The regulation of Branched–chain amino acids in metabolic and respiratory diseases, and in response to exercise

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Abstract

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Introduction

The increased life expectancy and global epidemic of diseases such as obesity and chronic obstructive pulmonary disease (COPD) in the last three decades has resulted in burgeoning socioeconomic burdens. Features of metabolic diseases (such as obesity) are commonly associated with insulin resistance (IR), reduced muscle mass and function, which is also observed in those with COPD. The heterogeneity of disease in individuals requires biomarkers as a diagnostic and prognostic marker which, ideally respond to nutritional and exercise-based interventions. Plasma branched chain-amino acids (BCAAs), leucine, isoleucine and valine represent such potential candidate since they are abundant in the circulation due to bypassing of the gut and liver and have accordingly shown promise in identifying as a signature of diseases, such as insulin resistance and obesity. The present thesis mostly describes the impacts of non-pharmacological interventions (nutrition, exercise) on branched-chain amino acids (BCAA) in plasma and muscle, which have long standing associations with scores of insulin resistance and obesity, despite being essential for whole body and muscle health.

Chapter three, published in *Nutrients* (2020), focuses on the impacts of a longitudinal resistance exercise (RET) programme across age and the response of plasma BCAAs with exercise, and reveals firstly no association with age and BCAA. Secondly, BCAAs did not associate with IR at any point, and instead displayed significantly positive

associations with strength and lean mass. This suggests that the BCAA/ IR paradigm does not hold within healthy individuals and demonstrates for the first time a unique relationship with muscle health providing a novel finding to build upon.

Chapter four describes the effects of severe calorie restriction (~600kcal) in obese, non-diabetic men for six-weeks, with a selection of individuals undergoing RET and high intensity interval training (HIIT) programmes. Despite profound weight loss, plasma BCAAs did not change with diet-alone or track with scores of IR (despite significant reductions following the intervention) at any point in either group. Calorie restriction alone was not sufficient to reduce plasma BCAAs, although those who performed exercise in addition to VLCD experienced a decrease in BCAAs. Further, although significant loss in fat mass was observed, this was concomitant with reductions in lean mass which is an unfavourable outcome. Future studies of this nature may benefit from increased protein content within the diets to negate the losses of lean mass.

Chapter five describes the effects of 8 weeks endurance exercise (EE) in those with stable COPD. This study, with retrospective analysis, found that aerobic capacity and strength was not improved in those with COPD, suggesting an intolerance to exercise, compared to their healthy, age-matched counterparts in which improvements in aerobic capacity and strength were seen. This was reflected in the BCAA profiles of the groups, whereby those with COPD showed no response, whereas the healthy individuals demonstrated increased BCAAs post-EE. The relationship between increased strength and BCAAs was observed again, however only in the healthy control group, supporting the results produced in the RET study (chapter three)

In **chapter six**, individuals admitted to hospital with an exacerbation of COPD were enrolled into a study comparing standard care following exacerbation, to an early rehabilitation programme comprised of various aerobic and resistance-based exercises for six weeks to attempt to alleviate the effects of an acute exacerbation. Measures of lung function, quadriceps size, thickness and strength were measured, however there was no response to the intervention. Plasma BCAAs were also quantified in these participants since increased catabolism of AA is a common feature in severe COPD compared to those with stable COPD (participants the EE study), however, there were no response in BCAAs in either group. Plasma BCAAs in exacerbated COPD patients are however decreased compared to stable state. In the 3-month follow-up period, the plasma BCAA concentrations reached the range values expected in the healthy. Unsurprisingly, BCAAs did not associate with quadriceps function, size, or thickness in either group. This study suggests that the time of admission is not optimum in assigning unique interventions and that perhaps it is more effective for routine care to take place to ensure a more stable state is established prior to further interventions aimed at improving exercise tolerance and quality of life.

In conclusion, the data from the present thesis challenges the commonly reported associations of IR and BCAAs in conditions of normal health as well as in obesity, and reveals a novel association with lean mass and strength following RET. The association of increased strength and BCAAs are also observed in healthy individuals undergoing both RET and EE, however not in those with COPD undergoing the same intervention suggests a novel finding that warrants further investigation. Taken together, these data suggest plasma BCAAs as markers of improved metabolic health following both resistance and endurance-based exercise, as opposed to insulin resistance.

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CHAPTER ONE: INTRODUCTION

1.1. Why study branched chain amino acids (BCAA) in health and disease

Branched chain amino acids (BCAAs), leucine, isoleucine and valine, are the most abundant amino acids in protein (Dill 1990). As with all other essential AA, BCAAs are diet-derived meaning they cannot be synthesised endogenously by humans, therefore are of fundamental importance for cell survival maintenance and turnover of proteins. Thus, their utilisation/metabolism must be balanced by their intake. The molar relative abundance of BCAAs in protein to each other is approximately 1.6: 2.2: 1.0 (valine: leucine: isoleucine) (Neinast, Murashige, and Arany 2019), which reflects the linked nature of their metabolism. BCAAs are almost always consumed and initially oxidised together (unless isolated for nutritional supplementations; Bassit et al. 2000; Marchesini et al. 2003), hence why they are regularly investigated together despite distinct differences in their biological effects e.g., leucine is ketogenic, valine is glucogenic and isoleucine is both (Yoshiharu Shimomura et al. 2001; Brosnan and Brosnan 2006). Although most AA are metabolised in the liver, BCAAs are three of six AA (asparagine, aspartate and glutamate) which are initially metabolised within skeletal muscle (Harper, Miller, and Block 1984), and can be used for maintaining the nitrogen in muscle pools of glutamate, alanine and glutamine for subsequent release and uptake by the liver, kidneys and intestines for energy that can be returned to peripheral tissues . The initial site of BCAA metabolism is skeletal muscle, largely due to the high activity of regulatory enzyme, BCAA aminotransferase (BCAT), which catalyses the first step to form each respective keto-acid (Lynch and Adams 2014) therefore BCAA in the bloodstream appear rapidly following ingestion of protein and become readily available to peripheral tissues. The second, irreversible step, is via the branched chain α-keto acid dehydrogenase complex (BCKDH) (Sitryawan et al. 1998), and since BCKDH catalyzes an irreversible flux-controlling step, the initial oxidation

rates of all three are linked. Figure 1 outlines the expression of BCAA-related enzymes in multiple organs and highlights the nitrogen recycling which occurs with BCAA oxidation. As such, BCAAs are key regulators in the synthesis of body proteins, energy metabolism and are major nitrogen donors for glutamate, glutamine and alanine synthesis (Blomstrand et al. 2006; Brosnan and Brosnan 2006). For example, BCAAs (particularly leucine) are known growth-regulatory signals to skeletal muscles (largely via promotion of mTORC1 activity) following feeding, exercise or both, resulting in cell growth (Rennie et al. 2006; Atherton, Smith, et al. 2010), through phosphorylation of the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1) and the p70 ribosomal S6 kinase (p70–S6K1) to promote mRNA translation (Laplante and Sabatini 2009). BCAAs also regulate *de novo* glutamine synthesis in peripheral tissues (Sakai et al. 2004), particularly skeletal muscle (Harper, Miller, and Block 1984). The most abundant free AA in the bloodstream, glutamine, contains two nitrogen groups therefore serving also as a nitrogen 'shuttle' between tissues taking excess ammonia to the liver to form urea for excretion. In addition, BCAA-derived nitrogen sources are also particularly important for the synthesis of alanine, which, when catabolised by the muscle is utilised as a key protein-derived gluconeogenic substrate (Felig 1973). Glutamine and alanine are concentrated in muscle (Gln 20mM (Rennie et al. 1981), Ala 2.8mM (Adibi et al. 1973)) and are the two major AA released by the muscles during instances of prolonged fasting, when *de novo* formation of these AA are increased (Chang and Goldberg 1978). This highlights the importance of BCAAs and related AA in healthy muscle metabolism, energy production. However they are also implicated in conditions of ill–health, and although crucial for protein synthesis, catabolic intermediates (such as 3-HIB; Jang et al. 2016) formed during BCAA metabolism can be toxic at high concentrations within the plasma, therefore efficient

clearance of excess BCAAs are equally important for maintaining normal physiological function. Chronic elevation of blood concentration of BCAAs in conditions such as cardiovascular disease (Ruiz-canela et al. 2016), cancers (Mayers et al. 2014; Budhathoki et al. 2017), obesity, IR and T2DM (Newgard 2017; Wang et al. 2011) and their role in inborn errors of metabolism are also documented (Mackenzie and Woolf 1964) whereby aberrations in a subunit of the BCKDH multi-enzyme complex results in increased plasma concentrations (Mitsubuchi, Owada, and Endo 2005). Thus, the present thesis aims to investigate the role of plasma and intracellular BCAAs, during ageing, obesity and chronic obstructive pulmonary disease (COPD), and in response to exercise interventions. The relationship between BCAAs and insulin sensitivity is investigated, and whether changes in circulating concentrations of BCAAs correlate with IR improvements in response to exercise interventions known to promote good health.

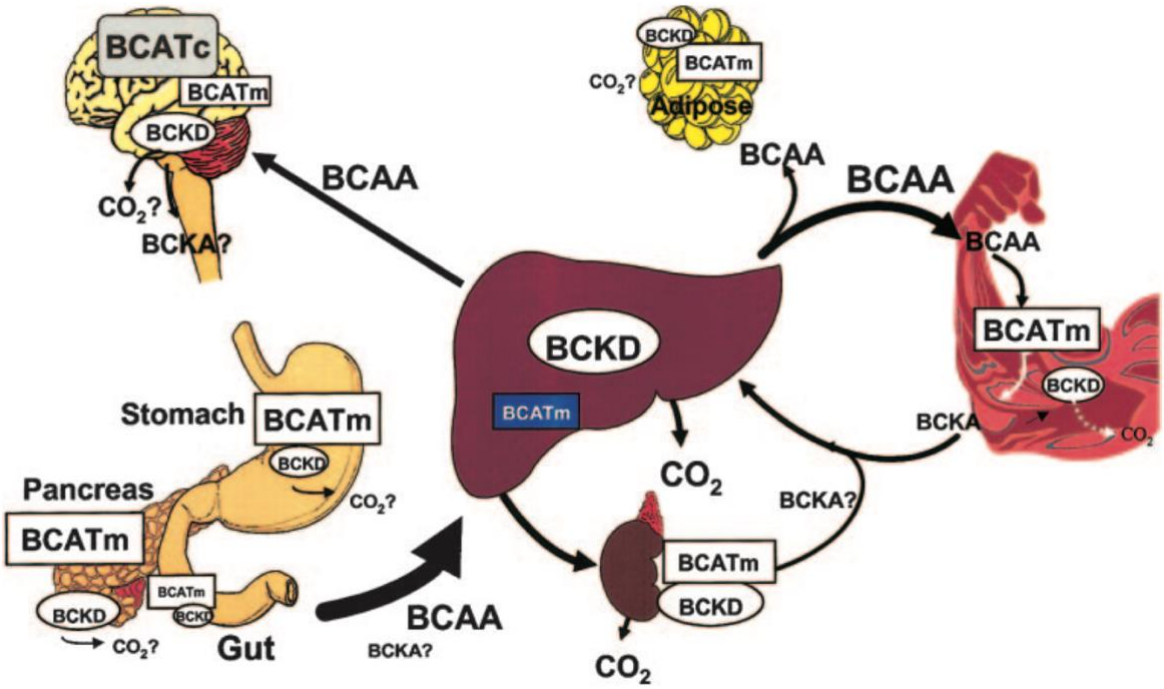


FIGURE 1. REGULATION OF BCAA-METABOLISING ENZYMES THROUGHOUT THE BODY AND ACROSS MULTIPLE ORGANS. SOURCE: HUTSON ET AL 2005.LOCALIZATION OF BCAA-RELATED ENZYMES THROUGHOUT THE BODY. BCATC (BCAT1) IS FOUND PRIMARILY IN THE BRAIN, HOWEVER ALSO IN THE PERIPHERAL NERVES, WHEREAS BCATM (BCAT2) IS FOUND IN ALL TISSUES (EXCEPT LIVER). THE EXPRESSION OF REGULATORY ENZYMES THROUGHOUT THE BODY PERMITS NITROGEN CYCLING AND ALLOWS FOR ENERGY PRODUCTION VIA CARBON SKELETON OF CERTIN AA. BCAT, BRANCHED-CHAIN AMINOTRANSFERASE; BCKD BRANCHED CHAIN KETO ACID DEHYDROGENASE; BCATM, BCAT MITOCHONDRIAL; BCATC, BCAT CYTOSOLIC

1.2. BCAA structure and function

Branched-chain amino acids, with an amino and carboxyl group and functional R groups in their chemical structures are branched hence their name (Dill 1990), make up ~35 % of the nine amino acids that are essential to whole-body health (Brosnan and Brosnan 2006), and must be obtained from the diet as opposed to non-essential amino acids which can be synthesised within the body (Reeds 2000). As well as established biochemical regulators and precursors of complex metabolic reactions, BCAAs also serve as essential substrates in the initiation of muscle protein synthesis (MPS; (Wilkinson et al. 2013), for example leucine is a known stimulator of mammalian target of rapamycin (mTOR) (Atherton, Smith, et al. 2010), and is also a potent insulin secretagogue. BCAAs serve also as a metabolic fuel during times of injury (Askanazi et al. 1980) starvation (Felig et al. 1969), and exercise (Rennie et al. 2006). Administration of BCAAs has also shown therapeutic benefits in treating cognitive impairment following injury (Cole et al. 2010), sepsis (De Bant and Cynober 2006) and chronic liver disease (Kawaguchi et al. 2011). In the gastrointestinal tract and fat depots, BCAA (along with glucose; Delgado et al. 1995) can influence the regulation and release of hormones (e.g., GLP-1 and ghrelin) which affect food intake and glycaemic control (Potier, Darcel, and Tomé 2009; Salehi et al. 2012; Chen and Reimer 2009). In addition, supplementation of BCAAs as an ergogenic aid is commonplace in the context of exercise recovery, performance and muscle growth (Y Shimomura et al. 2010; Crowe, Weatherson, and Bowden 2006; Watson, Shirreffs, and Maughan 2004; Duan, et al. 2016).

Unlike other AA which are oxidised in the liver (Harper, Miller, and Block 1984), BCAAs are uniquely metabolised in muscle by branched-chain aminotransferase (BCAT) to form α–ketoglutarate (yielding glutamate) and respective keto acids (α–

ketoisocaproate, KIC; α–keto–β–methylvalerate, KMV and α–ketoisovalerate, KIV) which is the first enzyme in the BCAA metabolism pathway (figure 2). BCAT1 (or BCATc) encodes the cytoplasmic protein and is primarily expressed in the brain. Once keto–acids are formed, the multi-enzyme branched-chain keto–acid dehydrogenase complex (BCKDH), located on the inner mitochondrial membrane and ubiquitously expressed in all tissues, is responsible for the oxidative decarboxylation, to form acyl-CoA esters for entry into the citric acid cycle (TCA). It is at this rate-limiting second step where deficiency of BCKDH enzyme leads to an increase in plasma BCAAs and accumulation of harmful by-products that are implicated in diseases such as maple syrup urine disease (MSUD); whereby a build-up of BCAAs and BCAA-related keto-acids are seen in the blood and urine (Yudkoff et al. 2005; Burrage et al. 2014; Lynch and Adams 2014). These reactions occur within the mitochondrial matrix, rendering all intermediate metabolites ‘trapped’ within the matrix as an acetyl-CoA adduct with the exception of the valine catabolite, 3-HIB, which can be secreted and is detected at 10–40μM concentrations in plasma (Neinast, Murashige, and Arany 2019).

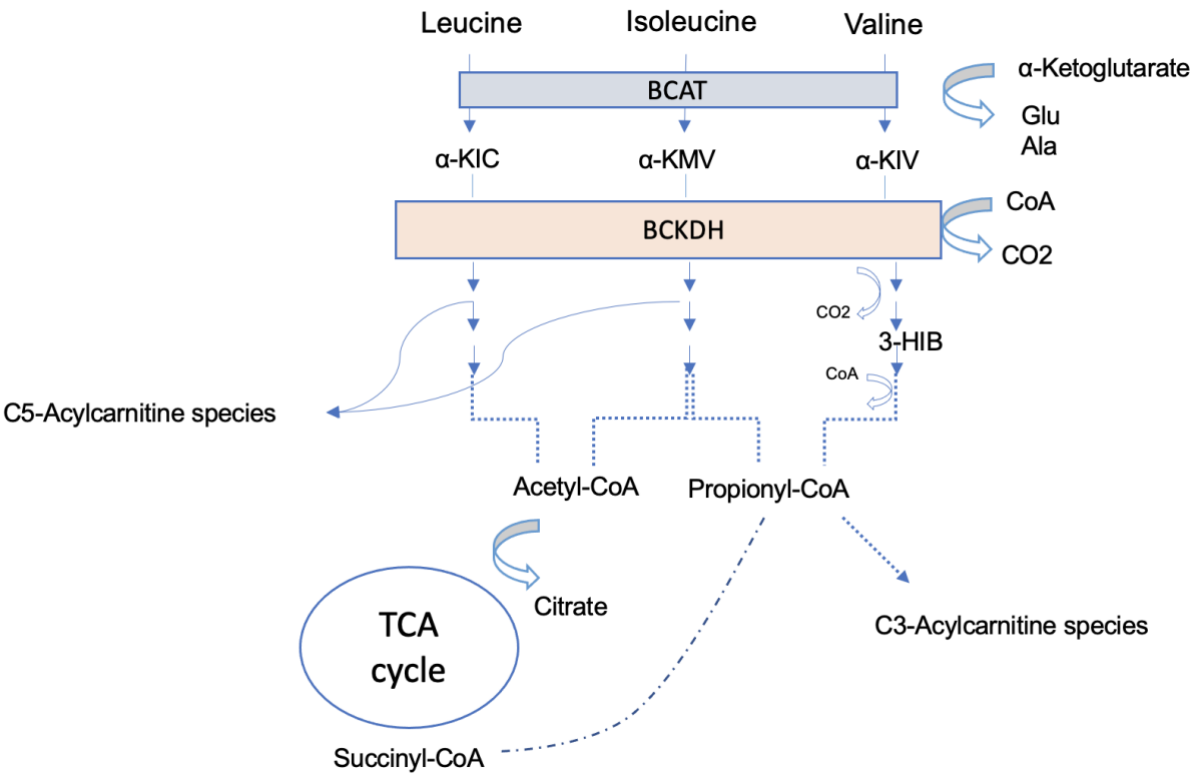


FIGURE 2. THE SIMPLIFIED PATHWAY OF BCAA METABOLISM IN MUSCLE. THE FIRST TWO STEPS OF BCAA METABOLISM ARE THE SAME WITH ALL THREE BCAA. INITIAL TRANSMINATION TO RESPECTIVE KETO-ACIDS BY BCAT IS FOLLOWED BY DECARBOXYLATION BY THE BCKDH MULTI-ENZYME COMPLEX, WHICH IS RATE-LIMITING FOR BCAAS A-KIC, ALPHA-KETO-ISOCAPROATE; A-KMV, ALPHA-KETO METHYL VALERATE; A-KIV, ALPHA-KETO ISOVALERATE; 3-HIB, 3-HYDROXYISOBUTYRATE; BCAT, BRANCHED-CHAIN AMINOTRANSFERASE; BCKDH, BRANCHED-CHAIN KETO-ACID DEHYDROGENASE.

1.3. Skeletal muscle and AA

In humans, skeletal muscle comprises ~40 % total body weight and as the largest organ of the body has a significant impact on whole body homeostasis and metabolism. As the largest reservoir of protein in the body, skeletal muscle therefore characterises the major storage of AA, including BCAA (Bergstrom et al. 1974). Not only are AA essential for providing energy, but they are also in a constant state of turnover and metabolic flux to meet demands (e.g., precursors for protein synthesis in muscle, and other tissues, as energy substrates) placed on the body and are sensitive to factors such as hormonal imbalance, physical activity, injury and disease.

From a mechanical perspective, skeletal muscles main function is to convert chemical potential into energy to generate force and power (Walter R. Frontera and Ochala 2015) i.e., muscle contraction that is largely governed by sliding contractile filaments (actin and myosin) (Herzog et al. 2015; Huxley and Simmons 1971).

Accordingly, skeletal muscles are crucial for locomotion and supporting posture in everyday activities. In contrast, age-related declines in muscle mass and function, as demonstrated by ~40 % decline of total muscle cross-sectional area (CSA) between the ages of 20–80 years with the decline accelerated more so at 50 years of age (Lexell, Taylor, and Sjostrom 1988), lead to functional dependence and impaired quality of life. Significant losses of muscle also occur with various diseases including, COPD (Bernard et al. 1998), cachexia (Evans 2010) and critical illness (Preiser et al. 2014). The loss of muscle also occurs in other settings, which necessitate continued muscle disuse, for example recovery from injury or illness which require prolonged bed rest (Dirks et al. 2016), where muscle atrophy is significantly accelerated (~3.5 % decline by day 5 and 8.4 % by day 14; Wall et al. 2014), with accompanied declines in strength ~0.3 % (Paddon-Jones et al. 2004) to 4.2 %/ day (Thom et al. 2001). It is

unsurprising then that of relevance to reducing risk of disease and optimising health, maintenance of muscle mass is crucial, and reduced muscle mass impairs the body's response to counter the effects of stress and chronic illness. The most effective, non-pharmacological counterbalance for preserving or restoring loss of muscle mass in young and older populations is physical activity (Law, Clark, and Clark 2016; DeFreitas et al. 2011). The physiological benefits of chronic bouts of exercise effects on muscle mass, strength and quality are well-defined (Binder et al. 2005; Moore et al. 2005; Halverstadt et al. 2007), in addition to the benefits on whole body metabolism (Brook et al. 2016), optimising fuel oxidation (Van Loon et al. 2001), body composition (Slentz et al. 2004) and mental health (Deslandes et al. 2009). Thus, preservation of skeletal muscle integrity is paramount in maintaining health and quality of life (Woods et al. 2012) particularly in old age where a progressive decline in muscle mass (Lexell, Taylor, and Sjostrom 1988), termed sarcopenia (Rosenberg 1997), is common.

1.3. The role of AA in skeletal muscle protein turnover

BCAAs are implicated in several features of skeletal muscle homeostasis, for example as a stimulatory effect on translation initiation and subsequent muscle protein synthesis (MPS), in addition to exerting inhibitory effects of muscle protein breakdown (MPB) although mechanisms responsible for this are not yet fully elucidated (Abdulla et al. 2016). Over the years, studies have shown that feeding of essential AA's as the core driver of stimulating muscle protein synthesis (Rennie 1985; Atherton, Smith, et al. 2010) and work from the early 1990's (Smith et al. 1992) confirmed certain EAA, particularly leucine are the most potent stimulators of MPS. This was evidenced when in contrast, large boluses of arginine, glycine and serine (all non-essential) did not elicit an anabolic response, highlighting the importance of EAA in stimulating MPS (Smith et al. 1998).

Above the other EAA's, leucine stimulates MPS (approximately ~1.6-fold greater than other EAA; Atherton, Smith, et al. 2010) through the activation of mammalian target of rapamycin pathway (mTOR; Norton and Layman 2006) and subsequent phosphorylation of 4E binding protein-1 (4EBP1) and ribosomal s6 kinase (p70S6K1) (Buse and Reid 1975; Baar and Esser 1999). Furthermore, an ergogenic role for metabolites related to leucine, namely β–hydroxy–β–methylbutyrate (HMB) has also been described, whereby ~2.4 g supplementation of pure free-acid HMB resulted in acute myofibrillar protein synthesis (150 min) and p70SK61 phosphorylation to a similar extent as leucine (Wilkinson et al. 2013) in the acute post prandial stage. Although other pathways have been described (Proud 2007), the mTOR pathway is an established significant regulator of muscle protein translation and synthesis. The regulation of skeletal muscle is determined by the relative turnover rate of its constituent processes (i.e., MPS and MPB) (Atherton and Smith 2012) which follows as part of a diurnal equilibrium. In conditions of anabolism, for example as that seen within resistance-based exercise or anabolic agents, the net balance of MPS exceeds that of MPB, whereas in situations of prolonged inactivity such as bed rest or critical illness, net MPB is greater than MPS resulting in muscle loss.

1.4. Whole-body oxidation of BCAAs

Stable isotope infusions of BCAAs over the years have determined the rate of flux of BCAA transamination and oxidative decarboxylation in humans. When a protein meal is ingested, oxidation of BCAAs can triple basal levels and thus accounts for a greater fraction of BCAA disposal (Boirie et al. 1997). The appearance of free BCAAs in circulation is determined by dietary intake and the rate of proteolysis and processes that remove i.e., MPS or oxidation/catabolism. Dietary BCAA are typically taken up by the small intestine, however unlike most other AA, BCAAs bypass metabolism in the liver and gut due in part to the low transamination activity within the liver (Hutson, Wallin, and Hall 1992).

Briefly, digestion of protein begins with chewing to increase the surface area of the foods (Rémond et al. 2007), and after swallowing, gastric mixing induced by stomach contractions facilitates further breakdown by gastic acids and pepsin enzyme before subsequent gastric emptying and delivery to the small intestine (duodenum, jejunum and ileum) (Hellström, Grybäck, and Jacobsson 2006). Following absorption, a substantial quantity of ingested AA escape first-pass splanchnic extraction and the majority of absorbed AA are released into the circulation for peripheral tissue uptake (Volpi et al. 1999; Gorissen et al. 2020). The fraction AA which are not absorbed by the small intestine, reaches the large intestine where although not absorbed, they are deaminated and metabolised by the microbiota (Smith and MacFarlane 1998; van der Wielen, Moughan, and Mensink 2017). The rate of digestibility of the protein consumed (e.g., whey, soy, casein) is dependent on the quality and content of EAA, which is an important factor with regards to the transport across the gut, and subsequent availability to peripheral tissues and extraction by the splanchnic tissues (i.e., liver, gut, pancreas, etc).

The rate of AA absorption has been studied in a variety of protein sources; revealing fast absorption and release from consumption of whey/ milk protein compared with less soluble casein fraction (Luiking et al. 2016). In addition, soy-protein, which despite more rapid digestibility and uptake in the gut, results in a greater proportion of AA from soy sequestered by the splanchnic tissues. Thus, greater urea production is observed – reducing the availability of AA to peripheral tissues such as skeletal muscle (Fouillet et al. 2002; 2009).

Upon consumption, absorption follows two phases, an initial fast absorption and delayed slow due to the casein element (Boirie et al. 1997). Casein, a “slow” absorption source (Boirie et al. 1997) results in gastric coagulation in the acidic environment, requiring further hydrolysis and thus slowing gastric emptying rates and subsequent plasma AA availability (Symons et al. 2009). Accordingly, studies of human digestion models reveal coagulation of casein with no coagulation of whey within the gut (Luiking et al. 2016), and despite containing similar amount of EAA, appearance of EAA in plasma increase faster and to a higher level upon dietary intake of whey. This highlights the marked differences in the kinetics of absorption and so consideration is needed when selecting protein source. Further, whey protein has been shown to induce greater AA absorption (as measured by appearance in circulation), whereby the MPS response was greater (0.15%h) compared to casein (0.08 %h) and casein hydrolysate (0.1 %h) (Pennings et al. 2011). Further, a special case has been made for leucine, whereby comparison of low-dose (3 g) leucine enriched AA and a large (20 g) bolus of whey has been shown to elicit similar response in myofibrillar FSR following RET (Bukhari et al. 2015).

Most organs are capable of BCAA oxidation, however skeletal muscle, liver and brown fat are the sites where the majority of BCAA oxidation occurs (Neinast et al. 2019).

That the liver, gut and skeletal muscle are well-known BCAA sites, adipose is a more recent revelation for its capacity to catabolise BCAA (Herman et al. 2010). More recently, a role for brown fat as a novel contributor to BCAA metabolism has also been described, whereby genetic disruption of BCKDH or of a brown fat specific BCAA transporter detail results in increased plasma levels of BCAA (Yoneshiro et al. 2019). This highlights the heterogeneity of global BCAA metabolism in healthy individuals. In situations of perturbed metabolism however, such as underfeeding (Pedrini et al. 1996), overfeeding (Fraipont and Preiser 2013) and metabolic disease e.g. obesity and diabetes (Solon-Biet et al. 2019; Tai et al. 2010; Karusheva et al. 2019) alterations in BCAA metabolism and circulating BCAA have been described.

1.5. Regulation of BCAA metabolism: nutrition, exercise and ageing

1.5.1. Nutrition

Ingestion of dietary protein, and constituent AA, provide the key stimulus for increased muscle protein synthesis (Blomstrand et al. 2006) particularly when combined with exercise which results in a prolonged net balance leading to eventual protein accretion (Phillips et al. 1997; Kumar et al. 2009). Nitrogen balance studies have been employed over the years to determine dietary requirements, for example the recommended daily allowance (RDA) of protein is ~0.65 g • kg^{-1} • d^{-1}, 0.8 g • kg^{-1} • d^{-1}, and 1.2 – 1.7 g • kg^{-1} • d^{-1} in young , adult and athletic populations, respectively (Rand, Pellett, and Young 2003; ACSM and ADA 2009). In the postabsorptive state, muscle protein breakdown (0.08–0.11 %/ h; (Tipton et al. 1999; Phillips et al. 1997) dominates rates of MPS (0.04-0.07 %/h; Kumar et al. 2009; Cuthbertson et al. 2005; Welle, Thornton, and Statt 1995) creating a negative net balance of protein turnover and thus representing loss of muscle protein and eventual loss of muscle, if prolonged.

Consumption of a protein-containing meal, and ensuing bioavailability of AA in the bloodstream, results in acute (~2h) increases in MPS and suppression of breakdown (MPB) (Atherton, Etheridge, et al. 2010; Bohé et al. 2001), which ensures the replacement of muscle protein lost during postabsorptive conditions, and maintains a general equilibrium despite diurnal fluctuations i.e. periods of fasting and feeding. As mentioned, the potency of MPS increases can be attributed to essential AAs, particularly leucine. The anabolic capacity of MPS is finite and thus begins to decline ~ 1.5 h after protein consumption (Atherton, Etheridge, et al. 2010), irrespective of continued nutrient availability i.e., "muscle full" effect (Bohé et al. 2001; Atherton, Etheridge, et al. 2010). At rest, no observed differences in net muscle protein turnover are present between young and old (Phillips et al. 2017; Brook, Wilkinson, Mitchell, et al. 2016), however with ageing, resistance to feeding (i.e., amino acids) becomes apparent and this anabolic resistance may result in loss of muscle over time (Cuthbertson et al. 2005; Phillips et al. 2017; Smeuninx et al. 2017). Prior to this, it was thought that reduced postabsorptive rates of MPS were responsible for the muscle loss with ageing (Welle, Thornton, and Statt 1995; Yarasheski, Zachwieja, and Bier 1993). This phenomenon has been extended to show that regulatory signals which maintain muscle mass and muscle protein homeostasis are dysregulated irrespective of nutrition or exercise (Kumar et al. 2009) – and that increases in MPS to exercise stimuli are less in older compared to young individuals (Atherton and Smith 2012) – this metabolic blunting has been implicated as a potential driver of muscle loss in ageing and the progression towards sarcopenia. Whilst the idea of muscle nutrient resistance is poorly understood, it is understood that physical activity can work as a tool to influence this 'set' point (Atherton and Smith 2012).

1.5.2. Exercise

Although the age-related loss of muscle (sarcopenia), and strength is inevitable, several factors (rate of muscle loss and age that decline starts) may determine the impact sarcopenia has on functional ability and quality of life. Typically, muscle loss begins around the fifth decade of life and thereafter proceeds at a rate of 0.6 % yearly (Janssen 2010; Lexell, Taylor, and Sjostrom 1988). This rate is perhaps made more concerning, since sedentary lifestyles involving muscle disuse or periods of confined bedrest (via illness or hospitalisation) can exacerbate the degree of muscle loss, thereby rendering recovery of lost muscle difficult. Undoubtedly, exercise, in particular resistance exercise training (RET) holds potent anabolic potential and has been shown to increase muscle mass and function in various populations, including older adults (Law, Clark, and Clark 2016) and cachectic cohorts (Strasser et al. 2013). RET been shown to stimulate up to 3-fold increase in MPS following a single bout of training (Phillips et al. 1997; Kumar et al. 2009). Unsurprisingly, there is a homologous relationship between RET and MPS, whereby in the 4 h following RET at near maximal efforts (~70-80 %) the rate of MPS peaks (Kumar et al. 2009; 2012) representing a nutrient sensitive window for EAA to promote protein accretion. Alternative modes of promoting greater muscle accretion have also been described such as low-load and high volume RET either alone (Burd et al. 2010; Ogasawara et al. 2013) or in conjunction with vascular occlusion techniques (Yasuda et al. 2015; Sieljacks et al. 2019) that have described comparable rates of MPS. Promisingly, this suggests a range of RET protocols which provide a means to stimulate MPS, however it is important to note that volitional fatigue may be necessary to facilitate maximal muscle fibre recruitment in order for the stimulatory threshold to be surpassed (Henneman, Somjen, and Carpenter 1965). Therefore, maintaining independence is important for

healthy ageing and adequate nutrition combined with physical activity represents an excellent approach to mitigate the physiological challenges facing individuals as they age.

1.5.3. Ageing

In coming years, ageing populations will represent a major socioeconomic and healthcare burden in society, due to increased life expectancy as a result of healthcare and technological (Christensen et al. 2009a; Rice and Fineman 2004). One of the major changes from an anthropometric standpoint is the loss of muscle mass with age, whereby beyond the fifth decade of life, a progressive decline in skeletal muscle mass and strength is observed (Lexell, Taylor, and Sjostrom 1988). Despite well–orchestrated mechanisms regulating nutrient-intake in young individuals, an alteration the balance between MPS and MPB likely contributes to muscle loss in older age. As such, it has been shown that supplementation with protein is needed in older populations to maximise the anabolic response to feeding (Moore et al. 2015; Volpi et al. 2000). Explanations to the less pronounced capacity of MPS in older individuals have included decreased capacity of digestion and absorption of AA's (Boirie et al. 1997), concomitant with greater AA retention by splanchnic tissues after intestinal absorption (Volpi et al. 1999) resulting in less AA availability for MPS stimulation. Indeed, work from our lab has defined a role for 'anabolic resistance' in older age (Brook, Wilkinson, Mitchell, et al. 2016; Kumar et al. 2009; Cuthbertson et al. 2005) in the face of continued nutrient availability (Atherton, Etheridge, et al. 2010; Bohé et al. 2001). However, an attractive prospect for protein (whey) supplementation with RET in older populations has been shown. Pennings and workers (Pennings et al. 2012) report RET combined with post-exercise protein supplementation (40 g whey) results in higher MPS (91 % greater myofibrillar MPS over placebo) while those consuming

just 20 g of whey displayed 44 % increase compared to volunteers who performed without supplementation. Other workers (Rondanelli et al. 2016) have shown positive results with EAA supplementation with regular physical activity in 130 sarcopenic individuals (mean age 80 years) and 22 g whey (~4 g leucine) 68 % of the elderly participants became non-sarcopenic through gains in fat free mass (~1.7 kg). These data demonstrate that whilst ageing is an inevitable event in the natural course of life, and concomitant reductions in muscle mass are to be expected, there is a potential therapeutic intervention by way of nutrition/ exercise to slow the declines commonly observed in those with more sedentary behaviours.

1.6. BCAAs in genetic metabolic diseases

Thus far, the regulation of BCAAs in healthy humans has been discussed and the decades of investigations has generated a deeper understanding of how BCAAs are metabolised in humans. Inborn errors of metabolism, such as maple syrup urine disease (MSUD), highlight the potential importance of orderly regulation of BCAA metabolism and the potential impact of elevated BCAA. A consistent finding however, aside from hereditary diseases has been the association of concentrations of plasma and intracellular BCAA activity with metabolic comorbidities.

1.7. BCAAs in obesity and diabetes

Despite the beneficial effects of BCAAs on whole-body health, there exists relationship with elevated circulating AA, including BCAA and obesity, which was first reported in the 1960's (Felig, Marliss, and Cahill 1969b), and which has re-emerged more recently and has been further supported using highly accurate analytical approaches, such as metabolomics (Newgard et al. 2009; David et al. 2019; She, Reid, Bronson, et al. 2007). Further, a role for BCAA-metabolites, particularly of valine (3-HIB), has been

shown to be elevated in diabetic individuals and facilitates increased fatty acid transport within skeletal muscle leading to glucose intolerance (Jang et al. 2016). Then, other workers showed that not only are BCAAs elevated in obese and type-2 diabetic (T2DM) individuals (Mihalik et al. 2010; Tai et al. 2010), but that they are also strong predictors of future risk of these conditions regardless of ethnicity (Wurtz et al. 2013; Wang et al. 2011; Lu et al. 2016). A proposed model of how elevated BCAAs contribute to IR is displayed in figure 3

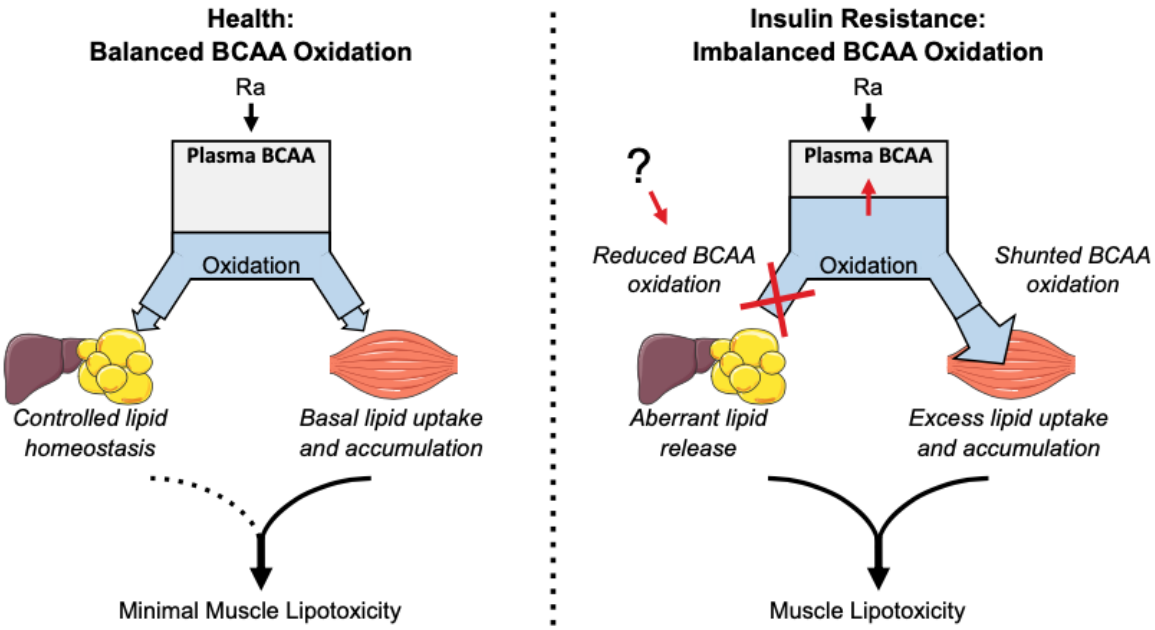


FIGURE 3. GENERAL OVERVIEW OF HOW INCREASED PLASMA BCAAS ARE IMPLICATED IN THE PATHOGENESIS OF IR, HEALTHY OXIDATION OF BCAAS IS DISPLAYED BY THE DIAGRAM ON THE LEFT. IN INSULIN RESISTANT INDIVIDUALS, INEFFICIENT CLEARANCE OF PLASMA BCAAS RESULTS IN INCREASED APPEARANCE OF BCAAS, AND RELEASE OF HYDROXYACIDS SUCH AS 3-HIB, WHICH FACILTATE ENTRANCE OF EXCESS LIPIDS IN THE MUSCLE. FIGURE ADAPTED FROM NEINAST ET AL 2019.

A number of studies in this area has led to evidence around two central themes. Firstly, that chronic activation of mTOR (of which, leucine is a potent stimulator; Yang et al. 2010; Melnik 2012), which promotes cellular growth in most cells, was demonstrated (Newgard et al. 2009). In this study, rats were fed with a high-fat diet enriched in BCAA which led to significant increase plasma levels of BCAA and increased mTOR phosphorylation with concomitant reduced Akt expression in muscle. The same rats were insulin resistant compared to their counterparts (who were fed standard chow) despite equal body weights. The IR and impaired glucose tolerance of the non-obese BCAA/ high fat rats were equal to those of obese high-fat diet-fed rats, an effect which was counteracted by mTOR inhibitor rapamycin – indicating an interaction between BCAA and dietary fat (Newgard et al. 2009). In support of this, BCAA deprivation in mice has been shown to increase liver AKT activation leading to improved insulin sensitivity (Xiao et al. 2014; 2011). Sustained activation of mTOR is known to promote IR through serine phosphorylation of insulin receptor substrate (IRS-1) and IRS-2 which may be caused by aminoacidemia, especially leucine (Macotela et al. 2011) i.e., sustained nutrient availability associated with obesity. The basic premise here suggests that increased demand of insulin over time elicits hyperinsulinemia and exhaustion of pancreatic β-cells to the point where euglycaemia is no longer maintained and T2DM ensues (Lynch and Adams 2014). However, it is unclear how other potential mediators besides BCAAs contribute to this mechanism. Importantly, although BCAAs are associated with the mTOR activation, exercise is also a potent stimulator which is a known measure of counteracting the obesity and T2DM characteristics, through improvements in IS (Holloszy 2005). Indeed, data demonstrating that supplementation of BCAAs improves metabolic markers of health despite increased mTOR activity (Macotela et al. 2011; She, Reid, Bronson, et al.

2007). Further, studies have shown normalised levels of plasma BCAA following gastric bypass surgery (She, Reid, Huston, et al. 2007; Laferrère et al. 2011; Magkos et al. 2013) and others have shown this has no effect on mTOR activation longitudinally (Magkos et al. 2013). Isoleucine alone has been reported to reduce plasma glucose levels via skeletal muscle stimulation of glucose uptake by an undefined mechanism (Doi et al. 2005; 2007). Taken together, these studies raise doubts about the notion of chronic mTOR activation promoting IR or T2DM. Indeed, plasma BCAAs are consistently shown to be elevated in these individuals and the cause of this is not well-defined. An obvious observation would be that in general participants of this demographic consume more food (and protein), however higher plasma BCAAs have been observed in individuals even after matching protein and BCAA intake or after an overnight fast (Newgard et al. 2009; Tai et al. 2010; Wang et al. 2011), and are complimented by studies demonstrating that whole body protein breakdown measurements do not seem to differ to healthy counterparts (Luzi, Petrides, and De Fronzo 1993; Biolo et al. 1992).

The second theme is that impaired BCAA catabolism could contribute to the increased build-up of BCAAs in circulation. In support of this, is evidence of lower BCKDH protein expression in the liver or adipose tissue of obese, diabetic or diet-induced obese mice (She, Reid, Huston, et al. 2007). An elegant study by Zhou et al (Zhou et al. 2019) demonstrated that treating obese mice or diet induced mice with 3,6,-dichlorobenzo[b]thiopene-2-carboxylic acid (BT2) which is a pharmacological inhibitor of BCKDH reduces plasma BCAA (and BCKA), attenuates IR and thus suggesting that catabolic capacity of BCAA is critical decreasing plasma concentrations. Unfortunately, few studies exist in which regulatory BCAA mechanisms are investigated to establish cause–effect. Others have explored systemic

hyperinsulinemia, by intravenous and/ or hyperinsulinemic euglycemic clamp lowers circulating BCAA to a lesser extent in obese or diabetic individuals, suggesting IR may be involved in elevating BCAAs (Schauder et al. 1983; Caballero and Wurtman 1991; Forlani et al. 1984). More recently, an elegant study by Jang et al (2016) provide important data linking 3-HIB (valine metabolite) as a paracrine regulator of cellular fatty acid uptake into skeletal muscle. These authors found that, like BCAAs, 3-HIB was elevated in *db/db* mice and in humans with T2DM, and by administering to rodents, resulted in increased vascular fatty acid transport into skeletal muscles leading to muscle lipotoxicity. Although supporting data is lacking, these aforementioned processes are likely exacerbated by accumulation of fatty acids induced by IR contributing to lipoxicity in the muscle tissues. Therefore, it has been proposed that development of IR is driven by the reduced catabolic capacity of BCAA across multiple organs (Newgard 2012). Indeed, this is supported by studies demonstrating attenuated postabsorptive rate of MPS compared to healthy controls (Murton et al. 2015), accompanied by greater mTOR activity (Tran et al. 2018), suggesting the muscles display an altered response to MPS despite continued AA availability.

It is important to note however that the increase seen in aforementioned studies are not due to recent (e.g., within a few hours) of eating, as studies cited were measured following an overnight fast. However, that is not to say that overeating and sustained nutrient disposal *per se* is not important in obesity. Although numerous studies over the years have promoted BCAA supplementation in promotion of lean body mass and as an ergogenic aid for promoting weight loss in the obese, the negative associations with increased levels of BCAA in metabolic disease have been demonstrated and this raises important questions as the cause-effect relationship between BCAA and metabolic disease and muscle health.

1.8. Role muscle health and BCAAs in chronic respiratory diseases

COPD is the manifestation of a multifactorial and complex interplay of long-term exposure to noxious gases and particles leading to chronic, irreversible airflow limitation (Ghosh et al. 2016b; Singh et al. 2017), and a host of systemic effects (discussed in section 1.8.4). Inhalation tobacco smoke is the most common risk factor of developing COPD, has been consistent in numerous investigations in both animals (Churg, Cosio, and Wright 2008) and humans (Celli and Macnee 2004; Buist et al. 2007). It is defined by the Global Initiative for COPD (GOLD), as a preventable and treatable disease characterised by progressive airflow limitation caused by prolonged exposure to noxious particles or gases (Strategy et al. 2017; Celli et al. 2015). In 2001, the World Health Organisation (WHO) predicted COPD to be the fifth leading cause of death, and this is expected to rise to the third leading cause of death by 2020 (Lopez, Shibuya, et al. 2006; Lopez, Mathers, et al. 2006). Moreover, approximately 3 million people died as a consequence of COPD in 2012, which accounts for 6% of deaths worldwide (Strategy et al. 2017). The onset of COPD is gradually progressive in its nature, with patients typically diagnosed during midlife (Buist et al. 2007), and is responsible for a growing cause of morbidity and mortality worldwide, largely due to its association extra pulmonary effects (Strategy et al. 2017; Lopez, Shibuya, et al. 2006; Lopez, Mathers, et al. 2006).

Patients with COPD display dominant features, chronic bronchitis (Pauwels and Rabe 2004) and emphysema, which are often referred together with airflow obstruction being the cardinal feature (Fischer, Pavlisko, and Voynow 2011). Further, the natural course of COPD is accelerated with frequent bouts of exacerbations (Gulcev et al. 2016), which accelerate lung function decline and thus limits physical activity. Consequently, quality of life is reduced and the risk of death is increased (Scioscia et

al. 2016). In addition, episodes of exacerbations represent the second most common cause of emergency hospital admissions (Bartolome R Celli et al. 2015; Guarascio et al. 2013), placing a great strain on healthcare professionals. Taken together, with an ageing population (Lopez, Shibuya, et al. 2006) and continued exposure to COPD risk factors (Strategy et al. 2017), the burden is substantial and predicted to increase in coming decades, and therefore places a significant economic burden on healthcare systems worldwide (Gulcev et al. 2016; Pauwels et al. 2001).

1.8.1. Methods of assessment of COPD

To date, classification of patients with COPD has relied upon measurement of airflow limitation through forced expiratory volume in 1 second (FEV_1) (Barton 2011), which is traditionally used to evaluate its diagnosis, progression and subsequent treatment (Vestbo and Anderson 2008). Pulmonary function mechanics such as FEV_1 and FVC provide a snapshot which only reflects the cumulative changes in all affected biological compartments (Fischer, Pavlisko, and Voynow 2011). However, COPD is now widely recognised as a heterogeneous disease, and so FEV_1 is unable to capture many features such as perturbed BCAA metabolism, and including disease progression, especially in the early stages (Vestbo and Anderson 2008). For example, similar FEV_1 results between patients may show different underlying pathologies, e.g., increased inflammation, muscle dysfunction and cardiovascular disease are all features associated with COPD which cannot be detected by spirometry (Ghosh et al. 2016a), therefore identifying biomarkers reflecting the underlying pathology of different COPD subtypes, and in response to therapeutic interventions (exercise or nutrition) are needed.

1.8.2. Causes of COPD

1.8.3. Genetic susceptibility to COPD

The risk of developing emphysema is increased with a rare (Pauwels and Rabe 2004), but well-known genetic disorder resulting in plasma deficiency of protease inhibitor, α_1-anti-trypsin (ATT) (Laurell and Eriksson 1963). The deficiency is a result of a mutation in the α1AT gene, and is a potent anti-protease produced in the liver which functions as an inhibitor of neutrophil elastase (NE), released by the leukocytes.

1.8.4. Systemic effects of COPD

As mentioned in section 1.8. COPD causes hallmark features such as progressive airflow limitation as a consequence of inflammation and remodelling of airways, however there are systemic features accompanying the disease such as reduced body/ muscle mass and undernutrition resulting in a greater disease burden.

1.8.5. Skeletal muscle wasting

Health outcomes and survival rates of patients with COPD are adversely affected by concomitant chronic comorbidities such as skeletal muscle wasting and reduced functional capacity. As such, a large number of COPD patients die from non-respiratory failures (Fabbri and Rabe 2007). Indeed, there is an appreciation of these clinically relevant systemic consequences such as weight loss, accompanied muscle loss, reduced strength and malnutrition (Wilson et al. 1989; Creutzberg et al. 2003; Nishimura et al. 1995) often leading to reduced functional capacity, and how they may contribute to better understanding and management of the disease. Of these systemic features, reduced skeletal muscle mass is a prominent and important one because it exacerbates the declines in muscle mass comprises physical activity (Fabbri and Rabe 2007; Agustı 2007) which predicts morbidity and mortality independent of lung

deterioration (Marquis et al. 2002; Swallow et al. 2007; Vestbo et al. 2006). A significant portion of COPD patients are characterised by fat-free mass (FFM) wasting and altered muscle (and plasma) amino acid profiles, which suggest perturbations at the metabolic level (M. P. K. J. Engelen, Deutz, et al. 2000; Yoneda et al. 2001). This is important because skeletal muscle represents the largest organ in the body, (Bonaldo and Sandri 2013) and earlier sections highlighted its important roles, such as in insulin-mediated glucose disposal and muscle protein turnover, and as a reservoir of amino acids that can be utilised during times of stress and starvation (Rennie 1985; Rennie 1990). Indeed, studies on patients with severe COPD have reported reduced thigh cross-sectional area, and significant loss of skeletal muscle represent a contributing factor to developing metabolic diseases and impaired glucose tolerance (Parr, Coffey, and Hawley 2013), as well reduced oxidative capacity making physical activity an arduous task (Wagenmakers 1999).

Significantly reduced FFM in COPD patients suggest perturbations in intermediary metabolism, and several studies report significant disturbances in plasma AA of patients with stable and severe COPD. For example, Schols and workers (Schols et al. 1993) demonstrated reduced FFM in COPD patients, despite being of normal whole body mass. Bernard et al. (1998) examined thigh CSA of COPD (~83 cm^2) patients and found a significantly reduced mass compared to healthy controls (~110 cm^2). Reduced CSA was shown to be accompanied with reduced skeletal muscle strength, contributing to exercise intolerance whilst also shown to be associated with healthcare utilisation (Decramer et al. 1997). Specifically, a role for decreased plasma levels of BCAA in COPD has been described (Yoneda et al. 2001), whereby they have significantly associated with weight loss, decreased muscle mass % FEV_1.

To this end, studies by Ubhi and co-workers (Ubhi, Cheng, et al. 2012; Ubhi, Riley, et al. 2012) report metabolomic data in support of increased muscle loss (measured by increased 3-methylhistidine concentration) in COPD patients of various stages, i.e., markers of increased protein turnover in all patients, however losses were most profound in those with predominant emphysema and accompanied with decreased concentrations of (BC)AA's. For example, well-characterised COPD patients with predominant emphysema (as per CT scan) were revealed to have significantly lower BCAAs and 3HIB compared to severe (GOLD III) COPD patients. In addition, these same workers (Ubhi, Riley, et al. 2012) used partial least squares (PLS) multivariate analysis to reveal increased CRP (suggestive of systemic) inflammation in all patients which uniquely correlated with selected AA (i.e. phenylalanine, histidine and valine). Further, they were able to stratify COPD patients with cachexia who had significantly increased concentration of phenylalanine, glutamine and serine ($p < 0.0001$ compared to non-cachectic; Ubhi et al. 2012). A common feature in both studies were the increased concentrations of 3-MH, a surrogate marker suggesting of increased MPB (Elia et al. 1981).

Several authors (Ubhi, Riley, et al. 2012; Ubhi, Cheng, et al. 2012), plus others before them (Yoneda et al. 2001; Pouw et al. 1998) have consistently shown losses of lean mass associate with COPD across varying stages, as well as lower BCAAs in plasma compared to age-matched controls (M. P. K. J. Engelen et al. 1998; Engelen et al. 1999). In particular, low leucine concentrations in plasma have been shown by Engelen et al (M. P. K. J. Engelen, Deutz, et al. 2000) and Hofford et al (Hofford et al. 1990) which was the main driver of decreased sum total BCAA. The majority of BCAAs and glutamine reside in the muscle free amino acid pool, and are therefore key to regulating lean mass, protein metabolism and degradation. Since muscle loss

is a prominent feature in COPD patients, it is unsurprising that we observe consistent features, such as increased AA, including BCAA and markers of increased protein breakdown in plasma (discussed below). BCAA are not only important in whole-body health (key players in protein turnover (Neinast et al. 2019) but may also offer prognostic value (i.e., metabolic 'signature'; Newgard et al. 2009) and these more accurate techniques of disease classification provide a basis to work upon, since traditional measures of disease classification (such as FEV_1) do not fully capture systemic manifestations (Engelen, Schols, et al. 2000; Schols et al. 1993).

1.10. Impacts of exercise on muscle mass and function in COPD

Loss of Skeletal muscle function leads to reduced activity in COPD patients, therefore various exercise training modalities may be an essential component in pulmonary rehabilitation, especially since muscle is a potential therapeutic target to alleviate dyspnea, fatigue and exercise intolerance (Man et al. 2009).

1.12. Endurance exercise

Endurance exercise (EE) training, for example ground walking or cycling, improves peak oxygen uptake in healthy individuals, in young and old (Kohrt et al. 1991; Wibom and Hultman 1990) to 4.9 ml•kg•min in comparison to untrained controls (Milanović, Sporiš, and Weston 2015). Unsurprisingly, EE has recognised benefits for those suffering with COPD (McConnell and Gosselink 2014). The magnitude of improvements in aerobic capacity are limited in these individuals due to reduced ventilatory capacity (Plankeel, McMullen, and MacIntyre 2005), however even in those who are unable to reach their peak, submaximal capacity stands to improve, independent of lung function (Spruit et al. 2002). A major concern in the use of EE in COPD patients is selection of intensity, since sufficient (usually high) intensities are problematic for patients to complete (LeBlanc et al. 1996). Negating this limitation,

Vogiatzis and coworkers (2005) report interval training in conjunction with pulmonary rehabilitation resulted in increases in peak power, quadriceps capillary: fibre ratio and citrate synthase activity in comparison to work-matched continuous cycling, suggesting that adaptation occurs however with lower reports of leg discomfort and dyspnoea. Others have shown that unilateral training in COPD patients to be superior compared to bilateral cycling (Dolmage and Goldstein 2008; Bjørgen et al. 2009). The likely explanation here is the less relative burden on ventilation and therefore more stimulation of cardiovascular system to aid in exercise adaptation.

1.13. Resistance exercise

Resistance exercise training (RET) has shown some promise in COPD patients, and may be preferred since cross-over (aerobic capacity) effects are observed (Frontera et al. 1990). The specificity of resistance exercise exploits the plasticity of skeletal muscle remarkably well, representing an effective and therapeutic strategy to counter the loss of muscle mass seen in chronic diseases, even in old age (Fiatarone et al. 1990). Implementation of RET programmes has demonstrated consistently positive results regarding lung function (Clark, Cochrane, and Mackay 1996; Richard Casaburi et al. 2004), which is also reflected in muscle strength and CSA (Bolton, Bevan-Smith, and Blakey 2014). Other works detail promising interventions involving supplementation of testosterone (Richard Casaburi et al. 2004) and ergogenic (leucine, vitamin D and omega-3 fatty acids) aids (van de Bool et al. 2017) in the context of increasing muscle mass, strength and aerobic capacity. Studies looking at the role of cell signalling proteins in muscular hypertrophy in COPD patients has shown promising results. Constantin et al (2013) provide supportive findings with temporal data of a range of molecular signalling targets response to resistance (knee extension) exercise. These authors found thigh lean mass increased from baseline

(6.2 %-fold-change) and was maintained up until the final time point (8-weeks) in COPD, which was matched by healthy controls (5.4 %-fold-change). This was supported by an exercise–induced increases in the ratio of phosphorylated: total protein expression in those regulating MPS (AKT, P70S6K, 4EBP1, Redd1) and MPB (20sproteoasome, MAFbx, MuRF1) from baseline compared to 8-weeks, although the magnitude of increase was greater in the healthy controls suggesting an impairment of the protein synthetic machinery in COPD patients, despite the similar increases in strength and thigh lean mass. These data suggest that the responsiveness to RET in COPD is preserved (albeit from a depleted baseline), and that physical inactivity resulting in disuse is a key factor in muscle wasting and deconditioning in COPD, a notion which has been supported by other workers (Vonbank et al. 2012; O' Shea, Taylor, and Paratz 2009).

Undoubtedly, low levels of physical activity is common in COPD cohorts (Pitta, Troosters, and Probst 2006; Pitta et al. 2005; Walker, Burnett, and Flavahan 2008) and a positive association between reduced physical activity and increased mortality has been shown (Waschki et al. 2011). In contrast, increasing physical activity is associated with more favourable outcomes in COPD cohorts, such as increased independence and reduced risk of hospital admission (Garcia-Aymerich et al. 2003; 2006). However, it is important to recognise that since individuals with COPD are generally more sedentary compared to age-matched controls (Cavalheri et al. 2016), exercise will likely induce improvements in muscle function and adaptation.

1.9. Summary and aims

Skeletal muscle and constituent proteins/ amino acids, serve important metabolic and physiological roles both during normal health but also when the body is perturbed such

as in disease states. In these instances, such as with COPD, and advancing age, patient outcomes will worsen, for example reduced muscle size is a risk factor from hospitalisation of an exacerbation of chronic respiratory diseases (Greening 2014). As discussed, BCAAs are indispensable in their role as key players in protein homeostasis, for exercise-induced muscle gains and fuel where required. The rationale for measuring BCAAs is that since loss of mass, particularly of muscle in those with COPD, and poor nutritional behaviours commonly observed in the those with IR and obesity, plasma BCAAs are ideal to measure since they have been shown to relate to markers of disease and respond to functional outcomes (such as increased mass and function) as well as metabolic markers such as insulin sensitivity. Furthermore, exercise responses have been well-studied in those with COPD, the response of plasma BCAAs under such interventions are yet to be defined. Consistent reports of BCAA association with obesity and insulin resistance, a marker of muscle/ protein loss in COPD cohorts suggests their role is not fully elucidated.

Therefore, present thesis will make attempts to add knowledge in this area, principally by investigating plasma concentrations of BCAAs in separate studies, during normal health, ageing, calorie restriction (+/-) exercise regimes and also in two distinct COPD phenotypes (stable vs exacerbated). The specific aims of each chapter are as follows:

Chapter 3 (Longitudinal RET study): The aims of this retrospective study were to evaluate the effects of a longitudinal exercise intervention on advancing age with regards to lean mass and strength. A further aim was to determine whether a relationship between IS and BCAA holds in healthy individuals, and if there exists an association between advancing age and plasma BCAAs. Finally, the effects of a structured RET regime was tested to determine whether this would impact a relationship between BCAAs and markers of metabolic health. The hypothesis of this study was that ageing would be associated with increased BCAA, and that RET would reduce BCAA. It was also hypothesised that reduced BCAA would be associated with improvements in IR.

Chapter 2 (Six-weeks calorie restriction, alone or combined with exercise): Next, we sought to confirm the relationship between plasma BCAAs and IS in a separate study in a cohort of obese men. Additionally, the relationship between IS and BCAA-related handling enzymes and candidate genes were investigated, both following a period of severe calorie restriction. Further, the cohort was divided into groups comprising of severe calorie restriction (VLCD) alone and VLCD + RET and VLCD + HIIT to investigate the effects of weight loss and exercise on BCAAs and its association with IS. The hypothesis of this study was that BCAA would be elevated in the obese, and that ensuing weight loss and reductions in IR would be associated with a 'normalisation' of BCAA, which would relate to improvements in IS.

Chapter 5 (Eight weeks of endurance exercise in individuals with stable COPD): Next, the aim of this study with retrospective analyses was to evaluate the effects of 8-weeks supervised EE in the form of cycling exercise on markers of lung function and aerobic capacity in individuals with stable COPD compared to age-matched controls. Since selective wasting of lean mass and alterations in BCAAs commonly observed

in those with COPD, a further aim was to evaluate whether plasma BCAAs can reflect changes in respiratory health in patients with chronic respiratory disease. A further aim was to determine whether an exercise response would correlate to changes in plasma BCAAs, and whether these would relate to lean mass and aerobic capacity. The hypothesis of this study was that individuals with COPD will experience smaller changes to aerobic capacity compared to control, and this exercise response would be reflected in the plasma BCAA.

Chapter 6 (Six-week early rehabilitation programme commenced in individuals admitted to hospital with an acute exacerbation of COPD): The aims of this study, a retrospective analysis, was to determine whether a novel, early rehabilitation programme, consisting of RET and aerobic-based exercises (supervised initially) would improve functional outcomes in patients with COPD (muscle CSA, thickness, strength), whom had been admitted to hospital following a bout of exacerbation. Secondly, since muscle wasting is a dominant feature of individuals who experience exacerbations, and decreased BCAAs have been reported compared to stable COPD and healthy individuals, it was investigated whether improvements in plasma BCAAs would reflect changes in functional improvements with a more severe form of chronic respiratory disease. The hypothesis of this study was that those with severe COPD would display decreased BCAAs compared to stable COPD, and an early rehabilitation intervention would elicit greater improvements in muscle function and size resulting in normalisation of plasma BCAAs

CHAPTER TWO: GENERAL METHODS

2.1. Participant recruitment and medical screening

With the exception of the results presented chapter 4 (VLCD intervention), the thesis contains data generated from retrospective analyses – two of which, chapter 3 (Phillips et al. 2017) and chapter 6 (Greening et al. 2014), have been published. Prior to commencement onto any studies, all participants had the study explained to them, including any risks, before providing written consent. Participants of chapters 3 and 4 performed activities of daily living and were recruited from the Derbyshire and Nottinghamshire areas by poster advertisements and letters sent to individuals of required age and health categories. Participants in these two studies were excluded if they had had evidence of cardiovascular disease, cerebrovascular diseases including previous stroke, respiratory disease including pulmonary hypertension or COPD, T2DM, musculoskeletal diseases or any disease requiring long-term drug treatment. Participants of chapter 5 (COPD) were who had obstructive spirometry with lung function FEV_1 <80 % predicted, MRC grade 3–5 but stable COPD with no episodes of exacerbation in the last 4 weeks were enrolled into the cycling exercise intervention. Participants of chapter 5B were enrolled within 48 h of admission to hospital for an episode of exacerbation of COPD and randomised into one of two groups. Exclusion criteria for the COPD studies were the presence of acute cardiac events, any musculoskeletal, neurological or psychiatric comorbidities and all participants had capacity to consent to study interventions.

2.2. Participant characteristics and ethical approval

In the RET study, three participants consisting of young (18–28, *n* = 8, BMI: 24±1 kg/m^2), middle–aged (45–55 years, *n* =9, BMI: 27±1 kg/m^2) and older (65–75 years, *n* = 15, BMI: 27±1 kg/m^2) were recruited for 20-weeks progressive RET (men and

women, ~50:50were screened by medical questionnaire and physical examination, where clinical chemistry blood profiles and ECG were conducted.

The VLCD study consisted of a total of 26, non-diabetic men whom followed 6-weeks VLCD (mean age 43.9 ± 9 y BMI 32.2 ± 2.9 kg/m^2) intended for weight loss and improvements in metabolic health. Participants were overweight, but non-diabetic and participants who suffered from any cognitive or metabolic disorders were not enrolled on this study. Individuals for both of the aforementioned studies were not taking any prescribed medications, were normotensive (< 140/ 90 mmHg) and were instructed to continue activities of daily living but not participate in any formal exercise. Study sessions for both chapters 3 and 4 were performed at the University of Nottingham Medical School at the Royal Derby Hospital centre and were fully supervised by a single member of research staff. The University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (D/2/2006–B12092016) reviewed and approved these studies, and both complied with the 2013 Declaration of Helsinki.

With the exception of the healthy, older controls, all participants of chapter 5 suffered from COPD of varying severity (MRC grade 3-5) with a selection enrolled onto the study after 48 h of admission to hospital following a bout of exacerbation (Greening et al. 2014). These COPD studies were reviewed by the NHS National Research Ethics Services (West Midlands – Coventry and Warwickshire; 13/WM/0075) and Nottingham REC1 committees (09/H0403/76), steady COPD and exacerbated individuals, respectively. Elderly, steady state COPD (*n* = 18 6 male and 12 females, mean age 68.8 ± 5.6 years, BMI: 31.1 ± 6.6) were recruited via pulmonary rehabilitation waiting lists and mailshot respondents. Patients were clinically diagnosed with COPD and obstructive spirometry with lung function FEV_1 <80& predicted, with no episodes of exacerbation of COPD in the last 4 weeks. Patients who had episodes of exacerbation

were randomised within 48 h to a usual care group (*n* = 10, mean age 68.2 ± 12.1, BMI: 23.8 ± 5.9 kg/m^2) or early rehabilitation group (*n* = 10, mean age 72.4 ± 3.7, BMI: 25 ± 6.3 kg/m^2). Participants who were unable to consent to these studies, or had acute cardiac events or the presence of any musculoskeletal or neurological comorbidities were excluded from the study. In addition, participants were excluded if more than four emergency hospital admissions had occurred in the previous 12 months of the study enrolment.

2.3. Conduct of the studies

2.3.1. Progressive RET programme

The progressive RET programme required all participants to report to the laboratory at 090:00 h, following overnight fast from 21:00 h the evening before. Participants were instructed to refrain from strenuous exercise (72 h prior), alcohol or caffeine (24 h prior). The RET programme was designed to achieve muscular hypertrophy following previously published recommendations (Singh 2002) for exercise intensity and duration. Therefore, supervised members of the research team were present at every session to deliver 60-minute sessions for 3 times a week over the course of 20 weeks. These sessions included two sets of 8–12 repetitions for three upper and three lower body exercises. To adhere to progressive overload principles and maintain 70 % intensity, testing of 1-RM was conducted every 4 weeks.

2.3.2. Six-weeks of VLCD +/- exercise

A total of 26 volunteers participated in a 6-week calorie restriction study (VLCD), with *n* = 16 of those randomised into RET and HIIT arms of the VLCD study. Every participant followed a diet consisting of 4 meals per day, which totalled ~600 kcal/ day with an additional 200kcal/ day permitted (in the form of fruit, vegetables or meat) to maintain motivation of adherence. The meal replacement diets were provided by

Lighter life (Harlow, Essex, U.K.), and provided a total of 50 g protein, 50 g carbohydrate, and ~17.3 g fat which delivered 100 % RDA of vitamins and minerals. Those in the RET group were tested for 1RM at baseline and prescribed a combination of machine-based upper and lower body exercise (3 each), following a warmup, supervised by a member of research staff. The frequency of sessions were three times per week, with strength tests carried out every 14 days to ensure relative intensity remained constant. Baseline CPET was conducted on a cycle ergometer (Lode Croival, Groningen) with inline gas analysis system (ZAN 680, nSpire Health, Colorado, USA) using a standard 15 w/ min ramp protocol following two min period of unloaded cycling. Participants were instructed to maintain ~55 RPM cadence and verbally encouraged throughout. The test was complete once the participant had indicated their maximum has been reached, via the Borg scale. ECG, blood pressure and pulse were monitored throughout. HIIT training sessions were performed on the same cycle ergometer and were prescribed a protocol involving 60 seconds of high intensity cycling at ~95 % watt max, repeated 5 times and interspersed with 90 s of recovery. Sessions started with 2 minutes of warm up and cool down, and the intensity was calculated from pre-determined maximal aerobic capacity. Session frequency was 3 times/ week and supervised by a trained member of research staff.

Collection of muscle biopsy

Participants of the VLCD study described in chapter 4 had baseline fasting biopsies of *m*. vastus lateralis taken under sterile conditions via the conchotome biopsy method (Dietrichson et al. 1987) under local anaesthetic conditions (1 % lidocaine; B. Braun Melsungen, Melsungen, Germany). Once collected, muscle was rapidly dissected free of fat and connective tissue, washed in ice-cold phosphate-buffered saline (PBS),

blotted dry before it was frozen in liquid nitrogen. All samples were stored at -80ºC until further analysis.

2.3.3. Endurance cycling intervention in stable COPD patients

Patients with stable COPD attended Glenfield Medical centre for baseline assessments and familiarisation split over two days. Testing included quadriceps strength (MVC) and CPET measurements. Exercise tests were separated by a minimum of 30 minutes recovery time, which is in-line with published guidelines (Holland et al. 2016), i.e. heart rate, ventilation and respiratory capacity exchange ratio returned to resting values before commencement of a second test. Peak exercising capacity was achieved by a ramp protocol of 5 W/ minute to 30 W/ minute with increments of 1 W until exhaustion. Tests were terminated if participant was unable to maintain a 60 RPM cadence. VO_2^{PEAK} was defined as the highest oxygen uptake (L/ min) during loaded pedalling. Participants then commenced an 8-week (three sessions a week) of aerobic cycling, with a gradient of 1 w/ minute to a maximum of 65 % intensity with cadence encouraged to be maintained at 60 RPM. Cycling resistance was adjusted at 4 weeks to ensure intensity remained constant. Measurements of body composition, where described, were achieved via DXA (Lunar Prodigy II, GE Medical systems, Buckinghamshire, United Kingdom) and regions automatically assessed by integrated software packages (Encore software, GE Healthcare). Fasted blood samples were obtained from the antecubital vain and collected into vacutainers containing lithium-heparin. Once collected, samples were centrifuged at 2000 g of force for 20 minutes at 4 ºC.

2.3.4. An early rehabilitation programme in COPD patients following an episode of acute exacerbation

Exacerbated COPD patients who were randomised in the usual care group received standard hospital care delivered by a respiratory physiotherapist which included techniques for airway clearance, assessment and supervision of mobility and advice on smoking cessation. Participants within the early rehabilitation group commenced the intervention within 48 hours of admission. Accompanied by the standard usual care, they also received daily supervised volitional (strength and aerobic) training. These were performed by the patient bedside and following discharge participants were provided with a home-based programme and monitored by telephone consultations. The exacerbated patients received 7 days of supervised delivery, with the remaining 5 weeks performed by the patient, unsupervised by research staff.

2.4 Analytical methods

Determination of plasma AA concentrations of each participant were determined by the addition of stable isotopically labelled internal standards and prepared according to previously published protocols from our lab (Wilkinson et al. 2013). Fasted plasma samples (100–200µL) were precipitated with 1 ml ice-cold ethanol and centrifuged at 10000 rpm for 5-min, the supernatant removed and evaporated to dryness under nitrogen at 90°C, followed by re-suspension in 0.5M HCI. Ethyl acetate was then added to aid in separation, and samples were vortexed thoroughly before the upper ethyl acetate layer (containing lipids) was removed. The aqueous AA-containing layer was decanted and evaporated to dryness under a steady flow of nitrogen at 90^{o}C. A pooled plasma QC sample was prepared with each batch and injected throughout to monitor instrument performance. This was achieved by pooling small aliquots of each study sample and thoroughly mixing. Aliquots of study-specific samples were used to

closely mimic metabolite composition of samples being measured, with the aim to account for analyst and analytical intra- and inter- batch variations.

To permit GC-MS analysis, AAs were derivatized as their *t*-BDMS forms. Dried residue samples were suspended in equal volumes of acetonitrile and N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), followed by thorough vortex. Samples were then allowed to react by incubation at 90^{o}C for 45 min, thus converting the AA's to their *t*-BDMS derivatives (Wilkinson et al. 2013). Amino acid samples were then ready for GC-MS analysis, and concentrations determined with reference to a calibration curve of standard amino acids of known concentration. A representative example of the chromatogram searching for masses of BCAAs is displayed in figure 4

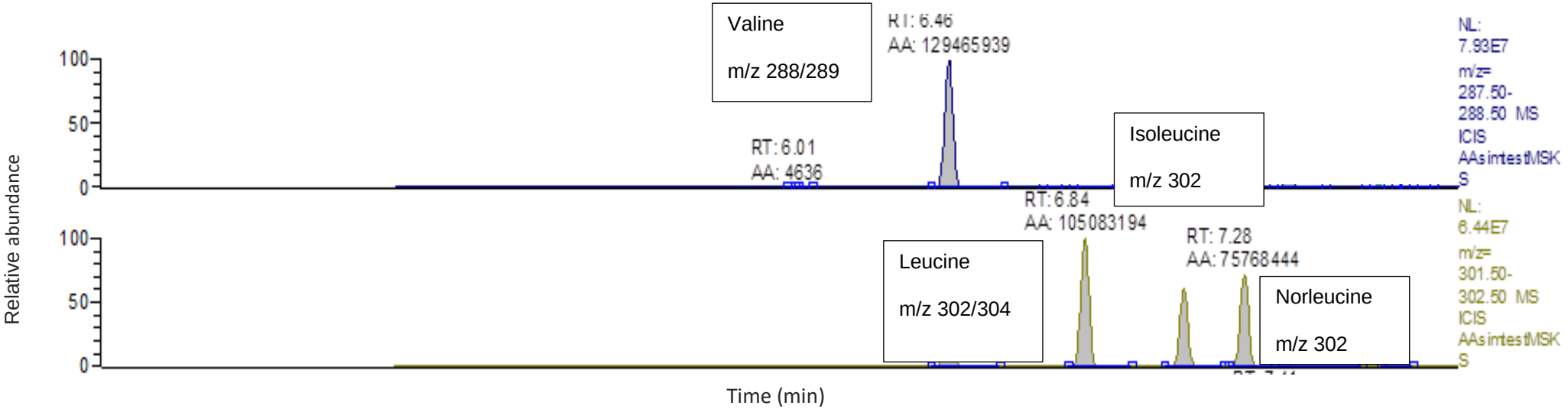


FIGURE 4. REPRESENTATIVE EXAMPLE OF A TOTAL ION CHROMATOGRAM (TIC) DISPLAYING MASS FRAGMENTS OF BCAAS AND CORRESPONDING ISOTOPICALLY LABELLED INTERNAL STANDARDS, NORLEUCINE WAS USED FOR QUANTIFICATION OF ISOLEUCINE.

2.5. GC-MS conditions

A total of 0.5µl of sample (*t*-BDMS) was injected into an ISQ Trace 1300 single quadrupole GC-MS (ThermoFisher Scientific Hemel Hempstead, UK) for plasma total AA. BCAAs from stable COPD patients were determined using a TSQ 9000 Triple quadrupole GC-MS (ThermoFisher Scientific Hemel Hempstead, UK). Split injection mode (1:10) was used, at an initial oven temperature of 100°C, held for 1 min, with a temperature ramp of 12°C/ min to 300°C and held for 5 min. Helium was used as a carrier gas at a flow rate of 1.5mL/ min, and separation was achieved on a 30 m Rxi-5MS (0.25mm internal diameter, 0.25µm film-thickness) fused silica column (Restek, Bellafonte, Pennsylvania). A selected ion monitoring scan (SIM) was created to search specifically for amino acid standards for Leucine (m/z 302), isoleucine (m/z 302) and valine (m/z 288), with corresponding isotopically labelled internal standards (304 and 289 for leucine and valine, respectively), or Norleucine (m/z 302) for isoleucine quantitation, included in the SIM.

2.6. Immunoblotting procedures

Immuoblotting was performed on muscle samples for participants in the VLCD studies only. For BCAA-related enzymes, muscle samples (~10 mg were muscle) were used to extract muscle protein content in radioimmunoprecipitation (RIPA) buffer (50 mM Tris HCI pH 7.4, 150 mM NaCI, 1 % Triton X-100, 0.5 % Sodium deoxylcholate, 0/1 % sodium dodecyl sulfate). To prepare samples, 50 µl of RIPA buffer was added per mg of wet weight muscle and homogenised with scissors, followed by sonication and centrifugation. Supernatant was carefully collected for protein quantification using the bicinchoninic acid (BCA) assay (Smith et al. 1985) with a commercially available BCA assay kit (Thermo Finnigan, Thermo Scientific, Hemel Hempstead, U.K.). Once

protein concentrations were determined, samples were standardized to 1 mg/ by dilution with x3 Laemmli loading buffer and heated for 5 min at 95 ºC. Samples containing 10 µg / ml protein and cross-gel quality controls were loaded on to a Criterion XT Bis-Tris 12 % SDS-PAGE precast gel (Bio-Rad, Hemel Hempstead, U.K.) for electrophoresis at 200 V for 60 min. Following protein separation (Bass et al. 2016), samples were transferred to polyvinylidene difluoride (PVDF) membranes for electro-transfer at 100 V for 45 min.

Membranes were subsequently blocked with non-fat dry milk (2.5 % diluted in Tris-buffered saline Tween 20 [TBST]) 1 hour at ambient temperature. Membranes were then incubated overnight at 4 °C under gentle agitation, in the presence of the following antibodies (all diluted 1:200 in 2.5 % bovine serum albumin in TBST) branched chain amino acid transaminase 1 (BCAT1), branched chain amino acid transferase mitochondrial (BCAT2), branched chain alpha-keto acid dehydrogenase complex (BCKDH-E1α) and Phospho-BCKDH-E1α (p-BCKDH)^{Ser293} (all Cell-Signaling technology, Leiden, The Netherlands). The following day, membranes were washed (3 x 5 minutes) with TBST and incubated for 1 hour at room temperature soaked in horseradish peroxidase-conjugated anti-rabbit secondary antibody (New England Biolabs' 1:200 in 2.5 % BSA in TBST). Finally, membranes washed for 3 X 5 min in TBST and incubated for exactly 5 min with enhanced Chemiluminescent HRP reagent (Millipore Corp., Billerica, MA, USA). Bands were quantified on a Chemidoc MP (Bio-Rad, Hemel Hempstead, U.K.) by peak density and normalized to Coomassie brilliant blue staining of the membranes (Welinder and Ekblad 2011) and software measures were taken to prevent band saturation and protein-loading anomalies were corrected to total Coomassie protein (Welinder and Ekblad 2011). Values were subsequently normalised to corresponding baseline value before statistical analysis. An example of

the immunoblotting images is displayed in figure 5.

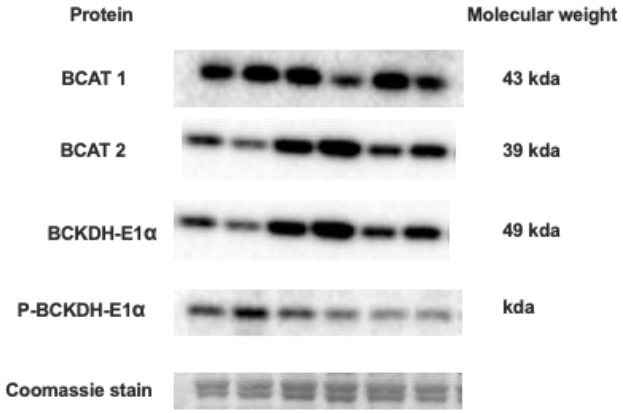


FIGURE 5. REPRESENTATIVE EXAMPLE OF IMMUNOBLOT OF BCAA-RELATED ENZYMES

2.7. Muscle gene expression analysis

To investigate changes in muscle gene expression following VLCD, ~10-15 mg of muscle tissue was homogenised for RNA content in TRIzol™ (Invitrogen, Thermo Fisher Scientific) with the addition of a stainless-steel bead (Tissue Lyser II, Quigen, U.K.) for 2 minutes at the frequency of 30 s, according to manufacturer's instructions. RNA quality and quantity were then measured by spectrophotometry (NanoDrop 2000, Thermo Scientific). A high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific) was used to reverse transcribe 500 ng of total RNA which were then diluted 1:10. Precisely 1 µl of 1:10 diluted cDNA (in triplicate) was individually added onto a 384 optical well plate (Life Technologies). Exon-exon boundary specific primers were mixed with SYBR Select Master Mix (Thermo Fisher Scientific), and RNase-free water and addition of 6 µl mixed solution with 1 µl of each cDNA, were added to the wells. Real-Time quantitative PCR (qPCR) was performed on a ViiATM 7 PCR System (Life Technologies). The ΔΔCt method (Schmittgen and Livak 2008) was used to quantify target mRNA expression, with RPL13A being used for normalisation. Primer sequences for each of the probed genes are listed in table

1. Primer validation of target genes were carried out prior to analysis and a melt curve plot of this is displayed in figure 6. Serial dilutions of cDNA were performed in triplicate (1:5–1:80) to generate a standard curve before efficiency of each amplification was calculated based on the slope of the curve generated by each primer. Primers reaching 90–110 % were accepted for subsequent analysis.

Table 3. The following primer sequences were used to measure gene expression described above.

RPL13A	Fwd	5’-TAAACAGGTACTGCTGGGCCG-3’
	Rev	5’-CTCGGGAAGGGTTGGTGTTC-3
BCAT 1	Fwd	5’-GGCTACGACCCTTGGGATCT-3’
	Rev	5’-GTCCCCACCACCTCTTTTGA-3’
NDUFB3	Fwd	5’-GCTGGCTGCAAAAGGGCTAA-3’
	Rev	5’-CAGCTCCTACAGCTACCACAA-3’
PPM1K	Fwd	5’-CCGCTTTGACTGCTTGCTTC-3’
	Rev	5‘-GAGGAGCTTTCTTGGTCGGT-3’
PCCB	Fwd	5’-AGGAGTGGAGTCTTTGGCTG-3’
	Rev	5‘-TCTGTTAGGGCTGGGGAGTA-3’
SDHB	Fwd	5’-GCTACTGGTGGAACGGAGAC-3’
	Rev	5’-GCGCTCCTCTGTGAAGTCAT-3’
ALDH6A1	Fwd	5’-GAGCTGATCTTGGCCCTCTG-3’
	Rev	5’-GCTCCCTCCTTTGTTCCACT-3’
HIBADH	Fwd	5’-ATGGATGCCCCTGTTTCTGG-3’
	Rev	5’-CCACAGTACACCACGTTGGA-3’
KLF15	Fwd	5’-GGGAGAGAGGTGAAAAGCGT-3’
	Rev	5’-TTGTCTGGGAAACCGGAGGA-3’

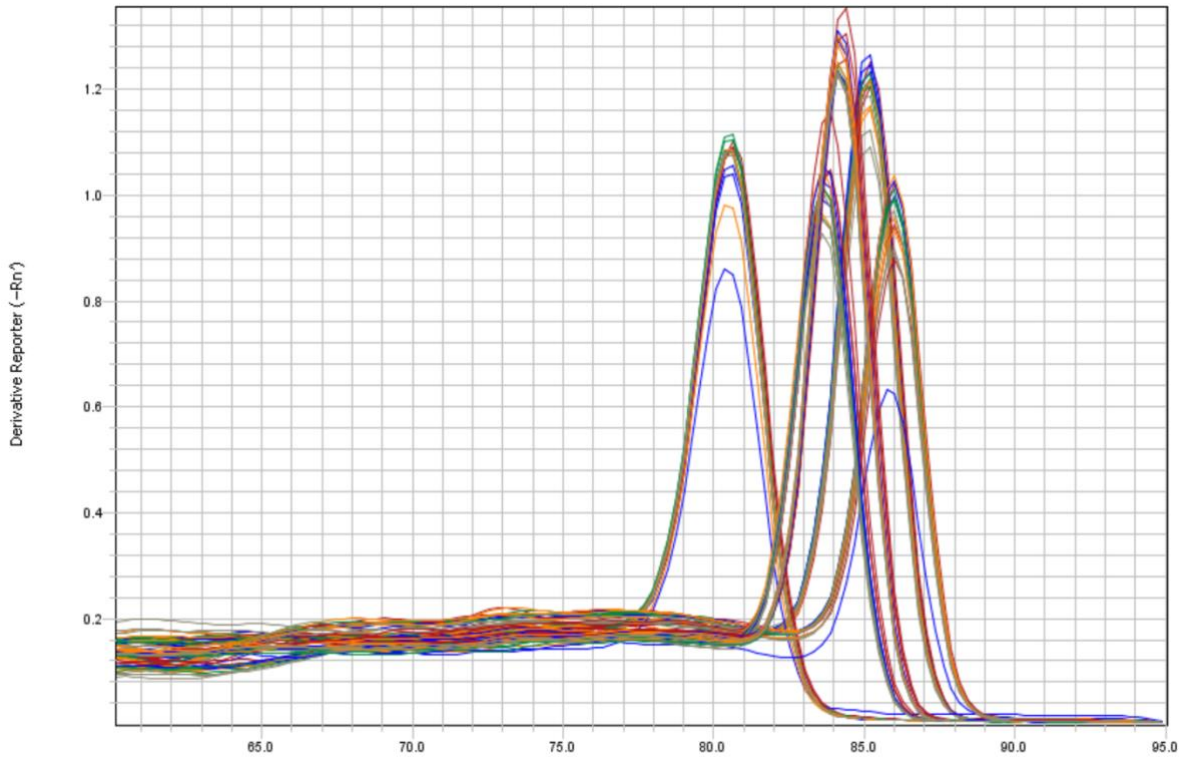


FIGURE 6. PCR VALIDATION OF MELT CURVE PLOT OF TARGET GENES RELATED TO BCAA METABOLISM. NORMALISED REPORTER (Y-AXIS) IS PLOTTED AGAINST THE TEMPERATURE (X-AXIS) WHICH HIGHLIGHTS THE FLUORESCENCE VISUALISED DUE TO DYE PRESENT IN SYBR MASTER MIX

2.8. Insulin and glucose concentrations

Plasma insulin and glucose concentrations were determined for RET and VLCD studies, as well as lipoprotein in RET study only. Samples were analyzed at baseline and following respective interventions, as reported (Phillips et al. 2017). In brief, plasma insulin and glucose were measured in duplicate, using undiluted samples. Insulin was assessed via a high-sensitivity human insulin ELISA (DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's instructions. Plasma glucose was measured using a clinical chemistry analyser (ILAB 300 Plus Clinical Chemistry System, Warrington, Cheshire, UK) against commercial standards. Plasma glucose was measured using a separate clinical chemistry analyser (YSI 2950, Biochemistry analyser, YSI Life Sciences, Ohio, USA) in results described in RET

study. Insulin sensitivity was calculated using the homeostatic model assessment of insulin resistance (HOMA-IR) and the following Formula:

$$(\text{HOMA-IR} = \text{plasma glucose concentration } (\text{mmol.l}^{-1}) \times \text{plasma insulin concentration } (\text{mU.l}^{-1}))/22.5$$

CHAPTER THREE: ASSOCIATIONS BETWEEN FASTING PLASMA BRANCHED CHAIN AMINO ACIDS AND HEALTH BIOMARKERS IN RESPONSE TO 20 WEEKS RESISTANCE EXERCISE TRAINING ACROSS AGE

Published chapter: *Nutrients, MDPI* (2020)

3.1. Abstract

Background: Leucine, isoleucine and valine (i.e., the branched chain amino acids, BCAA) play a key role in the support of tissue protein regulation and can be mobilized as energy substrates during times of starvation. However, positive relationships exist between elevated levels of BCAA and insulin resistance (IR). Thus, we sought to investigate the links between fasting plasma BCAA following a progressive resistance exercise training (RET) programme, an intervention known to improve metabolic health.

Methods: Fasting plasma BCAA were quantified in adults (young: 18–28 y, *n* = 8; middle-aged: 45–55 y, *n* = 9; older: 65–75 y, *n* = 15; BMI: 23–28 kg/m^2, both males and females (~50:50), in a cross-sectional, intervention study. Participants underwent 20-weeks whole-body RET. Measurements of body composition, muscle strength (1-RM) and metabolic health biomarkers (e.g., HOMA-IR) were made at baseline and post-RET.

Results: BCAA concentrations were determined by gas-chromatography mass spectrometry (GC-MS). No associations were observed across age with BCAA; however, RET elicited ($p < 0.05$) increases in plasma BCAA (all age-groups), while HOMA-IR scores reduced ($p < 0.05$) following RET. After RET, positive correlations in lean body mass ($p = 0.007$) and strength gains ($p = 0.001$) with fasting BCAA levels were observed.

Conclusions: BCAA do not correlate with age, nor are elevated BCAA a robust marker of IR in those with a healthy BMI; rather, despite decreasing IR, RET was unexpectedly associated with increased BCAA. A novel link with increased BCAA and LDL warrants further investigation.

3.2. Introduction

Insulin resistance (IR) is a core pathophysiological mechanism which manifests in concert with β-cell failure, leading to type 2 diabetes mellitus (T2DM) (Guariguata et al. 2013) a disease that is expected to increase in prevalence to affect 592 million by 2035 (382 million in 2013;Wild et al. 2004). Given that this disease is most apparent in older individuals (>65 years) (Harper, Miller, and Block 1984) in whom life expectancy has increased (Wang et al. 2016), this has, and will continue, to result in a burgeoning healthcare burden. While the aetiology of IR and T2DM are complex, there has been long-standing curiosity in relation to a potential link between the branched chain amino acids (BCAA) leucine, isoleucine and valine, which account for 35% of the essential amino acids (EAA) in muscle proteins (Wang et al. 2016), and IR.

In pioneering studies, Felig et al. (Felig, Marliss, and Cahill 1969b) demonstrated strong correlations between elevated circulatory BCAA concentrations and insulin levels in human obesity, that were corrected by weight loss. More recently, reports of elevated BCAA in obese and/or IR individuals have been substantiated in longitudinal (Wang et al. 2011) and cross-sectional (Huffman et al. 2009) studies, as well as across various ethnic groups (Tillin et al. 2015). For example, Newgard and colleagues (Newgard et al. 2009) reported positive associations between circulating BCAA concentrations and increased risk of IR and T2DM, and metabolomic profiling of >100 plasma analytes revealed elevated levels of BCAA and select other metabolites as a metabolic “footprint” of IR (Newgard et al. 2009). This was based on five principle components that accounted for the greatest differences between obese and lean subjects; a combination of BCAA, aromatic AA (AAA, phenylalanine and tyrosine), Glx

(glutamine and/or glutamate) and acyl-carnitines (C3 and C5), indicating interplay between AA and lipid metabolism (Newgard 2012). Moreover, the role of BCAA in IR was further cemented with observations that lower BCAA levels correlated with improved markers of insulin sensitivity (IS) following weight-loss interventions enhancing glycaemic control (Shah et al. 2012; Wang et al. 2017). In addition to this, more recent studies have reported that BCAA-mediated IR may be compounded by IR, further exacerbating BCAA accumulation (Mahendran et al. 2017; Batch et al. 2013). Finally, pre-clinical (Huffman et al. 2009; Yamakado et al. 2015; Wurtz et al. 2013) and clinical (Newgard 2012; Neinast et al. 2019) studies have shown that BCAA-mediated IR, at least in part, lies at the level of skeletal muscle (Shah et al. 2012; Smith et al. 1992).

Nonetheless, that circulatory BCAA positively links to IR does not alter the positive effects that dietary BCAA have upon skeletal muscle metabolism. The role of BCAA in stimulating muscle protein synthesis and supporting muscle hypertrophy following resistance exercise training (RET; Kumar et al. 2009; Atherton, Smith, et al. 2010) is well-documented. Further, leucine acts not only as a substrate for newly synthesised proteins and as a regulatory signalling metabolite activating anabolic pathways (Drummond et al. 2009; Flakoll et al. 1989), but is also a potent insulin secretagogue with the potential to enhance peripheral glucose uptake and to inhibit whole-body and muscle protein degradation via inducing insulin secretion (Fryburg et al. 1990; Petrides, Luzi, and DeFronzo 1994; Moller-Loswick et al. 1994; Chow et al. 2006; Lynch and Adams 2014). These properties of BCAA demonstrate the importance of their role in maintaining and increasing skeletal muscle mass (Petrides, Luzi, and DeFronzo 1994; Moller-Loswick et al. 1994). Yet despite these key metabolic roles, sustained elevated levels of circulating plasma BCAA remain widely implicated in the

pathophysiology of IR (Newgard et al. 2009; Mahendran et al. 2017; Yoon 2016), and the ensuing development of T2DM.

Collectively, previous studies implicate BCAA in the pathogenesis of IR and T2DM. Nonetheless, there remain few intervention-type studies (weight-loss, drugs, exercise, etc.) examining such links under circumstances promoting IS and metabolic health, e.g., exercise. Specifically, RET is one such powerful countermeasure to improve metabolic health and to mitigate age-associated declines in muscle mass and function across the lifespan (Phillips et al. 2017), even in frail elderly individuals (Yarasheski et al. 1999; Binder et al. 2005). As such, we investigated the effects of 20 weeks of fully supervised RET in relation to fasting plasma BCAA and AAA concentrations and metabolic/physiological health parameters. We hypothesised that: (1) ageing would be associated with increased BCAA, AAA (2) RET would reduce BCAA, AAA and (3) that this would be associated with improvements in IR (i.e., HOMA-IR) and/or or other indices of metabolic health.

3.3. Materials and Methods

3.3.1. Ethical Approval

The present study samples originated from previously published work by our research group (Phillips et al. 2017). This study was reviewed and approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (D/2/2006) and complied with the 2013 Declaration of Helsinki. All procedures and risks were thoroughly explained to volunteers and written consent was obtained prior to participation.

3.3.2. Participant Characteristics

Three participant cohorts were studied, consisting of young (18-28 years, *n* = 8, BMI: 24±1 kg/m^2), middle-aged (45–55 years, *n* = 9, BMI: 27 ± 1 kg/m^2) and older (65–75 years, *n* = 15, BMI: 27 ± 1 kg/m^2) men and women (~50:50). All participants were screened by a medical questionnaire (past and existing medical conditions, lifestyle choices), physical examination, clinical chemistry blood profiles (liver function tests (LFTs), thyroid function tests (TFTs), full blood count (FBC), urea and electrolytes (U and E's), fasting glucose, fasting insulin, clotting factors and lipid profiles) and a resting ECG. Participants were not taking any medication at the time of study, had normal blood chemistry, were normotensive (BP <139/89) and did not smoke (nor had they in the past 5 years). Participants were excluded from the study for any metabolic, respiratory or cardiovascular disorders including insulin resistance, dyslipidaemia, uncontrolled asthma, or family history of heightened cardiovascular disease risk (cardiovascular event <55 years). Participants performed activities of daily living but did not participate in formal aerobic exercise training and had not participated in structured RET in the last 2 years. All study sessions were performed at the University of Nottingham Medical School at the Royal Derby Hospital Centre. The exercise intervention was conducted at two sites, based on geographical proximity to the volunteers. All exercise sessions were fully supervised by a single member of research staff.

3.3.3. Participation Overview

Before study days (before and after RET), volunteers were instructed to refrain from strenuous exercise (including the RET intervention) for 72 h and from alcohol or caffeine for 24 h. On each study day, volunteers reported to the laboratory at 09:00 h,

following an overnight fast (water ad libitum) from 21:00 h the evening before. Body composition was assessed via dual-energy X-ray absorptiometry (DXA; Lunar Prodigy II, GE Medical Systems) with all regions automatically assessed by the integrated software package (Encore software, GE Healthcare). Blood samples were taken from the antecubital vein and collected into lithium-heparin containing vacutainers for measures of plasma metabolites, insulin and glucose concentrations, with the plasma-fraction collected following centrifugation at 2000 × G for 20 min at 4 °C. All samples were stored at −80 °C from collection until further analysis.

The RET programme was designed to achieve muscle hypertrophy based on previous recommendations (Singh 2002). As such, the RET programme comprised fully supervised exercise sessions, 3 times each week, with each session lasting approximately 60 min. Two sets of 8–12 repetitions of three upper and three lower body exercises were performed in each session. To achieve progressive overload, training intensity was increased from 40% to 60% 1-RM (repetition maximum) during 4 weeks of induction training (to ensure adoption of correct technique and exercise familiarisation) and was then set at 70% 1-RM for the remainder of the training with 1-RM re-assessment every 4 weeks to ensure progression and consistency of training intensity.

3.3.4. Analytical Methods

To determine plasma AA concentrations, we added stable isotopically labelled internal standards and prepared samples according to our standard methods (Wilkinson et al. 2013). Briefly, heparinized plasma proteins were precipitated with 1 mL ice-cold ethanol and centrifuged at 10,000 rpm for 5 min, the supernatant was removed and evaporated to dryness under nitrogen at 90 °C, followed by re-suspension in 0.5M

HCI. Ethyl acetate was then added, and samples were vortexed thoroughly before the upper, ethyl acetate layer (containing lipids) was extracted. The aqueous AA-containing layer was evaporated to dryness under a steady flow of nitrogen at 90 °C. Derivatization of the dry residue was achieved via addition of equal volumes of acetonitrile and N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), and incubated at 90 °C for 45 min, thus converting the AA to their *t*-BDMS derivatives [19]. A pooled plasma QC sample was prepared with each batch and injected throughout the batch run to monitor instrument performance over time. This was achieved by pooling small aliquots of each study sample and thoroughly mixing. Aliquots of study-specific samples were used to closely mimic metabolite composition of the samples being tested, with the purpose being to account for analyst and analytical variation during sample preparation and batch run, respectively. AA concentrations were determined with reference to a calibration curve composed of a standard AA mix of known quantity and analysed by GC-MS.

3.3.5. GC-MS Conditions

To quantify plasma AA concentrations, 0.5 µl of sample was injected into an ISQ Trace 1300 single quadrupole GC-MS (ThermoFisher Scientific, Hemel Hempstead, UK). A split injection mode (1:10) was used, at an initial oven temperature of 100 °C held for 1 min, with a temperature ramp of 12 °C/ min to 300 °C and held for 5 min. Helium was used as a carrier gas at a flow rate of 1.5 mL/min, and sample separation was achieved on a 30 m Rxi-5MS (0.25 mm internal diameter, 0.25 µm thickness) fused silica column (Restek, Bellafonte, Pennsylvania). A selected ion monitoring scan (SIM) was created to search AA standards for leucine (mass 302), isoleucine (mass 302) and valine (mass 288), with corresponding isotopically labelled internal standards (304

and 289 for leucine and valine, respectively), or norleucine for isoleucine quantitation, included in the SIM.

3.3.6. Insulin and Glucose Concentrations

Plasma insulin and glucose concentrations, as well as lipoprotein content, was assessed in samples from before and after RET, as reported (Phillips et al. 2017). In brief, plasma insulin and glucose were measured in duplicate, using undiluted samples. Insulin was assessed via a high-sensitivity human insulin ELISA (DRG Instruments GmbH, Marburg, Germany) according to the manufacturer's instructions. Plasma glucose was measured using a clinical chemistry analyser (ILAB 300 Plus Clinical Chemistry System, Warrington, Cheshire, UK) against commercial standards. Insulin sensitivity was calculated using the homeostatic model assessment of insulin resistance (HOMA-IR) and the following Formula:

$$(\text{HOMA-IR} = \text{plasma glucose concentration } (\text{mmol.l}^{-1}) \times \text{plasma insulin concentration } (\text{mU.l}^{-1}))/22.5$$

Circulating plasma lipoprotein concentrations (low-density lipoprotein (LDL) and high-density lipoprotein (HDL)) were analysed by the Clinical Pathology Laboratory at the Royal Derby Hospital.

3.3.7. Statistical Analysis

Principal component analysis (PCA) was used as multivariate analysis, firstly to reduce the number of variables to principal component clusters with scores of IS (HOMA-IR) and then with the addition of additional clinical variables (i.e., insulin, glucose, HDL, LDL, etc). Multiple linear regression (MLR) analyses were first used to identify which variables correlated with scores of IS, and then stepwise regression

analyses were performed to reveal any potentially novel associations with BCAA concentrations. These correlations were performed at baseline and post-RET. Relationships that were identified were then isolated and further correlation was determined to describe the strength and significance of the interaction. Statistical analysis was performed in R-Studio employing in-house R scripts. Subsequent statistical analyses were confirmed in Prism v8.3 (GraphPad, La Jolla, California, USA) version 7. All data are reported as mean ± SEM, with significance set at $p < 0.05$. Data were tested for normality to determine appropriate analysis. Paired t-tests were used to assess the effects of RET, with Pearson's correlation used to explore relationships between fasting plasma BCAA concentrations and clinical parameters, such as body composition, fat mass, fat free mass and IS.

3.4 Results

3.4.1. Muscle Mass and Function

The characteristics of our participants at baseline and following RET are listed in table 1. As previously reported, 20 weeks of our whole-body RET programme elicited improvements in strength irrespective of age. However, whole-body lean mass gains were only seen in the young and middle-aged groups (Phillips et al. 2017), with a significant negative correlation between age and hypertrophy (Phillips et al. 2012).

TABLE 1. PARTICIPANT DEMOGRAPHICS M: F DENOTES *N* OF MALES TO FEMALES PER GROUP

Participant ID	Baseline (BL)	Post-RET
Sex	Young (4: 4 M: F) Middle (5: 4 M: F) Old (8: 7 M: F)	
Age (years)	53 ± 19	
BMI (kg/m^2)	26 ± 3	26 ± 2
Fasting Glucose (mg/dL)	5.6 ± 0.6	5.3 ± 0.7
Fasting Insulin (µU/mL)	4.9 ± 2	4.5 ± 1.5
HOMA-IR (AU)	1.4 ± 0.9	1.1 ± 0.4 *

3.4.2. Circulating BCAA Levels

No correlation was seen with age and BCAA concentrations either at baseline or post-RET (figure 7A and figure 7B, respectively). Additionally, no relationship existed between HOMA-IR and age either at baseline (6C) or post-RET (6D). For each of the invididual age-groups, and when all age-groups were collapsed into a single cohort, RET resulted in significantly elevated BCAA concentrations (leucine, P = 0.0011;

isoleucine, *P* = 0.0004; valine, *P* = 0.03 (Figure 8). Pooled QC throughout the instrumental run yielded ~5 % CV for each AA. Furthermore, there were no apparent sex interactions, with both sexes responding similarly to the RET programme. Therefore, data from all the age groups were collapsed into a single cohort (*n* = 32)

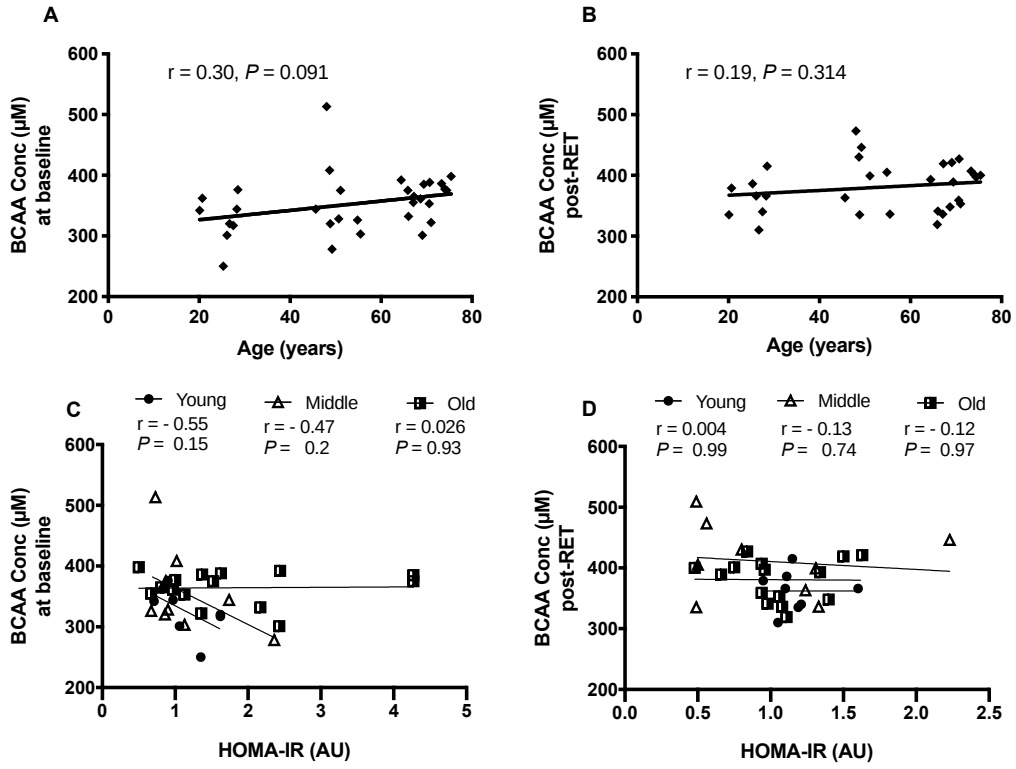


Figure 7 The relationship between circulating BCAA concentrations ang age at baseline (A) and after (B) 20-weeks supervised RET, and in distinct age groups at baseline and post-RET (D)

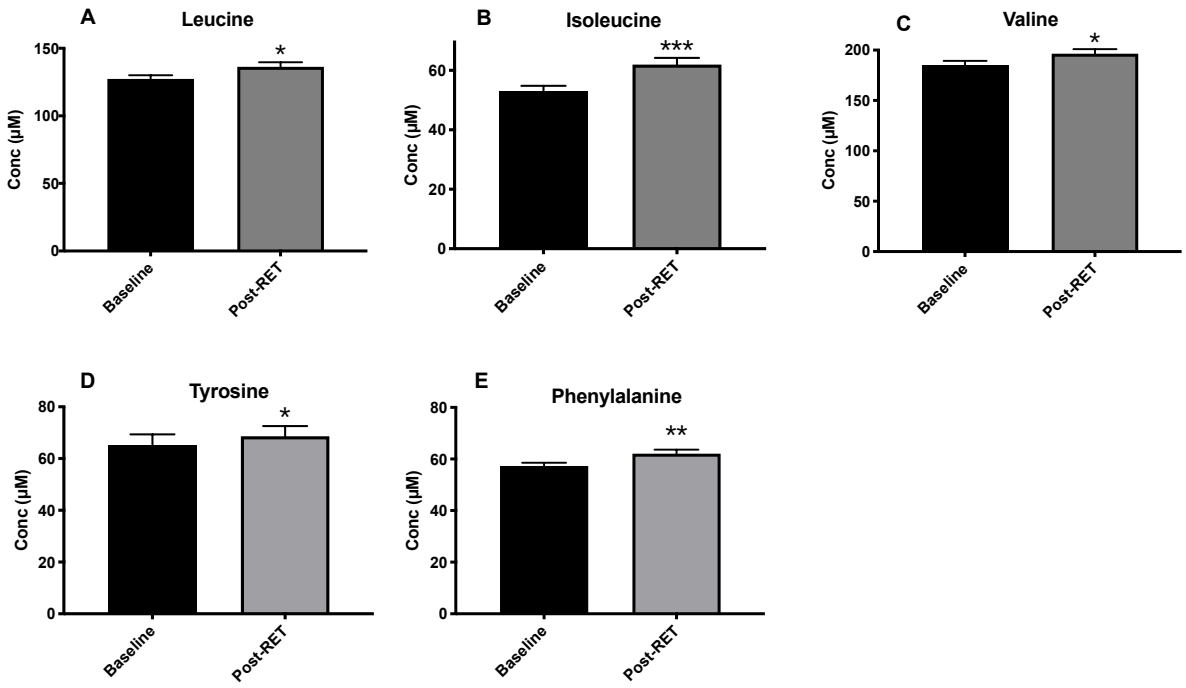


Figure 8 Circulating plasma leucine (A), isoleucine (B) valine (C), tyrosine (D) and phenylalanine (E) at baseline and after 20-weeks of RET (*n* = 32). Bars represent mean and SEM. Statistical analysis via paired t-tests. * *p* < 0.05; * *p* < 0.001 vs. baseline.**

3.4.3. HOMA-IR and Fasting Plasma BCAA and AAA Concentrations

Both body mass index (BMI) and HOMA-IR were significantly reduced ($P < 0.05$) after RET, suggesting improved IS in our volunteers (Figure 9A). However, there was no correlation between BCAA concentrations and HOMA-IR in any group either before (Figure 9B) or after RET (Figure 9C), despite significant alterations in each. To investigate whether other AA's which are associated with increased prevalence of IR and obesity, Pearson's correlation was performed on HOMA IR and aromatic AA's (phenylalanine and tyrosine), however these again showed no significant associations (figure 9D–G). Although a negative trend with phenylalanine at baseline was observed (figure 9E), this diminished with RET (figure 9G).

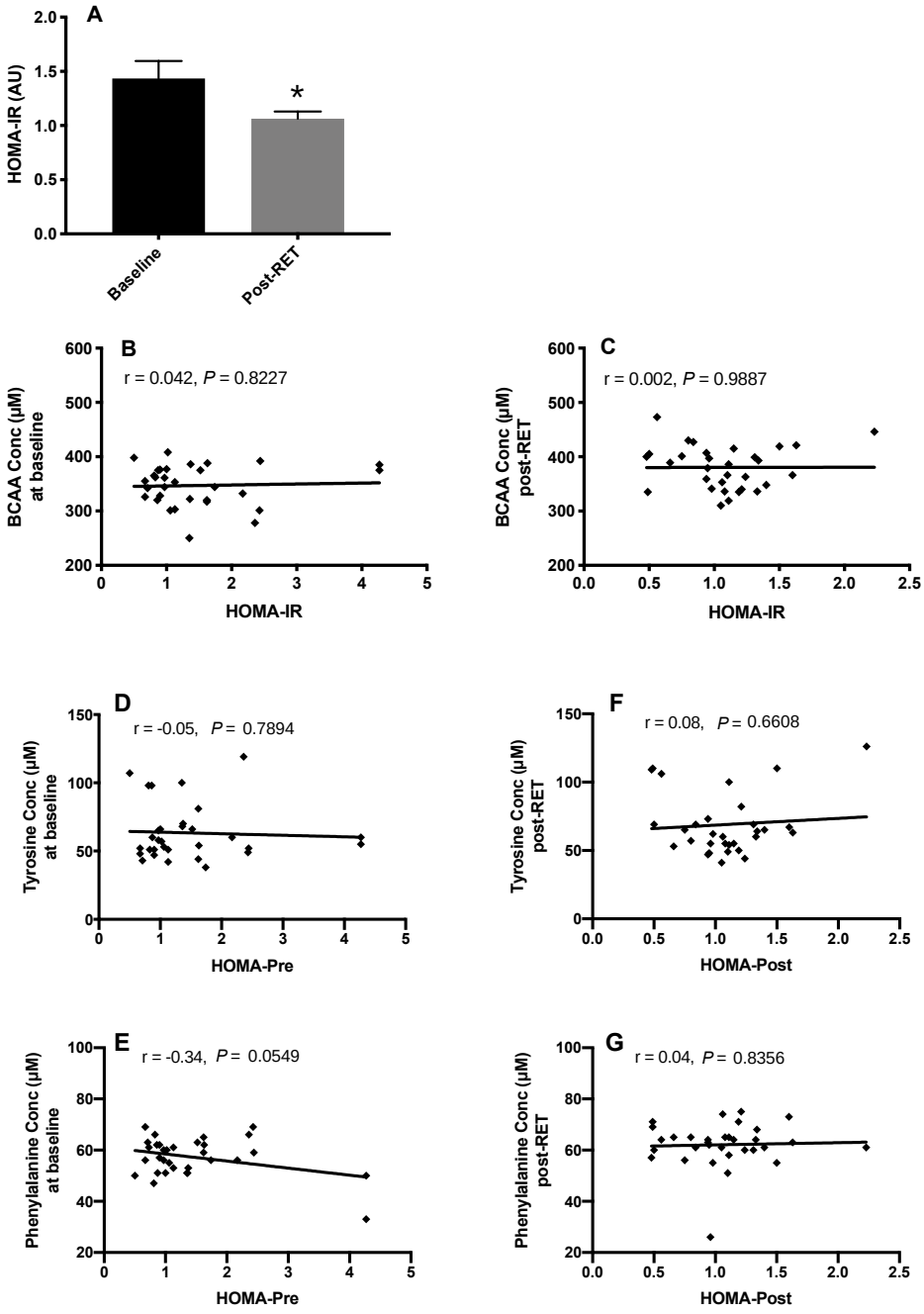


Figure 9 Insulin resistance (via homeostatic model assessment of insulin resistance; HOMA-IR) at baseline and after 20-weeks, whole-body resistance exercise training RET (A) and the relationship between IR and circulating BCAA concentrations at baseline (B) and after (C) RET. * $p < 0.05$ vs. baseline.

3.4.4. Relationships between BCAAs and Clinical Variables of Health

To visually explore whether there were any novel relationships from our study, data were log transformed for PCA analysis (Figure 10A) to investigate whether there were

metabolite clusters which could illustrate differences in variables either at baseline or with RET, however this revealed that there was no distinct clustering of metabolites that co-vary (at baseline or post-RET). Using the same variables and fasting plasma BCAA concentrations, we aimed to explore potential links that may be of interest with a correlational matrix in the form of a heatmap (Figure 10B). MLR was first used to test whether HOMA-IR or other clinical variables of health could predict BCAA concentrations in our healthy participants. At baseline, the results of the linear model predictors explain 29% of the variance with a residual standard error (RSE) of 40.06 on 9 degrees of freedom (DOF) and of 21 (adjusted $R^2 = -0.006$, F = 0.978 {on 9 and 21 DOF}, $p = 0.484$). Following RET, the results describe 73% of the variance with a RSE of 30.15 on 21 DOF (adjusted $R^2 = 0.616$, F = 0.653 {on 9 and 21 DOF}, $p = 0.0002$) which revealed strength as a significant ($p = 0.002$) associated variable with BCAA and other co-variates. Following this, stepwise regression was used to uncover which combination of our measured co-variates would best predict post-RET levels of plasma BCAA, and the stepwise regression model explains 69% of the variance with an RSE of 28.42 on 27 DOF (adjusted $R^2 = 0.659$, F = 20.37 {on 3 and 27 DOF}, $p = 4.19$) which revealed strength ($p = 0.001$), LDL ($p = 0.001$) and BMI ($p = 0.1$) as significant and promising variables in predicting post-RET levels of BCAA.

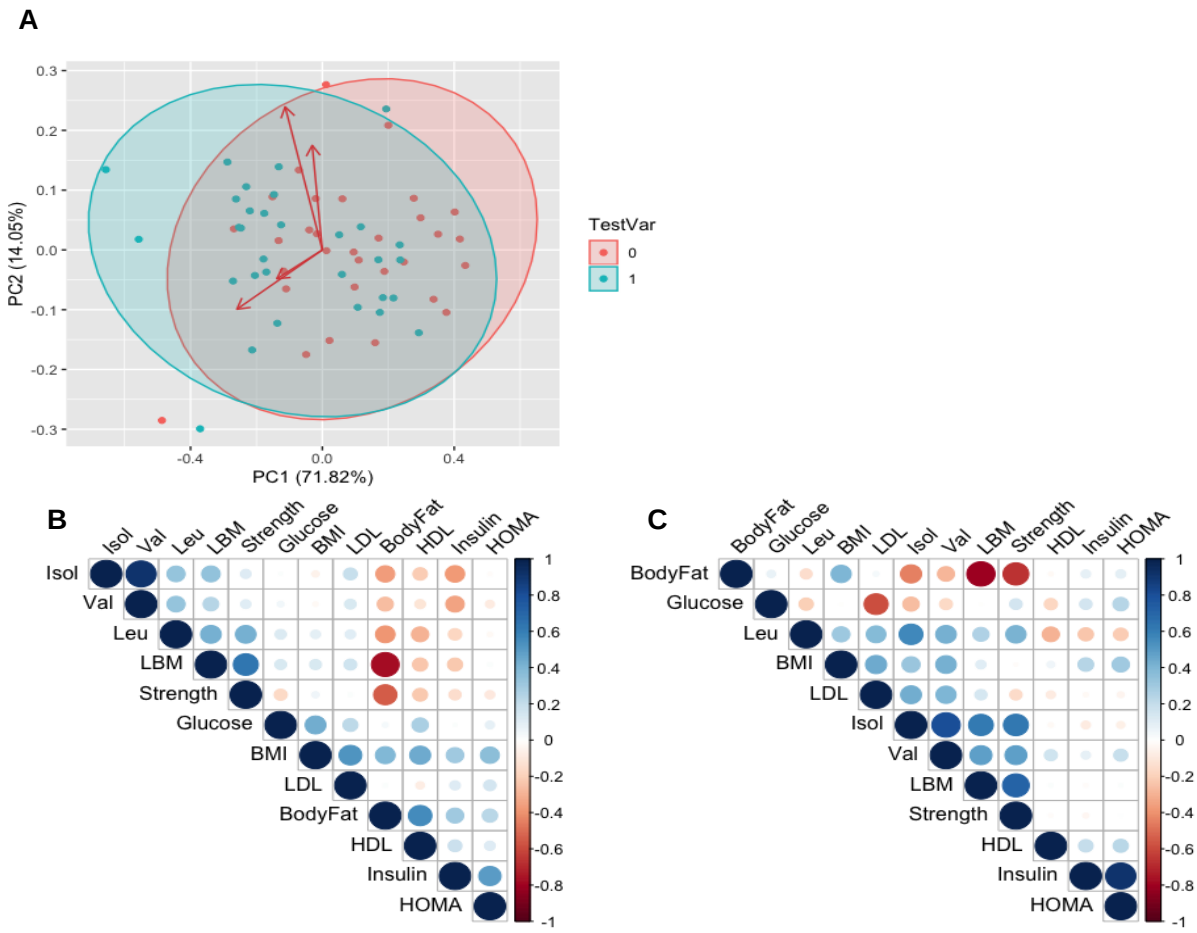


Figure 10 Principal component analysis (PCA) plot visually displaying overlap of clinical variables of health(A). Heatmap of correlations that are predictive of circulating BCAA concentrations at baseline (B) and after RET (C). The strength of relationships is based on a scale of −1 (red), representing a negative relationship and 1 (blue) a positive relationship. The strength of the relationships is depicted by the size of the circle.

3.4.5. Muscle Mass, Strength, and Circulating BCAA

Although there was no relationship between plasma BCAA concentrations and muscle strength (r = −0.04, p = 0.846) or mass (r = 0.14, p = 0.447) prior to RET (Figure 11A, C), increases in muscle strength and mass with RET resulted in significantly positive relationships between BCAA concentrations and both strength (r = 0.53, p = 0.001) and mass (r = 0.47, p = 0.007) post-RET (Figure 11B, D).

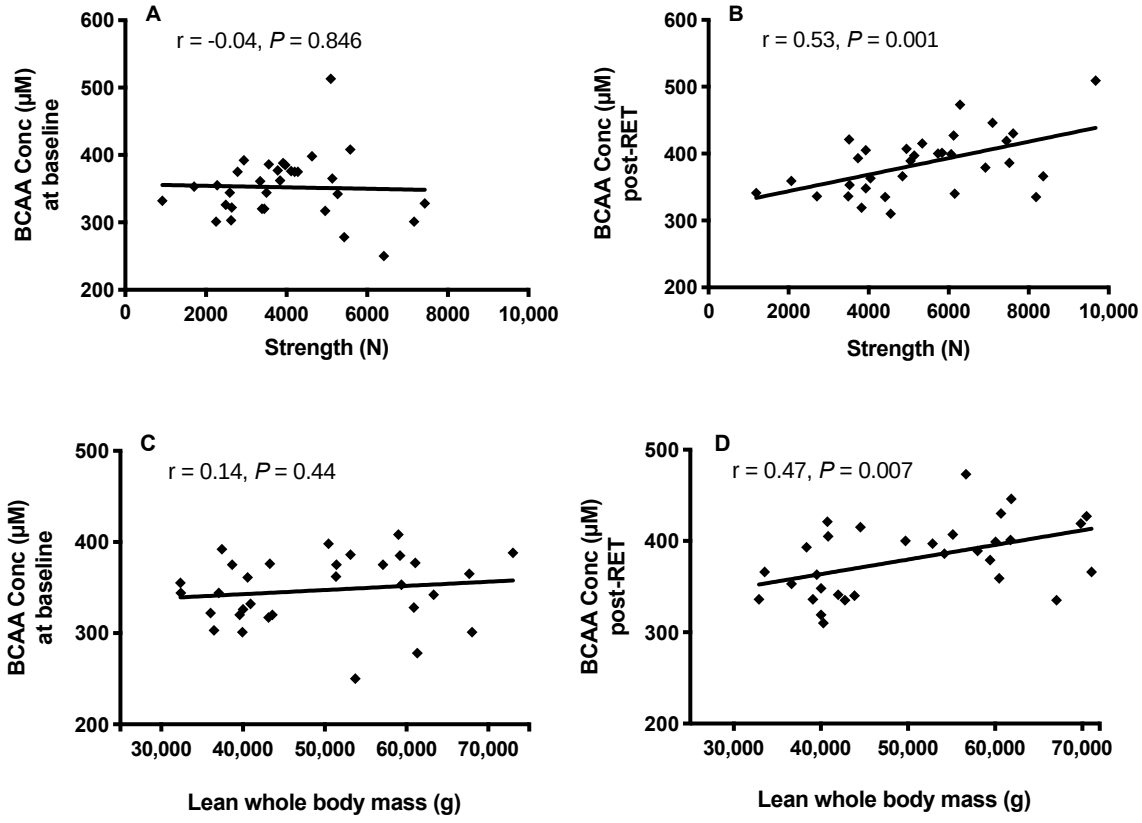


Figure 11 The relationship between circulating branched chain amino acid (BCAA) concentrations and muscle strength (A, B) and mass (C, D) at baseline and after 20-weeks, whole-body RET.

3.4.6. HDL, LDL and Plasma BCAA

LDL and HDL levels were unchanged by RET. However, a positive trend between fasting plasma BCAA concentrations and LDL was observed at baseline (r = 0.3, *p* = 0.08; Figure 12A), which was significant following RET (r = 0.48, *p* = 0.008; Figure 12B). A summary of the findings from this chapter are displayed in figure 13.

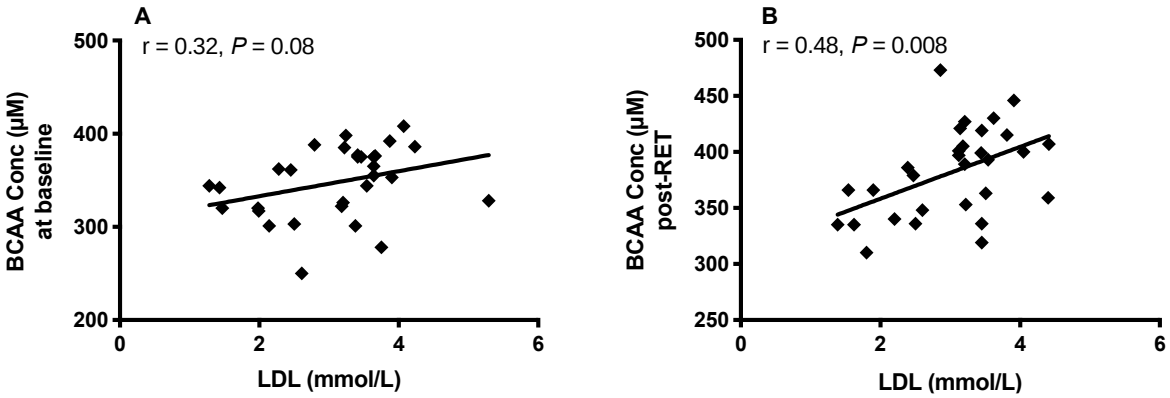


Figure 12. The relationship between circulating BCAA levels and low-density lipoprotein (LDL) before (A) and after 20-weeks of RET (B).

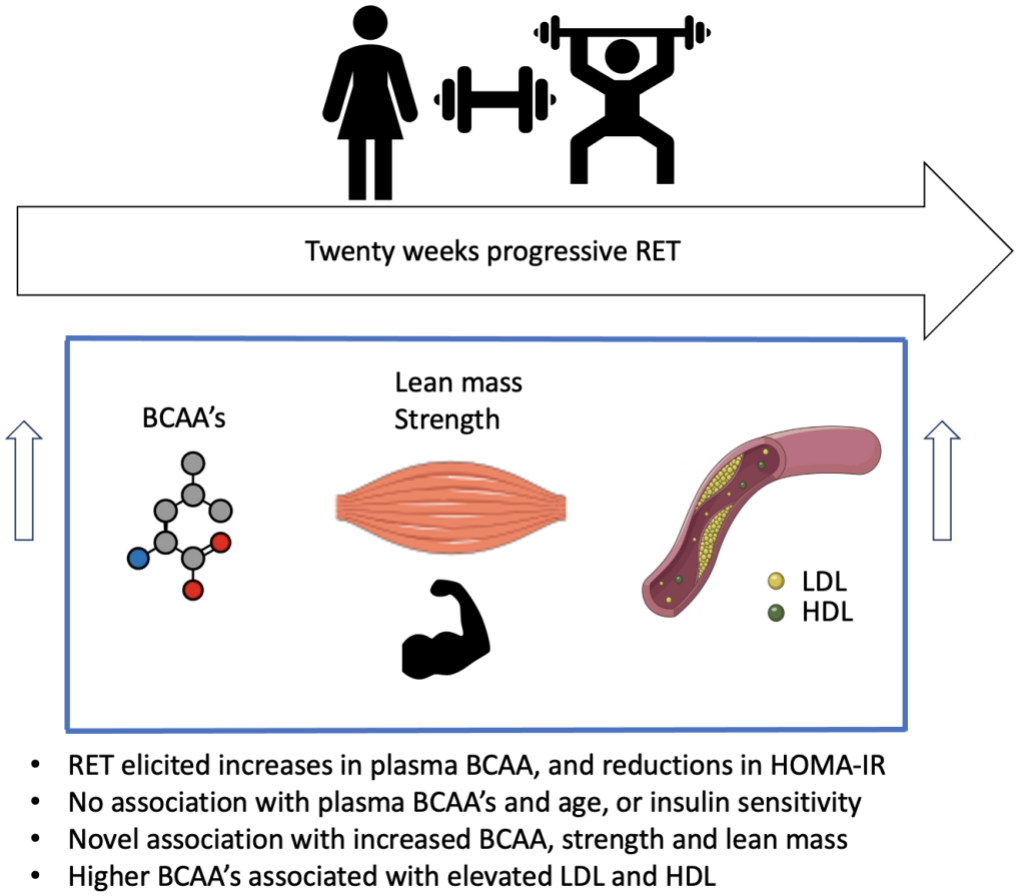


FIGURE 13. A SUMMARY OF THE FINDINGS OF THE PRESENT CHAPTER

3.5. Discussion

In the present study, we did not observe the commonly reported associations between HOMA-IR and BCAA or AAA's (Newgard et al. 2009; Wurtz et al. 2013). These data likely demonstrate that whilst elevated BCAA (Newgard et al. 2009) and AAA (Menge et al. 2010) have been linked to increased risk of T2DM and are a hallmark of obesity (Newgard 2012; Huffman et al. 2009), this relationship may not hold in individuals within a healthy BMI range. We also did not detect any relationships between IS and the AAA, phenylalanine and tyrosine which have been implicated in IR pathogenesis (Newgard et al. 2009; Mook-Kanamori et al. 2014), despite significantly elevated concentration post-RET. Elevated plasma AAA are a common trait in those with inborn errors of metabolism such as phenylketonuria (PKU) (Williams, Mamotte, and Burnett 2008) and have been postulated to contribute to IR by way of competing for cell

transport through large amino acid transporter (LAT1; Fernstrom 2005), with concomitant elevations in BCAA said to impair this transport. However, participants in the present study were within healthy BMI ranges and following an intervention promoting improved health, therefore it may be that hypoinsulinemia during fasting collection of samples contributes to the increased rate of appearance of AAA (Pozefsky et al. 1976). Further, since majority of phenylalanine metabolism occurs in the liver (hydroxylation to form tyrosine; Moss and Schoenheimer 1940), and the only metabolic fate of AAA in muscle is MPS or MPB, and that net muscle AA release increases with fasting (Pozefsky et al. 1976; Fryburg et al. 1990), and this is evidenced by the lack of relationship of AAA to strength and lean mass (as opposed to BCAAs) shown here. Moreover, our data do point to the notion that elevated BCAA are not an inevitable hallmark of “healthy ageing”. In support of this, in a previous study investigating the links between BCAA and cardio-metabolic risk factors, it was noted that reduced, rather than increased BCAA were evident in very old age (Sun et al. 2017).

While this may be due to chronic alterations in diet, the ageing gastrointestinal system, and dietary behaviours may have some influence, it once again highlights that elevated fasted plasma BCAA are not an all-encompassing biomarker of metabolic risk. Interestingly, recent studies (Le Couteur et al. 2019) have shown that BCAAs robustly correlate with HOMA-IR, as well as cardio(metabolic) risk factors and mortality, and so it has been proposed that lowered BCAAs (particularly in older individuals) are inversely associated with health risk factors. This has further been expanded by other studies showing that short-term (28 days) dietary overconsumption increases plasma BCAAs (namely driven by isoleucine and valine) in line with weight gain (Elshorbagy et al. 2018). However, nutritional intervention studies alone are not

able to account for the perturbation in BCAA metabolism which RET can induce, although clearly the link between circulating BCAAs and health continues to remain a paradoxical one. Promisingly, studies have shown that the relationship between plasma BCAAs, dietary intake and their regulation on whole-body weight are comparable in both humans and mice (Ribeiro et al. 2019), which may provide the potential for future studies examining the role of BCAAs in human health. Although, clearly BCAAs are essential for humans and exercise is beneficial to overall health, therefore caution ought to be taken when proposing whether plasma BCAAs are a positive or negative marker of health, as this relationship is likely to be dependent on context and the demographics of the individuals studied.

It is noted that lean mass gains were not evident in our older participants, and work from our lab shown this may indeed be due to anabolic resistance (Brook, Wilkinson, Mitchell, et al. 2016), which itself is multi-faceted (reduced translational capacity, hormonal efficiency and MPS with increased MPB), however RET still remains the most effective method of counteracting the age-related declines in loss of lean mass (Peterson, Sen, and Gordon 2011), particularly if it is combined with, for example, exogenous testosterone administration (Gharahdaghi et al. 2019) or protein supplementation (Esmarck et al. 2001; Tieland et al. 2012).

Based on previous correlations between BCAA and IR, we predicted that RET would improve biomarkers of metabolic risk and concomitantly reduce BCAA concentrations. Instead, we noted a reduction in HOMA-IR, in the face of a systematic increase in each of the BCAAs. The lack of this relationship was first highlighted in our principal components' analyses, and its absence was confirmed with Pearson's regression. These findings demonstrate that lowering of BCAA is not an inevitable consequence of improved metabolic health, i.e., HOMA-IR. Instead, following RET, our PCA matrix

plots comparing a number of variables with plasma BCAA levels revealed the most positively correlated facets to circulatory BCAA to be muscle mass and strength, thus illustrating novel positive links between muscle mass and circulatory BCAA (following RET). Basal (i.e., not under circumstances of exposure to a muscle growth regime) relationships have also been reported; Borg et al. (Borg et al. 2019) report data on 227 older (>65 y) volunteers from a cross-sectional study showing reduced levels of BCAA correlating with lower skeletal muscle mass, strength and longer sit-to-stand times. These data were also consistent with previous studies (Mcdonald et al. 2016) supporting the notion that low BCAA concentrations, particularly leucine, correlate with diminishing lean mass and sarcopenia. Our data in healthy individuals are in-line with studies suggesting that BCAA are a marker of muscle mass/strength (Borg et al. 2019; Mcdonald et al. 2016), and may also indicate that relationships with obesity could in fact reflect the notion that obese individuals have greater anti-gravity muscle mass than healthy weight counterparts (Bosco, Rusko, and Hirvonen 1986; Hulens et al. 2001; Maffiuletti et al. 2007). In other words, links to fat mass may be a misconception, i.e., with heightened fat mass reflecting heightened lean mass in obese individuals.

An interesting finding of this study was a positive correlation between BCAA and LDL after RET. Increased plasma LDL, particularly in older individuals, is a recognised risk factor for the development of conditions such as metabolic dyslipidaemia and coronary heart disease (Seals et al. 1984), particularly in the face of reduced HDL levels (Fukushima et al. 2019), making this link between elevated BCAA and LDL in the present study an intriguing, though paradoxical, finding given the improvements in other markers of metabolic health. A potential explanation for elevated LDL may be the established link between leucine and valine and cholesterol metabolism. Leucine and valine constituent metabolites (such as α-ketoisocaproate (α-KIC), β-Hydroxy β-

methylbutyric acid (HMB), mevalonate and 3-hydroxyisobutyrate (3-HIB)) contribute to an increased cholesterol metabolism (Wilkinson et al. 2013; Nissen and Abumrad 1997; Duan et al. 2016; Rudney and Ferguson 1957), which may be the case with our study participants given the increased concentration of BCAA following RET. Increased BCAA concentrations are reported to increase insulin action in hepatic cells (Xiao et al. 2011), resulting in prolonged gluconeogenesis leading to impaired hepatic lipid homeostasis, and subsequent accumulation of triglycerides and other fatty acids (Donnelly et al. 2005). Given that adipose tissue is effective in converting BCAA carbon skeletons to de novo fatty acid synthesis ex-vivo (Rosenthal, Angel, and Farkas 1974) and constitutes a major site where excess BCAA may be converted to lipid species, it would be reasonable to propose that inter-organ metabolism is implicated in the rise of lipid species in plasma. Both liver (Xiao et al. 2011) and adipose tissue (Herman et al. 2010) may therefore be important organs in defining the relationship between BCAA and dyslipidemia due to their central roles in glucose and lipid metabolism, respectively. Positive associations between plasma BCAA and dyslipidaemia have been reported previously, particularly for circulating LDL (Fukushima et al. 2019), and in both diabetic and non-diabetic follow-up studies (Yamakado et al. 2015; Mook-Kanamori et al. 2014). Moreover, even when adjusted for BMI, BCAA remain significantly correlated to triglyceride levels (Mook-Kanamori et al. 2014), suggesting at least a partial role of BCAA on circulating lipid species. Alternatively, the changes seen with LDL here could be due in part to the training modality used in the present study because, while the effects of endurance exercise in eliciting reduction of plasma LDL is well-known (Halverstadt et al. 2007), the effects of RET on the same parameters are not as well established.

The present study is not without its limitations. For example, although our participants are well-matched in terms of lean mass at baseline, our low sample size is an acknowledged limitation, although performing much larger highly controlled interventional trials are clearly a major undertaking. In addition, intra-group variability with regards to daily activity levels may pose potential confounding variables, as high levels of physical activity can lead to inadvertent stimulation of muscle remodelling (Aoyagi and Shephard 2013; DiPietro 2001). Also, since our participants were healthy and within a normal BMI range, our results should be extrapolated to cohorts fitting of similar criteria. Additionally, investigation into the effects of dietary intake in both sexes would provide some insight into the regulation of plasma BCAAs; however, absorption rates as well as the quantity of ingested dietary BCAA that eventually reaches blood circulation (Rietman et al. 2014; Cortiella et al. 1988; Biolo et al. 1992; Meguid et al. 1986) is unclear, as is whether plasma levels of BCAAs reflect short-term or long-term dietary intake (Rietman et al. 2014). Rodent studies (Solon-Biet et al. 2019) looking into the long-term effects of dietary BCAA control on health and lifespan have proposed mechanisms that exist for the elevation of BCAAs which involve notable interactions with tryptophan and threonine. Thus, studies looking into the temporal basis of dietary BCAA intake, and the influence of the gut microbiome (Meslier et al. 2020), could provide an insight into the causal relationships of this link.

3.6. Conclusions

In summary, twenty weeks RET in a tightly controlled and longitudinal intervention elicits significant increases in aromatic AA’s and all of the BCAA, which are commonly reported to be markers of poor IS. These increases do not correlate with indices of IS. However, BCAAs alone significantly correlate to strength and lean whole-body mass

changes (post-RET) irrespective of age or sex, highlighting a novel link that warrants further investigation.

CHAPTER FOUR: RELATIONSHIPS BETWEEN FASTED PLASMA BRANCHED-CHAIN AMINO ACIDS AND INSULIN SENSITIVITY AFTER 6-WKS VERY-LOW CALORIE DIET (VLCD) ALONE OR ADJUVANT TO EXERCISE

4.1. Abstract

Background: Elevated levels of plasma fasting branched-chain amino acids (BCAA: isoleucine, leucine and valine) are associated with an increased risk of obesity, insulin resistance (IR) and type 2 diabetes. Here, we evaluated the effects of 6-weeks very-low calorie diet (VLCD) with or without exercise training upon fasting BCAA levels in overweight non-diabetic individuals, to explore possible causal associations between elevated BCAA levels, weight loss and IR.

Methods: Fasting plasma BCAAs, muscle BCAA enzyme protein and muscle gene expression were quantified in twenty-six overweight, non-diabetic men (mean BMI 32.2 ± 2.9 kg/m2), middle-aged men (mean age 43.9 ± 9 y). Fasting plasma BCAA was also determined in a separate control group of non-obese healthy volunteers (*n* = 26, mean age 32 ± 12.3 y, BMI 24 ± 3.1). Participants were randomised into three groups and underwent 6-weeks of VLCD (600 kcal/day allocated, with the option of additional 200 kcal/day), VLCD + resistance exercise training (RET) or VLCD + high-intensity interval training (HIIT) exercise regimes. Measurements of BCAA, insulin sensitivity (HOMA-IR) and body weight/composition (DXA) were made at baseline and post VLCD. BCAA concentrations were determined by gas-chromatography mass spectrometry.

Results: BCAAs (isoleucine: p = 0.03; valine: p = 0.003) and sum circulating BCAA were higher (p = 0.01) in individuals who were obese. Following VLCD, significant losses in body weight (all p < 0.0001) and fat mass (kg) loss were observed (all p < 0.0001). Despite significant weight loss in all groups (diet-alone 13 %, VLCD+RET 14 %, VLCD+HIIT 15 %, all *p* < 0.0001), no changes were observed in sum BCAA concentrations in diet-only group. However, significant reductions were observed in BCAA concentrations following VLCD+RET (Leucine, p = 0.03; Isoleucine, p = 0.04;

Valine, p = 0.03) and VLCD+HIIT (Leucine, p = 0.01; Valine, p = 0.01). Although HOMA-IR (diet-only 1.2 ± 0.6, VLCD + RET 2.3 ± 1.2, VLCD + HIIT 1.9 ± 1.3) was reduced across all groups (VLCD alone 0.5 ± 0.21, p = 0.002; VLCD + RET 1.2 ± 1 p = 0.0002; VLCD + HIIT 1 ± 0.6, p = 0.04) there were no associations with BCAAs and HOMA-IR at baseline or after VLCD/exercise interventions. Muscle gene expression revealed increased expression of in muscle SDHB (p = 0.04) and muscle HIBADH (p = 0.002) genes in VLCD +RET group.

Conclusion: VLCD, coupled with RET or HIIT result in reductions in body-mass and plasma BCAAs, and though expectedly VLCD improves insulin sensitivity, no correlations were observed between plasma BCAAs and HOMA-IR. Possible causal links between BCAA and IR in the context of weight loss by means of calorie restriction and exercise training are lacking.

4.2. Introduction

Advances in medicine and technology have led to improved healthcare contributing to increased life expectancy (Wagner and Brath 2012). However, individuals are becoming less physically active in their lifestyles and adopting poor nutritional behaviours leading to increased instances of obesity and insulin resistance (IR; Warram et al. 1990; Lillioja et al. 1988). For example, the global prevalence of obesity (BMI of ≥30 kg/m2) among adults is predicted to rise from 33% in 2005 to ~58% by 2030 (Kelly et al. 2008), leading to an estimated doubling in the prevalence of T2DM, from ~7.7 % in 2010 (285 million) to ~439 million in 2030 (Shaw, Sicree, and Zimmet 2010). Moreover, obesity in older populations (>65 years of age) has also increased, which decreases the probability of good health and promotes disability in old age, as well as reducing the chances of recovery from diseases (Doblhammer and Hoffmann 2009; Reynolds, Saito, and Crimmins 2005), including COVID-19 (Cai et al. 2020). Additionally, the ageing demographic is the fastest growing subgroup of our population and is becoming a significant challenge to maintaining overall global health (Christensen et al. 2009b). Alarmingly, the effect of obesity on the lifetime risk of developing T2DM in paediatric populations (Narayan et al. 2007; Chen, Magliano, and Zimmet 2011) is also strong and has emerged as major public health issue (Wang et al. 2016; Flegal et al. 2016), thus novel insights into the pathophysiology of IR and effective treatments are needed.

While the pathophysiology of obesity and IR are multifactorial (Stumvoll, Goldstein, and van Haeften 2005; Defronzo 2004), recent and historic evidence suggest a role for plasma branched-chain (BCAAs; leucine, isoleucine, valine). BCAAs are diet-derived nutrients that are established regulators of skeletal muscle proteostasis, and act as critical anabolic signals positively regulating muscle and whole-body protein

synthesis (Lynch and Adams 2014; Wang et al. 2011).Yet, excess circulating BCAA have been associated with adverse metabolic health; for example several studies have shown that dietary BCAA restriction (White et al. 2016) and pharmacological reduction of plasma BCAAs (White et al. 2018). can improve insulin sensitivity (IS). Over decades, studies have consistently shown that elevated blood concentrations of BCAAs are common diagnostic and prognostic features for IR and risks of T2DM, with historical (Felig, Marliss, and Cahill 1969b), and more recent data (Newgard et al. 2009; Tobias et al. 2021), proposing BCAA to be a root-cause of IR across liver, skeletal muscle and adipose tissue sites (Newgard 2012). The proposed mechanisms by which BCAA-induce insulin resistance centre upon two themes: (i) that excess dietary BCAA lead to sustained activation of mTORC1 (of which, leucine is a trigger (Wilkinson et al. 2013)) via serine phosphorylation of insulin receptor substrate (IRS-1) and IRS-2 , and (ii) that impaired BCAA metabolism results in accumulation of metabolic intermediates (such as α-ketoisocaproate (KIC) α-ketoisovaleric (KIV) and 3-hydroxyisobutyrate (3HIB); Bridi et al. 2005; Jang et al. 2016), suppressing insulin action and resulting in lipid accumulation (Newgard 2012). It may thus seem paradoxical since BCAAs are also indispensable in their role in promoting metabolic and muscle preservation. For example, BCAAs, in healthy individuals are known to improve metabolic health, particularly in older individuals (D'Antona et al. 2010) and as presence of nutrient availability for increased MPS following exercise (Phillips et al. 1997; Kumar et al. 2012). Irrespective of these apparent positive or negative effects (e.g., IR conditions), relationships between elevated BCAA and prognostic risk have been derived from cross-sectional studies.

However, a limitation of this area of research is the lack of an observed "cause-effect" relationship between elevated BCAA and IR in humans. Longitudinal weight loss

studies which improve aspects of IS, whilst tracking BCAA abundance, may provide greater insight. Non-pharmaceutical approaches are an important first step in the management of obesity and diabetes. Reflecting this, lifestyle modifications, such as very low-calorie diets (VLCD) represent a safe and effective means to reduce IR with short-term (~8 week) VLCD being sufficient to normalise hepatic IS and β-cell function in people with T2DM (Lim et al. 2011), whilst also proving efficacious in obese (Hong et al. 2005) and adolescents (Willi et al. 2004) individuals.

As such, the aim of this study was to investigate the relationship between fasting plasma BCAA and measures of IS following 6 weeks of VLCD. We further examined the effects between plasma BCAAs and related gene and intracellular targets in participants who underwent 6-weeks of VLCD with and without exercise. We hypothesised that BCAA would be elevated in the obese volunteers, and that ensuing weight loss and reductions in IR would be associated with a normalisation of BCAA, that would be correlated with improved IS.

4.3. Methods

4.3.1. Ethical approval

This study was reviewed and approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (D/2/2006) and complied with the 2013 Declaration of Helsinki. All procedures and risks were thoroughly explained to volunteers and written consent was obtained prior to participation.

4.3.2. Participant characteristics

Twenty-six overweight, middle-aged men (mean BMI 32.2 ± 2.9 kg/m^2, mean age 43.9 ± 9 y) without diabetes were recruited to participant in this 6-week calorie restricted study. Volunteers who suffered from respiratory, cardiovascular or metabolic

disorders, or any cognitive impairments that precluded exercise training were not enrolled in this study. Individuals were not taking any prescribed medications and were normotensive (< 140/ 90 mmHg). Prior to enrolling on the study, participants were screened by means of a medical questionnaire and physical examination, including resting ECG and blood chemistry tests. Participants were instructed to continue activities of daily living but not participate in any formal exercise. Study exercise sessions were performed at the University of Nottingham Medical School at the Royal Derby Hospital centre and supervised by a trained member of research staff.

4.3.3. Study procedures

On study days, volunteers were nil-by-mouth (except for clear water) from midnight and reported to our research laboratory ~09:00 h. Fasting blood samples were taken from the antecubital vein and collected into lithium-heparin containing vacutainers for measures of plasma BCAA, insulin and glucose concentrations, with the plasma-fraction collected following centrifugation at 2000 x G for 20 min at 4^{o}C. We also compared our VLCD participants at baseline with age-matched, healthy control volunteer plasma, samples of which were from a participants decribed in the RET study of chapter 3 (Phillips et al. 2017).. Baseline fasting biopsies of *m*. vastus lateralis were taken under sterile conditions via the conchotome biopsy method (Dietrichson et al. 1987) under local anaesthetic conditions (1 % lidocaine; B. Braun Melsungen, Melsungen, Germany). Once collected, muscle was rapidly dissected free of fat and connective tissue, washed in ice-cold phosphate-buffered saline (PBS), blotted dry before it was frozen in liquid nitrogen. All samples were stored at -80^{o}C until further analysis.

4.3.4. Conduct of the study

In this 6-week VLCD study, participants (n=26) were randomly assigned to a VLCD only group (n = 10), a VLCD + Resistance exercise training for 6 weeks (VLCD+RET, n=8) group and a VLCD + High Intensity Interval Training for 6 weeks (VLCD+HIIT, n=8) group. All three groups followed meal replacement diets (Total VLCD), designed to aid in weight management, provided by Lighter Life (Harlow, Essex, U.K.). The diet consisted of 4 meals per day, providing ~600 kcal/ day (participants were permitted 200 kcal/ day extra, in the form of fruit, vegetables or meat), providing a total of 50 g protein, 50 g carbohydrate and ~17.3 g fat, complete with 100 % RDA of vitamins and minerals.

4.3.5. Exercise test

Prior to commencement of RET, participants were tested for 1 repetition maximum (1RM). A combination of upper and lower body movements (3 of each) was selected, on weight training machines. Following a warm-up, participants were verbally encouraged to perform maximally. Baseline cardiopulmonary exercise tests (CPET) were conducted to determine each individual's fitness and set the appropriate intensity for subsequent HIIT exercise. CPET was performed on a cycle ergometer (Lode Corival, Lode, Groningen) with inline gas analysis system (ZAN 680, nSpire Health, Colorado, USA), using a standard 15 W/min ramp protocol, following a 2-min period of unloaded cycling and instructed to maintain a ~55 revolutions per minute (RPM) cadence. Participants were verbally encouraged to exercise to a respiratory exchange ratio (VCO_2/VO_2) above one and the test was complete once the participant indicated, via the Borg scale, their maximum effort had been reached. ECG, blood pressure and pulse were monitored throughout CPET sessions.

RET comprised of 6-weeks of fully supervised whole-body exercises involving upper and lower body exercises, adopting a progressive overload model and calculated from their pre-determined 1RM. The format of the exercises were 8 sets of 12 repetitions at 70 % 1RM, with 2 minutes rest between sets following a thorough warm up. The frequency of the sessions was 3 times/ week, with muscle strength assessed (via 1RM) every ~14 days to ensure relative intensity of sessions remained constant throughout. The HIIT training sessions were performed on a cycle ergometer. The protocol used involved 60 seconds of high intensity cycling at ~95 % watt max, repeated 5 times, interspersed with 90 seconds of recovery following 2 minutes of warm up and a cool-down. The intensity was calculated based on pre-determined maximal aerobic capacity and performed 3 times per week, supervised by a trained member of the research staff.

4.3.6. Analytical methods

To determine plasma AA concentrations, we added stable isotopically labelled internal standards and prepared samples according to our standard methods (Wilkinson et al. 2013), which are described in detail in chapter two (2.4).

4.3.7. GC-MS conditions

Plasma concentration of AA were determined by GC-MS and the conditions of the instrument were according to methods described in chapter two (2.5).

4.3.8. Immunoblotting procedures for BCAA handling enzymes

Approximately 10 mg of wet muscle weighed was used to extract muscle protein content in radioimmunoprecipitation (RIPA) buffer (50 mM Tris HCI pH 7.4, 150 mM NaCI, 1 % Triton X-100, 0.5 % Sodium deoxylcholate, 0/1 % sodium dodecyl sulfate). To prepare samples, 50 µl of RIPA buffer was added per mg of wet weight muscle and

homogenised with scissors. Once homogenate consistency was achieved, samples were vortexed before gentle agitation for 5 minutes followed by sonication for 5 minutes. Samples were then centrifuged for 5 minutes at 7000 x g to pellet matrix debris. The supernatant was then carefully collected for protein quantification. Protein concentration was determined via the bicinchoninic acid (BCA) assay (Smith et al. 1985) using a commercially available BCA assay kit (Thermo Finnigan, Thermo Scientific, Hemel Hempstead, U.K.). From each sample, 25 µl was pipetted into a 384 well plate (Life Technologies) in triplicate, with reference to a 9-point calibration curve with a working range of 20 – 2 000 µg/mL). The plates were then sealed with foil, before incubation for 30 min at 37 °C and the cooled for 5 minutes at room temp. Absorbance was measured at 562 nm. Immunoblotting was performed on RIPA buffer extracted muscle samples following protein quantification with BCA assay, after which they were diluted and boiled with 3x Laemmli loading buffer (Laemmli 1970). Samples (10 µg/ ml), and cross-gel quality controls (QC) made up of study-specific samples, were loaded on to a Criterion XT Bis-Tris 12 % SDS-PAGE precast gel (Bio-Rad, Hemel Hempstead, U.K.) for electrophoresis at 200 V for 60 min. Following protein separation (Bass et al. 2016), samples were transferred to polyvinylidene difluoride (PVDF) membranes for electro-transfer at 100 V for 45 min. Then, PVDF membranes were subsequently blocked with non-fat dry milk (2.5 % diluted in Tris-buffered saline Tween 20 [TBST]) 1 hour at ambient temperature. Membranes were then incubated overnight at 4 °C under gentle agitation, in the presence of the following antibodies (all diluted 1:200 in 2.5 % bovine serum albumin in TBST) branched chain amino acid transaminase 1 (BCAT1), branched chain amino acid transferase mitochondrial (BCAT2), branched chain alpha-keto acid dehydrogenase complex (BCKDH-E1α) and Phospho-BCKDH-E1α (p-BCKDH)^{Ser293} (all Cell-Signaling technology, Leiden, The

Netherlands). The following day, membranes were washed (3 x 5 minutes) with TBST and incubated for 1 hour at room temperature soaked in horseradish peroxidase-conjugated anti-rabbit secondary antibody (New England Biolabs' 1:200 in 2.5 % BSA in TBST). Finally, membranes washed for 3 X 5 min in TBST and incubated for exactly 5 min with enhanced Chemiluminescent HRP reagent (Millipore Corp., Billerica, MA, USA). Bands were quantified on a Chemidoc MP (Bio-Rad, Hemel Hempstead, U.K.) by peak density and normalized to Coomassie brilliant blue staining of the membranes (Welinder and Ekblad 2011) and software measures were taken to prevent band saturation. Values were subsequently normalised to corresponding baseline value before statistical analysis.

4.3.9. Gene expression analysis of BCAA metabolism-related enzymes

To determine muscle RNA content, approximately 10 – 15 mg of muscle tissue was homogenised in TRIzol™ (Invitrogen, Thermo Fisher Scientific) with the addition of a stainless steel bead (Tissue Lyser II, Quigen, U.K.) for 2 minutes at the frequency of 30 s, according to manufacturer's instructions. RNA quality and quantity were then measured by spectrophotometry (NanoDrop 2000, Thermo Scientific). A high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific) was used to reverse transcribe 500 ng of total RNA which were then diluted 1:10. Precisely 1 µl of 1:10 diluted cDNA (in triplicate) was individually added onto a 384 optical well plate (Life Technologies). Exon-exon boundary specific primers were mixed with SYBR Select Master Mix (Thermo Fisher Scientific), and RNase-free water and addition of 6 µl mixed solution with 1 µl of each cDNA, were added to the wells. Real-Time quantitative PCR (qPCR) was performed on a ViiATM 7 PCR System (Life Technologies). The ΔΔCt method (Schmittgen and Livak 2008) was used to quantify

target mRNA expression, with RPL13A being used for normalisation. Primer sequences for each of the probed genes are listed in table 1.

TABLE 2 GENE NAMES AND PRIMER SEQUENCES USED IN PCR

Gene code	Forward	Reverse
RPL13A	5′-TAAACAGGTACTGCTGGGCCG-3’	5′-CTCGGGAAGGGTTGGTGTTC-3′
BCAT 1	5’-GGCTACGACCCTTGGGATCT-3’	5’-GTCCCCACCACCTCTTTTGA-3’
NDUFB3	5’-GCTGGCTGCAAAAGGGCTAA-3’	5’-CAGCTCCTACAGCTACCACAA-3’
PPM1K	5’-CCGCTTTGACTGCTTGCTTC-3’	5‘-GAGGAGCTTTCTTGGTCGGT-3’
PCCB	5’-AGGAGTGGAGTCTTTGGCTG-3’	5‘-TCTGTTAGGGCTGGGGAGTA-3’
SDHB	5’-GCTACTGGTGGAACGGAGAC-3’	5’-GCGCTCCTCTGTGAAGTCAT-3’
ALDH6A1	5’-GAGCTGATCTTGGCCCTCTG-3’	5’-GCTCCCTCCTTTGTTCCACT-3’
HIBADH	5’-ATGGATGCCCCTGTTTCTGG-3’	5’-CCACAGTACACCACGTTGGA-3’
KLF15	5’-GGGAGAGAGGTGAAAAGCGT-3’	5’-TTGTCTGGGAAACCGGAGGA-3’

4.3.10. Insulin and glucose concentrations

Plasma insulin and glucose concentrations, were assessed in duplicate samples from before and after VLCD interventions, as reported (Phillips et al. 2017), Insulin sensitivity was calculated using the homeostatic model assessment of insulin resistance (HOMA-IR) and the following formula:

(HOMA-IR=plasma glucose concentration (mmol.l^{-1}) x plasma insulin concentration (mU.l^{-1}))/22.5

4.3.11. Statistical analysis

Fasting BCAA concentrations of obese participants were compared with healthy-weight controls using Student's unpaired *t*-test. BCAA concentrations of obese participants who underwent 6-weeks of VLCD were compared using Student's paired *t*-test. Data were tested for normality before Student's paired *t*-test was used in obese participants to detect changes in plasma BCAA levels at baseline or following VLCD. One-way ANOVA was performed using Tukey's post-hoc analysis to determine whether insulin sensitivity following VLCD intervention declined to within the range observed in healthy-weight control volunteers. The relationship between HOMA-IR and BCAAs at baseline and following VLCD were investigated using Pearson's correlation. The significance level was set to $P< 0.05$ and presented as mean ± SEM. All analyses were performed using GraphPad Prism 8.3 (La Jolla, CA, USA).

4.4. Results

4.4.1. Effects of VLCD on body weight and BMI

The effects of VLCD on body mass and effects of exercise on strength and cardiovascular improvements are shown in table 3. Significant weight loss (VLCD-only ~13 %; VLCD + RET ~14 %; VLCD + HIIT ~15 %) was observed across the groups. Therefore, unsurprisingly BMI was also reduced in all participants (p< 0.0001) of all three groups. Uniform improvements in VO2 max were also achieved in the VLCD-only (p = 0.01), VLCD + RET (p = 0.006) and VLCD + HIIT groups (p = 0.009). Strength gains were observed in the VLCD + RET group only (p = 0.007)

TABLE 3 PARTICIPANT CHARACTERISTICS AND FUNCTIONAL IMPROVEMENTS FOLLOWING VLCD + EXERCISE INTERVENTIONS

	VLCD (only)		VLCD + RET		VLCD + HIIT	
	Baseline	**Post-VLCD**	**Baseline**	**Post-RET**	**Baseline**	**Post-HIIT**
Body mass (kg)	103.9 ± 12.3	92.9 ± 9.6 ****	98.6 ± 9.7	87.8 ± 9.2 ****	100.6 ± 12.4	89.0 ± 12.3 ****
BMI kg/m^2	32.2 ± 4.02	28.9 ± 3.6 ****	32.0 ± 1.5	28.5 ±1.5 ****	32.9 ±2.9	29.1 ± 3.2 ****
Lean mass (kg)	65.2 ± 6	60.9 ± 4.8 ****	61.1 ± 3.7	57.2 ± 3.3 ***	59.9 ± 5.1	55.9 ± 4.3 ****
Fat mass (kg)	35.3 ± 7.46	28.54 ± 6.9****	34.1 ± 7.1	27.2 ± 7.5****	37.9 ± 7.9	30.1 ± 8.7 ****
Strength (N)	4624 ± 774	4882 ± 640	4567 ± 814	5831 ± 1063 **	4401 ± 903	4699 ± 1102
VO_{2max} (mL/kg/min)	28.3 ± 8.5	31.2 ± 9.3 *	29.6 ± 5.7	33.3 ± 6.5 **	24.77 ± 5.3	29.9 ± 8.1 **

4.4.2. Circulating BCAA

In order to determine whether our participants have elevated circulating BCAA concentrations, in line with the literature, we compared them to age-matched healthy (normal weight, normal BMI) controls. Table 4 shows that at baseline, the BCAA concentrations of our VLCD participants were significantly higher than their healthy, age-matched counterparts ($p = 0.018$) with the changes driven primarily by valine ($p = 0.003$) and isoleucine ($p = 0.036$).

TABLE 4. COMPARISON OF INDIVIDUAL BCAAS OF VLCD STUDY PARTICIPANTS COMPARED TO BCAAS OF HEALTHY, AGE-MATCHED CONTROLS

Concentration (μM)	VLCD	Age-matched control
Leucine	134 ± 30	121 ± 30
Isoleucine	70 ± 20	53 ± 18 (*)
Valine	253 ± 38	193 ± 45 (**)
Total BCAA	457 ± 85	365 ± 78 (*)

In addition, we saw no significant changes in individual BCAA in the VLCD only group. However, significant reductions in individual leucine ($p = 0.034$), isoleucine ($p = 0.047$) and valine ($p = 0.039$) figure 14C and BCAA (figure 14D) were observed following VLCD+RET; while significant reductions in leucine ($p = 0.0167$, Figure 14A) and valine ($p = 0.0163$, Figure 14C) were also observed following VLCD+HIIT and sum BCAA (figure 14D).

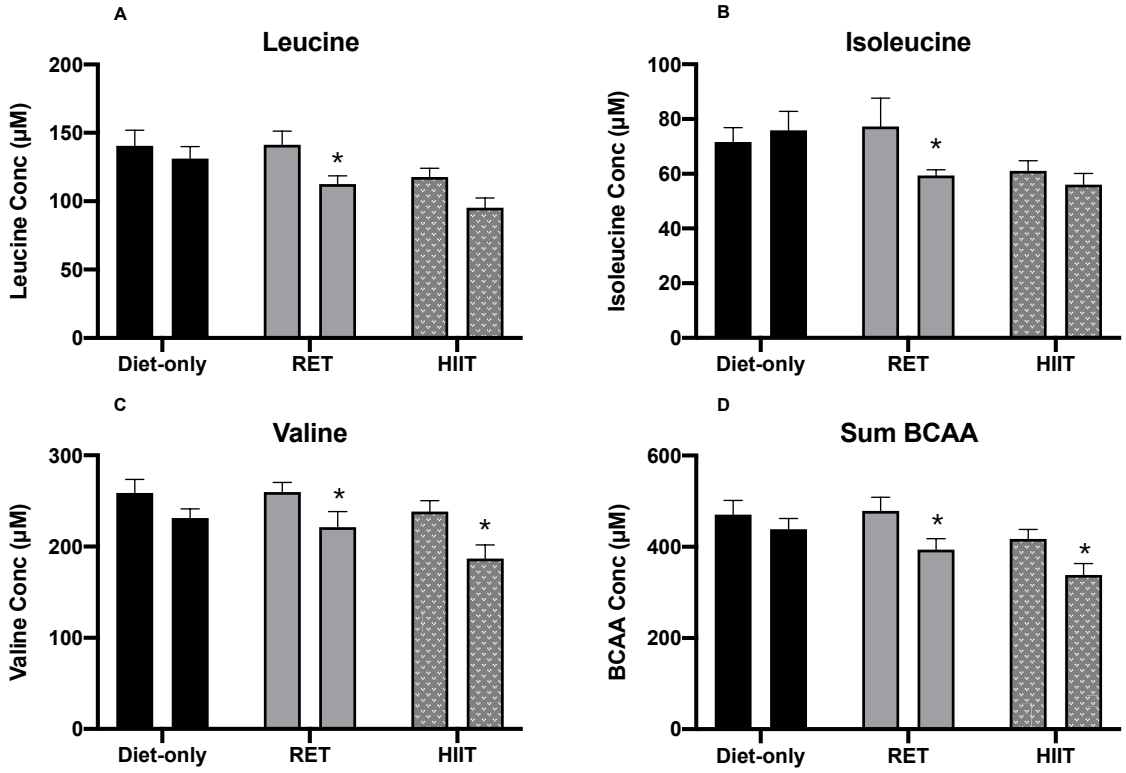


FIGURE 14. COMPARISON OF BRANCHED-CHAIN AMINO ACIDS IN ALL GROUPS AT BASELINE AND FOLLOWING VLCD INTERVENTIONS ALONGSIDE RET AND HIIT EXERCISES.

4.4.3. Relationships between fasting BCAAs and HOMA-IR

Given significant reductions in total body mass, including fat mass, it was anticipated that HOMA-IR would significantly reduce in all VLCD cohorts (VLCD-only figure 15A, $p = 0.002$; RET group figure 15B, $p = 0.0002$; HIIT group figure 15C, $p = 0.046$). There were however no associations between HOMA-IR and BCAA concentrations in either of the three groups, neither at baseline nor following our dietary and exercise interventions, figures 15 D – F). To further determine whether the small *n* within each group may limit the opportunity to detect this relationship, we collapsed all of our VLCD participants into one group ($n = 26$) to assess if there was a correlation with HOMA-IR and baseline BCAA concentrations at baseline. Still, there was no relationship between HOMA-IR or BCAAs (figure 15 G). To further investigate if the expectant correlation to BCAA and insulin sensitivity may exist across a broad range of BMI, we compared our collapsed VLCD groups at baseline, with a healthy BMI cohort of a

range of ages (Sayda et al. 2020) from our lab (total *n* = 58). Still, there were no associations with HOMA-IR and circulating BCAAs (figure 15 H).

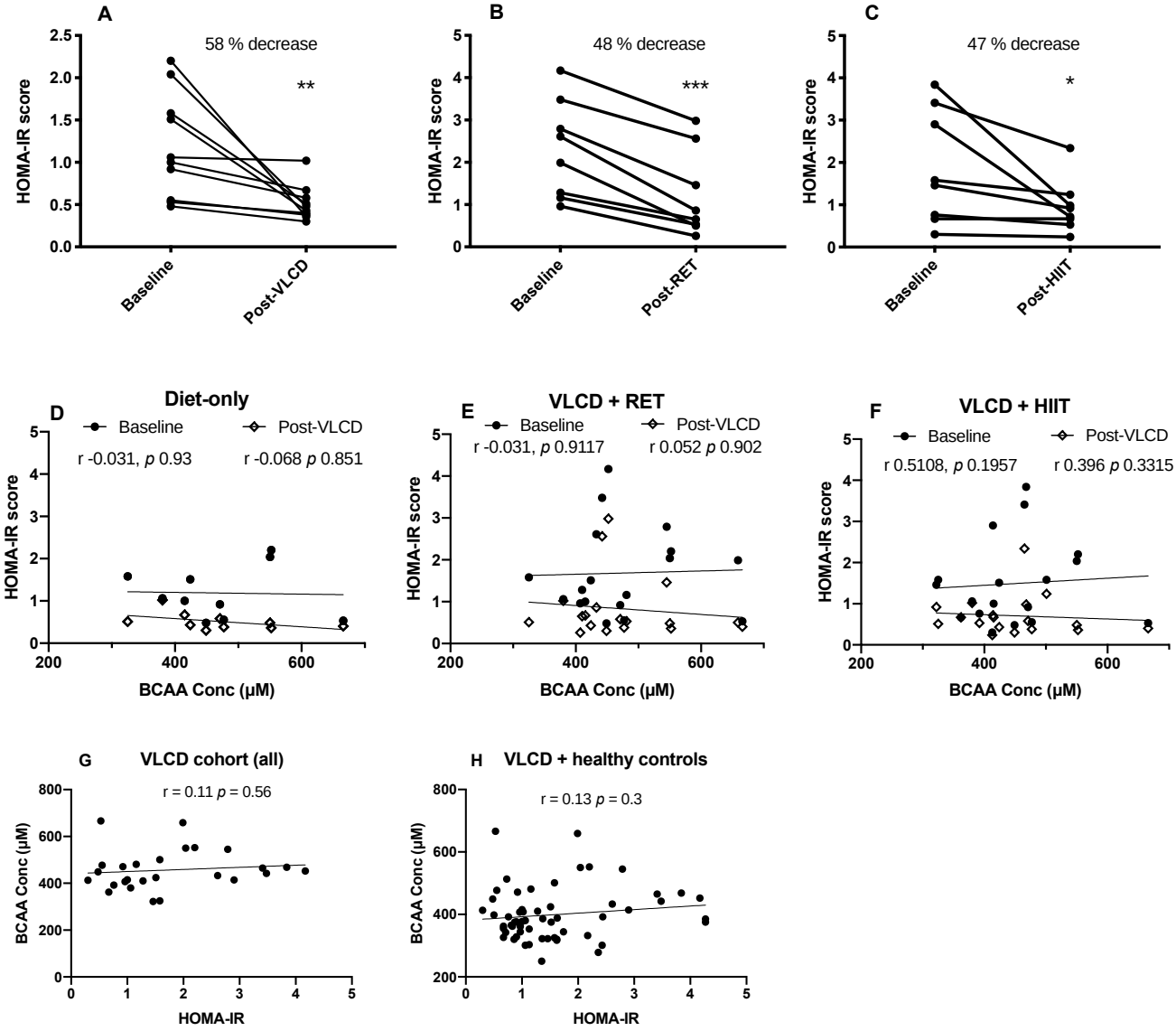


FIGURE 15. PAIRWISE COMPARISONS OF HOMA-IR SCORE AFTER VLCD, DIET-ONLY (15A), VLCD + RET (15B) AND VLCD + HIIT (15C). BCAA ASSOCIATIONS WITH INSULIN SENSITIVITY WERE INVESTIGATED AND ARE DISPLAYED IN FIGURE 15D (DIET-ONLY), 15E (VLCD + RET) AND 15F (VLCD + HIIT). ASSOCIATIONS BETWEEN BCAA AND HOMA-IR IN ALL VLCD PARTICIPANTS ARE DISPLAYED IN 15G (*N* = 26). THE RELATIONSHIP BETWEEN HOMA-IR AND BCAA IN A GREATER RANGE OF BMI ARE DISPLAYED IN FIGURE 15H (*N* = 58).

4.4.4. BCAA metabolising enzyme expression

Skeletal muscle is the main site of BCAA metabolism (namely due to the presence of the BCAT enzyme) which initiate reversible transamination reaction to facilitate transfer of an α-amino group to α-ketoglutarate yielding glutamate and each of the BCAAs respective keto-acid (Brosnan and Brosnan 2006). As such, BCAT1 contributes to catabolism of BCAA by generating nitrogen required for glutamate synthesis and accounts for BCAT activity particularly in the brain (Hall et al. 1993) and peripheral nerves (Sweatt et al. 2004). BCAT2, which plays a main role in peripheral BCAA catabolism and is expressed ubiquitously but especially in skeletal muscle (Lynch and Adams 2014a; Papathanassiu et al. 2017). Therefore, we studied whether expression and activity of these enzymes (increased flux) could provide insight into differences between plasma BCAAs that would emerge following VLCD. Further, total BCKDH (known to increase following exercise) and its phosphorylation of BCKD E1a provide rate-limiting steps giving rise to acyl coenzyme A adducts eventually feeding into the TCA cycle (She, Reid, Huston, et al. 2007; White et al. 2018). For BCAT 1 expression, our intervention resulted in a trend towards increased BCAT 1 content in all study groups (figure 16A). In contrast, BCAT 2 content remained unchanged in all groups (figure 16B). BCKDH expression was similar at baseline post-VLCD only (figure 14C; $p = 0.99$) and in VLCD+HIIT (figure 16C; $p = 0.99$) but showed a strong trend towards increasing in the VLCD+RET group (figure 16C; $p = 0.11$). VLCD alone was unable to induce any changes in phospho-BCKDH (figure 16D; $p = 0.96$), whereas an increased trend was observed for both VLCD+RET (figure 16D; $p = 0.083$) and VLCD+HIIT (figure 16D; $p = 0.093$) cohorts.

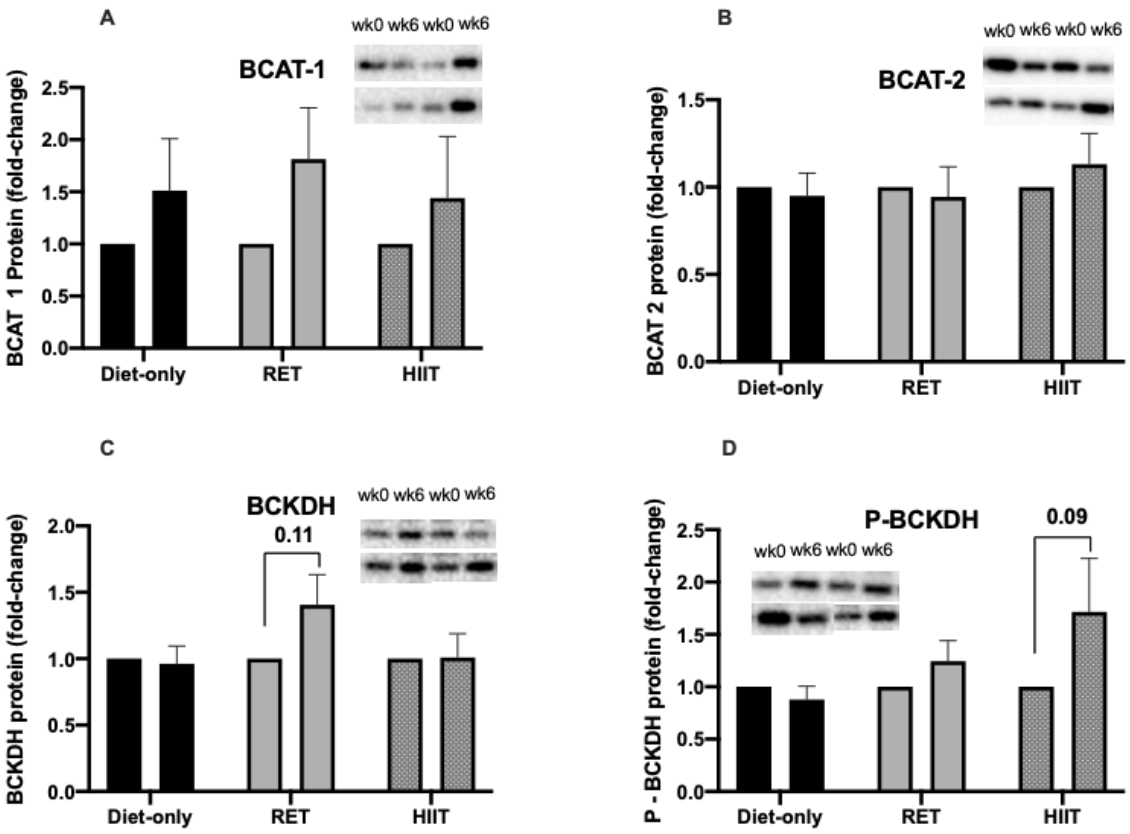


Figure 16. RIPA extracted western blot analyses on muscle in VLCD, VLCD + RET and VLCD + HIIT interventions.

4.4.5. Muscle BCAA-related gene expression

The expression of a number of genes intimately involved in branched-chain amino acid metabolism were also probed (listed in table 2). In total, 8 BCAA-related genes were analysed, significantly increased expression of mitochondrial respiratory chain complex subunit succinate dehydrogenase complex subunit B (SDHB) and 3-hydroxyisobutyrate dehydrogenase (HIBADH), which is implicated in valine catabolism, were observed only in the VLCD+RET group (figure 17 E & G). The other 6 genes remained unchanged pre- and post-6-weeks of VLCD, figure 17. A summary of this chapter's findings is displayed in figure 18.

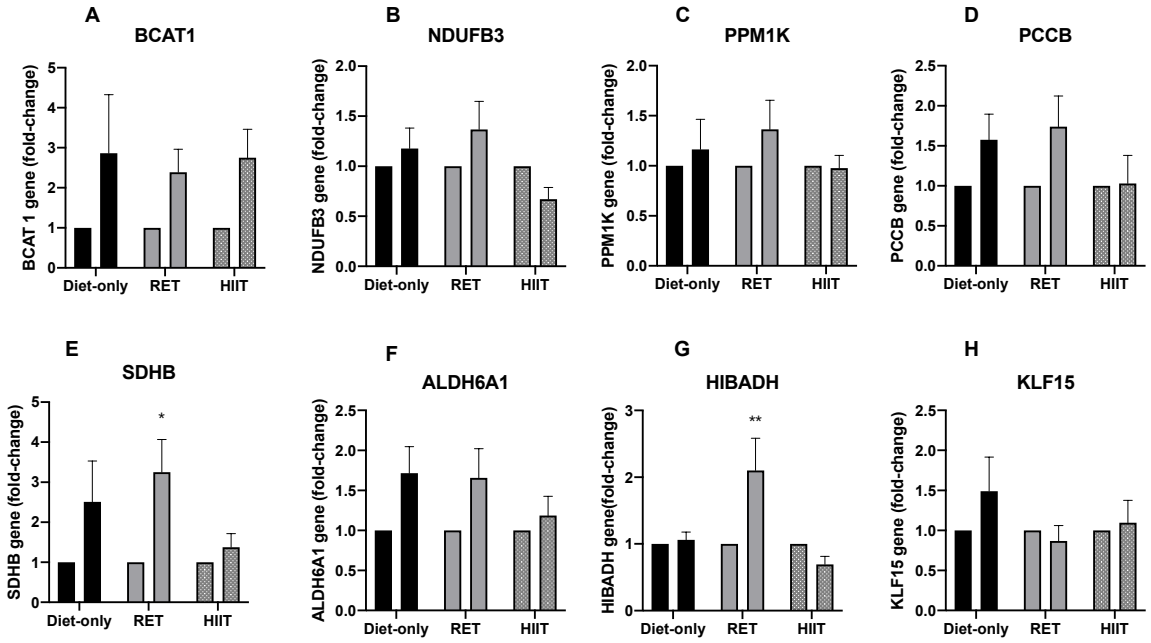


Figure 17. Muscle gene expression analyses of BCAA-related genes pre- and post-intervention in the VLCD groups. Data were analysed via two-way ANOVA and Tukey's post-hoc test ($p < 0.05$).

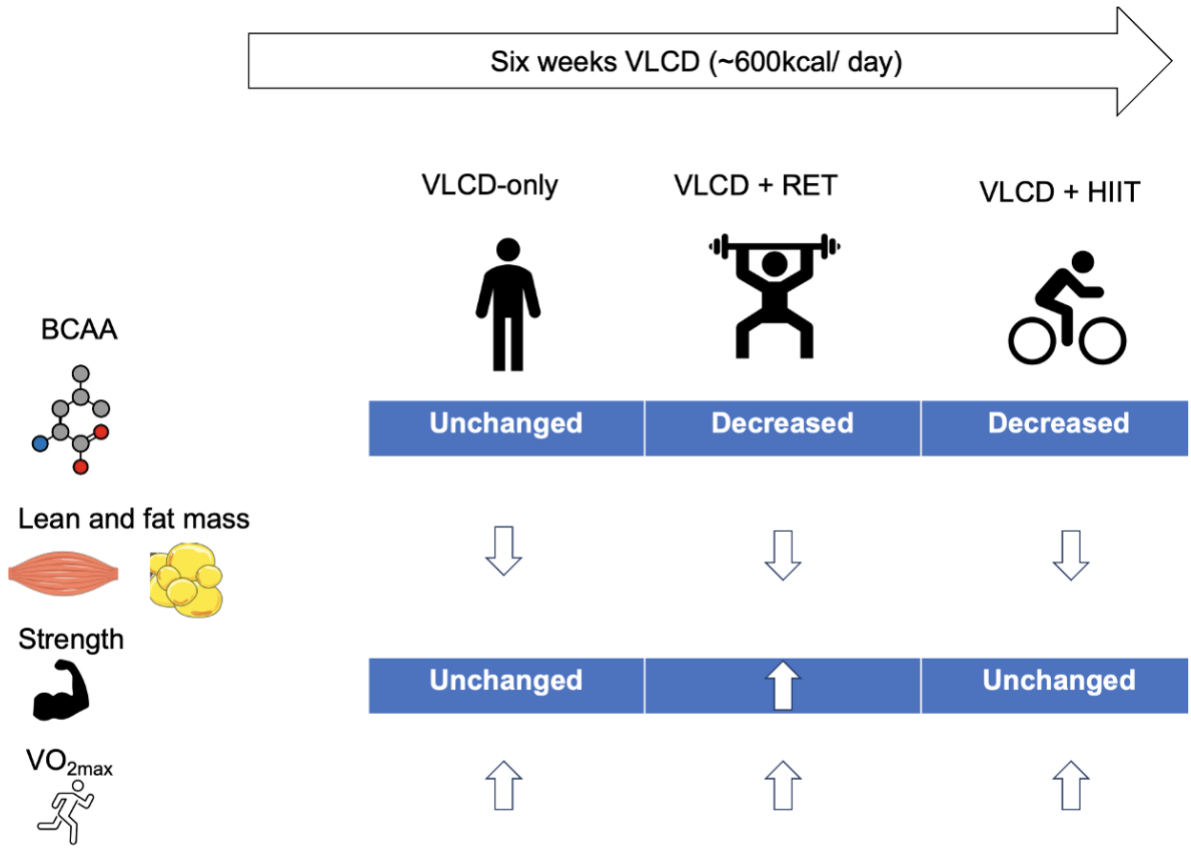


FIGURE 18. SUMMARY OF THE FINDINGS OF THE PRESENT CHAPTER

4.5. Discussion

In accordance with numerous other studies (Newgard et al. 2009; Felig, Marliss, and Cahill 1969b; Le Couteur et al. 2019; Siddik and Shin 2019), including in monozygotic twins (Pietiläinen et al. 2008), BCAA were elevated in our obese participants, and they decrease following VLCD and exercise, but not VLCD alone. To account for potential limitations in statistical power, we combined our entire study population baseline BCAA measures (which are elevated compared to healthy (BMI) controls of a similar age range to IS scores to determine if there is a trend towards the commonly reported associations, however, again this was unfounded. Further, we also included BCAA concentrations from a previous study of our lab (Phillips et al. 2017) to increase the spectrum of BMI, as well as study numbers, however this also failed to uncover any robust relationship between fasting plasma BCAA and variables of HOMA-IR. Indeed, combing recent data from our lab reveals that this relationship with HOMA-IR and BCAA does not appear to hold with normoglycemic, non-obese and healthy populations either, but rather the relationship appears to track more closely with increases lean body mass and strength following a period of RET (Sayda et al. 2020). The present study supports this, in the context that BCAAs are not a robust marker that track with changes in HOMA-IR, despite our exercise and nutritional interventions significantly reducing the circulating plasma BCAA levels of our volunteers undergoing a stringent weight loss programme.

The magnitude of weight loss (~11 %) in this study was comparable to those in other calorie restriction studies (~9.5-16 %; Colleluori et al. 2019; La Vignera et al. 2020) and was accompanied by improvements in insulin sensitivity across all groups, reduction of whole-body mass and BMI, which is indicative of compliance with the intervention and overall improved metabolic health. Our data suggest that whilst the

role of BCAAs in IR (Newgard et al. 2009) are routinely reported, these relationships do not hold in our obese participants at baseline nor following VLCD or VLCD with exercise training. However, the possibility of low *n* as a contributing factor cannot be ruled out.

Recent studies indicate BCAA alone or in combination with other metabolites (Wang et al. 2011), such as aromatic AA (Phe/Tyr) and acylcarnitine species (Newgard et al. 2009) alanine and proline (Tai et al. 2010) may hold value as metabolic 'signatures' of obesity and as predictors of T2DM. Nonetheless, relationships between plasma BCAA and metabolic 'signature' have been shown to vary across the lifespan. For example, elevated BCAA are not associated with obesity in adolescents (Mihalik et al. 2012), indicating that any relationship with plasma BCAA may be context-dependant (Le Couteur et al. 2019). Further these associations have also been shown to differ between sexes. Reflecting this, in females, it was shown that BCAAs, and phenylalanine, BCAA metabolite, 3-methyl-2-oxovalerate (Menni et al. 2013) when combined revealed strongest associations with IR; conversely, in males it was BCAA, alanine, proline, glutamine and aromatic AA that associated most strongly (Tai et al. 2010). Therefore, elevations in BCAA do not wholly indicate underlying metabolic dysfunction and show variability across the lifespan and between genders.

We conclude that while BCAA in conjunction with other metabolites may robustly relate to IR, a causal relationship is lacking despite recent studies suggesting a 'clogging' model of impaired BCAA catabolism, including 3-hydroxyisobutyrate (3-HIB) a valine catabolite (Jang et al. 2016). These authors report increased muscle 3-HIB secretion in IR facilitates fatty acid uptake resulting in lipid accumulation, a 'synergistic' viewpoint which is supported by other workers (Newgard 2012). Indeed, genetic markers (e.g. reduced BCAA dehydrogenase complex and its regulatory phosphatase PPM1K in

obese individuals; Wang et al. 2017) have been suggested as mechanistic explanations to describe the variation in studies of the long-standing associations between BCAA and IR/ T2DM. Taken together, the lack of correlation between reductions in IR and BCAA seen here, despite improved overall metabolic health of expected weight/ fat loss, suggests that reductions in BCAA *per se* is not required to improve IS, but rather a metabolic reprogramming of insulin-sensitive tissue(s) in response to VLCD causes improvement in IS which precede detectable changes in plasma BCAAs, particularly since no correlation was observed between total mass/ fat loss and indices of IS.

Contrary to previous observations between food over-consumption with increases in BCAA, we observed that BCAA levels was not reduced following 6-weeks of VLCD-induced weight loss. This observation is in-line with some reports (Lips et al. 2014; Laferrère et al. 2008) but is in contrast with other reports of calorie restriction induced weight loss via bariatric surgery (Shah et al. 2012; Magkos et al. 2013). While it is likely that subject characteristics may account for these contrasting results, some evidence suggest that reduction in BCAA occurs directly as a result of bariatric surgery *per se,* independent of weight loss due to increase in BCAA catabolic gene expression in adipose tissue (She et al. 2007). Therefore, while the causes of elevated plasma levels of BCAA are incompletely elucidated (i.e. the impact of age, dietary intake, or impaired catabolism; (Elshorbagy et al. 2018; Jang et al. 2016)), the present study suggests improvements in body composition and HOMA-IR occur independently of changes in plasma BCAA.

That BCAAs reduced with exercise may not be particularly surprising, given the demand placed on the body of those within exercise groups i.e., increased catabolism (Refsum and Strömme 1974; Haralambie and Berg 1976) without sufficient nutrient

availability and delivery to working muscles; particularly since the anabolic capacity of food/ exercise is dose-dependent (Witard et al. 2014; Rennie and Tipton 2000). It could be argued that by the data presented here and elsewhere (Magkos et al. 2013) diet alone is not enough to reduce plasma BCAAs and the introduction of exercise, and ensuing hyperaminoacidemia, is crucial in normalising BCAA metabolism particularly in obese individuals. Although BCAAs were significantly lowered with VLCD ± RET/ HIIT, and a significant increase in IS was observed, despite no association between BCAAs or HOMA-IR, or even when all data were combined.

One aim was to determine whether there was a mechanistic or genetic component in this study. For example, studies have shown decreased expression of the main BCAA enzyme, BCAT2 which deaminates BCAAs to their corresponding keto-acids, and BCKDH E1α in muscle from insulin resistant participants (Serralde-Zúñiga et al. 2014). In the present study, no change was observed in measured enzymes, in either total or phosphorylated form, in any groups after VLCD. Given that our participants are obese, it may be possible that changes in adipose tissue BCAA metabolism contribute to the change in circulating fasting BCAA (Herman et al. 2010), a site of BCAA transamination and decarboxylation described recently

Additionally, PCR analyses revealed higher expression higher expression of mitochondrial genes SDHB and HIBADH in the VLCD+RET group only. SDHB, a subunit of the succinate dehydrogenase complex (SDH), is a membrane protein which plays a key role in glucose and FA metabolism and is thought to be an important site of mitochondrial reactive oxygen species production (Anderson et al. 2009), and been shown to influence insulin action in animal models. Therefore, it may be that bouts of RET, coupled with low calorie intake, are primarily responsible for the increase in SDHB expression changes seen here. Muscle HIBADH, a gene implicated in 3-HIB

degradation (valine metabolite) has also been shown to increase expression following exercise (Jang et al. 2016). Decreased expression of HIBADH has been observed in the muscle of diabetic individuals, whilst changes in expression in the liver has not been reported (Chen et al. 2013), thereby offering a possible explanation as to the decrease in the plasma BCAAs observed in this group. The potential for gene expression analyses in offering mechanistic insight is promising, notably, genome-wide association studies have revealed PPM1K (Goni et al. 2017) and BCKDHA (Tiffin et al. 2006) as candidate genes with causal links to obesity and IR. In support, studies in weight-discordant monozygotic twins reveal differential expression of a range of genes involved in BCAA metabolism, including BCAT2, HIBADH and ALDH6A1 (Pietiläinen et al. 2008). Alongside this, recent studies (Zhou et al. 2019) have centred on defects in BCAA catabolism and provide evidence of the importance of BCAA dysregulation in obesity-associated IR and T2DM, which propose keto-acids (namely 3-HIB; Jang et al. 2016) metabolites giving rise to a 'clogging' model to explain that the accumulation and reduced clearance capacity of metabolic by-products of BCAA metabolism

There are some study limitations that warrant discussion, including low sample numbers reducing statistical power that make it difficult to detect mild associations between HOMA-IR and BCAA. Further, it may be that the 'low severity' of our volunteers IR was a factor. Previous studies which have shown correlations with BCAA, document HOMA-IR of ~3.5 – 5.5 in individuals with obesity (Newgard et al. 2009; Tai et al. 2010) compared to ~1.7 in this study. Further, a means to mitigate the loss of muscle mass in interventions of this nature are crucial to maximise the therapeutic benefits, with the manipulation of protein intake one such option. Six weeks VLCD resulted in significant weight loss and improved metabolic health in the

form of reduced IR. Moreover, despite the observation of elevated fasting BCAA in the overweight group, neither reductions in BCAA nor correlations between BCAA and HOMA-IR were observed following VLCD or VLCD + exercise. That VLCD alone does not markedly reduce circulating BCAA, questions this association. Furthermore, it should be noted that whilst whole body, fat and lean mass, including BMI significantly reduced (28.8 ± 2.9 kg.m^2), the reduction was not to within the healthy BMI range (~25 kg. m^2). Indeed, others have shown reduction of BMI to healthy ranges with VLCD (Hammer et al. 2008) suggesting a longer duration or repeated bouts of VLCD would be required to observe normalisation of BMI and possible plasma BCAAs.

4.6. Conclusion

Our study shows that 6-weeks VLCD alone or with RET or HIIT leads to significant weight loss and improvements in HOMA-IR and that all improve aerobic capacity, likely because of weight loss alone. Despite observations of elevated fasting BCAA in the obese confirmed at baseline, VLCD alone does not markedly reduce circulating BCAAs which questions this seemingly established association. These data suggest that elucidating the role of catabolic by-products in plasma and the enzymatic activity in adipose tissue may be potential underlying causes for this elevated BCAA phenotype commonly observed in the obese.

CHAPTER FIVE: 8-WEEKS OF ENDURANCE EXERCISE IMPROVES CIRCULATING BCAAS IN HEALTHY OLD INDIVIDUALS IN CONTRAST TO INDIVIDUALS WITH COPD

5.1. Abstract

Introduction: Chronic obstructive pulmonary disease (COPD) is characterised by lung injury which causes progressive airflow limitation because of lung inflammation and destruction of alveoli. The most prominent characteristic in COPD is chronic dyspnea (shortness of breath) in addition reduced muscle function and strength, resulting in reduced quality of life. Studies of exercise endurance (EE) training in COPD cohorts has shown promise in improving functional capacity and the role of plasma branched-chain amino acids (BCAA) in these patients are not well-defined but could provide a more reliable marker of disease severity and responsiveness to rehabilitation interventions.

Methods: COPD patients (*n* = 14 men and women, mean age 70.4 ± 6, BMI, 28.1 ± 27.8, FEV_1 1.2 ± 0.3) with stable COPD underwent EE on a cycle ergometer, comprising of 65 % 1-RM, 3 times per week. Load was adjusted throughout to maintain intensity, and the intervention lasted for a total of 8 weeks. Measurements of lean body mass, strength and lung function were collected. Plasma BCAA profiles were determined using gas chromatography-mass spectrometry (GC-MS)

Results: COPD patients displayed a blunted response to 8-weeks EE as demonstrated by VO_{2peak} 13.95 L/min/kg at baseline which remained unchanged. In contrast, age-matched controls displayed improvements (+ 3.3 L/min/kg, p = 0.001). Similarly, no strength improvements were seen within the COPD group, in contrast to age-matched control who demonstrated strength improvements (baseline 127 nM, post-EE 144 nM, P = 0.01). Unsurprisingly, no BCAA changes were observed within the COPD group, in contrast to control group who displayed significant elevations of sum BCAA (343µM ± 61 at baseline, compared to post-EE 389 ± 57 µM p = 0.02)

Conclusion: Eight weeks of progressive EE was not sufficient to improve aerobic capacity in stable COPD participants. Age-matched, older controls maintain the capacity to response to EE interventions.

5.2. Introduction

COPD is a disease which is characterised by alveolar abnormalities that cause progressive airflow limitation and often result in breathing difficulty (Vestbo et al. 2013). Two main terms are used to broadly define COPD, that is chronic bronchitis (long-term inflammation of the airways) and emphysema (destruction of the alveolar walls within the lungs) (Snider 1989), leading to the presence of a persistent cough, wheezing and sputum production (Müllerova et al. 2015). The diagnosis of airflow obstruction can be typically measured by forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC), and the global initiative for obstructive lung diseases (GOLD) suggests a ratio of 0.70, based on post-bronchodilator FEV_1 to define airflow limitation (Rodriguez-Roisin et al. 2017). The most obvious characteristic symptom of COPD is chronic dyspnoea (Ferguson et al. 2000), however despite clear deleterious effects on the lungs, COPD is also widely recognised as a systemic disease (Bernard et al. 1998) that includes (but not limited to) skeletal muscle dysfunction e.g. low muscle mass and strength, metabolic diseases (obesity, cardiovascular disease), gastroesophageal reflux and clinical depression (Barnes and Celli 2009). These extrapulmonary co-morbidities contribute significantly to the decline in quality of life and result in multiple symptoms, placing a significant burden on both the patient and healthcare systems (Donaldson et al. 2015). Skeletal muscle dysfunction in particular is associated with a poor quality of life, for example reduced quadricep muscle force in COPD patients are a significant factor associated with increased hospital admissions over a 6 month period (Decramer et al. 1997) and mortality (Celli et al. 2015). Decreased proportion of type I muscle fibers, reduced oxidative capacity, muscle cross-sectional area and endurance are hallmark features of COPD patients, which culminate in overall reduced working capacity and exercise tolerance (Casaburi 2001; Zeng et al. 2018). Exercise

training is regarded as the cornerstone of pulmonary rehabilitation and is a common clinical intervention to improve mobility and limb function. In addition to obvious roles in locomotion, skeletal muscle mass serves as a reliable prognostic marker of mortality, independent of either FEV_1, BMI or age (Swallow et al. 2007). Fat free mass (FFM) is therefore often used as a surrogate marker for skeletal muscle mass, and unsurprisingly lower FFM is associated with greater risk of both COPD and all-cause mortality (Vestbo et al. 2006), therefore improving muscle mass and function, may serve as an attractive therapeutic target, since there are few alternative interventions that can directly reverse the damage that has occurred or remodel of lung tissue.

As COPD progresses, advancing age becomes another risk factor for COPD, in a population where physical inactivity is already a prominent feature (Kohl et al. 2012), however exercise is arguably the most effective intervention at increasing exercise tolerance and quality of life (Spruit et al. 2002), including in those suffering from COPD regardless of disease severity (Vestbo et al. 2013; Neder et al. 2019).

For example, despite progressive loss of muscle with age (sarcopenia; Rosenberg 1997) and strength (dynapenia; Skelton et al. 1994), skeletal muscle maintains remarkable responsiveness to exercise stimuli (Goldspink 1999). Studies investigating the role of exercise in COPD patients have shown that maximal aerobic capacity (VO_{2max}) is up to ~50 % lower, when compared to healthy counterparts of similar age (LeBlanc et al. 1996; Vaes et al. 2011). Despite this, there is evidence to suggest efficacy in aerobic training in COPD patients whereby it has been shown exercise capacity (VO_{2max}) can be increased following tailored exercise regimes (Gosker et al. 2006; Brønstad et al. 2012), hence exercise as a nonpharmacological intervention is a cornerstone of clinical pulmonary rehabilitation programmes (Bolton, Bevan-Smith, and Blakey 2014; Singh 2002). Some studies investigating the impact of endurance

exercise in COPD cohorts have shown no improvements in aerobic capacity compared to healthy controls (Radom-Aizik et al. 2007), whereas others have shown that, when matched for relative intensity, COPD patients experience reduced VO_{2max} gains compared to their health age-matched counterparts (Sala et al. 1999; Rabinovich et al. 2001; Radom-Aizik et al. 2007). Further, Gouzi et al. (2013) have shown, following 6-weeks moderate endurance exercise, the response is significantly blunted (+0.96 mL• kg^{-1} • min^{-1}) compared to age-matched healthy controls (+2.9 mL• kg^{-1} • min^{-1}), whereas others have shown that 12-weeks of HIIT (2 times p/week) is effective in increasing peak power and increased quadriceps thickness and CSA, suggesting adaptation to exercise is preserved in the muscles in individuals with COPD.

As presented in chapter 1.8.1, measurement of airflow obstruction represents a simplistic diagnostic in a complex, multifactorial pulmonary disease. Therefore, there is an urgent need for more accurate diagnostic (and prognostic) measures of disease status and progression, because intervention during the early stages of COPD disease development likely represents an opportunity when interventions are likely to be most effective (Vestbo and Anderson 2008).

Studies of circulating plasma branched chain amino acids (BCAA) have revealed significant perturbations in the metabolism of these compounds, wherein patients with COPD present with decreased concentrations when compared to healthy age-matched non-smoker controls (Ubhi, Riley, et al. 2012), particularly for leucine (~107µM/L, compared to 147µM non-smoker control; Morrison et al. 1988). As shown in chapter 3, plasma BCAAs track with improvements in lean mass and strength following exercise interventions, even in older individuals, which provides us with a relatively accessible fluid to sample and from which to gather information on, and an

alternative means to measure functional changes of an intervention. Therefore, the aims of this chapter are firstly to determine how patients with COPD, and healthy age-matched men and women respond to 8-weeks of supervised endurance exercise (EE). Secondly, in addition to investigating EE responses in aerobic capacity and lean mass, the aim is to also determine whether these changes which are driven by the intervention reflect in the plasma BCAA profiles of these individuals which could predict clinical markers of health.

5.3. Materials and Methods

5.3.1. Ethical approval

Ethical approval for this study was granted by the NHS National Research Ethics Service (West Midlands – Coventry and Warwickshire, Reference 13/WM/0075).

5.3.2. Participant characteristics

A total of *n* = 14 (5 males and 9 females, mean age 70.4 ± 6 years, BMI: 28.1 ± 5.2) with existing COPD (MRC grade >3 ex-smokers, mean smoking pack years 40.6 ± 14.3) were recruited for this study. Participant recruitment was achieved through Pulmonary Rehabilitation waiting lists and mailshot respondents, performed by researchers at the University of Leicester and Glenfield Hospital, Leicester, U.K. Additional inclusion criteria were age >60 and <80 years with clinical diagnosis of COPD and obstructive spirometry with lung function FEV_1 <80% predicted, FEV_1 / FVC <70% Medical research council grade >3 and clinically stable with no exacerbation within last 4-weeks. COPD patients were excluded if participating in any regular exercise regime. Age-matched healthy volunteers *n* = 8 (50: 50, male: female, mean age 70.6 ± 5.8, BMI: 28.2 ± 3.6) were included with normal lung function FEV_1 >80% predicted, FEV_1 / FVC >70% with no history of respiratory diagnosis. No participants engaged in any structured exercise regime and were excluded from the study if any metabolic comorbidities were present (such as type II diabetes) or inflammation (e.g., rheumatoid arthritis), or had impaired muscle function. Participants were also not receiving any medication, such as systemic corticosteroid, anticoagulation therapy or current smoker (ex-smokers > 1 year were accepted). All participants had the capacity to provide informed consent and all exercise sessions were supervised by a member of research staff.

5.3.3. Conduct of the study

Volunteers of the study attended Glenfield Medical Centre for baseline physiological assessments and testing split over two-days to allow familiarisation. Baseline testing included incremental cardiopulmonary testing (CPET) and quadriceps strength (maximal voluntary contraction; MVC) tests for adjustment of intensity for subsequent exercise sessions. Exercise tests were separated by a minimum of 30 minutes recovery time, in-line with guidelines for exercise testing in COPD cohorts (Spruit et al. 2013), whereby it was ensured that heart rate, ventilation and respiratory exchange ratio had returned to resting values before commencement of second test. CPET tests were performed on a calibrated Lode cycle ergometer (Groningen, Netherlands) and monited using a CPET card (Ergocard Professional, Medisoft, Sorinnes, Belgium). Continuous cardiac monitoring was achieved using 12 lead electrocardiogram (ECG), oxygen saturation (SPO_2) measured by pulse oximetry (Nonin Medical B.V. Europe, Amsterdam, Netherlands) and blood pressure monitored using an automatic sphygmomanometer, all connected to the computer coordinating the CPET. Peak exercising capacity was measured using incremental cycling to voluntary exhaustion. Pedalling resistance was progressively increased by increments of 1 watt, with a ramp protocol of 5 W/ minute to 30 W/ minute. Participants were instructed to maintain 60 revolutions per minute (RPM) until local muscular fatigue or dyspnoea prevented them from continuing. Tests were terminated if participant was unable to maintain a 60 rpm cadence. VO_2^{PEAK} was defined as the highest oxygen uptake (L / min) acheived during loaded pedalling.

Body composition was measured using Dual Energy X-ray absorptiometry (DXA) scan (Lunar Prodigy; GE Healthcare, Buckinghamshire, United Kingdom) and regions of the body were automatically integrated by the software package (Encore softwards,

GE Healthcare). QMVC was measured on an isokinetic dynamometer (Cybex NORM II; CSMi, Stoughton, United States). The QMVC protocol was conducted in a seated position with knee and hip flexion at 90^{o} and one set of three repetitions at a rotational velocity of 60^{o} / s performed at 50 % effort to ensure familiarisation. Then, two sets of three maximal repetitions were performed separated by 30 seconds recovery with verbal encouragement – the highest value for peak torque was recorded as isokinetic QMVC. Blood samples were taken from the antecubital vein and collected into heparinized vacutainers for measures of plasma AA, with the plasma fraction collected following centrifugation at 2000 x g for 20 min at 4 ºC. These measures were collected at baseline and again following successful completion of training programme.

5.3.4. Endurance exercise intervention

Following a minimum of seven days recovery, participants commenced aerobic training for a total of eight weeks and three sessions per week on a Lode cycle ergometer (Groningen, Netherlands). Training commenced with 3 minutes of resting recordings, and 3 minutes of unloaded pedalling. Work rate was then ramped up by 1 W / minute to reach a 65 % intensity of what maximum, with cadence being maintained at 60 rpm. Physiological measures were taken during the test and averages calculated which excluded the first three minutes of pedalling to produce data representative of steady state exercise. Where a participant was unable to complete 30 minutes continuously, they were allowed a short break (< 5 minutes) before resuming to achieve 30 minutes. Cycling resistance was adjusted at 4-weeks to ensure intensity of the sessions were maintained. Once 8-weeks of exercise sessions were reached, participants were instructed to resume their habitual physical activity levels.

5.3.5. Analytical methods

To determine plasma AA concentrations, samples were prepared according to the methods described in chapter two.

5.3.6. GC-MS conditions

Plasma AA concentrations were determined according to methods previously described in chapter two.

5.3.7. Statistical analyses

Once data had passed normality, Student's *t*-test were used to determine plasma BCAA changes at baseline and following 8-week EE. Pearson's correlation was used to determine associations between plasma BCAAs and measures of exercise and health were present at baseline or following the intervention.

5.4. Results

5.4.1. Participant characteristics

Baseline and post-EE characteristics, including lung function and strength measurements are displayed in table 5 for those participants who completed the study. As shown, the older healthy control group had normal lung function (average 2.3 L Residual volume; RV) capacity whereas the COPD counterparts demonstrated obstructive lung spirometry (average 3.4 L RV).

Table 5 Baseline characteristics of individuals who underwent 8-wks endurance exercise

	COPD (*n* = 14)	**Age-matched (*n* = 8)**
Sex	5: 9 (M: FM)	4: 4 (M: FM)
Age (years)	70.4 ± 6	70.6 ± 5.8
Height (m)	1.6 ± 0.1	1.7 ± 0.1
Weight (kg)	73.7 ± 16.3	78.8 ± 15.4
FEV_1 (L)	1.2 ± 0.3	2.6 ± 0.4 ***
FEV_1 % predicted	54.2 ± 15.7	114 ± 22.5****
Smoking history	Current (*n* = 14)	Current: Ex (4: 4)
Pack years	38.8 ± 17.6	18.3 ± 21.5
MRC grade	3.2 ± 0.4	1.1 ± 0.4 ***
RV (L)	3.46 ± 1.2	2.3 ± 0.6 **
TLC (L)	6.5 ± 1.5	5.9 ± 1.1
Alcohol (units/ wk)	4.3 ± 6.8	10.6 ± 9.4

	Baseline	**Post-EE**	**Baseline**	**Post-EE**
BMI ($kg•m^2$)	28.1 ± 5.2	27.8 ± 5.1	28.5 ± 3.7	28.2 ± 3.6
Lean mass (kg)	45.3 ± 11.4	45.3 ± 11.4	50.3 ± 10.1	49.7 ± 10.0
Strength (Nm)	95 ± 42	99 ± 43	127 ± 34	144 ± 48 *
VO_{2peak} (L/min/kg)	14.0 ± 4.5	13.9 ± 4.1	17.8 ± 3.1	21.2 ± 2.8
VO_{2peak} (L/min/kg) Lean mass	24.2 ± 8.1	23.9 ± 7.3	29.5 ± 3	35.4 ± 2.1
Heart rate (max)	127 ± 14	132 ± 16	137 ± 12	142 ± 10
RER	1.05 ± 0.1	1.04 ± 0.09	1.19 ± 0.1	1.21 ± 0.05

*Data are mean (SD). BMI, body mass index; COPD, Chronic obstructive pulmonary disease. FEV1, forced expiratory volume in 1 s; RV, residual volume; TLC, total lung capacity; HR, heart rate; RER, respiratory exchange ratio

5.4.2. Exercise response

The response of both groups to the same EE protocol, is shown in Table 5. Individuals with COPD were not able to improve upon exercise capacity from baseline measurement (baseline 14 ± 4.5, post-EE 13.9±4.1L/min/kg, *P* = 0.82), in contrast to the older healthy control cohort in whom exercise capacity showed significant increase following EE (17.8 ± 3.1 post-EE 21.2±2.8 L/min/kg, *P* = 0.001). A similar trend is observed for changes in strength, as measured by isometric torque, whereby the COPD group did not differ after the intervention (baseline 95 ± 42, post-EE 99 ± 42 nM, *P* = 0.38), whereas their healthy counterparts showed significant improvements (baseline 127 ± 34 post-EE 144 ± 48 nM, *P* = 0.01). In terms of changes in lean mass, neither the COPD group (*p* = 0.84), nor the age-matched controls (*P* = 0.08), showed any significant changes (table 1). At 8 weeks, older participants were able to exercise at a higher heart rate max compared to COPD (132 compared to 142 BPM: ~10 % difference). There were no significant changes of RER at VO_{2peak} between the groups.

5.4.3. Circulating BCAA levels

Next, we assessed whether changes seen in aerobic capacity were matched by changes in plasma BCAAs. In COPD volunteers, baseline (leucine 113 ± 31 µM; isoleucine 64 ± 20 µM; 205 ± 56 µM; valine 205 ± 56 µM; sum BCAA 381 ± 105 µM) and post-EE (leucine 111 ± 26µM, *p* = 0.89; isoleucine 60 ± 15µM, *p* = 0.47; valine 205 ± 56µM, *p* = 0.97; sum BCAA 376 ± 94µM, *p* = 0.86) remained unchanged (figure 19). In contrast, age-matched healthy controls showed marked increases in all BCAA following EE figure 19 baseline leucine (102 ± 18 µM), isoleucine (51 ±12 µM), valine (190 ± 33 µM) and sum BCAA (343 ± 61 µM) all increased with endurance exercise (leucine 115 ± 20µM, *p* = 0.05; isoleucine 62 ± 12µM, *p* = 0.02; valine 212 ± 27µM, *p* = 0.03; sum BCAA 389 ± 57µM, *p* = 0.02), figure 19.

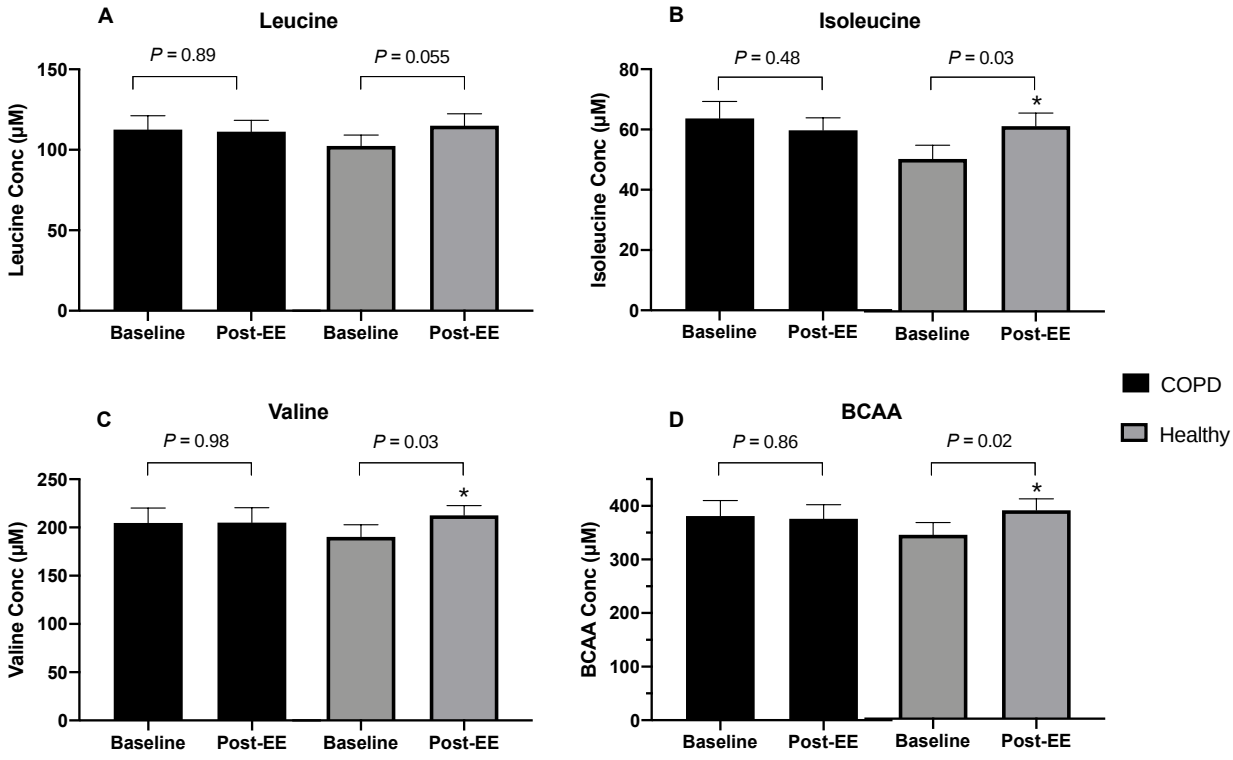


FIGURE 19. BCAA RESPONSES TO 8 WEEKS ENDURANCE TRAINING IN *N* = 14 COPD PATIENTS AND *N* = *8* HEALTHY, AGE-MATCHED CONTROLS.

5.4.4. Associations between BCAAs and variables of lung function, lean mass and strength

Pearson's correlation between BCAAs and FEV_1 was performed to determine if BCAAs could be an associative variable with some measures of lung function as potential for a surrogate biomarker for function, however no associations were present in the COPD group, however a positive trend was noted in the healthy control group, although it is likely the lack of statistical power limits the validity of this association (figures 20).

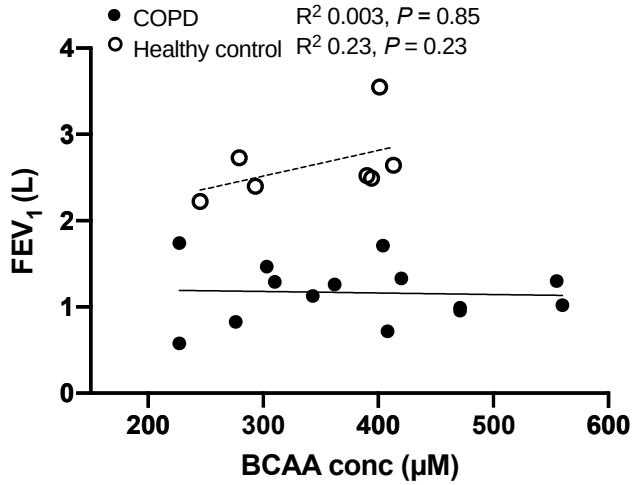


FIGURE 20. ASSOCIATION BETWEEN BCAAS AND FEV_1 IN COPD AND HEALTHY CONTROL PARTICIPANTS.

Pearson's correlation was also used to determine if BCAAs could predict the changes in lean mass, however no significant relationship was detected either at baseline or post EE in either group (figure 21B& figure 21D). With regards to strength changes, COPD group showed no significant associations with strength and BCAA (figure 21A), which is unsurprising given lack of changes in each variable with EE. In contrast, age-matched controls showed significant positive associations with BCAA and strength, which remained significant throughout (figure 21C). A schematic summary of this chapter's findings is also displayed in figure 22.

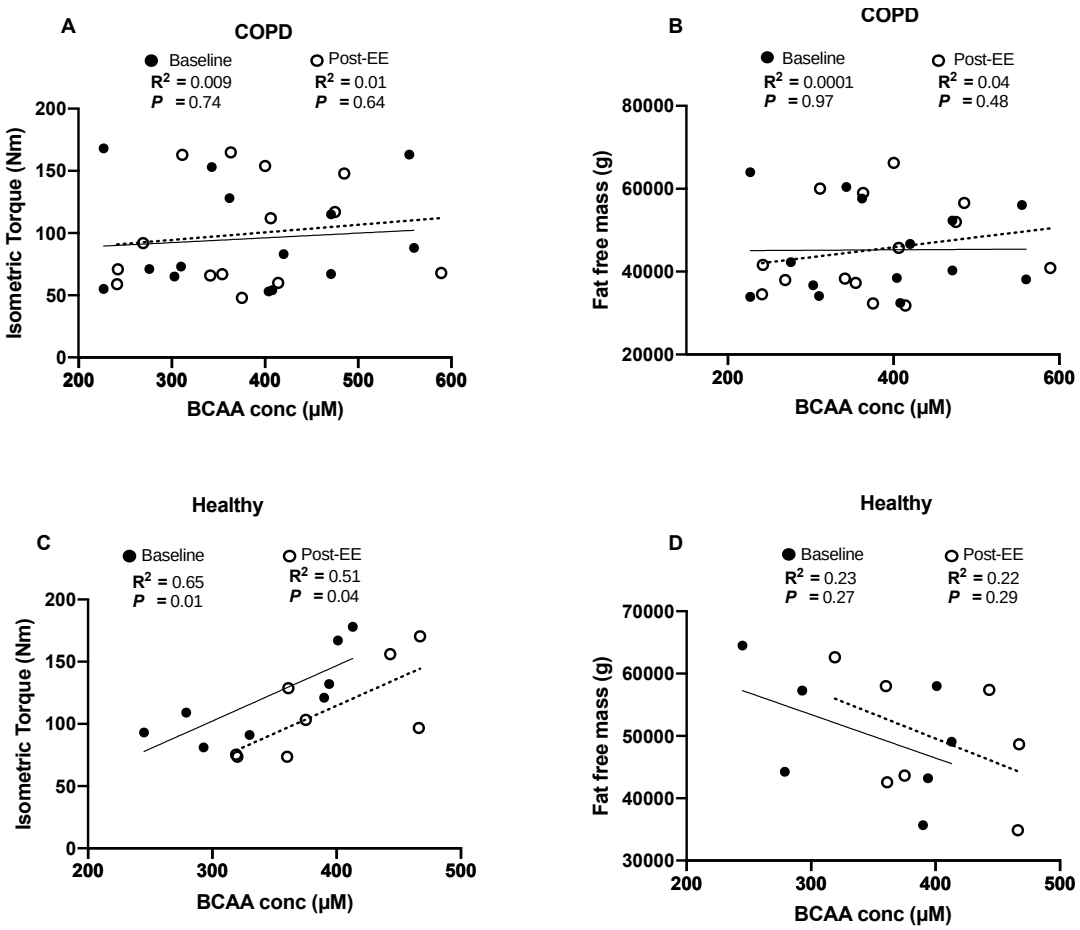
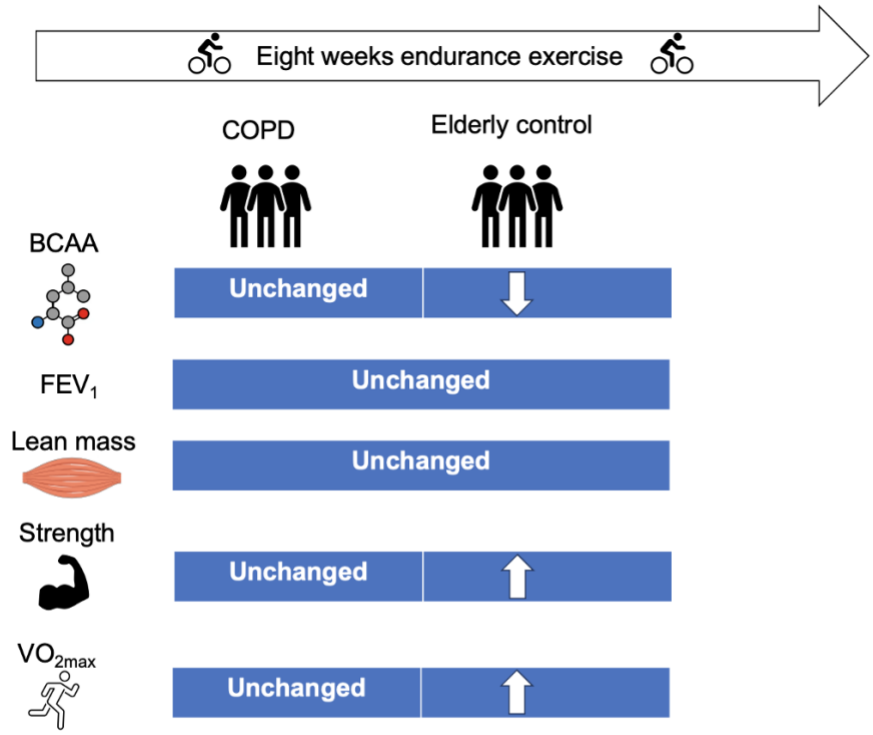


FIGURE 21. THE RELATIONSHIPS BETWEEN BCAA LEAN MASS AND STRENGTH IN COPD PATIENTS (21A AND 21B) AND HEALTHY CONTROLS (21C AND 21D) AT BASELINE AND FOLLOWING 8-WEEKS EE.



- EE did not result in exercise capacity or strength in COPD
- Age-matched controls demonstrated improved aerobic capacity and strength
- Plasma BCAA's were not altered with EE in those with COPD, in contrast to controls
- Increases in BCAA's correlated with strength, in controls only
- Lean mass remained unchanged in both groups

FIGURE 22. SUMMARY OF THE FINDINGS OF THIS CHAPTER

5.5. Discussion

The main aims of this study were to examine the effects of endurance exercise training on aerobic capacity, lower limb strength and lean mass, in individuals with COPD and compared to in age-matched controls without COPD. At baseline, those with COPD had lower (-27± %) oxygen uptake compared to age-matched healthy counterparts and no increase was observed in response to 8 weeks of supervised EE. In contrast, older age-matched individuals demonstrated an increase in peak oxygen uptake following EE (19 %). These values (24.2 mL·min·kg lean mass) are similar to previous studies (Vaes et al. 2011) defining oxygen uptake in COPD patients (24.6 mL·min·kg lean mass) of similar age and BMI. That COPD participants did not respond to exercise

training is concerning; however, this is likely due to exercise intolerance, caused by low muscle mass and dysfunction of the lower limbs, over the course the 8-week training intervention. Indeed, studies have shown that even in patients with normal body weight (Vermeeren et al. 2006), muscle atrophy remains present, and given that strength is mostly determined by muscle size (Bernard et al. 1998; Hamilton et al. 1995), weakness of the muscles are highly prevalent in COPD populations.

Several workers (Bernard et al. 1998; Schols et al. 1993; Simpson et al. 1992; Hamilton et al. 1995), have shown that skeletal muscle dysfunction, underpinned by morphological and biochemical abnormalities and correlating with functional impairments, are largely responsible for exercise intolerance in COPD cohorts, however, unlike the impairments in lung function common in COPD (hence no improvement in FEV_1 seen here), deficits in muscular function are amenable to therapy by rehabilitative strategies, at least in age matched, and otherwise healthy individuals. Indeed, the healthy individuals demonstrated clear adaptation to the intervention as evidenced by increases in muscle strength, aerobic capacity, and increased max heart rate. Although the mechanisms responsible for these improvements were not measured herein, it is likely due to increased cardiovascular adaptation and oxygen delivery to the exercising muscles (Murias, Kowalchuk, and Paterson 2010) as well as neural adaptations (Gabriel, Kamen, and Frost 2006) given the controls were untrained at baseline. The present study is in accordance with other studies in sedentary populations i.e. ‘trainability’ of individuals persists in older age (Houmard et al. 1996; Fujimoto et al. 2010; Milanović, Sporiš, and Weston 2015), therefore, these data suggest that the traditional VO_{2peak} as an all-conquering marker of healthy ageing is context-dependent (Pollock et al. 2015). The lack of increases in lean mass seen in the both COPD and older controls are a concern given the

importance muscle mass in advancing age, therefore efforts (such as alternative exercise modalities, calorie restriction) to maintain or reduce the rate of losses in strength and lean mass (Peterson, Johannsen, and Ravussin 2012). For example, alternative exercise modes i.e. RET/, implementation of 'concurrent' training paradigms as well as nutritional manipulation (i.e., protein; van de Bool *et al.*, 2017) have shown promise. When compared with EE, resistance training combined with EE twice weekly for twelve weeks has been shown to produce similar gains in maximal strength, power and 6-minute walk test in COPD (Zambom-Ferraresi et al. 2015), a notion which is supported by other researchers (Mador et al. 2004; Ortega et al. 2002). The caution related with abrupt increases in heart rate and arterial pressure associated with isometric contractions (such as RET) are acknowledged, however several studies have reported strength training to be well tolerated in COPD patients (Clark, Cochrane, and Mackay 1996; Simpson et al. 1992), in addition to being effective in reducing the strength deficits of COPD patients compared to healthy, age-matched participants (Clark et al. 2000).

Given the lack of changes in strength, lean mass, or exercise capacity in the COPD group, it is perhaps unsurprising that no changes were seen in plasma BCAA following EE. In contrast, given the beneficial changes in exercise performance and strength in the older, healthy controls, BCAAs show increases following 8 weeks EE in the absence of COPD. Uniquely, this correlated with strength gains in the older cohort and is in accordance with other researchers (Borg et al. 2019; Mcdonald et al. 2016), as well as the data presented in the RET chapter (three) of this thesis, where BCAAs show a positive association with strength across the lifespan. Therefore, it seems that in the context strength and lean mass gains with exercise, BCAAs do indeed hold potential in reflecting the response of muscle to RET (when adequately stimulated),

which are sensitive to improvements in metabolic health compared changes in aerobic capacity (VO_{2max}) in the presence COPD.

The present study reports improvements in aerobic capacity and strength of older individuals in comparison to patients with COPD, despite this, sample size limitations ($n = 8$ in control group) are acknowledged as are limitations regarding the extrapolation of the results to more general populations. However, the associations with strength and BCAA in the control participants show clear promise for BCAAs as a potential marker of functional gains. The results of this chapter are supportive of the findings presented in chapter 3, where strength gains correlated with changes in BCAA following an exercise intervention. These results support the work of others, in that in untrained individuals, advancing age should not be a barrier to exercise participation for the purpose of aerobic improvements which is an important conclusion of this study.

This chapter has described on the impact of exercise in elderly individuals with stable COPD and compared them to age and BMI matched controls. The next chapter (6) will investigate a larger cohort of COPD patients, who present to hospital with an acute exacerbation of COPD and their response to an alternative (relative to disease severity) exercise intervention, evaluating a novel, early rehabilitation programme consisting of aerobic, resistance exercises, in addition to neuromuscular electrical stimulation, designed to enhance recovery from a bout of exacerbation from COPD.

CHAPTER SIX: PLASMA BRANCHED CHAIN AMINO ACID RESPONSES TO AN EARLY REHABILITATION INTERVENTION UPON HOSPITAL ADMISSION OF AN EXACERBATION OF COPD

6.1. Abstract

Introduction: The progressive nature of COPD results in sudden worsening of symptoms, i.e., exacerbations (sudden worsening of COPD symptoms) which are characterised by prolonged bedrest, associated muscle loss due to inactivity, and consequently increased risk of mortality. This retrospective, randomized control trial used a progressive exercise-based rehabilitation programme initiated immediately following an exacerbation of COPD.

Methods: Individuals were enrolled on to this study, 48 h following admission to hospital for an acute exacerbation and randomised into standard care (mean age 68.2 ± 13.9, mean BMI 23.8 ± 5.9 kg/m^2, FEV_1 1.3 ± 0.3) or early rehabilitation (Mean age 72.4 ± 3.7, mean BMI 25 ± 6.3 kg/m^2, FEV_1 1.0 ± 0.5), *n* = 10 each. The early rehabilitation received standard care plus 6-weeks additional exercises, including knee extensions, sit-to-stand and step-ups. Participants were followed up at 3 months. Measures of strength, thickness, and cross-sectional area (CSA) of the quadriceps and circulating fasting plasma BCAAs were determined by gas chromatography-mass spectrometry.

Results: Usual care group showed no significant changes in muscle strength, thickness or CSA over the 6 weeks, or at 3 months follow-up. Similarly, early rehabilitation group showed no significant changes in strength or thickness, however a significant increase in muscle /quadriceps CSA (+ 0.8 µM ± 1.1) was observed at 3 months. No changes were seen in plasma BCAA levels after the intervention at 6 weeks. However, at 3 months follow up, only those in standard care showed increases in plasma BCAAs (316 ± 58µM at baseline, 449 ± 134 µM at 3 months). Baseline comparisons demonstrate exacerbated COPD patients with significantly lower plasma BCAAs compared to those with stable COPD.

Conclusion: These data suggest enrolment onto novel a rehabilitative programme is not optimal during the acute stage. From a depleted baseline, usual care is sufficient in increasing plasma BCAAs at 3 months follow-up to within a normal range, however it is not clear whether this is due to 6-weeks standard care.

6.2. Introduction

Chronic obstruction pulmonary disease (COPD), characterised by progressive airway limitation which is not fully reversible (Rodriguez-Roisin et al. 2017) and comprising of chronic bronchitis and emphysema, is a leading cause of chronic morbidity and mortality worldwide (Halbert et al. 2006). For example, the Global Burden of Disease (GBD) study estimate that 328 million individuals worldwide suffer from COPD (Vos et al. 2012), and predicted that COPD was the sixth leading cause of mortality in 1990 (Murray and Lopez 1997), fourth in 2000 (Lopez, Shibuya, et al. 2006) and projected to rise to third leading cause by 2030 (Murray et al. 2007) supported by recent World Health Organization (WHO) estimates. COPD affects lung function by a variety of mechanisms, for example oxidants present in tobacco smoke once inhaled can stimulate production of reactive oxygen species (ROS) from macrophages and neutrophils, which have been shown to be highly present in smokers compared to non-smokers (Rahman and Adcock 2006; Rahman and MacNee 1996). The progression of disease severity is punctuated by sudden worsening of symptoms such as increased sputum or dyspnoea, termed exacerbations (Scioscia et al. 2016), important events in the course of COPD associate with a more rapid decline in lung function, reduced exercise capacity whilst representing a significant (Vestbo et al. 2013; Müllerova et al. 2015). Most exacerbations are triggered by infections, either bacteria or viral infections (including COVID-19 [Alqahtani *et al.*, 2020]). These episodes represent a particularly worrying situation as they become more frequent with time, increase in inflammation, already present in the stable-state, leading to an accelerated decline in lung function loss of muscle mass – and worsening of health-related quality of life (Donaldson and Wedzicha 2014; Donaldson et al. 2002). In addition to sudden worsening, hospital admission will often include prolonged periods of reduced mobility

and functional movement, i.e., bedrest., resulting in reduced muscle mass and strength (Dirks et al. 2016). The prescription of antibiotics or bronchodilators to decrease respiratory symptom intensity and duration of exacerbation are cornerstones of COPD treatment (Donaldson et al. 2015).

As in other chronic inflammatory conditions, alterations of body composition including muscle and fat wasting is a significant cluster of systemic manifestations contributing to COPD disease burden. Specifically, muscle wasting and altered amino acid profiles is a frequent occurrence, the prevalence of which also increases airflow severity and suggest perturbations at the metabolic level (Yoneda et al. 2001). For example, Seymour et al (2010) reported that prevalence of muscle weakness increased from 25 % to 38 % in patients with GOLD severity 1 to GOLD severity 5. Further, profound weight loss, including selective wasting of fat free mass has been reported in COPD patients, and has been shown to adversely affect peripheral muscle function and exercise capacity (Baarends et al. 1997; Palange et al. 1998; Engelen, Schols, et al. 2000). The presence of muscle wasting suggests perturbations in intermediary metabolism; accordingly studies have demonstrated reduced BCAA profiles in COPD patients which associate with weight loss, arm-muscle circumference and % FEV_1 (Yoneda et al. 2001). Further, others have shown BCAAs are decreased in COPD patients with predominant emphysema (characterised by computed tomography), whereas other AA (phenylalanine and glutamine were increased (Ubhi, Riley, et al. 2012). Further, the same study found a combination of AA could even predict levels of C-reactive protein and fibrinogen, which are clinically relevant biomarkers of low-grade systemic inflammation (Leuzzi et al. 2017; Gan et al. 2004) further demonstrating the utility of blood-born biomarkers in helping to define individual phenotypes. Finally, other studies have proposed biomarkers of episodes of

exacerbation including decreased plasma levels of tryptophan (Gulcev et al. 2016), further highlighting the potential use of blood-born biomarkers of disease state and progression.

As demonstrated in chapters the previous chapter, as well the RET chapter (three) three, plasma BCAAs hold promise in tracking with changes in strength and lean mass (following RET), which are reliable markers of adverse health outcomes, even in individuals who experience exacerbations who require hospital admission (Greening et al. 2014). Therefore, the aim of this retrospective, randomised control trial was to investigate whether an early rehabilitation programme, compared to usual care, ameliorates plasma BCAA profiles in patients who had been admitted to hospital following an acute exacerbation of COPD, and whether these would relate to indices of health such as muscle strength, thickness, and size in the quadriceps. The hypothesis of this study was that early rehabilitation would improve muscle function and strength, and that these would be accompanied by increases in plasma BCAAs.

6.3. Materials and methods

6.3.1. Ethical approval

COPD REACH trial (Current controlled trials ISRCTN05557928).

This was a prospective, single-blinded randomised control trial conducted at the Glenfield and Kettering general hospitals, which some samples of the present study originate from (Greening et al. 2014). This study was reviewed and approved by the National Research Ethics Service, Nottingham REC 1 committee (09/H0403/76). Participants enrolled on this study were all admitted to hospital following an exacerbation of COPD and randomised within 48 hours of admission into two groups:

a usual care group and an early rehabilitation group. Participants in the early rehabilitation group underwent a six-week exercise intervention.

6.3.2. Study population

Inclusion criteria were diagnosis of chronic respiratory disease, self-reported breathlessness on exertion when stable (Medical Research Council dyspnoea grade 3 or worse), and over 40 years or older in age. Exclusion criteria were inability to consent to study, acute cardiac events, presence of any musculoskeletal, neurological or psychiatric comorbidities which would prevent delivery of the rehabilitation intervention or more than four emergency hospital admissions in the past 12 months.

6.3.3. Usual care group

Participants in this group (*n* = 10, mean age 68.2 ± 12.1, BMI: 23.8 ± 5.9 kg/m^2, MRC grade >3) received standard care from the clinical physiotherapy ward, as directed by responsible clinical team. Care for this group were delivered by a respiratory physiotherapist with techniques included for airway clearance, assessment and supervision of mobility and advice on smoking cessation. No supervised or progressive exercise programme was provided during the admission or immediately after discharge, however outpatient pulmonary rehabilitation was offered to all participants three months after discharge as per standard care.

6.3.4. Early rehabilitation group

Participants in this group (MRC grade >3) started early rehabilitation within 48 hours of hospital admission. In addition to usual care, they also received daily, supervised volitional (strength and aerobic training) and non-volitional (neuromuscular electrical stimulation techniques; NMES) techniques. Early rehabilitation was performed on the acute medical ward and by patient bedsides. Following discharge, participants were

provided with a home-based programme which was performed unsupervised but supported by telephone consultations. Those readmitted after the six-week intervention period did not receive a further early rehabilitation intervention.

6.3.5. Aerobic and strength training

Daily walking was performed at a set walking speed predetermined by the endurance shuttle walk test at 85 % oxygen consumption (VO_2) max which was calculated from predetermined incremental shuttle walk test. Walking time was progressed at the prescribed walking speed to achieve and maintain a Borg breathlessness score of between 3 – 5 (0 for no breathlessness to 10 for the most severe breathlessness) and a Borg exertion score for rating perceived exertion <13 (from 6 for no exertion at all to 20 for maximal exertion). Participants in this group also completed daily strength training, comprising three sets of eight repetitions with weights. This consisted of bicep curls, tricep curls, knee extension, sit-to-stand, and step-ups, based on predetermined one repetition max (1RM). Once the rate of perceived exertion was <13, the weight was increased to maintain intensity. Participants further received neuromuscular electrical stimulation (NMES, Empi 300PV, Minnesota, USA), which was initially supervised until participants were competent to use independently. NMES was applied to both quadriceps for 30 minutes daily, consisting of biphasic pulse at 50 Hz, pulse duration 300 ms, 15 seconds on 5 seconds off. Intensity was increased according to tolerance and continued throughout the inpatient and outpatient intervention periods.

6.3.6. Post-discharge training

Following discharge, participants were advised to follow a progressive walking-based home exercise programme and to continue daily NMES. Post-discharge training was supported by telephone consultations from the pulmonary rehabilitation intervention

team using motivational interviewing techniques, at 48 hours, two weeks and four weeks.

6.3.7. Analytical methods

To determine plasma AA concentrations, stable isotopically labelled internal standards were added and prepared samples according to standard methods (Wilkinson et al. 2013), described in detail in chapter two.

6.3.8. GC-MS conditions

To quantify plasma AA concentrations, 0.5 µl of sample was injected into an ISQ Trace 1300 single quadrupole GC-MS (ThermoFisher Scientific, Hemel Hempstead, UK), and performed according to the methods described in chapter two.

6.3.8. Statistical analyses

To determine whether baseline plasma BCAAs would change with 6-weeks early rehabilitation and at 3 months follow up, two-way ANOVA was used. Pearson's correlation was used to determine any associations which could predict BCAA levels in usual care, or early rehabilitation groups. Statistical analyses were performed in Prism v8.3 (GraphPad, La Jolla, California, USA) version 7. All data are reported as mean ± SEM, with significance set at $p < 0.05$.

6.4. Results

6.4.1. Participant characteristics

Baseline and post-intervention characteristics, including lung function and strength measurements are displayed in table 6 for those who completed the study. As expected, both groups display similar characteristics in terms of lung function ($FEV_{1)}$ smoking history and MRC grades of disease severity, as well as sex distribution between the two groups.

TABLE 6. BASELINE CHARACTERISTICS OF PATIENTS ENROLLED INTO THE REACH TRIAL (GLENFIELD, LEICESTER).

	Usual care (*n* = 10)			Early Rehab (*n* = 10)		
Sex	3: 7 (M:F)			5: 5 (M:F)		
Age (years)	68.2 ± 12.1			72.4 ± 3.7		
Height (m)	1.6 ± 0.1			1.7 ± 0.1		
Weight (kg)	62.3 ± 13.9			70.7 ± 23.5		
Smoking history	Current: Ex (2: 8)			Current: Ex (1: 9)		
Pack years	48.9 ± 53.5			50.8 ± 13.3		
MRC grade	4.6 ± 0.7			4.8 ± 0.4		
	Baseline	**6 weeks**	**3 Months**	**Baseline**	**6 weeks**	**3 Months**
BMI (kg/m^2)	23.8 ± 5.9	23.8 ± 6.1	24.1 ± 6.2	24.9 ± 6.3	25.5 ± 6.8	25.7 ± 6.7
FEV_1 (L)	1.3 ± 0.5	1.1 ± 0.4	0.99 ± 0.35	1.02 ± 0.51	1.02 ± 0.47	1.01 ± 0.42
FEV_1 % predicted	60 ± 15	47 ± 15	48 ± 19	44 ± 15	46 ± 16	46 ± 15
Q-strength (nM)	14.4 ± 5	14.3 ± 5.8	13.8 ± 5.7	17.8 ± 6.8	18.3 ± 7.4	20.8 ± 10.9
Q-Thickness (mm)	21.6 ± 6.5	17.4 ± 4.7	19.8 ± 5.3	18.2 ± 4.8	18.4 ± 4.1	17.9 ± 4.9
Q-CSA (μM)	4.7 ± 1	4.6 ± 0.6	5.1 ± 1.3	4.1 ± 1.2	4.7 ± 1.4	4.9 ± 1.1

Data are mean (SD). BMI, body mass index; FEV_1, forced expiratory volume in 1 s. Q, quadriceps; Q-CSA, quadriceps cross-sectional area. MRC, medical research council.

6.4.2. Changes in quadricep size, strength, and thickness

Despite the acute sickness of these patients, all completed the study. However, not all responded positively to the intervention. Those in the usual care group showed no significant changes in quadriceps strength (baseline 14.4 ± 5, post 13.7 ± 5.7 Nm), thickness (baseline 21.6 ± 6.5, post 19.8 ± 5.3 mm) or CSA (baseline 4.7 ± 1 μM, post 5.1 ± 1.3 μM). Similarly, in the early rehabilitation group, baseline quadriceps strength (17.8 ± 6.8 Nm) or thickness (18.2 ± 4.8 mm) did not differ to post-intervention (strength 20.8 ± 10.9; thickness 17.9 ± 4.9). However, a significant increase in

quadriceps CSA was seen in the early rehabilitation group (baseline 4.1 ± 1.2, post 4.9 ± 1.1 μM, p = 0.01). But no change in strength or thickness.

6.4.3. Changes in plasma BCAAs from admission and 3 months follow up

The first aim was to determine whether exacerbated plasma BCAA profiles (all participants, *n* = 20) were significantly different to those with stable COPD at baseline. Participants admitted to hospital with an acute exacerbation of COPD displayed significantly decreased BCAAs (leucine 100 ± 28μM, valine 179 ± 40μM, sum BCAA 332 ± 77μM) compared to those with stable COPD (leucine 115 ± 21μM *P* = 0.04, valine 204 ± 44μM *P* = 0.05, sum BCAA 378 ± 79μM *P* = 0.04), figure 23. No significant differences between isoleucine were observed at baseline stable vs exacerbated.

Next, we looked at the temporal effect of training and standard care on BCAA levels to determine the effects of standard care and early rehabilitation on plasma BCAAs over 3 months. No differences between the groups existed at baseline, or at week 6 (cessation of intervention). In the standard care group, however, significant increases in from baseline (leucine, 95 ± 20μM; isoleucine, 51 ± 10μM; valine 171 ± 32μM, total BCAA, 316 ± 58μM) and 3 months of follow up (leucine, 136 ± 40μM; isoleucine, 76 ± 25μM; valine 237 ± 71μM, total BCAA, 449 ± 134μM) were measured, figure 24. Concurrently, no changes were observed in the early rehabilitation group, figure 24, at any time point.

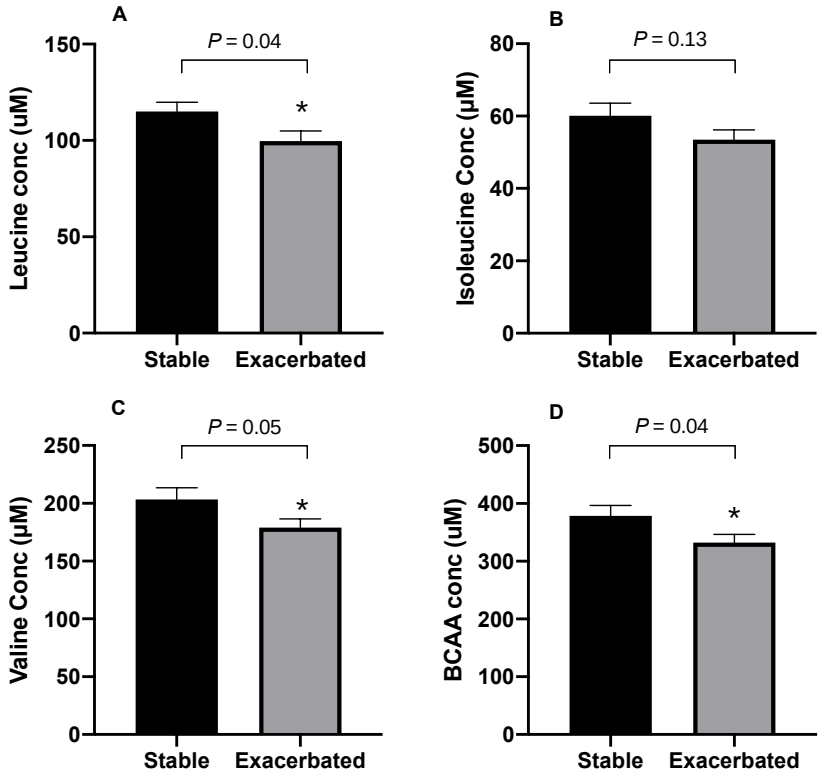


FIGURE 23. COMPARISON OF BCAA CONCENTRATIONS BETWEEN STABLE AND EXACERBATED COPD PATIENTS AT BASELINE.

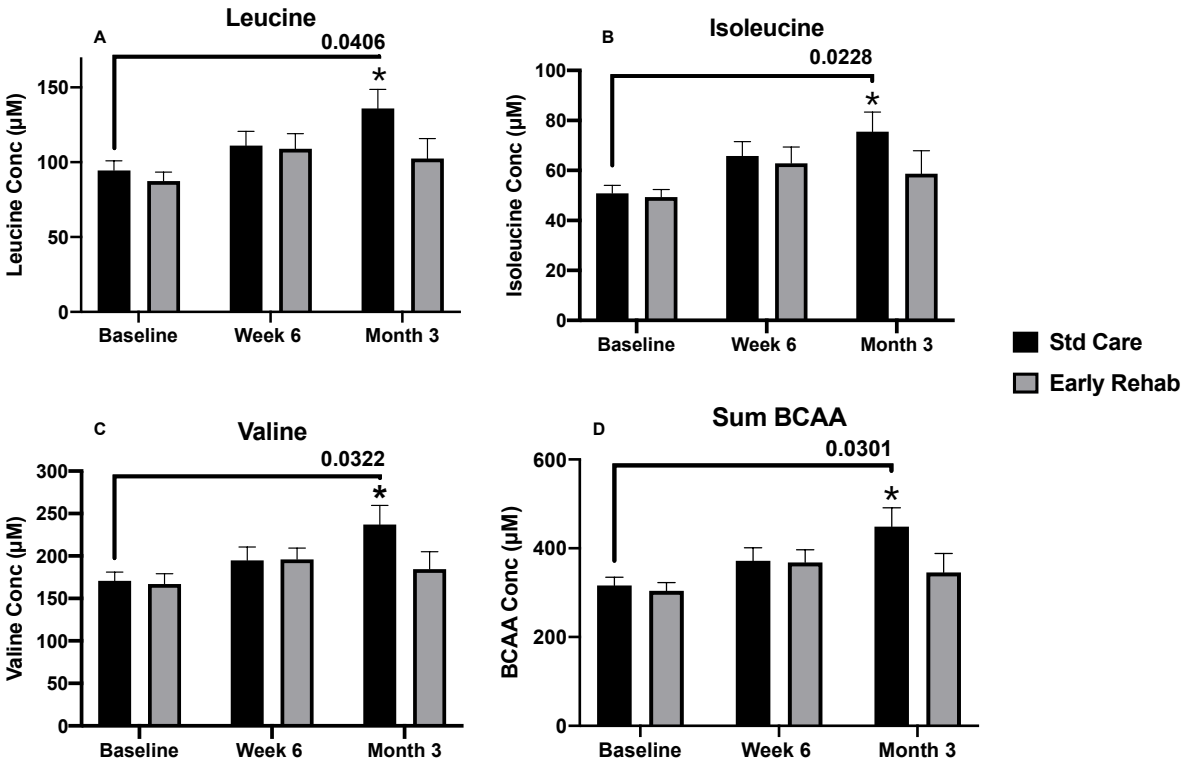


FIGURE 24. BRANCHED CHAIN AMINO ACID RESPONSES TO STANDARD CARE AND EARLY REHABILITATION INTERVENTIONS, *N* = 10 EACH.DATA ANALYSED BY TWO-WAY ANOVA WITH TUKEY'S POST-HOC ANALYSIS.

6.4.4. Associations of plasma BCAA to lung function or quadricep size, function or strength

Next, we aimed to determine associations between plasma BCAAs and measures of quadriceps size and functionality. In the usual care group, no significant associations existed either at baseline, or at 3 months follow up (figure 25). Similarly, in the early rehabilitation group, no significant associations existed with BCAAs or strength or thickness (figure 25A & B). A significantly positive association was present at baseline (figure 26C) with BCAAs and quadriceps CSA which weakened with early rehabilitation (figure 26D). In both groups, BCAAs were not associated to measures of airflow obstruction (FEV_1). A summary of this chapter's findings is displayed in figure 27.

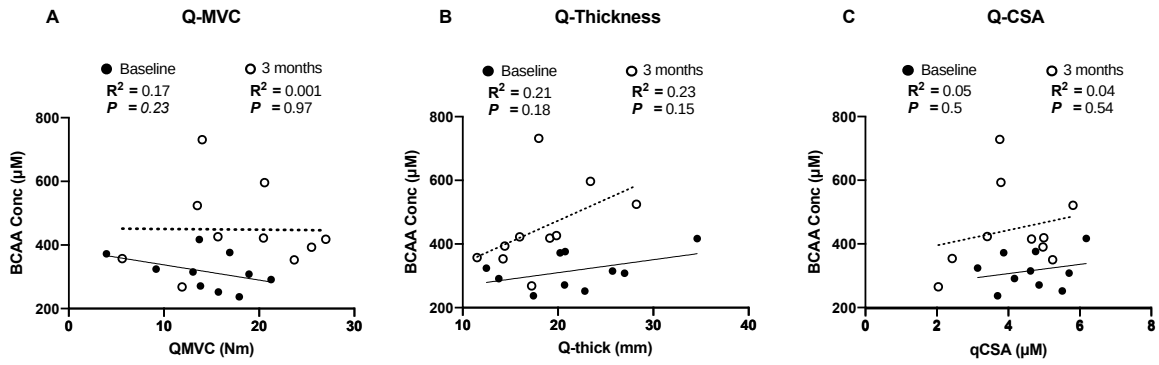


FIGURE 25. BASELINE AND 3 MONTHS FOLLOW UP OF BCAA ASSOCIATIONS WITH QMVC (25A), Q-THICKNESS (25B) AND Q-CSA (25C) IN STANDARD CARE.

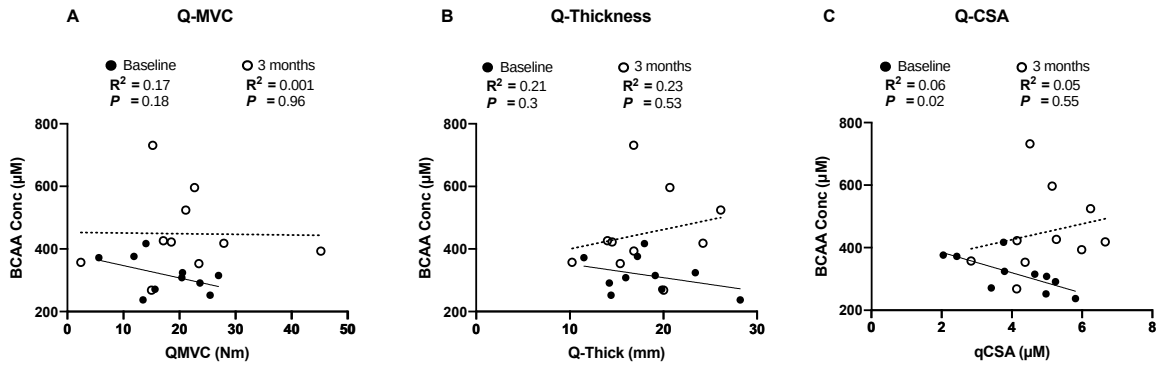
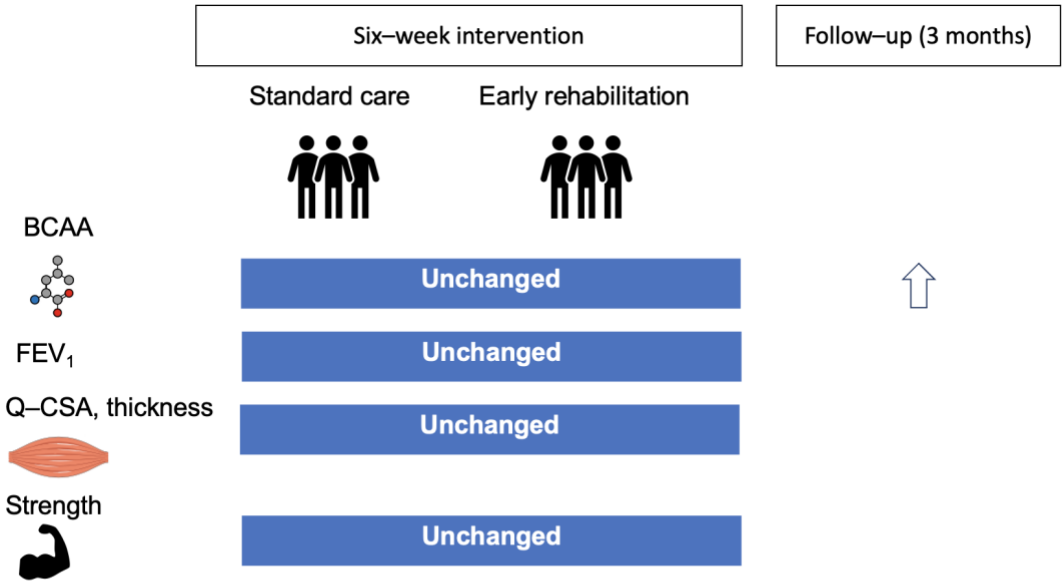


FIGURE 26. PEARSON'S CORRELATION OF EARLY REHABILITATION GROUP BASELINE AND 3 MONTHS FOLLOW UP OF BCAA ASSOCIATIONS WITH QMVC (26A), Q-THICKNESS (26B) AND Q-CSA (26C).



- Six weeks of early rehabilitation or standard care was not sufficient to elicit changes in muscle size or function
- An increase in Q-CSA in the early rehabilitation group was observed at 3 months follow-up
- Patients with an exacerbation of COPD display reduced BCAA compared to stable COPD
- Plasma BCAA's increase to the range of normal values by 3-month follow-up in the standard care group only

FIGURE 27. SUMMARY OF THE FINDINGS FROM THIS STUDY

6.5. Discussion

The main aims of this study were first to determine whether plasma BCAAs of exacerbated COPD patients are significantly decreased at baseline compared those with stable state COPD, and secondly whether 6 weeks of an early intervention programme would result in 'normalisation' of BCAAs measured at 3-months follow-up. This study suggests that a 6 weeks of usual standard care programme may be more effective in restoring BCAAs closer to normal values in those who have been admitted to hospital with an exacerbation of COPD, compared to those partaking in a structured early rehabilitation programme.

Interestingly, the baseline BCAA concentrations in exacerbated COPD patients (310 µM) are significantly lower than BCAA concentrations of those with stable COPD (381 µM). Since involuntary weight loss and associated muscle wasting is a common extrapulmonary feature of COPD (Morrison et al. 1988), especially in severe stage, it is possible these differences are attributed to increased loss of muscle in severe COPD, particularly for leucine (Hofford et al. 1990), which could be due to increased proteolysis related to hyperaminoacidemia (Castellino et al. 1987). Firstly, because the instances of hospitalisation from an episode of exacerbation results in periods of reduced mobility and functionality (Brown, Friedkin, and Inouye 2004) a known contributor of muscle wasting (i.e., bedrest; Atherton et al., 2016; Dirks et al., 2016). Further, selective wasting of fat free mass commonly seen COPD (Engelen et al. 1999), combined with and increased mobilisation of AA as substrates (Brosnan and Brosnan 2006) suggests an imbalance caused by inadequate dietary intake, inactivity or both. In support, lower levels of BCAA, particularly leucine have consistently shown to be lower in COPD patients (Morrison et al. 1988; Hofford et al. 1990; Kutsuzawa et al. 2009), the rate of appearance of which has been shown to be significantly lower

compared to other AA (e.g., phe) suggesting a disproportion of release from body proteins to the plasma contrary to the behaviour of EAA's under normal conditions. In support, arteriovenous balance studies demonstrate a fall in muscle protein synthesis in individuals with severe emphysema (Morrison et al. 1988) and higher post-absorptive muscle protein turnover rate in stable COPD patients (M. P. K. J. Engelen, Deutz, et al. 2000) compared to age-matched controls.

Although plasma BCAAs have shown considerable potential to be able to discriminate between disease severity (e.g., decreased BCAA and increased 3-methylhistidine, glutamine and arginine in GOLD stage IV compared to control; Ubhi, Riley, et al. 2012), temporal data (sampling over a prolonged period or with disease progression) are lacking, therefore it is difficult to draw unequivocal conclusions from 'snapshot' measurements. Further, COPD is a disease with several systemic manifestations at varying stages of severity (e.g., severity of airflow limitation, malnutrition), and it is not known whether plasma BCAAs track accordingly.

The lack of change in in quadriceps function and strength in COPD patients undertaking a structured RE regime is a concern and may explain why no associations were observed with plasma BCAAs, unlike previous associations between RET and BCAA (chapter three), wherein fasting plasma BCAAs appear to track with strength and mass improvements, following exercise interventions. Given that the intervention was delivered during acute phase of recovery (~48 h from admission), it was not in keeping with pulmonary rehabilitation guidelines (Spruit et al. 2013), i.e., intensity could have adjusted to be lower to ensure tolerance and completion. This is reflected in the bedside measures of quadriceps, whereby no significant difference was observed in quadriceps strength, or thickness in either group. Previous studies have demonstrated that assessment of skeletal muscle function is an important prognostic

measure to predict mortality, independently from the severity of the lung function impairment (Decramer et al. 1997; Swallow et al. 2007), for example patients with reduced quadriceps muscle size (CSA) at time of admission are more likely for unscheduled readmission to hospital over the subsequence twelve months (Greening et al. 2015). Troosters and co-workers (2010) provide promising data in delivery of resistance training intervention, whereby daily knee extension exercises at 70 % max combined by usual care resulted in significant improvements in 6-minute walk test (6MWT) and quadriceps force (supported by upregulation of anabolic markers) and therefore may offer protection against the loss of skeletal muscle function associated with exacerbations. They also found that muscle force remained higher at 1 month follow up, compared to the control group, with no adverse effects on readmission, therefore it is plausible to suggest altered behavioural patterns with regards to physical activity, since muscle force and 6MWT are a marker of physical activity in COPD (Pitta et al. 2005). Since resistance training has a relatively low ventilatory stimulus compared to EE (Probst et al. 2006), this provides an alternative and perhaps preferred form of muscle loading during an episode of exacerbation.

Hospital admissions, and readmissions (Donaldson et al. 2002) remain a significant healthcare burden, as well as a high risk scenario for increased muscle wasting (i.e., reduced quadriceps thickness; McAuley *et al.*, 2020), the frequency of which can be reduced by pulmonary rehabilitation. Although, results of the present study suggest timing is an important factor in the delivery and efficacy of interventions. Accordingly, the results demonstrated here i.e., lack of improvements in physical function suggest some intolerance to exercise and potential harm considering the early rehabilitation group did not show any promising advantages.

In conclusion, the present study found that an early rehabilitation programme, commenced soon after hospital admission was not effective in improving markers of muscle health. Interventions of this nature would probably benefit in those who have exacerbated, once they have had sufficient time to research stable state of disease, thus the timing of the present intervention was not optimal. Standard Usual care seems more appropriate in COPD patients of this severity in the context of normalising plasma BCAAs by the 3-month follow up, however the explanations for this are not clear.

CHAPTER SEVEN: OVERVIEW AND FUTURE DIRECTIONS

Dysregulated plasma BCAAs are a common feature of ageing, metabolic, cardiac, and respiratory diseases. However, they are also indispensable in their role of muscle health and as supportive nutrition in maximising benefits of exercise, particularly resistance exercise, thus our understanding of the regulation of BCAAs in these conditions are incompletely understood. With no signs of declining, obesity and obesity-related comorbidities will remain in epidemic proportions particularly in developed countries, continuing to pose a significant socioeconomic burden. In 2020, the Sars-CoV-2 (COVID 19) and obesity pandemics collided, and soon after the outbreak, IR (Apicella et al. 2020), obesity (Simonnet et al. 2020) as well as T2DM (Cai et al. 2020) were recognised as strong independent determinants of 'severe' COVID and recovery from infection. Whilst the risk of infection from such diseases are difficult to avoid without social isolation, the onset of obesity and its host of comorbidities represent a stage whereby an intervention such as exercise or nutrition may thrive. Particularly, since paediatric (~8-fold increase in age-standardised prevalence since 1975), and adult (>30 $kg.m^2$ estimates of 10.8 % men and 14.9% women in 2014; (Bentham et al. 2017)) obesity, combined with increased life expectancy) represent significant healthcare challenges for the future (Christensen et al. 2009b; Rice and Fineman 2004; Harper 2014). Therefore, promotion of lifestyle modifications such as exercise inclusion and supportive nutrition are powerful and cost-effective measures by which to promote good health and reduce the risk of metabolic diseases such as obesity and IR.

Despite many changes in body composition throughout life, a significant feature is the loss of muscle mass and strength with ageing, displaying a gradual and progressive decline, typically from the fifth decade of life (Lexell, Taylor, and Sjostrom 1988). The mechanisms underlying this loss are multifactorial (e.g., anabolic resistance, reduced

MPS response to exercise/ nutrition; Cuthbertson *et al.*, 2005; Kumar *et al.*, 2009; Atherton and Smith, 2012), consequences of which include reduced independence and greater risk of falls (Janssen 2006; Janssen and Ross 2005), resulting in a reduced quality of life. Although we will all experience muscle loss with age, the rate of loss is a potentially modifiable factor. As such, the most effective strategy to counterbalance this decline in exercise and supportive nutrition (Fiatarone et al. 1994). Accordingly, the present thesis supports the prescription of exercise in conditions of normal health, obesity and in COPD, albeit with a lesser response compared to those free of disease.

The aims of the present thesis were to probe the longstanding association of BCAAs with IS under various conditions, including their regulation in individuals within a healthy BMI range during a longitudinal RET programme, in obese individuals. Further, the regulation of BCAAs and their association to variables of lung and muscle health were evaluated in those with stable COPD, and acutely exacerbated COPD patients. The major conclusions of this thesis were that a relationship between plasma BCAAs and IS across age did not exist, either at baseline or following RET in healthy individuals. Further, no relationship existed with IS at baseline or following a longitudinal RET programme, despite improvements in IS. Interestingly however, a novel positive association with plasma BCAAs, lean mass and strength was described. Further, the relationship between IS and plasma BCAAs was not present even in our obese participants, nor did any association appear throughout the six-week intervention period despite significant reductions in BMI and improvements in IS. Although, consistent with other studies, elevated BCAAs are indeed present in obese individuals. In the presence of COPD, the response to eight weeks endurance exercise is blunted compared to individuals without COPD, and this was reflected by the

response in plasma BCAAs. For example, BCAAs did not change in COPD participants, however, increases in BCAAs were observed in healthy controls, which positively correlated to the increase in strength as a result of the endurance exercise intervention. During episodes of acute exacerbation, patients were shown to present with depleted baseline BCAAs when compared to those with 'stable' COPD, and an early rehabilitation programme was not sufficient to normalise BCAAs either during the six-week intervention or indeed in the three-month follow-up period. In contrast, standard, routine care was shown to increase plasma BCAAs to within normal range over the same duration, although it is not clear from the present study whether the standard care was responsible for the normalisation of plasma BCAAs. This suggests timing of interventions in severely sick individuals is an important consideration for future studies since the benefits of the exercise are not maximised if they are unable to efficiently perform. Future studies in patients with acute sickness may benefit strategical timing of interventions, such as commencement when disease is more stable. The present thesis suggests that evaluation of plasma BCAAs as markers of disease or in tracking with variables to measure efficacy of an intervention (such as RET) are context dependent. For example, in individuals with IR and obesity, BCAAs are known to be elevated, whereas in those who have experienced an exacerbation of COPD display significantly reduced BCAAs compared to those with more stable COPD. Therefore, the decrease in plasma BCAAs seen with severely sick COPD patients likely point to altered protein metabolism, which is typically associated with low FFM (Engelen et al. 2000; Yoneda et al. 2001)

The mechanisms underlying IR and the association with BCAA have been recently revisited, and their role in diseases beyond obesity and T2DM are also well-documented. However, how elevations in IR associate to elevations in BCAAs, and

whether elevated circulating BCAAs are a contributing cause, or a secondary effect of IR progression remains unclear. Despite providing useful insight into the role of plasma and muscle BCAAs in under distinct circumstances of health, the present thesis is not without limitations, particularly the lack of direct quantification of BCAA kinetics in the studied conditions. Although the retrospective nature of the studies (besides VLCD), and original primary endpoints limited this possibility. In the case of results presented in chapter 3 (RET ageing study), only samples with sufficient volume of plasma were used for the analysis, which perhaps creates an unintentional bias in that it is not representative of the entire cohort recruited to the original study. Additionally, the baseline comparisons of plasma BCAAs between healthy and obese individuals made in chapter 4 (VLCD study) would ideally be from samples collected at a similar time to ensure greater control of the study with regards to sample collection, storage, and freeze-thaw cycles, providing a more robust analysis. Further limitations are sample size, particularly in the stable COPD study (chapter five), where the association between strength and increased BCAAs within the healthy controls were consistent with the RET ageing study (chapter three), however with a low *n,* which lacks adequate statistical power.

The association between elevated circulatory BCAAs and IR has been reinvigorated since the initial studies within the area in the 1960s and remains an area of active investigation since the role of BCAA regulation in conditions of IR are incompletely understood. Recent data point towards tissue-specific suppression of catabolites, both in adipose and hepatic tissues, suggesting that a ‘snapshot’ of global BCAA changes in plasma does not necessarily reveal underlying mechanisms. For example, in adipose tissue of obese individuals, enzymes involved in the catabolic pathway of BCAAs are suppressed at the transcriptional level (She et al. 2007; Herman et al.

2010) a mechanism which does not seem active within the other BCAA catabolising sites such as liver, whereby instead hepatic BCAA catabolism is suppressed by increased inhibitory phosphorylation of BCKDH (She et al. 2007). Interestingly, these two mechanisms do not appear consistent within skeletal muscle, where BCKDH activity is in fact increased obese compared to lean rats (White et al. 2016). Rodent studies have also revealed a role for BCAA catabolic intermediates, such as 3-HIB, into the contribution of IR via excess accumulation of lipid species within skeletal muscle (Jang et al. 2016; Nilsen et al. 2020), suggesting the critical role of both BCAAs and BCAA–related metabolites in maintaining BCAA homeostasis. Further, genetic links between BCAA catabolic pathways and IR (BCKDHA and protein phosphatase 2Cm; PP2Cm) have also been described in the context of dysregulated glucose metabolism (Tiffin et al. 2006; Taneera et al. 2012; Xu et al. 2013; Goni et al. 2017), as well as a genetic Mendelian randomisation study that identified the PPM1K gene which associated with increased BCAA and incidences of diabetes, suggesting increased BCAA levels lead to disease prediction (Lotta et al. 2016). These studies demonstrate that additional upstream targets, in addition to plasma information should be considered for future studies investigating the complex relationship between BCAAs and IR.

Determination of the mechanisms underlying the pathophysiology of obesity and IR are therefore crucial in the potential use of BCAAs as robust biomarkers diagnosis and prognosis. Other biomarkers such as C–reactive protein, gamma-glutamyl transpeptidase or adiponectin are established as risk factors in the development of IR and T2DM (Lee et al. 2009; Pradhan et al. 2001; Li et al. 2009; Fraser et al. 2009; Sattar et al. 2008). The heterogeneity of obesity, IR and T2DM and the lack of determining the exact mechanisms contributing to these states, and how they

associate with plasma levels of BCAAs are likely limiting universal adoption of elevated BCAAs as clinical biomarkers. Studies are needed which aim to determine the magnitude of association between BCAAs and IR/ obesity, and this should include factors such as age, sex, ethnicity, and family history of such diseases and whether BCAAs as biomarkers remain predictive when all factors are combined for consideration. Early identification via potential biomarkers represents an opportunity for the introduction of preventative interventions (such as exercise) to halt or delay disease onset (Abbasi et al. 2012; Knowler et al. 2002) or to inform development of new therapeutic drug targets for preventative or therapeutic interventions (Atkinson et al. 2001). Nonetheless, in addition to the reflection of IS, BCAAs have also shown promise in feedback of drug effects, an important criterion when proposing biomarkers. In a randomized, double-blinded control study (Irving et al. 2015), patients using insulin sensitiser therapy displayed improved IS and reduced metabolites including BCAA, compared to placebo treatment over three months. Further, Walford et al (Walford et al. 2013) demonstrated that administration of glipizide and metformin (drug therapies for T2DM) can influence acute response of BCAA: aromatic AA ratio, with the magnitude of change dependent on the IR state of participants. These demonstrate utility of elevated plasma BCAAs as promising candidates for monitoring the early response of therapeutic interventions. Thus, elevations in plasma BCAAs and associated metabolites (Newgard et al. 2009; Felig, Marliss, and Cahill 1969a) are noteworthy observations in individuals with IR, since unbiased metabolomics studies have shown these increases can be detected in individuals over a decade before development of T2DM (Wang et al. 2011) which other longitudinal follow–up studies have supported (Liu et al. 2017). Future work is needed to elucidate the role of BCAA clearance and how BCAA intermediates contribute to the progression of IR

and obesity in humans. Strategies aimed at manipulating BCAAs and trace of their kinetics in conditions of IR or obesity are lacking, and the use of stable isotopes in elucidating the relative contribution of each tissue is useful. Indeed, *in vivo* tracing of BCAA–kinetics using stable isotopes demonstrates that specific rates of BCAA oxidation occur in most tissues, including muscle, brown fat, pancreas, and the heart (Neinast et al. 2019), which all likely have a role to play in resulting changes of circulatory BCAAs, suggesting future studies investigating the role of BCAAs and IS require analysis of several tissues. These additional insights may identify new targets to increase our understanding of mechanisms leading to IR and contribute to this burgeoning field.

COVID Impact statement

The outbreak of the COVID–19 pandemic posed unforeseeable impacts on the research activity of many PhD students including myself. Since much of my work was retrospective, bench-side analyses and conducted on samples already collected, the impact of lockdown on the university closures ruled out the prospect of any further experiments taking place. As discussed above, the area of BCAA-related catabolic products (such as keto and hydroxy-acids) have shown promise in elucidating the mechanisms contributing to insulin resistance in obese individuals. Analytical method development for such experiments were underway when the nationwide lockdown was imposed, for example standards for analysis of branched-chain hydroxy-acids had been bought, individually weighed out and run through GC–MS for peak identification (mass-to-charge ratio) and retention time adjustment. Further, established methods within our lab to quantify branched-chain keto-acids (such as KIC) were practised prior to analytical run on VLCD plasma samples, however the lockdown halted the progression of this experiment. Further, despite quantification of BCAAs in the plasma of those who had experienced an episode of exacerbation of COPD, untargeted/ unbiased metabolomics experiments were agreed with supervisors to be performed on these precious samples for hypothesis generation and global comprehensive metabolite analysis, studies of this nature are lacking and would have proved highly valuable for inclusion into the present thesis. Data of which would be novel, resulting in contention for an additional first-author publication resulting from this thesis. In addition, the mental health challenges posed by the pandemic in general impacted on my focus during writing (working from home was particularly challenging in the initial stages) and preparation for the viva examination as it was not how I envisaged the conclusion of my doctoral studies or time at the University of Nottingham.

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