Amino acid modulation of gut hormone release and defining roles of GLP-1 and insulin in muscle microcirculation and metabolism in older age

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Thesis Abstract

Background: Skeletal muscle's role as an organ extends beyond its classical function of locomotion. It represents an important metabolic repository for both glucose and amino acids. Its responses to nutrition, ageing and hormonal intervention continue to be an area of renewed debate. In addition, the ageing process is known for its multisystemic impact on human physiology. Little is known though on its effect on gastrointestinal gut hormone release in response to amino acids stimuli.

Aims: To investigate whether essential amino acids are capable of eliciting an "incretin effect" and assess whether the ageing process does impact EAA stimulated gut incretin release; to define the role of glucagon like peptide -1 on skeletal muscle microcirculatory parameters and glucose uptake under different concentrations of insulin; to define role of glucagon like peptide - 1 role on skeletal muscle protein metabolism under different insulin concentrations and to assess the role of insulin in regulation of human skeletal muscle protein metabolism.

Design: 40 volunteers divided in 5 groups were recruited to take part. 8 young men (20-40 yrs) were given 15 g of oral essential amino acids beverage and were compared to two other groups of 9 young men (20-40 yrs) who had 15g infusion of EAA and 8 older men (56-75 yrs) who had the same oral beverage. Further 2 groups of 7 and 8 older men (aged 65-75) underwent hyperglycaemic postabsorptive and postprandial insulin clamps respectively with and without glucagon like peptide – 1 (GLP-1).

Results: Essential amino acid delivered an approximate 45% incretin effect on calculating insulin response in the first 60 min following feeding. Glucose-dependent insulinotropic polypeptide levels increased following oral intake (399 $pmol.L^{-1}$ vs 205 $pmol.L^{-1}$, $p<0.05$) coinciding with insulin elevations.

At postprandial insulin concentration, GLP-1 increased acute myofibrillar protein synthesis, expressed as fractional synthesis rate in the fed state (mean 0.063 ± 0.004 with GLP-1 vs 0.102 ± 0.005 %.h.$^{-1}$ without GLP-1, p < 0.001). GLP-1 infusion produced a significant increase in microvascular blood flow under postabsorptive and postprandial insulin levels (p < 0.05) with an increase in microvascular blood volume only under postabsorptive insulin levels (p < 0.05). On an assessment of the role of insulin on human skeletal muscle protein turnover, insulin significantly reduced MPB (WMD −15.46 [95% CI −19.74, −11.18], p<0.001) without effect on muscle protein synthesis (WMD 3.90 [95% CI −0.74, 8.55], p=0.71).

Conclusion: Age related decline in muscle mass and strength is related to blunting of microvascular and intracellular responses to hormonal and nutritional stimuli. Essential amino acids are able to deliver an "incretin effect" that is not affected by the process of physiological ageing. Glucagon like peptide -1 could potentially improve muscle microcirculation and anabolic response to nutrition in older age but not glucose uptake. Insulin effect on human skeletal muscle metabolism is more towards inhibition of muscle protein breakdown and facilitation of muscle protein synthesis.

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Abbreviations

4E-BP1	4E-Binding protein 1
AA	Amino acids
AI	Acoustic intensity
ADP	Adenosine di-phosphate
Akt	Protein kinase B
AMPK	Adenosine monophosphate (AMP)-activated protein kinase
ATP	Adenosine tri-phosphate
BCAA	Branched-chain amino acids
cAMP	Cyclic adenosine mono-phosphate
CEUS	Contrast-enhanced ultrasound
CHO	Carbohydrates
CCK	Cholecystokinin
DPP-4	Dipeptidyl peptidase 4
EAA	Essential amino acids
EHC	Euglycaemic hyperinsulinaemic clamp
eIF4E	Eukaryotic initiation factor 4E
eNOS	Endothelial nitric oxide synthase
EPAC2	Exchange protein activated by cAMP2
FFA	Free fatty acids
FGF	Fibroblast growth factor
FSR	Fractional synthesis rate
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT-2	Glucose transporter-2
GLUT-4	Glucose transporter-4

IGF-1	Insulin growth factor-1
IMGD	Insulin-mediated glucose disposal
K_{ATP}	ATP (adenosine tri-phosphate) sensitive potassium channels
HGP	Hepatic glucose production
IRS	Insulin receptor substrate
LAT 1	L-type AA transporter 1
MAFbx	Muscle atrophy F-box
MAPK	Mitogen-activated protein kinase
MPS	Muscle protein synthesis
MPB	Muscle protein breakdown
mRNA	Messenger ribonucleic acid
mTORC	Mammalian target for rapamycin complex
MBF	Microvascular blood flow
MBV	Microvascular blood volume
MFV	Microvascular flow velocity
MVU	Microvascular unit
MuRF-1	Muscle ring finger-1
NEAA	Non-essential amino acids
NFκB	Nuclear factor κB
NO	Nitric oxide
PC-1/3	Pro-hormone convertase 1/3
PKA	Protein kinase A
PKC	Protein kinase C
PI3-K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PRO	Proteins
RET	Resistance exercise training

RHEB	Ras homologue enriched in brain
ROI	Region of interest
ROS	Reactive oxygen species
SGLT	Sodium-glucose transporter
SNAT 2	Sodium-coupled neural AA transporter 2
TNF	Tumour necrosis factor
TSC	Tuberous sclerosis complex

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Chapter 1: Introduction and review of the literature: Functional aspects of skeletal muscle glucose and protein metabolism and skeletal muscle responses to insulin, GLP-1 and advancing age

1.1 Chapter overview - Introduction

Skeletal muscle represents a crucial metabolic milieu for glucose disposal and protein turnover. The mode via which these functions are regulated is multi-level in its nature. This involves processes that start following the absorption of nutrients and extends to nutrient-induced vascular responses, crossing the vascular endothelial layer to skeletal muscle tissue, receptor-substrate interaction and intracellular signalling to stimulate metabolic function. Functional decline in these processes closely follows advancing age.

Nutrients, hormones and exercise are the principal metabolic stimulants for skeletal muscle. However its sensitivity to these stimulants becomes less evident with ageing and metabolic resistance becomes a signature as age advances.

The main objectives of this chapter are to review the current body of knowledge with regard to the processes regulating skeletal muscle metabolic functions in health, sarcopenia and its risks and effects on morbidity and mortality. Current evidence on hormonal regulation of skeletal muscle glucose and protein metabolism and the impact of advancing age will also be presented, as well as a review of novel hormonal interventions will also be presented.

1.2 An overview of skeletal muscle function

The primary function of skeletal muscle is to provide the necessary contractile structure that maintains posture and perform the task of locomotion. In addition, it has a vital metabolic role in handling all three macromolecules (Carbohydrates (CHO), proteins (PRO) and lipids). Under experimental conditions, skeletal muscle is responsible for up to 85% of insulin mediated glucose disposal (IMGD) [1]. Given the fact that bone tissue is metabolically inactive when it comes to macromolecular substrate handling, whole body peripheral glucose disposal is in fact a reflection of skeletal muscle glucose uptake [2,3]. Consequently, impaired metabolic functions at the level of skeletal muscle contribute significantly to whole body glycaemia in individuals with diabetes. Besides glucose utilization, skeletal muscle stores glycogen and provides carbon in the form of alanine and glutamine as a substrate for gluconeogenesis [4].

Proteins constitute approximately 15% of body weight [5]. They are the primary macro-molecular constituents of skeletal muscle both structurally and functionally. Skeletal muscle, in turn, contains approximately 30–45% of total body protein and contributes to 20–35% of whole-body protein turnover [6]. Amino acids (AA) and insulin have been shown to play crucial roles in regulating diurnal changes in skeletal muscle protein turnover [6]. Therefore, both tissue size and functionality are primarily dictated by the fine balance between anabolism (in the fed state) and catabolism (in the postabsorptive state). Imbalances between the rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) have important consequences for muscle size, quality and function [7].

Hypertrophy occurs when synthesis exceeds breakdown. Conversely, muscle wasting results from decreased synthesis, excess breakdown or a combination

of the two.

Skeletal muscle cells also contain intramyocellular lipids. Through free fatty acids (FFA) oxidation, which takes place in mitochondria, fuels for exercise are provided. This makes skeletal muscle the most dominant single organ that contributes to energy expenditure [8,9].

As with most body organs, skeletal muscle is subject to age-related decline in mass and function. Muscle mass decline reaches 1% per year after the age of 60 [10]. Indeed, in older individuals over the age of 65, this decline is a strong predictor of morbidity and mortality [11].

1.2.1 Skeletal muscle and protein metabolism

Skeletal muscle mass is maintained through continuous processes of synthesis and degradation. Net protein balance is the difference between MPS and MPB. The anabolic effects of nutrition are driven by the uptake and incorporation of AA captured from dietary protein sources into skeletal muscle proteins. Further, this anabolic effect is entirely regulated by essential amino acids (EAA) and hormones, predominantly insulin. This balance of gain during the fed state and loss during the fasted state is required to ensure the stability of lean mass in healthy mobile individuals.

This continuous physiological process is tightly regulated at the cellular level where changes in sensing, signalling and transcription could have vast clinical consequences. MPS is positively impacted by both aerobic and resistive exercise and nutrition. On the other hand, when rates of proteolysis exceed MPS rates, such as in the disuse or denervation of skeletal muscle, atrophy ensues. Hence, the development of successful interventions that would attenuate skeletal muscle loss and wasting and improve structure and function is highly reliant on understanding intracellular pathways that regulate MPS and

MPB. The two processes are not simply the reverse of each other, though there are various levels of cross talk between them.

1.2.2 Molecular pathways signalling MPS

Protein synthesis is a complex and highly regulated and integrated process that involves interaction between various stimulating and inhibiting intracellular molecules. The ultimate goal of these interactions is mRNA translation initiation and elongation. Insulin and nutrients (particularly AA leucine) regulate protein synthesis by mediating messenger ribonucleic acid (mRNA) translation. Insulin mediates the activation of the insulin-like growth factor-1 (IGF-1) receptor /phosphatidylinositol 3-kinase (PI3-K)/ protein kinase B (Akt) pathway [12] which results in the phosphorylation of the mammalian target of rapamycin complex (mTORC). mTORC exists in two forms, mTORC1 and mTORC2. mTORC1 phosphorylation and downstream signaling involve multiple molecules leading directly or indirectly to MPS. See figure 1-1.

While mTORC1 remains the central molecule, the activation or the inhibition of which controls MPS, other pathways are also involved such as the mitogen-activated protein kinase (MAPK) signalling pathway [13]. mTORC1 stimulation is an energy depleting process and therefore MPS is down-regulated in the postabsorptive state, periods of prolonged fasting and situations of sustained energy deficit [14]. On the other hand, combining the anabolic effect of exogenous AA acid intake and repetitive mechanical strain, either through resistive or endurance type exercise, potently stimulates mTORC1 signalling [15]. Therefore, mTORC1 leads to MPS through four different stimuli and pathways: 1) insulin and IGF-1 through the IRS/ PI3-K pathway; 2) AA through PI3-K; 3) energy level through the AMPK (AMP-activated protein kinase) pathway, and; 4) resistance exercise through the phospholipase D pathway [16].

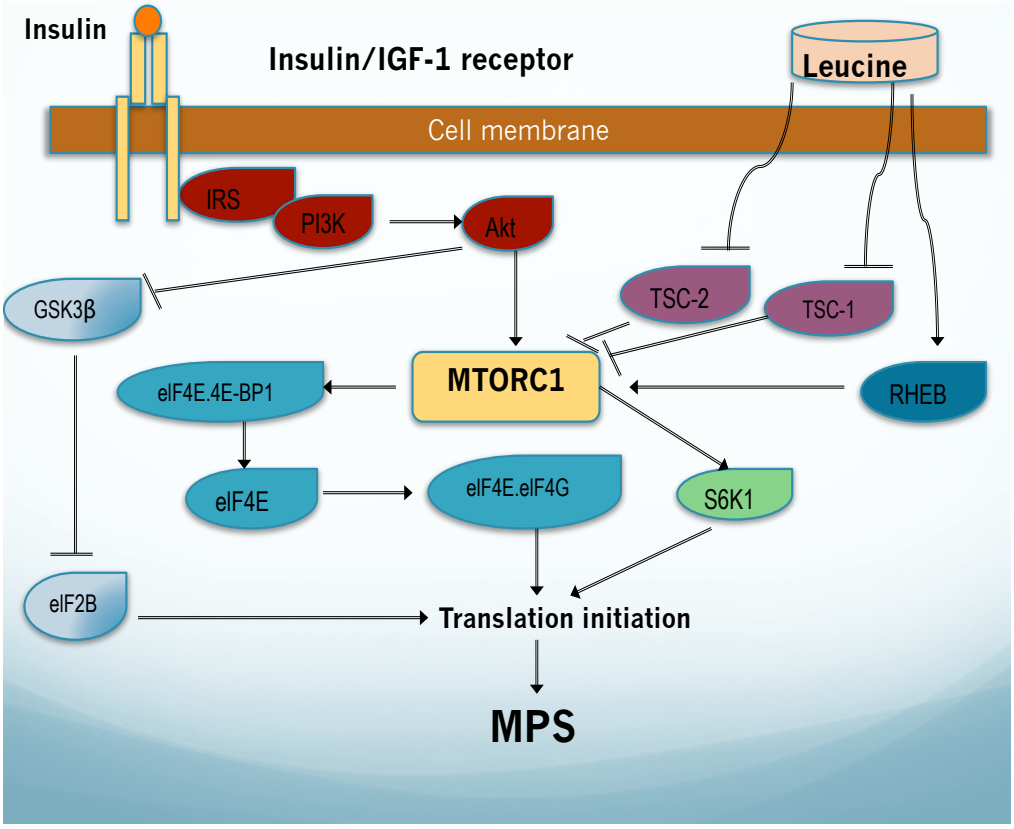


Figure 1-1: Molecular pathways signalling muscle protein synthesis (MPS).

Insulin and leucine drive MPS via different mechanisms involving mammalian target of rapamycin complex 1 (mTORC1). Phosphorylation of mTORC1 stimulates translation initiation by phosphorylating 4E-binding protein 1 (4E-BP1), leading to the dissociation of eukaryotic initiation factor 4E (eIF4E) from the inhibitory eIF4E ·4E-BP1 complex and the formation of the translation-promoting eIF4E ·eIF4G complex. In addition, mTORC1 also stimulates translation via activation of the serine-threonine kinase 1 (S6K1). It has been proposed that leucine stimulates mTORC1 via inhibition of tuberous sclerosis complex 1 and 2 (TSC1 and TSC2) or via activation of the Ras homologue enriched in brain (RHEB) protein. Therefore the two pathways for the two stimulants (insulin/ leucine) converge at the level of mTORC1. Protein kinase B (Akt) also stimulates protein

synthesis via the mTORC-independent mechanism. Akt mediates phosphorylation of glycogen synthase kinase 3 beta (GSK3β) to inhibit its activity, which is to block protein translation initiated by the eukaryotic initiation factor 2 B (eIF2B) protein. IRS: insulin receptor substrate, PI3-K: phosphatidylinositol 3 kinase. Figure 1-1 was developed based on a number of references [17–20].

Muscle satellite cells have a central role in the process of MPS and muscle hypertrophy, especially in terms of repair and regeneration. The activation of these "muscle stem cells" occurs in response to the same stimuli as those of mTORC1 such as muscle damage as a result of exercise, increased circulation of IGF-1, fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) [21].

There is an evolving understanding of the role of amino acid transporters in the regulation of muscle protein metabolism that continues to be exploited. The two main identified transporters are sodium-coupled neural AA transporter 2 (SNAT2) [22] and L-type AA transporter 1 (LAT1) [23]. These molecules are vital in facilitating the transport of AA inside the cell from extracellular space in response to AA availability. The internalisation of AA activates intracellular AA sensing components lucyle-tRNA synthase and vacuolar H^+ ATPase, which are responsible for mediating AA-induced localisation of mTORC1 to the lysosomal surface. In addition to its role in facilitating AA transport intracellularly, SNAT2 seems to play a dual role as a transporter and receptor for AA [24]. Its binding to AA on the cell surface during the process of internalisation triggers a signalling cascade of reactions that ultimately stimulates mTORC1 and therefore acts as a sensor that monitors extracellular AA availability as well as an advanced system that primes the cell for subsequent AA influx [25].

1.2.3 Molecular pathways signalling MPB

Proteolysis is a key process for human tissue metabolic balance. The advantages of this include the elimination of defective proteins and prevention of the deleterious effects of their accumulation. The process of protein breakdown also provides skeletal muscle with free AA, which is necessary and readily available for reuse for MPS or indeed energy production. Further, proteolysis is vital to the tissue's immune response and elimination of various pathogens [26].

The major pathway that regulates catabolic activities leading to atrophy is the ubiquitin-proteasome pathway. The activation of this system is stimulated by nuclear ligases. Other proteases including protease-3, calpains and cathepsins might also be involved [27]. In addition, the activation of the ligand receptor FAS/ tumour necrosis factor (TNF) leads to caspase-3 enzyme activation [28]. This enzyme is also activated by calpain and caspase-9, which are both stimulated via reactive oxygen species (ROS) mediated sarcoplasmic reticulum stress, leading to increased cytosolic calcium. The activation of the caspase-3 enzyme leads to actomyosin dissociation and caspase-3 dependent DNA fragmentation, and represents the initial, and possibly the rate-limiting step, in proteolytic degradation [29,30]. See figure 2-1.

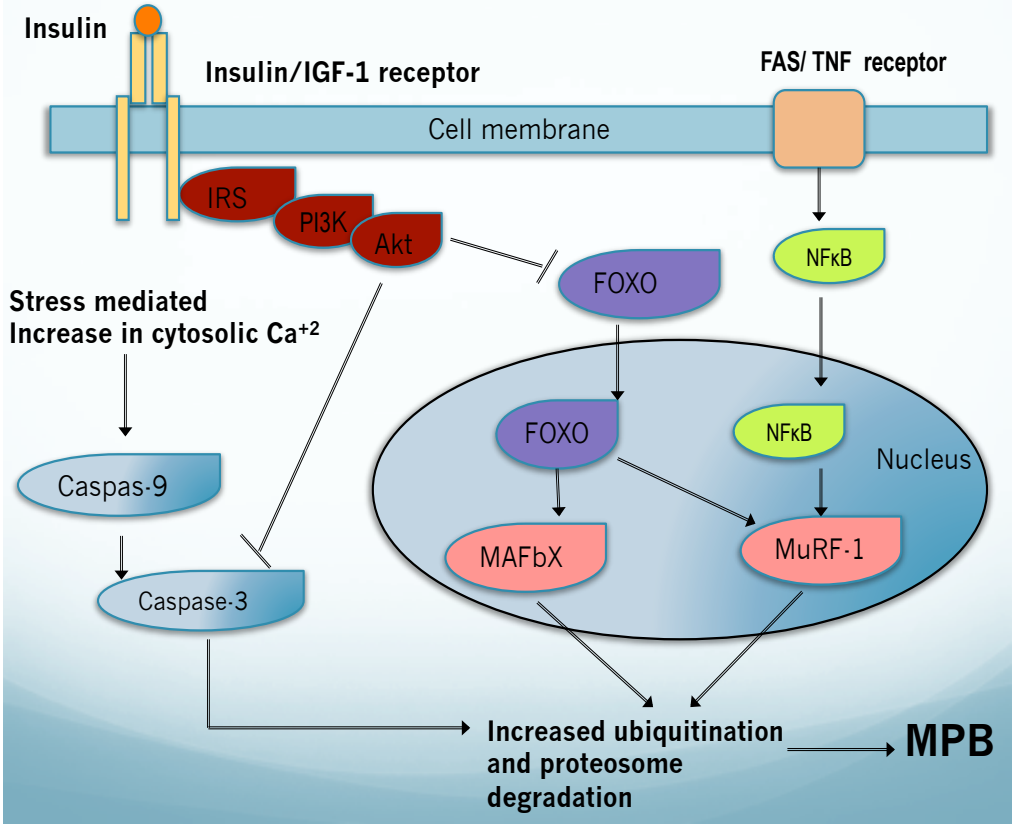


Figure 1-2: Molecular pathways signalling muscle protein breakdown (MPB).

The major ligases involved are muscle atrophy F-box (MAFbx) and muscle ring finger-1 (MuRF-1). The expression of these two ligases, in addition to ubiquitin, increases with increased rates of muscle degradation. The transcription of both MAFbx and MuRF-1 is dependent on the transcription factor FOXO with MuRF-1 further regulated by FAS/tumour necrosis factor (TNF) receptor-mediated activation of nuclear factor κB (NFκB). In addition, stress mediated rises in cytosolic calcium increase ubiquitination activities through caspases. Both FOXO and caspase-3 are negatively regulated by the PI3-K/ Akt pathway, giving it an indirect way of enhancing muscle anabolism. PI3-K: phosphatidylinositol 3-kinase, Akt: protein kinase B. Figure 1-2 was developed based on several references [17,28–31] .

The proteolytic process is largely dependent on the release of myofilaments from actin and myosin. This is because actomyosin complexes represent 50-70 % of skeletal muscle proteins. However, the main protein degradation system

(the ubiquitin-proteasome pathway) can only degrade monomers in the form of actin and myosin and is incapable of breaking down actomyosin complexes. Hence, an initial step is needed before ubiquitination takes place [29]. Once ubiquitin is covalently conjugated to the lysine residues of target proteins via sequential activities of ubiquitin-activating and conjugating enzymes and ubiquitin-protein ligases, the target protein becomes identifiable by the 26S proteasome. This latter protein acts as the degrading machinery [32].

1.3 Skeletal muscle and glucose metabolism

1.3.1 Molecular aspects of glucose regulation in skeletal muscle

The binding of insulin to its receptor in skeletal muscle leads to the phosphorylation of insulin receptor substrate (IRS), which in turn leads to the activation of PI3-K. See figure 1-3. Intracellular glucose is either converted to glycogen for storage and use during exercise or enters the glycolytic pathway for oxidation resulting in its classic role of energy production. Signalling through the PI3-K/Akt pathway is attenuated by PIP_3 (phosphatidylinositol 3,4,5- triphosphate) phosphatases, which catalyse the dephosphorylation of PIP_3 to PIP_2 and stop skeletal muscle glucose disposal [33].

The insulin receptor also regulates cell growth-related gene expression via the Ras/ MAPK pathway. The activated IRS interacts with adaptor protein Grb2 and recruits the Son-of-Sevenless (SOS) protein at the plasma membrane leading to the activation of the Ras protein. Once activated, Ras leads to a stepwise series of phosphorylation/ activation reaction cascades leading to the activation of MAPK, which in turn phosphorylates various other substrates leading to an increase in gene transcription and expression of proteins involved in cell growth and survival [34,35] (see Figure 1-3).

The intracellular regulatory mechanisms that are involved in insulin-mediated glucose uptake are complex, though the current body of knowledge suggests that glucose transport is the rate-limiting step [36]. Furthermore, under physiological hyperinsulinaemia, glucose phosphorylation by hexokinase II becomes an equally important rate-limiting step, suggesting that the regulation of insulin-mediated skeletal muscle glucose uptake is indeed a multi-level barrier system that controls glucose influx into the cell [37].

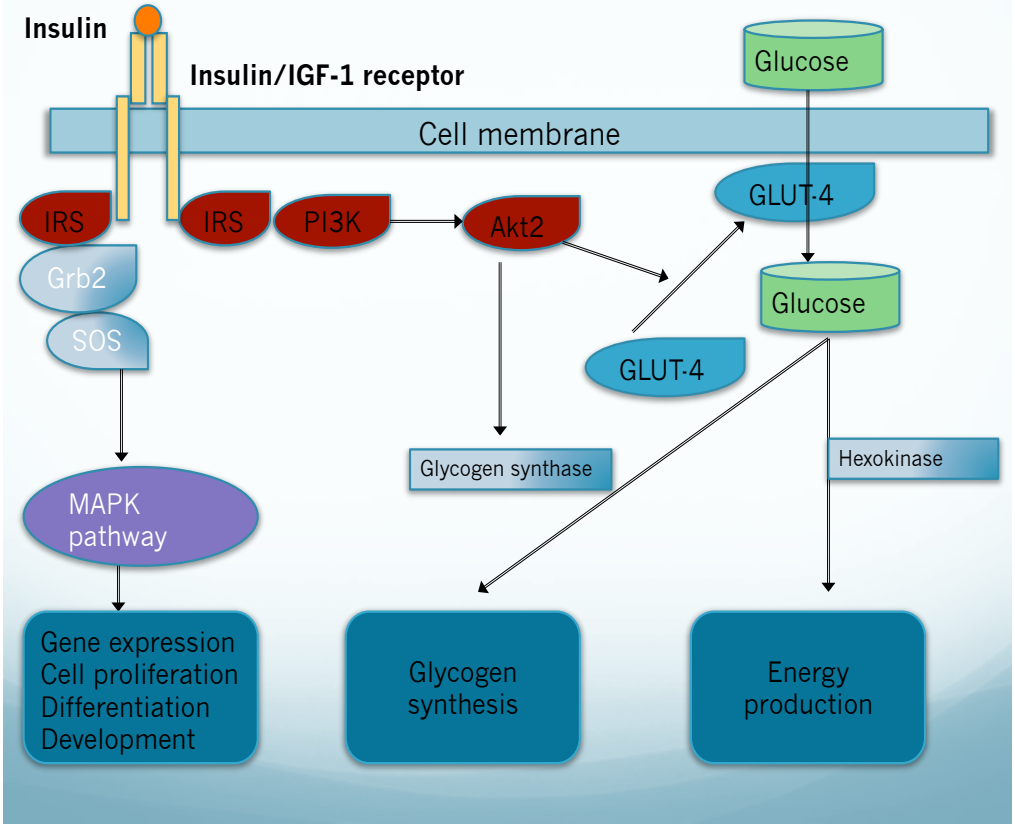


Figure 1-3: Insulin, glucose, and MAPK signalling.

Insulin-mediated activation of IRS and PI3-K leads to an increase in the level of intracellular phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 activates Akt, which promotes glucose transporter-4 (GLUT-4) translocation to the cell membrane to uptake glucose into the cell. While Glycogen synthase and hexokinase mediate energy production and storage respectively, signalling down the MAPK pathway regulates cell growth. Figure 1-3 was developed based on several references [33–35].

1.3.2 Insulin release

Rises in the concentration of plasma glucose, and to a lesser extent that of some AA and fatty acids, are sensed by pancreatic β-cells. In response, β-cells release the appropriately required amount of insulin that offsets the caloric

load. β-cells are organised and function in a distinctive way that allows rapid and continuous nutrient sensing. They are closely clustered around blood vessels such that they receive ten times more blood supply than their neighbouring exocrine cells [38]. Furthermore, β-cells do not contain membrane-bound receptors and the process of equilibrating glucose intracellularly is mediated by glucose transporter-2 (GLUT-2) facilitated diffusion. The migration of GLUT-2 to the cell membrane is not insulin dependent, unlike glucose transporter-4 (GLUT-4) migration observed in skeletal muscle and adipose tissue cells [39]. Once inside, β-cell glucose follows its normal glycolytic fate of producing adenosine tri-phosphate (ATP). The change in ATP/ adenosine di-phosphate (ADP) ratio will then lead to the opening of ATP sensitive potassium (K_{ATP}) channels, Ca^{+2} influx and exocytosis of insulin-containing granules [38]. The rate-limiting step in pancreatic β-cells' handling of glucose is phosphorylation of glucose by the enzyme glucokinase (a subtype of hexokinase only present in β-cells, hepatic cells, enterocytes and glucose-sensitive neurons). In β-cells, it is distinctive for its lower affinity to glucose and the lack of feedback inhibition by subsequent metabolites down the glycolytic pathway [38].

Insulin releases follow a distinctive biphasic pattern. Once plasma glucose reaches ~7 $mmo.L^{-1}$, an initial phase starts with the insulin release rate peaking at around 1.4 $nmol.min^{-1}$. This lasts for 10 minutes and is followed by a second phase of sustained insulin release at a rate ~0.4 $nmol.min^{-1}$ [40]. Insulin is secreted by pancreatic β-cells into the portal vein. The majority of secreted insulin gets extracted by the liver such that, at any given time, the peripheral insulin concentration accounts for one-third of total pancreatic β-cell secretion [41]. Insulin secretion in the postabsorptive state accounts for 50% of total daily insulin secretion. The rate of this varies between 0.25-1.5 $units.hour^{-1}$, enough to produce insulin levels between 5-15 $\mu u.mL^{-1}$ [41]. In the

postprandial state, insulin levels increase, stimulated by nutrient intake; mainly glucose and to lesser extent AA and lipids [42]. This process of the tight regulation of insulin concentration is subject to glucose changes between the fasted and fed state. Keeping glucose within its narrow physiological ranges in both postabsorptive and postprandial conditions is mostly a bi-hormonally regulated process. In the fed state, insulin-stimulated glucose-6-phosphatase and glycogen synthase carry out the tasks of energy production and glycogen formation, respectively. While in the fasted state, glucagon-stimulated glycogen phosphorylase generates glucose [43].

Insulin release is also subject to other hormone influences and regulations. Incretin hormones (Section 1.5) have a potent stimulatory effect on insulin secretion. Data also suggest a positive influence from oestradiol and melatonin. In contrast, growth hormone and leptin exert an inhibitory effect on insulin secretion [39].

1.3.3 Postabsorptive glucose regulation

Blood glucose levels in the postabsorptive state generally range between 3.4-6.2 $mmol.L^{-1}$. Despite this population variation, within a single individual, the range of blood glucose is strictly maintained within ±0.3 $mmol.L^{-1}$ [44]. Even in the postprandial state, blood glucose levels rarely experience excursions of more than 2 $mmol.L^{-1}$ and under normal physiological conditions rarely exceed levels beyond 7.8 $mmol.L^{-1}$ [44,45]. During postabsorptive conditions, both the liver and kidneys are the major sources of glucose production, with 70-75% of glucose disposal of non-insulin being mediated and taking place in the brain, erythrocytes and splanchnic tissues [46]. As a result, only 25-30% of glucose uptake under these circumstances is in fact insulin-mediated. It is in postprandial conditions that insulin secretion allows IMGD in insulin-sensitive tissues (i.e., muscle and adipose tissue) in order to maintain normal glucose

homeostasis. Besides promoting glucose uptake, insulin suppresses glycogenolysis and stimulates glycogenesis, suppresses gluconeogenesis in addition to stimulating FFA and glycerol synthesis and suppressing lipolysis [44].

In non-exercise conditions, the liver is the predominant source of glucose production in the postabsorptive state. Another contribution comes from the kidneys. The liver produces glucose through two processes; the first is through glycogenolysis, which normally occurs two to six hours into the postabsorptive state. The second source is through the de novo synthesis of glucose from non-glucogenic sources, namely lactate, AA and glycerol (gluconeogenesis). Gluconeogenesis is of vital importance during prolonged fasting. Its rates are controlled by a series of enzymes, whose genes are directly under the opposing influence of insulin and glucagon at the transcriptional level. In view of changing eating habits during the day, particularly in modern societies, postabsorptive hepatic glucose production (HGP) becomes very evident during night-time and is a major source of hyperglycaemia in diabetes [47,48] .

1.3.4 Skeletal muscle: the major site for postprandial glucose disposal

Euglycaemic hyperinsulinaemic clamp (EHC) is regarded as the gold standard method for investigating insulin action in peripheral tissues in vivo [49]. Under these experimental conditions, nearly 90% of IMGD takes place in the peripheral tissues rather than the splanchnic bed (i.e., the liver and gut), HGP is 50-60% suppressed [50] and less than 5% of peripheral IMGD occurs in adipose tissue, leaving skeletal muscle accounting for 80% of IMGD [51]. During postprandial conditions, insulin secretion leads to the suppression of HGP and stimulation of glucose uptake in target tissues (i.e., skeletal muscle and adipose tissue). See figure 1-4.

Splanchnic circulation is another key player that comes into effect under more physiological conditions. About 28% of oral glucose load is extracted by splanchnic bed (See figure 4-1). Skeletal muscle deals with around 60% of the glucose that escapes splanchnic extraction. If only IMGD is taken into account, then skeletal muscle disposes of up to 85% of that. Given the fact that brain uptake of glucose is constant during both the postabsorptive and postprandial conditions, glucose levels during EHC are a reflection of glucose disposal and insulin resistance within skeletal muscle. On the other hand, glucose levels during OGTT reflect both hepatic and skeletal muscle insulin resistance [1].

As human skeletal muscle represents 40-50% of lean body mass (i.e., non-fat tissue) and contributes to up to 85% of IMGD (bone tissue is metabolically inactive with regard to all major substrate metabolism), whole body peripheral glucose disposal is, in fact, a reflection of skeletal muscle glucose uptake [2,52]. Consequently, impaired metabolic functions either within or around skeletal muscle cells contribute significantly to whole body hyperglycaemia in individuals with type 2 diabetes.

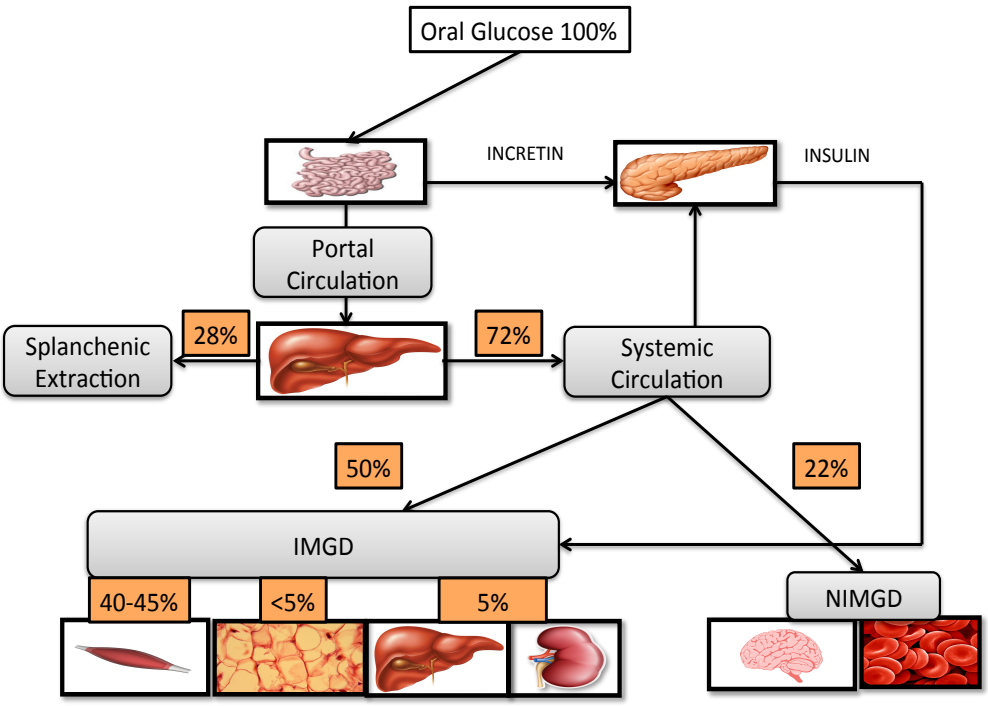


Figure 1-4: Oral glucose disposal and role of splanchnic circulation.

Following oral glucose challenge, the splanchnic bed extracts 28% of oral glucose in a non-insulin dependent manner. Around 72% will reach systemic circulation. Of this, non-insulin-mediated glucose uptake by the brain and red blood cells account for about 22%. Of the remaining glucose that escapes splanchnic extraction and reaches systemic circulation, 45% will be taken up by skeletal muscle, less than 5% by adipose tissue, and around 5% by both the liver and kidneys, all in an insulin-mediated fashion. IMGD: insulin-mediated glucose disposal, NIMGD: non-insulin mediated glucose disposal. Figure 1-4 was developed based on several references [50,51,53].

Glycogen synthesis represents the majority of non-oxidative glucose metabolism. Failure to incorporate intracellular glucose into glycogen is regarded as one of the principal steps in the development of insulin resistance. Insulin resistance is closely linked to a reduction in glycogen synthase activity. However, defects in glucose transport and hexokinase II are regarded as the

major contributors to insulin resistance [54,55]. On the other hand, the liver plays a vital role in limiting postprandial hyperglycaemia by the reduction of HGP and the enhancement of hepatic glucose uptake. Hepatic glucose uptake is maximally increased when glucose is taken orally and arrives to the liver through the portal system compared to intravenous glucose administration [56].

1.4 Vascular regulation of skeletal muscle metabolism

1.4.1 Microvascular unit within skeletal muscle and microvascular recruitment (MVR)

The functional organization of capillaries within skeletal muscle has come to be understood in terms of the microvascular unit (MVU). This represents the smallest unit of muscle volume to which blood supply is delivered. Typically, it arises from a single terminal arteriole, giving rise to 12-20 capillaries, which run along muscle fibres, i.e. parallel to it, towards collecting vessels [57]. A typical MVU will supply a muscle volume of ~0.1 mm^3 thus supplying segments of 20-30 fibres, each contacting 2-7 capillaries [58]. At rest, entire MVUs are devoid of blood due to the high tone in their terminal arteriole. Typically, two-thirds of capillaries are empty at any given time under rested and fasted conditions [59] such that resting muscle blood flow is just ~3 $mL \cdot min^{-1} \cdot 100g^{-1}$, representing only 3-5% of that in the kidneys, liver or intestine [60].

The relationship between terminal arterioles and capillaries varies but seems to control blood flow overall in MVUs. Both arterioles and capillaries respond in a coordinated fashion to pharmacological vasodilatory and vasoconstrictive stimuli. Rhythmic changes within arterioles are usually associated with changes in blood flow within corresponding capillaries. Further, in neighbouring MVUs, capillaries could run in parallel as well as overlap with each other. This allows different flows within a single MVU; its own and those from a neighbouring MVU such that up to 50% of blood flow in a single MVU at a given time could be a countercurrent flow from a neighbouring MVU. This concurrent countercurrent nature of blood flow within MVUs was suggested to permits homogeneous tissue perfusion [61].

Following nutrient intake, insulin not only mediates vasodilatation at the level of the skeletal muscle vasculature but also recruits more microvessels. Both of

these physiological functions precede the classical function of insulin to mediate glucose uptake into the cell. Insulin resistance significantly blunts MVR in skeletal muscle. This at least partially explains deregulations in skeletal muscle substrate and insulin uptake in individuals affected [62]. Therefore, MVR is key to the postprandial actions of insulin and substrates (mainly glucose and AA). Through these vital physiological capabilities of insulin, more microvasculature and hence more endothelial surface is made readily available for the delivery and trans-endothelial transport of oxygen, insulin and nutrients for postprandial metabolic processes [63,64].

1.4.2 Hormone and substrate delivery and transendothelial transport

The initial step towards nutrients and hormones exerting their anabolic actions on skeletal muscle cells is the intact delivery to the cell surface. This relies on intact blood vessels for delivery and then nutrients and hormones being able to exit capillaries and transverse into the interstitial space. These steps are reliant on adequate postprandial hyperemia triggered by vasodilatory stimuli such as insulin stimulating nitric oxide (NO) [3,65].

Blood vessels are lined with an endothelial mono-layer of cells that is unique in its ability to permit the exchange and transport of nutrients and hormones. While the entire endothelium in human blood macro-vasculature covers roughly 6 m^2, the microvascular endothelium covers about 700 m^2, 85% of which is present in capillaries. Therefore, in the skeletal muscle of a 70 Kg lean active adult, it is estimated that the microvascular endothelium would cover an area equivalent to at least 400 m^2. This wide exchange surface area is designed to meet metabolic requirements following food intake and exercise [66]. Increases in insulin concentrations following meal ingestion lead to a 50-80% increase in resting skeletal muscle MBV [67]. The resultant increase in capillary recruitment is a key postprandial event as it leads to the delivery and

redistribution of fuel into the skeletal muscle, affirming its position as a central repository for both glucose and AA.

The mode via which glucose is transported across the endothelium to the interstitial space around the cells is thought to be a facilitative, energy independent and saturable transport process via glucose transporter 1 (GLUT1) [61] with a concentration gradient across the endothelium acting as a driving force [68]. While information seems to be lacking on the mode in which AA are transported through the endothelial membrane, FAT/ CD36 is regarded as the fatty acid transporter responsible for the transport of FFA into the muscle interstitial space [69]. The transendothelial transport of insulin to the interstitium is preceded by insulin-mediated stimulation of endothelial nitric oxide synthase (eNOS), leading to the nitric oxide-mediated vasodilatation of the microvasculature. An increased concentration of NO in the endothelial cells directly promotes insulin transendothelial transport to the interstitial space. This process requires intact downstream signalling through both the PI3-K/Akt and MAPK pathways. The fact that increased activity of downstream intracellular insulin signalling enhances endothelial NO production reinforces the concept that insulin facilitates its own uptake into endothelial cells [70].

Building on these facts, the ability of the muscle to recruit greater microvasculature during postprandial conditions allows for a wider surface for hormone and substrate delivery and exchange. Hence, it is expected that endothelial dysfunction leading to changes in muscle microvascular endothelial surface area is likely to significantly impair insulin delivery and action in skeletal muscle [71]. Therefore, both delivery and trans-endothelial transport are rate-limiting factors for the metabolic actions of both substrate and insulin.

1.5 The incretins

1.5.1 General overview

Following the proposal by Bayliss and Starling in 1902 that 'secretin' was a blood-borne factor coordinating the arrival of food in the upper part of the small gut [72], Moore suggested in 1906 that intestinally originating substances are able to reduce glycosuria of diabetes [73]. The term "incretin" itself first came to life in 1932 when La Barre suggested that postprandial insulin secretion is regulated by intestinally produced substances [74]. The first scientific proof of the concept of the "incretin effect" however came later in 1964 [75,76].

The major incretins are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) [77,78]. GIP was the first incretin to be described when Brown and Dryburgh initially isolated the peptide and injected it into the blood stream of dogs with the resultant inhibition of gastric acid [79]. Later in 1973, they described its insulinotropic properties [77]. In late 1985, GLP-1 was the second and final incretin [78] to be discovered. GLP-1 and GIP are released from the L and K cells of the intestine respectively after nutrient ingestion. The initial rise in blood incretin levels following glucose uptake coincides with its arrival to the duodenum. However, L-cells seem to be also present (in even higher density compared to the duodenum) further distally in the small intestine and colon. Given the time that is needed for nutrients to arrive at distal parts of the intestine where most of the L-cells exist, the distal L-cell contribution to early rises in GLP-1 levels is unclear [80].

Healthy humans and individuals with diabetes have similar postprandial incretin concentrations, with GIP concentrations being much higher compared to those of GLP-1. However, in individuals with diabetes and impaired glucose tolerance, there is a reduced or absent incretin effect from a reduced pancreatic β-cell response to both incretins. This reduced response seems to

respond to pharmacological doses of GLP-1 but not GIP. This latter fact made incretin-based therapy in the form of GLP-1 receptor agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors (the degrading enzyme) be at the very frontier of tackling hyperglycaemia of type 2 diabetes mellitus [81,82].

1.5.2 Incretin formation and storage

The GLP-1 peptide is derived from post-translational processing of the pro-glucagon gene by pro-hormone convertase 1/3 (PC-1/3) in the intestinal L-cells. In the L-cell, pro-glucagon processing generates a 31-residue peptide, designated as GLP-1 (7-37). This peptide can then be amidated at its C-terminal glycine to give rise to a second form, GLP-1 (7-36) NH_2. In humans, the majority of the active peptide released from the L-cells is in the amidated form. In the case of GIP, PC-1/3 processing of pro-GIP within entero-endocrine K-cells results in the formation of a bioactive 42-residue peptide: GIP (1-42). In the intestinal entero-endocrine cells, both GLP-1 and GIP are stored as intact peptides within granules in L and K-cells respectively until their secretion is stimulated [83].

Evidence suggests that L and K-cells overlap in their distribution from the upper small intestine down to the distal ileum and large intestine. In the proximal small intestine at the duodenum, K-cells predominate with a small presence of L-cells. As nutrients travel distally, K-cell levels diminish gradually such that at the distal ileum and large intestine only L-cells are present [84,85].

1.5.3 Signalling pathways underlying L and K-cell nutrient sensing

All major macronutrients (CHO, PRO and lipids) have a proven capability of being able to trigger incretin secretion [86–88]. Mechanisms underlying their nutrient sensing continue to be scrutinised. Both L and K-cells are located in the epithelial layer of the gastrointestinal tract. Both have apical surfaces

opening into the gut lumen and basolateral poles containing the secretory vesicles and sit in close proximity to blood circulation [80].

1.5.3.1 Glucose sensing

Similar glucose sensing mechanisms function in both L and K-cells. The arrival of blood glucose in the gut lumen stimulates its coupled transport into the L and K-cells via the sodium-glucose transporter-1 (SGLT-1). The flux of sodium into cells generates enough membrane action potential with a subsequent rise in intracellular calcium, which in turn is regarded as the principal trigger for GLP-1 exocytosis from its basolateral cell vesicles [80]. SGLT-1 dependent L and K-cell glucose sensing is further supported by data from animal models. In global SGLT-1 knockout mice, GIP rises as a result of the glucose tolerance test were entirely negated with the resultant delivery of large amount of glucose to the distal intestine. In this same model, the arrival of glucose to the distal intestine, however, did manage to stimulate GLP-1 secretion, suggesting that alternative mechanisms do exist for L cell-sensing [89].

Other mechanisms including translocation of GLUT-2 to the apical membrane in response to elevated luminal glucose and/or closure of the K_{ATP} channels in response to increased ATP production from intracellular glucose metabolism have been suggested as possible stimulants for GLP-1 exocytosis [90]. While these latest observations were largely from the enteroendocrine cell line and perfused rat intestine, in vivo evidence is still lacking. Further, it was noted that glibenclamide (K_{ATP} inhibitor) had no effect on GLP-1 and GIP concentrations [91,92], throwing further doubt on the role of K_{ATP} in incretin hormone exocytosis.

1.5.3.2 Protein sensing

In animal experiments, both oral whey [93] and intraluminal infusion of meat hydrolysate [94] were able to stimulate incretin hormone release. Orally consumed mixed AA in humans delivered a similar effect [95]. Amongst AA, L-glutamine showed a consistent ability to stimulate GLP-1 secretion in healthy individuals and those with type 2 diabetes [96,97].

The mechanism via which entero-endocrine cells sense gut proteins leading to incretins secretion is unclear. Stimulation of the calcium-sensing receptor (CASR) and/or activation of MAPKS by meat hydrolysate have been suggested as a mechanism via which enteroendocrine cells secrete incretin hormones in response to protein load in the gut [90].

1.5.3.3 Lipid sensing

Several mechanisms that might underlie enteroendocrine cell fat sensing have been proposed, however, evidence from in vivo studies is lacking. The G-protein coupled receptor (GPCR) in the enteroendocrine cells seems to sense FFA formed from digested triglycerides. The resultant increase in cyclic adenosine monophosphate (cAMP) and intracellular calcium leads to GLP-1 release [80].

1.5.4 Incretin release and degradation

Once released into the circulation following a stimulus, N-terminal degradation by their targeting protease (DPP-4) takes place to form the metabolites, GLP-1 (9-37) / GLP-1 (9-36) NH2 and GIP (3-42), which account for the majority of the circulating forms of the two incretins. Incretin degradation by DPP-4 is a rapid process, such that the intact incretin peptides have short half-lives of just 1-2 minutes (GLP-1) and 2-3 minutes (GIP) [83]. Approximately 75% of GLP-1

leaving the gut is already in the inactive form following rapid degradation by DPP-4 inhibitor. Given the fact that further degradation takes place in the liver, the actual amount of active GLP-1 that reaches the systemic circulation is only 10-15% of the secreted hormone [98].

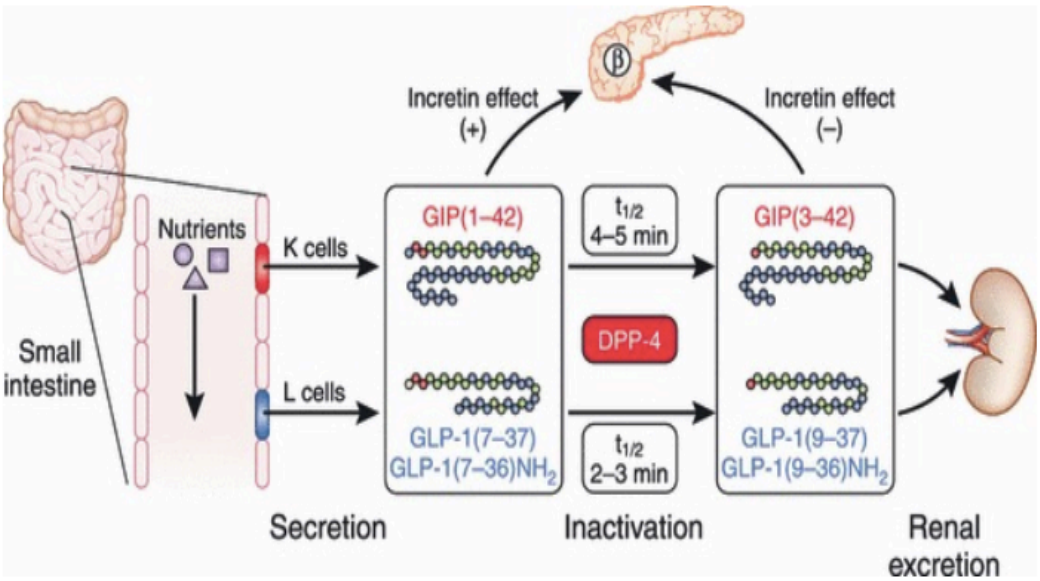


Figure 1-5: Secretion and metabolism of incretin hormones.

Sensing of nutrients by K and L-cells leads to release of GIP and GLP-1 respectively from their stored vesicles in the basolateral membrane of the intestine. Both undergo rapid inactivation by dipeptidyl peptidase 4 (DPP-4) to form inactive forms of the hormones before getting excreted by the kidney. Figure form Seino Y et al. J Diabetes Investig. 2010 [98]

1.5.5 Modes of physiological interplay between the gut and pancreatic islet cells

The physiological interplay between the gut and pancreatic β-cells involves several mechanisms. With regard to incretins, rapid pancreatic β-cell sensing of the two hormones must occur due to their short half-life. It is widely accepted

that the principal mode of connection between the gut and pancreas takes place in the form of circulating substrates, autonomic nervous system and hormonal factors [99]. Other mechanisms have also been proposed as additional modes of interplay between the gut and the pancreas. Gastrointestinal microbiota respond to changes in meal composition and these changes were found to impact GLP-1 secretion. In addition, changes in bile acid secretion, absorption and exposure to ingested nutrients were found to play a role in the fine-tuning of glucose within its narrow postprandial physiological range. Further, there is increased acknowledgement that HGP, which is largely a postabsorptive phenomenon, tends to be attenuated by bile acid receptors. Some of the bile acid receptors (namely TGR5) were also linked to the enhanced secretion of GLP-1 [82].

These multiple modes of communication seem to culminate together to respond to different meal compositions and maintain glucose homeostasis in the postprandial phase and might extend further to have some regulation of postabsorptive glucose levels. This is further consolidated by incretin's biological function of suppressing pancreatic alpha cells limiting postprandial rises in glucagon levels, in addition to its extra-pancreatic functions of slowing gastric emptying and enhancing the sense of satiety by acting on satiety control neurons in the hypothalamus [82].

1.5.6 Molecular mechanisms underlying incretin stimulated insulin secretion

Both GLP-1 and GIP are potent stimulants for insulin secretion from pancreatic β-cells. Molecular mechanisms underlie GIP and GLP-1 mediated pancreatic β cells insulin secretion involves activation of cAMP [100] leading to series of reactions resulting in release of insulin into the circulation. See figure 1-6.

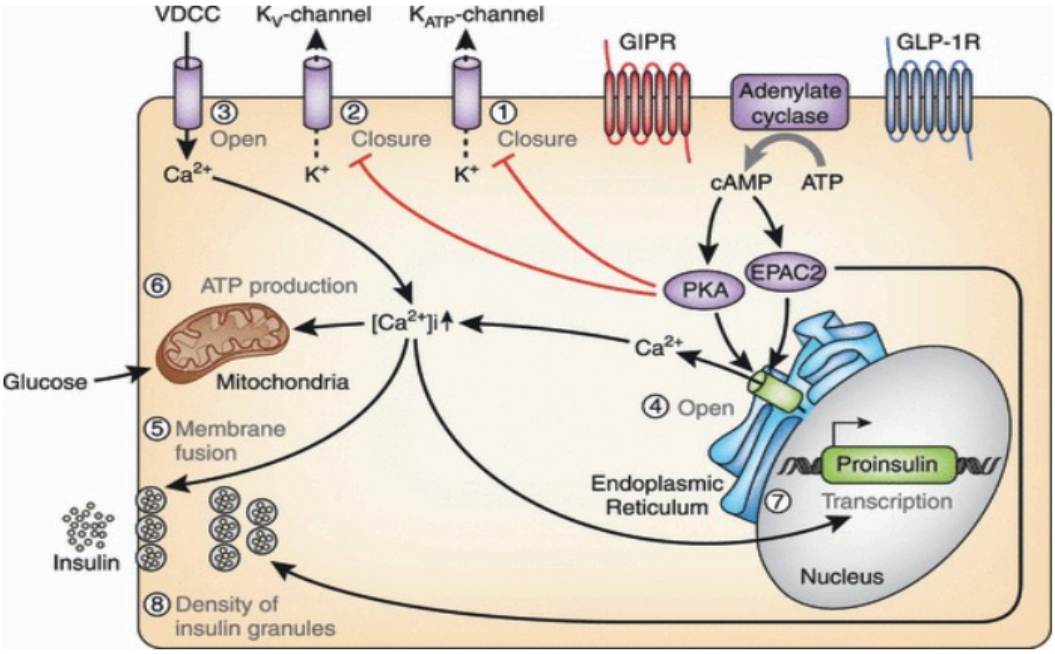


Figure 1-6: Molecular mechanisms derive incretin-mediated insulin secretion from pancreatic β cells.

The binding of the GLP-1 and GIP to their specific receptors (GLP-1R and GIPR respectively) in pancreatic β-cells leads to the activation of adenylate cyclase with a subsequent rise in cAMP levels [100]. This, in turn, leads to the activation of two vital molecules in this process: protein kinase A (PKA) and exchange protein activated by cAMP2 (EPAC2)/cAMP-guanine nucleotide exchange factor (GEF) II. This step initiates the traditional cascade of insulin release starting with the closure of ATP sensitive potassium (K_{ATP}) channels resulting in membrane depolarization, opening of voltage-gated calcium channels (VGCC) and increase in intracellular calcium concentrations. Intracellular rises in calcium levels trigger the fusion of insulin-containing granules with plasma membranes resulting in the exocytosis of insulin from its secretory vesicles and its subsequent release into the circulation [98]. EPAC2 activation directly increase the density of insulin-containing granules adjacent to the plasma membrane leading to the enhancement of the insulin secretory capacity of β-cells [98]. Figure 1-6 is from Seino et al. J Diabetes Investig. 2010 [98]

Both protein kinase A (PKA) and EPAC2 are key to the process of GLP-1 and GIP mediated insulin secretion into the circulation. It has been proposed that both molecules (PKA and EPAC2) directly affect regulators of insulin vesicle exocytosis, with PKA having a further additional stimulatory effect on vesicle priming [101]. Further, activation of GLP-1R per se leads to increased intracellular Ca^{+2} through Ca^{+2} releasing receptors located in the endoplasmic reticulum [102]. This was further supported by the fact that pre-treatment of pancreatic β-cells with inhibitors to endoplasmic reticulum receptors completely blocks rises in intracellular calcium as a result of the receptor-mediated effect [103] indicating that the receptor directly modulates the endoplasmic reticulum. Moreover, rises in intracellular calcium also stimulate transcription of the pro-insulin gene, further increasing the insulin reserve within β-cells. In addition, the activation of EPAC2 seems to increase the density of insulin-containing granules adjacent to the plasma membrane leading to the enhancement of the insulin secretory capacity of β-cells [98].

1.6 The incretin effect

The incretin effect is due to glucose eliciting a markedly greater increase in plasma insulin compared to an iso-glycaemic infusion [104]. Although a number of gut hormones are known to boost glucose-dependent insulin release into the circulation, the two incretins known to date inducing an incretin effect are GLP-1 and GIP. The incretin effect is expressed as a percentage of the insulin secretory response after oral glucose ingestion and represents the difference between the responses after oral and iso-glycaemic intravenous glucose stimulation, relative to the response seen after oral glucose ingestion. Together, both GIP and GLP-1 are responsible for up to 70% of insulin release following meal ingestion [105].

1.6.1 Contribution of GLP-1 and GIP hormones to the incretin effect

To date, GIP and GLP-1 are the only known incretins to be responsible for the total magnitude of the incretin effect observed following food intake. Several observations suggest that GIP is the main incretin that contributes to the incretin effect, at least under physiological conditions. GLP-1 (9-39) is a GLP-1 receptor blocker, the administration of which makes only minor changes to insulin secretory capacity, suggesting that blocking GLP-1 action might not have significant consequences on the overall incretin effect [106]. Similar observations are obtained when physiological concentrations of GIP and GLP-1 are infused [107]. Furthermore, when infusing GIP together with glucose to produce equivalent levels seen after oral glucose load, insulin rises were observed that are almost identical to those observed after oral glucose intake [107].

Studying how much insulin secretory capacity would change after total blockade of GIP is not possible, at least currently, as there is no available

receptor antagonist. However, available data strongly suggest that GIP is the main contributor towards the incretin effect by a magnitude of up to 80% [108].

The incretin effect of GIP is almost completely lost in individuals with type 2 diabetes mellitus. The cause for this is yet to be clearly understood. However, it is generally accepted that this could be due to GIPR desensitisation as a result of prolonged periods of hyperglycaemia ultimately leading to low levels of GIPR [100]. On the other hand, the incretin effect of GLP-1 is defective in this same group, but GLP-1 insulinotropic properties are largely intact supporting the presence of a fully functioning GLP-1R [81].

1.6.2 Other macro-nutrients and the incretin effect

Incretin hormone secretion not only occurs in response to CHO intake but also to the two other main macronutrients as well. While glucose's contribution to the incretin effect has been well documented, whether oral proteins or lipid load would give rise to the same phenomenon has only recently been described.

1.6.2.1 Proteins and the incretin effect

An ingestion of 6.5g of mixed AA can successfully lead to an increase in GIP but not GLP-1 compared to an equivalent intravenous mixture [95]. Higher rises in insulin were observed and deemed likely as a result of these GIP increments. It is not known whether higher oral protein content would elicit an incretin effect through GLP-1 stimulation as previous experiments have demonstrated an L-cell response to 2mg.kg^{-1} of mixed protein meal [87] and 30g of glutamine [96]. The effect of AA on the incretin effect will be explored on chapter 3.

1.6.2.2 Lipids and the incretin effect

Lipids are known to stimulate insulin secretion via various mechanisms that include direct effect on β-cells [109] or via efferent vagal stimulation in the upper intestine [110]. Giving oral lipid load to human volunteers successfully increases GLP-1 and GIP with resultant higher plasma insulin concentrations when compared to an intravenous equivalent load. Both oral and intravenous lipid loads achieved similar levels of triglycerides, FFA and glucose [111].

This provides further evidence of the ability of other macronutrients to deliver an incretin effect acknowledging the fact that other mechanisms involving β-cells responding to a lipid stimulus might be involved.

1.7 Classical versus non-classical roles of GIP and GLP-1

Classical incretin action acts to limit postprandial hyperglycaemia by enhancing β-cell mediated insulin secretion such that it contributes to 70% of postprandial insulin [105], suppressing α-cells glucagon secretion [112] and slowing gastric emptying [113]. These factors, together with inducing a sense of satiety in the hypothalamus [114] make incretin-based therapy the first to address many pathological aspects in type 2 diabetes in which there is a defective incretin effect, slow and inadequate postprandial rises in insulin concentrations and a lack of postprandial glucagon suppression. While the extrapancreatic actions of incretins in the hypothalamus and stomach are well described, both GIP and GLP-1 have direct non-classical biological functions in other extrapancreatic organs. See figure 1-7. Further, GLP-1R was found to be ubiquitously expressed in skeletal muscle [115], the cardiovascular system [116] and vascular endothelium [117]. These findings suggested exploring the possibility that GLP-1 might exhibit novel non-classical, insulin independent extra-pancreatic effects within the skeletal muscle and its microvascular endothelium. With skeletal muscle being the major site for glucose disposal and protein turnover, these effects would be of crucial importance.

Pharmacologically available GLP-1 receptor agonists have already been shown to have an added beneficial effect on cardiovascular outcome beyond that exerted through glycaemic control solely. These include reduced systolic blood pressure, reduced pulmonary artery wedge pressure, improved lipid profile, reduced endothelial dysfunction and improved myocardial salvage following ST-segment myocardial infarction [118]. Benefits related to other extrapancreatic tissues include weight loss, slowed gastric emptying, improved cognitive function and improved daytime somnolence in obese individuals with type 2 diabetes [118].

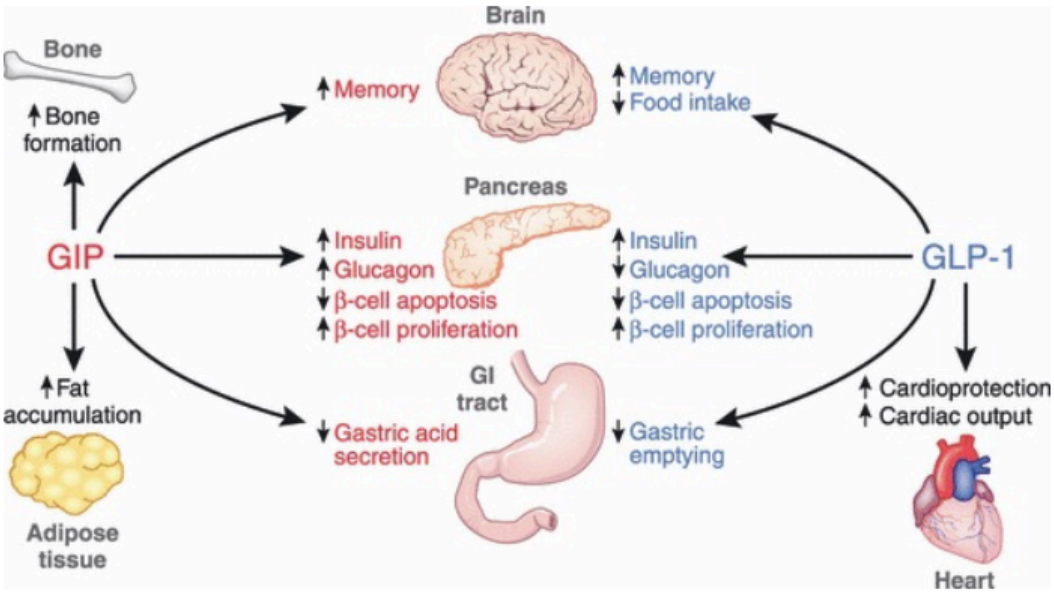


Figure 1-7: Direct classical and non-classical biological incretins action.

Both GIP and GLP-1 share similar biological functions in the pancreas and brain but different in their other extrapancreatic characteristics. Figure 1-7 is from Seino Y et al. J Diabetes Investig. 2010 [98]

1.7.1 GLP-1 receptor within skeletal muscle

GLP-1 receptors (GLP-1R) are ubiquitously expressed in both skeletal muscle [115] and vascular endothelium [117]. GLP-1R in skeletal muscle cells is a single G-protein coupled receptor and is expressed in both myocytes and satellite cells in amounts corresponding to those found in pancreatic β-cells [119]. An inositol phosphoglycan (IPG) has been suggested as the second messenger for GLP-1 action in skeletal muscle [120] as opposed to its classic cAMP messenger in pancreatic β-cells [121]. In vitro studies and studies in normal and diabetic animals have demonstrated the ability of GLP-1 to stimulate glycogen synthesis, enhance glucose oxidation and increase the activity of glycogen synthase [122], targeting fundamental cellular defects in the pathogenesis of insulin resistance within skeletal muscle [52,123,124] .

The effect of hyperglycaemia on receptor expression within myocytes and satellite cells is somehow not fully clear. While some studies showed an up to 50% reduction in the amount of receptor expressed under hyperglycaemic conditions [125], such a degree of downregulation was not observed in other studies [119].

1.7.2 Non-insulinotropic effects of GLP-1 on skeletal muscle MVR

Following food ingestion, MVR in human muscle is reported to increase by up to 100% from the baseline compared to an increment in MVR of only up to 40% during EHC [126]. As insulin levels during these investigative experiments are well within the ranges seen in postprandial conditions or are even higher, a potential incretin role beyond their pancreatic β-cell stimulation in postprandial conditions is thought to contribute to the heightened increases in MVR seen following food ingestion.

Chai et al. [127] demonstrated that GLP-1 infusion, with a concomitant inhibition of endogenous insulin via somatostatin infusion, resulted in a significant increase in MVR in the skeletal muscle of rats with a 2.3 fold increment in MBV and MBF without any significant change in MFV. In comparison, physiological postprandial insulin levels in rats were reported to induce lesser effects on these parameters: 1.7 fold for MBF and 2.1 for MBV. Intriguingly, inhibition of nitric oxide synthase, which is normally released from the endothelium, negated all these vital metabolic gains. This suggests that GLP-1, at least to a greater extent, delivers its biological functions related to skeletal muscle microcirculation through endothelium NO. Moreno et al. [128] reported that the glucose uptake benefits extend to rats affected by insulin resistance but not those with type 2 diabetes. Furthermore, increased GLP-1 bioavailability via DPP-4 inhibition resulted in increased expression and translocation of GLUT-4 [129].

Sjoberg et al. [130] were the first to report GLP-1 mediated increases in MVR in human skeletal muscle. After 60 min of continuous GLP-1 infusion (1 pmol.kg^{-1}.min^{-1}) into the femoral artery of young individuals, MVR increased by 60%. The GLP-1 concentration and insulin increased by a factor of 20 and 80 respectively. To adjust for the confounding effect of GLP-1 on insulin secretion and the effect of the latter on MVR and blood flow, the same experimenters repeated this study with an infusion of octreotide, which successfully reduced the insulin concentration to below fasting levels. Despite this, a 50% increase in MVR was still demonstrated in the infused leg where GLP-1 concentration was 30 times above the baseline and well above normal postprandial physiological ranges. Of greater interest was the 40% increase in MVR in the contralateral leg where GLP-1 systemic concentrations were within physiological postprandial ranges.

Significant GLP-1 effects on microvascular parameters were also demonstrated when it was infused to achieve no more than postprandial levels. Subaran et al. [131] showed 32% and 43% increases in MBF and MBV respectively after 150 minutes of systemic GLP-1 infusion in young volunteers.

Following these findings, researchers continued to exploit GLP-1 ability induce biological effects in extrapancreatic tissues independent of insulin. Insulin resistance is often closely linked to endothelial dysfunction and obesity. Hence, insulin-induced eNOS activity and signalling down the PI3-K and Akt is significantly blunted. Indeed, MVR is attenuated in obese individuals [132] and in healthy individuals following intravenous lipid infusion to induce insulin resistance [126]. Testing the effect of acute and chronically induced insulin resistance following 5 days and 8 weeks infusion of lipids respectively in rats revealed a promising role for GLP-1 in reversing blunted MVR induced by insulin resistance. While infusion of insulin alone did not fully restore MVR compared to pre-insulin resistance state parameters, co-infusion of GLP-1 did

in both groups [133]. Indeed, sustained activation of GLP-1R using the pharmacological preparation Liraglutide (GLP-1R analogue) in dolly rats fed with a high-fat diet to induce insulin resistance was able to alleviate microvascular insulin resistance and preserve skeletal muscle capillary density [134].

Despite recent reports suggesting that DPP-4 inhibition is linked to better tolerance to brain ischemic insult [135] and improved retinal microcirculation [136], the data is lacking regarding their effects on skeletal muscle microvasculature.

1.7.3 Non-insulinotropic effects of GLP-1 on limb macrovascular parameters

GLP-1 exerts a vasodilatory effect through its action on the endothelium leading to a 30% increase in limb blood flow in healthy subjects [137]. The conduit artery diameter increases by 3% with a 12% increase in arterial blood flow following 60 min of GLP-1 infusion with no further increase when the rate is doubled [130]. With a longer infusion duration (150 min) under physiological postprandial GLP-1 concentrations, conduit artery blood flow is 30% higher compared to the baseline as a result of increases in both artery diameter and flow velocity [131].

1.7.4 Non-insulinotropic effects of GLP-1 on skeletal muscle glucose uptake

Evidence to suggest that the role of GLP-1 in skeletal muscle glucose metabolism is independent of its insulinotropic role was first suggested when researchers reported that GLP-1 was involved in facilitating intracellular glucose transport and promoting the activity of glucagon synthase in both rats and human muscle cells. This was thought to be through mechanisms involving protein kinase C (PKC) [138]. Indeed, specifically in rat cells, the addition of GLP-

1 was found to enhance the activity of PI3-K, PKB, p70S6K and extracellular signal-regulated kinase (ERK); all are vital components of glucose metabolism and signalling pathway [120]. In addition, satellite cells isolated from healthy lean young men showed an ability to uptake glucose in response to GLP-1 [119]. Furthermore, in-vitro studies involving myocytes from obese human subjects showed a normalisation of glucose uptake following exposure to GLP-1 [139].

Animal experiments have consolidated these findings further showing that the GLP-1 receptor regulates endogenous HGP and skeletal muscle glucose uptake independent of its insulinotropic role [140]. Furthermore, intra-portal infusion of GLP-1 in fasted dogs where glucose was clamped at postprandial levels leads to increased non-hepatic glucose uptake with no changes in insulin and glucagon levels [141]. Using rat models, Chai et al. [127] measured glucose uptake using a femoral arterial-venous (A-V) balance method. Within 30 min of systemic GLP-1 infusion, there was a 3-fold increase in glucose uptake. Their results, however, could not be replicated by Sjoberg and colleagues [130] despite using a higher dose of GLP-1. The same group of researchers tested muscle glucose uptake in rats in the context of high-fat diet-induced insulin resistance. GLP-1 infusion restored muscle glucose uptake after five days of high-fat diet but failed to do so after eight weeks [133].

In human skeletal muscle, however, the evidence of the ability of GLP-1 to stimulate glucose uptake independent of insulin is still lacking. GLP-1 infusion does not stimulate glucose uptake either in young healthy subjects following femoral artery infusion [130], or in subjects with metabolic syndrome following brachial artery infusion [142].

It is still unclear as to why responses to GLP-1 are different in human studies compared to animal and cell culture models. Glucose uptake does not increase in human studies even after supraphysiological concentrations of GLP-1 are

reached in the bloodstream [130]. Even in animal studies, this response seems to be attenuated or negated by various degrees of experimental glucose dysregulation and insulin resistance. Interestingly, when Subaran and colleagues [131] studied MVR using physiological GLP-1 concentrations in healthy young human subjects, a small but significant reduction in glucose concentrations was observed following GLP-1 infusion. This observation is highly important and may be accounted for with a wide range of explanations, which their study did not have the scope to investigate. Possible explanations could include an increase in insulin-mediated glucose reduction as a result of increased MVR, inhibition of glucagon or indeed pure GLP-1 mediated glucose uptake.

The ability of hyperglycaemia to inhibit IMGD seems to even involve insulin independent GLP-1 mediated glucose uptake. Intriguingly this does not seem to be related to receptor underexpression, [119] and therefore, the involvement of other mechanisms is highly likely.

Thus, the incretin hormones could potentially play an important role in the regulation of skeletal muscle metabolism. We will focus specifically on GLP-1, which appears to be responsible for the majority of the novel extra-pancreatic incretin effects, likely due to the ubiquitous expression of its receptor (GLP-1R). Indeed, from our pilot studies we can confirm that the GLP-1R protein is robustly expressed in human skeletal muscle biopsy homogenates. See figure 1-8).

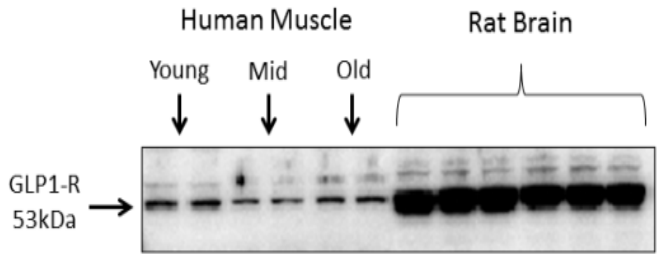


Figure 1-8: GLP-1 receptor expression in human skeletal muscle.

GLP-1R expression across different ages in human skeletal muscle cells compared to rat brain cells.

This observation is important since this study's intent is to focus on uncovering novel GLP-1 actions in skeletal muscle and associated microvasculature. Our rationale for focusing our efforts upon investigating GLP-1 effects on muscle is based on evidence that GLP-1 regulates key aspects of postprandial metabolism. This is relevant because nutrient-mediated anabolic effects in muscle occur through EAA-induced increases in MPS and insulin-mediated suppression of MPB, with the former being quantitatively more important. The anabolic effects of nutrition are driven by the uptake and incorporation of AA, captured from dietary protein sources, into skeletal muscle proteins.

Table 1-1 provides a summary of recent studies that have focused on skeletal muscle microcirculation and glucose uptake. Figure 1-9 summarises the overlapping roles of incretins, insulin and AA on skeletal muscle metabolism and microcirculatory responses.

No studies have examined the role of incretins in skeletal muscle protein metabolism. This is part of the focus of this study aiming to exploit this virgin area of research.

Table 1-1.

No.	Study	Year	Character of subjects	GLP-1 infusion	Measured GLP-1 fraction	Study conditions	Glucose uptake	Microvascular parameters			
								MVR	MBV	MFV	MBF
1	Chai et al.	2012	Rats	30 pmol.kg^{-1}.min^{-1}		Euglycaemic clamp + somatostatin	3 fold increase (AV balance)		2.3-fold increase	No change	2.3-fold increase
2	Dong et al.	2013	Rats	30 pmol.kg^{-1}.min^{-1}			2-3 fold increase (AV balance)		2-fold increase	No change	2-fold increase
3	Sjoberg et al.	2014	Rats	100 pmol.kg^{-1}.min^{-1}	Total	Euglycaemic clamp	No change (2-DG uptake in muscle)	28 % increase			
4	Sjoberg et al.	2014	Humans (healthy young males)	2 pmol.kg^{-1}.min^{-1}	Total	Euglycaemic clamp + Octreotide	No change (AV balance)	50% increase			
5	Subaran et al.	2014	Humans (healthy young)	1.2 pmol.kg^{-1}.min^{-1}	Total	No clamp	Not tested		43% increase	No change	32% increase
6	Chai et al.	2014	Insulin resistant rats	30 pmol.kg^{-1}.min^{-1}			30% increase (AV balance)		Increase	No change	Increase

Table 1-1: Recent Studies involved GLP-1 intervention effect on microcirculation and glucose uptake

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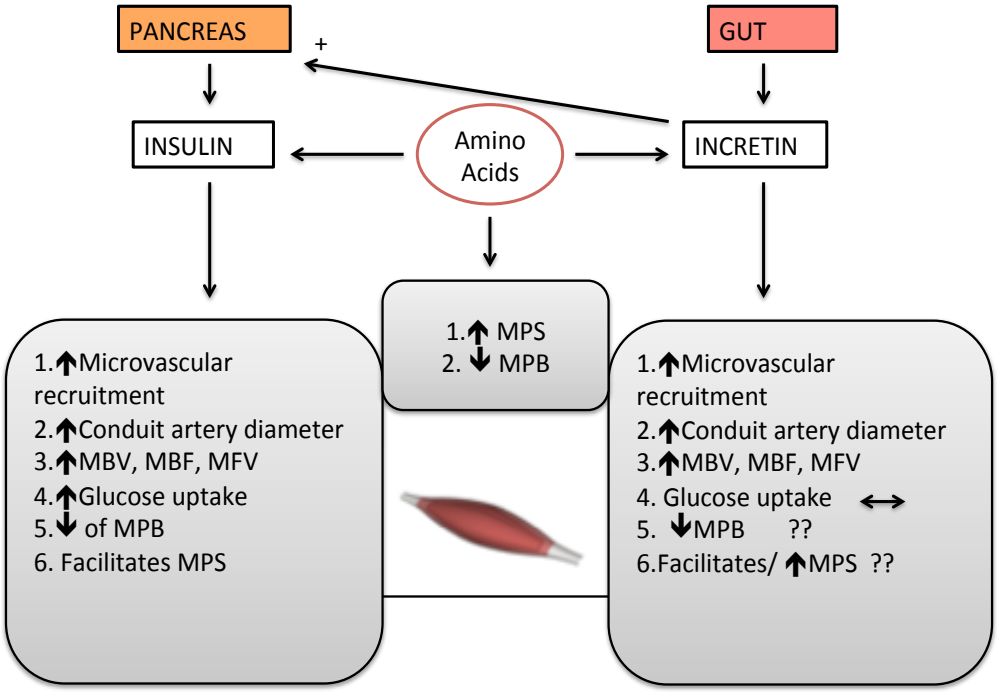


Figure 1-9: Insulin, AA and GLP-1 effects on skeletal muscle.

MBF: microvascular blood flow, MBV: microvascular blood volume, MFV: microvascular flow velocity, MPS: muscle protein synthesis, MPB: muscle protein breakdown.

1.8 Advancing age and skeletal muscle

1.8.1 Sarcopenia: its risk factors, burden and effects on frailty and disability

The definition of sarcopenia is controversial and there is no universal consensus. It could be described as the loss of skeletal muscle mass and strength that occurs with advancing age [143], eventually leading to reduced muscle function (dynapenia) [144]. The ageing process in itself is characterised by the incipient development of sarcopenia, where a steady decline in lean mass (and associated function) of approximately 1% per year beyond 60 years of age has been reported [10]. This is usually a consequence of a chronic imbalance between MPS and MPB [145]. The aetiology of sarcopenia, however, is not yet fully understood, with different factors seeming to contribute to its development including inactivity, inflammation and dysfunctional neuronal physiology [146].

While the prevalence of sarcopenia is estimated to be 30% in the population over 60 years, this estimate rises to more than 50% in those who are over 80 years old [147]. Due to its far-reaching consequences and the growing ageing population in the western world, sarcopenia represents a huge challenge to healthcare planners and providers in terms of morbidity [148], disability [149], high cost of care [150] and indeed mortality [151].

It should be noted that accelerated muscle loss is not an age exclusive phenomenon. It is seen in other examples of ill health and musculoskeletal dysfunctions. In critically ill patients [152] for example, it is characterized by reduced protein turnover and lack of stimulation of mitochondrial protein synthesis [153]. Another example is type 2 diabetes in which it is related to poor muscle strength and function, with an accelerated rate of decline in muscle quality and strength in

older subjects of up to 30% [154,155].

Functionally, sarcopenia is associated with several burdens and negative outcomes including frailty, falls, disability, fractures, increased body fat, reduced mobility and loss of independence, [11,156–158] leading to significantly increased mortality [151].

The risk factors for the development of sarcopenia include advanced age and its consequences of relative inactivity, lack of optimal diet and low body mass index. Other risk factors include low birth weight, female sex and impaired or loss of lower limb function [159,160]. Not all of these risks are modifiable and they represent the major culprits that lead to age-related progressive loss of skeletal muscle tissue and functional capacity.

Various theories have been proposed to explain the development of sarcopenia. Anabolic resistance (see below) is regarded as a significant contributing factor to skeletal muscle age-related decline [161]. Others include loss of myofibre innervation as part of wider age-related neuronal decline [162] and increased oxidative stress from the accumulation of ROS [163].

1.8.2 Advancing age and anabolic resistance

Ageing in human muscle starts from the fourth decade of life onwards. This is related to the decline in muscle mass, function and quality, and also oxygen uptake [164–167]. These changes in muscle composition together with other risk factors (e.g. reduced mobility, genetic susceptibility, etc.) mark the metabolic disturbances seen in age-related insulin resistance, obesity, type 2 diabetes, dyslipidaemia and hypertension, all of which significantly increase the incidence of cardiovascular mortality [168].

The term anabolic resistance refers to the diminished ability to respond adequately to AA provided either as an oral beverage or an intravenous infusion. The term is not an age exclusive phenomenon but also characterises many hypercatabolic conditions such as acute critical illness, liver failure, chronic obstructive pulmonary disease and immobilization [161].

Currently available data suggest that skeletal muscle loss that characterises the process of ageing is to a great degree due to the inability to elicit the expected anabolic response rather than enhanced protein breakdown. In young individuals, muscle protein mass remains constant due to a dynamic equilibrium in protein turnover where MPB exceeds MPS in fasted periods but MPS exceeds MPB in fed periods. When eliminating hormone-amino acid dose-response through euglycaemic insulin clamp, the ability of AA to stimulate MPS is shown to be significantly impaired in older individuals when compared to younger ones subjected to the same quantity of amino acid beverage [169]. Further, concerning a protein beverage in older individuals, the time required to reach positive protein balance is longer compared to younger individuals. In addition, a return to the point of negative balance is reached sooner such that the ability to sustain adequate positive equilibrium is impaired even when higher concentrations of AA are achieved in the circulation [170,171].

Ageing is associated with reduced IMGD [172] with a tendency to have higher insulin concentrations as a result [173]. Due to reduced peripheral insulin clearance, this rise is not associated with increased secretion [174].

An anabolic response requires the activation of the mTORC1 pathway. This pathway requires signalling through the insulin/Akt pathway for complete activation. Insulin sensitivity declines with age. However, experiments in older

individuals showed over-expression of mTORC1 at baseline and it is unclear whether this is just a compensatory mechanism to offset resistance to both insulin and nutrients or if it is in itself a contributory factor to anabolic resistance [175]. On the other hand, insulin's ability to attenuate protein breakdown is also impaired with advancing age. Normal insulin suppression of proteolysis following ingestion of a moderate sized meal is blunted in older age. Raising insulin from 5 to 15 $\mu U.mL^{-1}$ successfully reverses protein metabolism in younger but not older individuals [176] (see figure 1-10). Even when applying hyperinsulinaemic conditions, balances of AA tracer kinetics remain in the negative for older while becoming positive for younger individuals [177] (see figure 1-11). These experiments further explain the blunted response to nutrients in this group compared to younger counterparts. Since skeletal muscle is the major site for glucose and protein metabolism, it is no surprise that sarcopenia contributes heavily to whole body glycaemia and dysregulation in protein turnover, i.e. synthesis and breakdown.

It is worth noting that other anabolic hormone levels are affected by age. Oestrogen deficiency is one of the hallmarks of advancing age in women. In men, testosterone levels decline, and in both sexes, growth hormone and insulin growth factor-1 (IGF-1) are low. While there is no evidence to support a link between oestrogen deficiency and sarcopenia [178], muscle loss is strongly linked with a decline in testosterone and growth hormone levels [179–181].

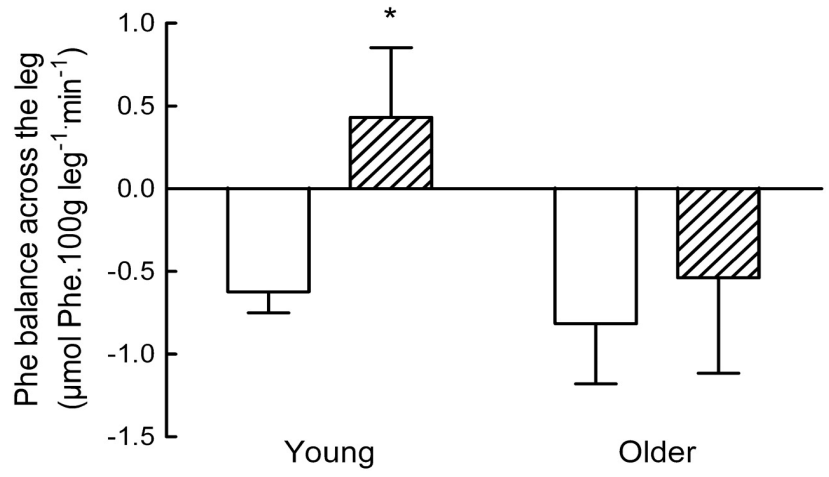


Figure 1-10: Blunting of proteolysis in response to moderate insulin.

Phenylalanine across the leg after clamping insulin at ≈5 μU.mL-1 (open bars) and ≈15 μIU/mL (hatched bars). In older individuals, proteolysis failed to respond to insulin. Figure from Wilkes EA et al. AJCN 2009 [176].

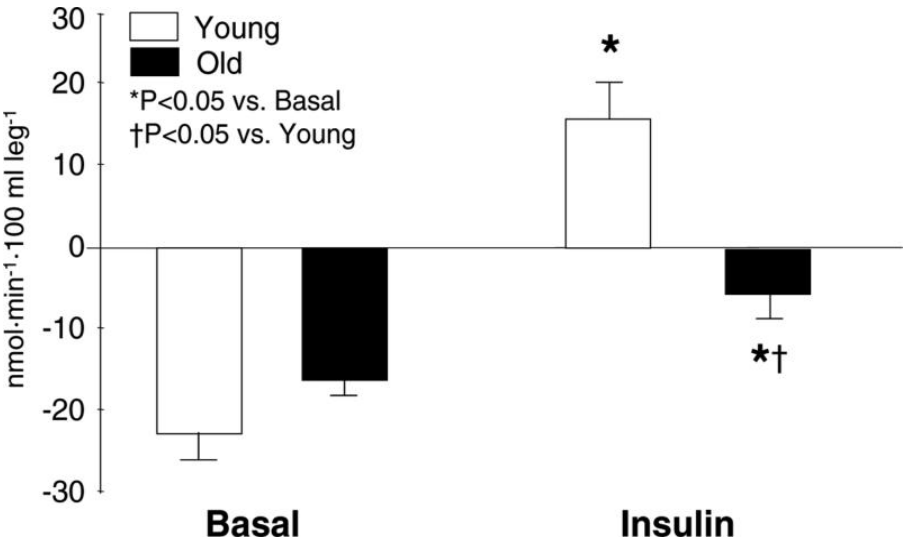


Figure 1-11: Blunting of proteolysis in response to high insulin.

Phenylalanine kinetics across the leg after local hyperinsulinaemia. Failure of protein kinetics to move to positive balance following physiological hyperinsulinaemia. Figure from Rasmussen BB et al. FASEB J 2006 [182].

1.8.3 Mechanisms underlying the development of anabolic resistance

Postprandial aminoacidaemia is a key event that switches protein balance from a negative to the positive state. Impaired digestion and absorption of protein and AA respectively adversely affect plasma substrate levels. In addition, enhanced splanchnic extraction, as opposed to young population, seems to further limit the appearance of AA in the systemic circulation. These factors contribute to depriving skeletal muscle of its major anabolic stimulus [183,184]. Moreover, postprandial insulin-mediated capillary recruitment is also impaired, which would ultimately limit the cellular capture of nutrients; therefore, less substrate is available for synthetic processes and anabolism [177]. Another possible contributory factor is the downregulation of amino acid transporters in the older population [185].

An intracellular anabolic signalling deficit was also suggested as a contributory factor to anabolic resistance. The expression of mTORC1 and its downstream targets p70S6K and eIF2 have been attenuated in older individuals [169]. The mechanisms that underlie downregulation of mTORC1 are yet to be fully understood. Under normal physiological conditions, the activation of mTORC1 requires crosstalk between several regulatory proteins leading to the phosphorylation of S6K1 and 4E-BP1, followed by the initiation of protein translation. In addition, leucine (facilitated by insulin) leads to translation initiation and elongation through the phosphorylation of S6K1 [186]. The ageing process is associated with the blunting of the AA-induced activation of S6K1 and therefore attenuated anabolic response [187]. There is also strong evidence to suggest the presence of dysregulation of energy metabolism, disturbed energy balance and reduced cell response to stress as a result of ageing [188]. See figure 1-11.

As much as this downregulation in anabolic signalling could be an intrinsic age-related phenomenon, it could also be a response to the attenuated availability of AA due to the above factors taking place prior to the influx of AA into the cell [189].

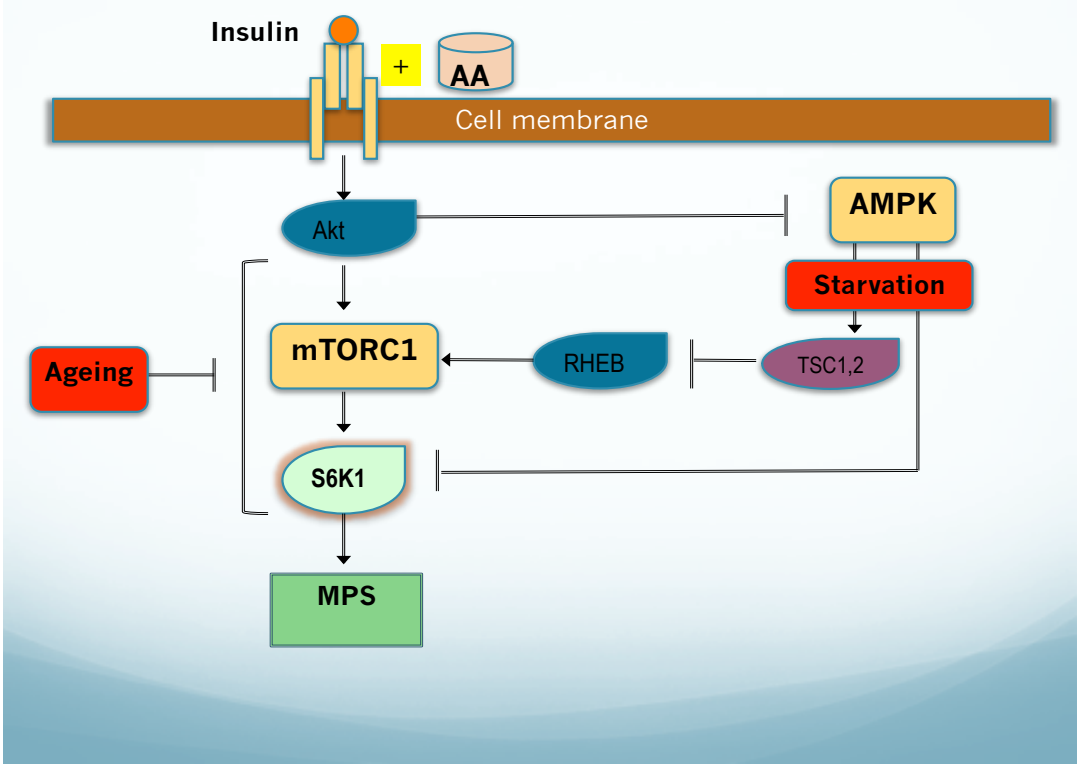


Figure 1-12: Ageing and energy balance role in anabolic resistance.

Ageing downregulates mTORC1 and downstream signalling. Normally, mTORC1 activation following AA intake requires the blockade of certain inhibitory proteins. These proteins are normally downregulated in circumstances of nutrient depletion (e.g. starvation) in order to conserve energy. AMPK, TSC1 and TSC2 are of notable importance in the inhibition of mTORC1 during stress and energy deprivation. AMPK under energy depletion conditions deactivates S6K1 directly and indirectly via activation of TSC1,2. mTORC1 stimulation following food intake is permitted upon inhibition of AMPK/TSC1,2 through increases in ATP and increased signalling down the insulin/PI3-K/Akt pathway. The inhibition of TSC1,2 leads to upregulation of the Ras homologue enriched in brain (RHEB) protein, the molecule that directly interacts with mTORC1. The ageing process is associated with blunted upregulation of RHEB and impaired signalling. mTORC1: mammalian target of rapamycin complex 1,

AMPK: adenosine mono-phosphate activated protein kinase, TSC: tuberous sclerosis complex, PI3-K: Phosphatidylinositol 3-kinase, Akt: protein kinase B. Figure 1-12 was developed based on a number of references [187,188,190,191].

1.8.4 Hormone and substrate delivery in anabolic resistance

The appropriate control of skeletal muscle protein and glucose metabolism is very closely linked to blood supply. Subjects with anabolic resistance exhibit 20-30 % reductions in limb conduit artery blood flow under both postabsorptive [192,193] and postprandial [194] conditions. As recruiting muscle microvasculature is central to maintaining protein [195] and glucose [62] homeostasis, these vascular defects could well be a contributory factor to impaired muscle anabolism and glucose uptake. Following femoral artery insulin infusions, Timmerman et al. reported increased MPS related to increased AA delivery via the enhancement of microvascular flow in an insulin availability-dependent manner [195].

The morphology and structure of ageing large arteries differ considerably compared to younger counterparts. Ageing large arteries tend to be long, tortuous and thick, with increased capillary permeability. Plasma proteins tend to cross easily into the medial layer of vascular smooth muscle due to impaired endothelial function as a barrier to plasma proteins. This causes increased vascular thickness. Molecules such as collagen infiltrate the subendothelial space. The endothelial mono-layer tends to be irregular in shape and might be infiltrated by vascular smooth muscle cells [196].

Functionally, ageing vessels have impaired distensibility with increased endothelial permeability and reduced responses to NO. These functional changes have been observed in older individuals with no evidence of atherosclerotic disease or

hypertension [196,197]. However, atherosclerosis generally is regarded as an accelerated form of ageing vessels. There is strong evidence to suggest that ageing and endothelial dysfunction progress in parallel to each other. Endothelial responses to insulin are attenuated leading to defective NO synthesis and an increased vascular tone and reactivity and predisposition to atherosclerosis [198]. Evidence also suggests that age-related endothelial dysfunction is associated with impaired endothelial cell division leading to the irreversible arrest of cell division [199].

This age-related decline in endothelial function ultimately manifests itself in the form of attenuated angiogenic and regenerative capacity thought to be due to the impaired secretion of growth factors or reduced sensitivity to them [200]. Moreover, the number of bone marrow-derived endothelial progenitor cells that are thought to carry out the regenerative processes in the endothelium is significantly reduced, reflecting the possible relative failure of migration of these cells from the bone marrow [200,201].

All of the above changes significantly impair the delivery of hormones and substrate to ageing muscle and would affect its responses to feeding and exercise. However, evidence suggests that resistance exercise training (RET) per se might be able to positively modulate vascular responses in older age and restore microvascular circulation within skeletal muscle [202]. RET was reported to improve age-related impairment in vascular reactivity in skeletal muscle arterioles and to improve the disturbed redistribution of blood flow between white and red fibres [203]. Further, RET was able to reverse age-related endothelial dysfunction linked to NO deficiency secondary to underexpression of eNOS [204].

1.8.5 Microvascular recruitment response to advancing age

MVR is a key aspect to the skeletal muscle response to both exercise and postprandial rises in insulin [63,67]. Feeding robustly stimulates the vascular response mainly through the vasodilatory effect of insulin via NO. Recruiting more vasculature provides a wider endothelial surface that allows the delivery and transendothelial transport of insulin and nutritional substrates. Further, downstream microvascular vasodilation is one of the mechanisms for microvessels network communication. In addition, microvessels are also able to increase their blood flow in direct response to endothelial NO and prostaglandins [205].

In healthy ageing, microvascular network resistance, especially arterioles, accounts for the majority of peripheral resistance under fed conditions. While pressure in arteries lying external to skeletal muscle is about 80% of systemic circulation, pressure just before entry to the capillaries is only about 30% of systemic circulation. This microvascular reactivity to fed conditions changes with age. Age-induced adaptation that follows this predominantly arteriolar resistance, however, is heterogeneous and different across muscle fibre types [205,206].

Physiological ageing is associated with the blunting of insulin-mediated vasodilatory stimulation. Skilton and colleagues demonstrated that postprandial microvascular dilatation is significantly impaired even after adjusting for baseline parameters between old and young individuals [194]. While the response to NO is thought to be intact, it is the underexpression of eNOS in response to insulin that seems to be the major contributor to blunted insulin response, amongst other causes, at both ends of the stream [182,205,207].

Several reasons have been proposed for this blunted postprandial response to feeding. These include a decline in gross limb blood flow [193], insulin resistance and underexpression of eNOS [182,207], increased degradation of NO by oxygen-derived free radicals, increase release of vasoconstrictors [194], reduction in vascular responsiveness [208], reduced capillary density [209] and endothelial dysfunction [210]. All of these factors jeopardise the ability of skeletal muscles to respond to vasodilatory stimuli such as insulin (exogenously infused or in response to feeding) and exercise.

The resistance to insulin action at the microcirculatory level is thought to impair insulin's movement to the interstitial space through the endothelium, which is another rate-limiting factor. The duration taken by insulin to cross into the interstitial space is longer in ageing skeletal muscle [211]. Further, insulin uptake by endothelial cells is impaired, possibly due to raised levels of cytokines [212]. These facts are further supported by the observation that insulin concentration in the interstitium is significantly lower than in plasma, with a consequent delayed insulin action at the myocyte level [211].

Contrast-enhanced ultrasound (CEUS) using microbubble contrast agents is the gold standard technique for providing real-time assessment of the muscle microvascular response to different vasodilatory stimuli [213]. Age-related attenuation of vascular responses to feeding is now well-documented. In respond to feeding, microvascular blood volume (MBV), microvascular blood flow (MBF) and microvascular flow velocity (MFV) have all been shown to be blunted in older compared to young men. Feeding young and old men a mixed EAA meal showed a 130% increase in MBF from a baseline at 195 min in young individuals, however, no significant increase was observed in older participants [214]. Even when

supraphysiological increases in insulin were achieved via hyperinsulinaemic euglycaemic clamp, MBV failed to increase in both elderly and sedentary individuals [61]. These examples provide strong evidence for age-related blunting in the microvascular response. Further, these age-related changes represent a cornerstone in the development of type 2 diabetes due to an initial increase in insulin resistance and increased glucose excursions both in the postabsorptive and postprandial states [215]. The overall consequences of such resistance could potentially expose skeletal muscle to compromised substrate and hormone delivery, hypoxia and increased oxidative stress [216].

Therefore, ageing negatively impacts skeletal muscle metabolism in many ways: 1. Anabolic resistance manifests as a blunting of the increase in MPS following the intake of nutrition; 2. Older adults exhibit reduced insulin-mediated suppression of MPB [176] ; 3. Studies using isotope tracer approaches reveal a decreased glucose rate of disappearance and forearm glucose uptake rate following the glucose challenge in older adults [217]; 4. Finally, even when providing increased insulin levels via insulin clamps, glucose disposal rates still remain depressed in the elderly individuals[218]. This "metabolic resistance" is thought to be multi-factorial owing to a decline in insulin secretory capacity (β-cell function) [219], insulin action (muscle and vascular insensitivity to insulin) [220] and reduced GLUT4 levels [221] , with the latter defects occurring within and around skeletal muscle.

1.8.6 Intervention strategies in sarcopenia

Lifestyle adjustments, improved diet and the provision of nutritional supplements are currently some of the ways used to tackle sarcopenia. Sarcopenia is likely an interplay of factors that occur both early and later in life. Factors like birth weight and early nutrition affect peak mass attained earlier in life [222], while general ill

health and immobility lead to accelerated muscle loss at later stages of life. Developing strategies that tackle both fronts could be promising to prevent the progression of sarcopenia in the future.

1.8.6.1 Lifestyle and exercise interventions

Of all forms of exercise, RET is by and large the most studied due to its proven efficacy. High-intensity RET for a duration of 3-6 months achieves up to a 40% increase in muscle strength and function in both frail and otherwise healthy older people and in those with other co-morbidities [223]. To maximally achieve its goal, RET would need to take into account intensity, volume and velocity [224]. Training with high intensity, volume and velocity has better outcomes in terms of strength and function compared to conventional training. The latter, however, has the advantage of being better endured [224,225]. This improvement seen with RET could well be at least partially explained by vascular adaptation with improved limb blood flow and vascular conductance [202]. Goodpaster et al. [226] reported a preventative effect when intervening with a moderate increase in physical activity on age-related dynapenia (loss of muscle strength) and increased fatty infiltration. Moreover, nutritional intervention in combination with exercise also stimulates MPS in older individuals. However, the response is diminished when compared to younger subjects. This is thought to be related to reduced activation of upstream molecules involved in mTOR signalling, in addition to elevated AMPK activity compared with young individuals after resistance exercise [169,227].

Chronic smoking and alcohol consumption are both related to muscle loss and reduced responses to anabolic stimuli. Chronic tobacco consumption leads to

chronic inflammation, oxidative stress and the blockade of neuromuscular transmission with resultant imbalances between MPS and MPB [228]. Alcohol exposure leads to impaired skeletal muscle MPS and increased autophagy [229]. Furthermore, chronic alcoholic myopathy, characterized by a selective atrophy of type II muscle fibres due to decreased protein synthesis, is one of the known features of chronic alcohol abuse [230].

1.8.6.2 Dietary intervention

In contrast to younger individuals, high protein supplements are needed to elicit positive net protein balance in older individuals. This is mainly attributed to anabolic resistance. Adequate protein supplementation of 25-30 g is needed to achieve this aim. Distributing protein intake through the day is equally important. A total daily protein intake in the range 1-1.2 $g.kg^{-1}.day^{-1}$ would meet the recommended daily intake [231]. Consumption of too large amount of protein at just one time of the day is a less efficient strategy. The molecular content of the protein itself is another vital element. EAA are the main anabolic stimulant, and more specifically, their branched-chain amino acids (BCAA) sub-types (i.e., leucine, iso-leucine and valine). Of the latter, leucine and its metabolite HMB have a notable effect. This needs to be consolidated by long-term studies to prove its long-term efficacy and favourable clinical outcome in tackling sarcopenia [232–234].

The role of other naturally occurring nutritional supplements has also been explored. Creatine and omega-3 fatty acids have proven to have an additive effect when combined with resistance exercise in older people [235,236]. Vitamin D supplements in older age are associated with increased muscle strength and up to a 20% reduction in the rate of falls [237,238]. A combined supply of vitamin D and leucine-enriched whey was also found to be effective [239].

1.8.6.3 Hormones

Testosterone replacement in older men with low levels is related to increased muscle mass, and to a lesser extent muscle strength, improved function and reduced hospital admissions. However, adverse cardiovascular outcomes go against adopting this approach to tackle sarcopenia [240]. Growth hormone replacement could lead to increased muscle mass but not strength or function [241]. Its significant side effect profile makes it an unsuitable option [242]. Both oestrogen and dehydroepiandrostenedione (DHEA) replacement are of no proven added benefit to muscle mass or strength [241,243].

1.9 Conclusions

Skeletal muscle as an organ has significant biological functions that extend far beyond its traditional function of locomotion. It contains up to 45% of total body protein and contributes heavily to the body's entire protein turnover. Skeletal muscle represents a metabolic milieu for glucose disposal and lipid oxidation and is regarded as the most dominant organ in the body that contributes to energy expenditure.

The metabolic functions of skeletal muscle are tightly regulated under both postabsorptive and postprandial conditions. Insulin is key in regulating the entrance of all major micronutrients to the cell. Its microcirculatory functions are mediated through NO. Insulin regulates its own delivery and transendothelial transport. Once insulin is bound to its receptor, it stimulates a cascade of intracellular signalling processes leading to energy production and positive protein balance. However, insulin's exact role in MPS and MPB is heavily disputed. Both animal and human studies have provided different results. Its action and levels in relation to AA availability still need to be further scrutinised.

Age-related decline in skeletal muscle size and function is multi-factorial. Muscle size is generally genetically determined, however, factors in early and late life tend to contribute to this age-related decline. Birth weight, early life nutrition, gender, relative inactivity later in life and poor nutrition are amongst the leading factors in this decline.

There is no consensus on the definition of sarcopenia but in broad terms it describes the age-related decline in muscle strength and function. It affects about 50% of octogenarians and contributes significantly to morbidity and mortality.

Sarcopenia is marked by the development of anabolic resistance to protein supplements, vascular compromise, impaired endothelial transport and resistance to anabolic hormones such as insulin. These dysfunctions come on top of an already compromised nutritional input and impaired digestion and absorption. The evidence also suggests blunted intracellular anabolic signalling as a mechanism that underlies muscle wasting in older age.

The incretins (GIP and GLP-1) are intestinally produced hormones secreted predominantly in response to CHO, and to a lesser extent, proteins and lipids. Their classical action is to stimulate insulin secretion in anticipation of the arrival of nutrients into the circulation. Moreover, they reduce glucagon secretion, slow gastric emptying and enhance satiety in the brain. Today, GLP-1 based therapy represents one of the vital therapeutic interventions tackling type 2 diabetes-related pathophysiological processes.

Incretin actions however extend beyond these classical roles. Their receptors are expressed in other extrapancreatic organs, e.g., heart, vascular endothelium and skeletal muscle. GLP-1 was shown to improve skeletal muscle microvascular functions in both humans and rats independent of insulin. To date, there is no evidence to suggest that GLP-1 improves glucose uptake independent of insulin in human skeletal muscle. This is despite the positive results shown in cell cultures and animal models.

GIP and GLP-1 are the only two gut hormones known to induce an "incretin effect" where oral glucose stimulates higher insulin release as opposed to an equivalent glycaemic infusion. There is a scarcity of available data suggesting that AA elicit an incretin effect. However, this needs to be further characterised.

Nutritional inputs are one of the vital strategies that have been researched to tackle age-related decline in skeletal muscle mass and function. The evidence supports the use of leucine-enriched EAA due to their beneficial effect on stimulating MPS and regulating anabolic signalling. HMB (a leucine metabolite) is a promising agent used to attenuate MPB. It is not clear whether its provision would add further benefits as opposed to leucine alone.

Tackling sarcopenia needs a deeper understanding of its different molecular aetiologies and then identifying novel strategies for interventions. Furthermore, developing better biomarkers to try to monitor specific interventions would be of equal importance. Current successful strategies emphasise lifestyle adjustment in the form of exercise, diet, smoking cessation and reduced alcohol intake. The role of RET is to attenuate the decline in muscle strength and function and the promotion of a healthy diet at all stages of life with leucine-enriched EAA is a vital component to this. Intervention with omega-3 oil and creatine have an additive effect on RET. The evidence on vitamin D is still not certain, however, maintaining normal vitamin D intake is important to bone health and general wellbeing.

To date, GLP-1 studies that have been performed in human skeletal muscle have concentrated on testing its biological functions in healthy young individuals. Studying GLP-1 biological functions in the context of advancing age has not been performed before. It would, therefore, be interesting to try to establish whether GLP-1 has any effects on tackling sarcopenia and age-related metabolic resistance. Bringing this piece of scientific information into knowledge domain would be key to developing strategies to address pathophysiology related to sarcopenia, insulin resistance and endothelial dysfunction.

1.10 Gaps in knowledge and understanding

This study aims to address current gaps in the literature in regard to our understanding of certain metabolic principles related to AA, gut hormones, skeletal muscle metabolism, and skeletal muscle microcirculation in the context of advancing age. At the centre of this study are the roles played by EAA in modulating gut hormones, in addition to establishing physiological functions exerted by both insulin and GLPL-1 in skeletal muscle glucose and protein metabolism and MBF.

Firstly, despite the fact that it is well reported in the literature that AA stimulate the secretion of both insulin and GLP-1, data on their ability to spark an incretin effect, i.e. gut-derived increase in insulin release, is very scarce. Studies on AA have not tested EAA on their own. Furthermore, the latest evidence suggests that the incretin effect is achieved only through K-cell stimulation and not L-cell stimulation. It is not known whether an EAA beverage would have a different effect.

Secondly, it is not known whether the ageing process has an impact on gut incretin secretory ability in response to EAA.

Thirdly, GLP-1 is increasingly found to have benefits that extend beyond its classic insulinotropic role in stimulating pancreatic β-cell insulin secretion. There is well-documented evidence supporting the role of GLP-1 in skeletal muscle MVR in health. Its role in skeletal muscle MVR in the context of advancing age under postprandial glucose concentrations and different levels of insulin is yet to be explored.

Fourthly, the classic insulin role is known to promote skeletal muscle glucose uptake via its effects on MVR and starting intracellular signalling processes that end in GLUT-4 migration to the cell membrane leading to glucose internalisation. Data from cell culture and studies in rats showed the insulin-independent role of GLP-1 in skeletal muscle glucose uptake. However, studies in young humans using postabsorptive glucose concentrations failed to confirm findings from cell cultures and rat studies. It remains to be established whether GLP-1 has an insulin-independent effect on glucose uptake under postprandial conditions.

Fifthly, no study has so far investigated the effect of GLP-1 on skeletal muscle protein metabolism. Therefore, the non-insulinotropic effect of GLP-1 on skeletal muscle protein synthesis and breakdown remains to be elucidated.

Finally, despite being a fiercely debated issue, insulin's role in human skeletal muscle continues to raise controversy. Further scrutiny is needed through a well-designed systematic review and a meta-analysis would shed more light on this area.

1.11 Hypothesis

Incretin release following nutrient ingestion is the fundamental step that ultimately determines quantitative postprandial insulin release. We contend that understanding the basis by which GLP-1 regulates muscle turnover and the effect of the ageing process on GLP-1 release is important in identifying strategies to halt the progression of sarcopenia and promote healthier ageing. GLP-1 and insulin are key elements in postprandial metabolism. We argue that identifying their dependent and independent roles in muscle metabolism and microvascular function is key to determining any future role for GLP-1 in related pathophysiology and promotion of healthy ageing. We, therefore, hypothesize that:

I. EAA, like CHO, possess an incretin effect capability. The degree to which EAA could stimulate incretin secretion and hence deliver an incretin effect is likely to be affected by the ageing process.
II. GLP-1 positively affects skeletal muscle protein metabolism under postprandial conditions independent of insulin levels.
III. GLP-1 positively affects skeletal muscle microcirculation under postprandial conditions independent of insulin levels.
IV. GLP-1 has a role in enhancing skeletal muscle glucose uptake under postprandial conditions independent of its insulinotropic role.

1.12 Objectives of the study

I. To identify age-related effects of EAA ability to stimulate gut hormone secretion and whether and to what extent EAA induce a true incretin effect (gut-mediated increases in plasma insulin).

II. To define the extra-pancreatic effects of GLP-1 effects on postprandial muscle protein and glucose metabolism under low and high levels of insulin concentration.

III. To define the extra-pancreatic effect of GLP-1 on skeletal muscle microvascular parameters under low and high levels of insulin concentration.

IV. To characterize the role of insulin on human skeletal muscle protein synthesis and breakdown.

Chapter 2: Materials and Methods

2.1 Framework of studies

2.1.1 General

This work as a whole addresses various research questions through different study methodologies. It assesses gut hormone release in response to EAA in young and older individuals. It also assesses acute metabolic responses of human skeletal muscle in response to hormone (insulin and incretins) intervention under the experimental fed state. Furthermore, it investigates macrovascular and microvascular response recruitment via the use of ultrasound Doppler and CEUS respectively. The assessment of protein and glucose metabolism utilises the infusion of labelled AA, glucose, blood samples and muscle biopsies. Dual energy x-ray absorptiometry (DEXA) scans is used to estimate total body lean mass and fat content to aid the quantification of parameters of protein metabolism.

2.1.2 Volunteers

Healthy volunteers aged 18-40 (Chapter 3) and 60-75 (Chapter 3, 4 and 5) were recruited. Recruitment was conducted through local advertisements at the university or in the surrounding community using mail shots, posters and research awareness events organized by the medical school. Each target volunteer received the designated study protocol information sheet. This explains in layman terms the study's experimental protocol, inclusion and exclusion criteria and what each study visit encompasses. A further verbal explanation of the experimental protocol was conducted at the first point of contact, usually screening or pre-screening visits before the subjects gave their consent to be part of the study. Prior to participation in each study, all volunteers filled out a health questionnaire, underwent a face-to-face clinical assessment (medical history and physical

examination) and had a blood test for full blood count, clotting screen, kidney and liver function, fasting blood glucose and thyroid function. An electrocardiogram was also taken.

Volunteers were excluded in case of frailty, active malignancy, and any medical condition making their participation not suitable or unsafe, e.g. a history of cardiac, pulmonary, liver, kidney, neurological, vascular or autoimmune disease, clotting disorders, uncontrolled hypertension (>160/90 mmHg), metabolic disorders including diabetes, uncontrolled thyroid disorders, obesity, anaemia, alcohol abuse or visually obvious muscle wasting or skeletal deformity. Volunteers taking medications that would affect the results or deemed unsafe to stop were also excluded, e.g. corticosteroids.

A total of 40 healthy volunteers were studied to complete the protocols detailed in Chapter 3, 4 and 5. All studies were approved by The Nottingham University Ethics Committee and were conducted according to the Helsinki Declaration.

2.1.3 Measurement of body composition

Dual-energy photon X-ray absorptiometry (DEXA; Lunar Prodigy II, GE Medical Systems) was used to measure the total skeletal muscle mass, leg lean mass and fat mass of each volunteer before their first acute study as per the specific protocol. These measurements were used to estimate the parameters of skeletal muscle metabolism, i.e. MPS and MPB.

2.1.4 Conduct of studies

All volunteers reported to the study site (Human Physiology laboratories – clinical room- at the Division of Medical Sciences and Graduate Entry Medicine – Royal

Derby Hospital Centre) at 08:00 in the morning after 10-14 hours of overnight fasting.

A DEXA scan was performed as per the appropriate protocol to estimate body composition. Following this, the acute study started with the insertion of polyethylene catheters (BD, Oxford, UK) into both arms for blood sampling or the infusion of amino acid tracers, contrast agents, intravenous fluids, drugs and hormones. A femoral vein and femoral artery catheters were inserted for blood sampling and hormonal infusion. Each study lasted for 2 hours (Chapter 3) or 6 hours (Chapter 4 and 5). After the first baseline blood sample was taken, primed, continuous infusions of L-[ring-$^{13}C_6$]-phenylalanine began and were maintained until the end of the study.

The constant tracer infusion method was used which allowed the incorporation of L-[ring-$^{13}C_6$]-phenylalanine into the m. vastus lateralis of the quadriceps group of muscles. This facilitated the measurement of the myofibrillar fraction of muscle protein. With this tracer infusion method, it usually takes about 150 min for tracers to reach a steady state in the plasma[244]. Therefore, 2 baseline muscle samples were obtained at 1 hour and 3 hours from the time the tracer was started. After this intervention, a further 2 muscle biopsies were taken at 1.5 hour intervals.

Blood samples were taken at 15-30 min intervals. Samples were immediately stored on ice and the plasma was separated from the whole blood by centrifugation during or immediately after the study. Muscle biopsies, ultrasound Doppler scans and CEUS were performed during the studies, as per the specific protocols. After the study, all catheters were removed and volunteers were fed and monitored for 30-90 min before leaving the study site.

2.2 Techniques and procedures

2.2.1 Infusion of stable isotope tracers

Stable isotope tracers are naturally occurring atoms (e.g. carbon, oxygen, nitrogen and sulphur) containing extra neutrons. Chemically, they behave identically to original molecules, and importantly, they are non-radioactive, i.e., they do not undergo decay ensuring the safety of use in humans. They allow us to explore how different stimuli (e.g. nutrition, exercise and hormones) affect substrate metabolism in different tissues. When infused into a vein, tracers enter the arterio-venous (AV) pool, reach the tissue, are transported by AA transporters, calibrate into the intracellular pool, bind and charge their cognate tRNA, and incorporate into peptide. By measuring the incorporation (enrichment) of labelled tracer into the peptide in tissue sample, the rate of synthesis could be estimated. Furthermore, by looking at the enrichment of the tracer in the artery and the vein in conjunction with blood flow, the rate of disappearance or appearance of the tracer could be estimated which corresponds to the rate of MPS/ tissue uptake and MPB/ tissue release respectively.

2.2.2 L-[ring-$^{13}C_6$]-phenylalanine

L-[ring-$^{13}C_6$]-phenylalanine (Cambridge Isotope Ltd, Cambridge, MA, USA) is a stable isotope-labelled tracer. Phenylalanine is not metabolized by skeletal muscle and therefore the rates of its intracellular use and appearance correspond to MPS and MPB respectively [245]. Following its purchase, it was prepared aseptically under sterile conditions for infusion by the Clinical Trials Sterile Production Unit (Clinical Trials Pharmacy, Queens Medical Centre, Nottingham, UK).

The prescription for the infusion was calculated according to the volunteer's weight and height during the screening visit. These infusions were made a day before the study day in The Clinical Trial Sterile Production Unit by weighing the tracer as per prescription, dissolving it in 250 mL of 0.9% saline and then allowing it go through two-staged filtering through a 0.22µm filter in syringes labelled with an expiry time to remove pathogens.

Infusions were transported to the clinical pharmacy at the Royal Derby Hospital (Derby, UK) in cool boxes before being collected by a member of the study team on the morning of the acute study. They were infused via a Baxter Colleague 3 infusion pump (Baxter, Thetford, UK) at $0.4mg.kg^{-1}$ (priming dose) and $0.6mg.kg^{-1}.h^{-1}$ (constant infusion rate), which was maintained throughout the period of the study.

2.2.3 Muscle biopsies

The conchotome biopsy technique was used for obtaining muscle biopsies as described by Deitrichson and colleagues [246]. After sterilizing the skin over the m. vastus lateralis of the quadriceps muscle, the skin and deep tissues were anaesthetized using 5mL of 1% Lidocaine (Hameln pharmaceuticals Ltd, Gloucester, UK). After 5-10 minutes, a 0.5-1 cm incision was made through the skin, subcutaneous tissue and muscle fascia. Using the conchotome, 200-300 mg of muscle tissue was obtained. Muscle samples were immediately washed in ice cold 0.9% saline solution to remove blood, visible fat and connective tissue. Immediately afterwards, the biopsy was frozen in liquid nitrogen before being stored at -80 °C until further analysis was carried out.



Figure 2-1: Muscle biopsy tray.

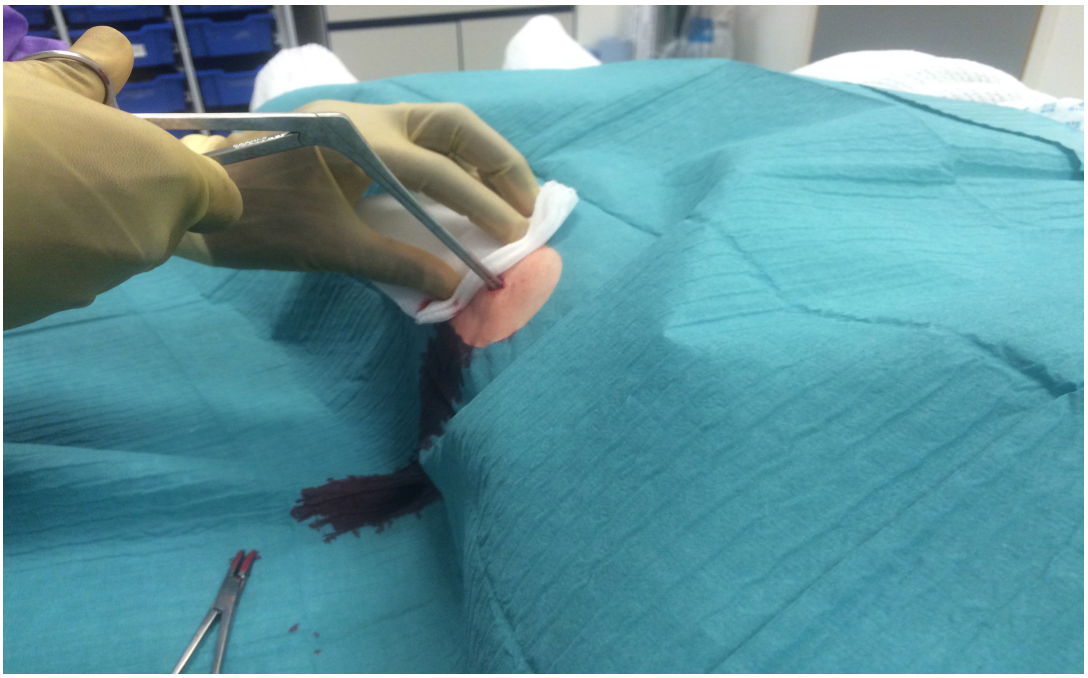


Figure 2-2: Taking muscle biopsy using conchotome forceps.

2.2.4 Contrast-enhanced ultrasound (CEUS)

The CEUS (Philips iU22 Ultrasound, Bothell, WA, USA) method was used for the quantification of vastus lateralis muscle perfusions. This method is based on the analysis of microbubble replenishment kinetics after their destruction by a high-energy (high-MI) ultrasound pulse. Replenishment kinetics describe the refilling of microbubbles from outside a region of interest (ROI) where the contrast agent is still present.

2.2.5 Sonovue^{TM} microbubbles

Sonovue^{TM} (Bracco, Milan, Italy) is a contrast agent in the form of 25 mg of lyophilized powder in a sulphur hexafluoride (SF_6) atmosphere. Its use to evaluate skeletal muscle microvasculature was recently validated [214]. Upon reconstitution with 0.9% saline and vigorous shaking for 20 seconds, it forms a dispersion with $\sim 2 \times 10^8$, or 8 µL of SF_6 microbubbles mL^{-1}. One single microbubble has a diameter of 2.5 µm with a median effective half-life of 0.97 min in men and 1.23 min in women at relevant dose [247]. After reconstitution, the microbubbles were delivered via a Vueject oscillating infusion pump (Bracco, Milan, Italy) and a low volume connection line (Acist, Eden Prarie, MN, USA) to a 20GA catheter (BD, Oxford, UK) inserted into a left antecubital fossa vein. When the microbubbles are infused long enough to fill the small vessels, the blood flow of the capillaries can be visualized. The contrast agent has no significant impact on the flow, even at the capillary level, [248] and the microbubbles resonate to the insonated frequency allowing detection of their signal while the signal reflected off the surrounding tissue is suppressed [214].

2.2.6 Application of transducer to region of interest (ROI) and imaging protocol

Using a polyethylene-housing unit (University of Nottingham Medical Engineering Unit, Nottingham, UK) and Velcro^{TM} strips (Amsterdam, Netherlands), the ultrasound transducer was secured onto the thigh over the ROI (overlying m. vastus lateralis). This position was maintained throughout the study. The L9-3 probe in the Philips iU22 scanner was used to perform the contrast imaging. The scanner was defaulted to perform cine recording at 21 Hz. sec^{-1}, contrast resolution C30, gain of 96%, cursor gain constant throughout, depth 4 cm with a 2-4 focus, working low mechanical index (MI) of 0.08 to achieve microbubbles resonation without being destroyed, high MI of 1.12 to destroy microbubbles and prospective 30 seconds video recording thereafter.

The contrast microbubbles were infused at a priming rate of 2 $mL.min^{-1}$ for 1 min followed by 1 $mL.min^{-1}$ for 2 min. The oscillating pump insured homogeneity of the microbubbles. A steady state concentration was achieved after 1 min. This was followed by 30 sec of dead space (no recordings) to allow altering the rate setting. At 1.5 min, 3 successive back-to-back 30 sec recordings were made of flash/ replenishment cycles. This made a total recording of 90 sec and a total of 3 min contrast infusion.

2.2.7 Ultrasound Doppler scans

Using Doppler ultrasound (Philips iU22 Ultrasound, Bothell, WA, USA), serial measurements were taken for conduit artery diameter and flow velocity. Femoral artery blood flow was calculated as artery diameter multiplied by flow velocity.

2.3 Analytical methods and calculations

2.3.1 Extraction of myofibrillar protein fraction and isolation of amino acids

The extraction of myofibrillar protein was done in accordance with the local standard technique described previously [249,176]. From the Eppendorfs containing aliquots of frozen muscle samples, 20-30mg of muscle tissue was removed. A homogenization buffer (HB, 10 $\mu L.mg^{-1}$ tissue) was added to the muscle tissue before it was chopped with scissors. The resulting homogenate was put in a Vibrax shaker at room temperature at a speed of 1500 rpm for 10 min. This was then centrifuged at 11000 g at 4°C for 15 min. The supernatant (containing the sarcoplasmic fraction) was stored at -20°C for further analysis on amino acid enrichment. The pallet (containing myofibrils, collagen and mitochondria) was kept on ice, washed in HB and centrifuged at 11000g twice. This was then homogenized in Mito buffer and centrifuged at 1000g. The supernatant (containing mitochondria) was stored at -20°C. The pallet (containing myofibrils and collagen) was solubilized in 0.3% NaOH and centrifuged at 13000 rpm at 4°C for 10 min. The pallet was washed with 70% ethanol and centrifuged at 3200 rpm to collect collagen. The supernatant (containing myofibrils) was further centrifuged at 3000 rpm, washed with 70% ethanol and collected by centrifugation.

The myofibrillar extract was hydrolysed in 0.05 M HCL Dowex 50W-X8-200.L^{-1} (Sigma Aldrich, Pool, UK) at 110°C overnight. The liberated AA were purified by ion exchange chromatography on Dowex H^+ resin and eluted in 2 mol of NH_4OH. AA were subsequently derivatised as their N-acetyl-N-propyl ester.

2.3.2 Determination of muscle protein-bound and intracellular phenylalanine enrichment

Determination of L-[ring-$^{13}C_6$]-phenylalanine incorporation into myofibrillar protein was analysed by gas chromatography combustion isotope ratio mass spectrometry (GC–C-IRMS), (Delta-plus XP, Thermo Fisher Scientific, Hemel Hampstead, UK). Separation was achieved on a 25 m · 0.25 mm · 1.0 µ-film DB 1701 capillary column (Agilent Technologies, West Lothian, United Kingdom). [250,251]

Gas chromatography mass spectrometry (GC-MS - Agilent-5977a, California, USA) was used to determine muscle intracellular L-[ring-$^{13}C_6$]-phenylalanine enrichment. For this, the sarcoplasmic fraction separated as described above was precipitated and the aqueous supernatant was purified using Dowex H+ resin and derivatised as its tert-Butyldimethylsilyl (t-BDMS) before being transferred to the GC-MS.

2.3.3 Calculation of fractional synthesis rate (FSR)

FSR was calculated via measurement of the increase in L-[ring-$^{13}C_6$]-phenylalanine enrichment into myofibrillar protein between two chronological biopsies. The calculations used the standard equation: FSR [expressed as $\%.h^{-1}$] = $(\Delta E_m/E_p \times 1/t) \times 100$, where ΔE_m is the change in labelling of muscle protein L-[ring-$^{13}C_6$]-phenylalanine between 2 biopsy samples, E_p is the mean L-[ring-$^{13}C_6$] free phenylalanine enrichment in the intramuscular pool, and t is the time in hours between biopsies.

2.3.4 Determination of plasma phenylalanine enrichment

Concentrations and enrichments of arterial plasma phenylalanine were determined as follows, plasma proteins were precipitated with 100% ethanol, centrifuged and the supernatant was removed and dried under nitrogen then reconstituted in 0.5 M HCl, the lipid content was removed by extraction with ethyl acetate; the aqueous phase was then dried under nitrogen, the amino acids were converted to their *tert*-butyldimethylsilyl (*t*-BDMS) derivatives using N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), and the labelling [atom per cent excess (APE)] of arterialized and venous [$^{13}C_6$]phenylalanine was determined using GC-MS, by measuring the ratio of labelled to unlabelled Phe (m/z 240 to 234). Concentrations for arterial plasma phenylalanine were determined using a [^{15}N] phenylalanine internal standard (m/z 235), with reference to standard curves of known concentrations and enrichment was confirmed with reference to a standard enrichment curve (0-10 APE).

2.3.5 Calculation of rate of appearance (protein breakdown), net protein balance and rate of disappearance (protein synthesis)

Leg protein breakdown, rate of appearance (Ra), was calculated from the arterio-venous dilution of [$^{13}C_6$] phenylalanine using the following: [(Ea/Ev) − 1] × Ca × blood flow, where Ea and Ev are the values of Phenylalanine enrichment, under steady state conditions, in both arterial and femoral venous plasma, and Ca is the average concentration in the arterial blood, blood flow (expressed as ml/100 ml leg) is adjusted for the haematocrit (Hct) to calculate plasma flow, and leg muscle volume, assessed using dual-energy X-ray absorptiometry (DXA; Lunar Prodigy, GE Medical Systems). Net protein balance (NPB) was calculated from the arterial-venous concentration difference of phenylalanine, multiplied by the plasma flow,

as corrected for Hct, also expressed per 100 ml leg muscle volume. Under steady state conditions, the rate of disappearance (R_d) i.e. synthesis, is calculated as the sum of net balance and rate of appearance (R_a). Four plasma samples were analysed to calculate postabsorptive, early and late fed phenylalanine kinetics.

2.3.6 Determination of plasma AA concentrations

To analyse AA concentrations, internal standards were added to plasma samples, before addition of urease solution at room temperature for 20 min. Samples were then de-proteinised with ice-cold ethanol for 20 min at 4°C, before centrifugation at 13,000g. The plasma AA containing supernatant was then decanted into boiling tubes and evaporated at 90°C under N_2. Dried AAs were votex mixed in 0.5M HCl, and lipids extracted in Ethyl Acetate, before evaporation to dryness. AAs were derivatised as their N-acetyl-N-propyl esters through the addition of equal volumes of acetonitrile (ACN) and MTBSTFA and heated to 90°C for 60 min. Samples were allowed to cool before transfer to autosampler vials. AA concentrations were quantified against a standard curve of known concentrations using GC-MS.

2.3.7 Determination of muscle glucose uptake

Glucose concentrations from venous and arterial samples were measured via GC-MS. Muscle glucose uptake was determined using the A-V balance method where A is the concentration of glucose in the femoral artery and V is the concentration of glucose in the femoral vein.

2.3.8 Determination of plasma hormone

Plasma insulin concentrations were measured in undiluted plasma samples using a high sensitivity human enzyme-linked immunosorbent assay: ELISA. Specific ELISA used is detailed in each chapter as appropriate. Incremental area-under-curve (AUC) analysis estimated the total hormone response to intervention and was calculated for each individual with a baseline equating the fasting mean and different means at regular intervals post intervention. Blood for GLP-1 and GIP analysis was sampled in ice-chilled P800 tubes and immediately spun down; the plasma was then stored at −20°C until analysis. Plasma samples were analysed for total and active GLP-1 plus total GIP. Samples for ghrelin were collected in ice-chilled tubes containing PHMB solution (polyhexa methylene biguanide) and HCL. Samples for PYY were collected in ice-chilled tubes containing DPP-4 inhibitor and aprotinin.

2.3.9 Calculation of microvascular and macrovascular parameters

Two video recordings were obtained (baseline and post intervention) by the end of each study. Video data were then exported to the quantification software (QLab, Philips, Andover, MA, USA) for analysis. Regions of interest were drawn avoiding areas of connective tissue and large vessels and copied into each file to ensure that regions were identical for each recording. The period immediately following flash (0.57 sec) was used to calculate the background acoustic intensity (AI), an arbitrary unit, attributable to rapidly filing larger non-exchange vessels and tissue echogenicity. The mean AI during this was calculated and subtracted from all subsequent values during that replenishment period. Then the mean AI across all three flash/ replenishment recording cycle was calculated after background correction and was curtailed at 24 sec.

The software automatically carries out calculations as described previously [252,253] where AI versus time curves were fitted to the exponential function: $y = A[1 - e^{-\beta(t - Bt)}]$, where *t* is time in seconds, *Bt* the time used for background subtraction, y is the AI at any given t, A is the plateau AI defined as MBV in mL in the ROI, and β is the flow rate constant (1itres.s^{-1}) that determines the rate of rise of AI and corresponds to the mean blood flow velocity or perfusion rate in mL per sec. of all vessels in the ROI. Then, the blood flow in mL per sec. can be defined as blood volume · blood flow velocity ($A \cdot \beta$) [254].

Leg blood flow (LBF) was calculated using measurements obtained from ultrasound Doppler scan where LBF is defined as femoral artery blood volume · blood flow velocity in the femoral artery.

Chapter 3: Characterising the incretin effect of essential amino acids (EAA) and advancing age outcome on EAA stimulated incretin hormone release

3.1 Chapter overview

Background: The oral ingestion of glucose triggers greater insulin release than a comparable glucose challenge delivered intravenously. However, whether this phenomenon - known as the 'incretin effect' - applies to ingested volumes of dietary essential amino acids (EAA) during intake of meals is ill-defined. Furthermore, since EAA (and insulin) are important components of skeletal muscle protein anabolism and muscle loss is accelerated with ageing, exploration of how ageing impacts any incretin effect of EAA is warranted. We thus explored: i) whether EAA could induce an incretin effect, and ii) whether ageing impacted this incretin effect.

Methods: A 15 g mixed EAA beverage was administered orally to two groups of younger (N=8; mean age 25.6 ± 1.5 y) and older (N=8; mean age 69.1 ± 1 y) healthy volunteers. Another group of younger volunteers (N=9; mean age 21±1 y) were given an equivalent intravenous (IV) beverage aiming to achieve matched plasma AA profiles. Oral EAA were given as a drink dissolved in an aqueous solution and consumed over 2 min. For IV delivery, 15 g of mixed EAA were infused at a rate of 133 $mg.min^{-1}$ for 15 min followed by a rate of 289 $mg.min^{-1}$ for a further 45 min. Plasma concentrations of AA, insulin, glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) were quantified over a period of 120 min. Analytically, AA were derivatised as their N-acetyl-N-propyl esters and their concentrations determined against a standard curve of known concentrations using gas chromatography mass spectrometry (GC-MS). Milliplex Map, commercial ELISA kits, were used to determine insulin and gut hormone abundance. Statistical analysis was performed in Graphpad Prism 7 via ANOVA and t-tests as appropriate.

Results: EAA produced rapid insulinaemia and aminoacidaemia with total AA, sum EAA and sum branched-chain AA matched between oral and IV groups. Insulin levels peaked at 39 ± 29 pmol.L^{-1} at 30 min following oral feeding compared to 22 ± 9 pmol.L^{-1} at 60 min following IV feeding. EAA peaked at 3395 μM at 45 min during IV infusion compared to 2891 μM following oral intake (*p*: NS). There was an approximate 45% incretin effect on calculating insulin response in the first 60 min. GIP increased following oral intake (399 pmol.L^{-1} vs 205 pmol.L^{-1} , $p<0.05$) coinciding with insulin elevations. No differences were observed between younger and older groups in the profile of insulin or incretins following oral EAA intake

Conclusion: In response to oral vs. IV plasma essential aminoacidemia, oral EAA intake induced an incretin effect associated with augmented GIP, but not GLP-1. No ageing effects were observed on EAA stimulated incretin hormone release. Thus, postprandial levels of EAA induce a GIP-mediated incretin effect, unaffected by age, supporting the notion of EAA as acute nutritive therapeutics in Diabetes and ageing.

3.2 Introduction

3.2.1 Amino acids and incretin effect

The incretin effect originally described in relation to glucose is a phenomenon where by oral glucose stimulates higher insulin release compared to a comparable glucose challenge given intravenously [104] and measured as the difference between insulin attributed to oral versus intravenous glucose load. This incretin effect is attributed to secretions of GLP-1 and GIP once glucose comes in contact with L and K cell of the gut respectively accounts for up to 70-80% of insulin secreted following orally consumed glucose. However whether such effect applies to ingested volumes of dietary EAA remains unclear.

It was previously demonstrated that consumption of oral AA does stimulate both GLP-1 and GIP secretion. Consumption of Milk, casein and whey proteins is associated with rises in GLP-1 that could extend from two to seven hours postprandially [255]. Further, individual AA such as leucine, iso-leucine, alanine and arginine are reported to stimulate insulin secretion [256]. Preliminary data from our laboratory (see Figure 3-1, a) provide evidence that EAA in the absence of other nutrients cause a sustained increase even in the active form of GLP-1 (7-36 amide), which is quickly degraded by DPP-4 while at the same time robustly stimulating insulin secretion (see Figure 3-1, b). This pilot data confirms that AAs likely represent a valid nutritional avenue by which incretin (and insulin) secretion could be stimulated, and thus underline the significant but untapped therapeutic potential for exploiting the use of AAs in order to stimulate incretins.

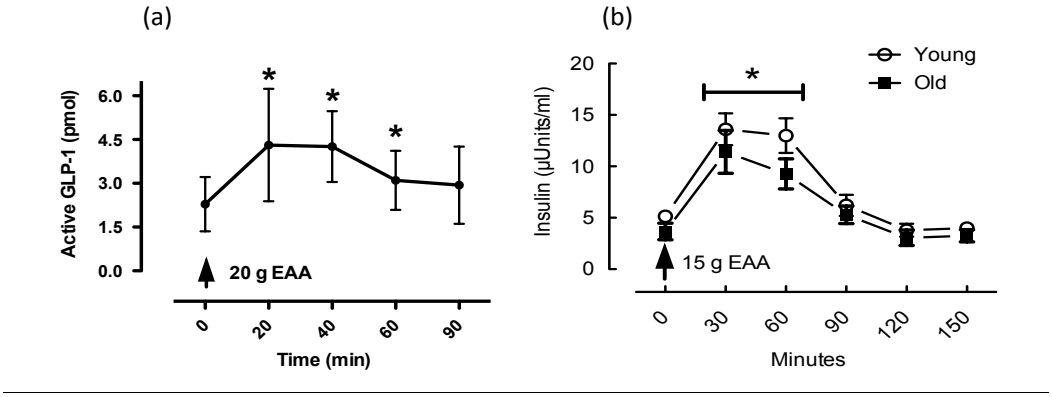


Figure 3-1: Pilot data showing plasma GLP-1 in response to EAA feeding.

Plasma insulin GLP-1 levels in response to EAA feeding in young (a) and insulin levels in young and older individuals in response to EAA oral feed (b). *=p <0.05 vs. baseline.

Taken collectively, data on AA on their own merit would not provide proof for AA capability to induce an incretin effect. However it represents solid ground to exploit the idea. The availability of commercial intravenous mixed AA made this possible. Lindgren and colleagues [95] reported incretin effect of 25% attributed mainly to rises in GIP but not GLP-1 following consumption of oral mixed AA beverage compared to a matched intravenous equivalent. In contrast, orally ingested lipids has been shown to be associated with incretin effect driven by both GLP-1 and GIP [111].

Given this body of evidence, providing optimised AA mixtures could represent a strategy for individuals with impaired glycaemia. Having the advantage of superior 'anabolic profile' compared to other dietary protein mixes [257], EAA represent a tempting target for investigating whether they possess antiglycaemic properties in the form incretin effect stimulation. If proven, this is likely to represent a key

strategy in tackling anabolic resistance seen in ageing and individuals with impaired glucose tolerance.

3.2.2 Inter-regulations between gut hormones

The regulatory effect of gut hormones on each other is still an area under exploration and ongoing research. While GLP-1 is primarily secreted in response to nutrient ingestion, other GI hormones are involved in regulating and optimising its physiological functions. GIP and insulin enhance GLP-1 secretion from L-cells. Indeed, insulin resistance has a negative impact on the L-cell secretory response [258,259]. Further, in obese subjects having reduced pre-meal ghrelin levels, ghrelin infusion is associated with improved L-cell secretion following glucose ingestion [260].

This level of interaction between GIP, GLP-1, insulin and ghrelin in addition to leptin is ultimately thought to regulate appetite through signals carried via the afferent vagal nervous system [261]. GLP-1 receptor agonist therapy is at the frontier of type 2 diabetes treatment. It remains to be investigated whether exogenous GLP-1, which escapes normal physiological regulation, would have any effect on the way these hormones interact. It is also not clear whether the ageing process is associated with altered levels of inter-regulation between gut hormones. The same perhaps applies for exogenous insulin either when delivered for therapeutic purposes or administered under a controlled experimental and investigative environment.

3.2.3 Ageing and gut hormone secretion

The regulation of appetite and satiety originate from signals initially triggered by gastric distension and then via nutrient interaction with components of the gastrointestinal tract further distally leading to the secretion of regulatory hormones. To this end, hormones including cholecystokinin (CCK), GLP-1, GIP, peptide tyrosine tyrosine (PYY) and amylin are released to limit further food intake while ghrelin release is inhibited during the prandial state; the opposite occurs in the hunger state [262,263].

The ageing process is associated with altered dietary habits related to reduction in protein and energy intake [264]. This is multi-factorial and due to changes in the choices related to macronutrient consumption, physiological changes in appetite regulation and gut endocrine physiology. These factors ultimately lead to weight loss. This per se is not necessarily an undesirable outcome, especially in middle age where weight gain is mostly fat mass. However, weight loss that occurs beyond the age of 70 is predominantly lean body mass leading to sarcopenia and its related undesirable outcomes [265].

Changes in habits related to food intake are further complicated by changes in functional aspects of gastrointestinal (GI) physiology that are likely to play an important role in nutrient deficiency and predisposition to age related sarcopenia. The expression of hunger is reduced in healthy older subjects [266]. Further, gastric distension and emptying are reduced and sense of satiety is augmented compared to younger individuals [267,268]. Delayed nutrient migration distally as a result of slowed gastric emptying leads to a sense of fullness on the one hand and a delay in appetite regulatory hormones on the other. Although these two phenomena are

at different ends of the appetite regulatory spectrum, the overall effect results in the reduction of appetite and food intake [269].

Most studies in this regard have been predominantly undertaken in response to mixed meals or glucose load. The outcome, therefore, is conflicting of whether physiological ageing affects the mode and magnitude of gut hormone secretion in response to food intake.

CCK is secreted from the I cells in duodenum and jejunum in response to nutrient presence [270]. It stimulates gallbladder contraction, pancreatic exocrine functions, a delay in gastric emptying and the overall effect of inhibition of food intake. Older populations have higher CCK levels in response to mixed meal intake and they are more sensitive to its satiating effect [271,272]. GLP-1 concentrations were reported to be similar between young and old individuals both at fasting and after infusion of intraduodenal lipids and glucose, indicating the same magnitude of L cell stimulation [271]. A more recent study suggested that older individuals have more elevated levels of GIP in comparison to young in response to mixed AA load [273].

Ghrelin is produced by the enteroendocrine cells within the epithelial layer of the stomach [274]. Its circulating levels are increased in fasting, malnourished and underweight individuals, and in those undergoing weight losing dietary regimens. Ghrelin levels fall following food intake, in particular, CHO and fats and in individuals with excess body fat contents [272,275]. In regards to PRO consumption, however, levels may go up. Up to a 35% reduction in circulating postabsorptive Ghrelin levels have been reported in older people [276], however other studies have reported no significant difference between old and young in both postabsorptive and postprandial ghrelin levels following mixed meal ingestion [277,278].

Postprandial PYY concentrations were reported to be elevated in older females [278], however other reports suggested no difference in both older men and women [267,279].

There are scarce reports in the literature on gut incretin hormone secretion in response to proteins in older people. Moreover, gut hormones responses to EAA in both young and older people have never been studied before. Further, the incretin effect has never been studied in relation to a pure EAA beverage.

The aim of this study, therefore, was to examine i) whether EAA could stimulate an incretin effect via quantification of gut incretin hormone response to an oral EAA load compared to an intravenous iso-equivalent, and ii) whether ageing impacted this incretin effect.

3.3 Subjects and methods

3.3.1 Study design

The study was approved by The University of Nottingham ethics committee and was conducted in line with the Declaration of Helsinki. Total of 25 healthy volunteers were recruited to the study. Seventeen healthy young male volunteers and eight healthy older males were recruited via local advertisement in the two sites of the study (University of Nottingham - Derby – United Kingdom and McMaster University - Hamilton – Canada). All volunteers singed an informed consent. All participants had no history of diabetes, liver disease or gastrointestinal abnormalities. They were assigned to: young group to take oral EAA, N = 8 (Y.O group), young group to take equivalent intravenous EAA, n = 9 (Y.IV group) and older group to take same oral EAA as O.Y group, n = 8 (O.O group). Volunteers were studied after overnight fasting of 8-14 hours. Please see table (3-1) for demographic details of study volunteers.

All groups reported to the study sites following overnight fasting of 8-14 hours. An 18-20-g cannula was inserted for baseline (0 time) measurement and later blood sampling. Samples were collected at 15, 30, 45, 60, 75, 90, 105 and 120 min post consumption of either 15 grams of oral EAA solution (Y.O. and O.O. groups) or following initiation of intravenous 15 grams of equivalent EAA infusion (group Y.IV.). After baseline samples, volunteers (Y.O. and O.O. groups) were asked to consume a pre-prepared oral EAA beverage. The solution was prepared the day before in 250 ml of aqueous solution, kept refrigerated overnight and warmed to room temperature in the morning of the study. The compositions of the EAA beverage were: L-histidine: 1.21 g, L-isoleucine: 1.73 g, L-leucine: 3.59g, L-lysine: 3.07g, L-methionine: 0.95g: L-phenylalanine: 0.91 g, L-threonine: 1.13 g, L-tryptophan: 0.48 g and L-valine 1.86 g [280]. Following the last sample (120

min), cannula was removed and volunteers were fed. They were kept in the study site for 30 min observation before being allowed to leave. The same protocol was applied to the eight older (65-75 years) volunteers (O.O). The final group of nine young males were recruited to have an intravenous equivalent of 15g EAA (Stepping Hill Hospital pharmacy, Stockport, UK). They reported to the study site following overnight fast of 8-14 hours. Two cannulae were inserted; one for blood sampling as described above and the second for infusion of EAA. The EAA infusate contains equivalent amount of EAA in the oral beverage. EAA were infused for a total of 60 min. Following sampling for baseline measurements, EAA infusion was started at a rate of 133 $mg.min^{-1}$ for 15 min after which the rate of infusion was increased to 289 $mg.min^{-1}$. At the start of EAA infusion, volunteers (Y.IV group) were given 250 ml H_2O to compensate for stomach distension. The aim of this infusion protocol was to try to match concentration of EAA appearance in the plasma to that of the oral EAA solution and was derived with modifications to adjust for total EAA infused from Lindgren and colleagues [95]. At the end of infusion (60 min), blood sampling continued every 15 min for another hour to complete total 120 min of sampling. At the end of the study, cannulae were removed and volunteers were fed. They stayed for monitoring period as described above. See figure 3-2 for schematic representation of study protocol.

3.3.2 Measurement of plasma AA concentrations

Plasma AA concentrations were determined as described in section 2, page 95.

3.3.3 Measurement of insulin and gut hormones concentrations

A commercial ELISA kits (Milliplex Map kit, EMS Millipore, Germany) was used to determine insulin and gut hormones concentrations. The analytes were: insulin, amylin total, C-peptide, ghrelin, GIP total, GLP-1 total, glucagon and PYY.

Table 3-1.

Parameter (mean ± SD)	Y.O. (n=8)	Y.IV. (n=9)	O.O. (n=8)	*P*
Age (year)	25.6 ± 4.4	21.6 ± 1.9	69.1 ± 3.8	****
Height (m)	1.80 ± 0.1	1.79 ± 0.0	1.7 ± 0.1	*
Weight (kg)	81 ± 11.8	78.1 ± 8.8	75.8 ± 5.1	NS
BMI ($kg.m^{-2}$)	25.04 ± 2.8	24.4 ± 2.8	25.5 ± 1.9	NS

Table 3-1: Characteristics of study participants.

Y.O: young group consumed oral EAA, Y.IV: young group given intravenous EAA and O.O: older group consumed oral EAA. **** $p < 0.0001$ O.O vs Y.O and O.O vs Y.IV. NS: not significant.

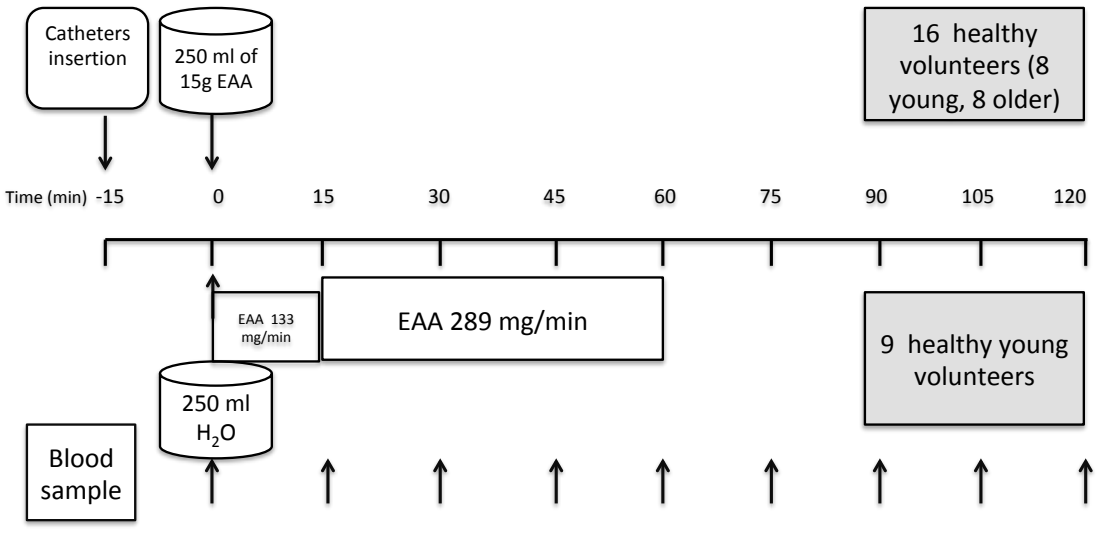


Figure 3-2: Schematic representation of the study protocol.

8 young and 8 older male volunteers had total of 15g EAA orally. Another group of 9 young male volunteers had 15g of intravenous EAA infusion.

3.3.4 Statistical analysis

Analysis was conducted using Prism 7 (Graphpad, San Diego, CA). Data are presented as mean ± standard deviation (SD). Normality of distribution was tested using D'Agostino and Pearson Omnibus normality tests. Comparison between two measures made at times before and after feeding or same time point between two groups were made via repeated measures 2 way-ANOVA with Bonferroni post-test analysis to determine significance. AA concentrations and AUC change from baseline was calculated as the ratio between time point measurement and calculated average baseline value.

3.4 Results

3.4.1 Plasma concentrations of AA

For both Y.O and Y.IV groups, profiles folds of change from baseline and AUC change from baseline were matched between the two groups for total AA, EAA and BCAA. See figure 3-3. There was no difference in the absolute concentration of total AA, EAA and BCAA between the two groups. Total AA, total EAA and total BCAA were significantly higher than correspondent baseline values at 15 min (p <0.05) in both groups with exception of total AA in Y.O group, which started to become significantly higher at 30 min. Total AA reached a peak of 5170 µM in Y.O group compared to a peak of 4523 µM in Y.IV group at 45 min (p: NS). EAA peaked at 3395 µM at 45 min during intravenous infusion compared to 2891 µM following oral intake (p: NS). BCAA reached a peak of 1678 µM in Y.O group compared to 1330 µM in the Y.IV group at 60 min. Non-essential amino acid (NEAA) concentrations remained unchanged from baseline in both groups throughout the 120 min duration of the study. Total AA concentration returned to baseline at 120 min in Y.O group, as opposed to 105 min in the Y.IV group. Total EAA and total BCAA were back to baseline at 105 and 120 min respectively. See figure 3-4.

There was no significant difference between individual EAA concentrations at all time points with the exception of threonine and histidine in the Y.IV at 45 and 60. However, change from baseline was not different for threonine at same time points.

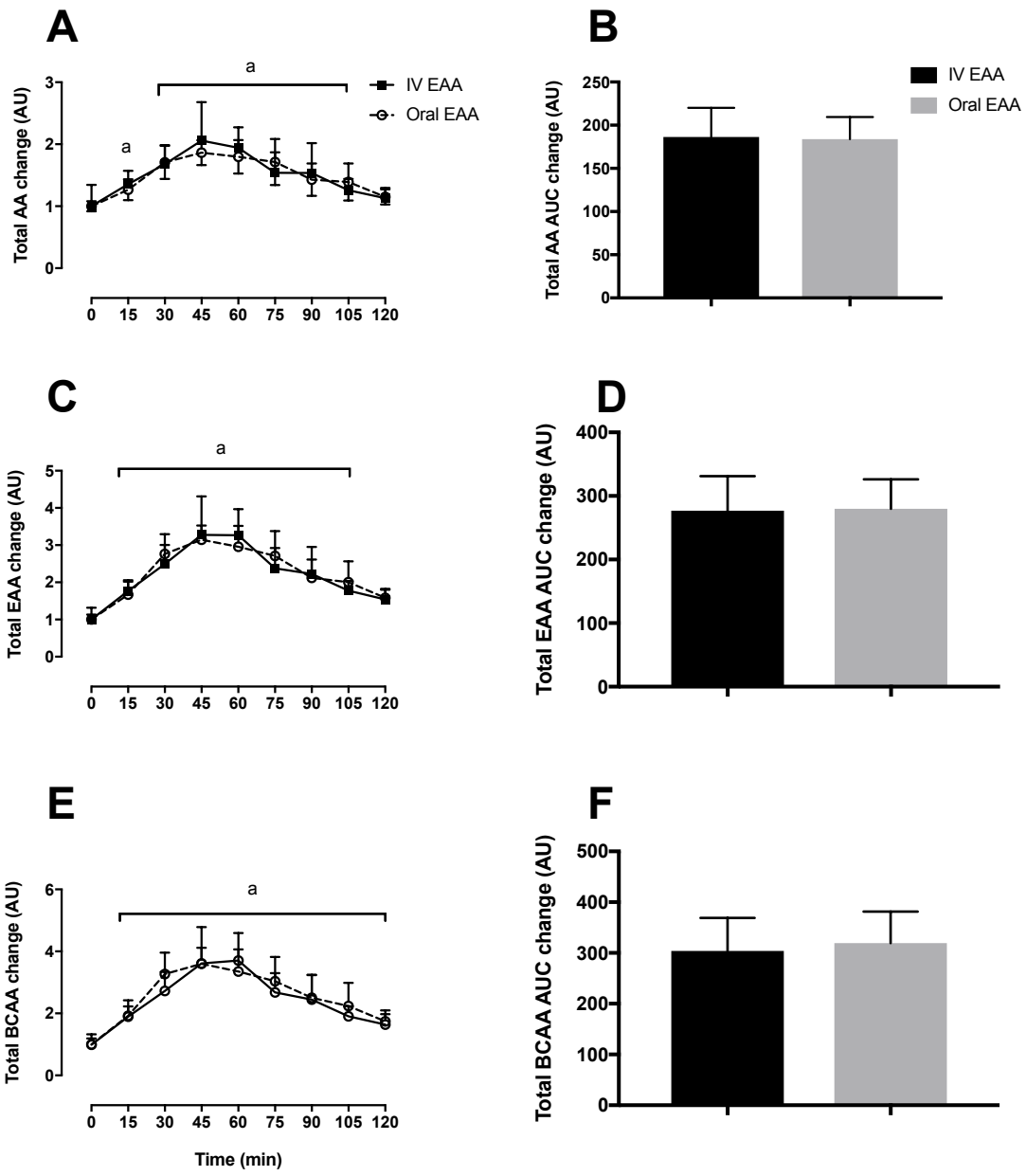


Figure 3-3: Oral vs iv EAA- AA concentration and AUC change from baseline.

A & B: Plasma total AA concentration and AUC change from baseline respectively, C & D: Plasma total EAA concentration and AUC change from baseline respectively, and E & F: Plasma total BCAA concentration and AUC change from baseline respectively. [a] Greater than respective baseline ($p < 0.05$). Main effect of feeding, $p < 0.0001$, effect of feeding type NS, Interaction $p < 0.05$. AA: amino acids. EAA: essential amino acids, BCAA: branched-chain amino acids.

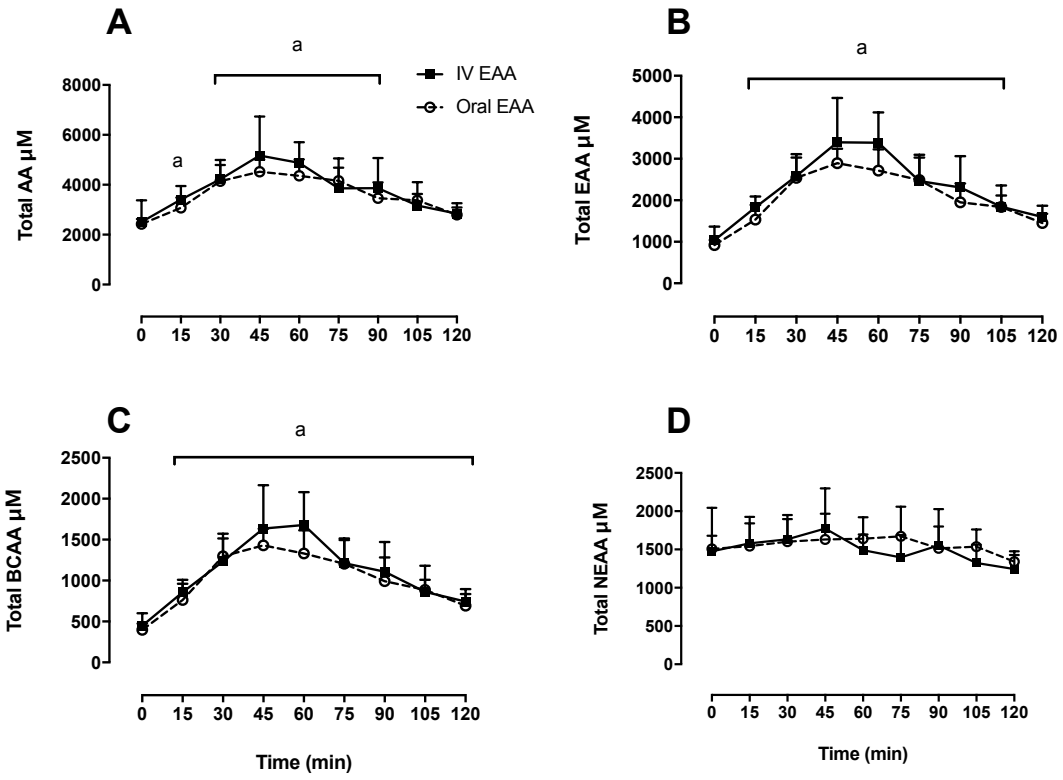


Figure 3-4: Oral vs IV EAA- Plasma AA concentrations.

A: Plasma total AA concentrations, B: Plasma total EAA concentrations, C: Plasma total BCAA concentrations, D: Plasma total NEAA. [a] Greater than respective baseline (p<0.05). Main effects of feeding, p<0.0001, effect of feeding type NS, Interaction p<0.05. AA: amino acids, EAA: essential amino acids, BCCA: branched-chain amino acids, NEAA: non essential amino acids.

3.4.2 Glucose, Insulin, C-peptide and Glucagon

Analyses were conducted in Y.O and Y.IV groups. There was no difference in glucose concentration between the two young groups at baseline. Insulin peaked at 39 ± 29 pmol.L^{-1} at 30 min in the Y.O group, which was significantly elevated compared to baseline (225% rise from baseline, p < 0.05). In comparison, insulin concentrations in Y.IV group peaked at 22 ± 9 pmol.L^{-1} at 60 min (38% rise from

baseline) and overall remained not different from baseline throughout the study. The difference in insulin rise between the two groups (the incretin effect) was 45% calculated as the difference between AUC for the first 60 min and was 11% calculated as AUC difference for the entire 120 min duration of the study. C-peptide change from baseline was 1.7 ± 0.7 folds ($p < 0.5$ vs. baseline) in the Y.O group compared to 1.3 ± 0.8 folds change from baseline (p: NS against baseline) in the Y.IV group. This rise corresponded to that seen in insulin concentrations. Glucagon concentrations rose significantly at 30 min for both groups (45 ± 22 $pmol.L^{-1}$ in Y.O group and 60 ± 23 $pmol.L^{-1}$ in Y.IV group) and continued to rise until 60 min for the Y.IV group where its concentrations were significantly higher than corresponding ones in the Y.O group (68 ± 24 vs. 31 ± 16, $p < 0.05$). See figure 3-5.

3.4.3 Incretin hormones

Total GLP-1 concentrations rose significantly compared to baseline at 30 min (83% rise) in the Y.O group (136 ± 41 vs. 74 ± 20 $pmol.L^{-1}$, $p < 0.05$). In comparison, GLP-1 concentrations in the Y.IV group stayed unchanged through the 120 min duration of the study. No difference was observed between feeding types in GLP-1 concentrations. Total GIP concentrations rose significantly following oral consumption to peak at 30 min (158% rise from baseline) in the Y.O group, while remaining unchanged throughout the study in the Y.IV group. This rise was significantly different between the two groups (399 ± 173 vs. 205 ± 68 $pmol.L^{-1}$, $p < 0.05$). GIP peak in the Y.O group coincided with insulin peak. See figure 3-5 and 3-6.

3.4.4 Amylin, Ghrelin and PYY

No difference was observed in response to the mode of feeding of EAA to concentrations of Amylin, Ghrelin and PYY. Amylin rose in both groups to peak at 30 min and 60 min in response to oral EAA and intravenous EAA feed respectively. There was no significant change from baseline in the concentration of Ghrelin and PYY in both groups. Ghrelin concentrations experienced gradual rise after 60 min in both groups. See figure 3-7.

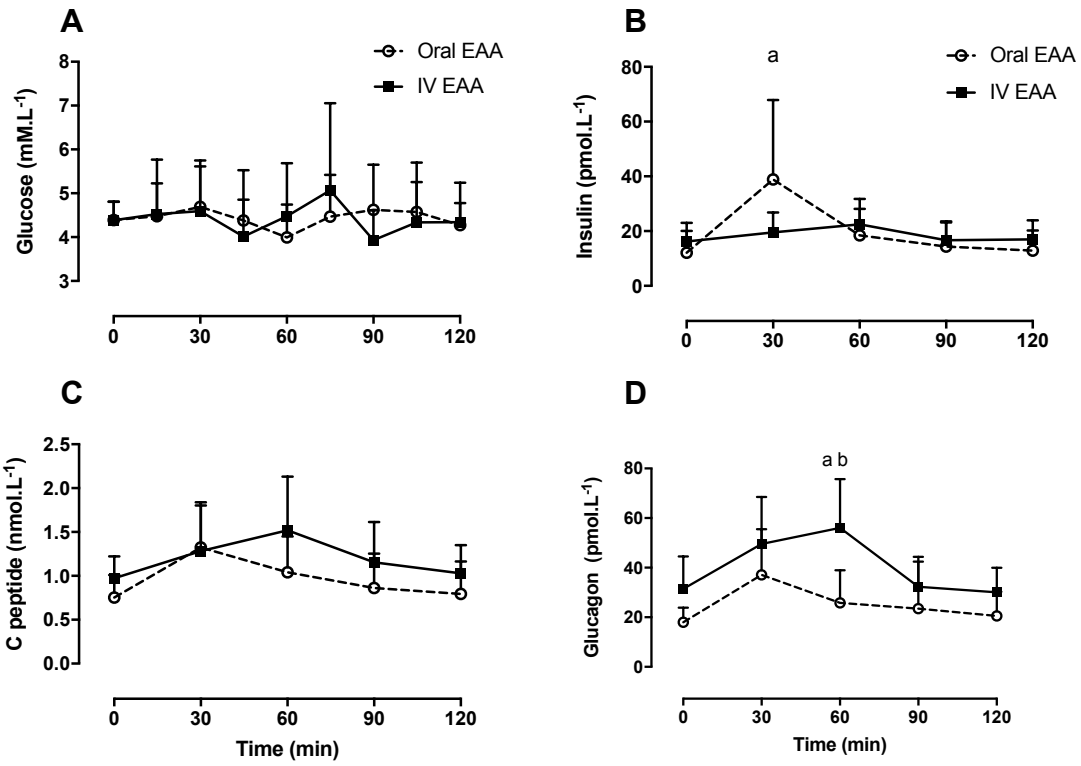


Figure 3-5: Oral vs IV EAA– Plasma hormones and glucose concentrations.

A: glucose, B: insulin C: C-peptide, D: Glucagon. [a] Greater than respective baseline (p<0.05). [b] Between feeding types (p<0.01). Main effects of feeding, p<0.0001. EAA: essential amino acids

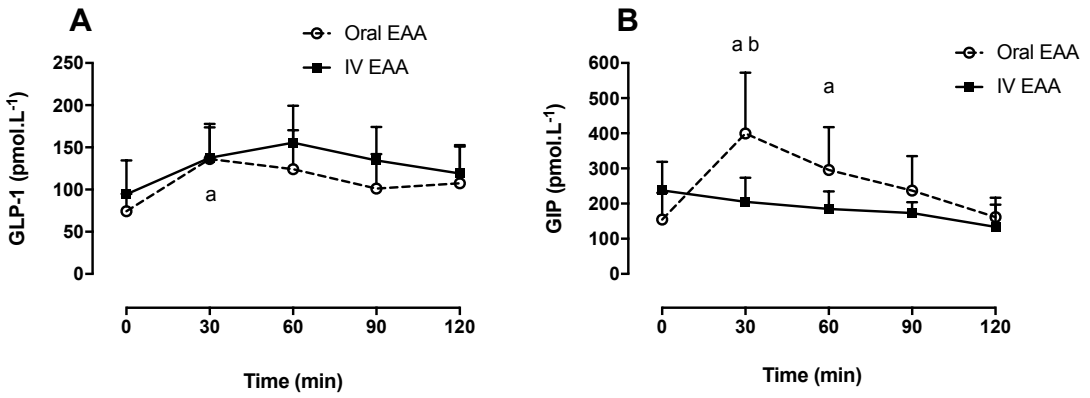


Figure 3-6: Oral vs IV EAA. Plasma incretin hormone concentrations

A: GLP-1, B: GIP. [a] Greater than respective baseline (p<0.05). [b] Between feeding types (p<0.05). Main effects of feeding, p<0.0001. Interaction NS. EAA: essential amino acids.

3.4.5 Ageing and incretin hormone release

Insulin, GLP-1 and GIP rose similarly between Y.O and O.O groups in response to oral consumption of EAA. All hormones peaked at 30 min (67 ± 50 vs. 68 ± 35 pmol.L^{-1}, 246 ± 101 vs. 261 ± 109 pmol.L^{-1} and 594 ± 264 vs. 538 ± 99 pmol.L^{-1}; Y.O vs. O.O for insulin, GLP-1 and GIP respectively, p: NS) with a slower return to baseline in the O.O group. See figure 3-8.

3.4.6 Ageing and AA acid profile

Following oral consumption of EAA beverage in the Y.O and O.O groups, AA profiles were similar between the two groups at all time points. At 15 min, total AA, EAA and BCAA were significantly elevated compared to baseline. Total AA, EAA and BCAA peaked at 60 min in both groups (Y.O vs. O.O: 4357 ± 659 vs. 4868 ± 1402 μM, 2716 ± 513 vs. 3214 ± 858 μM and 1330 ± 284 vs. 1632 ± 386 μM respectively, p: NS). See figure 3-9.

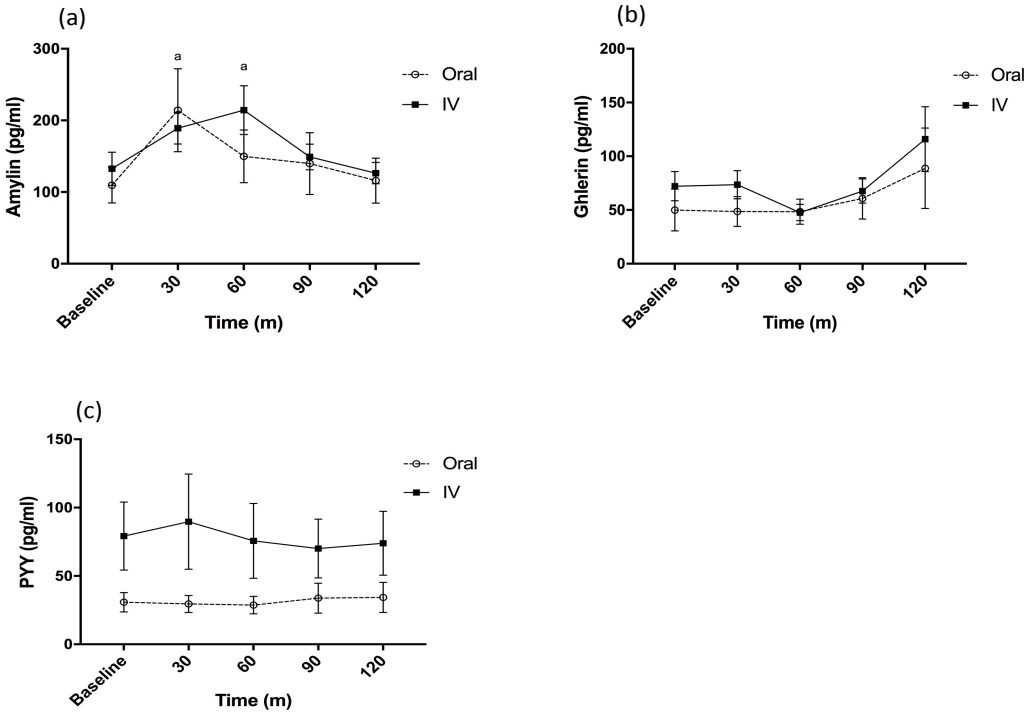


Figure 3-7: Oral vs IV EAA– Plasma gut hormones concentrations.

(a): Amylin, (b): ghrelin and (c): PYY. [a] Greater than respective baseline (p<0.05). Main effects of feeding, p<0.0001, effect of feeding type NS, Interaction NS. EAA: essential amino acids.

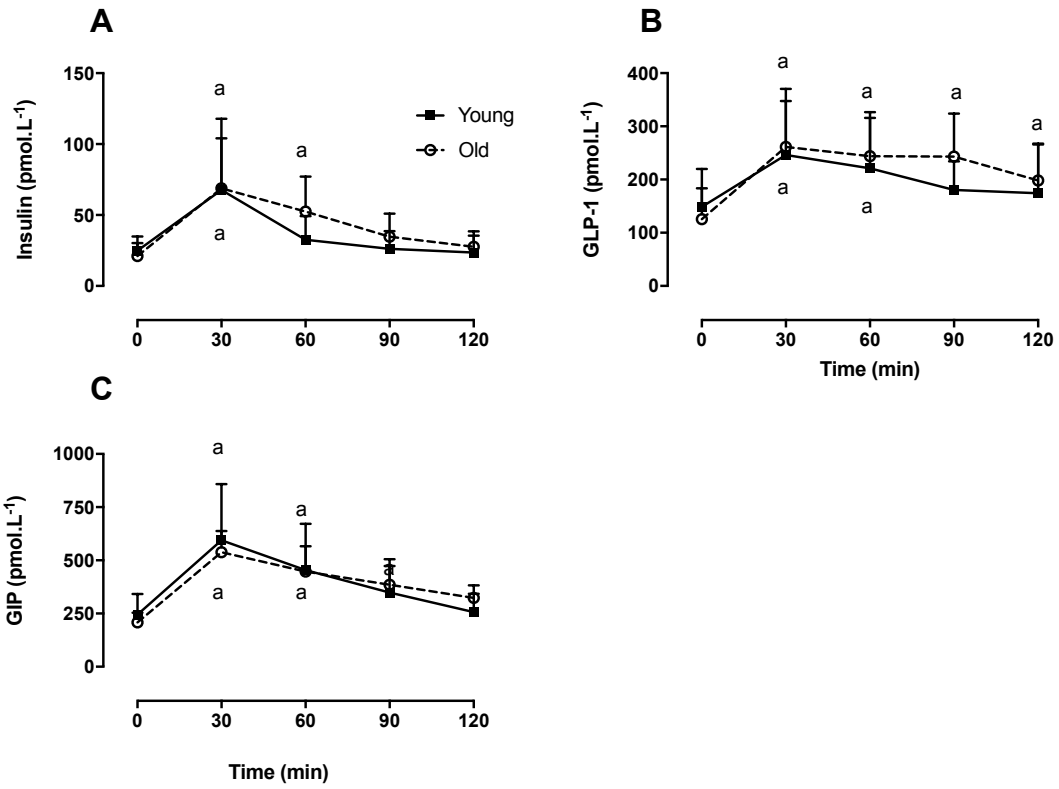


Figure 3-8: Young vs. Old– Plasma Insulin and incretin release.

A: Insulin, B: GLP-1 and C: GIP concentrations. [a] Greater than respective baseline (p<0.05). Main effects of feeding, p<0.0001, effects of age, NS, Interaction, NS. Measured by 2way ANOVA.

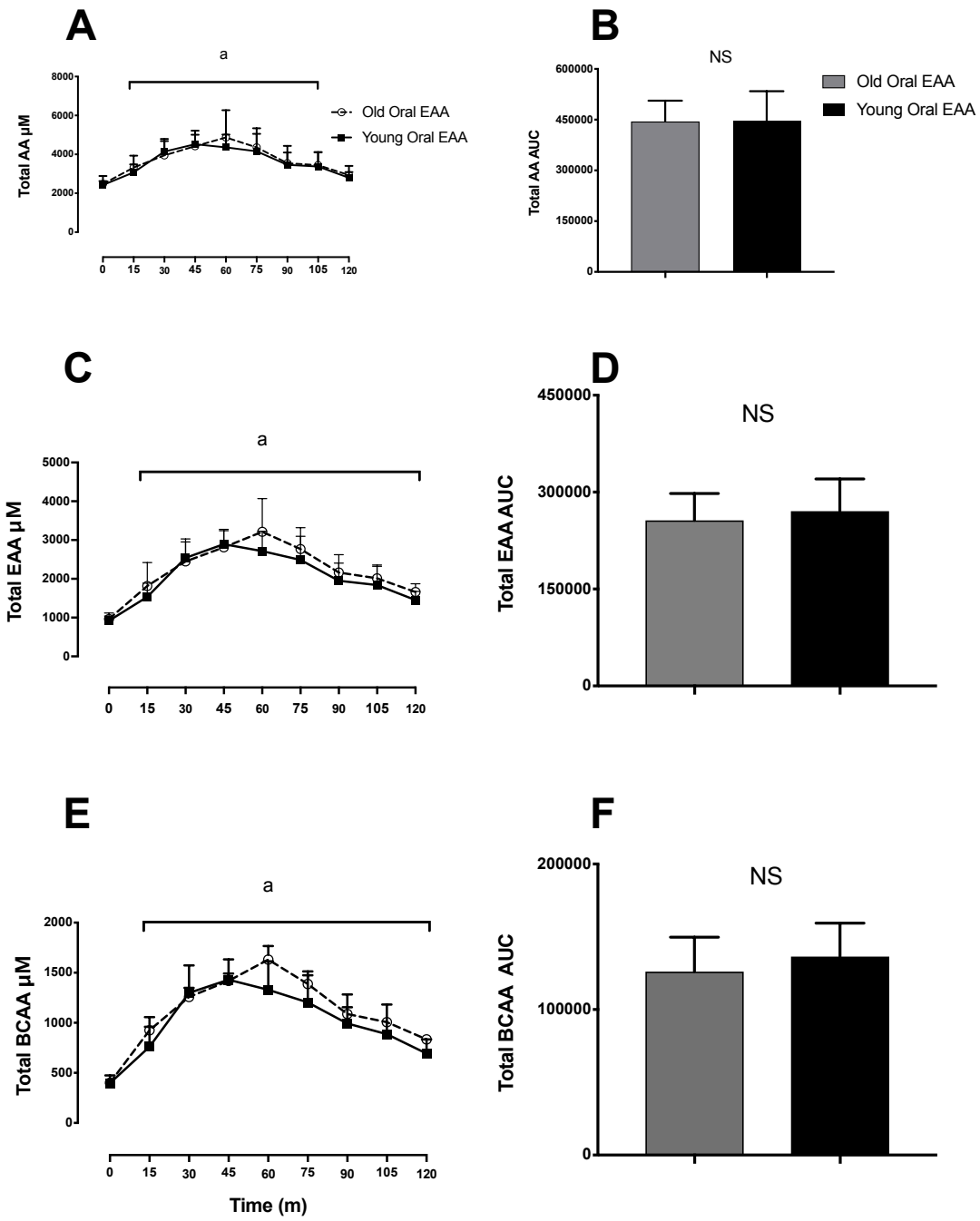


Figure 3-9: Young vs. Old plasma AA profiles.

Plasma concentrations of total AA (A), total EAA (C) and BCAA (E) plotted alongside AUC in B, D and F respectively. [a] Greater than respective baseline (p<0.05). Main effects of feeding, p<0.0001, effects of age, NS, Interaction, NS. Measured by 2way ANOVA.

3.5 Discussion

This study shows an insight into how EAA modulate gut hormone release in young and older individuals. For the first time, we tested the incretin effect of EAA and its magnitude in response to oral EAA compared to an intravenous equivalent. The study also adds to the very few studies to report on the ageing effect on incretin hormone release in response to EAA beverage. We have matched total AA, total EAA and total BCAA between Y.O and Y.IV groups.

Incretin and insulin rises in response to protein are well reported [281]. Examining the incretin effect of AA, however, needs to overcome a few hurdles. Firstly, currently used AA mixtures, especially infusions, have both essential and non-essential components of AA. This is slightly problematic when it comes to testing the incretin effect. Some NEAAs are known to be gluconeogenic precursors, namely alanine (kidney) and glutamine (liver and intestine). Indeed, glutamine is the main source of energy for the gut, especially small intestine, through gluconeogenic production of glucose [282]. Therefore, any quantification of an incretin effect thereafter would be very difficult to be ascribed solely as an AA-mediated one. Using EAA in the current study represents a novel approach to avoid this issue. Secondly, matching the mode of appearance of intravenously infused AA in plasma over time compared to that of orally consumed mixed AA beverage could be challenging. Thirdly, even when matching is achieved, it would likely lead to an exact or close match of total AA concentration between oral and intravenous modes of delivery; however, achieving a match between every single AA is difficult and unpredictable.

In this study, we showed that 15 g of EAA orally consumed could significantly increase insulin concentration compared to baseline. This rise in insulin was

matched by a significant rise in GIP between the groups, though not GLP-1. GLP-1 rose following oral intake of EAA, however concentrations were comparable compared to those in the Y.IV group. The incretin effect attributed to this rise in insulin and likely GIP was 11% calculated as total AUC over 120 min and 45% calculated as AUC for the peak period of 0-60 min. Previous reports using mixed AA have reported 25% incretin effect over a 120 min period of feeding [95]. This higher incretin effect compared to this study could be explained by an added effect from metabolism of gluconeogenic AA such as glutamine.

Stimulation of GIP but not GLP-1 by protein beverage has been reported previously [87]. Enteroendocrine cells are scattered along the length of the entire gut making it the largest endocrine organ in the body. The lack of GLP-1 stimulation might be related to absorption sites in the bowel. K-cells are predominantly present in the duodenum and ileum. By the time AA reach the jejunum, where L-cells are more predominant, there might be very little substrate left to act as a stimulus for L-cells. Another explanation could be that L-cells are not as sensitive to AA compared to K-cells. In contrast to this, data from mice and cell cultures reported good evidence of AA stimulated GLP-1 release, namely glutamine, glycine, alanine and phenylalanine [90].

In comparison to previously reported data on lack of GLP-1 stimulation by protein, our study showed that GLP-1 rose transiently at 30 min following oral consumption of EAA. It is likely that the contribution of this GLP-1 rise to the incretin effect seen is very minimal if any. However, a GLP-1 mediated neuroendocrine effect at the gut level on pancreatic β cells cannot be excluded. Therefore within the scope of this study and reported results, a contributory effect on insulin secretion from GLP-1 release cannot be entirely excluded.

Our results show that providing 15 g of EAA stimulates glucagon production as previously reported following mixed AA and whey protein [42,283]. It was previously suggested that AA and glucagon regulate each other in a feedback loop that invoves α-cells and hepatocytes [284]. In comparison, orally or intravenously delivered glucose suppresses glucagon secretion, with this effect being more pronounced with the latter mode of delivery [82]. Incretin hormones have a contrasting effect on glucagon secretion. While GIP stimulates glucagon secretion from pancreatic α-cells, GLP-1 inhibits its release. Further, pancreatic α-cells are known to have a very small number of GLP-1 receptors compared to receptors of GIP, which are ubiquitously expressed [285]. Therefore, when compared to glucose consumption, the contrasting glucagon response seen in this study is likely a combination of indirect stimulation via GIP and direct glucagon rise in response to elevated plasma amino acids [284]. In our experiment, glucagon transiently rose at 30 min (NS compared to baseline) following oral intake of EAA. However, it continued to rise in response to IV EAA until the infusion was discontinued at 60 min. This data is suggestive of the fact that EAA stimulate glucagon secretion, and this stimulation could be dose and duration dependent. However more studies needed to characterise this effect further.

We have not detected differences in insulin, GLP-1 and GIP secretion between young and older individuals in response to the dose of EAA given. This indicate that enteroendocrine cells are unaffected by ageing and remain responsive to EAA. The slower retain of hormones to baseline seen in older group could suggest slower rate of clearance, however, this needs to be further confirmed.

The incretin effect is crucial in alleviating postprandial hyperglycaemia in normal individuals. In individuals with type 2 diabetes where this effect is impaired [104], strategies that potentiate insulin secretion are vital in controlling postprandial

blood glucose excursions. Further, postprandial glycaemia has proven to be a better predictor for HbA1c compared to fasting blood glucose [286]. Therefore, optimised AA mixtures could represent a strategy for individuals with impaired glycaemia. The amount of 15 g of EAA also ensures that a sufficient meal portion is delivered per meal in a daily AA requirement of 24-56 g of protein per day for a 70 kg adult. In addition to this antiglycaemic property showed in our study, EAA have the advantage of superior 'anabolic profile' compared to other dietary protein mixes [257].

AA profiles were also similar between both age groups, indicating that digestion, absorption and splanchnic extraction are not affected by the ageing process. Therefore, nutritional strategies aimed at improving health performance and skeletal muscle health in the older population should focus on the quality of diet and appetite enhancement.

Future directions could aim at further scrutinising of the mechanisms via which EAA stimulate L-cells and whether this could be translated into more potent stimulation and hence higher GLP-1 concentration as a result of EAA beverage. We have shown that there were no differences related to gut hormones secretion in response to EAA between young and older groups. Therefore, looking at whether signals regulating appetite and anabolic resistance could represent a promising strategy to tackle sarcopenia of old age and insulin resistance state.

Our study has some limitations. The incretin effect was tested in two different groups. This was mainly related to difficulty surrounding obtaining approval to infuse EAA in one of the study sties. Despite significant increase in insulin levels from baseline in the O.Y as opposed to the Y.IV group, between group levels were not significantly different. An increase in insulin clearance following oral

consumption may explain this. We managed to achieve matching between total and individual AA with exception of threonine and histidine.

3.6 Conclusion

We have shown that EAA are able to induce an incretin effect when given orally and matched with an intravenous equivalent. Our results add to those from series of studies previously proved that the incretin effect exists following consumption of glucose, mixed AA and lipid beverage. Our results also suggest that an EAA-induced incretin effect is delivered likely through rises in GIP levels. However, a small contributory effect from GLP-1 could not be entirely excluded at least from gut mediated neuroendocrine stimulation of pancreatic β cells. We have also demonstrated that the process of physiological ageing does not adversely affect EAA-induced incretin hormone release and therefore incretin effect remains intact in old age.

Further research is needed to elucidate the mechanism via which L and K-cells sense AA in the gut, EAA mediated GLP-1 release and appetite signalling regulation in the older population. This could give way to novel nutritional and pharmacological interventions to boost or suppress incretin secretion in conditions such as diabetes in the former and post-bariatric surgery hypoglycaemia in the latter. It would also help give more understanding of nutritional needs related to the ageing process.

Chapter 4: Effect of GLP-1 on skeletal muscle protein metabolism under low and high insulin concentrations

4.1 Chapter overview

Background: Nutrition, hormones and exercise remain the main drivers for skeletal muscle protein turnover. Glucagon like peptide-1 (GLP-1), an insulinotropic hormone, is proven to have insulin-independent physiological effects on skeletal muscle metabolism but its biological effects on muscle protein synthesis and breakdown in the elderly however, have never been explored before.

Methods: 15 older men were randomised into two groups to receive postabsorptive (PA INS, n = 7, age 70 ± 0.94- mean ± SEM) and postprandial (PP INS, n = 8, age 71 ± 1.04- mean ± SEM) levels of insulin with and without GLP-1. Each study lasted 6 hours where baseline measurements were taken during the first 3 before undergoing insulin and glucose clamps. Participant received co-infusion of octreotide, vamin 14-EF with or without GLP-1. 4 muscle biopsies were taken at 90-120 min intervals. MPS was estimated via GC-MS. GLP-1 and insulin were measured using Milliplex Map, commercial ELISA kits. Statistical analysis were performed using graph pad prism 7. Data comparison was made using t-test or Repeated Measures analysis of variance (ANOVA) as appropriate, with Benforroni post tests.

Results: At post-prandial insulin concentration, GLP-1 significantly increased acute myofibrillar protein synthesis (MPS), expressed as fractional synthesis rate (FSR) in the fed state (mean 0.063 ± 0.004 with GLP-1 vs 0.102 ± 0.005 %.h.$^{-1}$ without GLP-1, $p < 0.001$); i.e. 60% vs 29% increase in MPS with GLP-1 and without GLP-1 respectively (P<0.001). No significant difference was observed between fasted and fed state in the PA INS group.

Conclusion: The observed favourable effects of GLP-1 on increasing MPS in the postprandial condition needs further research to elucidate the therapeutic potentials of GLP-1 treatment on sarcopenic clinical outcomes in elderly men.

4.2 Introduction

Skeletal muscle mass is maintained by the fine equilibrium between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Increases in MPS and suppression of MPB underlie the trophic effect of nutrition on maintaining skeletal muscle metabolism and maintenance. The positive stimulus effect that nutrients deliver on skeletal muscle is largely due to EAA. NEAA seem to play no role. Furthermore, there is strong evidence to suggest that the anabolic effect of insulin on skeletal muscle is through the suppression of MPB rather than the stimulation of MPS [169,287].

Besides the effects of both insulin and EAA, GLP-1 is emerging as a key regulator of skeletal muscle metabolism through its ubiquitously expressed receptor (GLP-1R). It is secreted from the neuro-endocrine L-cells of the gut in response to oral food intake in any form of the three major macro-nutrients (CHO, PRO, fats) [86–88]. Reports from both human and animal data suggested that GLP-1 can increase MBF and increase glucose uptake in an insulin independent manner [126,127,130,131]. This is highly relevant as both direct and indirect anabolic effects of nutrients and hormones are contingent upon postprandial hyperemia triggered by vasodilatory stimuli [3,65].

GLP-1R is ubiquitously expressed in skeletal muscle tissue. There is evidence to suggest its involvement in fundamental aspects that regulate glucose metabolism such as activation of glycogen synthase [115,122,127]. However, it has not yet been established whether the receptor is regulating any aspects of muscle protein metabolism, and therefore, it has also not yet been established whether GLP-1 in its active (7-36 and 7-37) amides or inactive (9-36 and 9-37) amides has any role

in the way and mode by which skeletal muscle handles AA from transport to the translational processes involved in muscle synthesis and breakdown.

Advancing age negatively impacts skeletal muscle leading to sarcopenia and dynapenia. This is predominantly due to anabolic resistance to protein ingestion, blunted response to suppression of muscle breakdown by insulin (metabolic resistance) and delayed glucose disposal [169,171,176,288–292]. Therefore, GLP-1 through its suggested non-insulinotropic actions on skeletal muscle might represent an important physiological intervention that could tackle age-related muscle decline. While its action has been studied in young individuals and animal models, it has not yet been explored in the context of ageing. Moreover, to date, no study has exploited GLP-1 non-insulinotropic potentials in skeletal muscle protein metabolism. This is particularly relevant in the context of ageing due to age-related decline in muscle size, strength, function and increase muscle fat deposition; all feeding into anabolic resistance of older age [143,144,169,293].

The aims of this study were to test whether GLP-1 can have an acute impact on muscle protein metabolism. We contended that GLP-1, independent of its insulinotropic properties, might have a positive impact on skeletal muscle protein metabolism such that nutritional and pharmacological strategies that enhance GLP-1 could be explored to see their impact on physiological ageing-related muscle decline.

4.3 Subjects and methods

4.3.1 Study design

The study was approved by The University of Nottingham Ethics Committee and was conducted in line with the Declaration of Helsinki. A total of 15 healthy older volunteers (aged 65-75 years) were involved in the study. They were randomised into two groups to receive low (postabsorptive) or high (postprandial) insulin concentration with or without GLP-1. They were recruited through targeted mailshots sent to a population in the local area. All volunteers were studied following overnight fasting of 8-14 hours. See Table 4-1 for the demographic details of the study volunteers. Each volunteer was studied twice at least 3 weeks apart. In one visit, volunteers received GLP-1, while in another visit the contralateral leg was studied for comparison. Volunteers were blinded regarding insulin group and in which visit they would receive a GLP-1 infusion.

On the morning of the study, volunteers reported to the Clinical Physiology Laboratory, Division of Medical Sciences and Graduate Entry Medicine – Derby-UK. Following a DEXA scan, volunteers were made to lie on a bed. Three cannulae for intravenous infusions were inserted distributed between the two forearms. This was followed by the insertion of femoral venous and arterial cannulae in the femoral artery and vein of the leg designated for study, respectively. Cannulation of femoral vessels was conducted under local anaesthesia. The area below the inguinal ligament was anaesthetised before introduction of wire guided femoral catheters under ultrasound scan guidance (Philips iU22 Ultrasound, Bothell, WA, USA).

At 0 time, infusions of $^{13}C_6$ phenylalanine was started at a prime rate of 0.4mg.kg^{-1}. This was followed by a rate of 0.6mg.kg^{-1}.h^{-1} and was continued until the end of

the study (total 6 hours). Baseline muscle biopsies were taken at 60 min (time of steady state in isotope enrichment) and 180 min after the start of the infusion. Regular venous and arterial blood samples were taken at fixed intervals. At 180 min and after taking the second baseline muscle biopsy and last baseline blood samples, infusions of insulin Actrapid (Novo Nordisk, Gatwick, UK), 20% glucose (Baxter –UK), octreotide (Novartis, Surry, UK) and mixed AA (Vamine 14-EF, Fresenius Kabi Ltd, manor park, Runcorn, UK) ± GLP-1 infusion (Bachem AG, Bubendorf, Switzerland) were started. Glucose and insulin were infused as described before [49] aiming to clamp glucose at 7-7.5 mol/l. Insulin concentrations were aimed to be kept at fasting or postprandial level depending on randomization. Octreotide was infused at a rate of 30 $ng.kg^{-1}.min^{-1}$. Vamine 14-EF was started at a prime rate of 34 $mg.kg^{-1}$ followed by a constant infusion rate of 102 $mg.kg^{-1}.hour^{-1}$ for 3 hours. GLP-1 was infused in the applicable visits at a constant rate of 1.2 $pmol.kg^{-1}.min^{-1}$ This infusion rate was shown previously to achieve physiological postprandial GLP-1 levels [131].

Regular arterial and venous samples were taken throughout the 6-hour duration of the study. A further 2 biopsies were taken at 90 min and 180 min after the start of the insulin and glucose clamps, making total biopsies equal 4 biopsies/ study/ leg. Femoral arterial blood flow was checked before second, third and fourth muscle biopsy for calculation of $^{13}C_6$ across the leg. The volunteers returned after around 3-5 weeks for the contralateral leg to be studied. See Figure 4-1 for a schematic demonstration of the protocol.

4.3.2 Specific methods

Preparation of isotope amino acid tracer, performing DEXA scan, insertion of femoral venous and arterial catheters and obtaining muscle biopsies were

performed as described in Chapter 2, page 86-89. GLP-1 (Bachem AG, Bubendorf, Switzerland) was bought in powder form and stored in −20 °C until infusion day. It was diluted in 0.9% saline solution and infused intra-arterially through the femoral artery catheter at quantities of 1.2 pmol.Kg^{-1}.min^{-1} aiming for infusion rates of 25-50 mL.h^{-1} to compact femoral artery pressure.

4.3.3 Measurements

Measurement of muscle protein-bound and intramuscular phenylalanine enrichment and rates of myofibrillar protein synthesis, leg blood flow, plasma AA, insulin and GLP-1 hormone concentrations, was performed as described in Chapter 2, page 93-96.

4.3.4 Statistical analysis and power of calculation

The sample size for this experiment was prospectively determined. This was done with the power of calculation taking a population variance of 15% based on previous laboratory data. Also taken into account was a CV of laboratory techniques of 15% to detect changes in primary endpoints (e.g., difference between rates of muscle glucose uptake between two groups under different insulin concentrations) with 80% confidence at the 5% significance level. A post hoc sample size calculation was performed with the actual data.

Graph pad prism 7 was used to conduct statistical analysis with the use of Repeated measure (RM) ANOVA and t-tests as appropriate. Data is presented as mean ± SEM. Comparison between measures was made via t test or ANOVA as appropriate with use of the Bonferroni post test to perform post test analysis of variance.

Parameter (mean ± SEM)	PA INS (n=7)	PP INS (n=8)	*P*
Age (year)	70 ± 0.94	71 ± 1.04	NS
Height (m)	1.73 ± 0.01	1.77 ± 0.03	NS
Weight (kg)	75 ± 3.24	83 ± 4.25	NS
BMI ($kg.m^{-2}$)	25.3 ± 1.04	26.2 ± 0.63	NS
Sarcopenic index	7.72 ± 0.18	8.13 ± 0.18	NS

Table 4-1: Demographic characteristics of study participants.

PA INS: postabsorptive insulin group, PP INS: postprandial insulin group.

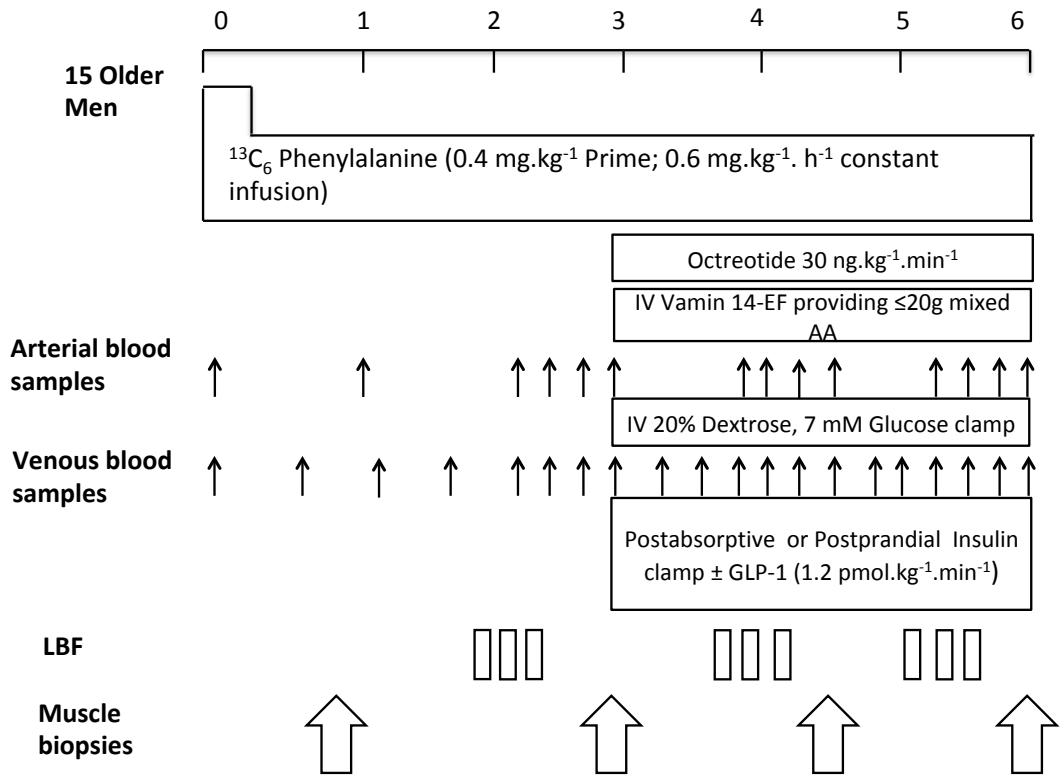


Figure 4-1: Schematic representation of the study protocol.

15 older men were studied in two groups. Muscle metabolism was assessed using IV tracer, muscle biopsies, LBF, and arterial and venous blood samples . At the midpoint insulin and glucose clamp started with simultaneous infusion of Octreotide, IV AA ± GLP-1. LBF: Leg blood flow.

4.4 Results

4.4.1 Insulin and GLP-1

Octreotide blocked endogenous insulin production within the first 5 minutes of infusion. Mean insulin concentrations achieved in the PA INS group were 6.77 ± 1.93 and 8.68.52 ± 1.83 μIU.mL^{-1} without and with GLP-1 respectively. In the PP INS group, mean insulin concentrations achieved were 25.40 ± 3.67 ($p < 0.001$ vs baseline) and 31.60 ± 5.55 μIU.mL^{-1} ($p < 0.001$ vs baseline) without and with GLP-1 respectively. In PA INS group, when GLP-1 was infused and following an initial drop in insulin concentrations, levels started to rise gradually to become significantly different from baseline values from 110 min onwards ($p < 0.05$ vs baseline). In the same group, insulin levels remained not significantly different from baseline for the entire length of the clamp period in the absence of GLP-1. In the PP INS group, no difference was observed between insulin concentrations with and without GLP-1. (Figure 4-2, A, B and C).

GLP-1 concentrations were comparable across groups in study visits when it was not infused (15.51 ± 2.59 and 17.44 ± 3.62 pmol.L^{-1} in PA INS and PP INS groups respectively) and on visits where it was infused (56.65 ± 8.60 and 63.26 ± 15.05 pmol.L^{-1} in the PA INS and PP INS groups respectively, $p < 0.01$ against baseline values). GLP-1 infusion caused marginal increase in insulin concentrations however, there was no significant difference across time points and in overall insulin AUC analysis

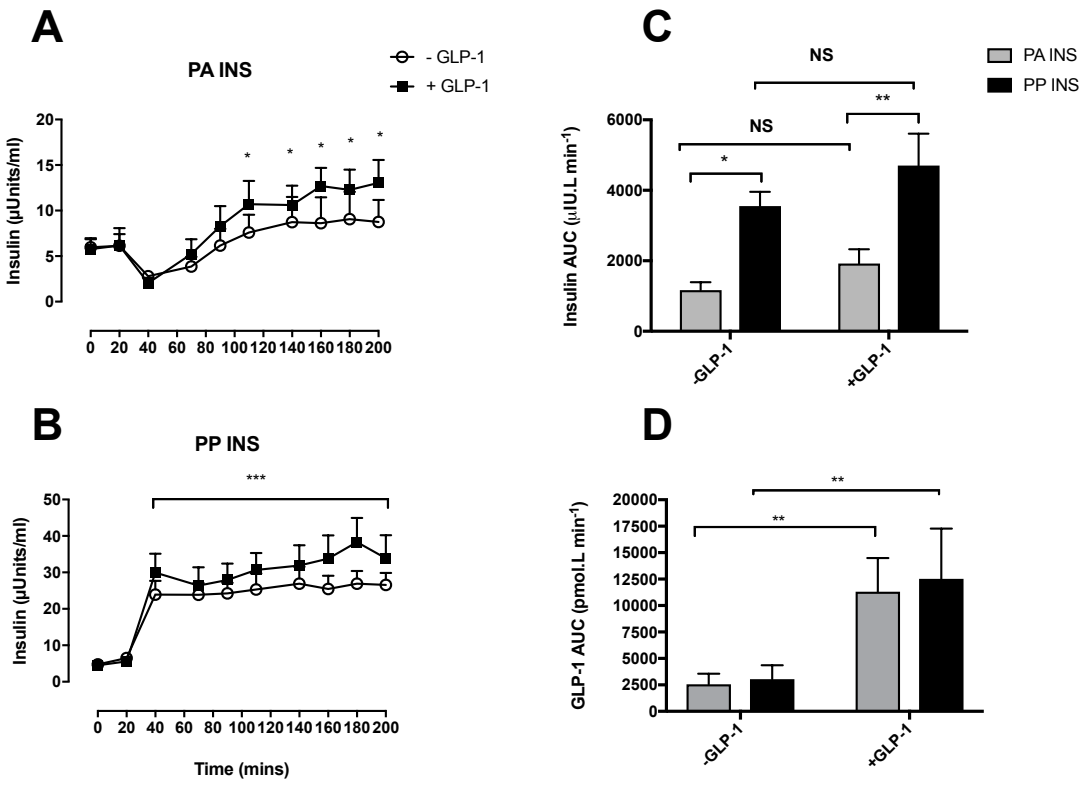


Figure 4-2: Insulin and GLP-1 profiles in both groups.

PA INS (A) and PP INS (B) profiles and AUC (C) over 180 min period with and without GLP-1. D: GLP-1 AUC in both PA INS and PP INS groups. Insulin, glucose, mixed AA and octreotide infusions started at 20 min time point. *p < 0.05, **p < 0.01, ***p < 0.001; A & B: against retrospective basal. Data presented as mean ± SEM. Measured by 2 Way ANOVA.

4.4.2 Muscle protein synthesis

Myofibrillar protein synthesis, expressed as fractional synthesis rate (FSR), was not different between fasted and fed state in the PA INS group. In the PP INS group, no difference was shown in response to postprandial levels of insulin alone (mean 0.058 ± 0.009 vs 0.075 ± 0.008 %.h^{-1}, p 0.236). However FSR increased significantly compared to fasted state when GLP-1 was co-infused with

postprandial insulin (mean 0.063 ± 0.004 vs 0.102 ± 0.005 %.h.$^{-1}$, $p < 0.001$). See figure 4-3.

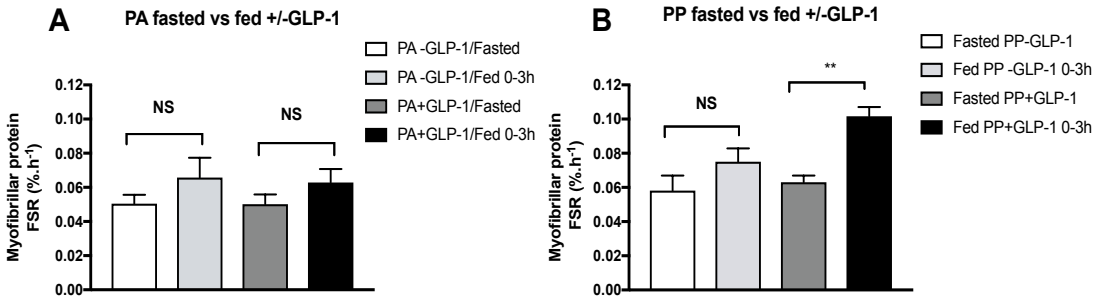


Figure 4-3: Fractional synthesis rate (FSR).

Muscle myofibrillar protein synthesis expressed as mean FSR (%/h) in PA INS group (A) and PP INS group (B) at fasted state and across 180 min postprandial period. Data presented as mean ± SEM. Measured by RM One way ANOVA.

4.4.3 Phenylalanine A-V balance

Muscle protein breakdown (MBP) was calculated as rate of appearance of $^{13}C_6$ Phenylalanine into the femoral vein. Following PA insulin infusion MPB fell in the first 90 min of fed state without and with GLP-1 compared to fasted state (32.77 ± 6.12 vs 57.91 ± 5.05 nmol.100 g leg^{-1}.min^{-1}, $p < 0.05$ and 32.11 ± 5.28 vs 67.44 ± 3.19 nmol.100 g leg^{-1}.min^{-1}, $p < 0.05$ respectively), figure 4-4 A & B. No effect was observed on protein synthesis in this PA INS group. However, protein net balance was significantly higher when GLP-1 was added, figure 4-4 B. On the other hand, in the PP INS group MPB continued to fall throughout the entire feeding period (180 min). It was significantly lower without and with GLP-1 at 180 min compared to fasted state (10.09 ± 6.05 vs 46.36 ± 1.74 nmol.100 g leg^{-}

^{1}.min^{-1} , p < 0.05 and 19.5 ± 4.48 vs 54.24 ± 5.57 nmol.100 g leg^{-1}.min^{-1}, p < 0.05 respectively), figure 4-4 C & D. Compared to insulin alone in this group, addition of GLP-1 seems to have favourable effect on protein balance in the first 90 min compared to baseline (38.99 ± 3.04 vs -13 ± 3.04 nmol.100 g leg^{-1}.min^{-1}, *p* <0.001), figure 4-4 D.

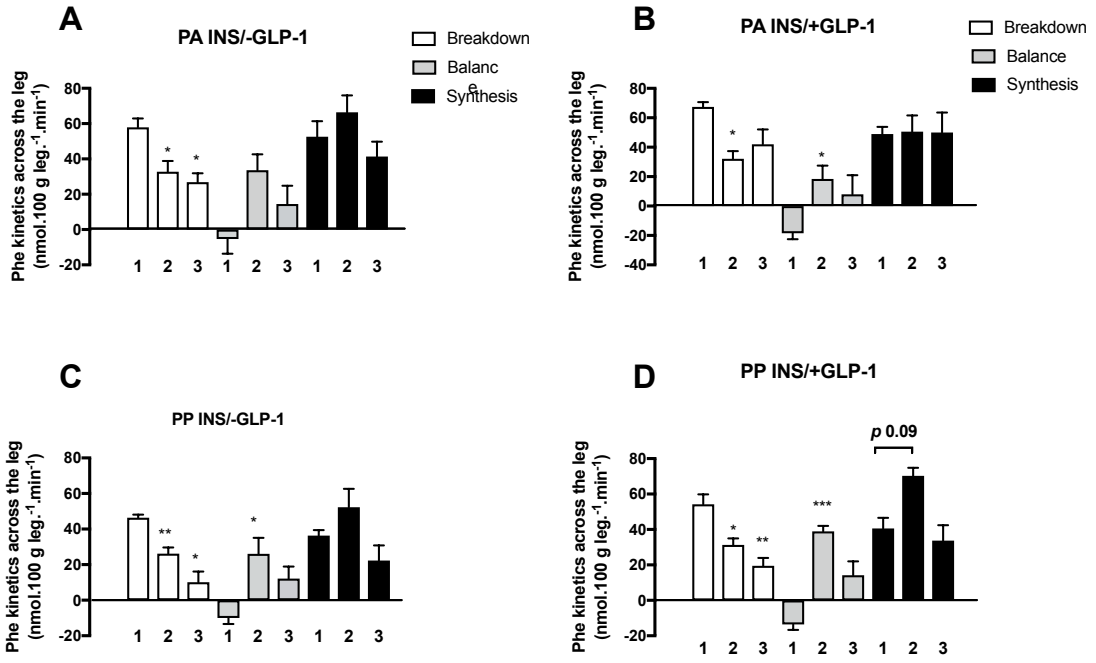


Figure 4-4: Phenylalanin kinetics across the leg.

Phe kinetics across the leg – At postabsorptive (A & B) and postprandial (C & D) insulin levels with and without GLP-1 at fated state, fed state 0-1.5 h and fed state 1.5-3 h (This is 1,2,3 respectively as shown in the figure). $^{*}p$ < 0.05, $^{**}p$ <0.001 vs fasted. Data presented as mean ± SEM. Measured by RM One-way ANOVA.

4.5 Discussion

From my knowledge and review of the literature, this was the first study undertaken to examine the effect of GLP-1 on skeletal muscle protein metabolism. This was conducted under different concentrations of insulin in experimental fed state conditions in older age. No study was conducted in this age group before to examine such effect.

In terms of major findings of this study, we have demonstrated that: a) Despite well-described age related anabolic resistance to substrates and hormones, there is positive response to GLP-1 under postprandial conditions in terms of muscle protein synthesis, b) phenylalanine kinetics across the leg indicate stronger effect for GLP-1 in muscle protein net balance likely due to combination of significant reduction in muscle protein breakdown (MPB) and an increase, albeit not significant, in muscle protein synthesis, c) as previously demonstrated by various research groups, insulin is able to suppress MPB when at both postabsorptive levels and postprandial levels, d) GLP-1 has no added benefits to postabsorptive insulin on overall muscle protein net balance.

The use of $^{13}C_6$ phenylalanine over the entire period of the study (6 hours) allowed estimation of FSR and temporal assessment of phenylalanine kinetics across the leg. This technique coupled with the use of gas chromatography - mass spectrometry enabled higher accuracy in studying the dynamic responses to muscle protein turn over in response to physiological stimuli (nutrition, exercise and hormones). The co-infusion of mixed AA ensured AA availability throughout the 3 hour fed period. The study showed no incremental effect on MPS over time. The same effect was also demonstrated when postprandial insulin levels were used with no GLP-1. This observation is compatible with anabolic resistance

observed with ageing [177]. In the younger population in comparison, lower levels of insulin in the presence of hyperaminoacidaemia are able to stimulate MPS [249].

The addition of GLP-1 delivered further stimulation of postprandial MPS compared to the fasted state. This effect and the signalling pathways underpinning it would need to be further investigated. We think this is likely to be a non-insulinotropic effect and represented 60% increase from baseline as opposed to 29% increase from baseline without GLP-1 at the same level of insulinaemia, albeit not significant.

MPB was suppressed for the entirety of the fed period, with the exception of when GLP-1 was given at postabsorptive insulin state during which MPB returned to levels comparable to the fasted state. In normal physiological conditions, GLP-1 is a postprandial hormone released upon arrival of nutrients to intestinal L-cells. Its effect on extrapancreatic peripheral tissues is likely to be a neuroendocrine one rather than hormonal due to rapid degradation by DPP-4. Therefore the presence of the combination of postabsorptive level of insulin and high GLP-1 levels are merely experimental to exploit the influence of insulin into its extrapancreatic functions. Having said that, current pharmacological preparations used for treatment of type 2 diabetes in the form of GLP-1 agonists and DPP-4 inhibitors could allow the presence of sustained elevated levels of GLP-1 in the postabsorptive state. Whether this sustained elevated levels of GLP-1 are of any added benefits to muscle protein kinetics, remains to be elucidated.

Sarcopenia remains a considerable challenge in advancing age due to adverse clinical outcome including mortality [11]. Certain chronic conditions such as diabetes represent an accelerated form of sarcopenia [155]. There is evidence also that patient with diabetes metabolically behave similarly to older individuals with

anabolic resistance [294]. In a recent study, it was shown that the antiglycaemic agents DPP4 inhibitors are associated with higher levels of GLP-1 in elderly patients with diabetes compared to other antiglycaemic medications. Those higher levels of GLP-1 were positively correlated to better sarcopenic outcome measures such as muscle mass, muscle strength and physical performance (hand grip and gait speed) [295]. It remains unclear whether this is a direct GLP-1, DPP-4 inhibitor effect or if other mechanisms are involved.

This study confirmed an acute effect of GLP-1 on postprandial MPS. This could inform further studies to look at the role of pharmacological GLP-1 preparations on muscle outcomes in elderly individuals. Another reason to study this is because energy deficit diets (including VLCD) is associated with lean muscle mass loss. Evidence of GLP-1 (a drug used to facilitate weight loss via energy deficit) to facilitate MPS will benefit future strategy to lose weight and treat obesity without detrimental effect on muscle health. With the infusion of native GLP-1 in this study proving to enhance muscle protein turnover acutely, the currently available pharmacological GLP-1 preparation (in the form of GLP-1-r agonist) might provide a further advantage due to their ability to achieve sustained elevated levels of the hormone. However, whether stimulation is directed to myocytes or vascular endothelium to elicit such effect remains to be further investigated. Three stages for sarcopenia were described by the EWGSOP: pre-sarcopenia, sarcopenia and severe sarcopenia [296]. The first stage only characterised by reduced muscle mass without change in muscle function and physical performance, and therefore, represents a crucial time window for intervention.

Limitations: The A-V balance technique relies on the measurement of amino acid that is of constant intracellular concentrations i.e., neither synthesised nor catabolized in skeletal muscle cells. This technique when performed solely relying

on concentrations of a labelled AA tracer (phenylalanine in this study) in the artery and vein, does not take into account cellular reutilization of AA released into the circulation by breakdown and therefore rates of synthesis and breakdown could be under estimated and may not correlate well with FSR data. Furthermore, A-V balance data presented are not necessarily coinciding with muscle biopsy times to allow timed parallel comparison to take place.

4.6 Conclusion

For the first time, the effect of GLP-1 on muscle protein turnover has been evaluated. There is no clear evidence to show that GLP-1 have positive effect on protein turnover parameters in the postabsorptive state. There was reduction in MPB related to insulin and nutrition. Crucially however, GLP-1 seems to have an acute positive effect on MPS under postprandial conditions. The mechanism for this is unclear and further studies are required to investigate the intracellular mechanisms underlying this. GLP-1 based therapy could represent an avenue for further clinical trials to elucidate its full potential on clinical outcomes and parameters to prevent sarcopenia of older age.

Chapter 5: Effect of GLP-1 on skeletal muscle microcirculation and glucose metabolism in older age under high and low insulin concentrations

5.1 Chapter overview

Background: GLP-1 is an insulinotropic gut hormone, previously shown to increase microvascular recruitment (MRV) in both rats and humans and glucose uptake in rats. However, its effects in both young and older age on postprandial muscle metabolism and glucose uptake have never been tested before.

Methods: Skeletal muscle microvascular recruitment (MVR) was investigated using real time contrast enhanced ultrasound (CEUS) in 15 older men randomised to receive postabsorptive (PA INS, n = 7, age 70 ± 0.94- mean ± SEM) and postprandial (PP INS, n = 8, age 71 ± 1.04- mean ± SEM) levels of insulin with or without GLP-1 following overnight fast. Following baseline measurement, volunteers were put under experimental clamp conditions with intravenous glucose, insulin, mixed amino acids and octreotide. Muscle glucose uptake was determined by measurement of arterial-venous (A-V) balance from samples obtained from femoral artery and vein and was analysed via GC-MS.

Results: GLP-1 infusion produced significant increase in microvascular blood volume (MBV) in PA INS group but not PP INS, $p < 0.05$. There was also significant increase in microvascular blood flow (MBF) in both PA INS ($p < 0.01$) and PP INS ($p < 0.05$) state. No effect was observed on microvascular flow velocity (MFV). Glucose uptake was unaffected by GLP-1.

Conclusion: GLP-1 under physiological postprandial levels positively affects MVR in older age. No effect shown on glucose uptake. This observation should form a basis for future studies to provide a deeper understanding of the relationship between skeletal muscle microcirculation and glucose uptake. The aim of this will be to try to explore how the demonstrated GLP-1 effect on MVR could be translated into improved glucose utilisation.

5.2 Introduction

GLP-1 is a postprandial hormone secreted upon direct nutrient contact with L-cells of the small intestine. Its metabolic effects are largely understood within the context of its ability to stimulate pancreatic β-cells to secrete insulin in anticipation of rises in blood glucose following nutrient intake. GLP-1 secretion also leads to inhibition of glucagon secretion from neighbouring α-cells. Insulin regulation of skeletal muscle glucose metabolism involves both pre and post-receptor signalling. It is involved in key functions of MVR and trans-endothelial transport. Once the insulin receptor is stimulated, translocation of GLUT-4 to the cell membrane follows to internalise glucose molecules. In addition, post-receptor signalling promotes key functions that promote cell proliferation and survival through the MAPK pathway [33–35].

However, GLP-1R is not solely expressed in pancreatic β-cells. Extrapancreatic receptor expression has been shown in the skeletal muscle, vascular endothelium, gastric cell, brain and heart. There is strong data to support the ability of GLP-1 to promote a sense of satiety in the prandial state, slow gastric emptying, and skeletal muscle MVR and also to improve myocardial survival following ischaemic insult [297] . Animal data suggest its ability to enhance skeletal muscle glucose uptake in rats examined under EHC experimental environment [112–118,127,130,298].

It is unclear whether the ability of GLP-1 to enhance skeletal muscle MVR would extrapolate to enhance glucose uptake independent of insulin levels. Further, whether GLP-1 could independently modulate insulin receptors at the surface of myocytes remains inconclusive. Being a prandial hormone, under normal physiological conditions, levels of GLP-1 at the postabsorptive are likely to be negligible where blood glucose levels are between 3.5 – 6.0 $mmol.l^{-1}$ and insulin

concentrations at 5-10 μIU.mL^{-1}. Among individuals with diabetes however, where blood glucose dysregulation resulted in higher postabsorptive glucose levels compared with normal individuals, due to insulin insufficiency and/ or insulin resistance. Further, since pharmacological data from individuals with diabetes receiving GLP-1R agonist-based therapy have shown improved postabsorptive glucose profiles [299], it is important to investigate whether GLP-1 enhances glucose metabolism even in insulin deficient individuals, provided glucose levels are at postprandial levels. Hence, the mode by which GLP-1 influences glucose metabolism could well be independent of insulin.

Both insulin and glucose stimulate skeletal muscle glucose uptake, albeit via different mechanisms. Post-receptor signals via PI3-Kinase lead to GLUT-4 migration to the cell membrane. On the other hand, the presence of significant hyperglycaemia stimulates glucose uptake via a Ca^{+2} dependent mechanism [300]. In addition, studies have also shown that the combination of hyperglycaemia and hyperinsulinaemia stimulates splanchnic and peripheral glucose uptake more than the effect of each in isolation [301]. Further, recent studies that identified the GLP-1 effect on peripheral glucose uptake have done so under hyperinsulinaemic euglycaemic conditions [130]. Therefore, there is increasing need to assess the effect of GLP-1 on peripheral tissue glucose uptake under postprandial (raised insulin and glucose levels) and postabsorptive (low insulin and glucose levels) metabolic conditions and indeed elucidate if GLP-1 has any further effect on peripheral tissue glucose utilisation and insulin effectiveness.

In the postabsorptive state, skeletal muscle microvascular blood supply is considerably less compared to that of other body organs (only 3-5% of that of the kidney, liver or intestine) [60]. Postprandially, the effectiveness of insulin action on skeletal muscle is largely dependent on postprandial hyperaemia for effective

delivery of nutrients and hormones (insulin) to the very terminal capillaries within skeletal muscle. The binding of insulin to vascular endothelium leads to nitric oxide (NO) dependent vasodilation of terminal arterioles and therefore enhancement of its own delivery and that of other nutrients. Insulin also plays a pivotal role in transendothelial transport of glucose to the interstitium wherein it is ready for trans-membrane internalization via insulin/IRS/PI3-K stimulated GLUT-4 translocation to the cell membrane.

Several studies performed in both human and rat skeletal muscle have suggested that the combination of GLP-1 and insulin has a synergistic effect when compared to insulin alone with regard to MVR. The mechanism by which GLP-1 modulates vascular tone or affects the vascular endothelium and transendothelial movement of nutrients and hormones is not fully understood but Wei et al. have previously shown that GLP-1 induced increase in skeletal muscle microcirculation are NO dependent. In their experiments on rats, the addition of NO synthase (NOS) inhibitor negated GLP-1 mediated MVR and glucose uptake [127]. In the same experiments, the inhibition of NOS via N^G-nitro-L-arginine methyl ester (L-NAME) lead to the abolishment of these metabolic benefits. In vivo studies and studies in cultured endothelial cells demonstrated an increase in PKA upon infusion and incubation of GLP-1 respectively. This was associated with the stimulation of endothelial NOS (eNOS) and increased levels of NO, an effect that was negated when H89 (PKA inhibitor) was added. This suggests that PKA is the main post GLP-1R signalling pathway, which activates eNOS phosphorylation and NO production.

Advancing age is reported to be linked to altered microvascular responses to insulin and exercise. Older individuals tend to have a reduced and/ or delayed response to insulin. Amino acid stimulated rises in insulin levels were associated

with significantly less remarkable microvascular response in older individuals compared to younger individuals. Further, older individuals' microvascular responses to mixed meals were significantly delayed, i.e. older individuals took 120 min post feed to generate significant microvascular responses compared to 60 min in younger individuals [302].

The direct link however between ageing and impaired glucose disposal remains controversial despite the close link between microvascular response and glucose disposal. Luzi et al. reported impaired glucose uptake in middle-aged individuals [292]. On the other hand, skeletal muscle glucose uptake was unchanged between young and old individuals in response to local hyperinsulinaemia [177]. In addition, young and older individuals showed similar whole body glucose uptake when subjected to hyperglycaemic hyperinsulinaemic conditions [303]. However, other age-related factors such as sedentarism, increased muscle fat deposition and disorders of the vascular system such as hypertension and hyperlipidaemia could well be strong contributors independent of the ageing process *per se*.

We hypothesise that GLP-1 regulates key aspects of postprandial skeletal muscle microcirculation that are likely to reflect positively on glucose utilisation. Therefore, the aims of this present study were to test whether GLP-1 would have added benefits in skeletal muscle glucose uptake and MVR in older individuals. The study also aimed to examine whether such an effect (if present) is affected by different degrees of insulinaemia.

5.3 Material and methods

5.3.1 Subjects and study design

The study was approved by The University of Nottingham Ethics Committee and was conducted in line with the Declaration of Helsinki. A total of 15 healthy older volunteers (aged 65-75 years) were involved in the study. They were randomised into two groups to receive low (postabsorptive) or high (postprandial) insulin concentration (PA INS and PP INS group respectively) with or without GLP-1. Volunteers were recruited through targeted mailshots sent to the population in the local area. All volunteers were studied following overnight fasting of 8-14 hours. See Table 5-1 for demographic details of study volunteers. Each volunteer was studied twice at least 3 weeks apart. In one visit volunteers received GLP-1 into the femoral artery in one leg, while in the other visit the contralateral leg was studied for comparison without GLP-1. Volunteers were blinded to which visit they would receive a GLP-1 infusion and which insulin level group they were allocated to.

On the morning of the study, volunteers reported to the Clinical Physiology Laboratory, Division of Medical Sciences and Graduate Entry Medicine – Derby-UK. Following a DEXA scan, volunteers were made to lie on a bed. Three cannulae for intravenous infusions were inserted distributed between the two forearms. This was followed by the insertion of femoral venous and arterial cannulae in the femoral artery and vein of the leg designated for study, respectively. Cannulation of femoral vessels was conducted under local anaesthesia. The area below the inguinal ligament was anaesthetised before introduction of wire guided femoral catheters under ultrasound scan guidance (Philips iU22 Ultrasound, Bothell, WA, USA).

At 0 time, venous and arterial blood samples were taken and continued afterwards at fixed intervals. A baseline measurement microvascular parameters was also under taken using CEUS. Following baseline measurements, infusions of insulin Actrapid (Novo Nordisk, Gatwick, UK), 20% glucose (Baxter –UK), octreotide (Novartis, Surry, UK) and mixed AA (Vamin 14-EF (Fresenius Kabi Ltd, Runcorn, UK) ± GLP-1 infusion (Bachem AG, Bubendorf, Switzerland) were started. Glucose and insulin were infused intravenously as previously described [49] aiming to clamp glucose at 7-7.5 mol/l. Insulin concentrations were aimed to be kept at postabsorptive or postprandial levels depending on randomisation. Octreotide was infused at a rate of 30 $ng.kg^{-1}.min^{-1}$. Vamin 14-EF was started at a prime rate of 34 $mg.kg^{-1}$ followed by a constant infusion rate of 102 $mg.kg^{-1}.hour^{-1}$ for 3 hours. GLP-1 was infused into the femoral artery in the applicable visits at a constant rate of 1.2 $pmol.kg^{-1}.min^{-1}$

Regular arterial and venous samples were taken throughout the 3-hour clamp period. A further measurement of CEUS was undertaken. At the end of the study volunteers participants were fed and observed for 0.5 an hour before allowed home. Volunteers returned after at least 3 weeks for the contralateral leg to be studied. See table 5-1 for subjects' characteristics and figure 5-1 for a schematic representation of the protocol.

Parameter (mean ± SEM)	PA INS (n=7)	PP INS (n=8)	*P*
Age (year)	70 ± 0.94	71 ± 1.04	NS
Height (m)	1.73 ± 0.01	1.77 ± 0.03	NS
Weight (kg)	75 ± 3.24	83 ± 4.25	NS
BMI ($kg.m^{-2}$)	25.3 ± 1.04	26.2 ± 0.63	NS
Sarcopenic index	7.72 ± 0.18	8.13 ± 0.18	NS

Table 5-1: Demographic characteristics of study participants.

PA INS: postabsorptive insulin group, PP INS: postprandial insulin group.

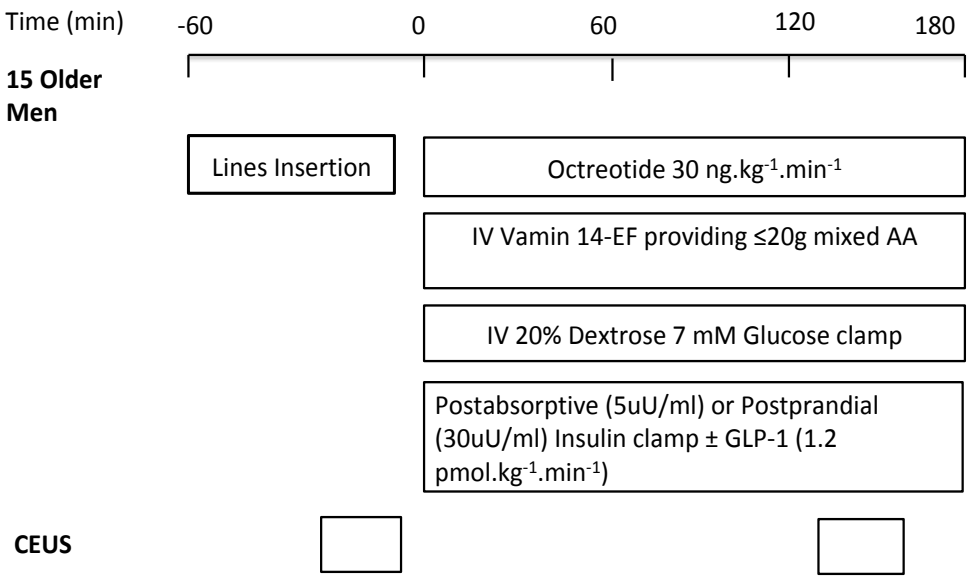


Figure 5-1: Schematic representation of the study protocol.

15 older men were studied in two groups of 7 and 8. MVR was assessed via CEUS. At the midpoint insulin and glucose clamp started with simultaneous infusion of Octreotide, IV AA ± GLP-1. MVR: microvascular recruitment. CEUS: contrast-enhanced ultrasound.

5.3.2 Specific methods

Preparation of Sonovue™ contrast agent and use of CEUS was performed as described in Chapter 2, page 90-91. GLP-1 (Bachem AG, Bubendorf, Switzerland) was bought in powder form and stored in −20 °C until infusion day. It was diluted in 0.9% saline solution and infused intra-arterially through the femoral artery catheter at quantities of 1.2 $pmol.Kg^{-1}.min^{-1}$ aiming for infusion rates of 25-50 $mL.h^{-1}$ to compact femoral artery pressure.

5.3.3 Measurements

5.3.3.1 Measurement of insulin, GLP-1, plasma AA

The measurement of plasma insulin, GLP-1 was performed as described in chapter 2, page 96.

5.3.3.2 Measurement of skeletal muscle microcirculatory parameters

Baseline CEUS measurement was performed before start of clamp period. A second CEUS measurement was performed at 120 min after start of insulin and glucose clamps. CEUS data recorded as videos where two recordings were made by the end of each study. Recordings were then exported to the quantification software (QLab, Philips, Andover, MA, USA) for analysis of MBV, MFV and MBF as described in chapter 2, page 96-97.

5.3.3.3 Glucose measurement

Glucose uptake was measured as A-V difference, where A is concentration of glucose in the femoral artery and V is glucose concentration in the femoral vein. Glucose concentrations for A-V balance were measured via GC-MS. During insulin and glucose clamp, venous glucose measurements were performed at intervals of 5-10 min using YSI machine (YSI UK limited, Hampshire, UK). The results from venous samples were used to adjust the rate of glucose infusion to keep levels between 7.0 – 7.5 mmol.l^{-1}.

5.3.3.4 Power of calculation and statistical analysis

The sample size for this experiment was prospectively determined. This was done with the power of calculation taking a population variance of 15% based on previous laboratory data. Also taken into account was a CV of laboratory techniques of 15% to detect changes in primary endpoints (e.g., difference between rates of muscle glucose uptake between two groups under different insulin concentrations) with 80% confidence at the 5% significance level. A post hoc sample size calculation was performed with the actual data.

The results are presented as means ± SEMs. The software Prism 7 (Graph Pad, San Diego, CA, USA) was used to analyse the data. Folds of change in microvascular parameters from baseline were calculated as difference between intervention values and baseline values divided by baseline values. Comparison between measures was made via Student's t test or ANOVA as appropriate with use of the Bonferroni post test to perform analysis of variance.

5.4 Results

5.4.1 Microvascular recruitment

GLP-1 significantly increases AI under PA and PP insulin and increases MBV under PA insulin with no effect on MFV on both groups

Acoustic intensity rose as expected following Sonovue^{TM} infusion. Replenishment curves, which correspond to MBV, following high mechanical index (MI) flashes are shown in figure 5-2. Following GLP-1 infusion in PA INS and PP INS groups, change in AI AUC was significantly higher compared to when same groups were studied without GLP-1 infusion (*p* 0.004 and 0.03 respectively) (Figure 5-3 A & B). MBV did not change from baseline values following fed conditions in both PA and PP insulin groups with or without GLP-1 (Figure 5-4 A & B). However, there was a significant change in the fold of change in MBV when GLP-1 was given to PA insulin group compared to when the same group did not have GLP-1 in the fed state (1.8 folds ± 1.2, *p* 0.018 – figure 5-5 A). MFV did not change in response to insulin or GLP-1 with exception of a significant change from baseline values in the fed state under both PP insulin and GLP-1 (Figure 5-4 D).

GLP-1 increases MBF under PA insulin and PP insulin

MBF changed significantly from baseline in response to GLP-1 in the PA insulin group (*p* 0.022) (Figure 5-4 E) and in response to insulin alone (*p* 0.014) and with GLP-1 (*p* 0.048) in the PP insulin group (Figure 5-4 F). Folds of change were also significantly increased when adding GLP-1 in both PA insulin and PP insulin groups (7.2 ± 4.7 vs 0.40 ± 0.26 folds, *p* 0.009 and 5.0 ± 2.08 vs 1.9 ± 0.73 folds, *p* 0.041 respectively), (Figure 5-5 E & F).

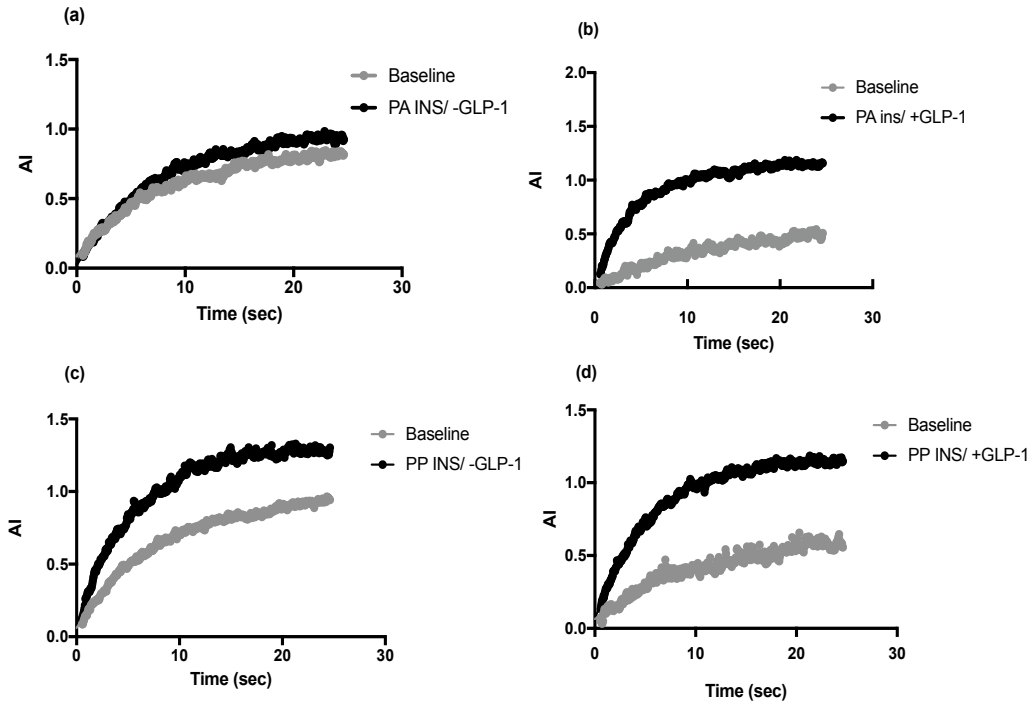


Figure 5-2: Microvascular parameters derived from microbubble replenishment.

Microbubble replenishment was measured following high MI flashes and showing vascular response as average AI replenishment curves. Measures were performed at baseline and following PA insulin in 7 older men without (a) and with (b) GLP-1 and following PP insulin in 8 older men without (c) and with (d) GLP-1. PA INS: postabsorptive insulin, PP INS: postprandial insulin. AI: acoustic intensity.

5.4.2 Insulin and GLP-1 levels

Octreotide blocked endogenous insulin production within the first 5 minutes of infusion. Mean insulin concentrations achieved in the PA INS group were 6.77 ± 1.93 and 8.68.52 ± 1.83 μIU.mL^{-1} without and with GLP-1 respectively. In the PP INS group, mean insulin concentrations achieved were 25.40 ± 3.67 (p < 0.001 vs baseline) and 31.60 ± 5.55 μIU.mL^{-1} (p < 0.001 vs baseline) without and with GLP-1 respectively. In PA INS group, when GLP-1 was infused, following an initial

drop in insulin concentrations, levels started to rise gradually to become significantly increased from baseline values from 110 min onwards when GLP-1 (p < 0.05 vs baseline). In the same group, insulin levels remained not significantly different from baseline for the entire length of the clamp period in the absence of GLP-1 infusion. In the PP INS group, levels with and without GLP-1 behaved similarly, i.e. no difference observed between insulin concentrations with and without GLP-1. See figure 5-6, A, B, C & D).

GLP-1 concentrations were comparable across groups in study visits when it was not infused (15.51 ± 2.59 vs 17.44 ± 3.62 pmol.l^{-1} in PA INS and PP INS groups respectively) and on visits where it was infused (56.65 ± 8.60 vs 63.26 ± 15.05 pmol.l^{-1} in the PA INS and PP INS groups respectively, p < 0.01 against baseline values). GLP-1 infusion caused marginal increase in insulin concentrations but, there was no significant difference across time points and in overall insulin AUC analysis. See figure 5-6 D.

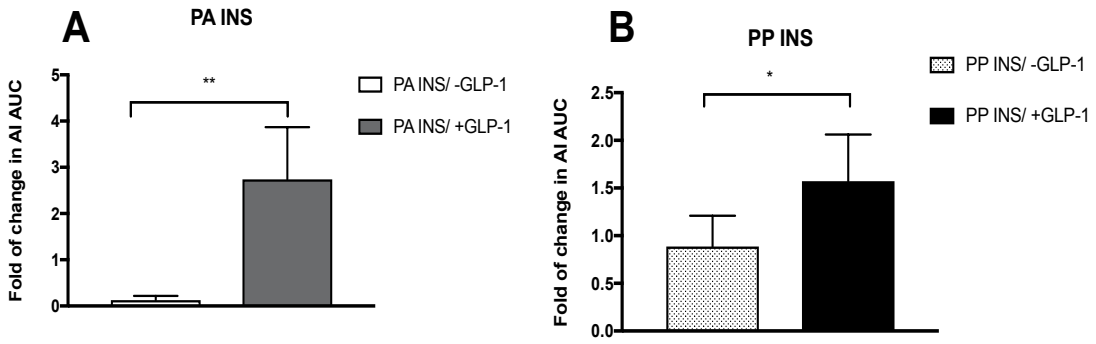


Figure 5-3: Change in microvascular MBV represented as AI AUC in each group.

Change was demonstrated as fold of change in AUC of AI replenishment curves when volunteers studied without or with GLP-1 in PA INS (A) and PP INS (B) groups. ** $p < 0.01$. PA INS: postabsorptive insulin, PA INS: postprandial insulin.

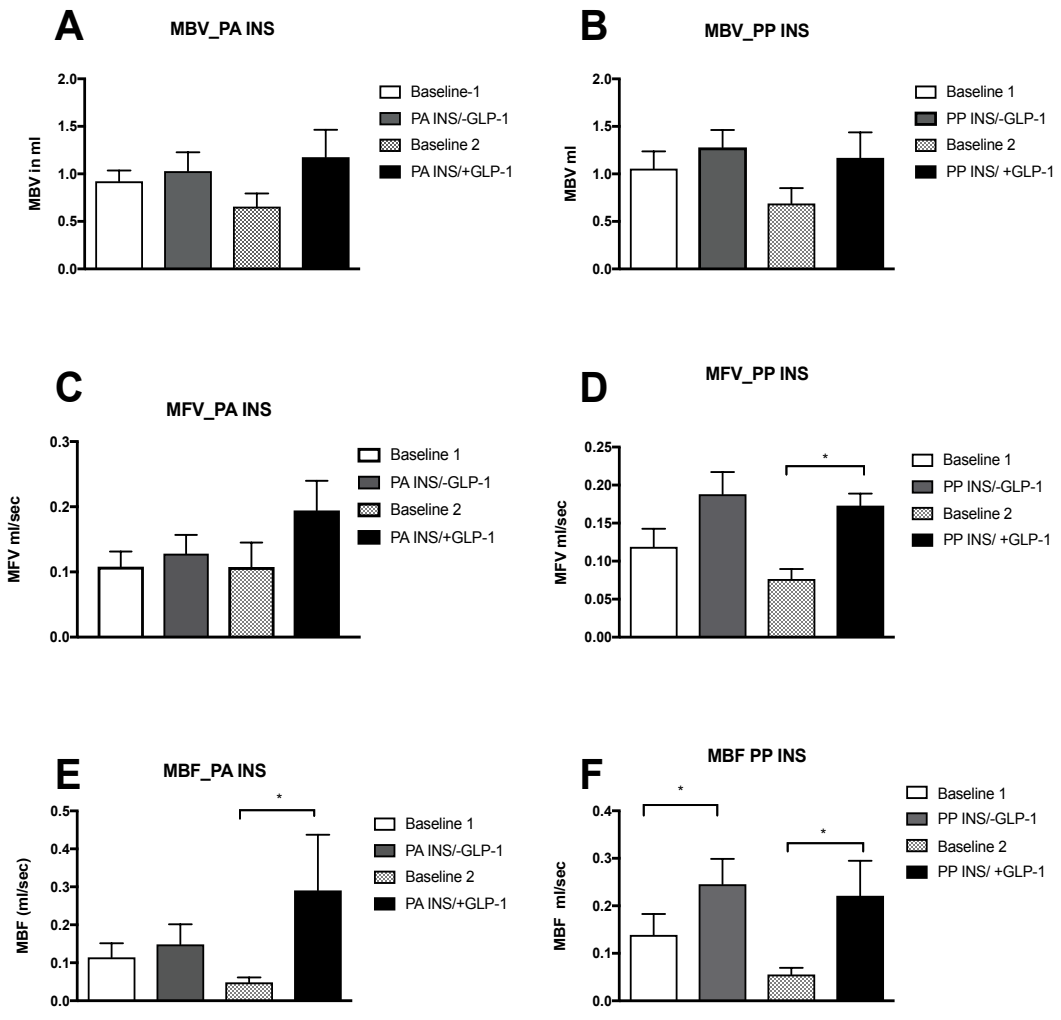


Figure 5-4: Change in absolute microvascular parametters from baseline in different visits.

Change in numerical measures within PA INS and PP INS groups from baseline in each visit in MBV (A and B), MFV (C and D) and MBV (E and F) with and without GLP-1. Baseline 1:

refers to visit without GLP-1 infusion, baseline 2: refers to visit with GLP-1 infusion. PA INS: postabsorptive insulin, PP INS: postprandial insulin, MBV: microvascular blood volume, MFV: microvascular flow velocity, MBF: microvascular blood flow.

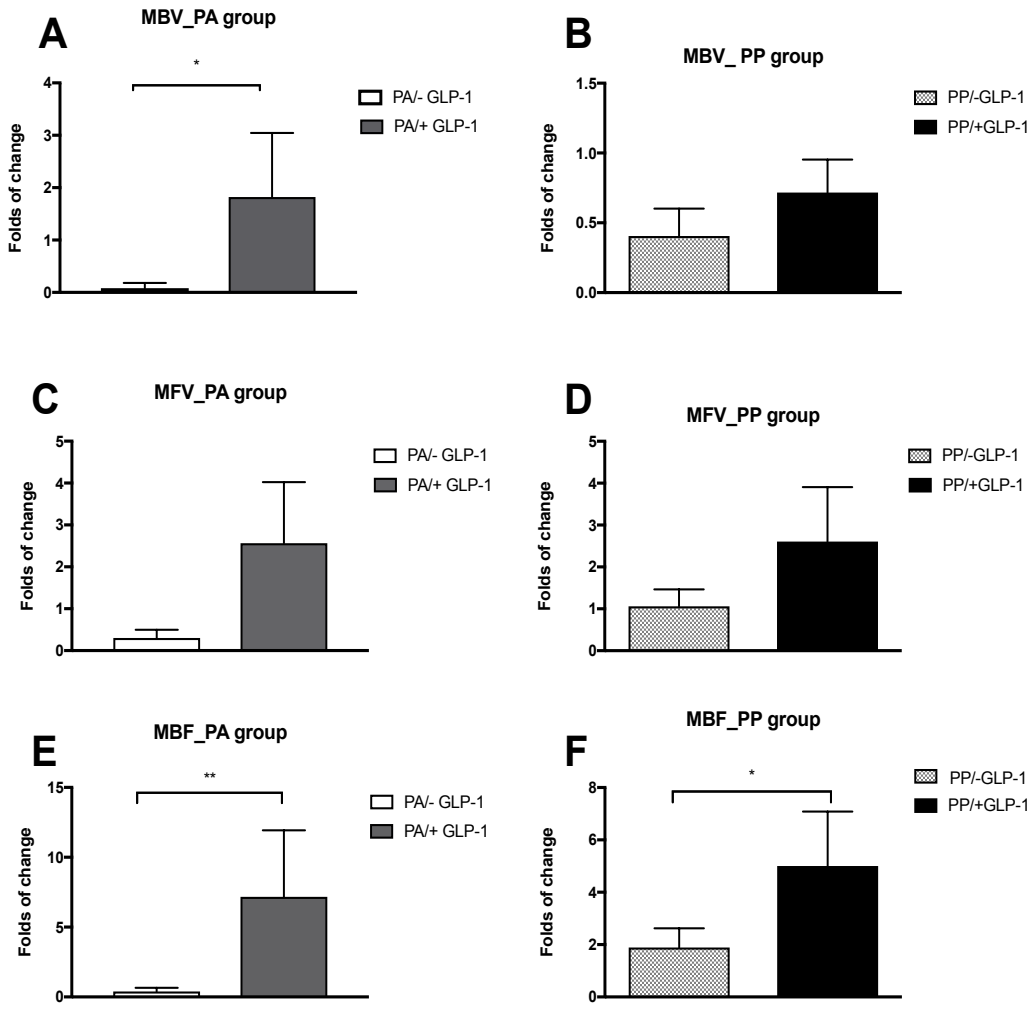


Figure 5-5: Change in detailed microvascular responses.

Fold of change within PA INS and PP insulin groups in MBV (A & B), MFV (C & D) and MBF (E & F) from baseline with and without GLP-1. * $p < 0.05$, ** $p < 0.01$. PA INS: postabsorptive insulin, PP INS: postprandial insulin. MBV: microvascular blood volume, MFV: microvascular flow velocity, MBF: microvascular blood flow.

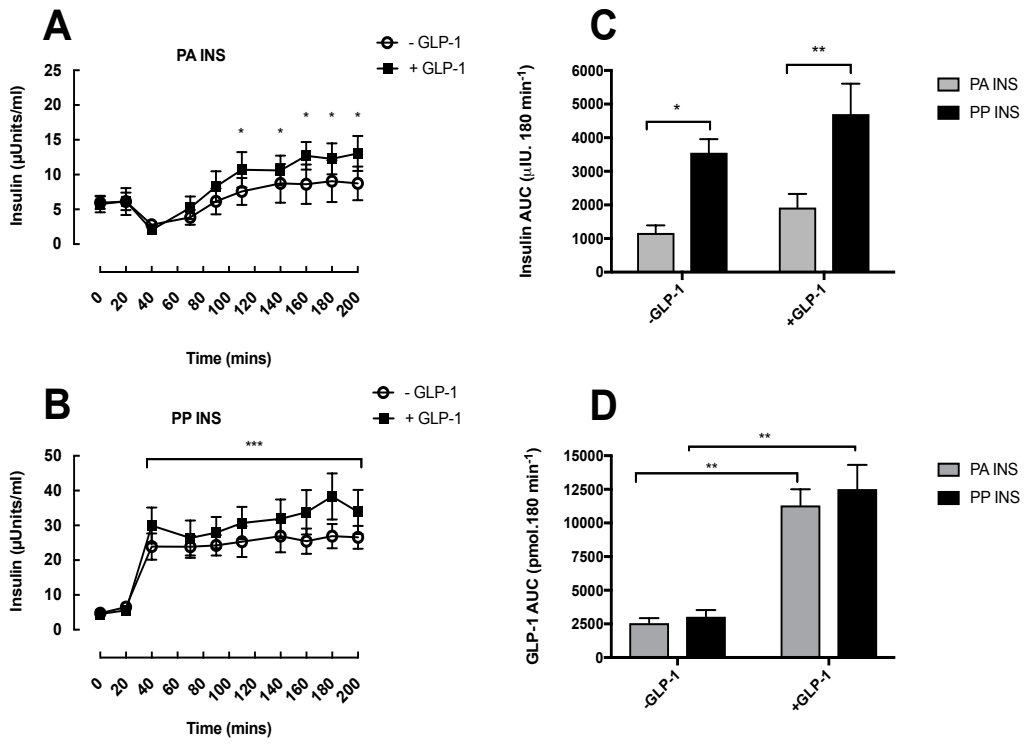


Figure 5-6 : Insulin and GLP-1 profiles in PA and PP groups.

PA INS (A) and PP INS (B) groups insulin profiles and AUC (C & D) over 180 min period with and without GLP-1. Insulin, glucose, mixed AA and octreotide infusions started at 20 min time point. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; A & B: against retrospective basal. Data presented as mean ± SEM. Measured by 2 Way ANOVA.

5.4.3 Glucose uptake

Glucose uptake was not different in both groups following GLP-1 infusion. This suggests that GLP-1 does not affect glucose uptake the in both low and moderately high insulin concentrations during hyperglycaemic postprandial conditions. See figure 5-7.

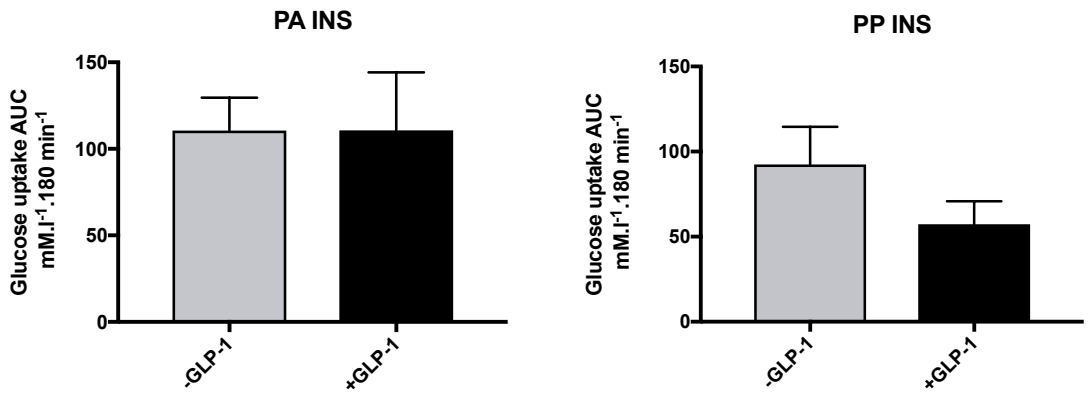


Figure 5-7: Glucose uptake under low and high insulin.

Both groups underwent hyperglycaemic clamp with low insulin concentrations on the PA INS group and relatively higher insulin concentrations on the PP INS group. PA INS: postabsorptive insulin, PP INS: postprandial insulin. Effect of feeding: NS. Measures expressed as mean ± SEM.

5.5 Discussion

This is the first study to report on the effect of GLP-1 infusion on skeletal muscle microvascular recruitment and glucose uptake in older age. Subjects were put under experimental fed conditions and sub-grouped to have either low or high insulin with or without GLP-1. The co-infusion of octreotide insured blockade of endogenous insulin production and therefore infused insulin levels stay as designated in both groups despite experimental fed state. In our pilot data, it was shown octreotide was able to inhibit endogenous insulin secretion within 6 minutes of infusion at a rate of 30 $ng.kg^{-1}.min^{-1}$. We have demonstrated that infusion of GLP-1 into femoral artery acutely improves MBV under postabsorptive insulin conditions and MBF under both postabsorptive and postprandial insulin levels. Under postprandial insulin without GLP-1, MBF increased with a significant added effect when GLP-1 was added. Increases in MBF delivered by GLP-1 were even more potent under postabsorptive insulin conditions. This suggests that GLP-1 effect on MVR within skeletal muscle is insulin independent and appears to be more pronounced under low insulin concentrations (postabsorptive state).

Mixed meal ingestion was shown to recruit more microvasculature compared to insulin infusion alone [126]. Therefore it is inconceivable that an added effect on MVR comes as a result of nutrient sensing the gut leading to incretin hormone release. Whether this biological effect of GLP-1 is a direct hormonal or a neuroendocrine one remains to be investigated. Under normal physiological conditions following food intake, a neuroendocrine effect is the likely possibility given the fact that the active hormone is rapidly degraded by DPP-4. In our study, GLP-1 was directly infused into the femoral artery, we conclude that the observed effect of GLP-1 on MVR in this current study is indeed a direct one.

In contrast, this study showed no direct effect for GLP-1 on glucose uptake in skeletal muscle under fed conditions and physiological GLP-1 postprandial GLP-1 concentrations. In fact, glucose uptake showed considerable variability in both groups throughout the entire duration of the experimental postprandial fed state dipping frequently to a negative balance. This finding is in line with previous reports that did not support incremental benefits over insulin for GLP-1 in regards to human skeletal muscle glucose uptake under both physiological and supraphysiological levels [130]. Therefore, the notion that GLP-1 increases muscle glucose uptake independent of insulin remains confined to rats [127] and muscle cell cultures [119]. However, it is expected that increased MVR would lead to increased endothelial surface and thus improved insulin and glucose delivery to target tissue [53,127]. Indeed, improvement in glucose delivery to muscle has been demonstrated using pharmacological preparations that are known to modulate peripheral microvascular tone such as losartan, an angiotensin II type I receptor blocker [304].

The ageing process is known to be linked to impaired microvascular responses to insulin and reduced insulin sensitivity in the microvasculature. Microvascular blood flow is reduced in response to oral beverage and reduction in resting MBV was also reported. The cohort of volunteers studied in this work are expected to have anabolic resistance, which involves resistance to insulin action. It is unclear whether insulin anabolic resistance of ageing is a direct ageing effect or a phenomenon rather linked to other risk factors such as sedentarism, increased adiposity and hypertension. Moreover, it remains debatable whether age related insulin resistance is also linked to impaired glucose disposal. While some reports showed both total body and skeletal muscle glucose disposal to be unaffected [182], others reported reduced glucose uptake in association with ageing [292]. The positive effect of GLP-1 observed in this study compliments previous reports

demonstrated in young individuals [130,131]. The lack of response in glucose uptake feeds more into the debate of where exactly GLP-1 receptor is located: vascular endothelium vs muscle cell surface. Given our findings and those of others, the latter seems increasingly less likely. This would explain the improved MVR without significant effect on glucose disposal.

Both insulin and GLP-1 mediate their microvascular action through NO dependent mechanism. In our results, GLP-1 appears more potent than insulin in recruiting microvasculature. Insulin resistance of type 2 diabetes closely linked to endothelial dysfunction. However, GLP-1 signalling in type 2 diabetes is intact and therefore, GLP-1 therapy remains a viable option that would likely improve substrate, hormones and oxygen delivery. The lack of positive response in glucose disposal in muscle cells might be related to lack of enough receptors to promote this function at myocytes surface.

Limitations: This study tried to explore glucose uptake at lower concentrations of insulin. However such data needs to be interpreted with caution. The advantage of hyperinsulinaemic clamp is switching off of other pathways that might affect glucose metabolism. In our study, infusion of octreotide would inhibit other pancreatic regulatory hormones such as glucagon, however, this was not checked. We did not report on insulin clearance as a further evidence of increased insulin delivery to skeletal muscle. The A-V balance method of reporting glucose uptake has it own limitations. The method is not robust enough to give a clear idea on quantitative glucose incorporation into muscle cells. Therefore, it is difficult to ascertain if there is a small GLP-1 related effect on glucose disposal that might have been missed. Furthermore, postprandial clamps of glucose and insulin under low or postabsorptive levels of insulin remain experimental, albeit, such scenario might exist in individuals with type 1 diabetes mellitus.

5.6 Conclusion

The current study demonstrates that GLP-1 acutely improves skeletal muscle MVR in older age under postprandial conditions. This action is preserved under low and high physiological levels of insulin. In comparison to insulin, GLP-1 does not affect muscle glucose disposal. Improving MVR in the postprandial state is crucial to insulin, substrate and oxygen delivery to the muscle by providing a more endothelial surface for transport across to muscle surface. This function is likely to contribute to improving the anabolic resistance of ageing and diabetes.

Chapter 6: Role of insulin in the regulation of human skeletal muscle protein synthesis and breakdown in health, sarcopenia and diabetes: A systematic review and meta-analysis

6.1 Chapter overview

Aims/hypothesis: We aim to investigate the role of insulin in regulating human skeletal muscle metabolism in health and diabetes.

Methods: We conduct a systematic review and meta-analysis of published data that examined changes in skeletal muscle protein synthesis (MPS) and/or muscle protein breakdown (MPB) in response to insulin infusion. Random-effects models were used to calculate weighted mean differences (WMDs), 95% CIs and corresponding *p* values. Both MPS and MPB are reported in units of nmol (100 mL leg vol.)$^{-1}$ min^{-1}.

Results: A total of 104 articles were examined in detail. Of these, 44 and 25 studies (including a total of 173 individuals) were included in the systematic review and meta-analysis, respectively. Our analysis suggests that overall, insulin did not affect MPS (WMD 3.90 [95% CI −0.74, 8.55], p=0.71), but significantly reduced MPB (WMD −15.46 [95% CI −19.74, −11.18], p<0.001). Overall, insulin significantly increased net balance protein acquisition (WMD 20.09 [95% CI 15.93, 24.26], p<0.001). Subgroup analysis of the effect of insulin on MPS according to amino acid (AA) delivery was performed using meta-regression analysis. The estimate size (WMD) was significantly different between subgroups based on AA availability (p=0.001). An increase in MPS was observed when AA availability increased (WMD 13.44 [95% CI 4.07, 22.81], p<0.01), but not when AA availability was reduced or unchanged. In individuals with diabetes and in the presence of the maintained delivery of AA, there was a significant reduction in MPS in response to insulin (WMD −6.67 [95% CI −12.29, −0.66], p<0.05).

Conclusion/interpretation: This study demonstrates the complex role of insulin in regulating skeletal muscle metabolism. Insulin appears to have a permissive

role in MPS in the presence of elevated AA, and plays a clear role in reducing MPB independent of AA availability.

6.2 Introduction

Skeletal muscle is the primary depository for protein metabolism. Both proteins and insulin interplay to control various functional aspects of skeletal muscle metabolism. Insulin and AA together collate to deliver the necessary intracellular signals that control protein turnover. A number of signalling molecules are involved in protein metabolism and they ultimately converge at the level of mTORC1, giving it its unique centrality. EAA availability is strongly linked to MPS initiation [10]. The activation of Akt by EAA and insulin receptor leads to mTORC1 phosphorylation. mTORC1 is also directly activated by signals from BCAA, specifically leucine. Further, insulin-mediated activation of the PI3-K/Akt signalling pathway negatively regulates markers involved in MPB; in other words, it indirectly stimulates MPS [31]. In addition, insulin has primary circulatory functions that are key to overall metabolic output. These functions include increased large vessel compliance and vasodilation of arterioles. Moreover, insulin via eNOS enhances relaxation of terminal arterioles, giving it an overall control over its own delivery, substrate delivery and therefore flux of substrates into the intracellular space [305].

The anabolic effects of nutrition are driven by the uptake of AA captured from dietary protein sources and their direct incorporation into skeletal muscle proteins. This process is entirely regulated by EAA. The protein accruing effects of nutrients are further driven by anabolic (i.e., increases in MPS) and anti-catabolic (i.e., decreases in MPB) processes. This balance of the "fasted-loss/ fed-gain" cycle in muscle proteostasis is required to ensure the stability of lean mass in healthy mobile individuals. Dysregulation in this cycle especially due to advancing age would lead to the development of muscle wasting with significant adverse morbidity and mortality consequences [184].

Provision of AA alone is able to stimulate MPS in the presence of a very modest concentration of insulin (7-10μU.mL^{-1}) [257]. This response of skeletal muscle to AA, however, is dose-dependent and seems to reach a state of saturation at which MPS can no longer be further stimulated beyond a given concentration of AA [10,306].

Nutritional intervention with AA with or without CHO is expected to cause a physiological rise in insulin concentration that peaks at 1-2 hours before returning to postabsorptive levels. This consequentiality may feed more into the debate of whether a yielding metabolic effect is ascribed to insulin or the given substrate in question. However, the evidence supports the fact that oral nutritional supplements do in fact induce MPS [307–309].

The quality of protein ingested is of vital importance. Whey proteins stimulate MPS more compared to soy or casein in young healthy men [232]. The difference between these protein elements lies in their EAA, their subset BCAA and NEAA constituents. Most of the stimulant effect of AA on MPS can be obtained by infusion of EAA [257] with NEAA having no incremental benefit [287]. Further, EAAs seem to elicit this effect in the presence of insulin concentrations mostly seen in the postabsorptive state [147]. Moreover, BCAA per se seems to be responsible for most of the anabolic effect of EAA. Within this subset, leucine and its metabolites β-hydroxy-β-methylbutyrate (HMB) were found to play a significant role in stimulating a positive metabolic balance [147,232,257,287]. Further, both leucine and HMB are critical in initiating MPS signalling through their stimulation of mTOR [310] and Akt phosphorylation [311] respectively.

In comparison to PRO supplements, CHO were shown to improve the muscle protein net balance following recovery from resistance exercise through a reduction in MPB [307] (an effect attributed to a rise in insulin levels). However,

protein supplements alone are capable of inducing such rises in insulin with the same effect on MPB and net balance [312]. Furthermore, when CHO are added to PRO, this does not result in a further increase in MPS in young [17,313,314] and older [291] healthy individuals. Therefore, irrespective of their glycaemic index, CHO are unlikely to induce a further increment in MPS when a protein substrate that maximally stimulates insulin secretion is supplemented [315].

Data from epidemiological and experimental studies have reported that type 2 diabetes is related to poor muscle strength and function, with an accelerated rate of decline in muscle quality and strength in older individuals of up to 30% [155]. In view of the rising prevalence of diabetes and the metabolic sequelae of age-related sarcopenia, there has been increased interest in the mechanisms by which type 2 diabetes exacerbates the age-related decline in muscle mass. Furthermore, since skeletal muscle is an important site for glucose disposal, quantitative reductions in appendicular muscle volume might potentially adversely affect glucose disposal and metabolism [316]. Increased understanding of the endocrine factors that regulate muscle mass is therefore important in terms of glycaemic control and countering sarcopenia. Many patients with type 2 diabetes eventually require insulin to achieve optimal glucose targets as the ability to produce endogenous insulin from pancreatic beta cells progressively declines [317]. However, insulin therapy is associated with weight gain [318,319], predominantly fat mass [320], which increases insulin resistance and necessitates the use of higher doses of insulin at the expense of further weight gain.

The exact role of insulin in human skeletal muscle metabolism, however, continues to raise debate. Experimental studies in animal models have reported that insulin promotes MPS. These studies were predominantly performed using growing animals [321,322]. The role of insulin in adult human skeletal muscle is more complex

and subject to an interplay between other factors such as AA availability, muscle blood flow and MVR [323,324]. This has led to various studies reporting opposing conclusions regarding the relationship between insulin and human skeletal muscle protein turnover [245,323–328]. We, therefore, undertook a systematic review and meta-analysis in an attempt to clarify the role of insulin in regulating muscle metabolism in humans.

6.3 Methods

A systematic search of MEDLINE and EMBASE, and of reviews and the references lists of relevant articles, was conducted using the terms 'muscle protein synthesis' OR 'fractional synthesis rate' OR 'muscle protein anabolism' OR 'muscle protein metabolism', paired with the term 'insulin'.

6.3.1 Study selection

We searched English-language studies published between 1946 and November 2013. Peer reviewed papers investigating the role of insulin on MPS and/or MPB were selected. All experimental studies that reported changes in muscle protein metabolism in humans in response to interventions with insulin were selected for the systematic review, regardless of the method of evaluation. Both the two-pool (compartment) and three-pool methods were used in reporting muscle protein metabolism, and both provide qualitatively comparable changes in protein metabolism from blood and intracellular enrichment of phenylalanine [328]. In the two-pool (artery and vein) model, phenylalanine enters and leaves the limb via the artery and vein, respectively. The rate of disappearance of phenylalanine from the artery is used to estimate MPS, and is derived from measurements of the rate of MPB and net balance (NB); MPB is determined by the rate of appearance of phenylalanine in the vein (i.e. the dilution of tracer enrichment across the limb), while NB is simply the concentration difference of phenylalanine across the limb. In the three-pool (artery, vein and muscle) model, phenylalanine enters and leaves the limb as described above. The unidirectional flow of free phenylalanine from the artery to the intramuscular compartment is determined as the rate of its inward transport. The rate of the intracellular appearance of phenylalanine defines

the rate of release from MPB. Since phenylalanine is not oxidised by skeletal muscle, the rate of its intracellular use corresponds to the rate of use for MPS [245].

In the studies we reviewed, the two-pool model was the most commonly used analytical method and phenylalanine was the most commonly used AA tracer. Therefore, in order to allow comparable quantitative analysis of the studies eligible for the systematic review and to prevent significant heterogeneity, the meta-analysis only included studies that used the two-pool model to analyse phenylalanine data, as opposed to other analytical methods or other AA tracers (e.g. leucine). Three-pool data were included in the systematic review. Due to a large overlap between studies that reported two and three-pool data (n=10) and the significantly fewer studies that exclusively reported three-pool data (n=5), we did not perform a meta-analysis of the three-pool data.

6.3.2 Data extraction and statistical analysis

All studies meeting the inclusion criteria were reviewed. The primary outcome was the change in MPS and/or MPB in response to insulin intervention. The published data were extracted from the studies and SDs of the means were calculated. Because of similar reporting methods of measurement between the included studies, random-effects models were used to calculate weighted mean differences (WMDs), 95% CIs and corresponding p values. Heterogeneity between studies was assessed using the I2 statistic, which describes the percentage of total variation across studies that is as a result of heterogeneity rather than chance [329]. Because AA are the principal substrate for protein synthesis, subgroup analyses were performed based on different levels of AA delivery to the muscle, and hence the amount available (i.e., increased, constant or decreased) for protein metabolism. Another subgroup analysis was performed with studies involving populations with

diabetes. In these studies, AA delivery did not change. Meta-regression analysis was conducted to test for differences in the pooled estimates between subgroups, and to test whether pooled estimates differed according to other covariants (e.g. insulin concentration levels achieved, age or lean body mass).

6.3.3 Statistical tests and publication bias

This was assessed by examining a funnel plot as a function of effect size. Statistical tests for the meta-analyses were performed using the statistical package STATA 13.0 (Stata Corp, College Station, TX, USA).

6.4 Results

6.4.1 Search results

After the removal of duplicates, 646 articles were retrieved from the search and the reference lists of selected articles (Figure 6-1). Title and abstract screening led to 455 articles being excluded because of irrelevance (i.e. in-vitro studies, studies investigating liver protein metabolism) and a further 87 articles were excluded because the studies were conducted in animal skeletal muscle.

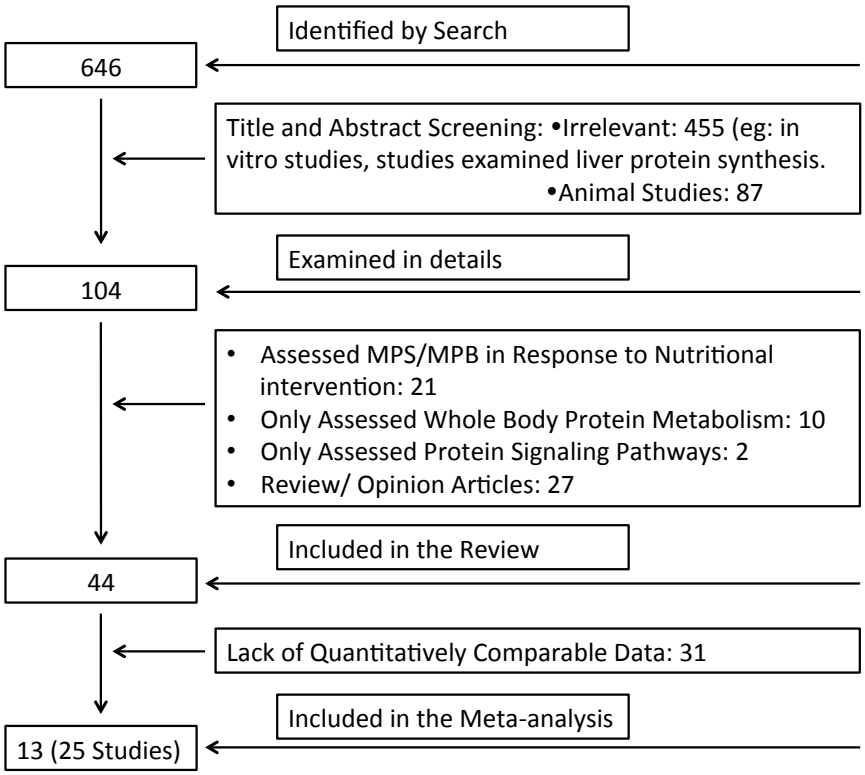


Figure 6-1: Flowchart representing the article retrieval process.

The flowchart represents the article-retrieval process and outlines the articles that met the criteria for systematic review and meta-analysis. Only studies using the two-pool model and phenylalanine tracers were included in the meta-analysis. A total of 104 potentially relevant articles were identified and evaluated in greater detail. Of these, a further 60 articles were excluded. The main reasons for exclusion were: (1) the studies assessed the role of

nutritional interventions but not insulin per se; and (2) the articles were reviews rather than research study articles. A total of 44 articles encompassing 75 studies met the criteria for the systematic review. Of these 44 articles, 13 (containing 25 studies) were included in the meta-analysis, as they represented the largest group that contained quantitatively comparable data. All of the 25 studies used phenylalanine as the AA tracer, reported MPS/MPB in units of nmol (100 mL leg vol.)$^{-1}$min^{-1} and used the two-pool (arterio-venous balance) approach to estimate outcome variables.

6.4.2 Meta-analysis of study characteristics

A total of 13 articles [177,195,323,326–328,330–336], containing 25 experimental studies using different insulin concentrations, were identified for inclusion in the meta-analysis; all analysed the effect of insulin on both MPS and MPB (Table 6-1). A total of 173 individuals were included in these studies. A total of 13 studies involved young adults, and three of these involved individuals with diabetes. Eight studies involved healthy older individuals, and there were no age data available for four studies. The average age of the participants ranged from 18 to 74 years. The majority of the studies (20 studies) used local intra-arterial insulin infusion to limit the development of systemic hypoglycaemia and an obligatory infusion of glucose, also to limit hypoaminoacidaemia[337]. This is of crucial importance, especially when supraphysiological insulin concentrations are used. Systemic insulin concentrations varied between 62.5 and 861.2 pmol/l. AA delivery (concentration of AA in the artery × arterial blood flow) was maintained in 14 studies and increased in eight studies. However, as a direct consequence of systemic insulin administration (see discussion below), circulating AA were decreased in three studies.

Table 6-1.

No.	Study [ref a]	Participants, *n* (M/F)	Age, years	Insulin conc., pmol/l	Insulin infusion	Limb studied	AA delivery[b]	MPS, nmol (100 mL limb vol.)$^{-1}$ min^{-1}	MPS summary[b]	MPB, nmol (100 mL limb vol.)$^{-1}$ min^{-1}	MPB summary	NB, nmol (100 mL limb vol.)$^{-1}$ min^{-1}	NB summary[b]
1	Louard et al. (b) 1992 [326]	7	–	277.8±34.7	Local	Forearm	↓	33±2	↔	31±2	↓	1±3	↑
2	Louard et al. (c) 1992 [326]	7	–	456.3±55.7	Local	Forearm	↓	36±5	↔	28±5	↓	9±3	↑
3	Louard et al. (d) 1992 [326]	8	–	861.2±76.2	Local	Forearm	↓	42±2	↔	33±5	↓	9±1	↑
4	Rasmussen et al. (a) 2006 [182]	7 (3/4)	25±2	279.9±6.9	Local	Leg	↔	71±19	↑	56±16	↔	15±4	↑
5	Rasmussen et al. (b) 2006 [182]	6 (5/1)	68±1	579.2±43.1	Local	Leg	↔	40±7	↔	44±9	↔	−4±2	↑
6	Fujita et al. (1a) 2006 [330]	6 (4/2)	29±3	284.1±53.5	Local	Leg	↔	36±7	↔	49±8	↔	−14±4	↔
7	Fujita et al. (1b) 2006 [330]	7 (3/4)	25±2	318.1±43.8	Local	Leg	↔	71±19	↑	56±16	↔	15±4	↑

Table 6-1, continued.

8	Fujita et al. (1c) 2006 [330]	6 (4/2)	28±2	586.9±34.7	Local	Leg	↔	41±7	↔	44±8	↔	−3±4	↑
9	Fujita et al. (2a) 2007 [328]	7 (5/2)	70±2	579.2±36.1	Local	Leg	↔	40±7	↔	44±9	↔	−2±2	↑
10	Fujita et al. (3a) 2009 [331]	8 (6/2)	69±1	552.0±41.9	Local	Leg	↔	45±8	↔	46±8	↓	−1±3	↑
11	Timmerman et al. (1b) 2010 [332]	6 (5/11)	74±3	394±124	Local	Leg	↔	52±9	↔	54±7	↔	−2±3	↑
12	Timmerman et al. (2a) 2010 [195]	7 (2/5)	32±2	255±33	Local	Leg	↔	47±7	↑	4±9	↔	3±3	↑
13	Timmerman et al. (2b) 2010 [195]	7 (3/4)	32±3	282±28	Local	Leg	↔	51±7	↔	46±4	↓	4±3	↑
	Gelfand and Barrett 1987 [323]	7 (7/0)	18–29	166.7±76.4	Local	Forearm	↑	42±2	↔	33±5	↓	9±3	↑
15	Louard et al. (a) 1992 [326]	10	–	229.2±27.8	Local	Forearm	↑	35±4	↔	35±5	↓	−1±3	↑
16	Biolo et al. 1995 [334]	6	29±5	534.8±62.5	Local	Leg	↑	35±5	↔	28±3	↔	7±2	↑
	Fryburg et al. 1995 [335]	10	20–32	606.0±36	Local	Forearm	↑	68±5	↔	42±5	↓	26±4	↑
18	Fujita et al. (2b) 2007 [328]	6 (5/1)	68±1	324.3±35.4	Local	Leg	↑	79±19	↑	70±19	↔	9±4	↑

Table 6-1, continued.

19	Fujita et al. (3b) 2009 [331]	6 (5/1)	72±2	864.4±224.5	Local	Leg	↑	55±7	↑	46±7	↔	9±4	↑
20	Timmerman et al. (1a) 2010 [332]	6 (2/4)	69±1	290.0±54.9	Local	Leg	↑	94±23	↑	69±13	↔	26±12	↑
21	Luzi et al. (a) 2000 [336] [c]	5	30±5	558.3±25	Systemic	Forearm	↔	12±1.3	↔	10.6±7.8	↔	1.4±8.4	↔
22	Luzi et al. (b) 2000 [336] [c]	4	46±4	533.4±69.5	Systemic	Forearm	↔	18.1±4.8	↓	14.8±11.3	↓	3.3±6.6	↔
23	Bell et al. (a) 2006 [327] [c]	6	40±4	144±30	Systemic	Leg	↔	42±7	↔	51±7	↓	−9±8	↑
24	Bell et al. (b) 2006 [327]	6	35±3	239.9±24	Systemic	Leg	↔	28±5	↔	33±7	↓	−5±5	↑
25	Newman et al. 1994 [333]	12 (5/7)	53±2	486.2±27.8	Systemic	Forearm	↑	52±7	↑	40±5	↔	12±7	↑

Table 6-1: Characteristics of the studies included in the meta-analysis ordered by mode of insulin administration and AA delivery.

Values are means ± SEM, unless otherwise stated. MPS: Muscle protein synthesis, MBP: Muscle protein breakdown, NB: Protein net balance. [a] Numbers alongside the author's name differentiate different trials by the same author; letters signify different interventions or participant characteristics within one study. [b] Versus baseline. [c] Participants with diabetes.

6.4.3 Effect of insulin on MPS

The meta-analysis data were pooled from 13 trials (25 studies or comparisons) involving 173 individuals. The WMD for MPS was 3.90 (95% CI −0.74, 8.55; p=0.71). Analysing the studies based on AA delivery revealed increased MPS (WMD 13.44 [95% CI 4.07, 22.81], p<0.01) in the studies where AA delivery was increased (eight studies, 63 individuals). However, MPS did not significantly change when AA delivery was reduced (three studies; 22 participants; WMD 1.57 [95% CI −3.97, 7.12], p=0.58) or maintained at baseline (11 studies; 73 participants; WMD 2.00 [95% CI −5.28, 9.28], p=0.59). Studies involving individuals with diabetes (three studies, 15 individuals) showed significant reductions in MPS in response to insulin, even though AA delivery was maintained (WMD −6.67 [95% CI −12.69, −0.66], p<0.05; Figure 6-2).

On meta-regression analysis, the estimate size (WMD) was significantly different between subgroups based on AA availability (p=0.001).

The I^2 for the overall effect of insulin on MPS was 49% (p=0.003). This significant moderate heterogeneity seemed to be primarily due to heterogeneity within the increased AA subgroup (I^2 59%; p=0.018). The other subgroups showed non-significant p values for heterogeneity, suggesting greater consistency among these studies compared with the overall MPS data (subgroups: maintained AA delivery: I^2 21%; p=0.241; decreased AA delivery: I^2 0%; p=0.811; individuals with diabetes: I^2 0%; p=0.605).

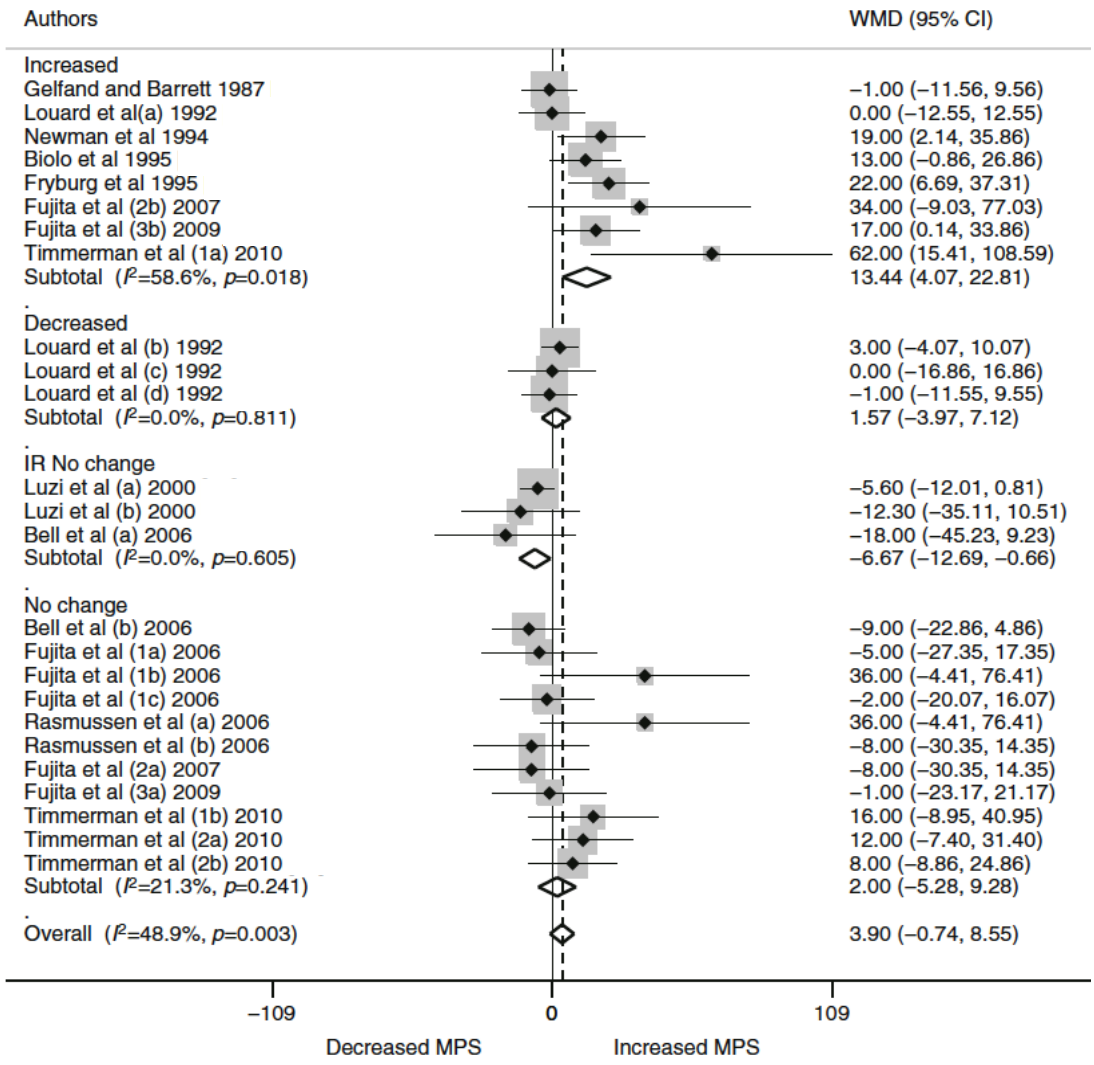


Figure 6-2: Forest plot: Effect of Insulin on MPS.

Forest plot of random effects meta-analysis of WMD (95% CI) on the effect of insulin on MPS. No significant increase in MPS was seen overall (p= 0.710). When stratified for AA delivery, increased MPS was seen where AA was increased (p<0.01), but no difference was observed where AA delivery was maintained (p=0.59) or decreased (p=0.58). In individuals with insulin resistance or diabetes (IR), MPS was significantly reduced (p<0.05) despite maintained AA delivery. Numbers alongside the author name differentiate different trials by the same author; letters signify different interventions or participant characteristics within one study. MPS: muscle protein synthesis. Studies references are detailed in table 6-1.

6.4.4 Effect of insulin on MPB

Data were pooled from the same 25 studies as for MPS. The WMD for MPB was −15.46 [95% CI −19.74, −11.18]; p<0.0001; (Figure 6-3). AA availability did not have a significant impact on the estimated size of MPB (p=0.754). The I^2 for overall insulin effect on MPB was 13% (p=0.282), pointing to non-significant heterogeneity (Figure 6-3).

6.4.5 Effect of insulin on protein NB

In a further pooled analysis of all 25 studies, insulin was found to significantly increase NB protein acquirement (WMD20.09 [95% CI 15.93, 24.26], p<0.0001; Figure 6-4).

6.4.6 Meta-regression analysis of other variables

A meta-regression analysis was conducted to test whether other confounding variables of interest would have any effect on the WMD (e.g. concentration of insulin infusion, age and lean body mass, where available). Differences in the infused insulin concentrations had no effect on MPS (p=0.955), MPB (p=0.713) or NB (p=0.621). There was also no effect for age differences (p=0.480, p= 0.159 and p=0.610, respectively) or for changes in lean body mass (p= 0.433, p= 0.936 and p= 0.617, respectively).

6.4.7 Publication bias

Funnel plots of the insulin effect on both MPS and MPB against SE showed no publication bias (Figure 6-5 and 6-6).

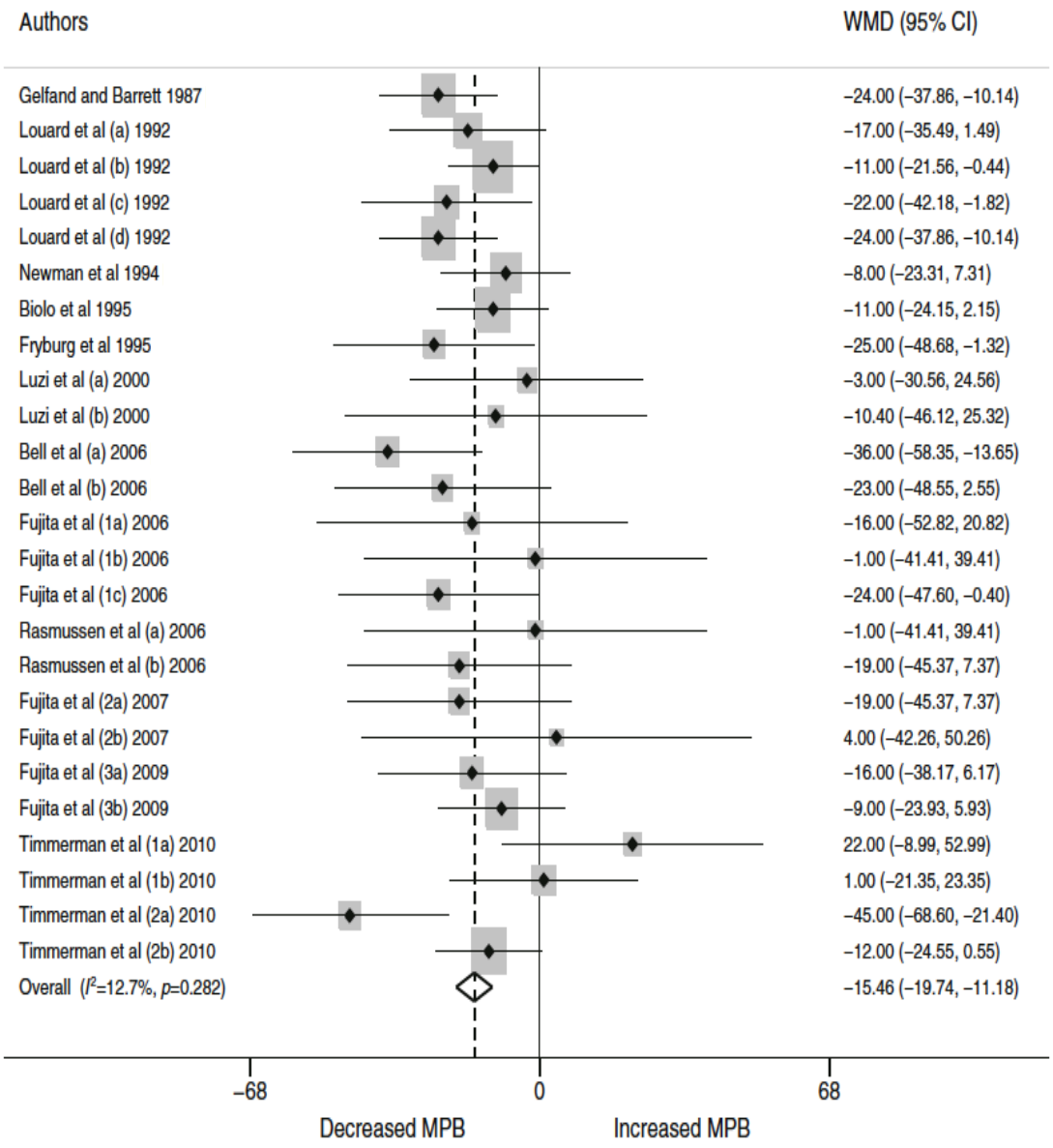


Figure 6-3: Forest plot: Effect of insulin on MPB.

Forest plot of random effects meta-analysis of WMD (95% CI) on the effect of insulin on MPB. A significant reduction in MPB was seen overall (p<0.0001). Numbers alongside the author name differentiate different trials by the same author; letters signify different interventions or participant characteristics within one study. MPB: muscle protein breakdown. Studies references are detailed in table 6-1.

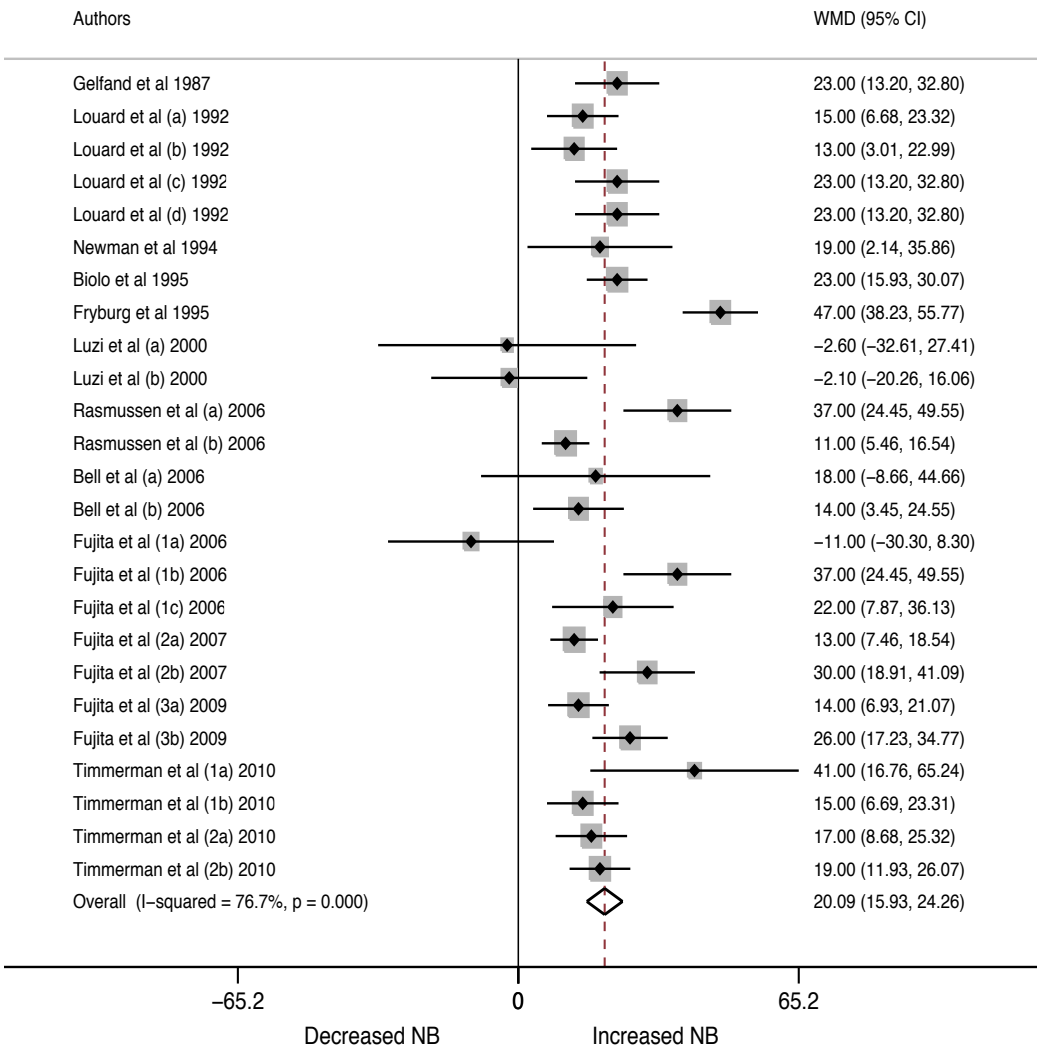


Figure 6-4: Effect of insulin on muscle protein net balance.

Forest plot of random effects meta-analysis of WMD (95% CI) on the effect of insulin on protein NB kinetics. Insulin had a strong positive effect on NB protein acquirement overall ($p<0.0001$). Studies are ordered chronologically. Numbers alongside the author name differentiate different trials by the same author; letters signify different interventions or participant characteristics within one study. Studies references are detailed in table 6-1.

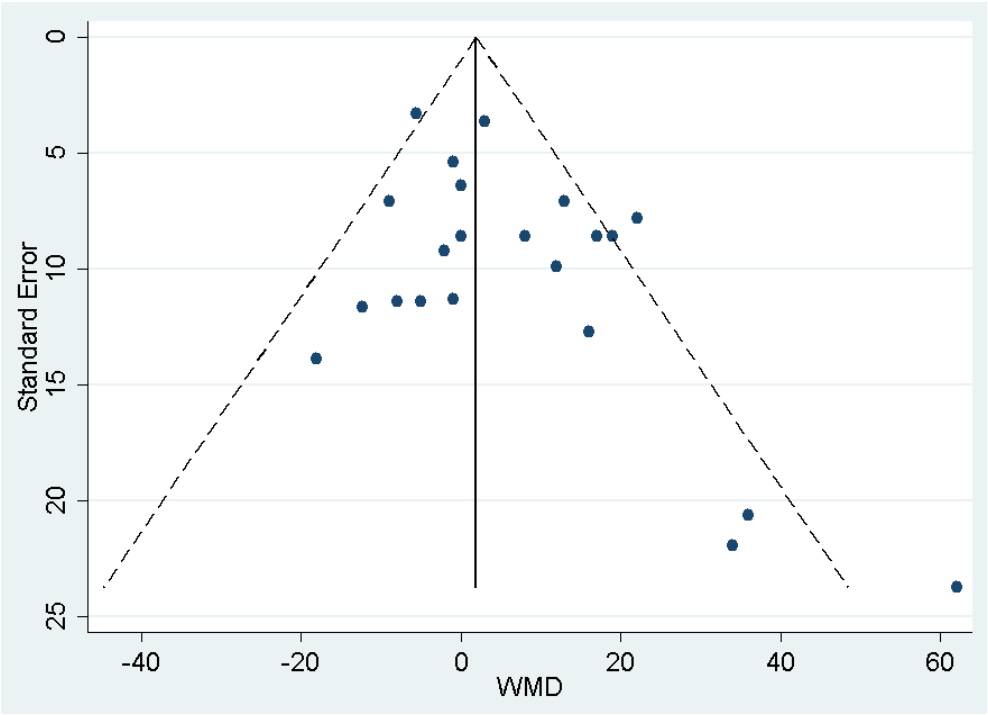


Figure 6-5: Funnel plot: effect of insulin on MPS.

Studies were plotted with weighted mean difference on the horizontal axis and the inverse standard error along the vertical axis.

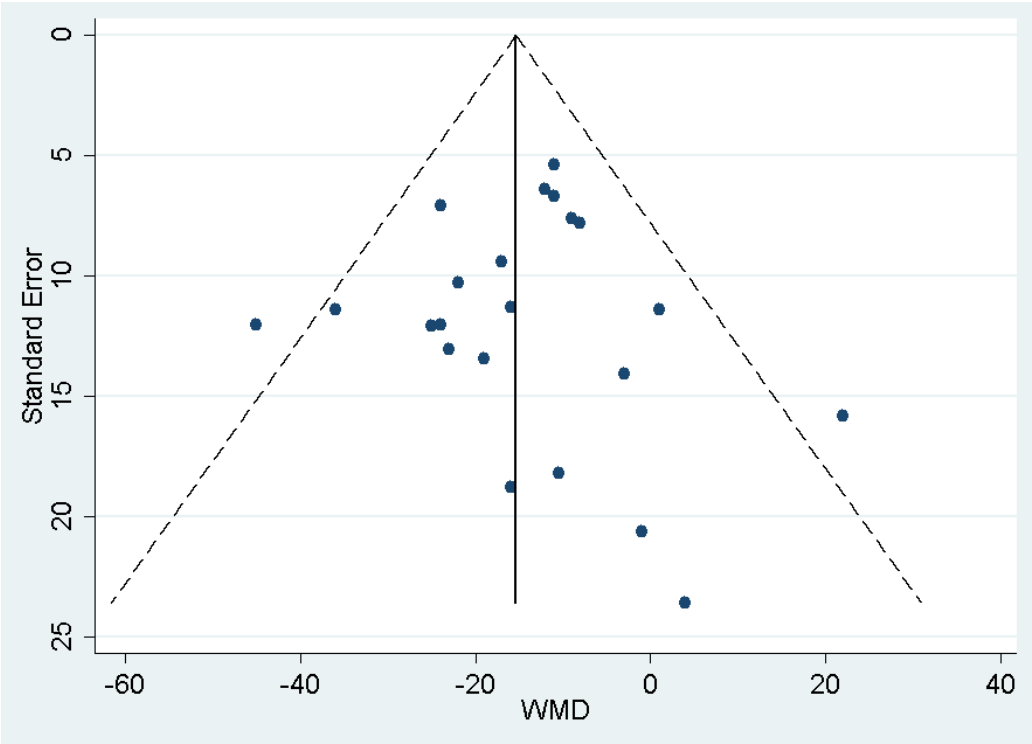


Figure 6-6: Funnel plot: Effect of insulin on MPB.

Studies were plotted with weighted mean difference on the horizontal axis and the inverse standard error along the vertical axis.

6.4.8 Other data

From the articles reviewed, 15 studies used three-pool data [177,195,328,331,332,334,337–339]. Two-pool data from ten of these studies were included in the meta-analysis, which by and large reported similar results with quantitatively different displays. All five studies that exclusively reported three-pool data showed that insulin improved NB protein acquirement [334,337–339]. The increment in NB in these studies was primarily driven by either a reduction in MPB[338] or an increase in MPS [334,337–339]. None of these studies involved a reduction in AA availability. Two of the

studies reported an increase in MPS in an experimentally induced hyperinsulinaemic state in seemingly insulin-resistant individuals with severe burns who were being treated in a high-dependency unit setting [337,339]. A summary of the systematic review of three-pool data is presented in Table 6-2.

Table 6-2.

No.	Study [ref a]	Participants, *n* (M/F)	Age, years	Insulin conc., pmol/l	Limb studied	Insulin infusion	AA delivery[b]	MPS, nmol (100 mL limb vol.)$^{-1}$ min^{-1}	MPS summary[b]	MPB, nmol (100 mL limb vol.)$^{-1}$ min^{-1}	MPB summary[b]	NB, nmol (100 mL limb vol.)$^{-1}$ min^{-1}	NB summary[b]
1	Biolo et al. (1) 1995 [334]	6	29±5	534.8±62.5	Leg	Local	↑	59±8	↑	52±6	↔	−16±2	↑
2	Biolo et al. (2a) 1999 [338]	5	–	359.8±48.6	Leg	Local	↔	51±4	↑	48±3	↔	3±3	↑
3	Biolo et al. (2b) 1999 [338]	5	–	443.8±55.6	Leg	Local	↔	64±9	↔	52±9	↓	12±5	↑
4	Ferrando et al. 1999 [339]	8	–	1680±305.6	Leg	Systemic	↔	347±64	↑		↔	37±9	↑
5	Gore et al. 2005 [337]	6	–	826.5±133.2	Leg	Local		68±14	↑	64±21	↔	4±12	↑
6	Rasmussen et al. (a) 2006 [182]	7 (3/4)	25±2	279.9±6.9	Leg	Local	↔	93±21	↑	78±19	↔	15±4	↑
7	Rasmussen et al. (b) 2006 [182]	6 (5/1)	68±1	579.2±43.1	Leg	Local	↔	57±12	↔	61±14	↔	−4±2	↑
8	Fujita et al. (2a) 2007 [328]	7 (5/2)	70±2	579.2±36.1	Leg	Local	↔	57±12	↔	61±4	↔	−2±2	↑
9	Fujita et al. (2b) 2007 [328]	6 (5/1)	68±1	324.3±35.4	Leg	Local	↑	89±23	↑	80±23	↔	9± 4	↑
10	Fujita et al. (3a) 2009 [331]	8	69±1	552.0±41.9	Leg	Local	↔	59±11	↔	61±12	↔	−1± 3	↑
11	Fujita et al. (3b) 2009 [331]	6	72±2	864.4±224.5	Leg	Local	↑	71±11	↑	61±10	↔	9±4	↑
12	Timmerman et al. (1a) 2010 [332]	6 (2/4)	69±1	290.0±54.9	–	Local	↑	107±23	↑	81±12	↑	26±12	↑

Table 6-2, continued.

13	Timmerman et al. (1b) 2010 [332]	6 (5/1)	74±3	394±124	–	Local	↔	58±10	↔	60±8	↔	−2±3	↑
14	Timmerman et al. (2a) 2010 [195]	7 (2/5)	32±2	255±33	Leg	Local	↔	56±10	↑	54±11	↔	3±3	↑
15	Timmerman et al. (2b) 2010 [195]	7 (3/4)	32±3	282±28	–	Local	↔	56±8	↔	52±5	↓	4±3	↑

Table 6-2: Characteristics of studies reporting three-pool data.

Values are means ± SEM, unless otherwise stated. MPS: Muscle protein synthesis, MBP: Muscle protein breakdown, NB: Protein net balance. [a] Numbers alongside the author's name differentiate different trials by the same author, letters signify different interventions or participant characteristics within one study. [b] Versus baseline.

6.5 Discussion

Systemic insulin infusion leads to hypoglycaemia and reduced AA availability (hypoaminoacidaemia) for protein synthesis [338]. To overcome these consequences, glucose and AA are co-infused to maintain target glycaemia and AA availability. Local intra-arterial insulin infusion appears to limit the effect of systemic hypoglycaemia and hypoaminoacidaemia, thereby avoiding the need for obligatory glucose (or AA) co-infusion [337].

Meta-analysis of these 25 studies showed no significant effect of insulin on MPS. Subgroup analysis, however, revealed that in healthy individuals, the effect of insulin on MPS only becomes significant when AA delivery to the skeletal muscle is increased. These results have been replicated by other researchers in studies where co-infusion of AA and insulin has successfully increased AA delivery to muscle [153,187,249,331,332,340–343]. In a study by Fujita et al., exercising for 45 min successfully increased AA delivery and MPS compared with non-exercising controls, although exercise per se has acute anabolic effects on muscle MPS [328]. In some studies, however, increased AA delivery has not produced an insulin-induced increase in MPS [323,344]. This is probably because the rises in AA delivery were very minimal and achieved mainly through increased blood flow, rather than an increased AA concentration. Older people display resistance to the anabolic effect of insulin compared with their younger counterparts, possibly through mechanisms related to endothelial dysfunction, reduced tissue perfusion and blunted anabolic signalling, rather than reduced glucose tolerance [331]. However, in the presence of increased AA delivery, insulin seems to preserve its anabolic effect in healthy older people. This seems to be the case whether the increments in AA delivery to muscle are achieved via physiological [187,333] or supraphysiological [331]

concentrations of insulin, pharmacologically using sodium nitroprusside [332] or via exercise [328].

In most [324,326,327,345,346] but not all [177,330] studies where insulin was infused with or without AA but with no concomitant increase in AA delivery to the muscle, MPS either did not change or even dropped compared to the baseline. Studies that closely simulated normal feeding where physiological insulinaemia and increased AA delivery to skeletal muscle were achieved, a positive impact on MPS was successfully enhanced [195,187,249,342,347]. Insulin with [153,249,343] or without [323,326,348] AA was able to suppress MPB in most studies. Greenhaff et al. [249] demonstrated that when insulin and AA were infused in young healthy subjects, increasing insulin concentration above 5 $\mu U.mL^{-1}$ (fasting levels) did not result in incremental benefits on MPS. Moreover, there was a significant reduction in MPB when the insulin concentration was increased to 30$\mu U.mL^{-1}$, though without any further significant reduction when the insulin concentration was further increased to higher physiological ranges in the presence of adequate AA delivery.

Increased insulin concentrations within the postprandial range do not appear to affect MPS. A previous study reported that, with incremental increases in AA concentrations, MPS responded positively to insulin concentrations of 139.0–194.5 pmol/l, rising by 22% from baseline and by 72% when higher AA concentrations were given [341]. On the other hand, another study reported that, in the presence of fixed concentrations of AA, increasing insulin concentration from 34.7 pmol/l to 500.0 pmol/l did not produce any further significant increments in MPS [249]. Insulin had no effect on MPS when AA delivery was unchanged from baseline. These results are also supported by other studies [176,177,324,327,330–332,338]. In all healthy human studies where AA availability was reduced, MPS was also reduced or

remained unchanged [325,326,341,346,349]. In such circumstances of AA availability, supraphysiological hyperinsulinaemia was able to deliver positive net protein balance in some studies [339,340,350] but failed to so in others [325].

The meta-analysis of the 25 studies showed that insulin exerts its regulation of lean muscle mass principally via an anti-catabolic effect in reducing MPB. This is more evident when looking at the data on NB, which showed a positive effect on muscle mass. Therefore, the pro-anabolic capabilities of insulin are predominantly driven by its ability to attenuate skeletal MPB, rather than any positive effect on MPS. This finding is in agreement with assessments from other researchers [323,330].

The insulin-induced reduction in MPB appears to be more potent when AA are scarce. These findings are consistent with other studies that have reported a reduction in response to insulin [176,195,249,331,341,343] with the exception of three studies, which did not observe any significant change in MPB [347,349,351]. This might well be due to anabolic resistance to insulin in a relatively older study population and the presence of diabetes mellitus. Interestingly, this maximal inhibition of MPB by insulin occurs in response to very modest elevations in insulin concentration (i.e. to 104.2 pmol/l) [176].

Diabetes has been reported to attenuate the positive effect of insulin on MPS in the presence of maintained AA delivery [324,327,352]. However, in response to long-term intensive sub-cutaneous insulin treatment, Halvatsiotis et al. found no difference in mitochondrial, sarcoplasmic or mixed MPS compared with healthy controls who did not receive insulin [353]. Whether increased AA intake in insulin-treated patients with diabetes will lead to increased muscle mass is not known. However, given the facilitative role of insulin in maintaining muscle mass, particularly in the presence of increased AA availability, it is tempting to speculate

on the need to advise patients with diabetes who are on insulin treatment to increase their AA intake in order to harness the positive effects of insulin on muscle metabolism. In critically ill patients, where significant insulin resistance is expected, increases in MPS have been observed, but only when supraphysiological concentrations of insulin have been used [337,350]. In a study of individuals with obesity [153] and heart failure [343], MPB has been shown to reduce in response to insulin. This reduction, however, was significantly less than that observed in healthy controls. Clearly, further studies are required to fully understand the role of insulin resistance in regulating MPS and MPB in type 2 diabetes.

These discrepancies of results between animal models and human studies could be related to the fact that the animals studied are by and large at growing stage and their response to insulin might be different to counterparts with fully grown muscles. It is to be determined whether insulin on its own has a real role to play in increasing MPS or whether its effect is simply a permissive one. On the other hand, modest postprandial insulin concentration could effectively suppress MPB. As the provision of AA alone could stimulate enzymes of MPB, [354] and insulin provision lowers AA concentration, it is conceivable that the combination (insulin and AA) is needed to strike a positive protein net balance.

Limitations:

This review has several limitations. Different methods of estimating skeletal muscle protein metabolism and the lack of available primary data made it difficult to perform a full quantitative assessment by meta-analysis for all studies that met the inclusion criteria of the systematic review. We also acknowledge that the number of research groups is small and disproportionate to the number of studies involved. In addition, the use of multiple studies per publication means that

pooling of the studies is not entirely independent. In addition, since this is a meta-analysis of experimental studies, a full assessment of bias—as would normally be conducted in meta-analyses of randomised controlled trials [355] (e.g. sequence generation and allocation concealment to check for selection bias; blinding to check for possible performance bias, incomplete outcome data, selective reporting bias and other sources of bias)—could not be performed, as none of the studies used these methods for patient allocation. Furthermore, since all of the studies were of similar size, the funnel plot analysis might not be completely reliable in informing us of any publication bias.

6.6 Conclusion

This systematic review and meta-analysis suggests that the main role of insulin in human skeletal muscle anabolism is facilitative and influenced by the rate of AA delivery. In situations where AA delivery is unchanged, supra-physiological concentrations of insulin are needed to achieve skeletal muscle anabolism. However, the role of insulin in reducing MPB is clearly evident in most studies. This effect is blunted in older people and in those with insulin resistance. This resistance is likely related to impaired insulin signalling of muscle protein metabolism and endothelial dysfunction, rather than glucose intolerance. Further evidence is required to translate these findings into strategies to maximise muscle mass among patients with insulin-treated diabetes.

Chapter 7: Thesis conclusion

My work has reviewed functional aspects of skeletal muscle handling of glucose and protein metabolism and the different ways it responds to vascular changes related ageing, nutrition and hormones, namely insulin and GLP-1. It also looked at the "incretin effect" as a physiological phenomenon that could be exploited to optimise nutritional options in advancing age.

Skeletal muscle represents the major repository for glucose and amino acids. This work provided comprehensive understanding to mechanisms that regulate vascular recruitment following nutrition, together with mechanisms that influence microvascular responses within and around skeletal muscle cells. Insulin is a potent regulator of nutrient entry to skeletal muscle cells. It delivers the necessary microvascular responses such as increased MVR and facilitating transendothelial transport for itself and postprandial nutrients. This ultimately allows anabolic signalling that facilitates nutrients entry into the cell and feedback to regulate pre-cellular responses.

Signalling deficits underlie the development of anabolic resistance to nutrition and hormones in older age. Early life nutritional gains, environmental, behavioural and lifestyle options culminate together to predispose to reduced muscle strength and function (sarcopenia) in older age giving sarcopenia a multifaceted and complex clinical entity that is difficult to address from just single approach.

There has been increasing interest in EAA and their subset BCAA as a distinct nutritional option that not only incorporates into muscle to increase net protein accruement but also influence signalling processes that lead to anabolic gain. Although the incretin effect was first described in relation to glucose, protein and lipid had proved to have varying degree of delivering such an effect. I have, for the first time, demonstrated that EAA could robustly elicit an incretin effect

following 15g of EAA beverage. Moreover, I have shown that this elicited incretin effect is associated with rises in GIP rather than GLP-1. I have shown evidence that the ageing process does not impact the ability of EAA to stimulate incretin hormone secretion and hence generate an incretin effect. Therefore, EAA can be looked at as an attractive option that impacts positively on hormonal responses and anabolic signalling following oral nutrition.

GLP-1 proved to have extrapancreatic non-insulinotropic characteristics that could potentially regulate key aspects of skeletal muscle function. In this work, I showed that under postprandial condition GLP-1 could potentially improve the anabolic resistance of older age. This work also proved that no key properties for GLP-1 on acute regulation of muscle protein synthesis under postabsorptive levels of insulin. The data provided by my work on hormonal regulation of muscle protein breakdown add to existing data demonstrated that insulin has stronger attributes in suppressing MPB down under levels seen in postabsorptive and postprandial state. GLP-1 provided no differential effect over insulin on suppression of MPB at least in older age.

As the current scientific knowledge stands, we have very little understanding of how GLP-1 could potentially regulate skeletal muscle protein turnover. More research needs to be deployed in this area. Perhaps understanding how GLP-1 interacts with myocytes in a non-insulinotropic fashion would be an initial step. Crucially, we also need to uncover any intracellular signalling as a result of this interaction. Moreover, elucidating how GLP-1 interacts with tissues around muscle cells such as vascular endothelium would be equally important. It remains to be clarified whether current pharmacological options of GLP-1 based therapy, which provides sustained levels of GLP-1 concentrations at both postprandial and

postabsorptive states, are beneficial beyond their insulinotropic characteristics in improving muscle metabolism and microcirculation.

Our current understanding of the way GLP-1 stimulates pancreatic insulin secretion is likely via combinations of both direct stimulations from hormone released from L-cells localised within the duodenum and via neuroendocrine stimulation from L-cells located more distally in the small and large bowel. Physiologically, given the very short half-life of the hormone, any extrapancreatic effects are likely to be neuroendocrine in nature. Whether the inactive form of the hormone, GLP-1 (9-36) amide, has any biological actions on pancreatic or extrapancreatic tissue remains to be investigated.

In this work, I have demonstrated that GLP-1 at physiological postprandial levels has a differential effect over insulin on recruiting microvasculature in skeletal muscle at different levels of insulin in the nutritive state. GLP-1 effects on MVR under fed conditions have never been investigated before. The results demonstrate GLP-1 ability to recruit vascular endothelium postprandially, same as insulin. Other research groups have demonstrated that this effect (examined under postabsorptive conditions) is NO dependent, suggesting physiological functions conducted via the same mechanism as insulin.

This work could not demonstrate an increase in glucose uptake into the skeletal muscle as a result of improved MVR. These findings are not different from other results shown in young individuals examined under hyperinsulinaemic euglycaemic clamp conditions.

The clinical and socioeconomic impact of sarcopenia represents a huge challenge to care planners. One of the mechanisms involved in the development of

sarcopenia is blunted vascular responses to hormone and nutrition. My work suggests that adopting certain nutritional approaches could result in hormonal profiles in plasma that might tackle vascular non-responsiveness seen in physiological ageing. Despite these promising results, experiments with GLP-1 failed to facilitate muscle protein synthesis compared to insulin alone but proved to have a positive effect compared to the fasted state. These positive results warrant further investigations to see GLP-1 effectiveness in addressing anabolic resistance in older age.

Age related decline in muscle function would continue to need better understanding and investigatory tools. More research will help the development of new more targeted therapeutic strategies and planning of optimal usage of current available physiological and pharmacological approaches that positively affect skeletal muscle vascular endothelium and improve anabolic responses to hormone and nutritional stimuli.

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Appendices

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28th February 2014

Dr Haitham Abdulla
Clinical Research Fellow
Division of Medical Science & Graduate Entry Medicine
School of Medicine
Royal Derby Hospital
Uttoxeter Road
Derby
DE22 3DT

Dear Dr Abdulla

Ethics Reference No: G12122013 MSGEM. – **Please always quote**
Study Title: Characterizing the Incretin Effect of Amino Acids (AA) and Defining the Effect of GLP-1 on Muscle Microvascular Blood Flow and Muscle protein and Glucose Metabolism in Older Age.
Short Title: Incretin
Chief Investigators/Supervisor: Dr P J Atherton, Associate Professor, Dr I Idris, Associate Professor and Consultant Physician, Division of Medical Science & Graduate Entry Medicine (MSGEM). School of Medicine, Royal Derby Hospital.
Lead /Co-Investigators: Dr K Smith, Principal Research Fellow, Dr D J Wilkinson, Research Fellow, Dr B Phillips, Research Fellow, Dr H Abdulla, Clinical Research Fellow, Mrs M Baker and Mrs A Gates, Senior Human Measurement Technicians, MSGEM, School of Medicine, Royal Derby Hospital
Duration of Study: 03/14-02/2016 3 years **No of Subjects:** 32

Thank you for your letter dated 18th February 2014 responding to the issues raised by the Committee and the following revised documents were received:

1. Incretin Study_Response to UoN Ethics Committee; 18/02/2014.
2. FMHS Incretin Study: Ethics Application Form, V.2, 18/02/2014.
3. Incretin Study: Detailed Protocol/Proposal, V.2 18/02/2014.
4. FMHS Incretin Study: Protocol 1; Younger Age Subject Information Sheet, V2 18/02/2014
5. FHMS Incretin Study: Protocol 1; Older Age Subject Information Sheet, V1 18/02/2014
6. FHMS Incretin Study: Protocol 2; Subject Information Sheet, V2 18/02/2014
7. Incretin Study GP Letter Protocol 1; Older Subjects, V1 18/02/2014
8. Incretin Study GP Letter Protocol 1; Younger Subjects V1 18/02/2014
9. Incretin Study: GP Letter, Protocol 2; V2 18/02/2014
10. Incretin Study Consent Form Protocol 1 V2, 18/02/2014
11. Incretin Study Consent Form Protocol 1 V1, 18/02/2014
12. DEXA Scan Information leaflet V1, 18/02/2014
13. Incretin Study: Protocol 1 Older Advert 18/02/2014
14. Incretin Study: Protocol 1 Young Advert 18/02/2014
15. Incretin Study Protocol 2 Advert 18/02/2014

These have been reviewed and are satisfactory and the study is approved.

Approval is given on the understanding that the Conditions of Approval set out below are followed.

1. You must follow the protocol agreed and inform the Committee of any changes using a notification of amendment form (please request a form).

2. You must notify the Chair of any serious or unexpected event.

3. This study is approved for the period of active recruitment requested. The Committee also provides a further 5 year approval for any necessary work to be performed on the study which may arise in the process of publication and peer review.

4. An End of Project Progress Report is completed and returned when the study has finished (Please request a form).

Yours sincerely



Dr Clodagh Dugdale
Chair, Nottingham University Medical School Research Ethics Committee

Faculty of Medicine and Health Sciences Research Ethics Committee Membership 2013/2014

*denotes attendance at 12th December 2013

Chair	Dr Clodagh Dugdale, Lecturer in Sports and Exercise Medicine, Division of Rheumatology, Orthopaedic and Dermatology, School of Medicine.*
School	**Representative**
School of Life Sciences	Dr Vince Wilson, Reader and Basic Scientist, Pharmacology Dr Liz Simpson, Chief Experimental Officer, Metabolic and Molecular Physiology.*
School of Medicine	Dr David Turner, Clinical Associate Professor in Microbiology, Division of Medical Sciences and Graduate Entry Medicine Dr Kimberly Edwards, Director of Postgraduate Courses/Epidemiologist, Division of Rheumatology Orthopaedics and Dermatology.*
School of Medicine	Dr Gillian Doody, Clinical Associate Professor & Reader in General Adult Psychiatry, Division of Psychiatry and Applied Psychology. Dr Neil S. Coulson,Associate Professor in Health Psychology, Division of Rehabilitation & Ageing.
School of Medicine-Non Clinical	Dr Philip Clark, Laboratory Manager, Pre-Clinical Cancer Studies, Division of Oncology Dr Charlotte Billington, Research Fellow, Division of Respiratory Medicine Dr Debbie Palmer-Green, Research Fellow, Division of Rheumatology Orthopaedics and Dermatology.*
School of Medicine Derby	Dr Caroline Chapman, Associate Professor, Breast Surgery, Division of Medical Sciences and Graduate Entry Medicine.* Dr Saoirse O'Sullivan, Associate Professor in Vascular Medicine, Division of Medical Sciences and Graduate Entry Medicine Derby.*
School of Medicine - Clinical	Dr Paul Maddison, Consultant Neurologist/Honorary Clinical Associate Professor, Queen's Medical Centre, NUH*
Division of Primary Care	Dr Daren Forward, Consultant Orthopaedic Trauma Surgeon, Honorary Consultant Lecturer Dr Richard Knox, General Practitioner/ Part-time Lecturer Dr Edward Tyrrell, General Practitioner/Part-time Lecturer Dr Jaspal Taggar, General Practitioner/Part-time Lecturer* Division of Primary Care, QMC Campus
School of Health Sciences	Dr Stephen Timmons, Associate Professor/Senior Lecturer, Division of Nursing.*
Lay (Out of Faculty)	Professor Nigel White, Professor of Public International Law, School of Law, University of Nottingham. Lydia Davies-Bright, PhD Student, School of Law. Dr Emma Hall, Research Fellow, SPMMRC, School of Physics and Astronomy.* Mary Beirne, Retired Mental Health Nurse, Birmingham and Solihull Mental Health Foundation Trust.
Medical Students nominated by Medical student Society	To be appointed.
Postgraduate Student Member	Peter Collins, PhD Student, Division of Psychiatry and Applied Psychology.*
Administrator	Mrs Louise Sabir, Division of Respiratory Medicine, School of Medicine*

School of Graduate Entry Medicine and Health
Royal Derby Hospital

The University of Nottingham

Healthy Volunteer's Information Sheet

Title of Project: **Characterizing the Incretin Effect of Amino Acids (AA) and Defining the Effect of GLP-1 on Muscle Microvascular Blood Flow and Muscle Protein and Glucose Metabolism in Older Age.**

Name of Investigators:

Dr PJ Atherton
Dr B Phillips
Dr H Abdulla
Dr K Smith
Dr J Williams
Dr DJ Wilkinson
Dr I Idris

You have been invited to take part in a research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives if you wish to. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you decide to take part you will be given a copy of your consent form and this leaflet.

Background:

Age related muscle wasting and weakness represents one of the most significant health problems of the developed world. With age, muscles tend to waste at rate of 0.5-1% per year, so that an 80 year old may have only 70% of the muscle possessed at the age of 50. Muscle loss makes it harder to carry out tasks that require strength, keeping the body balanced and prolonged periods of activity. Together these things may contribute to loss of independence and increased risk of falls.

As part of muscle mass maintenance, muscle is constantly broken down and re-made. This balance is lost with old age in a way that re-making is decreased. This is possibly due to a reduced response to amino acids, which are the building blocks of protein. This may, in part, explain why muscle is gradually lost with ageing.

It is known that muscle building in all age groups depends on good delivery of nutrients (largely sugars and amino acids) to the muscle and good capture of these nutrients by the muscle tissue. With advancing age there is a decline in muscle blood flow with consequent negative effects on muscle building and health.

It is also known that taking glucose (a form of sugar) in a meal leads to secretion of chemicals called incretins by the gut. Interestingly injecting glucose through veins doesn't lead to the same effect. Incretins positively impact various body tissues, including muscle uptake of glucose from the blood stream. We would like to test if amino acids would lead to the same effect as glucose does and also test whether gut hormone secretion in response to amino acids changes with old age. Furthermore, we would like to test if incretins improve blood flow to the muscles. If they do we would expect to see improved delivery of nutrients to the muscle and therefore better capture of these nutrients.

Establishing these facts will give an idea whether incretin therapy is a promising option to target age related declines in muscle performance and help improve activity and independence in older people.

Why have you been chosen?

In order for this study to be successful, volunteers have been chosen who fit the entry criteria and do not have on-going medical problems. We are inviting 16 participants aged between 65 and 75 years to take part.

Do you have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

Would an inconvenience allowance be paid?

You will receive £150.00 for completion of the study protocol.

What does the study involve?

The site of the study is The Medical School at the Royal Derby Hospital. The study consists of a screening visit, 2 acute study visits and 2 follow up visits.

Screening visit:

Before the first study day you will be asked to attend the study site without having eaten or drunk anything (except water) from 10pm the night before.

During this visit (approximately 60 minutes), we will:

(i) Ask you to sign a consent form for the study
(ii) Take blood samples
(iii) Conduct a health assessment, and
(iv) Ask you to complete an activity questionnaire

The health assessment will include:

1) Discussion of any past medical problems or illnesses (including any tablets you are taking and any allergies you might have), basic personal and social information (e.g. age, gender, marital status, employment) and a routine medical examination.
2) Perform an electric recording of your heart activity.
3) Measurement of your height, and weight.
4) Measurement of your blood pressure and pulse rate.
5) Perform an ultrasound scan of the muscles and blood vessels in your legs to check your suitability for the study procedures. This ultrasound scan is similar to that used to image babies during pregnancy and does not involve any x-rays.

If any medical abnormalities are found these would be discussed with you and, with your permission your GP.

Diet diaries:

Between your screening visit and acute study visit 1 and before acute study visit 2 you will be asked to complete a diet diary, recording all food and drink taken during a three day period (2 week days and 1 weekend day). You will be advised how to complete these records so that we can accurately determine your nutrient intake.

Acute study visits:

These will take place at least 7 days after your screening visit, once we have received your blood sample results.

First acute study (see Figure 1):

You will be asked to attend the medical school at 8am, having not had anything to eat or drink (other than water) from 10pm the previous evening. Your visit is expected to last 7-8 hours. Firstly you will have a DEXA scan (a form of x-ray) for the estimation of your body fat and muscle mass; please find information leaflet enclosed. You will then be asked to lie on a bed. After freezing the skin with local anaesthetic, small plastic tubes (cannulae) will be introduced into 3 veins in your forearms and a further 2 inserted into the two large vessels at the top of your leg. All these insertions will be performed by a doctor who is skilled in these procedures. These insertions allow us to take blood samples periodically during the day, and enable us to give the infusions used in the study. Over the first two hours blood sampling will take place every 30 minutes, with samples every 15 minutes in the last 4 hours. In addition to this, when the insulin infusion is started your blood glucose will be monitored every 5 minutes. Roughly 230ml of blood will be taken by the end of the study day.

The infusions we use during the study are:

i. saline (salty water) to stop blood clotting in the blood sampling tubes
ii. a solution containing labelled amino acids and glucose to allow us to measure your protein metabolism
iii. Octreotide; a medicine that will suppress insulin produced from your pancreas
iv. Glucose
v. Glamin®; mixed amino acids to simulate feeding
vi. Insulin at concentrations similar to those your body secrete when fasting or those secreted after feeding, and
vii. Sonovue®; a solution of tiny bubbles which will be infused to allow us to measure where the blood in your leg is going using a specialist ultrasound scanner.

You will receive the labelled amino acid and glucose infusion at the start of the study and this will run until the study finishes (6 hours/ 360 min). From 3 hours (180 min) into the study, Octreotide®, Glucose, Insulin and Glamin® infusions will run and will continue until the end of the study.

Ultrasound scanning will be performed twice at 100 and 285 min from the start of the study. It involves the doctor placing gel (which can feel a bit cool) on the skin of your upper leg, near the groin, and holding a smooth probe on the skin, above the vein. In addition, at these same time points, muscle blood flow will be measured by contrast enhanced ultrasound (CEUS) using Sonovue® microbubbles.

During the study 4 muscle samples (biopsies) will be taken from the leg that have the plastic tubes inserted in it. Each biopsy will be taken after local anaesthetic has been injected to numb the area. A small (0.5 - 1 cm) cut is then made through the skin and underlying tissue, before a small piece of muscle (about the size of an orange pip), is taken with specially designed tweezers. With the local anaesthetic you should feel no pain during this procedure but you may feel movement or pushing sensations. On a scale of 0-10, where 0 was ‘no pain’ and 10 was ‘excruciating pain’, previous volunteers have scored this procedure as an average of 1/10.

Initially, the biopsy instrument may return just a small amount of fatty tissue. This means the instrument is sometimes reintroduced through the incision several times to provide the sample of tissue we need for analysis; usually 2-3 “takes” are sufficient. A stitch is placed in the skin afterwards to aid healing, and a tight bandage will be placed around your thigh after the study. This should remain in place for ~6 hours, to reduce bruising.

The biopsies will take place at 60, 170, 270 and 360 min from the start of the study.

When the last biopsy is taken all cannulae will be taken out, with the exception of one, which is to be used for slowly reducing the glucose infusion to avoid your blood sugar going too low. You will then have a meal and a drink. The final cannula will be removed when your blood sugar level is deemed stable. With these precautions (having food and monitoring of your blood sugar after the study) the risk

of developing low blood sugar is minimised, however there is small risk this might happen after you leave the study site. The study staff will discuss this with you and advise you on what to look for and how to manage this. You will also be given a contact number for the study physician should you needed any help after you leave the study site.

Your heart's electrical activity will be monitored throughout the duration of the study and for 20 minutes after the study has finished.

Follow-Up Visit

This will take place approximately 7 days after your first acute study visit for the removal of stitches from biopsy sites and to check femoral insertion sites.

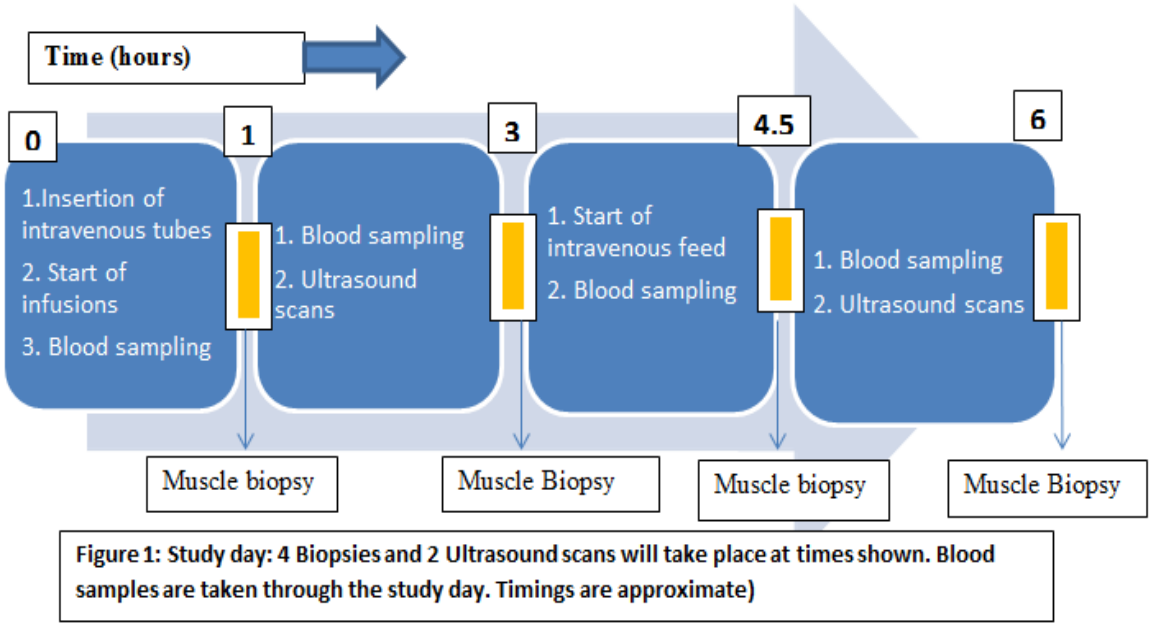


Figure 1: Study day: 4 Biopsies and 2 Ultrasound scans will take place at times shown. Blood samples are taken through the study day. Timings are approximate)

Second acute study:

This will take place 4 weeks after your first acute study visit. It will follow the same steps detailed on the first acute study visit with the exception that GLP-1 (a gut hormone) will also be infused from 3 hours into the study until the end. We are infusing this to test its effect on your muscle blood flow and metabolism.

Second follow-Up Visit

This will take place approximately 7 days after your second acute study visit for the removal of stitches from biopsy sites, to check femoral insertion sites and to allow payment of the inconvenience allowance for the study.

What do I have to do?

We are studying the effect of GLP-1 (an incretin hormone) on muscle blood flow and muscle health under feeding conditions. We are studying this in healthy adults, so we need volunteers who are eating and exercising as they would normally.

With the exceptions of fasting on the nights before the initial screening and the acute studies, please do not modify your usual diet or activity levels for this study.

What is the drug or procedure that is being tested?

This study assesses the potential novel function of incretins to improve blood flow to the muscles as measured by contrast-enhanced ultrasound (CEUS). It will assess the effect of incretins on muscle health under feeding conditions as mimicked by the infusion of amino acids and glucose.

What are the side effects or risk of any treatment or procedures received when taking part?

Research studies often involve some risks, not all of which may be currently known.

Infusions carry a risk of infection or irritation of the vein at the administration site when given repeatedly. As this is a short study side-effects are not expected.

Studies using labelled amino acids and glucose have been performed for over 20 years without side-effects.

In our experience, muscle biopsies are generally well tolerated by subjects. Scarring is hardly perceptible and fades with time to near invisibility. The muscle biopsies are usually painless at the time they are being taken, because of the local anaesthetic we use. However, some discomfort can be felt during the procedure, and at the biopsy sites when the anaesthetic has worn off (around 6-12 hrs later). However this is usually mild (rated as less than 2/10 by most past participants). Some muscle tenderness and stiffness is usually felt for 1 to 2 days afterwards and this can be alleviated by over-the-counter painkillers, such as Paracetamol. There will be some bruising associated with the biopsy procedure, though this should clear within a week. There is a slight risk of infection at biopsy sites, and damage to small nerves in the skin is possible during the biopsy procedure. These nerves usually grow back with restoration of normal sensation, although occasionally it can take up to a year for this to resolve.

The risks involved in arterial and venous cannulation and blood sampling are very small. The femoral vein lies adjacent to the femoral artery therefore to aid correct placement and reduce risks, ultrasound imaging will be used to locate both artery and vein. It is rare to cause damage to the vessels using this technique; however, should this occur, pressure would be applied over the site for five to ten minutes until bleeding had stopped. Occasionally however there may be bruising under the skin due to leakage from a blood vessel on withdrawal of the cannula, but this is uncommon with good practice.

Sonovue®, the fluid containing tiny bubbles, is widely used within the NHS for a range of diagnostic procedures. It has no commonly identified side effects. However, less common side effects include a rash, itch, dizziness, nausea (feeling sick), flushing, back pain and chest pain. Rare cases suggestive of hypersensitivity, include skin erythema (redness), bradycardia (slow heart rate), hypotension (low blood pressure) or anaphylactic shock (severe sensitivity reaction ~0.01%) have been reported following the injection of Sonovue®. More than 220 administrations of Sonovue® have been carried out by this research group without any problems. However, if you experience any problems when you are given this infusion, you should immediately inform the experimenters.

Side effects of drugs used in the study:

Insulin: Causes low blood sugar. If blood sugar levels drop too low you may experience sweats, shakiness, confusion and blurred vision, reduced concentration and in rare occasions difficulty with speech or arm movement. During the study insulin infusion will be accompanied by glucose infusion, the level of which will be tested every 5 min to avoid this risk. At the end of the study you will have a meal and your blood sugar will be monitored until it is safe for you to leave the study site. These precautions and the fact that you are not diabetic make an event of low blood sugar unlikely.

Glucagon Like Peptide-1 (GLP-1): May cause nausea (feeling sick), but this mostly occurs in much higher concentrations than those used in this study. Other side effects associated with long-term use are: diarrhoea, constipation, infections of the upper airways and headache, although we are giving this over a very short period in this study. Patients taking this over the long-term may also experience flu like symptoms, dizziness, sinusitis (inflammation of sinuses), back pain, and reactions at the injection site.

Octreotide®: May cause anorexia (reduced of appetite), nausea (feeling sick), vomiting, abdominal pain, bloating, flatulence and diarrhoea. Very rarely pancreatitis (inflammation of the pancreas) has been reported with long term use.

Glamin®: Rarely may cause fever, chills, hot flushes, changes in liver and kidney function tests, and reduced blood cell count.

The doctor supervising the study will monitor all of these symptoms closely. If it is judged that the study cannot be completed because of a particular side effect, the study will be terminated and you will be monitored until it's safe for you to leave the study site.

What are the possible benefits of taking part?

We cannot promise the study will help you, but the information we get from this study may help us design feeding strategies for seriously ill patients, and those undergoing major surgery, that will help preserve their muscles and strength. A better understanding of muscle aging may also help us understand the role of diet in staying healthy and active in advancing years, and may lead to better dietary advice and possibly even dietary supplements.

Participation would provide you with a medical check-up, including clinical examination, measurement of your blood pressure, fasting blood glucose and electric recording of your heart activity. We will also test your blood for kidney and liver function and you will have your bone mineral density checked. If you are interested in these results, a copy can be provided to you.

What are the possible disadvantages and risks of taking part?

Healthy volunteers have been chosen for this study; however, it is possible that the routine tests could detect unknown health problems. Examples of these include diabetes and high blood pressure, as they are common and often undiagnosed. Should this be the case, you will be informed and advised to attend your GP practice for further management. After your permission, your GP will be notified. Be advised, however, that future insurance status e.g. for life insurance or private medical insurance, could be affected by these diagnoses.

What are the exclusion criteria for this study?

We are recruiting healthy men between the ages of 65 and 75 years, who are normal weight or slightly overweight. If you have been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance; having an invasive procedure (e.g. blood sample >50ml), or exposure to ionising radiation, you would not be suitable to take part.

You would also be unsuitable if you have particular medical conditions, are taking certain medications, or have allergies to any of the products we use in the study. Please discuss these further with the study doctor.

What if something goes wrong?

If you suffer any symptoms or side effects you should report them immediately to the study doctor (see end of sheet for contact details). If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you have a complaint on your treatment by a member

of staff or anything to do with the study, you can initially approach the lead investigators, Dr I Idris and Dr PJ Atherton. If this achieves no satisfactory outcome, you should then contact the Ethics Committee Secretary, Mrs Louise Sabir, Division of Therapeutics and Molecular Medicine, D Floor, South Block, Queen's Medical Centre, Nottingham, NG7 2UH. E-mail louise.sabir@nottingham.ac.uk. In the unlikely event that you suffer injury to yourself or damage to your property as a result in taking part in this research, the University does have an insurance policy to cover harm arising as a result of the defect in the design of the study. In addition, all medical practitioners taking part in the research have personal medical negligence cover.

Will my taking part in this study be kept confidential?

We respect your right to privacy and we will take measures to safeguard confidentiality. A single form, on which you are asked to sign to give consent for involvement, carries details of your name and address, but no health related details. This is kept securely in a locked cabinet within the Medical School. Access to this cabinet is restricted to personnel directly involved in the study and to University staff with direct responsibility for ensuring the study is conducted appropriately.

We will follow current ethical and legal practice and all information about you will be handled in confidence. If you join the study, some parts of the data collected about you will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by people authorised to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept strictly confidential, stored in a secure and locked office, and on a password protected database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it with the exception of a letter sent to your GP advising of your participation in this study.

Your personal data (address, telephone number) will be kept for 3 years after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies (unless you advise us that you do not wish to be contacted). All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team will have access to your personal data.

What will happen to any samples I give?

We will use the samples you give us to explore how muscles respond to protein feeding.

We will also seek your consent for any blood samples remaining after analysis for this study has been completed, to be stored and used in future research. This is optional and you will be asked to give separate consent for this. The samples will be stored with a code unique to you and securely at the University of Nottingham under the Universities Human Tissue Research Licence (No. 12265).

Some of these future studies may be carried out by researchers other than the current research team of Dr Atherton and Dr Idris who ran the first study. This may include researchers working for commercial companies. Any samples or data used will be anonymised so that you could not be identified in any way. If you do not agree to this, any remaining samples will be disposed of in accordance with the Human Tissue Authorities codes of practice.

Will any genetic tests be done?

In the current study we will not be using your blood samples for genetic testing.

What will happen to the results of the research study?

Data collected during the study will be published in the scientific literature enabling other health professionals to use the information. You will not be identified in any publication. There is usually a delay of up to one year from the study completion before this occurs. Should you wish to be informed of publications resulting from this study, please inform the trial investigators.

Who is funding the research?

This research is funded by The University of Nottingham.

Who has reviewed the study?

This study has been reviewed and approved by the University of Nottingham Medical School Ethics Committee.

A questionnaire based audit of one of our studies involving biopsies in the past demonstrated that 95% of volunteers felt that they were given adequate explanation of the procedure. 83% experienced lesser or expected discomfort during the muscle biopsy and all of them said that they would be or may be willing to participate in a similar study in the future.

Contact for Further Information

Thank you for taking part in the study. For further information please contact **Dr Haitham Abdulla on 01332 724685/ Haitham.abdulla@nhs.net**. Alternatively **Dr Abdulla** is also contactable at The University of Nottingham, Division of Medical Sciences Graduate Entry Medicine, Royal Derby Hospital, Derby, DE22 3DT.

Division of Medical Sciences and Graduate Entry
Medicine, School of Medicine, Royal Derby Hospital

Title: ***Characterizing the Incretin Effect of Amino Acids (AA) and Defining the Effect of GLP-1 on Muscle Microvascular Blood Flow and Muscle Protein and Glucose Metabolism in Older Age***

Name of Investigators:
Dr Philip J Atherton (Associate Professor - Principal Investigator)
Dr Ken Smith (Principal Research Fellow)
Dr Idris Iskandar (Associate Professor, Honorary Consultant Physician)
Dr John Williams (Associate Professor and Consultant Anaesthetist)
Dr Bethan Phillips (Research fellow)
Dr Daniel Wilkinson (Research fellow)
Dr Haitham Abdulla (Clinical Research Fellow)
Mrs Margaret Baker/Mrs Amanda Gates (Senior Research Technicians)

Thank you for your interest in this study. I would be grateful if you would complete and return this form so that we may contact you with further information.

FULL NAME (Including title):

ADDRESS:

POST CODE:

TELEPHONE NUMBER:

EMAIL:

DATE OF BIRTH:

Are you taking any regular medication? YES / NO

If yes, please list:

Do you have a medical history of:

Diabetes	YES / NO	High Blood Pressure	YES / NO
Asthma	YES / NO	Heart Disease	YES / NO
Stroke	YES / NO	Epilepsy	YES / NO

Any other medical problems (please list below): YES / NO

HEIGHT: WEIGHT: GENDER:

Signature: Date:

Please note: Information submitted on this form will be kept **strictly confidential** and will not be shared with any additional parties.

University of Nottingham
School of Medicine
Division of Medical Sciences and Graduate Entry Medicine

Healthy Volunteers Consent Form

Title of Study: Characterizing the Incretin Effect of Amino Acids (AA) and Defining the Effect of GLP-1 on Muscle Microvascular Blood Flow and Muscle Protein and Glucose Metabolism in Older age

Name of Researchers:

Dr PJ Atherton PhD, Associate Professor
Dr I Idris DM FRCP, FRCP(Edin), Associate Professor and Consultant Physician
Dr K Smith PhD, Principal Research Fellow
Dr J Williams PhD, FRCA, Associate Professor and Consultant Anaesthetist
Dr DJ Wilkinson PhD, Research Fellow
Dr BE Phillips PhD, Research Fellow
Dr H Abdulla MRCP, Clinical Research Fellow

Name of Participant:
Recruitment ID: _____
Enrolment ID: _____

Please initial box

1.	I voluntarily agree to take part of **protocol 2** of the above study.	☐
2.	I confirm that I have been given a full explanation by the above named and that I have read and understand the information sheet given to me which is attached.	☐
3.	I have been given the opportunity to ask questions and discuss the study with one of the above investigators or their deputies on all aspects of the study and have understood the advice and information given as a result.	☐
4.	I agree to the above investigators contacting my general practitioner to make known my participation in the study where relevant.	☐
5.	I agree to comply with the reasonable instructions of the supervising investigator and will notify him immediately of any unexpected unusual symptoms or deterioration of health.	☐
6.	I authorise the investigators to disclose the results of my participation in the study but not my name.	☐
7.	I understand that information about me recorded during the study will be kept in a secure database. If data is transferred to others it will be made anonymous. Data will be kept for 7 years after the results of this study have been published.	☐

8. I understand that relevant sections of data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential ☐

9. I understand and agree that muscle samples will be taken for analysis of muscle protein metabolism and molecular repair mechanisms. I agree to the storage of my muscle samples for any future, unspecified research relating to muscle health and disease. ☐

10. Consent for storage and use in possible future research (Optional) ☐
I agree that the samples I have given and the information gathered about me can be stored by researchers at the School of Graduate Entry Medicine and Health, for possible use in future studies. I understand that some of these studies may be carried out by researchers other than current team of PJ Atherton and colleagues, who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and I will not be identified in anyway.

11. I authorise the investigators to disclose to me any abnormal test results. ☐

12. I understand that I can ask for further instructions or explanations at any time. ☐

13. I understand that I am free to withdraw from the study at any time, without having to give a reason for withdrawing. ☐

14. I confirm that I have disclosed relevant medical information before the study. ☐

15. I shall receive an inconvenience allowance of £150.00. If I withdraw from the study for medical reasons not associated with the study a payment will be made to me proportional to the length of the period of participation, but if I withdraw for any other reason, the payment to be made, if any, shall be at the discretion of the supervising investigator. ☐

16. I have not been a subject in any other research study in the last three months which involved: taking a drug; being paid a disturbance allowance**;** having an invasive procedure (eg venepuncture >50ml, endoscopy) or exposure to ionising radiation. ☐

17. I confirm that I have not been exposed to more than 5 mSv of ionising radiation in the last 12 months. ☐

Name of Participant:……..**Date:**……**Signature:**…….……

Name of Person taking consent:…….**Date:**…….**Signature:**……
(if different from Principal Investigator)

Name of Principal Investigator:……..**Date:**…….**Signature:**……

2 copies: 1 for participant, 1 for the project notes

Incretin Study
Protocol 2 Acute Study Sheet-
Visit 1

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Date: _____

Subject recruitment ID: _____
Subject enrollment ID: _____

Insulin: PA □ PP □
GLP-1 : + □ -- □

Min	Intended Time	Actual Time	Procedure/ Measurements	Monitoring/ Comments
-05	[redacted]	☐☐:☐☐	V0: EDTA,P800, Pax, SST YSI GLU: Hct: (from peripheral line)	BP: HR: DEXA Femoral lines R □ L □ Biopsies R □ L □ US Doppler R □ L □ CEUS R □ L □
0	[redacted]	☐☐:☐☐	Start Tracer Start 2-DOG	Prime: Inf: Prime: Inf:
30	☐☐:☐☐	☐☐:☐☐	V1: EDTA	Insert femoral lines (A and V)
45	☐☐:☐☐	☐☐:☐☐	Infiltrate local anaesthetic	
60	☐☐:☐☐	☐☐:☐☐	V2: EDTA A2: EDTA	BP: HR:
60	☐☐:☐☐	☐☐:☐☐	**Biopsy 1**	
70	☐☐:☐☐	☐☐:☐☐	Site CEUS probe	
90	☐☐:☐☐	☐☐:☐☐	V3: EDTA	
120	☐☐:☐☐	☐☐:☐☐	V4: EDTA A4: EDTA	BP: HR:
130	☐☐:☐☐	☐☐:☐☐	**US Doppler 1**	
140	☐☐:☐☐	☐☐:☐☐	V5: EDTA A5: EDTA	
145	☐☐:☐☐	☐☐:☐☐	**Start CEUS 1**	
150	☐☐:☐☐	☐☐:☐☐	**US Doppler 2**	
160	☐☐:☐☐	☐☐:☐☐	V6: EDTA A6: EDTA	
165	☐☐:☐☐	☐☐:☐☐	Infiltrate local anaesthetic	
170	☐☐:☐☐	☐☐:☐☐	**US Doppler 3**	

180	☐☐:☐☐	☐☐:☐☐	V7: EDTA, P800 A7: EDTA, P800	YSI Glu:
180	☐☐:☐☐	☐☐:☐☐	**Biopsy 2**	
0	☐☐:☐☐	☐☐:☐☐	Start Insulin, Dextrose, Octreotide & Vamine ± GLP-1	BP: HR: YSI GLU - Every 5 min until 180 min
20	☐☐:☐☐	☐☐:☐☐	V8: EDTA, P800 A8: EDTA	
40	☐☐:☐☐	☐☐:☐☐	**US Doppler 4**	BP: HR:
50	☐☐:☐☐	☐☐:☐☐	V9: EDTA, P800 A9: EDTA	
60	☐☐:☐☐	☐☐:☐☐	**Doppler 5**	
70	☐☐:☐☐	☐☐:☐☐	V10: EDTA, P800 A10: EDTA, P800	
75	☐☐:☐☐	☐☐:☐☐	Infiltrate local anaesthetic	
80	☐☐:☐☐	☐☐:☐☐	**US Doppler 6**	BP: HR:
90	☐☐:☐☐	☐☐:☐☐	V11: EDTA, P800 A11: EDTA	
90	☐☐:☐☐	☐☐:☐☐	**Biopsy 3**	
110	☐☐:☐☐	☐☐:☐☐	V12: EDTA, P800 A12: EDTA	
120	☐☐:☐☐	☐☐:☐☐	**Start CEUS 2**	BP: HR:
130	☐☐:☐☐	☐☐:☐☐	**US Doppler 7**	
140	☐☐:☐☐	☐☐:☐☐	V13: EDTA, P800 A13: EDTA	
150	☐☐:☐☐	☐☐:☐☐	**US Doppler 8**	
160	☐☐:☐☐	☐☐:☐☐	V14: EDTA, P800 A14: EDTA, P800	
165	☐☐:☐☐	☐☐:☐☐	Infiltrate local anaesthetic	
170	☐☐:☐☐	☐☐:☐☐	**US Doppler 9**	
180	☐☐:☐☐	☐☐:☐☐	V15: EDTA, P800 A15: EDTA	Hct:
180	☐☐:☐☐	☐☐:☐☐	**Biopsy 4**	BP: HR:

Subject recruitment ID: _____ Date: _____
Subject enrollment ID: _____

Notes:

Signature: _____ Date: _____

Subject recruitment ID: _____ Date: _____
Subject enrollment ID: _____

Use this this space to document any relevant data/ clinical event during the acute study.

Name _____ Signature _____
Date_____