

Determining the Effect of Glucagon-Like Peptide 1 (GLP-1) upon Skeletal Muscle Metabolism and Blood Flow in Older Age

Tereza Jandova BSc.

Thesis submitted to the University of Nottingham

for the degree of Master of Research

December 2016

Abstract

Age-related musculoskeletal and metabolic disorders often resulting from glucose intolerance due to age-related insulin resistance represent one of the most significant health problems in our ageing world. The incretin hormone glucagon-like peptide-1 (GLP-1) has shown to have positive effects on vascular function and glucose disposal as demonstrated by recent human and animal studies. This study, therefore, aimed to investigate whether GLP-1 can exert the same positive effect on muscle's postprandial glucose metabolism and vascular blood flow in old age. To examine such effects, the study was conducted in a cross-over design (± GLP-1) consisting of two acute study visits with a four week 'wash-out' period. GLP-1 was infused in the femoral artery in overnight fasted healthy old volunteers (n=7) along with glucose and insulin clamps in combination with Octreotide and amino acids to achieve constant postprandial conditions. Leg blood flow (LBF) was measured by Doppler ultrasound, computation of rates of total body glucose metabolism (M) during the clamps was calculated by glucose infusion rates minus urinary loss of glucose minus space correction and leg glucose disposal by arterial-venous (A-V) balance technique. Our results show that the infusion of GLP-1 increased overall LBF, which was significant when a t-test was applied ($P < 0.05$) but not significant using a 2-way ANOVA. Significant increases ($P < 0.05$) were also seen in measured whole body glucose metabolism (M). However, glucose leg uptake during the GLP-1 infusion did not significantly increase. We, therefore, conclude that GLP-1 infusion in healthy old people has a significant effect on whole body postprandial glucose metabolism and

vascular blood flow. However, this effect may be influenced by raised insulin concentrations and exerted possibly through an insulin-mediated mechanism and not by GLP-1 action alone. Nevertheless, GLP-1 effect could still contribute to the increased delivery of glucose to the muscle in the elderly and specifically combat the negative consequences of ageing i.e. impaired glucose uptake, vascular blood flow and age-related insulin resistance by further enhancing the action of insulin. However, further investigations are required.

Acknowledgements

I would like to express my sincere gratitude to my supervisor Dr Kenneth Smith for his mentorship and support throughout the learning process of this master thesis. I would also like to thank to my other co-supervisors Prof Philip Atherton and Prof Iskandar Idris for their time and engagement in this project. Furthermore, I am immensely grateful to Marie Limb for her help and advice through the whole year on matters relating to both laboratory work and this thesis. I would like to also thank to Jessica Cegielski for her general help and assistance in the laboratory research unit. My thanks also go to the staff at the Clinical, Metabolic and Molecular Physiology Unit, Division of Medical Sciences and Graduate Entry Medicine, Debbie Rankin, Beth Philips, Amanda Gates and others for their general assistance throughout the whole year of my study. Above all, I would like to thank my loved ones for their overall support and patience during the entire process.

List of Abbreviations

AA	Amino acids
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APE	Atom percent excess
aPKC	Atypical protein kinase C
ATP	Adenosine Triphosphate
AUC	Area under the curve
AV	Arterial - venous
BP	Blood pressure
BSTFA	N, O-bis (trimethylsilyl) trifluoroacetimidate with 1% trimethylchlorosilane
CEUS	Contrast enhanced ultrasound
CP	Creatine phosphate
ddW	Double distilled water
DPP-4	Dipeptidyl-peptidase-4
DXA	Dual-energy X-ray absorptiometry scan

EC	Endothelial cell
EDTA	Ethylenediamine tetraacetic acid
EHC	Euglycaemic hyperinsulinaemic clamp
ELISA	Enzyme linked immunosorbent assays
FDG	Fluorodeoxyglucose
FFA	Free fatty acids
F6P	Fructose 6-phosphate
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide 1
GLUTS	Glucose transporters
GCMS	Gas chromatography-mass spectrometry
GIR	Glucose infusion rate
g-6P	Glucose 6-phosphate
IMGD	Insulin mediated glucose disposal
IR	Insulin resistance
IRS-1	Insulin receptor substrate 1
LBF	Leg blood flow
MFI	Mean fluorescent intensity

MDs	Metabolic disorders
MSKDs	Musculoskeletal disorders
MTBSTFA	N-tert - butyldimethyl - silyl -N- methyltrifluoracetamide
MVR	Microvascular recruitment
MVU	Microvascular unit
NCDs	Non-communicable diseases
NIMGD	Non-insulin mediated glucose disposal
NO	Nitric oxide
PBS	Phosphate buffered saline
PDK1	Phosphoinositide-dependent protein kinase 1
PEP	Pyruvate to phosphoenol pyruvate
PI3-K	Phosphatidylinositol-3-kinase
QC	Quality control
ROS	Reacting oxygen species
SEM	Standard error of a mean
SIM	Selected ion monitoring
T2DM	Type 2 diabetes mellitus
YSI	Yellow Springs Instrument

Table of Contents

Abstract i
Acknowledgements iii
List of Abbreviations iv
Table of Contents vii
List of Figures and Tables ix
1. Introduction 1
1.1. The socio-economic burden of an ageing population 1
1.1.1. Musculoskeletal and metabolic disorders in older age 2
1.2. Skeletal muscles: locomotion and beyond 4
1.2.1. Skeletal muscle structure and function 4
1.2.2. Muscle energy sources and glucose production 9
1.2.3. Glycaemic control and the role of skeletal muscle 11
1.2.4. Glucose uptake by skeletal muscle 13
1.2.5. Skeletal muscle vascular architecture 15
1.2.6. Glucose delivery and blood flow in skeletal muscle 17
1.3. Skeletal muscle ageing 18
1.3.1. Ageing: insulin resistance and decreased blood supply 19
1.4. GLP-1 as a potential therapeutic for skeletal muscle ageing 22
1.4.1. Biological action of GLP-1 23
1.4.2. Role of GLP-1 in skeletal muscle glucose metabolism and glucose uptake 25
1.4.3. Effect of GLP-1 on skeletal muscle vasculature 26
1.5. Aims and hypothesis 28
2. Materials and Methods 30
2.1. Subjects and Ethics 30
2.2. Study design 31
2.3. Sample processing 34
2.4. Determination of glucose and 2-DOG concentration in plasma by GCMS . 35
2.4.1. Determination of glucose 36
2.4.2. Determination of 2-DOG 39
2.5. Determination of human insulin in plasma by enzyme immunoassay 41
2.6. Determination of GLP-1 in plasma 41
2.7. Determination of amino acids in plasma by phenylalanine labelling 43
2.8. Determination of total leg blood flow 43
2.9. Calculations 44

2.10. Statistical analyses 44
3. Results 46
3.1. General characteristics of study demographics 46
3.2. GLP-1 concentration in plasma 46
3.3. Phenylalanine concentration in plasma in fasted and fed clamp conditions 48
3.4. Leg blood flow 49
3.5. Insulin concentration in plasma 51
3.6. Glucose concentration in plasma measured by YSI and measured glucose metabolism (M) 53
3.7. Glucose infusion rates 55
3.8. Glucose concentration in plasma measured by GCMS and A-V balance 58
Discussion 62
Appendix A 70
Appendix B 72
References 75

List of Figures and Tables

Figure 1.1 Structure of a skeletal muscle.....5

Figure 1.2 Structure of skeletal muscle fibre and sarcomere.....7

Figure 1.3 Oral glucose clearance.....13

Figure 1.4 Mechanism of glucose uptake into skeletal muscle. 15

Figure 1.5 Schematic representation of muscle's vascular architecture. 16

Figure 1.6 GLP-1 biological actions in peripheral tissues. 24

Figure 2.1 A schematic diagram of the study protocol. 34

Figure 2.2 An example of generated glucose standard curve. 38

Figure 2.3 Chromatogram of the three monitored masses. 39

Figure 2.4 Chromatogram of 2-DOG and FDG peaks. 40

Figure 2.5 An example of generated GLP-1 standard curve. 42

Figure 3.1 Time course of plasma GLP-1 concentrations (pM/L) ± GLP-1 during 180 min post-clamp period. 47

Figure 3.2 Phenylalanine concentration in plasma ± GLP-1 during 180 min pre- and 180 min post clamp amino acid (AA) infusion period. 48

Figure 3.3 Comparison of leg blood flow ± GLP-1. 50

Figure 3.4Time course of plasma insulin concentrations (µU/mL) ± GLP-1 during 180 min post clamp period (A) and area under the curve (AUC) (B). 52

Figure 3.5Time course of glucose concentration measured by YSI (mM/L) (A) and measured glucose metabolism (M) (B) ± GLP-1 during 180 min post clamp postprandial period. 54

Figure 3.6Time course of glucose infusion rates (GIR; mg/kg/min) (A) and area under the curve (AUC) ±GLP-1 during 180 min post-clamp postprandial period (B). 56

Figure 3.7 Area under the curve (AUC) of glucose infusion rates (GIR) for 15 -90 (A) and 90 – 180 (B) min post clamp postprandial period ± GLP-1. 57

Figure 3.8 Time course of arterial and venous (A-V) glucose concentration measured by GCMS (mM/L) ± GLP-1 during 180 min pre- and 180 min post- clamp glucose infusion periods. 60

Figure 3.9 Glucose arterial and venous (A-V) balance in µM/min/100g of leg mass ± GLP-1. 61

Table 1. Medical examinations at screening session 30

Table 2. Study infusions, their site of administration and purpose. 32

Table 3 General characteristics of study volunteers. 46

1. Introduction

1.1. The socio-economic burden of an ageing population

The world is on the edge of a demographic revolution due to population ageing. According to data from World Population Ageing 2015 (1), the number of people in the world over the age of 60 is projected to rise from 901 million in 2015 to nearly 2.1 billion by 2050; a proportional rise from 12% to 22% (2). Moreover, the number of people over the age of 80, so called 'oldest-old', has been growing even faster and it is projected that in 2050 some countries will have nearly four times more (from 125 million to 434 million) oldest-old people than in 2015 (1–3). Such exponential increase of old people is largely due to an increase in life expectancy owing to better healthcare. This has been especially observed during the 20th century in the western world and has been undoubtedly considered as society's greatest achievement (1–3). However, will the growing numbers of older people enjoy their added years of life in good health or will these extra years come with poor health and disability? There is currently extensive debate among researchers over this question as some believe that there will be a compression of morbidity, whereas others think there will be an expansion of morbidity and some argue that with advancing medicine severe disabilities will be reduced (4,5).

Nonetheless, this transition towards low mortality has already brought demographic and epidemiologic changes in health and disease patterns such as the rise of chronic non-communicable diseases (NCDs); a phenomenon described as the 'epidemiological transition' (6,7). Recent evidence has shown (1–3,7) that in persons aged 60 and over, NCDs already account for nearly

90% of the disease burden worldwide, which carries profound medical as well as socio-economic consequences that are outlined in the same demographic studies (1–3,7). Particularly NCDs with strong age-dependent relation such as musculoskeletal (MSKDs) and metabolic disorders (MDs) have a negative impact on the quality of life of the elderly and worldwide economics.

1.1.1. Musculoskeletal and metabolic disorders in older age

MSKDs and MDs contribute significantly to the disease burden among the elderly as they are strongly age-related but also reflect changes in the lifestyle and diet of past decades (8–10). MSKDs in particular represent one of the major incapacitating condition among the elderly as they have a pronounced effect on the level of disability, quality of life and independence in older age. Data from the Global Burden of Disease study (11) show that MSKDs reached nearly 21% of global years lived with disability in 2013, and according to the World Health Organization are the second most common cause of disability worldwide (12). It was estimated that the disability due to MSKDs increased by 45% from 1990, and is likely to continue to rise as a result of the ageing population with increasing trends in sedentary life and obesity (12,13). In people 60 years and over, the prevalence of MSKDs currently range from 3% to 29% depending on the type of MSKDs condition itself (13). Of great concern is particularly age-related loss of skeletal muscle mass and strength, referred to as *sarcopenia*.

The peak mass of human male skeletal muscle is around the age of 25, about 10% of the muscle mass is lost by the age of 50, and another 30% by the age of 80 (14). It has been shown that even a 10% loss of lean muscle tissue in

previously healthy adults increases infection risk, impairs immunity, and is associated with increased mortality (15). This decline in the lean tissue along with an increase in body fat specifically in the abdominal region is commonly observed with ageing (8,9). It is known as *sarcopenic obesity* (16) and it is believed to contribute to the development of insulin resistance (IR) among the elderly (8,9,16).

IR along with obesity is a known risk factor for MDs, in particular for type 2 diabetes mellitus (T2DM). Estimates from 2010 have revealed that there were almost 347 million adults living with diabetes worldwide, which is by the way a 2-fold increase when compared to 3 decades ago (10,17), and 44% of this diabetes burden is ascribed to obesity (18). On the other hand, it has also been established that T2DM is a risk factor for physical disability and it is associated with development of sarcopenia in diabetic patients (16). Therefore, when one considers the aforementioned consequences related to advancing age and its interactions between them, it puts the elderly at an increased risk of stepping into a vicious cycle that accelerates the onset of MSKDs and MDs, which in turn would result in even larger cost burden to healthcare systems. Therefore, in the view of these trends, it is more important than ever to identify cost-effective interventions targeted at older people to maintain healthy muscle mass due to the relation of muscle mass to physical function, strength, and disease states. The following section 1.2 briefly reviews the structure and both mechanical and metabolic function of skeletal muscle.

1.2. Skeletal muscles: locomotion and beyond

Healthy skeletal muscle mass promotes muscle performance, maintains metabolic functions, supports longevity, enhances healthy life span and contributes to functional independence (19). From a mechanical perspective, the main function of skeletal muscle is to support *locomotion* or in other words to produce movement. From a metabolic point of view, skeletal muscle contributes to basal energy metabolism, maintains body temperature and stores and utilises important substrates such as amino acids (AA) and carbohydrates (glucose); it is therefore considered to be a key contributor to whole body metabolism, which makes it an essential tissue for life (19–21).

1.2.1. Skeletal muscle structure and function

Skeletal muscle is the largest organ in the body, constituting approximately 40-50% of total body mass (19,20). Skeletal muscles are attached to the bone by connective tissue called *tendons*; the muscle attachments at the moving bone are known as the *insertions*, and at the fixed bone they are the *origins*. When muscle contracts, it pulls the attachment point of insertion towards the point of origin (20). They also work in antagonistic pairs; i.e. in the action of elbow flexion the biceps is the agonist (prime mover) and triceps is the antagonist. Skeletal muscle is primarily composed of a heterogeneous population of muscle fibres (also known as myofibers or muscle cells) with varying contractile and metabolic characteristics that ultimately determine the whole muscle quality (19). A typical skeletal muscle such as the leg *vastus lateralis* (Figure 1.1, C) is a discrete entity characterized by a muscle mass called the *belly* with fibrous tendons at each end. It consists of muscle cells and three

layers of protective connective tissue *coverings*. The muscle and its coverings are packaged in deep *fascia* along with other muscles, nerves and vasculature network (Figure 1.1, D). The outer covering of the muscle is called *epimysium*. Each skeletal muscle is arranged into fascicles that are wrapped in *perimysium* (along with the nerves and neurovascular bundles) and finally each muscle fibre is surrounded by a thin sheath of fibrous tissue called *endomysium* (Figure 1.1, A-B) (19,20,22).

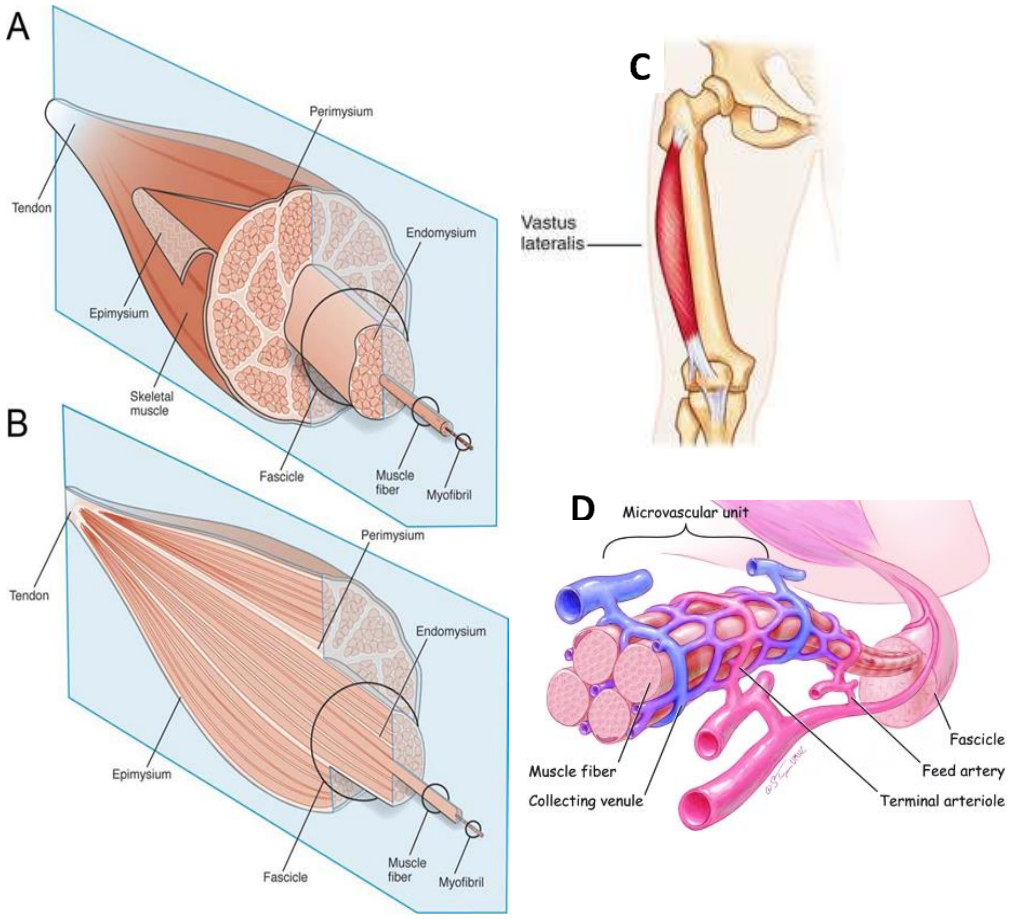


Figure 1.1 Structure of a skeletal muscle
A and B showing the structure of a skeletal muscle with its coverings; C showing a human leg vastus lateralis - its belly and tendons; D showing a vascular inflow to skeletal muscles. [Adapted from: (22–24)]

Anatomically, the skeletal muscle fibres have a striated appearance and are long, narrow and cylindrical often spanning along the entire length of the muscle. For this reason, they are multinucleated with *myonuclei* located on the periphery of the cells to meet the metabolic needs of the muscle (20,22). Furthermore, skeletal muscle fibres are post-mitotic incapable of further division (the nuclei within the muscle do not undergo post-natal cell division), however growth and regeneration does occur thanks to a population of stem cells called *satellite cells* (19,25,26). The cytoplasm of skeletal muscle fibre cells is known as the *sarcoplasm* (which contains important subcellular organelles such as mitochondria, intracellular fluid and variety of enzymes and proteins) and plasma membrane as the *sarcolemma* which comprises of a phospholipid bilayer and basal lamina (19,26). Skeletal muscle fibres are comprised of a collection of *myofibrils* – contractile proteins arranged in *sarcomeres*, which form the contractile machinery of the muscle fibre and are responsible for the striated appearance. The sarcomere is the basic contractile unit of muscle and makes up 70-80% of the total protein content in a single muscle fibre (19). It contains the two most abundant contractile protein filaments: *actin* (thin filament) and *myosin* (thick filament) that together with the *troponin* (C, I, and T) - *tropomyosin complex* (that is attached to the actin filament) permits the actual muscle contraction and thereby locomotion (19,26). Other proteins such as *titin*, *nebulin*, *α-actinin* and *desmin* are also involved and contribute to the integrity of the sarcomere (19). Locomotion itself is powered by actin-myosin *cross-bridge cycling* conforming to the sliding filament theory of skeletal muscle contraction proposed by Huxley in 1954 (27). The structure of the skeletal muscle fibre and of the sarcomere

along with the explanation of the sliding filament mechanism is presented in Figure 1.2. For cross-bridge cycling details, see Appendix A (Figure A).

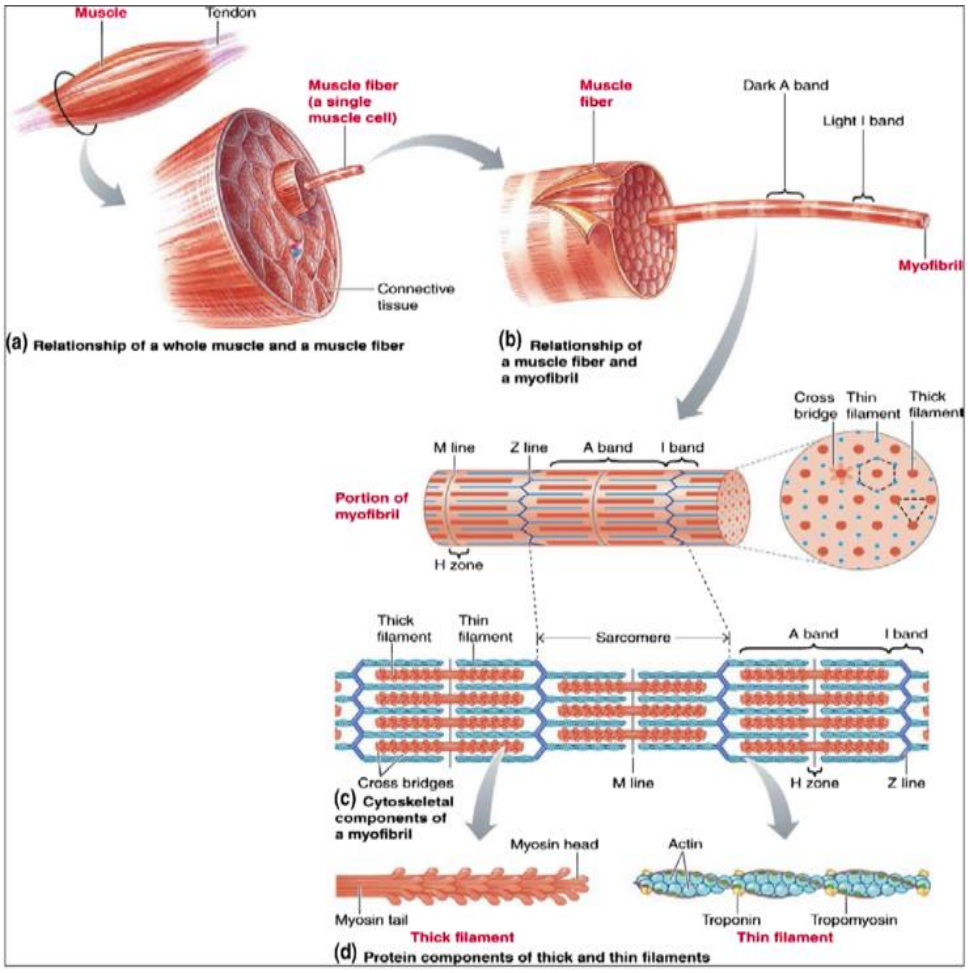


Figure 1.2 Structure of skeletal muscle fibre and sarcomere
Myofibrils are composed of repeating dark A and light I bands, which are responsible for the striation. As illustrated in the diagram, each fibril is composed of thick myosin (long rod-shaped with globular heads) and thin actin (pear-shaped) filaments joined together like two strings of beads intertwined at regular intervals with tropomyosin and troponin proteins forming a twisted helical structure. Myosin filaments run the length of the A band, whereas actin filaments run through the I band and peripheral portions, but not the central H zone, of the A band. Actin is anchored in the centre of the I band by the Z line, which consist of the structural nebulin, α actinin and desmin. Titin is also attached to the Z line but helps to stabilize the myosin filament. The portion of the myofibril between the two Z lines is the sarcomere, which is about 2.5µm long (19). The filaments interact through cross bridges which are bud-like extension of myosin. When muscle contracts, the I band shortens and the H zone shortens, but the length of the A band does not change. Thus, none of the actin or myosin change length; they simply slide past each other, increasing the overlap area (19–21). [Adapted from: (19), Fig. 3; original in (28), Fig. 8.2]

Furthermore, many components of the sarcomere exist as multiple isoforms due to a variety of fast and slow contracting fibre types and subtypes (24). Generally, depending on the classification technique (fibres can be classified by their histologic appearance, speed of contractions or ability to resist fatigue) there are four major types of muscle fibre: type I, IIa, IIx and IIb that determine the wide variety of individual's capabilities to perform aerobic and anaerobic activities (24,25,29,30). Briefly, type I fibres are slow (-twitch), fatigue resistant with a red appearance, whereas type II fibres are known as fast (-twitch) fibres (IIa intermediate and IIx/b the fastest), easily fatigable with a white appearance (19,24,25). The major differences between muscle fibre types relate to their myosin complement (and subsequent ATPase activity); i.e. isoforms of myosin light and heavy chains, which has been extensively reviewed by Schiaffino and Reggiani (21). Such variability partially explains differences between individuals in terms of physical performance as well as the relationship of skeletal muscle fibre type composition to certain chronic disease states, including sarcopenic obesity and insulin resistance (31). In addition, muscle fibres are also capable of adapting to changing demands or in other words adjusting their phenotypic properties by changing their size, type, or composition, allowing the muscle to perform a huge variety of contractions ranging from simple postural support to explosive sprinting (22). However, such muscle actions cannot occur without an energy source. The immediate source of chemical energy for muscle contraction is ATP (refer to Figure A, Appendix A).

1.2.2. Muscle energy sources and glucose production

The most remarkable attribute of skeletal muscle is its ability to increase its rate of energy consumption (ATP) during physiological challenges. For example, during high-intensity exercise the rate can be increased by up to 100-fold (29). This is possible through catabolism of stored substrates (and through increased uptake of them from the circulation, which will be discussed later). There are two main substrates (fuels) utilised by the muscle cells to produce ATP: 1) carbohydrates such as plasma glucose and muscle glycogen and 2) fats such as plasma free fatty acids and muscle triglycerides (32,33). In addition, there are three common metabolic pathways in the muscle fibres that generate ATP depending on the intensity and duration of the activity: 1) storage of ATP and creatine phosphate (CP) (refer to Figure A in Appendix A), which supports short (max. 15 sec) high intensity activities; 2) anaerobic glycolysis, which sustains muscle action for few minutes and 3) oxidative phosphorylation, which can sustain activity of a long duration (minutes to hours) (29). It is important to note that in real life, different activities trigger different pathways and use a combination of both fuels to produce ATP (19,32).

Glucose is the major source of energy in the skeletal muscle. Glucose is produced primarily by the liver and kidneys via processes known as *glycogenolysis* and *gluconeogenesis*. Glycogenolysis is the biochemical breakdown of glycogen in the muscle and liver cells, which provides energy via the process of *glycolysis*. Gluconeogenesis, on the other hand, is the new formation of glucose from non-carbohydrate precursors such as lactate, glycerol (liberated by the breakdown of triglycerides - lipolysis), and

glucogenic amino acids (especially alanine) via reverse glycolysis that takes place primarily in the liver and, to a lesser extent, in the renal cortex of the kidneys (19,33,34). Skeletal muscle can provide glucose for its own energy requirements by converting glucose-6-phospate (g-6P), which is a phosphorylated (by hexokinase on the mitochondria) form of glucose, back to glucose. At rest, muscles can store converted g-6P as glycogen, and then during activity (contraction) it can be converted back to g-6P but because muscle does not have the necessary enzymes to convert the g-6P to free glucose the glycogen can only be used for muscle's own needs (20,33,34). In oxygen abundance, glucose taken up from the blood is converted to g-6P and then to pyruvate (the oxidative phosphorylation pathway) by the enzymes of glycolysis (aerobic glycolysis) and oxidized completely to carbon dioxide and water by the enzymes of TCA cycle in mitochondria. The glycolytic breakdown of glucose yields 2 ATPs per one glucose molecule whereas the mitochondrial oxidation of pyruvate yields 38 ATP per glucose molecule, which can be utilised by the muscle for its contraction work. In the absence of oxygen, the muscle metabolizes glucose by converting the pyruvate to lactate (the anaerobic glycolysis pathway), which yields only 2 ATP molecules per glucose molecule and leads to a build-up of lactate in the muscles. When lactate builds up in the muscle, it is released into the blood where it is taken up by the liver, converted to pyruvate and glucose and returned to the muscle, this is known as the Cori cycle. Furthermore, there are also three futile cycles in glucose metabolism: 1) the glucose cycle (glucose $\rightarrow$ g-6P $\rightarrow$ glucose); 2) the fructose-6-phosphate (F6P) cycle (F6P $\rightarrow$ fructose diphosphate $\rightarrow$ F6P); and 3) the conversion of pyruvate to phosphoenolpyruvate (PEP) cycle (pyruvate

→ PEP → pyruvate), which has been reviewed in greater detail by Wolfe and Chinkes (33). The production of glucose is regulated by hormones. Glucagon is the leading stimulator of glucose production, whereas insulin is the primary inhibitor (33).

1.2.3. Glycaemic control and the role of skeletal muscle

The human body has many physiological mechanisms that ensure a relatively close homeostatic regulation of glucose uptake and glucose appearance in the bloodstream, which is reflected by a relatively constant blood glucose level. In a postabsorptive (fasting) state, blood glucose levels usually range between 3.4 and 6.2 mmol/L (60-100mg/dL). Despite this population variation, blood glucose levels within a single individual are usually controlled within a narrow range of ± 0.3 mmol/L (5 mg/dL). After a meal (in a postprandial state), blood glucose concentrations increase no more than 2 mmol/L, and rarely exceed levels of 7.8 to 8.4 mmol/L (140-150 mg/dL) (18,32). This strict maintenance of blood glucose levels is through a complex homeostatic mechanism governed by both glucose production and glucose uptake. It involves a set of interactions between hormones that act in a coordinated way on tissues such as the liver, pancreatic beta cells, and skeletal muscles, which has been reviewed in detail elsewhere (32).

During postabsorptive state, glucose is maintained via hepatic glycogenolysis or via gluconeogenesis. About 75% of blood glucose is metabolized in a non-insulin mediated glucose disposal (NIMGD) manner by the brain, splanchnic tissues (liver and gut), and red blood cells. Therefore, only about 25% of glucose uptake is required by insulin-sensitive tissues such as skeletal muscle

and fat (18). However, in a postprandial state, insulin mediated glucose disposal (IMGD) occurs to maintain normal glucose homeostasis. In the postprandial state, insulin is secreted from pancreatic beta cells into the portal vein of the liver, which inhibits approximately 50-60% of hepatic glucose disposal and consequently promotes glucose uptake into skeletal muscle and adipose tissue (18,34). Therefore, primary function of insulin in the postprandial state is to stimulate glycogenesis, glucose disposal (and storage). Insulin levels in the postabsorptive state range between 5-15uU/ml and in the postprandial state between 16 – 250 uU/ml (18,35). Stimulation of secretion is by glucose and to lesser extent amino acids (AA) (18,36).

The gold standard method for investigating the insulin action in peripheral tissues in vivo is the euglycaemic hyperinsulinaemic clamp (EHC) (37). The EHC method was reviewed by Abdulla et al (18) presenting that under these experimental conditions, nearly 90% of IMGD takes place in peripheral rather than in the splanchnic tissues, with about 5% of peripheral IMGD occurring in adipose tissue and the remaining 80% in skeletal muscle. Furthermore, based on a research that used an oral glucose challenge, the review has shown that the splanchnic tissues absorb 28% of oral glucose in NIMGD manner and the other 72% reaches systemic blood circulation. Of this 72%, 22% is taken up by the brain (which is constant under both postprandial and postabsorptive conditions) and red blood cells. The remaining is extracted in IMGD manner by skeletal muscle (45%), adipose tissue and by both liver and kidneys (5%) (Figure 1.3). Therefore, skeletal muscle is the major influence in the overall glucose utilisation under postprandial conditions dealing approximately with 60% of glucose that escapes splanchnic extraction.

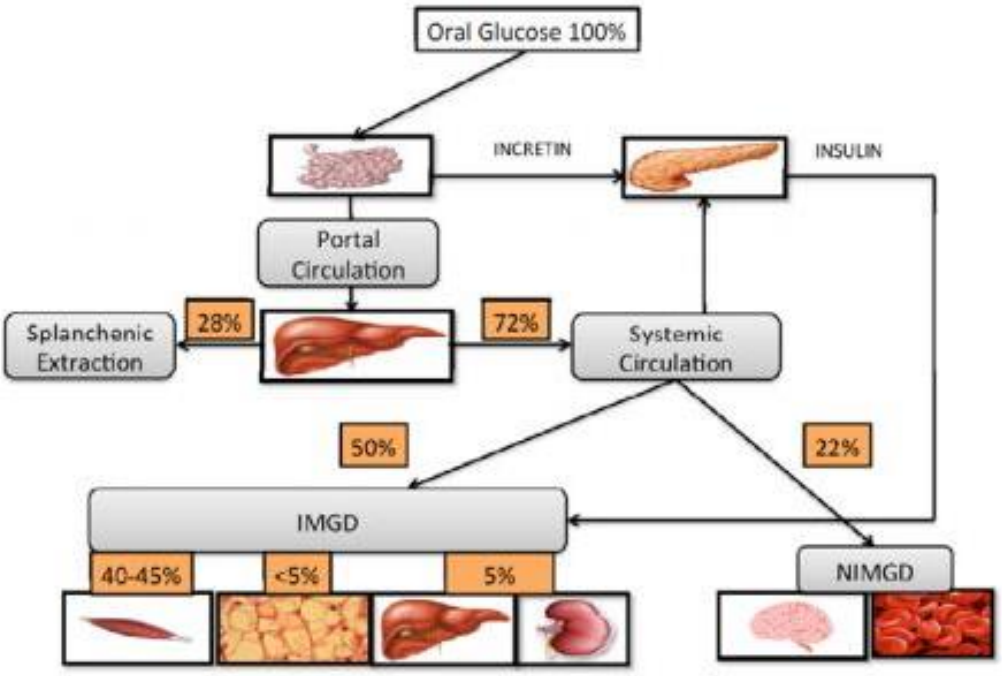


Figure 1.3 Oral glucose clearance.
(Taken from [18])

1.2.4. Glucose uptake by skeletal muscle

Glucose cannot passively diffuse into a cell and must be transported through the cell membrane by glucose transporters (GLUTS) (38,39). GLUT4 is the major glucose transporter isoform expressed in skeletal muscle, transporting glucose across the cell membrane via facilitative diffusion (30,38,39).When blood glucose levels increase, the rate of glucose uptake increases too. Insulin is the main regulator of muscle glucose utilisation and increases the ability of muscle fibres to uptake glucose. Insulin controls the rate of entry of glucose by increasing GLUT4 translocation to the cell membrane (GLUT4 is otherwise located intracellularly) (30,38,39). Insulin initiates this action by binding to its insulin specific glucose receptors located on the cell surface, which leads to subsequent tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1). This leads to an activation of phosphatidylinositol-3-kinase (PI3-K), which results in an activation of phosphoinositide-dependent protein kinase 1 (PDK1)

and atypical protein kinase C (aPKC). These kinases then stimulate the translocation of GLUT4 to the plasma membrane and subsequent glucose uptake (Figure 1.4) (40); this is known as the *insulin-dependent pathway* (38–40). It has been shown that the maximal metabolic effect of insulin can be elicited when only 2% of the insulin receptors are filled (33). Furthermore, there is also an *insulin-independent pathway* that involves AMP-activated protein kinase (AMPK), calcium, nitric oxide (NO), bradykinin, or reacting oxygen species (ROS), which is not discussed in detail in this thesis but elsewhere (30,38). Moreover, it has been shown (30,32,40) that muscle contraction can also facilitate glucose uptake into skeletal muscle by both insulin dependent and independent pathways. The mechanism of both insulin-stimulated and contraction-stimulated glucose uptake into skeletal muscle is illustrated by Figure 1.4. Disorders of glucose uptake by skeletal muscle such as T2DM and obesity are characterised by high levels of circulating glucose due to the inability of insulin to exert its effect on glucose metabolism (38). This is known as insulin resistance (discussed later) and can be assessed by the EHC and oral glucose tolerance tests (refer to 1.2.3) (36,37).

Of note, it has been also suggested (33) that there may be other additional mechanism that controls glucose uptake where insulin plays a secondary role. This mechanism is described as the glucose-ketone-fatty acid cycle and the basis of the theory is that free fatty acids (FFA) inhibit glucose oxidation and consequently glucose uptake. When insulin concentration is low (in a postabsorptive state), the levels of FFA are high. This inhibits glucose utilisation and subsequently glucose uptake and production of glucose, which then causes ketosis (during prolonged fasting). The ketones then compete with

glucose as the energy substrate in the brain, which causes a further fall in glucose production. However, experimental support of this theory is lacking. On the other hand, the importance of skeletal muscle vasculature in maintaining glucose uptake is well established and will be discussed in detail in the two following subsections.

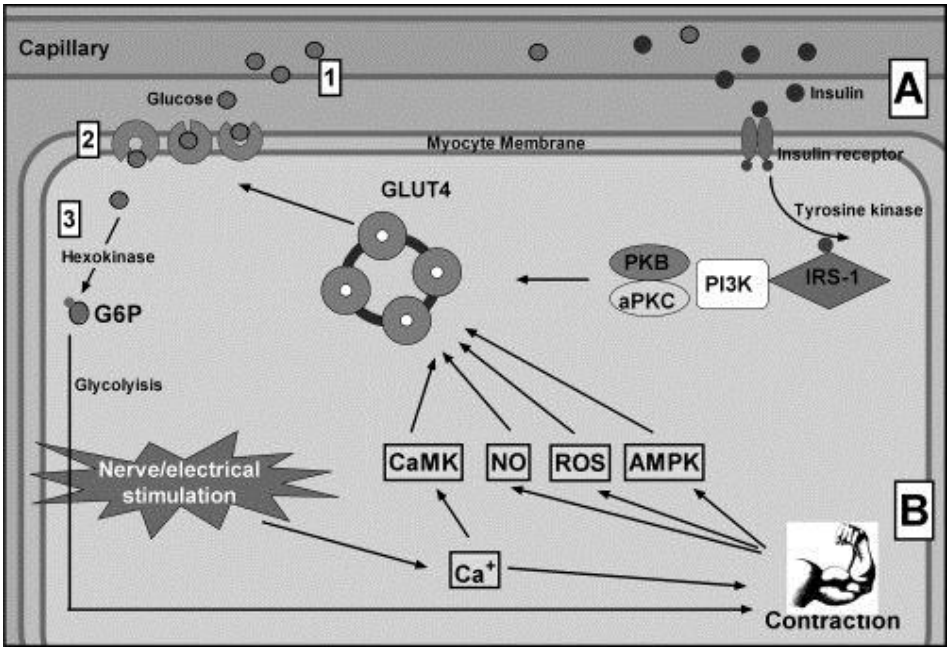


Figure 1.4 Mechanism of glucose uptake into skeletal muscle.
A) insulin-stimulated glucose uptake; B) contraction-stimulated glucose uptake; 1) Glucose delivery to the muscle cell, 2) glucose transport through the membrane and 3) glucose phosphorylation to g-6P. To note, the exact mechanisms how insulin and contraction signal GLUT4 translocation and glucose uptake are not yet completely understood (38,39) [Taken from (39)]

1.2.5. Skeletal muscle vascular architecture

Muscle tissue has highly organized vasculature comprising of primary (or conduit) arteries (macrovasculature), which branch off into arterioles down to terminal arterioles (microvasculature). Terminal arterioles are oriented vertically to the muscle fibres, supplying them at regular intervals of approximately 1 mm. Furthermore, each terminal arteriole supplies about 15-

20 capillaries that run alongside the fibres with many branches (anastomoses) (24,41). The functional organization of this rich capillary network is known as a microvascular unit (MVU). A typical MVU supplies segments of 20-30 fibres (volume of about 0.1 mm3), each contacting between 2-7 capillaries (18,41). Venules are arranged in the same way as arterioles located in between two terminal arterioles. In addition to this there is a network of lymph vessels within the muscle (24,41). Endothelial cells (ECs) form the inner, physical lining of the blood vessels and play an important role in skeletal muscle metabolism and blood flow (41). A schematic representation of muscle's vascular architecture is displayed in

Figure 1.5 and Figure 1.1 (D).

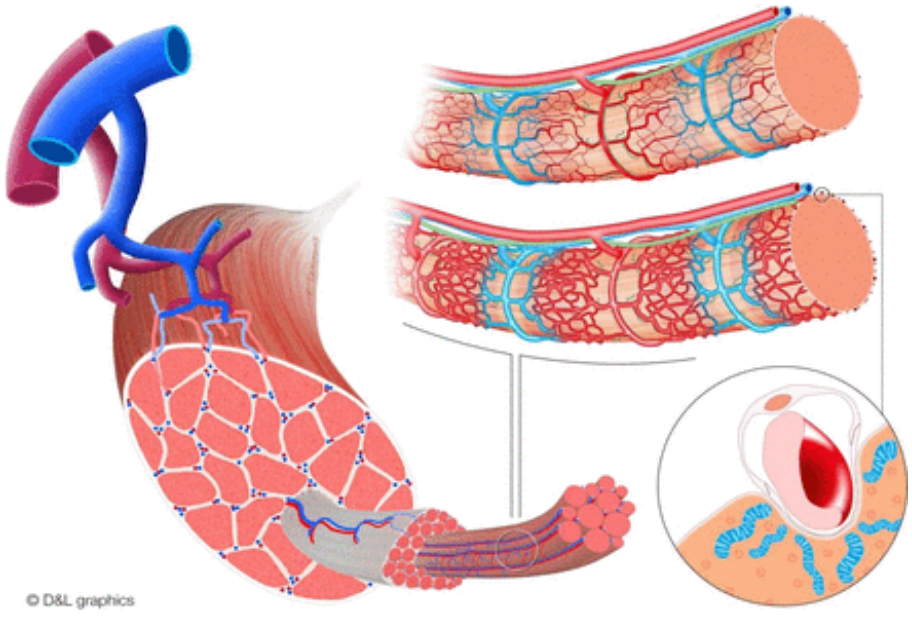


Figure 1.5 Schematic representation of muscle's vascular architecture.
The left image is showing the branching off arteries down to terminal arterioles with their vertical orientation to muscle fibres. The upper right image is showing two muscle fibres and their MVUs, with terminal arterioles in red feeding multiple capillaries (in red and blue) and lymph vessels with venules. The bottom right image is showing a capillary embedded in a sarcolemma with mitochondria clustered along it, which expands the contact area between muscle and endothelial cells and

> minimizes the diffusion distance for oxygen (41). It has been also shown that there are differences in microvascular architecture between anaerobic and aerobic muscles; for example, aerobic muscles have more and larger (lengthwise) capillaries per fibre to facilitate better gas and nutrient exchange (42,43). [Taken from (41)]

1.2.6. Glucose delivery and blood flow in skeletal muscle

Glucose (and other substrates and hormones) delivery to the muscle is reliant on intact blood vessels and transendothelial transport. The hierarchical vascular network described above (1.2.5) allows blood flow and glucose exchange to be regulated at numerous levels that are specifically dependent on postprandial hyperaemia triggered by vasodilatory stimuli (18,24,41). Vasomotion (rhythmic vessel diameter oscillations) provides a local mechanism for vascular adaptations to altered metabolic needs and its regulation impacts muscle perfusion (24,41,44). Vasomotion increases the number of ECs exposed to plasma for glucose exchange without total blood flow or cardiac output being changed (41,44). Only when perfusion demand exceeds flowmotion (periodic fluctuations caused by vasomotion) compensatory capacity can total blood flow increase (41,44). Insulin regulates the volume of microvasculature perfused within the muscle and is also capable of increasing total blood flow; for both effects, nitric oxide (NO) needs to be generated from the ECs to cause vasodilation (41,45). For more details regarding insulin (and exercise) vasodilation, see Appendix A (Figure B). Insulin, therefore, also regulates its own delivery, which precedes its classical function in mediating glucose uptake as discussed in 1.2.4.

MVU represents the smallest functional unit for blood flow regulation (18,24,41). At rest, whole MVU is unfilled with blood due to high tone in their terminal arteriole (18,24). Typically, two thirds of capillaries are empty at any

given time under rested and fasted conditions, such that resting muscle blood flow in overnight fasted humans is just about 3 ml·min-1·100g-1 (which is only 3-5% of that seen in kidney, liver or intestine) (18,24). Therefore, recruiting skeletal muscle microvasculature is crucial to the postprandial action of insulin to better facilitate glucose uptake and its own delivery. Insulin resistance significantly reduces microvascular recruitment in skeletal muscle and subsequent glucose uptake, which contributes to the pathogenesis of age-related metabolic disorders such as sarcopenia and T2DM (41,45). It is also worth mentioning that the measurement of skeletal muscle total blood flow can be assessed for example by Doppler ultrasound but there are other methods too such as venous occlusion plethysmography or thermal dilution; and the measurement of microvascular blood flow can be assessed by contrast enhanced ultrasound (CEUS) (for overview of each refer to (46)). The following section will briefly overview skeletal muscle ageing, insulin resistance and vascular circulation with ageing.

1.3. Skeletal muscle ageing

Muscle ageing process in humans starts around the age of 30, which is supported by studies involving both healthy individuals as well as healthy persons that died in car accidents using techniques such as computed tomography imaging or biopsies (47). Muscle mass decreases annually from the age of fifty by 1-2% and muscle strength similarly decreases, by 1.5% from the age of fifty to sixty and by 3% thereafter (also refer to 1.1.1) (48). The loss of skeletal muscle mass and strength, sarcopenia, was originally believed to be only due to the loss of muscle mass which then affects the muscle strength (49). However, it has been shown that the decline in muscle strength far

exceeds the expected consequences of the associated muscle mass decline, therefore it is the quality of the muscle that deteriorates too (49,50). The biggest concern is specifically the decline in the lower extremities as it affects the mobility status of the elderly. For example, the cross -sectional area of the vastus lateralis decreases by up to 40% between 20 – 80 years of age (51). The development of sarcopenia with ageing has been shown to be related to a variety of age-related factors such as: 1) neurodegenerative changes leading to denervation and loss of motor units and decline in satellite cells; 2) decline in anabolic hormones, specifically testosterone (about 1% per year from the age of 30); 3) decline in growth hormone leading to a decline in insulin-like growth factor-1; 4) anorexia of ageing; 5) decline in physical activity leading to muscle atrophy; 6) decline in levels of vitamin D due to a decrease ability of cholecalciferol in the skin to make it; 7) production of low-grade pro-inflammatory cytokines; 8) mitochondrial dysfunction leading not only to oxidative damage of the muscles but also to a reduction in the ability to generate energy to allow muscles to function properly; 9) decreased blood supply to the muscles due to atherosclerosis, which leads to muscle hypoxia and 10) insulin resistance associated with ageing that results in increased fat infiltration into muscles (48,52–54). However, since skeletal muscle maintenance in all age groups depends on good delivery of nutrients (glucose), decreased blood supply to the muscles and insulin resistance associated with ageing represent the two biggest concerns that are discussed in the next subsection.

1.3.1. Ageing: insulin resistance and decreased blood supply

Ageing in general is a very complex phenomenon accompanied mostly by negative changes in body composition of the elderly. It is important to understand these changes in body composition with ageing to recognise and interpret disease related alterations correctly. General agreement is that with age, body fat mass increases (and its location alter in such way that subcutaneous fat decreases and visceral fat increases) and fat free mass such as muscle or bone density decreases (47,50). It has been shown that this fat increase happens up to about 75 years of age, then it decreases or remains stable (15). Lean mass on the other hand by the age of 75 decreases to 25% of total bodyweight (compared to 50% in young adults) (50). It is this pattern of fat distribution along with decreased physical activity that is associated with age-related IR contributing greatly to the development of many MSKDs and MDs including cardiovascular diseases (15,47,50). For example, decreased blood supply in older age leads to coronary artery disease and hypertension (54,55).

IR can be described as decrease sensitivity to the metabolic actions of endogenous (and exogenous) insulin to increase whole body glucose uptake and utilisation, which is characterized by generally higher levels of insulin (36,56). IR in muscle fibres is believed to manifest through post-receptor defects in insulin signalling in any of the steps involving kinases as well as through abnormalities of GLUT4 function, which then results in impaired insulin mediated glucose transport (refer to Figure 1.4) (36,56). Glucose uptake into skeletal muscle is also dependant on insulin mediated vasodilation in muscle endothelium via activation of the P-I3K pathway (as discussed in 1.2.6 and Appendix A, Figure B). In IR there is a shift in balance between the

production of vasodilator and vasoconstrictor substances due to impaired activation of the P-I3K/Akt/NO pathway, which results in impaired insulin stimulated vasodilation due to decreased NO production (refer to Appendix A, Figure B), which then contributes to reductions in insulin stimulated glucose uptake (36,56).

It is well recognised that ageing is associated with glucose intolerance and increased insulin resistance, which is reflected by elevated levels in both fasting and postprandial glucose concentrations in the elderly (57). Studies using isotope tracer approaches revealed decreased rate of glucose disappearance and forearm glucose uptake rate following glucose challenge in older adults (58). Even when providing increased insulin levels via insulin clamps, glucose disposal rates still remain depressed in the elderly vs. younger individuals (59). Furthermore, the age related decline in muscle blood flow has consequent negative effects on muscle building and health. It has been shown that older individuals exhibit 20-30 % reductions in limb conduit artery blood flow under both postabsorptive (60,61) and postprandial conditions (62) as well as when challenged with various exercises (61). This is believed to be related to impaired glucose uptake that is associated with insulin resistance (18) but also for example to a reduction in capillary number (63) or increased AngII-mediated vasoconstriction (64). All these in turn contribute to age-related declines in anabolic responses to feeding (65) by reducing delivery and use of both insulin and AA to muscles (66–68). Thus, determining strategies to improve limb blood flow in older people is also very important for muscle mass maintenance. Fortunately, insulin is not the only hormone that can stimulate glucose uptake and recruit muscle microvasculature, which is central

for maintaining glucose homeostasis. Other hormones such as glucagon-like peptide 1 (GLP-1) can recruit muscle microvasculature in a similar fashion, yet through a different biological pathway, which is discussed in the following section.

1.4. GLP-1 as a potential therapeutic for skeletal muscle ageing

GLP-1 belongs to the group of insulinotropic hormones (along with gastric inhibitory polypeptide) called incretins that stimulate a decrease in blood glucose levels by inducing secretion of insulin from pancreatic beta cells (69). GLP-1 is secreted by the L cells of the proximal intestine (mostly in the ileum and colon) (69,70) and is derived from post-translational processing of the pro-glucagon gene by pro-hormone convertase 1/3 (PC-1/3) where it generates a 31-residue peptide – GLP-1 (7-37). This form can be then amidated at its C-terminal glycine to give rise to a second form known as GLP-1 (7-36) NH2 (69). In humans, most of the released hormone is in the amidated form. GLP-1 is stored in the intestinal enteroendocrine cells as an intact peptide within granules until its secretion is stimulated by the ingestion of food. It has been shown (69–71) that all three principal macronutrients: lipids, carbohydrates and proteins have stimulatory effects on GLP-1 release, with carbohydrates having the most robust incretin response. Upon release, a protease called dipeptidyl-peptidase-4 (DPP-4) forms metabolites GLP-1 (9-37) and GLP-1 (9-36) NH2, which are the major circulating forms (69). GLP-1 has a short half-life of only 1-2 minutes due to the fast degradation of GLP-1 by DPP-4 and exerts its effects by binding to its receptor called GLP-1R, which is a 463 amino acid G-protein-coupled receptor mostly present in the pancreatic beta

and alpha cells, gut, brain and in moderation in other tissues such as lung, kidney, heart, blood vessels (vascular endothelium) and skeletal muscle (69–71).

1.4.1. Biological action of GLP-1

The most defined physiological role of GLP-1 is to stimulate insulin secretion from pancreatic beta cells also known as 'classical' incretin effect, which depends on the levels of glucose in the blood. GLP-1 can exert its minimal effect when the levels of glucose are slightly raised above the basal level (71). In the fasting state, GLP-1 levels are low ranging from 1-10pmol/L, whereas in the postprandial state the levels rise to approximately 7-23 pmol/L (72,73). Besides stimulating insulin secretion, GLP-1 also inhibits glucagon release (due to its effect on the alpha cells), helping to maintain overall glucose homeostasis by lowering blood glucose levels (69,70). Furthermore, GLP-1 also exhibits extra pancreatic effects due to the presence of GLP-1 receptors in the other tissues. Due to its receptors in the brain, GLP-1 has been shown to further regulate short- and long-term energy homeostasis by 1) affecting (slowing) gastric emptying, which prevents glucose concentration rising too rapidly in the blood circulation (an important factor for the control of postprandial glycaemic excursions) and 2) decreasing gut motility and inducing satiety, all via the gut-brain axis mechanism (70–72). Due to all these effects, GLP-1 has been of great interest for the treatment of metabolic diseases; i.e. increasing GLP-1 bioavailability in the form of DPP-4 inhibitors or GLP-1 receptor agonist (such as liraglutide) is now a front-line drug treatment of T2DM and obesity (71,72,74). The development of these therapies originated from the observation that oral glucose ingestion resulted in

an amplified insulin response compared to intravenous administration, which is defined as a ‘true incretin’ effect (70). Additionally, GLP-1 has been further explored and has shown to exhibit also ‘novel’ insulin independent extra-pancreatic effects. Research into this area has revealed novel acute metabolic and longer health benefits of GLP-1 such as: 1) cardio protection (75,76); 2) beta cells protection (77); and 3) insulin-independent microvascular recruitment and glucose disposal in skeletal muscle (78). The latter is discussed in the following subsection. A schematic representation of GLP-1 biological actions in peripheral tissues is demonstrated in Figure 1.6.

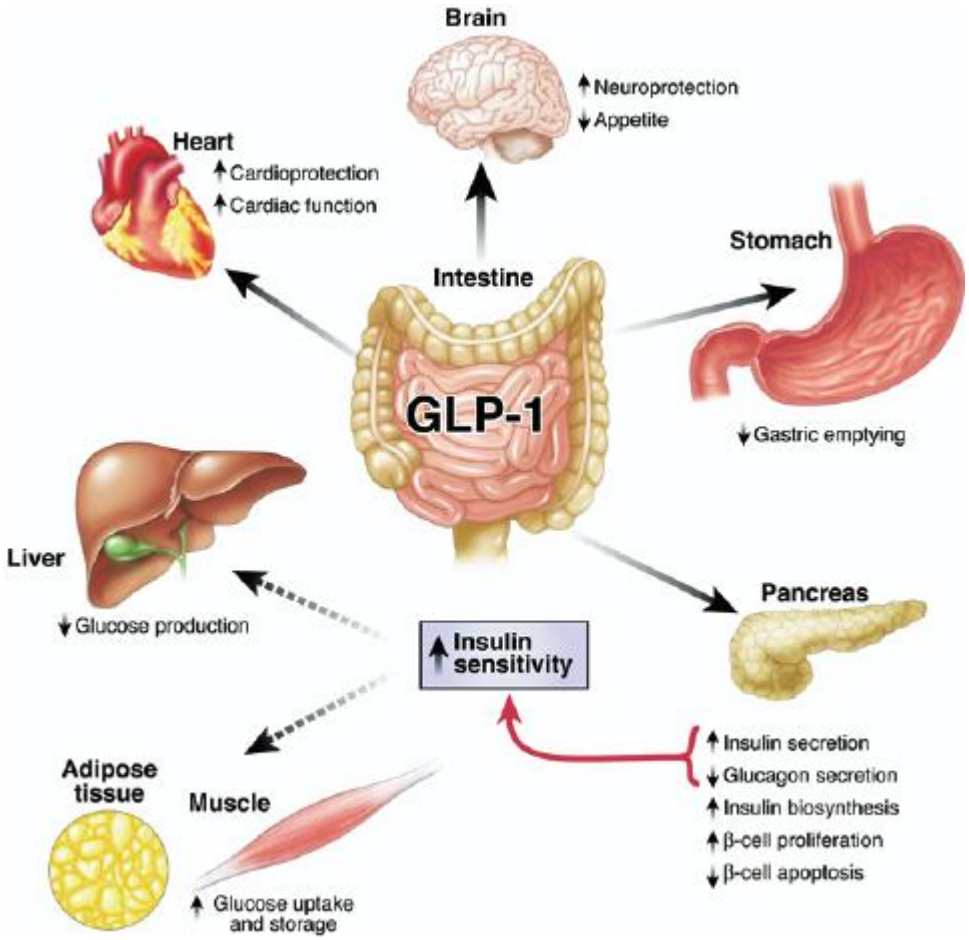


Figure 1.6 GLP-1 biological actions in peripheral tissues.

[Taken from (79)]

1.4.2. Role of GLP-1 in skeletal muscle glucose metabolism and glucose uptake

GLP-1 due to its receptors in both skeletal muscle and vascular endothelium has been shown to have insulin independent but insulin-like effects on glucose homeostasis. In vitro study (80) using human skeletal muscle strips and primary culture myotubes (developmental stage of muscle fibre) has shown the ability of GLP-1 to stimulate glycogen synthesis, increase the activity of glycogen synthase, glucose oxidation and utilisation in association with activation of the PI-3K pathway. These effects have been further confirmed by animal studies involving normal and diabetic rats (81–83). A study using hyperinsulinaemic-euglycaemic clamp and exercise in wild type and knock-out mice for GLP-1 receptor has shown that GLP-1 receptor regulates glucose production and muscle glucose uptake independent of its insulinotropic ability (84). Moreover, Chai et al. (78) measured glucose uptake in rats using a femoral arterial-venous (A-V) balance method and demonstrated that there was a threefold increase in glucose uptake within 30 minutes of systemic GLP-1 infusion in an insulin independent manner. This was demonstrated by inhibition of endogenous insulin through a somatostatin infusion. Furthermore, a study (73) involving fully-differentiated human satellite cells has proposed that GLP-1 stimulated increase of glucose uptake and glycogen synthesis is via the PI3-K pathway probably due to increased levels of GLUT4 , independent of the insulin signalling pathway. However, this was demonstrated only when the cells were differentiated in 5mM glucose media. Under hyperglycaemic (22.5mM glucose) conditions, the cells were both insulin and GLP-1 resistant

in terms of the glucose uptake and glycogen synthesis, providing evidence that hyperglycaemia may promote both insulin and GLP-1 resistance in skeletal muscle. Beside this, GLP-1 has been also shown to stimulate glucose uptake, lipogenesis, and lipolysis in adipocytes of normal rats and obese diabetic mice (85,86). In normal human adipocytes, GLP-1 was shown to increase glucose uptake in association with the PI-3K pathway, which was impaired in obese patients (87). All this suggests that GLP-1 plays an important role in both glucose and lipid metabolism independent of insulin, targeting fundamental cellular defects in the pathophysiology of metabolic disorders, specifically insulin resistance within skeletal muscle via the PI-3K pathway. However, the evidence of GLP-1's ability to stimulate glucose uptake independent of insulin in human skeletal muscle is still lacking. Two recent human studies (88,89) involving healthy young subjects (following femoral and brachial artery infusion) and one study (90) involving subjects with metabolic syndrome (following brachial artery infusion) have shown that GLP-1 infusion did not stimulate glucose uptake. However, all three studies did show that GLP-1 has positive vasodilatory effects on skeletal muscle vasculature, which will be discussed in the following section.

1.4.3. Effect of GLP-1 on skeletal muscle vasculature

In addition to the glycaemic actions of GLP-1 discussed above, studies in both animals and humans have repeatedly shown beneficial actions of GLP-1 on skeletal muscle vasculature. For example, in the Chai et al. study (78) the infusion of GLP-1 in skeletal muscle of rats was shown to increase MVR and volume with a 2.5-fold increase (which likely explains the increased glucose uptake discussed previously) via an NO-dependent mechanism as

demonstrated by inhibition of NO synthase, which prevented all these changes. In a similar fashion, a study by Richter et al. (91) has demonstrated that GLP-1 exerts its vasodilatory effect through its action on the endothelium, with GLP-1 losing this effect upon removal of the endothelium. The three human studies (88–90) have shown that GLP-1 exerts its vasodilatory action on both conduit arteries as well as on MVR. In the Sjøberg et al. study (89), GLP-1 increased MVR in human skeletal muscle by 60% following 60 min of continuous GLP-1 infusion into the femoral artery. Within that time both insulin and GLP-1 concentrations increased by 80- and 20-fold, respectively. This confirms the fact that GLP-1 also enhances the effects of insulin on MVR, which was demonstrated already by Liu et al. (92) study where after the ingestion of food, MVR in forearm muscles was increased by approximately 100% compared to an increase of only about 40% during a euglycaemic hyperinsulinaemic clamp. Nevertheless, to adjust for this insulin mediated effect of GLP-1, Sjøberg et al. (89) repeated the experiment with infusion of Octreotide to inhibit insulin and reduce its concentration below fasting levels and demonstrated that there was still 50% increase in MVR in the infused leg (where the postprandial levels of GLP-1 were physiologically high) and 40% increase in the contralateral leg (where GLP-1 systemic concentration was within postprandial physiological ranges). This effect was preserved also when the levels of GLP-1 were no more than postprandial. The other study by Subaran et al. (88) presented increases in both arterial blood flow (30% increase compare to baseline) and also microvascular blood flow (43% increase), after 150min of GLP-1 infusion at normal physiological postprandial levels. In the view of these data, we

propose that GLP-1 may facilitate glucose uptake under postprandial conditions through increased blood flow in older adults.

1.5. Aims and hypothesis

Only through this 'brief' overview, it is obvious that our understanding of skeletal muscle anatomy and physiology has increased greatly over the past decades and only continuous advances in this field can improve our understanding further in the future. At the same time, the need to understand the factors influencing muscle health in old age has never been more urgent and should be a research priority due to the worldwide increase in ageing of our populations. The significant contribution of age-related changes such as insulin resistance, impaired glucose metabolism and reductions in limb conduit artery blood flow that otherwise guarantee healthy muscle mass maintenance cannot be omitted and should be of a first rank in the research priority to develop interventions that ensure active and healthy ageing. Therefore, since functions of the GLP-1 receptor have been recently shown to extend beyond classical insulinotropic effects; i.e. GLP-1 ability to increase blood flow and muscle glucose uptake in an insulin-independent manner, these data provide strong evidence that GLP-1 may also play an important physiological role in postprandial muscle glucose metabolism in older age independent of insulin that could be exploited in the future in order to become a novel therapeutic target in ageing and age-related conditions such as sarcopenia and T2DM.

The role of GLP-1 in the skeletal muscle blood flow and glucose uptake has not been investigated under postprandial conditions and in aged individuals

yet; such that the efficacy of targeting incretin bioavailability for improving metabolic responses to nutrition is poorly defined. Therefore, the overall aim of this thesis was to define the extra-pancreatic 'novel' effect of GLP-1 on muscle postprandial glucose metabolism and vascular blood flow in older age. The specific objective was to assess 1) glucose uptake in skeletal muscle using 2-deoxy-D-glucose (2-DOG) by measuring 2-DOG phosphate in muscle by gas chromatography-mass spectrometry (GCMS) and 2) femoral artery blood flow by US Doppler. It was hypothesised that GLP-1 has a novel facilitative role in regulating muscle glucose metabolism and uptake under postprandial conditions in an insulin-independent manner in people 65 years old and over. We aimed to test the hypothesis by studying the feeding responses in the absence or presence of a GLP-1 in seven healthy older men with use of techniques including insulin and glucose clamps, the Doppler ultrasound and GCMS. Crucially, I.V delivery of both AA and glucose was administered to avoid the confounding gut derived incretin effect and to mimic the postprandial state. We investigated whether GLP-1 infusion would increase total leg blood flow to the muscle and if it does, would improve delivery of glucose to the muscle and therefore better capture of the glucose be seen. Establishing the results of these investigations would suggest whether GLP-1 therapy is a promising option to target age related declines in muscle performance and help to improve metabolic health and consequently quality of life.

2. Materials and Methods

All standard reagents and equipment used are specified within each experimental method below. Methods 2.7 and 2.8 were performed by technicians at the Human Physiology laboratory at Derby Medical School, University of Nottingham.

2.1. Subjects and Ethics

Seven healthy older male volunteers [aged 65-75, a body mass index (BMI) between 18-28 kg/m^2] were recruited for the study. All volunteers underwent a fasting (10-12 h) screening visit at the Clinical Physiology laboratory of the Division of Medical Sciences and Graduate Entry Medicine (University of Nottingham, Royal Derby Hospital) that comprised of 1) a health assessment questionnaire detailing previous medical history and medications and 2) a routine medical examination (Table 1).

Table 1. Medical examinations at screening session

A collection of blood samples for the analysis of: FBC, U&E's, LFT's, TFT's, coagulation, fasting glucose, fasting insulin and HbA1c
Measurements of height, weight, blood pressure (BP), heart rate (HR)
An electrocardiogram (ECG)

All volunteers were considered healthy and suitable for the study after meeting the inclusion and exclusion criteria detailed in Table A (Appendix B). The study was approved by the University of Nottingham Ethics Committee and conformed to the ethical standards set by the Declaration of Helsinki (93). All volunteers provided their informed and written consent to participate in the study, after being informed of the purpose of the study and of all the risks and procedures involved.

2.2.Study design

The study conducted in a cross-over design included two acute study visits with a four week 'wash-out' period between the two visits. For each of the two study visits, the volunteers arrived at the clinical physiology laboratory at 8am after an overnight fast (10-12 h). They had a dual-energy X-ray absorptiometry scan done (at first visit only) to estimate their body composition, i.e. fat and muscle mass (DXA; GE Lunar Prodigy II, GE Medical Systems, Chalfont, Buckinghamshire, UK). The volunteer then lay in a bed where three polyethylene catheters (Becton Dickinson, Oxford, UK) were inserted into peripheral veins distributed between both arms. Then under local anaesthetic using 1% Lidocaine solution (Pfizer, UK) and under ultrasound guidance (iU22, Philips, CA, USA) two further longer catheters were inserted into 1) a femoral vein for systemic blood sampling and 2) a femoral artery for GLP-1 infusion and arterial sampling of one leg (first visit - right leg, second visit - left leg; applies to biopsies too). All infusions, their purpose and blood sampling sites are detailed in Table 2.

After taking baseline samples at t = 0 min, 2-DOG infusion was started at a rate of 10 mg/kg prime followed by 6 mg/kg/h constant infusion. This was subsequently maintained for the entire duration of the study visit (6 hours). The three hours' post start of 2-DOG infusion was designated for baseline measurements. During this time, regular samples from femoral venous and arterial line were performed. Arterial and venous blood samples were collected into tubes containing either ethylenediamine tetraacetic acid (EDTA), an anticoagulant to stabilize the blood plasma (94), or P800 to stabilize and prevent the degradation of GLP-1 (83). In total, there were 16 samples taken

from the venous line (V0-V15) and 14 samples (A2-A15) taken from the arterial line collected in the EDTA tubes. In addition, venous samples V0 and V7 – V15 and arterial samples A7, A10 and A14 were collected in the P800 tubes. In addition, two muscle biopsies at t=60 and t=180 and three US Doppler measurements were also taken to measure baseline values.

Table 2. Study infusions, their site of administration and purpose.

Peripheral venous catheters distributed between two arms	➢ 2-Deoxy-D-glucose (2-DOG; Sigma-Aldrich, UK) to measure glucose metabolism ➢ 20% Dextrose (Baxter, UK) to maintain blood glucose at 7-7.5 mmol/L ➢ Actrapid Insulin (Novo Nordisk, UK) to achieve constant postprandial insulin levels ➢ Octreotide (Novartis, UK) to supress endogenous insulin ➢ Vamin EF-14 (Fresenius Kabi, UK) to provide AA's
Femoral artery catheter	➢ Glucagon-like peptide-1(GLP-1; Bachem, Switzerland) to test the effect of GLP-1 on muscle glucose metabolism and total leg blood flow ➢ Arterial blood sampling
Femoral vein catheter	➢ Venous blood sampling

At t = 180 min following the 2nd biopsy the glucose and insulin clamps were started in combination with Octreotide, which adequately supresses endogenous insulin release during glucose infusion (89), Vamin EF-14, and ± GLP-1 infusion as appropriate to study visit (refer to Table 2) . To achieve constant postprandial insulin levels, insulin Actrapid was used and diluted with 0.9 % saline to make a volume of 48 mL. Further 2 mL of volunteer's own blood was added to prevent insulin sticking to the syringe material making a

total volume of 50 mL solution (37). At the start insulin was primed for 10 min followed by constant infusion aiming to achieve postprandial levels at approximately 30 mU/mL (dose per body surface area per minute, during 180 min). 10% glucose was also primed for the first 15 min of the infusion aiming to raise glucose concentration from fasting levels to levels between 7-7.5 mmol/L. Octreotide was diluted in 0.9% saline and started as a constant infusion of 30 ng/kg/min and continued at a same rate for 180 min. Vamin EF-14 was infused at a priming rate of 34 mg/kg followed by constant infusion of 102 mg/kg/h. GLP-1 was diluted in 0.9% Saline and infused to provide 1.2 pmol/kg/min for 180 min.

Following the initiation of appropriate infusions detailed above muscle biopsies were taken t = 90 min and t = 180 min to provide measures of postprandial 2-DOG content in the muscle. Muscle biopsies were taken from the vastus lateralis using sterile procedures carried out by appropriately trained medical staff. After sterilising the skin using an alcohol wipe, Lidocaine 1% was used to provide local anaesthesia. The conchotome technique (96) was used to take 200-300 mg of muscle tissue. Blood pressure (BP), pulse, respiratory rate and oxygen saturation were also measured throughout the acute study day using Datascope Trio monitor (Mindray, Huntingdon, Cambridgeshire, UK). The glucose infusion rate was adjusted as necessary guided by measurement of the venous blood glucose concentration every 5-15 min using the Yellow Springs Instrument 2300 Stat Analyser (YSI; YSI Inc, Yellow Springs, Ohio, USA), which uses a steady state methodology by which a membrane based immobilised glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (97). After the study, catheters

were removed and the volunteers monitored for 30 min before departure. A schematic diagram of the protocol can be seen by Figure 2.1. The exact sequence of acute study visit events is detailed in Table B, Appendix B.

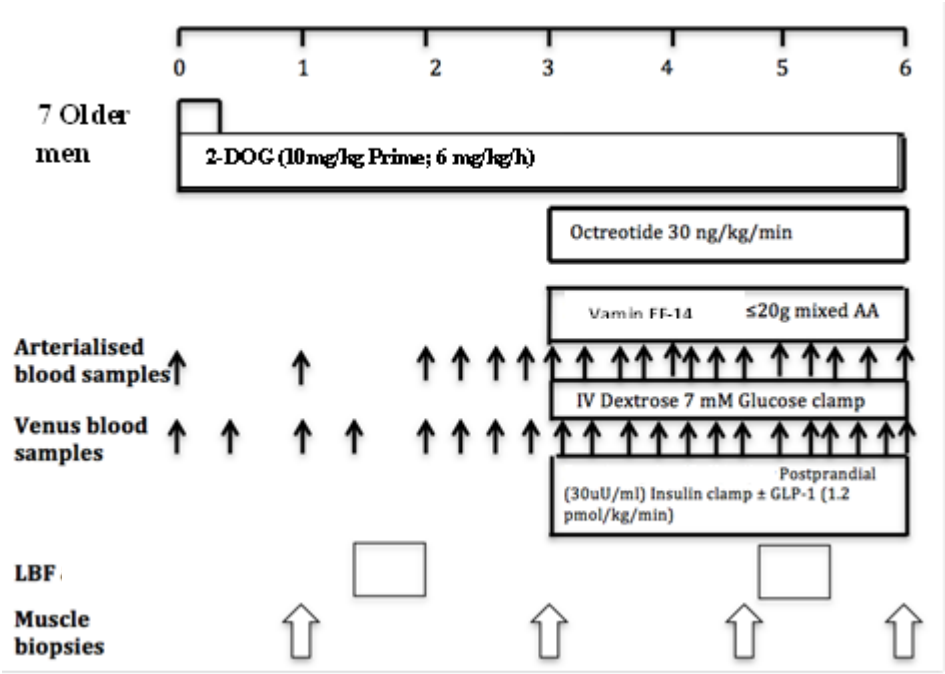


Figure 2.1 A schematic diagram of the study protocol.
2-DOG was used to measure glucose metabolism. Glucose and insulin clamps were initiated with combination of Octreotide, Vamin EF-14 ± GLP-1 infusions.

2.3.Sample processing

Blood samples and muscle biopsies were processed according to the University of Nottingham standard operating procedures (SOPs) for 'Handling of Human Body Fluids and Tissue' and 'Blood Samples'. Blood samples were centrifuged (IEC Centra CL3R, Thermo electron corporation, Waltham, Massachusetts, USA) at 3,200 rpm for 20 min at 4°C and the plasma was aliquot into appropriate corresponding Eppendorf tubes and stored at -80°C for further analysis. All muscle biopsies were immediately washed with ice cold phosphate buffered saline (PBS) to clear any visible excess of blood, fat or

connective tissue, snap-frozen in liquid nitrogen and stored at -80°C until further analysis for 2-DOG content.

2.4. Determination of glucose and 2-DOG concentration in plasma by GCMS

Gas chromatography coupled to mass spectrometry is an instrumental technique by which complex chemical mixtures can be separated (the GC component), identified and quantified (the MS component) (33,98). GCMS is widely used in metabolic profiling and can identify changes of different physiological states (98). The GC method requires the samples to be volatile which can be achieved by a processed termed *derivatization* (98,99). The gaseous sample is then carried through a column with an inert gas (such as helium) where its constituent parts are separated (100). The separation (and subsequent peak detection) can be affected by a range of factors such as the injection volume, temperature or the flow into the MS (98). Once in the MS, the sample is then ionised and fragmented, each ionised fragment can be detected by its mass to charge (m/z) ratio. In the GC the difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote their separation (98,99). The detector can be set to scan for only selected masses over different time periods which can be done by setting up a method known as selected ion monitoring (SIM). The following GCMS method to measure glucose and 2-DOG concentration is based on that reported by Shippee et al (101).

2.4.1. Determination of glucose

Standard preparation: Glucose standards were prepared by diluting 10mM glucose [180mg of D-Glucose anhydrous (Fisher Scientific, UK) in 100mL of double distilled water (ddW)] serially to 8, 6, 4 and 2mM with ddW. 25µL of 1mM U-13C6 [180mg of D-glucose U-13C6 (Cambridge Isotope Laboratories, Inc.) in 100mL of ddW] was added as an internal standard to each glucose standard. One sample of 6mM glucose was prepared without adding the internal standard to calculate its contribution to 323, as this 13C isotope is naturally occurring (102). Relative amounts of the tracer measured in A and V blood supply samples were used to calculate release of glucose in the muscle of interest (A-V balance). The concentration of glucose in the blood samples is measured by reference to a standard curve of known concentration normalised to the amount of internal standard.

Plasma sample preparation: Plasma samples were defrosted, vortex mixed (Genie-2, G-560E, Scientific Industries) and then spun for 2 min at 10,000rpm (Heraeus Fresco 17, Thermo Scientific) to pellet fibrin clots. 50µL of each plasma sample was aliquot into a fresh Eppendorf, followed by the addition of 25µL of U-13C6 glucose standard before vortex mixing.

Procedure: 1mL of ice cold 100% ethanol (Scientific laboratory supplies) was added to each samples and standards and placed in the fridge for 20 min to precipitate protein. The samples and standards were then spun for 2 mins at 10,000 rpm. After centrifugation, 100µL of the supernatant was removed and put into a corresponding 16X100mm labelled glass test tube (Fisherbrand, USA) and dried under nitrogen in the techne block (D13-3, Techne) at 90˚C

for approximately 2-3 mins. The tubes were subsequently derivatized to increase the volatility in two steps. First, 100µL of freshly prepared oxime reagent [dissolve 20mg of hydroxylamine hydrochloride (Sigma-Aldrich) per 1mL of pyridine (Fisher Scientific)] was added to each tube, tubes were capped, vortex mixed, and incubated at 85˚C for 30 mins in the techne block and allowed to cool. Then 70µL of N,O-bis (trimethylsilyl) trifluoroacetimidate with 1% trimethylchlorosilane (BSTFA, 1% TMCS, Sigma Aldrich) was added to each tube, capped, vortex mixed and incubated for another 30 mins at 85˚C in the techne block. After cooling, 70µL of the samples and standards were transferred into autosampler vials (Agilent technologies) and capped, ready for analysis by GCMS.

Pooled quality control (QC) samples were prepared from freshly thawed plasma by pipetting 25µL of 40 plasma samples from both venous and arterial line from two random subjects into a single Eppendorf tube to create a final volume of 1000µL (done 2x), divided into 100µL aliquots and processed alongside the rest of the samples. Each QC sample was then repeatedly injected (two injections near the start of the sequence, another two injections near the middle of the sequence and two injections near the end of the sequence) throughout each analysis run (same applies for the 2-DOG analysis) to assess machine stability and repeatability.

Analysis: The samples were ran on an Agilent 7890B (Agilent Technologies) GCMS using the selected ion monitoring (SIM) method. The SIM was set to detect and report incidence of m/z 319 for glucose, 321 (for deuterium D2 Glc – not measured), and 323 for U-13C6 glucose. Data reduction involved

recording the peak areas for the 3 masses of the largest peak (Figure 2.3). A concentration curve was generated from the 2, 4, 6, 8 and 10mM glucose standards. The internal standard mass 323 was corrected for the natural isotopomer background signal in the unlabelled sample. Ratio of 319 to 323 (corrected for carryover) was used to read off the concentrations from the standard curve (Figure 2.2).

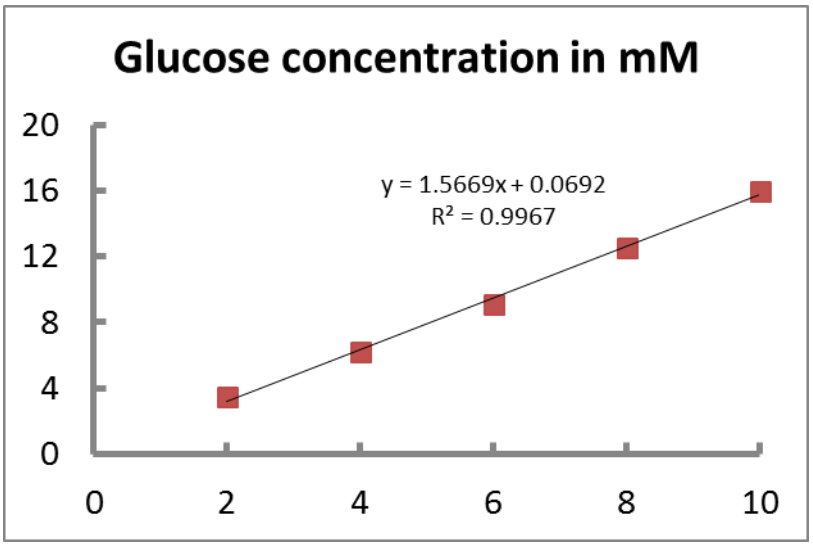


Figure 2.2 An example of generated glucose standard curve.
A standard curve was generated from the standards ran to allow calculation of glucose concentration in the samples.

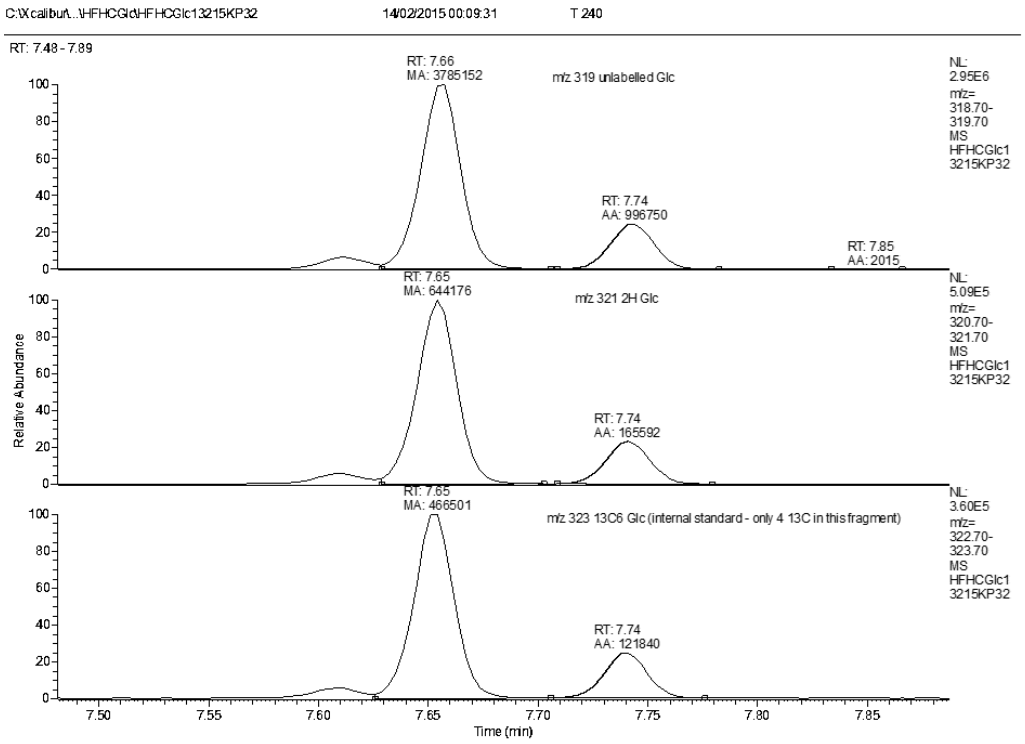


Figure 2.3 Chromatogram of the three monitored masses.
The 319 mass is the major peak of unlabelled glucose used for quantitation. The 321 is the deuterium tracer (not measured but included in the SIM). The 323 mass represents the internal U-13C6 standard added to quantify glucose.

2.4.2. Determination of 2-DOG

Standard preparation: 2-DOG standards were prepared by diluting 5mM 2-DOG [41mg of 2-DOG (Sigma-Aldrich), UK] in 50mL ddW] standard, 1 in 20 with ddW to make 250µM working standard which was then aliquot in 10, 25, 50, 75, 100, 125, 150µL into fresh Eppendorfs. This was followed by adding 50 µL of 100µM fluorodeoxyglucose (FDG) [1.8mg of FDG (Sigma-Aldrich) in 5mL ddW, diluted 1 in 4 to get 500µM and then again 1 in 4 to get 100µM) standard into each tube and vortex mixed.

Plasma sample preparation: After defrosting at room temperature, the plasma samples were vortex mixed and centrifuged at 10,000 rpm for 2 mins at 4˚C.

100µL of plasma was aliquoted into a fresh Eppendorf, followed by the addition of 50 µL of 100µM FDG working standard and vortex mixed.

Procedure: The procedure in 2.4.1 was followed except that all of the supernatant following ethanol precipitation was removed into labelled test tube and dried completely in the techne block at 90˚C for approximately 10 min.

Analysis: 2-DOG concentration was determined using 2DG SIM programme set for 231 and 232 m/z. 2-DOG m/z 232 peak is a doublet and elutes first and FDG m/z 231 elutes closely after the second 2-DOG peak (Figure 2.4). Samples were run again on the Agilent as in 2.4.1. Data reduction involved only the peak area for the first 2-DOG (major) peak and the FDG peak. The concentrations were calculated with reference to a standard curve of known concentrations prepared alongside each batch.

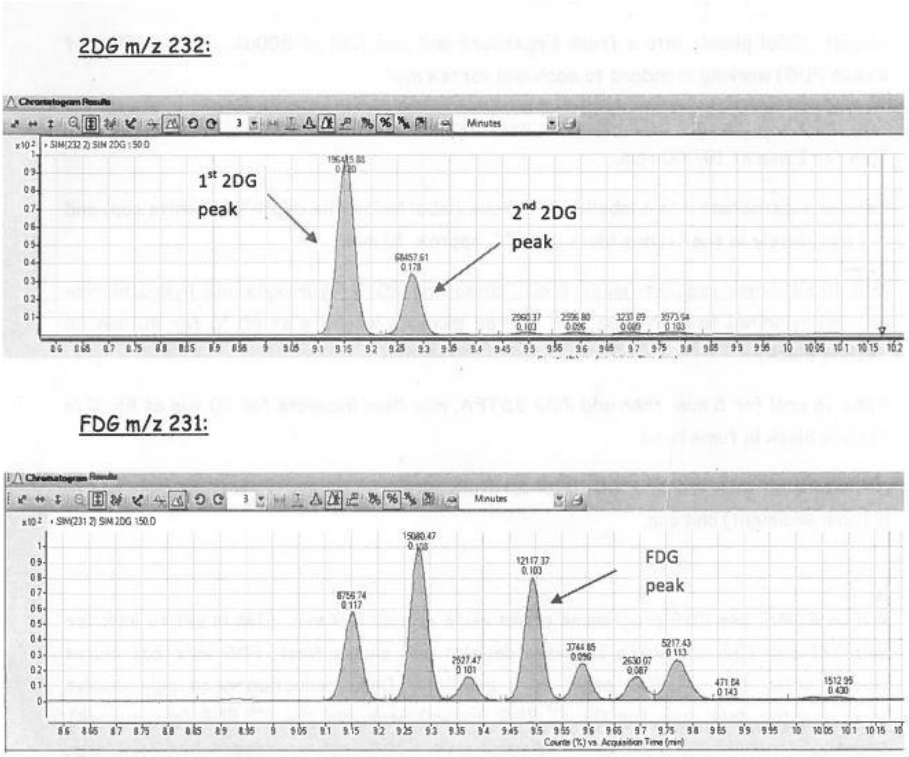


Figure 2.4 Chromatogram of 2-DOG and FDG peaks.

2.5. Determination of human insulin in plasma by enzyme immunoassay

Postprandial plasma (PP) insulin concentrations were measured in this study by high-sensitivity insulin enzyme linked immunosorbent assays (ELISA; DRG Instruments GmbH, Marburg, Germany) according to manufacturer's protocol (EIA-2337). Samples V0 to V7 baseline samples were ran undiluted whilst V8-V15, following commencement of clamps and infusions, were diluted 1:5 in ddw. The human insulin ELISA is a solid phase two-site enzyme immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule (103). In this assay, insulin in the sample during incubation period reacted with peroxidase-conjugated anti-insulin antibodies causing the antibodies to bind to the microplate. The bound conjugate was then detected by reaction with 3,3',5,5'-tetramethylbenzidine (103). The 96-well plates were then read spectrophometrically [filter (nm):450] on an automatic microplate reader (Multiskan ascent V1.24, Thermo electron corporation) interfaced to a computer with Ascent Software Version 2.6. Measurements were calculated against generated standard curve.

2.6. Determination of GLP-1 in plasma

Levels of active GLP-1 were measured alongside other gut hormones (leptin, GIP and PYY) using undiluted venous (V7-V15) samples collected in p800 tubes by MILLIPLEX MAP Kit - human metabolic hormone magnetic bead panel (EMD Millipore Corporation, Billerica, MA, USA; Cat. # HMHMAG-34K). The principle of this method is based on the Luminex XMAP technology (104) which uses proprietary techniques to internally colour-code

microspheres (beads) with two fluorescent dyes that through precise concentrations can create 100 distinctly coloured bead sets (each coated with a specific capture antibody). Samples were processed according to the manufacturer's instructions except that half volumes were used (12.5µL) of the beads and the detection antibodies. Measurements were performed on Luminex 200 system with xPONENT software, version 3.1 (Luminex, Austin TX) and values were interpolated from known standards. In this assay the mean fluorescent intensity (MFI) is plotted against known concentration of standards and a curve is fitted by the plate reader software (non-linear) (Figure 2.5). Each sample's MFI is then used to calculate the concentration from the standard curve (equation).

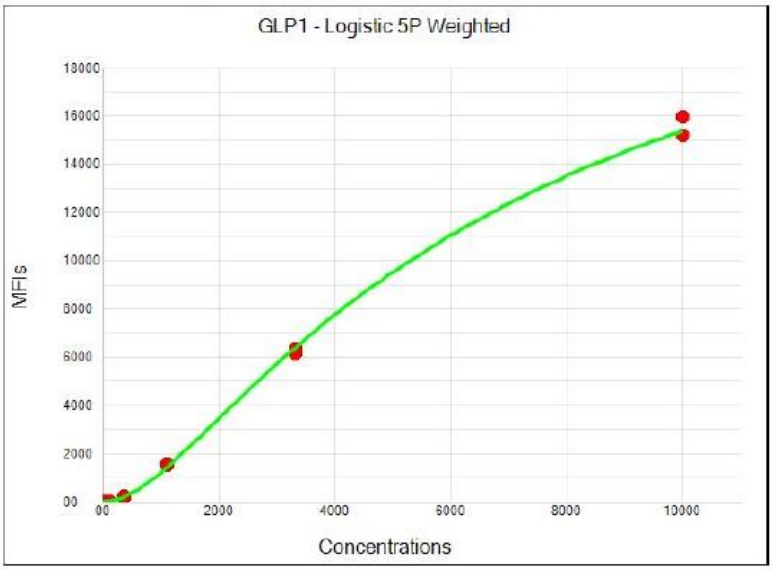


Figure 2.5 An example of generated GLP-1 standard curve.

2.7. Determination of amino acids in plasma by phenylalanine labelling

This method is based on Tipton *et.al.* (105,106). To determine labelling (atom percent excess; APE) and/or concentrations of arterialised venous phenylalanine, leucine, valine, and isoleucine: briefly, plasma protein was fractionally precipitated using 100% ethanol; the aqueous phase (containing the free amino acids) was dried under nitrogen, and then solubilised in 0.5 M HCl. Lipids were removed by extraction into ethyl acetate. The aqueous layer, containing the amino acids of interest was dried down and the N-tert - butyldimethyl - silyl -N- methyltrifluoracetamide (MTBSTFA) derivatives of phenylalanine, leucine, valine, isoleucine, and norleucine formed. l-[ring-13C6]Phenylalanine tracer-to-tracee (t/T) enrichment and amino acid (Phenylalanine, leucine, valine, and isoleucine) concentrations were then measured by GC-MS by selected ion-monitoring (SIM), m/z 302 for leucine, valine, isoleucine, norleucine and 234, 236, 240 for phenylalanine. Appropriate corrections were made for overlapping spectra contributing to the t/T ratio in enrichment measurement. Concentrations for plasma phenylalanine were determined using 2H2 phenylalanine, and norleucine (for the leucine, valine, and isoleucine) as the internal standards with reference to a standard curve.

2.8. Determination of total leg blood flow

Leg blood flow (LBF) was measured at regular intervals (as previously detailed in Table B, Appendix B) using a (US) Doppler ultrasound (iU22; Philips Ultrasound, CA, USA). Three measurements were taken to obtain a mean final value. All measurements were taken with the subject lying in a supine position. A linear array 9-3 MHz frequency probe (Philips Ultrasound)

was used to measure mean blood velocity (determined by pulse-wave Doppler) and arterial lumen diameter (by 2-D imagining as the distance between inner arterial walls) in the common femoral artery of one leg. LBF (l.min-1) was calculated from the diameter and the velocity measurements by the system as: mean blood velocity (cm.sec-1) × π × (femoral artery radius (mm))2)/1000 × 60 (107).

2.9. Calculations

Area under the curve (AUC) for insulin was calculated using the trapezoidal rule to assess individual statistical differences. Glucose metabolism (M) was calculated according to DeFronzo et al. (37) where M is equal to glucose infusion rate (GIR) minus urinary loss of glucose (which averages 0.2 mg/kg/min) minus space correction. In this study M was made for 15 min intervals using YSI glucose concentration values. The total M was calculated from the means of the twelve 15min study intervals. The M was also calculated for the three time periods (fasted and two fed). Total LBF data were corrected for plasma using haematocrit. Glucose concentration for A-V balance was analysed by GCMS (see 2.4.1) and A-V balance was calculated using the Fick principle (108) by subtracting venous concentration from arterial concentration, which was multiplied by blood flow, haematocrit and leg muscle mass (per 100g).

2.10. Statistical analyses

Statistical analyses were performed using Graph Pad Prism Version 6.00 (La Jolla, CA, USA). All data are reported as means ± standard error of a mean (SEM) with the threshold for significance set at $P < 0.05$. Statistical analyses

applied to the data consisted of a two-way analysis of variance (ANOVA) and paired student t-test as detailed within each result section.

3. Results

3.1. General characteristics of study demographics

General characteristics of 7 healthy old male volunteers including BMI, age, total lean leg mass, fat mass ratio – legs/total and appendicular skeletal mass (ASM) are shown in Table 3. All values (n=7) are expressed as means ± (SEM).

Table 3 General characteristics of study volunteers.

CHARACTERISTICS	Mean ± SEM
Age (in years)	70 ± 0.92
BMI (kg/m^2)	26.04 ± 0.65
Total lean leg mass (g)	18079 ± 469.55
Fat mass ratio – legs total (%)	0.27 ± 0.008
ASM (kg)	24 ± 0.68

3.2. GLP-1 concentration in plasma

The basal concentration of active GLP-1 in -GLP-1 group was 26 ± 4 pM/L and in the +GLP group 10 ± 5 pM/L (not significantly different based on 2-way ANOVA, Tukey's multiple comparison test). Following the initiation of the insulin and glucose clamps, the concentration in –GLP-1 group declined very slightly to 9 ± 2 pM/L and then maintained a steady-state throughout the whole 180 min period of the study with no significant differences observed (based on 2-way ANOVA, Sidak's multiple comparison test) between individual time points (see Figure 3.1). In the +GLP-1 group following

commencement of the GLP-1 infusion, the GLP-1 concentration rose steadily and plateaued from 45 – 135 min followed by a sharp increase from 135 min. Statistical significant differences were observed from 110 min ($P < 0.05$) when compared to the baseline value denoted by different letters (see Figure 3.1). The peak value of 378 ± 85 pM/L was reached at 180 min. Furthermore, the concentration at 160 min and 180 min differed significantly when compared to the earlier time points ($P < 0.05$). There was a significant difference ($P < 0.05$) between +GLP-1 and –GLP-1 groups (based on paired t-test) (Figure 3.1).

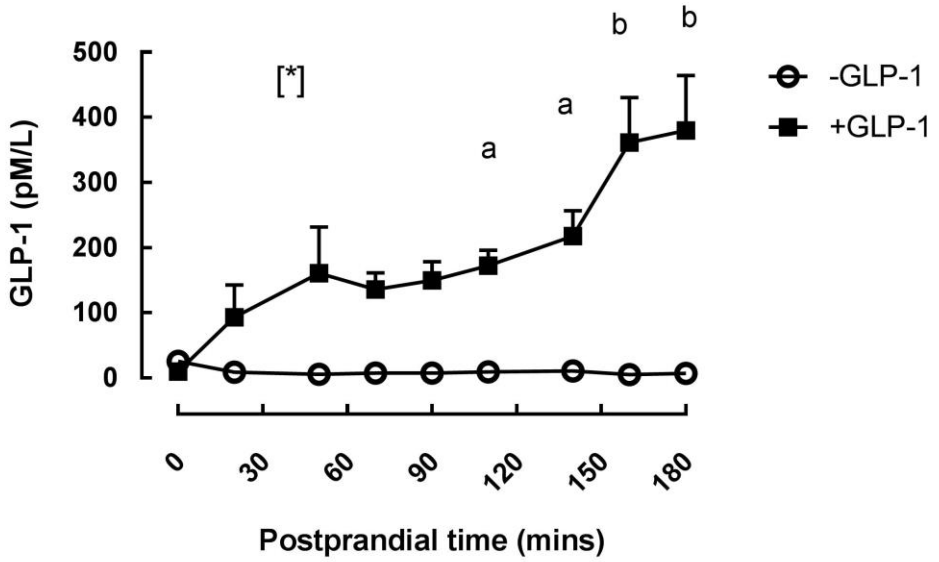


Figure 3.1 Time course of plasma GLP-1 concentrations (pM/L) ± GLP-1 during 180 min post-clamp period.
There were statistically significant differences between the two groups and between time points in the +GLP-1 group. Error bars denote SEM. (*$P < 0.05$; a and b letters denote significant differences when compared to the baseline; b denotes significant differences from previous time points). Statistical analyses via ANOVA – Sidak's multiple comparison test and via a student paired t-test.

3.3. Phenylalanine concentration in plasma in fasted and fed clamp conditions

Phenylalanine (Phe) was measured as a proxy of AA concentration in plasma. Following the primed constant AA infusion provided at t =0 (post clamp postprandial period) increases in plasma Phe concentration were observed in both groups when compared to the pre-infusion baseline values (56.7 ± 2.9 -GLP-1; 59.8 ± 2.2μM +GLP-1). (Figure 3.2). Plasma Phe concentration became elevated after 20 min (112.5 ± 5.5 -GLP-1; 111.4 ± 4.5μM +GLP-1) of AA infusion, reaching a peak concentration at 180 min (157.3 ± 5.1 –GLP-1; 156.1 ± 9.2μM +GLP-1) for both groups. There were no significant differences between the two groups (based on 2-way ANOVA, Tukey's multiple comparison test).

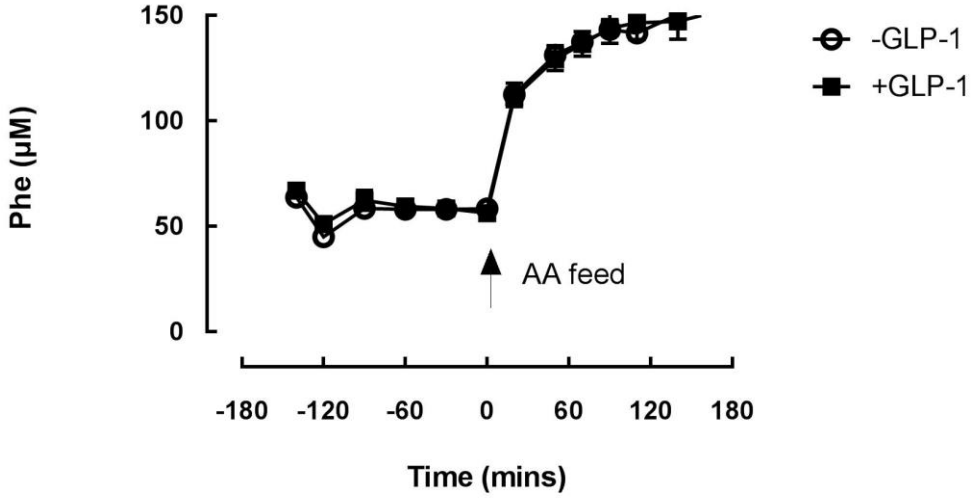


Figure 3.2 Phenylalanine concentration in plasma ± GLP-1 during 180 min pre- and 180 min post clamp amino acid (AA) infusion period.
Error bars denote SEM. No statistical differences observed between the groups. Statistical analyses via ANOVA – Tukey's multiple comparison test.

3.4.Leg blood flow

In both groups leg blood flow rose slightly during the clamp from 0.39 ± 0.04 to 0.44 ± 0.04 (-GLP-1) and 0.42 ± 0.03 and 0.48 ± 0.05 (+GLP-1) L/min (Figure 3.3, A), however that was not significant (based on 2-way ANOVA, Sidak's multiple comparison test). There were also no differences between the two groups. However, when LBF for the two fed periods was combined there was a significant difference observed between the two groups (based on a student paired t-test, $P < 0.05$) (Figure 3.3, B).

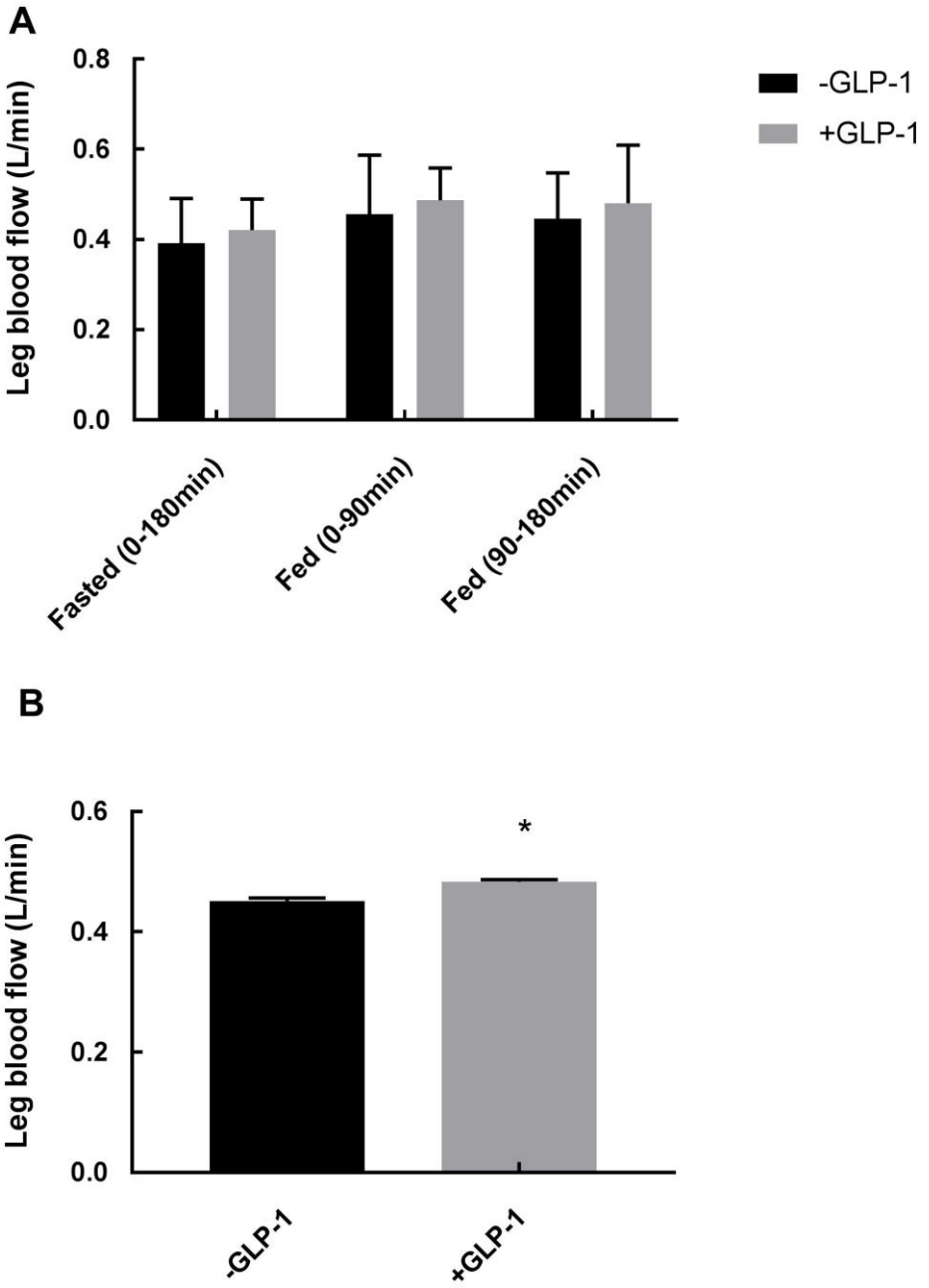


Figure 3.3 Comparison of leg blood flow ± GLP-1.
A) Comparison in between fasted (0-180 min) and two fed (0-90 and 90-180 min) periods. B) Comparison between fed periods ± GLP-1. Error bars denote SEM. (*P < 0.05). Statistical analyses via ANOVA – Sidak's multiple comparison test (A) and via a student paired t-test (B).

3.5. Insulin concentration in plasma

The basal plasma insulin concentration was 4.4 ± 0.7 for –GLP-1 and 3.9 ± 0.9 µU/mL for +GLP-1. Following initiation of the primed constant infusion of insulin a sharp increase in plasma insulin concentration was observed from 20 to 40 min for both groups (Figure 3.4, A). The –GLP-1 group achieved a steady state with average value of 22.7 ± 0.5 µU/mL, whilst insulin concentration in the +GLP-1 group rose gradually with average value of 26.5 ± 4.1 µU/mL and a peak value of 37.9 ± 7.7 reached at 160 min (Figure 3.4, A).

From 120 min, the values for plasma insulin concentration were statistically different between the two groups (based on 2-way ANOVA, Sidak's multiple comparison test, $P < 0.05$) as presented in Figure 3.4 (A). There was also a significant difference in the AUC insulin (Figure 3.4, B) between the groups (based on a paired t-test, $P < 0.0014$) with concentration values of 19.2 ± 7.4 (–GLP-1) and 25.4 ± 11.5 µU/mL (+GLP-1).

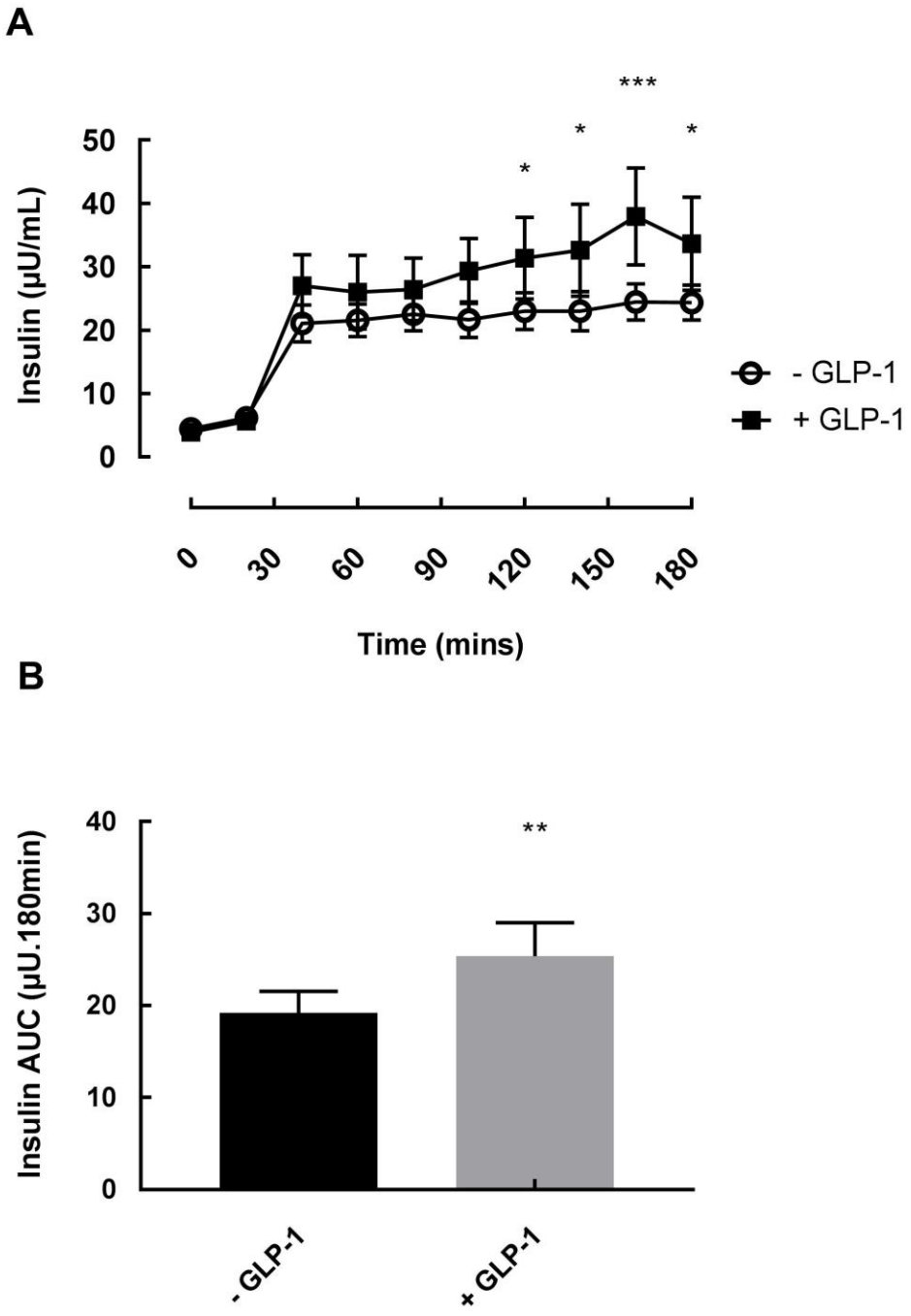


Figure 3.4Time course of plasma insulin concentrations (µU/mL) ± GLP-1 during 180 min post clamp period (A) and area under the curve (AUC) (B). Statistical differences were observed from 120 min between the two groups (A). There was statistically significant difference in the AUC between the groups (B). Error bars denote SEM. (*P < 0.05, **P < 0.02, ***P < 0.001). Statistical analyses via ANOVA - Sidak's multiple comparison test (A) and a student paired t-test (B).

3.6. Glucose concentration in plasma measured by YSI and measured glucose metabolism (M)

The basal arterial plasma glucose concentration measured by YSI was 6.5 ± 0.3 for –GLP-1 and 6.6 ± 0.3 mM/L for +GLP-1. Following the infusion of glucose with clamp at 7–7.5 mM/L an increase in plasma glucose concentration was observed from 15 min for both groups. In the –GLP-1 group the plasma glucose concentration rose significantly (based on 2-way ANOVA, Tukey's multiple comparison test, $P < 0.05$) when compared to the baseline from 45 – 90 min with average value of 7.7 ± 0.2 mM/L and then gradually decreased (average value of 7.3 ± 0.1mM/L) till the end of the study. In +GLP-1 group the plasma glucose concentration was 7.4 ± 0.2 mM/L (average value for 15-60 min period), which then slightly dropped to a plateau until the end of the study with average value of 7.2 ± 0.2 mM/L. There were no significant differences between the two groups (based on 2-way ANOVA, Sidak's multiple comparison test). The time course of the glucose concentration for both groups is shown in Figure 3.5 (A). The calculated amount of M (total body glucose metabolism) in mg/min/kg during 0 – 90 min period was 1.09 ± 0.2 (-GLP-1) and 1.30 ± 0.2 (+GLP-1), during 90 – 135 min the M was 1.03 ± 0.2 (-GLP-1) and 1.39 ± 0.2 (+GLP-1) and during 135- 180 min the M was 1.02 ± 0.1 (-GLP-1) and 1.52 ± 0.3 (+GLP-1) as shown in Figure 3.5 (B). There were significant differences found between the two groups in the last two fed time periods (based on 2-way ANOVA, Tukey's multiple comparison test, $P < 0.05$) (Figure 3.5, B).

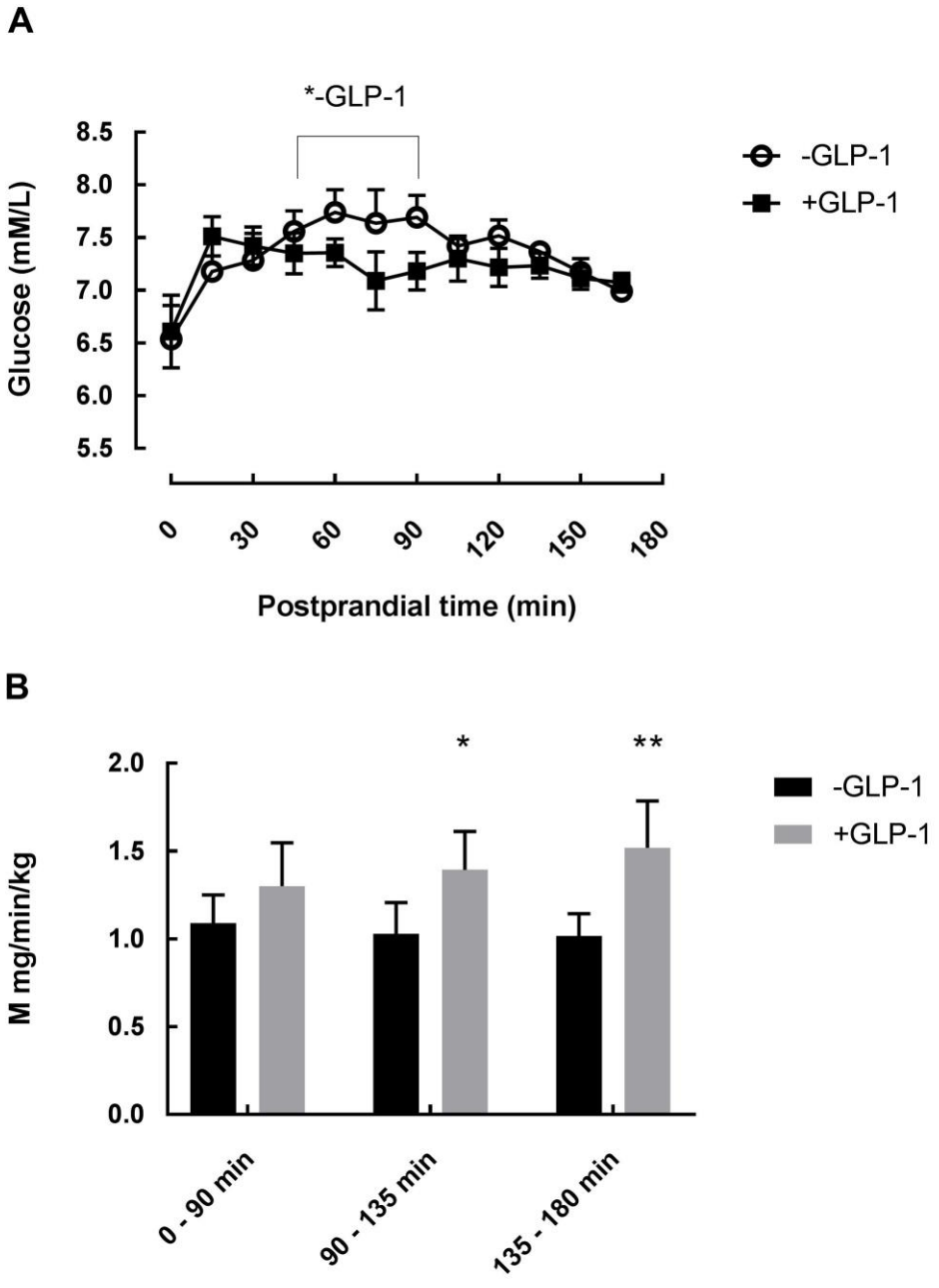


Figure 3.5Time course of glucose concentration measured by YSI (mM/L) (A) and measured glucose metabolism (M) (B) ± GLP-1 during 180 min post clamp postprandial period.
There was a statistical significant increase in plasma glucose concentration in the – GLP-1 group when compared to the baseline (A). M for the two late fed time periods (B) showed significant differences between the groups. Error bars denote SEM. (*P < 0.05, **P < 0.02). Statistical analyses via ANOVA – Tukey's and Sidak's multiple comparison test (A, B).

3.7. Glucose infusion rates

The effect of increasing or decreasing the glucose infusion rate (GIR) to maintain 7–7.5mM/L glucose levels is illustrated in Figure 3.6 (A). The initial GIR were observed to be 3.4 ± 0.7 (-GLP-1) and 3.83 ± 0.9 (+GLP-1) mg/kg/min per kg body weight following the priming rate infusion of glucose to establish the 7–7.5mM/L steady state. From 30 min post commencement of infusion the GIR in both groups maintained a steady state throughout with mean values of 1 ± 0.02 mg/kg/min (-GLP-1) and 1.4 ± 0.3(+GLP-1). Although there were no significant differences (based on 2 way ANOVA, Tukey's multiple comparison test) between the groups at any individual time point, the AUC over the whole fed period was significantly different (based on a paired t-test, $P < 0.05$) (Figure 3.6, B), which was due to the late (90 – 180 min) fed period with $P < 0.0001$ (Figure 3.7, B).

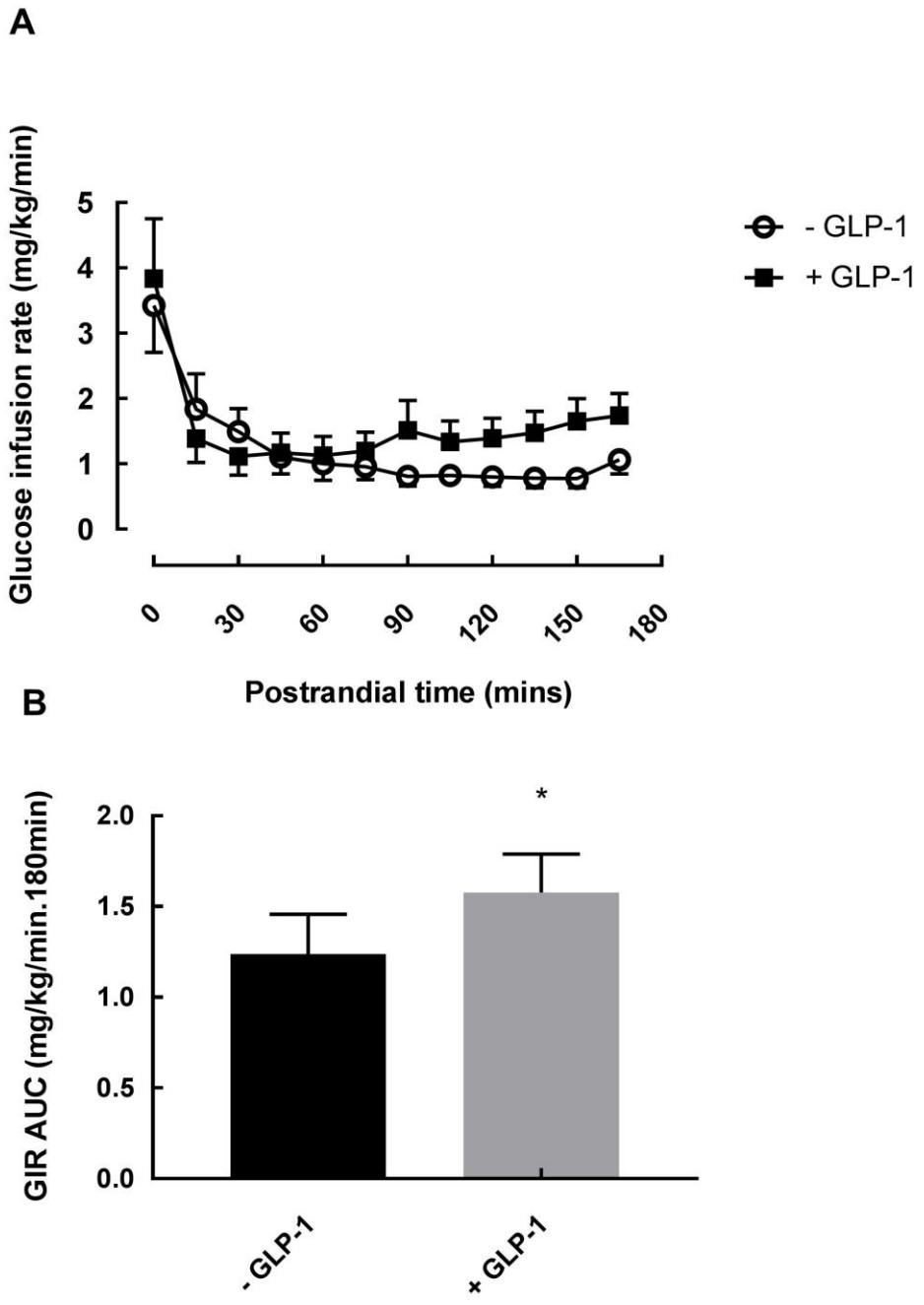


Figure 3.6Time course of glucose infusion rates (GIR; mg/kg/min) (A) and area under the curve (AUC) ±GLP-1 during 180 min post-clamp postprandial period (B).
There were statistically significant differences in the AUC of GIR between the groups (B). Error bars denote SEM. (*P < 0.05). Statistical analyses via ANOVA – Tukey's multiple comparison test (A) and a student paired t-test (B).

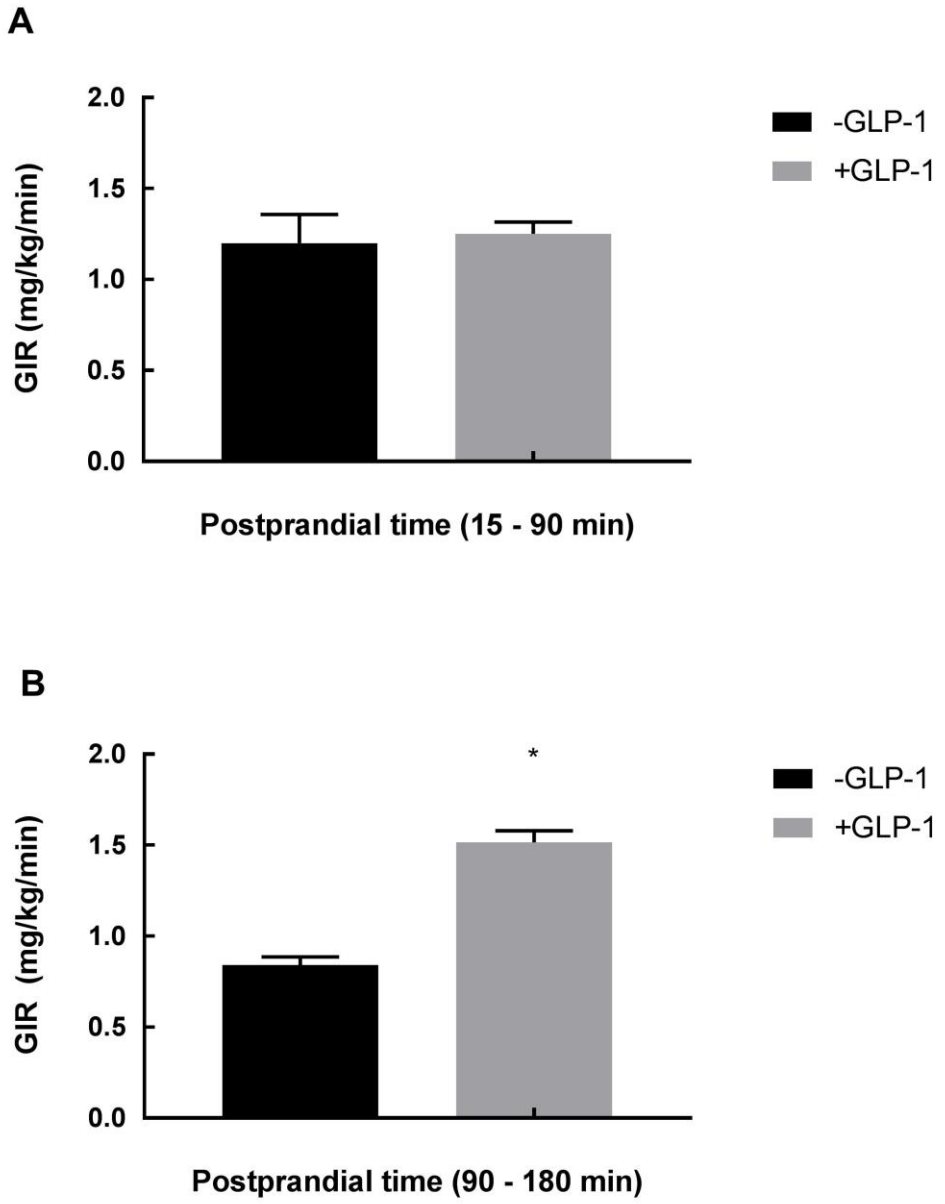


Figure 3.7 Area under the curve (AUC) of glucose infusion rates (GIR) for 15 -90 (A) and 90 – 180 (B) min post clamp postprandial period ± GLP-1. There were statistically significant differences in the AUC of GIR between the groups in the late (90 – 180 min) fed period (B). Error bars denote SEM. (*P < 0.0001). Statistical analyses via a student paired t-test (A, B).

3.8. Glucose concentration in plasma measured by GCMS and A-V balance

The basal venous plasma glucose concentration measured by GCMS at t = -180 was 5.5 ± 0.4 for –GLP-1 and 5.7 ± 0.4 mM/L for +GLP-1. The basal arterial glucose concentration at t = 0 (before the glucose infusion clamp) was 6.5 ± 0.4 for –GLP-1 (corresponding with the YSI value, see result section 3.6) and 5.9 ± 0.3 mM/L for +GLP-1 (0.7 mM/L lower when compared to the YSI value). Following the infusion of glucose clamped to achieve 7–7.5 mM/L sharp significant increases in both arterial and venous plasma glucose concentration were observed from 20 min to approximately 110 min for both groups (based on 2-way ANOVA, Tukey's multiple comparison test, $P < 0.05$) when compared to the arterial baseline values. The average arterial plasma glucose concentration for this time period was 8.0 ± 0.4 (–GLP-1) (0.3 mM/L higher when compared to the YSI results of a similar period) and 7.7 ± 0. 4 mM/L (+GLP-1) (~0.3 mM/L higher than the YSI measure for the similar period), and 7.7 ± 0.2 (–GLP-1) (0.4 mM/L higher than in YSI) 7.4 ± 0.4 mM/L 9+GLP-1) (0.2 mM/L higher than in YSI) for the last hour or so. In both groups a small decrease was observed towards the end of the study, which corresponds with the pattern of glucose concentration measured by YSI (refer to Figure 3.5, A and Figure 3.8). There were no significant differences observed (based on 2-way ANOVA, Sidak's and Tukey's multiple comparison test) between the A and V plasma concentration at any time point or between the two groups. The time course of the glucose concentration for both groups is shown in Figure 3.8.

The calculated amount of A-V balance in µM/min/100g of leg mass during the fasted period was 0.43 ± 0.2 (-GLP-1) and 0.40 ± 0.4 (+GLP-1), during the fed period (20 – 90 min) the amount was -0.97 ± 1.0 (-GLP-1) and -0.96 ± 1.7 (+GLP-1) and during the late fed period (110- 180 min) the amount was -2.07 ± 2.1 (-GLP-1) and 1.52 ± 1.0 (+GLP-1) as shown in Figure 3.9 (A). The total A-V balance amount for the fed periods in –GLP-1 group was -1.52 ± 1.6 and 0.28 ± 1.3 in +GLP-1 group (Figure 3.9, B). There were no significant differences found between the two groups based on 2-way ANOVA, Tukey's multiple comparison test and a student paired t-test).

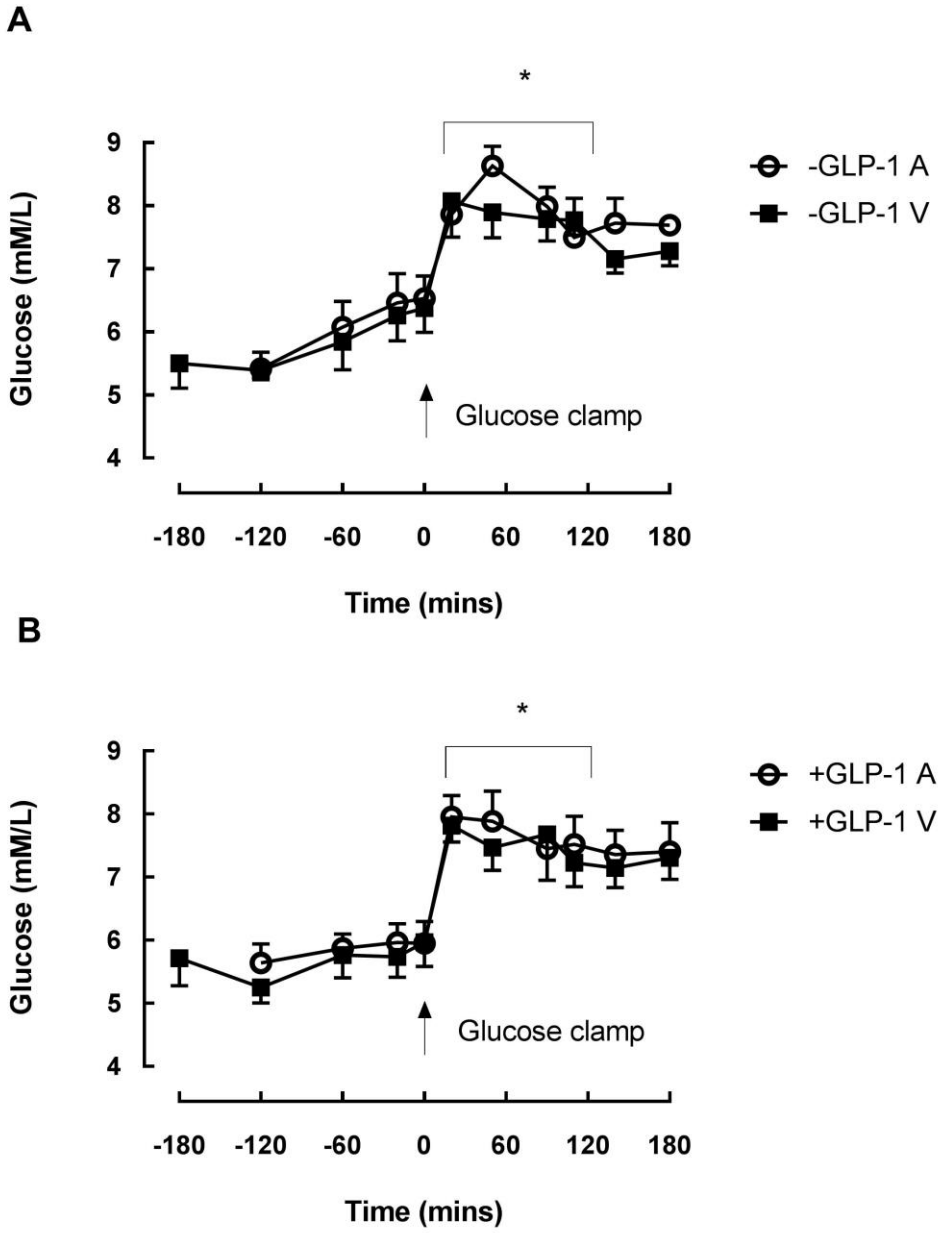


Figure 3.8 Time course of arterial and venous (A-V) glucose concentration measured by GCMS (mM/L) ± GLP-1 during 180 min pre- and 180 min post- clamp glucose infusion periods.
Significant increases were observed in both A-V plasma glucose concentration from 20 min until about 110 min in both groups (A, B) when compared to the baseline. (A) –GLP-1; (B) +GLP-1. Error bars denote SEM. (*$P < 0.05$). Statistical analyses via ANOVA – Sidak's and Tukey's multiple comparison test (A, B).

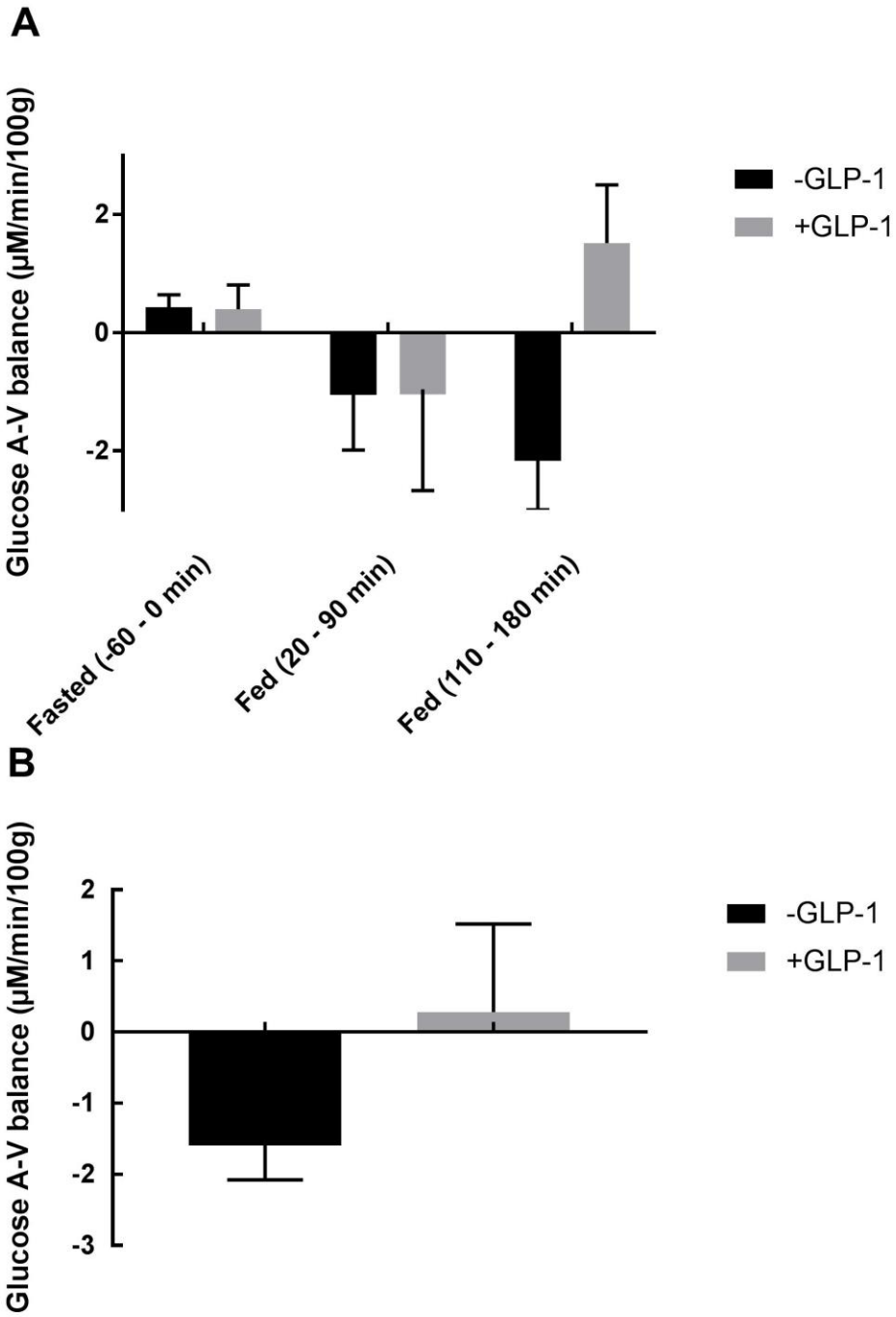


Figure 3.9 Glucose arterial and venous (A-V) balance in µM/min/100g of leg mass ± GLP-1.
A) Comparison in between fasted (-60 - 0 min) and two fed (20-90 and 110-180 min) periods. B) Comparison between fed periods ± GLP-1. There were no significant differences observed between the two groups. Error bars denote SEM. Statistical analyses via ANOVA – Tukey's multiple comparison test and a student paired t-test (A, B).

Discussion

This study was designed to define for the first time the extra-pancreatic ‘novel’ effect of GLP-1 on muscle postprandial glucose metabolism and vascular blood flow in older age since recent animal and human studies involving young subjects (78,81–90,92) have shown beneficial effects of GLP-1 infusions on skeletal muscle vasculature and glucose uptake. We, therefore, hypothesised that GLP-1 infusion would have the same positive effects on muscle glucose metabolism and vascular blood flow under postprandial conditions in the older population, which would improve delivery of glucose to the muscles. We chose to study healthy volunteers aged 65 years old and over (mean age in years 70 ± 0.92) since age-related metabolic dysregulations in postprandial muscle metabolism i.e. impaired glucose uptake and decreased blood flow to muscles are commonly observed phenomena in this age group (58–62). Our data revealed that GLP-1 ‘significantly’ increases total leg blood flow and has a significant effect on whole body postprandial glucose metabolism in healthy old male volunteers. However, no significant change in leg glucose uptake has been observed.

One consequence of ageing is that of impaired glucose uptake; both insulin- and non-insulin-mediated glucose uptake, which mostly occurs in skeletal muscle under both fasted and postprandial conditions (56,62,109,110). This impairment can be partly explained by decreased limb blood flow to the muscles causing decreased delivery of nutrients such as glucose and insulin (111) as well as by insulin resistance that comes with ageing (36,56). In fact, insulin resistance has been shown (112) to be often accompanied by

endothelial dysfunction as insulin action within the microvasculature is reduced in the phosphatidylinositol 3-kinase/Akt (PI3-K) pathway that is otherwise responsible for the activation of endothelial NO synthase (eNOS) and NO-mediated vasodilation (113) along with increasing GLUT4 translocation to the cell membrane (30,38,39). These age-related impairments have been then shown to be associated with metabolic disorders including cardiovascular (9,55,61,114) and may contribute to the pathophysiology of sarcopenia in the elderly (48,54). Here, it is shown that GLP-1, when infused peripherally in the femoral artery at rate of 1.2 pmol/kg/min 'significantly' increases total leg blood flow and has a significant effect on whole body postprandial glucose metabolism as indicated by increased values of measured glucose metabolism (M). Furthermore, observed significant increases in glucose infusion rates (GIR) that were needed to maintain the glucose clamp also suggest that GLP-1 infusion promoted glucose uptake.

The involvement of GLP-1 in insulin mediated glucose uptake is well defined (71,79), however, its role in non-insulin mediated glucose uptake particularly in skeletal muscle has still not been well established. Recently Chai et al.(78) demonstrated that GLP-1 infusion resulted in glucose uptake in rats using femoral arterial-venous (A-V) balance method. However, these data conflict with the most recent study by Sjøberg et al. (113) where intravenous infusion of GLP-1 did not result in increased skeletal muscle glucose uptake in rats. This observation supports their previous findings in both rats and humans where a direct GLP-1 infusion into the femoral artery also did not cause increases in leg glucose uptake (89). These observations are further supported by other two human studies that also did not find any increases in glucose

uptake following brachial artery GLP-1 infusions (88,90). However, all the above mentioned studies demonstrated increases either in arterial (88–90) or microvascular limb blood flow (78,88,89,113), which corroborate our findings. In our study, GLP-1 group demonstrated 7% increase in total limb blood flow which was significant (based on a student paired t-test) when compared to the control group. This is in accordance with the Sjøberg et al. (89) study (conducted under very similar experimental conditions as applied in this study), which showed 12% increase in total leg blood flow in healthy young men. Furthermore, most recent Danish study (115) also demonstrated sustained significant increases in leg blood flow after the commencement of GLP-1 infusion in healthy young men, suggesting that GLP-1 acts as a potent vascular stimulus in humans too. In our study, similar but smaller changes are seen in the old, perhaps as a result of the reduced vasodilatory response of decreased postprandial blood flow as demonstrated by Phillips et al. (116). Moreover, it is important to note that in both above mentioned studies (89,115) GLP-1 was infused in the femoral artery under fasted conditions, so it may be that the response to GLP-1 infusion under postprandial conditions would be much higher in the young because of the well-defined response of nutrient intake to an increased limb blood flow (62,117). Nonetheless, our 7% increase in the old still may have a positive impact on the impaired postprandial vasodilation that comes with ageing (62) positively affecting consequent glucose metabolism and uptake.

In our experiment the peripherally administered GLP-1 infusion at rate of 1.2 pmol/min/kg resulted in elevated GLP-1 plasma concentration levels as high as 378 pM/L that were significantly raised particularly in the last 30 min of the

180 min GLP-1 infusion period. One possible explanation for the significantly raised levels is probably the inability of dipeptidyl-peptidase-4 (DPP-4) to degrade GLP-1 due to oversaturation of its GLP-1R receptors (69). Furthermore, the plasma concentration levels of GLP-1 reached are beyond the physiological ranges (72,89). However, we speculate that probably even physiological plasma GLP-1 postprandial concentrations would be sufficient to increase leg blood flow in the elderly as Sjøberg et al. (89) demonstrated in the young. The rationale behind is that GLP-1 concentrations are not different between younger and older men, both under fasted and postprandial conditions (118).

The mechanisms by which GLP-1 could exert both vascular or glucose uptake effects still remain unclear too (we were also unable to seek a mechanism for our findings in this study). A mounting evidence indicates that these vasodilatory effects of GLP-1 are endothelial dependent (91), however a study by Nyström et al. (119) did not support this conclusion. Recently, Chai et al. (78) demonstrated that GLP-1 acts via a nitric oxide dependent mechanism, which is similar to that utilized by insulin (46,120) and that these GLP-1 effects could be diminished by a pre-infusion of the nitric oxide inhibitor L-NAME (78). These observations are further verified by cell culture experiments using human umbilical vein endothelial cells (121) demonstrating that incubation of cells with L-NAME resulted in attenuation of GLP-1-induced endothelial NO synthase, an enzyme that is important in regulating vascular tone (induces NO-mediated vasodilation) (39,46). Similarly, despite the presence of GLP-1 receptors in heart and vasculature of both humans and rodents (71,73), which corresponds with GLP-1 vasodilatory effects, its direct

effect on skeletal muscle glucose uptake is not fully understood (122). Some studies have shown effects of GLP-1 on muscle glucose uptake via increased activity of glycogen synthase (80,83,123), in association with activation of the PI-3K pathway, independent of insulin (80), which is a positive finding as in insulin resistance, impaired glycogen synthesis secondary to reduced activity of glycogen synthase have been shown to be the earliest detectable metabolic defects in the muscle (124,125). However, a study by Furnsinn et al. (126) did not find such effects in isolated rat soleus muscle incubated with either GLP-1 or insulin ± GLP-1. Moreover, in Tesauro et al. study (90) GLP-1 was co-infused with insulin in T2DM patients and despite the increased total forearm blood flow, glucose uptake did not change. Therefore, despite positive reports from animal and cell culture studies that demonstrated increases in glucose uptake in skeletal muscle, GLP-1 does not appear to have the same effect in humans (even when the concentration of GLP-1 is beyond physiological ranges). Saying that, despite not demonstrating significant differences between the groups also in our study, there was a positive trend seen in glucose uptake in the +GLP-1 group (as indicated by the overall positive A-V balance value), which lends itself to a suggestion that glucose was taken up by the muscles and it may be that the A-V balance method is not sensitive enough (discussed later).

This study aimed to assess the independent action of GLP-1 whilst insulin concentrations were clamped at postprandial levels; and as GLP-1 and amino acid infusions result in the release of endogenous insulin from pancreatic beta cells (18,72), Octreotide was infused to suppress such release (89). However, despite infusing insulin at the same rate under the two conditions i.e. with and

without octreotide, plasma insulin was unexpectedly significantly elevated in the +GLP-1 group. Assuming the octreotide clamp at the rate of 30 ng/kg/min was sufficient to prevent insulin release from the pancreas as previously evaluated by Krentz et al. (127) then it appears that insulin is perhaps protected from further metabolism in the presence of high GLP-1. As a result, the significantly higher insulin levels observed in our study in the GLP-1 group may influence the overall importance of the parameters we measured since increased blood flow and greater uptake of glucose is to be expected with higher insulin levels (34,41). It is not clear, however, what role GLP-1 is playing in this. Either GLP-1 is stopping the insulin being metabolized or we are getting insulin release from the pancreas despite the Octreotide infusion. In fact, a study by Ahrén et al. (128) demonstrated in mice using C-peptide deconvolution technique that GLP-1 reduces the clearance of endogenously released insulin. Whether this is the case of our study warrants future investigation but the C-peptide deconvolution technique suggests C-peptide is secreted in equimolar amounts to insulin but, unlike insulin, this peptide is not extracted in the liver itself and can therefore be used to determine if insulin is indeed released (128,129). The results of our study do not tell a conclusive story with regards to the effects of GLP-1 per se on glucose metabolism, glucose uptake and vascular blood flow in older age as we cannot dismiss the fact that due to the higher circulating insulin in the GLP-1 group the active biological component may be actually insulin but the results suggest that glucose is indeed being metabolised. However, whether it is being immediately stored (glucose to glucose-6-phosphate to glycogen) or undergoes glycolysis

(both oxidative and non-oxidative) and in which tissues and to what extent warrant future investigations.

Whilst these findings are generally in accordance with the findings of recent human studies (88–90,114) several methodological limitations need to be addressed before drawing any conclusion. Firstly, it is important to note that the A-V balance approach to assess glucose uptake was not the primary aim of this study due to its perceived limitations. In that we are looking for small changes in concentration (100-200 μM) across the leg with a prevailing high concentration (5-7.5 mM) and most glucose analysers are incapable of accurately measuring these changes (130); in addition problems with poor sample collection and handling during the study which can lead to erroneous glucose concentration measure, may have exacerbated this lack of sensitivity such that even more accurate techniques e.g. GC-MS are unable to robustly measure these small changes (131,132). Similarly, with the measurement of M, the study protocol and design were not optimised for this measurement and blood glucose was not measured every 5 min (37), therefore, we had to calculate the glucose concentration in between the actual sampling points in order to derive the M values. The M value calculation assumes that basal hepatic glucose production is suppressed by the infusion of euglycaemic hyperinsulinaemic clamp (EHC) (37) when the levels of glucose and insulin are raised to levels higher than our postprandial levels. Therefore, if hepatic glucose production is not abolished, the measurement of total body glucose metabolism may not be accurate in this situation. To determine glucose uptake by skeletal muscle more accurately in the future, the use of the 2-DOG method would be the best approach. Although we infused the 2DOG in these studies

and made measures of plasma 2DOG, to date, we have not been able to develop the measurement of 2DOG6P in muscle and hence muscle glucose uptake. A colorimetric commercially available kit for 2DOG6P was not sufficiently precise nor sensitive to make the measures of muscle 2DOG6P that we had planned. LC-MS methods are currently in development and will be applied to these samples in the future.

In summary, this study demonstrates that GLP-1 infusion in healthy old people has a significant effect on whole body postprandial glucose metabolism and vascular blood flow as demonstrated by the increased levels of glucose metabolism (M), glucose infusion rates (GIR) and insulin. However, this might be a consequence of the raised insulin concentrations and possibly through an insulin-mediated mechanism and not by GLP-1 action alone. However, GLP-1 effect could still contribute to the increased delivery of glucose to the muscle in the elderly and specifically combat the negative consequences of ageing i.e. impaired glucose uptake, vascular blood flow and age-related insulin resistance by further enhancing the action of insulin. These data form the basis for a future research and suggest that GLP-1 therapy may be a promising option to target age related declines in muscle performance by enhancing the action of insulin and may, therefore, improve metabolic health and consequently quality of life of the elderly. However, further analyses are required i.e. 2DOG6P and C-peptide to fully understand these data. The increased insulin in the GLP-1 group was unexpected and could not have been easily controlled. Further work may be required to unravel the effects of GLP-1 and provide a robust mechanism of its action, possibly using a dose response study design.

Appendix A

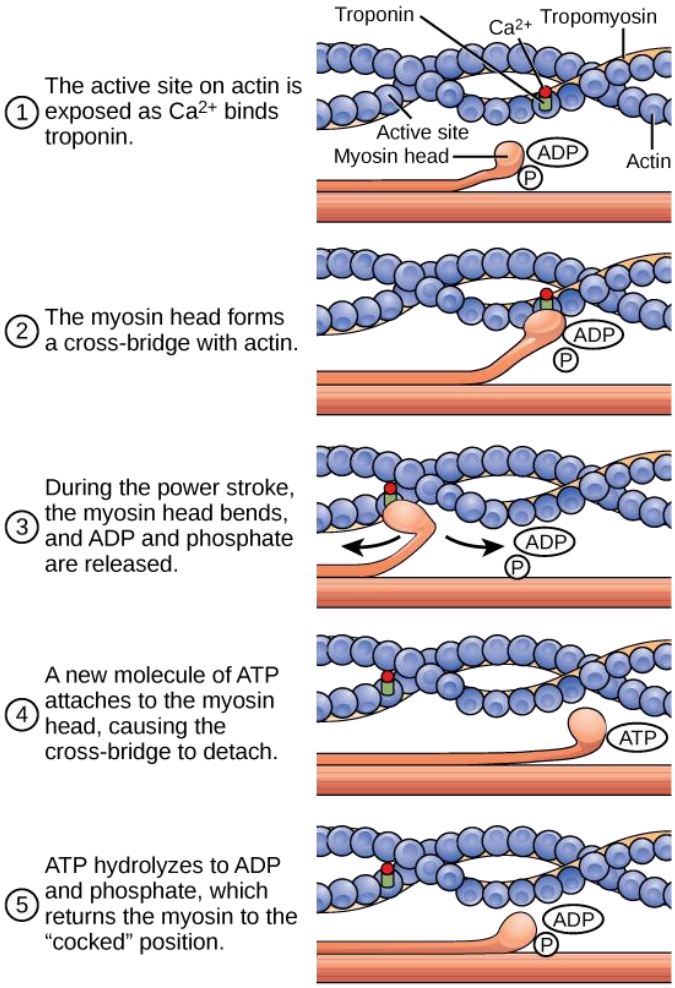


Figure A
Gross muscle movements are brought about by a cyclic reaction of the cross bridges, which in short consists of: attachment (to actin) → tilting (producing movement) → release and attachment to the next site. This can only occur with an energy source, which is ATP. Briefly, ATP attaches to the myosin head groups, where it is hydrolysed by myosin ATPase allowing the myosin heads to release the actin. Upon this, a 'high energy' phosphate (P), also called creatine phosphate (CP), is transferred from the ATP to the myosin, which becomes 'energized', while the original ATP becomes ADP. The energized cross-bridge will then attach to the actin, tilt and move the actin along, which is called a power stroke movement. If ATP is not available, the myosin heads remain locked to the actin filaments, so no sliding can occur. In addition to ATP, calcium is also required for the attachment phase of the cycle to move tropomyosin out of the way upon binding to the troponin, therefore exposing the attachment sites (19–21). [Taken from (20)]

Insulin (Figure B) and exercise are both able to stimulate muscle and induce vasodilation at the level of terminal arterioles, which enhances muscle perfusion and subsequent glucose uptake. Muscle due to the action of insulin has the ability to recruit greater microvasculature during postprandial conditions (without changing the total blood flow), which generates a wider surface for glucose delivery and transendothelial transport (41). It is worth nothing that during intense activity skeletal muscle blood flow can increase dramatically up to 80-100 ml min-1 100g-1 to meet the increased demand for oxygen (24). For example, blood flow to the human quadriceps can increase more than 50-fold during brief intense exercise and glucose extraction can increase more than six-fold (41). In terms of exercise, the increased bulk blood flow during exercise arises both from local factors such as nitric oxide (NO) release by the muscle as well as from neurovascular changes (30,40).

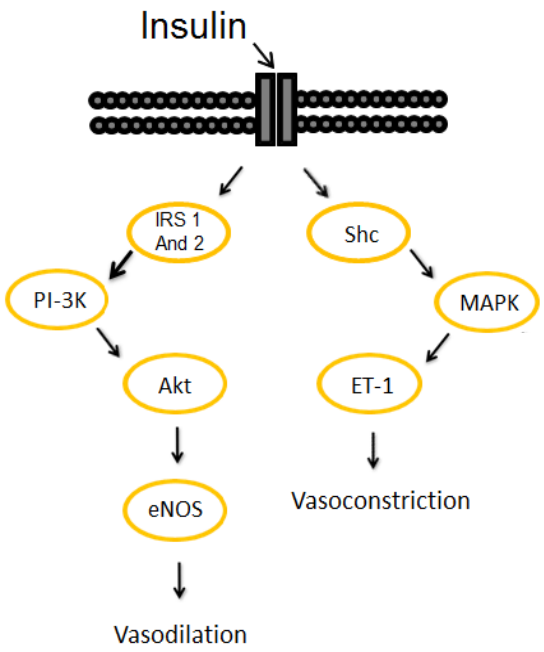


Figure B Insulin vasodilation and vasoconstriction pathways in the endothelium. Insulin stimulates two pathways in the endothelium through its binding to the insulin receptor on ECs: 1) the phosphatidylinositol 3-kinase/Akt (PI3-K) pathway that activates endothelial NO synthase (eNOS) and induces NO-mediated vasodilation; and 2) the MAPK pathway which mediates production of the vasoconstrictor endothelin-1 (ET-1) (38–40). These signalling pathways presented in this figure are closely parallel to insulin signalling pathways that regulate glucose uptake (refer to Figure 1.4), this is supported by studies that used limb balance methods combined with euglycemic insulin infusions (40). Following ingestion of food, insulin first mediates vasodilation at the level of terminal arterioles, thereby increasing microvascular capillary recruitment (MVR) (without changing the total blood flow) (41,44,46). This occurs within five minutes of insulin stimulation and causes subsequent relaxation of larger resistance vessels, resulting in an increase of total blood flow, which can be observed within 30 minutes of insulin stimulation (peak flow is reached after two hours) (46). Insulin-stimulated production of NO from endothelium leads to capillary recruitment, vasodilation, increased blood flow to skeletal muscle, and subsequent increase of glucose uptake and disposal. **(Taken from** (46)**).**

Appendix B

Table A. Inclusion and exclusion criteria for the study

Inclusion criteria for the study:
Aged between 65-75 years
A body mass index (BMI) >18 and <28 kg/m^2
Exclusion criteria for the study:
A BMI <18 or >28 kg/m^2
Active cardiovascular disease: uncontrolled hypertension (BP > 160/100), angina, heart failure (class III/IV), arrhythmia, right to left cardiac shunt or recent cardiac event
Cerebrovascular disease: previous stroke, aneurysm (large vessel or intracranial)
Respiratory disease including pulmonary hypertension, COPD, asthma or an FEV1 less than 1.5 l
Metabolic disease: hyper and hypo-parathyroidism, untreated hyper and hypothyroidism, Cushing's disease, types 1 or 2 diabetes
Active inflammatory bowel or renal disease
Malignancy
Recent steroid treatment (within 6 month), or hormone replacement therapy
Clotting dysfunction
Musculoskeletal or neurological disorder
Family history of early (<55y) death from cardiovascular disease
Overt muscle wasting i.e. muscle mass is more than 1 SD below normal muscle or fat-free mass for age
Taking beta-adrenergic blocking agents, statins or non-steroidal anti-inflammatory drugs
Known sensitivity any drug used in the study
Subject deemed unsuitable for femoral venous cannulation at screening visit

Table B. Timetable of acute study day 1 and 2.

Time (min)	Evaluation/Assessment/Procedure/Measurements
-60	Confirm name, DOB, address
-60	Assess if any AEs or SAEs occurred since screening visit
-60	Confirm appropriate period of overnight fasting
-60	Confirm subject has not taken any medications in the last 24 hours that might have effect on tests results
-60	Confirm normal physical activity in the last 7 days
-40	Subject would change own dress into a clinical gown
-30	Subject will have DXA scan performed (acute study visit1 only)
-20	Insert peripheral venous catheters across two arms, femoral artery and vein catheters
-5	Check BP, Pulse, Respiratory rate and Oxygen saturation Obtain baseline arterial and venous blood samples (V0: EDTA, P800)
0	Start primed constant infusion of 2-DOG. This will be maintained for 360 min (6 hours)
30	Check BP, Pulse, Respiratory rate Obtain AV blood samples (V1: EDTA)
45	Infiltrate local anaesthetic
60	Check BP, Pulse, Respiratory rate Obtain AV blood samples (V2, A2: EDTA) **Biopsy 1**
90	Obtain AV blood samples (V3, A3: EDTA)
120	Check BP, Pulse, Respiratory rate Obtain AV blood samples (V4, A4: EDTA)
130	**US Doppler 1**
140	Obtain AV blood samples (V5, A5: EDTA)
150	**US Doppler 2**
160	Obtain AV blood samples (V6, A6: EDTA)
165	Infiltrate local anesthetic
170	**US Doppler 3**
180	Check BP, Pulse, Respiratory rate and Oxygen saturation Obtain AV blood samples (V7, A7: EDTA, P800) **Biopsy 2**
0	Initiate Insulin, Dextrose, Octreotide and Vamin EF-14 +/- GLP-1

	YSI GLU – every 5 min until 180 min
20	Obtain AV blood samples (V8: EDTA, P800; A8: EDTA)
40	Check BP, Pulse, Respiratory rate **US Doppler 4**
50	Obtain AV blood samples (V9: EDTA, P800; A9: EDTA)
60	**US Doppler 5**
70	Obtain AV blood samples (V10, A10: EDTA, P800)
75	Infiltrate local anesthetic
80	Check BP, Pulse, Respiratory rate **US Doppler 6**
90	Obtain AV blood samples (V11: EDTA, P800; A11: EDTA) **Biopsy 3**
110	Obtain AV blood samples (V12: EDTA, P800; A12: EDTA)
120	Check BP, Pulse, Respiratory rate
130	**US Doppler 7**
140	Obtain AV blood samples (V13: EDTA, P800; A13: EDTA)
150	**US Doppler 8**
160	Obtain AV blood samples (V14, A14: EDTA, P800)
165	Infiltrate local anesthetic
170	**US Doppler 9**
180	Check BP, Pulse, Respiratory rate and Oxygen saturation Obtain AV blood samples (V15: EDTA, P800; A15: EDTA) **Biopsy 4**
+180	Subject monitoring

References

1. United Nations, Department of Economic and Social Affairs PD. World Population Ageing 2015. World Population Ageing 2015. 2015.
2. He W, Goodkind D, Kowal P. An Aging World : 2015 International Population Reports. Aging. 2016.
3. National Institute on Ageing. Global Health and Aging [Internet]. 2011. Available from: file:///C:/Users/reception/Downloads/global_health_and_aging (1).pdf
4. Chatterji S, Byles J, Cutler D, Seeman T, Verdes E, Crimmins E, et al. Health, functioning, and disability in older adults--present status and future implications. Lancet (London, England) [Internet]. 2015;385(9967):563–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25468158%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4882096
5. Prince MJ, Wu F, Guo Y, Gutierrez Robledo LM, O'Donnell M, Sullivan R, et al. The burden of disease in older people and implications for health policy and practice. Lancet [Internet]. 2015;385(9967):549–62. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-84922932233&partnerID=40&md5=e1ff2dfcc40e860dc2d43e5cbf97765e
6. Santosa A, Wall S, Fottrell E, Högberg U, Byass P. The development and experience of epidemiological transition theory over four decades: a systematic review. Glob Health Action [Internet]. 2014;7(5):23574. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4038769&tool=pmcentrez&rendertype=abstract
7. Bamford SM, Serra V. Non-Communicable Diseases in an Ageing World [Internet]. 2011. Available from: www.ilcuk.org.uk/files/pdf_pdf_185.pdf
8. Suastika K, Dwipayana P, Ratna Saraswati IM, Kuswardhani T, Astika N, Putrawan IB, et al. Relationship between age and metabolic disorders in the population of Bali. J Clin Gerontol Geriatr. 2011;2(2):47–52.
9. Barzilai N, Huffman D, Muzumdar R, Bartke A. The critical role of metabolic pathways in aging. Diabetes. 2012;61(6):1315–22.
10. Danaei G, Singh GM, Paciorek CJ, Lin JK, Cowan MJ, Finucane MM, et al. The global cardiovascular risk transition: Associations of four metabolic risk factors with national income, urbanization, and western diet in 1980 and 2008. Circulation. 2013;127(14):1493–502.
11. Collaborators GB of DS 2013. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2015;386(9995):743–800.
12. Storheim K, Zwart J-A. Musculoskeletal disorders and the Global Burden of Disease study. Ann Rheum Dis. 2014;73(6):949–50.
13. Fejer R, Ruhe A. What is the prevalence of musculoskeletal problems in the elderly population in developed countries? A systematic critical literature review. Chiropr {&} Man Ther. 2012;20(1):31.
14. Pattison JS, Folk LC, Madsen RW, Childs TE, Booth FW. Transcriptional profiling identifies extensive downregulation of extracellular matrix gene expression in sarcopenic rat soleus muscle. Physiol Genomics. 2003;15(1):34–

43.

15. Hickson M. Malnutrition and ageing. Postgrad Med J. 2006;82(963):2–8.

16. Umegaki H. Sarcopenia and diabetes: Hyperglycemia is a risk factor for age-associated muscle mass and functional reduction. Vol. 6, Journal of Diabetes Investigation. 2015. p. 623–4.

17. Danaei G, Finucane M, Lu Y, Singh G. trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country. Lancet. 2011;378:31–40.

18. Abdulla H, Phillips B, Smith K, Wilkinson D, Atherton PJ, Idris I. Physiological mechanisms of action of incretin and insulin in regulating skeletal muscle metabolism. Curr Diabetes Rev [Internet]. 2014;10(5):327–35. Available from: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=medl&NEWS=N&AN=25323297

19. Frontera WR, Ochala J. Skeletal muscle: a brief review of structure and function. Vol. 96, Calcified tissue international. 2015. p. 183–95.

20. Marieb EN, Hoehn K. Human Anatomy & Physiology. Vol. 7, Physiology. 2014. 906 p.

21. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. Physiol Rev [Internet]. 2011;91(4):1447–531. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22013216

22. Gillies AR, Lieber RL. Structure and function of the skeletal muscle extracellular matrix. Muscle Nerve [Internet]. 2011;44(3):318–31. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21949456%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3177172

23. Mosby. Mosby's Medical Dictionary. 9th editio. Elsevier; 2009.

24. Korthuis RJ. Skeletal Muscle Circulation. Vol. 3, Colloquium Series on Integrated Systems Physiology: From Molecule to Function. 2011. 1-144 p.

25. Westerblad H, Bruton JD, Katz A. Skeletal muscle: Energy metabolism, fiber types, fatigue and adaptability. Vol. 316, Experimental Cell Research. 2010. p. 3093–9.

26. Anderson JE. The satellite cell as a companion in skeletal muscle plasticity: currency, conveyance, clue, connector and colander. J Exp Biol. 2006;209:2276–92.

27. Szent-Györgyi AG. The early history of the biochemistry of muscle contraction. J Gen Physiol. 2004;123(6):631–41.

28. Sherwood L. Human Physiology: From Cells to Systems. Hum Physiol [Internet]. 2010;7th editio:766. Available from: http://books.google.com/books?id=gOmpysGBC90C&pgis=1

29. Egan B, Zierath JR. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. Vol. 17, Cell Metabolism. 2013. p. 162–84.

30. Ehrenborg EW a, Krook A. Regulation of Skeletal Muscle Physiology and Metabolism by Peroxisome Proliferator-Activated Receptor. Pharmacol Rev [Internet]. 2009;61(3):373–93. Available from: http://pharmrev.aspetjournals.org/content/61/3/373.abstract

31. Zierath JR, Hawley JA. Skeletal muscle fiber type: Influence on contractile and metabolic properties. Vol. 2, PLoS Biology. 2004.

32. Sinacore DR, Gulve EA. The role of skeletal muscle in glucose transport, glucose homeostasis, and insulin resistance: implications for physical therapy. PHYS THER [Internet]. 1993;73(12):878–91. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8248296

33. Wolfe R. CD. Isotope tracers in metabolic research. Principles and Practice of Kinetic Analysis. second edi. John Wiley & Sons., Inc.; 2005.

34. Aronoff SL, Berkowitz K, Shreiner B, Want L. Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. Diabetes Spectr. 2004;17(3):183–90.

35. Fallis A. WILLIAMS Textbook of ENDOCRINOLOGY. Elsevier. 2016. 1-1872 p.

36. Wilcox G. Insulin and insulin resistance. Clin Biochem Rev [Internet]. 2005;26(2):19–39. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16278749%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1204764

37. DeFronzo R a, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol. 1979;237(3):E214–23.

38. Alvim RO, Cheuhen MR, Machado SR, Sousa AGP, Santos PCJL. General aspects of muscle glucose uptake. An Acad Bras Cienc. 2015;87(1).

39. Merry TL, McConell GK. Skeletal muscle glucose uptake during exercise: A focus on reactive oxygen species and nitric oxide signaling. Vol. 61, IUBMB Life. 2009. p. 479–84.

40. Richter E a, Hargreaves M. Exercise, GLUT4, and skeletal muscle glucose uptake. Physiol Rev [Internet]. 2013;93(3):993–1017. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23899560

41. Kusters YHAM, Barrett EJ. Muscle's microvasculature's structural and functional specializations facilitate muscle metabolism. Am J Physiol Endocrinol Metab. 2015;1:epub ahead of print.

42. Glancy B, Hsu L, Dao L, Bakalar M, French S, David J, et al. within the Sarcolemma of Skeletal Muscle Fibers. 2015;21(2):131–47.

43. Kubínová L, Janáček J, Ribarič S, Čebašek V, Eržen I. Three-dimensional study of the capillary supply of skeletal muscle fibres using confocal microscopy. J Muscle Res Cell Motil. 2001;22(3):217–27.

44. M Intaglietta. Vasomotion and flowmotion: physiological mechanisms and clinical evidence. Vasc Med Rev. 1990;1:101–12.

45. Muris DMJ, Houben AJHM, Kroon A a, Henry RM a, van der Kallen CJH, Sep SJS, et al. Age, waist circumference, and blood pressure are associated with skin microvascular flow motion: the Maastricht Study. J Hypertens [Internet]. 2014;32(12):2439–49; discussion 2449. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25222377

46. Mikus CR, Laughlin MH. World â€™s largest Science , Technology & Medicine Open Access book publisher Insulin Resistance and Endothelial Dysfunction : Macro and Microangiopathy. :2–28.

47. Mccollum E V, Lecture A. Aging muscle 1–5. 2005;953–63.

48. Morley JE. Frailty and sarcopenia: The new geriatric giants. Rev Investig Clin. 2016;68(2):59–67.

49. Stenholm S, Harris TB, Rantanen T, Visser M, Kritchevsky SB, Ferrucci L. Sarcopenic obesity: definition, cause and consequences. Curr Opin Clin Nutr Metab Care [Internet]. 2008;11(6):693–700. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2633408&tool=pmcentrez&rendertype=abstract

50. Moore AZ, Caturegli G, Metter EJ, Makrogiannis S, Resnick SM, Harris TB, et al. Difference in muscle quality over the adult life span and biological correlates in the baltimore longitudinal study of aging. J Am Geriatr Soc. 2014;62(2):230–6.

51. Kalyani RR, Corriere M, Ferrucci L. Age-related and disease-related muscle loss: The effect of diabetes, obesity, and other diseases. Lancet Diabetes Endocrinol. 2014;2(10):819–29.

52. Deschenes MR. Motor Unit and Neuromuscular Junction Remodeling with Aging. Vol. 4, Current Aging Sciencee. 2011. p. 209–20.

53. Hangelbroek RWJ, Fazelzadeh P, Tieland M, Boekschoten M V., Hooiveld GJEJ, van Duynhoven JPM, et al. Expression of protocadherin gamma in skeletal muscle tissue is associated with age and muscle weakness. Journal of Cachexia, Sarcopenia and Muscle. 2016;

54. Marcell TJ. Sarcopenia : Causes , Consequences , and Preventions. J Gerontol. 2003;58(10):911–6.

55. Lind L LH. Decreased peripheral blood flow in the pathogenesis of the metabolic syndrome comprising hypertension, hyperlipidemia, and hyperinsulinemia. Am Hear J. 1993;May(125(5 Pt 2)):1494–7.

56. Evans JL, Goldfine ID. Aging and insulin resistance: Just say iNOS. Diabetes. 2013;62(2):346–8.

57. Pepersack T, de Breucker S. Could geriatric comprehensive assessment predict the outcome of pneumonia in the very old? [1]. Age Ageing. 2007;36(4):459–62.

58. Jackson RA, Hawa MI, Roshania RD, Sim BM, DiSilvio L, Jaspan JB. Influence of aging on hepatic and peripheral glucose metabolism in humans. Diabetes. 1988;37(1):119–29.

59. Lanza IR, Short DK, Short KR, Raghavakaimal S, Basu R, Joyner MJ, et al. Endurance exercise as a countermeasure for aging. Diabetes. 2008;57(11):2933–42.

60. Proctor DN, Shen PH, Dietz NM, Eickhoff TJ, Lawler LA, Ebersold EJ, et al. Reduced leg blood flow during dynamic exercise in older endurance-trained men. J Appl Physiol [Internet]. 1998;85(1):68–75. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9655757

61. Donato AJ, Uberoi A, Wray DW, Nishiyama S, Lawrenson L, Richardson RS. Differential effects of aging on limb blood flow in humans. Am J Physiol Hear Circ Physiol. 2006;290(1):272–8.

62. Skilton MR, Lai NT, Griffiths KA, Molyneaux LM, Yue DK, Sullivan DR, et al. Meal-related increases in vascular reactivity are impaired in older and diabetic adults: insights into roles of aging and insulin in vascular flow. Am J Physiol Heart Circ Physiol [Internet]. 2005;288(3):H1404-10. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/15513964

63. Coggan AR, Spina RJ, King DS, Rogers MA, Brown M, Nemeth PM, et al. Histochemical and enzymatic comparison of the gastrocnemius muscle of young and elderly men and women. J Gerontol. 1992;47(3):71–6.

64. Barrett-O'Keefe Z, Witman MA, McDaniel J, Fjeldstad AS, Trinity JD, Ives SJ, et al. Angiotensin II potentiates alpha-adrenergic vasoconstriction in the elderly. Clin Sci [Internet]. 2013;124(6):413–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22985469%5Cnhttp://www.clinsci.org/content/ppclinsci/124/6/413.full.pdf

65. Durham WJ, Casperson SL, Dillon EL, Keske MA, Paddon-Jones D, Sanford AP, et al. Age-related anabolic resistance after endurance-type exercise in healthy humans. FASEB J. 2010;24(10):4117–27.

66. Timmerman KL, Lee JL, Dreyer HC, Dhanani S, Glynn EL, Fry CS, et al. Insulin stimulates human skeletal muscle protein synthesis via an indirect mechanism involving endothelial-dependent vasodilation and mammalian target of rapamycin complex 1 signaling. J Clin Endocrinol Metab [Internet]. 2010;95(8):3848–57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20484484%5Cnhttp://jcem.endojournals.org/cgi/reprint/95/8/3848.pdf

67. Clark MG, Rattigan S, Barrett EJ. Nutritive blood flow as an essential element supporting muscle anabolism. Curr Opin Clin Nutr Metab Care. 2006;9(3):185–9.

68. Clark MG, Wallis MG, Barrett EJ, Vincent MA, Richards SM, Clerk LH, et al. Blood flow and muscle metabolism: a focus on insulin action. Am J Physiol Endocrinol Metab. 2003;284(2):E241-58.

69. Campbell JE, Drucker DJ. Pharmacology, physiology, and mechanisms of incretin hormone action. Vol. 17, Cell Metabolism. 2013. p. 819–37.

70. Kim W, Egan JM. NIH Public Access. Pharmacol Rev. 2008;60(4):470–512.

71. Rajeev SP, Wilding J. GLP-1 as a target for therapeutic intervention. Curr Opin Pharmacol [Internet]. 2016;31:44–9. Available from: http://linkinghub.elsevier.com/retrieve/pii/S147148921630090X

72. Burcelin R, Gourdy P. Harnessing glucagon-like peptide-1 receptor agonists for the pharmacological treatment of overweight and obesity. Obes Rev [Internet]. 2016; Available from: http://doi.wiley.com/10.1111/obr.12465

73. Green CJ, Henriksen TI, Pedersen BK, Solomon TPJ. Glucagon Like Peptide-1-Induced Glucose Metabolism in Differentiated Human Muscle Satellite Cells Is Attenuated by Hyperglycemia. PLoS One. 2012;7(8).

74. Denisa M, Žďárská J, Ph D. Liraglutid. 2009;116–20.

75. McCormick LM, Kydd AC, Dutka DP. Cardiac protection via metabolic modulation: an emerging role for incretin-based therapies?. Cardiovasc Hematol Agents Med Chem [Internet]. 2012;10(4):319–24. Available from: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=medl&NEWS=N&AN=22827290

76. Zhao TC. Glucagon-like peptide-1 (GLP-1) and protective effects in cardiovascular disease: a new therapeutic approach for myocardial protection. Cardiovasc Diabetol [Internet]. 2013;12(1):90. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3700838&tool=pmcentrez&rendertype=abstract

77. Puddu A, Sanguineti R, Durante A, Nencioni A, Mach F, Montecucco F, et al. Glucagon-like peptide-1 triggers protective pathways in pancreatic beta-cells exposed to glycated serum. Mediators Inflamm. 2013;2013.

78. Chai W, Dong Z, Wang N, Wang W, Tao L, Cao W, et al. Glucagon-like peptide 1 recruits microvasculature and increases glucose use in muscle via a nitric oxide-dependent mechanism. Diabetes. 2012;61(4):888–96.

79. Baggio LL, Drucker DJ. Biology of Incretins: GLP-1 and GIP. Gastroenterology. 2007;132(6):2131–57.

80. Luque MA, González N, Márquez L, Acitores A, Redondo A, Morales M, et al. Glucagon-like peptide-1 (GLP-1) and glucose metabolism in human myocytes. J Endocrinol. 2002;173(3):465–73.

81. Acitores a, González N, Sancho V, Valverde I, Villanueva-Peñacarrillo ML. Cell signalling of glucagon-like peptide-1 action in rat skeletal muscle. J Endocrinol. 2004;180(3):389–98.

82. Sancho V, Trigo M V., González N, Valverde I, Malaisse WJ, Villanueva-Peñacarrillo ML. Effects of glucagon-like peptide-1 and exendins on kinase activity, glucose transport and lipid metabolism in adipocytes from normal and type-2 diabetic rats. J Mol Endocrinol. 2005;35(1):27–38.

83. Morales M, López-Delgado MI, Alcántara A, Luque MA, Clemente F, Márquez L, et al. Preserved GLP-I Effects on Glycogen Synthase a Activity and Glucose Metabolism in Isolated Hepatocytes and Skeletal Muscle From Diabetic Rats. Diabetes. 1997;Aug(46(8)):1264–9.

84. Ayala JE, Bracy DP, James FD, Julien BM, Wasserman DH, Drucker DJ. The glucagon-like peptide-1 receptor regulates endogenous glucose production and muscle glucose uptake independent of its incretin action. Endocrinology. 2009;150(3):1155–64.

85. Lee YS, Shin S, Shigihara T, Hahm E, Liu MJ, Han J, et al. Glucagon-like peptide-1 gene therapy in obese diabetic mice results in long-term cure of diabetes by improving insulin sensitivity and reducing hepatic gluconeogenesis. Diabetes. 2007;56(6):1671–9.

86. Gao H, Wang X, Zhang Z, Yang Y, Yang J, Li X, et al. GLP-1 amplifies insulin signaling by up-regulation of IR??, IRS-1 and Glut4 in 3T3-L1 adipocytes. Endocrine. 2007;32(1):90–5.

87. Sancho V, Nuche B, Arnés L, Cancelas J, González N, Díaz-Miguel M, et al. The action of GLP-1 and exendins upon glucose transport in normal human adipocytes, and on kinase activity as compared to morbidly obese patients. Int J Mol Med. 2007;19(6):961–6.

88. Subaran SC, Sauder MA, Chai W, Jahn LA, Fowler DE, Aylor KW, et al. GLP-1 at physiological concentrations recruits skeletal and cardiac muscle microvasculature in healthy humans. Clin Sci (Lond) [Internet]. 2014;127(3):163–70. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4353574&tool=pmcentrez&rendertype=abstract

89. Sjoberg K a, Holst JJ, Rattigan S, Richter E a, Kiens B. GLP-1 increases microvascular recruitment but not glucose uptake in human and rat skeletal muscle. Am J Physiol Endocrinol Metab [Internet]. 2014;306(4):E355-62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24302010%5Cnhttp://ajpendo.physiology.org/content/ajpendo/306/4/E355.full.pdf

90. Tesauro M, Schinzari F, Adamo A, Rovella V, Martini F, Mores N, et al. Effects of GLP-1 on forearm vasodilator function and glucose disposal during hyperinsulinemia in themetabolic syndrome. Diabetes Care. 2013;36(3):683–9.

91. Richter G, Feddersen O, Wagner U, Barth P, Göke R, Göke B. GLP-1 stimulates secretion of macromolecules from airways and relaxes pulmonary artery. Am J Physiol [Internet]. 1993;265(4 Pt 1):L374-81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8238371

92. Liu Z, Liu J, Jahn LA, Fowler DE, Barrett EJ. Infusing lipid raises plasma free fatty acids and induces insulin resistance in muscle microvasculature. J Clin Endocrinol Metab. 2009;94(9):3543–9.

93. World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA [Internet]. 2013;310(20):2191–4. Available from: http://search.ebscohost.com/login.aspx?direct=true&db=edb&AN=61202708&site=eds-live&scope=site%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/24309077%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/24458102%5Cnhttp://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2013.2

94. Banfi G, Salvagno GL, Lippi G. The role of ethylenediamine tetraacetic acid (EDTA) as in vitro anticoagulant for diagnostic purposes. Vol. 45, Clinical Chemistry and Laboratory Medicine. 2007. p. 565–76.

95. Yi J, Warunek D, Craft D. Degradation and stabilization of peptide hormones in human blood specimens. PLoS One. 2015;10(7).

96. Dietrichson P, Coakley J, Smith PE, Griffiths RD, Helliwell TR, Edwards RH. Conchotome and needle percutaneous biopsy of skeletal muscle. J Neurol Neurosurg Psychiatry. 1987;50:1461–7.

97. Chua KS, Tan IK. Plasma glucose measurement with the yellow springs glucose analyzer. Clin Chem. 1978;24(1):150–2.

98. Pasikanti KK, Ho PC, Chan ECY. Gas chromatography/mass spectrometry in metabolic profiling of biological fluids. Vol. 871, Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences. 2008. p. 202–11.

99. Dettmer K, Aronov PA, Hammock BD. Mass spectrometry-based metabolomics. Vol. 26, Mass Spectrometry Reviews. 2007. p. 51–78.

100. Hoffmann E De, Stroobant V. Mass Spectrometry Principles and Applications [Internet]. Mass spectrometry principles and applications. 2007. 43-55 p. Available from: http://books.google.com/books?hl=en&lr=&id=pqhr5XQZDtUC&oi=fnd&pg=PR11&dq=mass+spectrometry+principles+and+applications&ots=6l9XvbS0r-&sig=FB4zmdUIyUrmKqG_Y7Hyl-jZ6Kk

101. Shippee RL, Johnson AA, Cioffi WG, Lasko J, LeVoyer TE, Jordan BS. Simultaneous determination of lactulose and mannitol in urine of burn patients by gas-liquid chromatography. Clin Chem. 1992;38(3):343–5.

102. Project LS, Nakagawa K, Miyagawa H, Kawana S. Quantitative Analysis of Stable Isotopes of Glucose in Blood Plasma Using Quadrupole GC-MS. 2015;(2):8.

103. Elisa UI, International DRG. AM. 2012;1–8.

104. Millipore M. The Power of Biomarker Analysis Why just multiplex.

105. Tipton KD, Rasmussen BB, Miller SL, Wolf SE, Owens-Stovall SK, Petrini BE, et al. Timing of amino acid-carbohydrate ingestion alters anabolic response of muscle to resistance exercise. Am J Physiol Endocrinol Metab. 2001;281(2):E197–206.

106. Tipton KD, Ferrando AA, Phillips SM, Doyle D, Wolfe RR. Postexercise net protein synthesis in human muscle from orally administered amino acids. Am J Physiol [Internet]. 1999;276(4 Pt 1):E628-34. Available from: http://ajpendo.physiology.org/content/276/4/E628.abstract

107. Phillips BE, Atherton PJ, Varadhan K, Limb MC, Williams JP, Smith K. Acute cocoa flavanol supplementation improves muscle macro- and microvascular but not anabolic responses to amino acids in older men. Appl Physiol Nutr Metab [Internet]. 2016;41(5):548–56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27120341%5Cnhttp://www.nrcresearchpress.com/doi/abs/10.1139/apnm-2015-0543?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub%3Dpubmed&

108. Zierler Kenneth. Whole body glucose. Am J Physiol. 1999;276(Endocrinol. Metab.39):E409–26.

109. Abdulla H, Phillips B, Smith K, Wilkinson D, Atherton PJ, Idris I. Physiological mechanisms of action of incretin and insulin in regulating skeletal muscle metabolism. Curr Diabetes Rev. 2014;10(5):327–35.

110. Meneilly GS, Mcintosh CHS, Pederson RA, Habener JF, Gingerich R, Egan JM, et al. Effect of glucagon-like peptide 1 on non-insulin-mediated glucose uptake in the elderly patient with diabetes. Diabetes Care [Internet]. 2001;24(11):1951–6. Available from: http://unicorn.novo.dk/netacgi/getref.pl?ref=C-171937800018

111. Meneilly GS, Elliot T, Bryer-Ash M, Floras JS. Insulin-mediated increase in blood flow is impaired in the elderly. J Clin Endocrinol Metab [Internet]. 1995;80(6):1899–903. Available from: http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L25170567%5Cnhttp://dx.doi.org/10.1210/jc.80.6.1899%5Cnhttp://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=0021972X&id=doi:10.1210%2Fjc.80.6.1899&atitle=Insulin-mediated+increase+in+blo

112. Kim JA, Montagnani M, Kwang KK, Quon MJ. Reciprocal relationships between insulin resistance and endothelial dysfunction: Molecular and pathophysiological mechanisms. Vol. 113, Circulation. 2006. p. 1888–904.

113. Sjoberg KA, Rattigan S, Jeppesen JF, Lundsgaard AM, Holst JJ, Kiens B. Differential effects of glucagon-like peptide-1 on microvascular recruitment and glucose metabolism in short- and long-term insulin resistance. J Physiol [Internet]. 2015;9(September):2185–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25688993%5Cnhttp://onlinelibrary.wiley.com/store/10.1113/JP270129/asset/tjp6594.pdf?v=1&t=i8f9q4dl&s=737fca7bc141bb14b70a6975c9a192cdd6dc992c

114. Jani B. Ageing and vascular ageing. Postgrad Med J [Internet]. 2006;82(968):357–62. Available from: http://pmj.bmj.com/cgi/doi/10.1136/pgmj.2005.036053

115. Asmar A, Asmar M, Simonsen L, Madsbad S, Holst JJ, Hartmann B, et al. Glucagon-like peptide-1 elicits vasodilation in adipose tissue and skeletal muscle in healthy men. Physiol Rep [Internet]. 2017;5(3):e13073. Available

from: http://physreports.physiology.org/lookup/doi/10.14814/phy2.13073

116. Phillips B, Williams J, Atherton P, Smith K, Hildebrandt W, Rankin D, et al. Resistance exercise training improves age-related declines in leg vascular conductance and rejuvenates acute leg blood flow responses to feeding and exercise. J Appl Physiol [Internet]. 2012;112(3):347–53. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21998269

117. Raitakari OT, Lai N, Griffiths K, McCredie R, Sullivan D, Celermajer DS. Enhanced peripheral vasodilation in humans after a fatty meal. J Am Coll Cardiol. 2000;36(2):417–22.

118. MacIntosh CG, Andrews JM, Jones KL, Wishart JM, Morris HA, Jansen JBMJ, et al. Effects of age on concentrations of plasma cholecystokinin, glucagon- like peptide 1, and peptide YY and their relation appetite and pyloric motility. Am J Clin Nutr. 1999;69(5):999–1006.

119. Nyström T, Gonon AT, Sjöholm Å, Pernow J. Glucagon-like peptide-1 relaxes rat conduit arteries via an endothelium-independent mechanism. Regul Pept. 2005;125(1–3):173–7.

120. Vincent M a, Barrett EJ, Lindner JR, Clark MG, Rattigan S. Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. Am J Physiol Endocrinol Metab. 2003;285(1):E123–9.

121. Ding L, Zhang J. Glucagon-like peptide-1 activates endothelial nitric oxide synthase in human umbilical vein endothelial cells. Acta Pharmacol Sin [Internet]. 2012;33(1):75–81. Available from: http://www.nature.com/doifinder/10.1038/aps.2011.149

122. Pyke C, Knudsen LB. The Glucagon-Like Peptide-1 Receptor—or Not? Endocrinology [Internet]. 2013;154(1):4–8. Available from: http://press.endocrine.org/doi/10.1210/en.2012-2124

123. Villanueva-Peñacarrillo ML, Alcántara AI, Clemente F, Delgado E, Valverde I. Potent glycogenic effect of GLP-1(7-36)amide in rat skeletal muscle. Diabetologia. 1994;37(11):1163–6.

124. Gulli G, Ferrannini E, Stern M, Haffner S, Defronzo RA. The metabolic profile of NIDDM is fully established in glucose-tolerant offspring of two Mexican-American NIDDM parents. Diabetes. 1992;41(12):1575–86.

125. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Vol. 32 Suppl 2, Diabetes care. 2009.

126. Fürnsinn C, Ebner K, Waldhäusl W. Failure of GLP-1(7-36)amide to affect glycogenesis in rat skeletal muscle. Diabetologia. 1995;38(7):864–7.

127. Krentz AJ, Boyle PJ, Macdonald LM, Schade DS. Octreotide: A long-acting inhibitor of endogenous hormone secretion for human metabolic investigations. Metabolism. 1994;43(1):24–31.

128. Ahrén B, Thomaseth K, Pacini G. Reduced insulin clearance contributes to the increased insulin levels after administration of glucagon-like peptide 1 in mice. Diabetologia. 2005;48(10):2140–6.

129. Van Cauter E, Mestrez F, Sturis J PK. Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. Diabetes. 1992;41(3)(March):368–77.

130. Tonyushkina K, Nichols JH. Glucose meters: a review of technical challenges to obtaining accurate results. J Diabetes Sci Technol [Internet]. 2009;3(4):971–80. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/20144348%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2769957

131. Gallen IW, Macdonald I a. The effects of underfeeding for 7 d on the thermogenic and physiological response to glucose and insulin infusion (hyperinsulinaemic euglycaemic clamp). Br J Nutr. 1990;64(2):427–37.

132. Webber J, Macdonald IA. Effects of fasting on fatty acid kinetics and on the cardiovascular, thermogenic, and metabolic responses to the glucose clamp. Clin Sci. 1994;706:697–706.