Enho expression is regulated by energy status and dietary nutrient content, and is altered with obesity (Kumar et al. 2008).
  - Butler et al. (2012) reported no circadian, meal or fasting related changes suggested adropin is not regulated by acute changes.

HEPATOKINE

LECT2

No

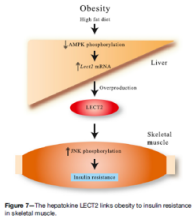


Figure 7—The hepatokine LECT2 links obesity to insulin resistance in skeletal muscle.

Main Functions:

Associated with obesity and IR. Increases in obesity and fatty liver and shown to have a link with obesity and skeletal muscle IR.

Reasons For:

- LECT2 is a hepatokine that impairs insulin signalling in skeletal muscle, by activating JNK. In animal models, AMPK negatively regulates LECT2.
  - Commonly related to obesity, as well as IR.
- In a sample of 200 individuals (BMI 22.9kg/m2, HOMA-IR 1.4) significant positive correlations were found between serum LECT2 and BMI, WC, HOMA-IR, HbA1c, SBP, SeP (hepatokine that induces IR) and negative correlations with Matsuda index (Lan et al. 2014). In another study, HDL, HOMA-IR, BMI, FINS and TG were significant independent determinants of LECT2 (Zhang et al. 2017).
- AMPK is reduced by physical inactivity (Bergouignan et al. 2011).
  ↓AMPK → ↑LECT2 → ↑JNK → ↑ IR in skeletal muscle (possible effects on lipids too)

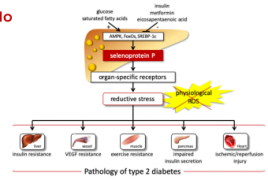
Reasons Against:

- Unsure of the effect of energy balance during bed rest on AMPK – we have no measurements of hepatic AMPK.

HEPATOKINE

SEPP-1

No



Main Functions:

Selenoprotein-P inhibits the insulin signalling pathway by inactivating AMPK.

Reasons For:

- Selenoprotein-P is the most abundant selenoprotein in plasma and is primarily produced in the liver. The expression of SELENOP is regulated by FoxOs and SREBP-1c. SeP expression is regulated positively by glucose and saturated FAs and negatively by insulin and cytokine levels (IL-1b, TNF-a, IFN-γ, and TGF-β).
- SeP has been found to be independently associated with adiponectin and insulin resistance (others reported obesity rather than IR ↑SeP)
- It is believed that SeP causes insulin resistance in the liver, at least in part, by inactivating AMPK.
  - AMPK is reduced by physical inactivity (Bergouignan et al. 2011).

Reasons Against:

- Unsure of the effect of energy balance during bed rest on AMPK – we have no measurements of hepatic AMPK.
- Mechanism responsible for SeP-induced IR in skeletal muscle is not known.

HEPATOKINE

ANGPTL3

No

Main Functions:

ANGPTL3 regulates TG levels by inhibiting LPL and may induce deterioration of glucose metabolism by increasing lipolysis in AT. Functionality depends on ANGPTL8 (increased with both)

True hepatokine of the ANGPTL family.

Reasons For:

- ANGPTL3 is a hepatokine that modulates the metabolism of TG-rich lipoproteins by inhibiting the activity of lipoprotein lipase (LPL). ANGPTL3 also stimulates lipolysis of adipose tissue and FFA release into the circulation, which can also affect hepatic IR. Animal studies have also proposed that ANGPTL3 inhibits the activity of endothelial lipase, an endothelial lipase implicated in the catabolism of HDL particles.
- Measured in response to HDTBR: Rudwill et al. (2015).
  - CTRL group ANGPTL3 at HDT60 (+29%; P = 0.02)
  - Moderate changes might show that volunteers did not reach severe stages of NAFLD and ↑TG may be due to defects in fat uptake at the muscle level.

Reasons Against:

- In mice, the N-terminal region of ANGPTL3 has been identified to be essential for the regulation of TG, while the C-terminal region containing a fibrinogen-like domain is important for angiogenesis.
- In some studies, the level of ANGPTL3 in plasma does not seem to correlate with plasma TG concentrations, which may be due to the samples being collected in the fasted state – ANGPTL3 expression does not seem to be substantially affected by fasting/refeeding but its activity on LPL may be more pronounced in the fed or re-fed state.
  - However, other studies have reported correlations with the lipid profile.

HEPATOKINE

Betatrophin/ ANGPTL8

No

Main Functions:

Inhibits LPL and regulated TG levels (muscle and BAT), and is associated with HDL.

Reasons For:

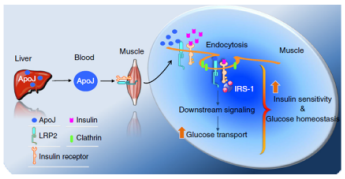
- ANGPTL3 serves as a binding partner and activator of ANGPTL8/betatrophin, which inhibits LPL thereby regulating TG levels. ↓fasting and ↑post-prandial state – stress and insulin can increase ANGPTL8.
- Irisin has been proposed to promote the expression of betatrophin – PCG-1α through a ↑FNDC5 secretes irisin, which acts on WAT to stimulate UCP1. This promotes the expression of betatrophin and ↓IR. The opposite effect is proposed to happen in aging and IR.
  - However, betatrophin shown to increase, while irisin was significantly decreased in T2DM compared to NGT, matched for age, sex and BMI.

Reasons Against:

- Some studies have mentioned that some of contradictory findings on betatrophin may be due to the lack of specificity of the ELISA's (full-length or N-terminal only), duration of long term storage and freeze-thaw cycles.

HEPATOKINE

Apo-J Yes



Main Functions:

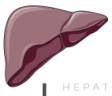
Found in HDL, LDL and VLDL. ApoJ has a key role as a co-factor in coordinating insulin-mediated signalling events - absence or high levels impair insulin signalling.

Reasons For:

- ApoJ acts as a hepatokine that plays a pivotal role in modulating muscle glucose metabolism through LRP2, highlighting an inter-organ communication network between liver and muscle.
  - This is either through a defect in the internalisation of the IR or ApoJ/LRP2 modulates P13K signalling – *studies in mice.*
- ApoJ has been found to negatively regulate gene expression of SREBP1c, a master regulator of the lipogenic pathway but the importance of hepatic ApoJ requires further investigation.
- ApoJ levels were a significant independent association factor for fasting insulin and HOMA-IR after adjusting for age, sex and BMI in a group of ND and T2DM.

Reasons Against:

- Seo and authors (2020) propose that a certain level of ApoJ in circulation is required to achieve glucose homeostasis, such that if ApoJ is beyond a certain threshold or lack of ApoJ, this hepatokine could desensitize insulin action.



HEPATOKINE

FGF-21

Yes

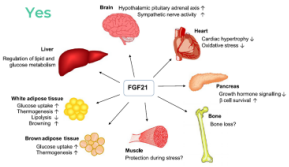


FIG. 2 An integrated view of FGF21 functions in humans.

Main Functions:

FGF-21 is an endocrine factor secreted by the liver which acts as a metabolic regulator.

Reasons For:

- Fibroblast growth factor 21 (FGF21) is a hormone that regulates important metabolic pathways. Considering the beneficial effects of FGF-21 in murine glucose and lipid metabolism, it is unexpected that levels of FGF-21 are increased in metabolic dysfunction. This has prompted the hypothesis of FGF-21 resistance, which is similar to insulin and leptin resistance.
- Elevated fasting FGF-21 levels in disease states characterised by increased liver fat.
  - Staiger et al. (2017): The strongest BMI-independent determinant of hepatic FGF-21 expression and circulating FGF-21 concentrations is liver fat content [review of 4 studies].

Reasons Against:

- The FGF21 function is complicated and well debated due to its different sites of production and action and different mechanisms are proposed in animals and humans.
- FFA and PPARα appear to be the most important regulators of FGF21 under physiological energy balance in humans. Fasting but not post-prandial FFAs are closely linked to human FGF-21 concentrations.
  - We saw no change in NEFA concentrations in response to HDTBR.

Appendix IX: Estimation of MVPA and Energy Cost of Reactive Jump Training

Using the methodology described by Le Roux et al., (2021), and assuming an equivalent of 9.0 MET/hour for reactive jump training (Ainsworth et al., 2000), we have estimated the duration of MVPA and energy cost of reactive jump training. The calculations and results are provided below.

Calculations

Estimation of MVPA

Total duration of HDT bed rest / days per week = 60 / 7 = 8.57 weeks in HDT bed rest

Sessions of RJT during HDT bed rest / weeks in HDT bed rest = 48 / 8.57 = 5.6 sessions per week

Duration of training (excluding breaks) =

12.5 minutes / 4 sessions = 3.125 minutes per session

3.125 minutes per session * 5.6 sessions per week = 17.5 minutes of MVPA/week

17.5 minutes of MVPA per week / 7 days = 2.5 minutes of MVPA/day

Duration of training (including breaks) =

49.5 minutes / 4 sessions = 12.375 minutes per session

12.375 minutes per session * 5.6 sessions per week = 69.3 minutes of MVPA/week

69.3 minutes of MVPA per week / 7 days = 9.9 minutes of MVPA/day

Estimation of Energy Cost

Energy cost of training (excluding breaks) =

17.5 minutes of MVPA per week / minutes per hour = 0.292 hours of MVPA per week

0.292 hours of MVPA per week * 9.0 MET/hour = 2.625 MET hour/week

2.625 MET hour/week / days per week = 0.375 MET hour/day

Energy cost of training (including breaks) =

69.3 minutes of MVPA per week / minutes per hour = 1.155 hours of MVPA per week

1.155 hours of MVPA per week * 9.0 MET/hour = 10.395 MET hour/week

10.395 MET hour/week / days per week = 1.485 MET hour/day

Results

Without breaks (total work time), subjects in the JUMP group performed 17.5 minutes of MVPA per week (or 2.5 minutes of MVPA per day). The estimated energy cost was 2.6 MET hours per week (or 0.4 MET hours per day). Over the 60 days of HDT bed rest, this equates to approximately 150 minutes (or 2.5 hours) of MVPA without breaks.

Including breaks (total exercise time), subjects in the JUMP group performed 69.3 minutes of MVPA per week (or 9.9 minutes of MVPA per day). The estimated energy cost of total exercise time was 10.4 MET hours per week (or 1.5 MET hours per day). Over the 60 days of HDT bed rest, this equates to approximately 594 minutes (9.9 hours) of MVPA including breaks.

Appendix X: Conferences and Courses Attended

1. Athlone Institute of Technology Postgraduate Poster Festival (Online), June 2020; Poster Presentation.
2. 15th Annual Biomarker Congress, Manchester, UK, February 2020; Conference Attendee.
3. Athlone Institute of Technology Postgraduate Research Conference, November 2019; Poster Presentation.
4. All-Ireland Postgraduate Conference in Sports Sciences, Physical Activity and Physical Education, Athlone Institute of Technology, May 2019; Oral Presentation.
5. Cell Symposia in Exercise Metabolism, Sitges, Spain, May 2019; Poster Presentation.
6. 39th International Society of Gravitational Physiology Meeting & European Space Agency (ESA) Life Sciences Meeting, ESA ESTEC, Netherlands, June 2018; Oral Presentation.
7. The Physiological Society H3 Symposium: The Integrative Physiology of Physical Inactivity across the Lifespan, UK, December 2017; Conference Attendee.
8. All-Ireland Postgraduate Conference in Sports Sciences, Physical Activity and Physical Education, Institute of Technology Carlow, April 2017; Oral Presentation.
9. ESA Human Space Physiology Training Course, Redu Centre, Belgium, February 2017; Course Attendee.
10. ESA Innovation Exchange: When Space Meets Health, ESA ESTEC, Netherlands, November 2016; Conference Attendee.
11. All-Ireland Postgraduate Conference in Sports Sciences, Physical Activity and Physical Education, Waterford Institute of Technology, April 2016; Poster Presentation.