The chronic effects of dietary (poly)phenols on mitochondrial dysfunction and glucose uptake in cellular models of the liver and skeletal muscle.

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Publications

Chapters 2, 3 and 4 contain work which has also been used in the following publications:

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Conference abstracts

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“If you’re going through hell, keep going.” – Winston Churchill.

“Try not. Do or do not. There is no try.” – Yoda.

Abstract

Background: Type 2 diabetes is characterised by chronic hyperglycaemia, insulin resistance and associated mitochondrial dysfunction. (Poly)phenols have been shown to attenuate cellular oxidative stress and restore glucose homeostasis, but the specific mechanisms and compounds responsible remain unknown.

Methods: HepG2 cells were used as an *in vitro* hepatic model, on which the effects of quercetin on high glucose-induced oxidative stress and mitochondrial dysfunction were investigated. Mitochondria were assessed for complex I activity, cellular redox status, mitochondrial respiration and PGC-1α expression. LHCN-M2 human skeletal myocytes were differentiated in various glucose and insulin concentrations and characterised for their use as a model to explore the effects of relevant (poly)phenol metabolites on glucose uptake and metabolism. Metabolic phenotype and the effects of metabolites derived from ferulic acid, flavonols, resveratrol and berry (poly)phenols were evaluated by Western capillary protein assays, uptake of 2-[1-^{14}C(U)]-deoxy-D-glucose and D-[^{14}C(U)]-glucose; respirometry and the ROS assay were also used for initial metabolic characterisation.

Results: Mitochondrial function was restored by quercetin in HepG2 cells exposed to high glucose, by reversing the increased cellular NADH, enhancing mitochondrial respiration and preventing proton leak, and upregulating PGC-1α, all of which led to restored complex I activity after 24 h. The LHCN-M2 model was established and cells differentiated in a normal or high glucose/insulin environment. Glucose transport was restored, and metabolism increased, in high glucose/insulin myotubes by various metabolites. Isovanillic acid 3-*O*-sulfate in particular elicited this effect by upregulating GLUT1, GLUT4 and PI3K protein expression, and acutely activating the insulin signalling pathway.

Conclusions: Quercetin protects against hepatic mitochondrial dysfunction through pleiotropic effects involving improved redox status and enhanced mitochondrial respiration and function. (Poly)phenol metabolites, including the gut microbiome catabolite isovanillic acid 3-*O*-sulfate, restore glucose uptake and metabolism in human skeletal muscle exposed to high glucose and insulin, via insulin-dependent pathways.

Table of contents

List of figures

List of tables

List of Abbreviations

(N/n)	(Number of biological replicates/number of technical replicates)
(p-)Akt	(Phosphorylated) Akt (protein kinase B)
^{14}C	Radioactive isotope of carbon
2-[1-^{14}C(U)]-DG	2-[1-^{14}C(U)]-deoxy-D-glucose
2-DG	2-deoxy-D-glucose
Acetyl-CoA	Acetyl coenzyme A
AGEs	Advanced glycation end products
AmA	Antimycin A
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ARE	Antioxidant response element
ATP	Adenosine triphosphate
AUC	Area under the curve
BCA	Bicinchoninic acid
C_{max}	Maximal plasma concentration
COMT	Catechol-*O*-methyl transferase
CoQ_1	Coenzyme Q 1, analog of coenzyme Q_{10}
CoQ_{10}	Conezyme Q_{10} (ubiquinone/ubiquinol (oxidised/reduced))
COX	Cyclooxygenase
CS	Catechol-*O*-sulfate
CVD(s)	Cardiovascular disease(s)
D-[^{14}C(U)]-Glc	D-[^{14}C(U)]-glucose
DAG	Diacylglycerol
DCF	2′,7′-dichlorofluorescin
DCFH-DA	2′,7′-dichlorofluorescin diacetate
ddPCR	Droplet digital PCR
DHFA	Dihydroferulic acid
DHFA-4S	Dihydroferulic acid 4-*O*-sulfate
DMEM	Dulbecco’s minimum essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

EC_{50}	Effective concentration at which activity is 50%
ENCODE	The Encyclopedia of DNA Elements
eNOS	Endothelial nitric oxide synthase
ETS	Electron transfer system
FA	Ferulic acid
FA4S	Ferulic acid 4-*O*-sulfate
FBS	Foetal bovine serum
FFA(s)	Free fatty acid(s)
FOXO	Forkhead box O
FPKM	Fragments per kilobase of transcript per million mapped reads
G3P	Glyceraldehyde 3-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Glc	Glucose
GLUT	Glucose transporter
GSH	Glutathione
GYS1	Glycogen synthase
H_2O_2	Hydrogen peroxide
HbA1c	(Glycated) haemoglobin A1c
HepG2	Human hepatocellular carcinoma, immortalised cell line
HG	High glucose (25 mM)
HO-1	Heme oxygenase-1
HUVECs	Human umbilical vein endothelial cells
IC_{50}	Inhibitor concentration at which activity is 50%
IKK	Inhibitor of nuclear factor κB kinase
IL	Interleukin
IMM	Inner mitochondrial membrane
IMS	Intermembrane space, mitochondrial
Ins	Insulin
INSR	Insulin receptor
IRS	Insulin receptor substrate(s)
IVA	Isovanillic acid
IVAS	Isovanillic acid 3-*O*-sulfate
JNK	c-Jun N-terminal kinase

K3G	Kaempferol 3-*O*-β-D-glucuronide
kDa	Kilodalton
kg bw	Kilograms per body weight
LDH	Lactate dehydrogenase
LHCN-M2	Human skeletal myoblasts immortalised with lox-hygro-hTERT ("LH") and Cdk4-neo ("CN"), well-differentiating subclone M2
MAPK	Mitogen-activated protein kinase
MCS	4-Methyl-catechol-*O*-sulfate
MEM	Eagle's minimum essential medium
MGS	4-Methyl-gallic 3-*O*-sulfate
miRNAs	Micro RNAs
MPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
MRP	Multidrug-resistant protein
mtDNA	Mitochondrial DNA
MW	Molecular weight
NAD^+	Nicotinamide adenine dinucleotide, oxidised
NADH	Nicotinamide adenine dinucleotide, reduced
NADPH	Nicotinamide adenine dinucleotide phosphate
NfκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG	Normal glucose (5.5 mM)
Normal/high GI	Normal/high glucose and insulin-differentiated myotubes
NOS	Nitric oxide synthase
NOX	NADPH oxidase
Nrf2	Nuclear respiratory factor (erythroid-derived 2)-like 2
OAT	Organic anion transporter
OXPHOS	Oxidative phosphorylation
PCA	Protocatechuic acid
PDK4	Pyruvate dehydrogenase lipoamide kinase 4
PGC-1α	Peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1-α
PGS	Pyrogallol *O*-sulfate
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase

PKC	Protein kinase C
PP-rich	(Poly)phenol-rich
PPAR	Peroxisome proliferator-activated receptor
Q	Quercetin
Q3G	Quercetin 3-*O*-β-D-glucuronide
Q3S	Quercetin 3′-*O*-sulfate
R3G	*trans*-Resveratrol 3-*O*-glucuronide
R3S	*trans*-Resveratrol 3-*O*-sulfate
R4G	*trans*-Resveratrol 4′-*O*-glucuronide
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
Rot	Rotenone
ROS	Reactive oxygen species
ROX	Residual oxygen consumption
SD	Standard deviation
SEM	Standard error of the mean
SGLT1	Sodium-dependent glucose transporter 1
SIRT	Sirtuin
SLC	Solute carrier family member
SULT	Sulfotransferase
$T_{½}$	Half-life
TBP	TATA-box binding protein
TCA	Tricarboxylic acid
TFAM	Mitochondrial transcription factor A
T_{max}	Time taken to reach C_{max}
TNFα	Tumour necrosis factor-α
TR-FRET	Time-resolved fluorescence energy transfer
TZD	Thiazolidinedione
UDP	Uridine diphosphate
UGT	UDP glucuronosyltransferase
VAS	Vanillic acid 4-*O*-sulfate
VCAM	Vascular cellular adhesion molecules
VEGF	Vascular endothelial growth factor

V_{max}	Maximum velocity, reaction rate when enzyme is saturated
WHO	World health organization
Z'	Z-factor, measure of statistical effect size

Chapter 1

Introduction and Literature Review.

1.1 Introduction

The incidence of diabetes is on the rise worldwide. It is estimated that 422 million adults are currently living with the disease, a number which has almost quadrupled in the last 35 years and is representative of 8.5% of the global population. This chronic metabolic disease is best known by elevated blood glucose levels and 3.7 million deaths were attributable to hyperglycaemia-related pathologies, 1.5 million of these directly caused by diabetes, in 2012 (WHO 2016). Type 2 accounts for around 90% of all diabetes cases and, worryingly, a further ~193 million people are undiagnosed, with increasing occurrences in children, too (Chatterjee, Khunti et al. 2017). The disease is no longer considered exclusive to the 'Western' world, with a substantial increase in the Asian population, for example (Lam and LeRoith 2012). Considerable physical, psychological and socioeconomic pressures are placed on patients and an overwhelming burden hangs over health care systems and economies, with an estimated cost of more than $825 billion US dollars per year, accounting for treatment as well as absenteeism, early retirement and social benefits for associated disabilities (Seuring, Archangelidi et al. 2015).

Management of blood glucose and lipid concentrations and blood pressure are required to minimise disease progression and complications such as cardiovascular disease (CVD). To lower prevalence and the associated huge costs, a better understanding of the genetic and metabolic risk factors, as well as early diagnosis, better therapies and disease management tailored to each patient, are still needed (Chatterjee, Khunti et al. 2017). For example, only in recent years has it been acknowledged that there is a need to treat the underlying metabolic disorder of individuals with CVD (Mellbin, Anselmino et al. 2010). Prevention is better than cure and therefore early changes in lifestyle and increasing education are key. Being overweight/obese is the biggest risk factor (though this can vary between populations; South-East Asians are at higher risk for example) (WHO 2016) and

therefore improvements in diet and increased physical activity can be effective in prevention and treatment. Excessive overall calorie intake, and notably free sugars, fats, and a lack of dietary fibre are correlated with increased risk of type 2 diabetes (WHO 2016). Epidemiological evidence suggests that increased consumption of foods rich in (poly)phenols, substances synthesized and stored in plants for their protection, is inversely correlated with incidence (Ley, Hamdy et al. 2014, Amiot, Riva et al. 2016, Kim, Keogh et al. 2016) and as such there is a focus on looking for novel means of using (poly)phenols to control carbohydrate and lipid metabolism to manage, or even prevent, diabetes.

A diet rich in plant-based foods and (poly)phenols over the course of a lifetime may lower the incidence of metabolic disorders (Kim, Keogh et al. 2016) and prescription of such a diet or applications with specific (poly)phenols may be used to provide new nutraceutical therapies for patients already diagnosed with disorders such as diabetes and CVD (Bozzetto, Annuzzi et al. 2015, Amiot, Riva et al. 2016). This literature review will draw on the current understanding of metabolic disease aetiology and the effects that dietary (poly)phenols can have on contributing risk factors, and identify gaps and shortcomings in the research. Focus will be placed on two central aspects of metabolic disease pathophysiology with potential to be influenced by (poly)phenols, and these will form the basis of the *in vitro* investigations in this project. These aspects are glucose metabolism and oxidative stress in the liver, and glucose uptake in the skeletal muscle.

1.2 Type 2 diabetes: a metabolic disease

Diabetes mellitus is a metabolic disease characterised by chronic hyperglycaemia (Krentz and Hompesch 2016). Symptoms include increased urination, thirst and hunger and, if left undiagnosed, untreated or poorly managed, long-term complications include CVD, stroke, kidney failure, gout and retinopathy. Hyperglycaemia and the encompassing syndrome arises either from the pancreas failing to produce sufficient insulin to maintain glucose homeostasis, referred to as type 1, or from the cells of the peripheral tissues involved in glucose homeostasis becoming insulin resistant, which in turn can also lead to pancreatic failure; this is type 2 and in this case early symptoms tend to develop slower and can be subtle or event absent. Gestational diabetes is another type, in which women develop hyperglycaemia during pregnancy, and it is typically resolved

after birth but comes with its own complications and increases the risk of eventually developing type 2 (WHO 2016, Chatterjee, Khunti et al. 2017).

Prevention and treatment involve a controlled diet and regular physical exercise and, for those with the disease, maintaining strict control of blood glucose homeostasis. Type 1 management requires insulin injections combined with diet control, while type 2 is managed by improvements in diet and lifestyle with drugs, with or without insulin injections. The direct causes of type 1 are still unknown (WHO 2016), though it is characterised by loss of β-cell function in the pancreatic islets of Langerhans and thus the compromised insulin secretion. As well as genetic and environmental factors, it is thought that an auto-immune attack can initiate the β-cell dysfunction (Rother 2007). Type 2 is caused initially by insulin resistance, resulting from preventable risk factors including obesity, poor diet and a sedentary lifestyle, in addition to other factors such as age, ethnicity and genetic predisposition (WHO 2016, Chatterjee, Khunti et al. 2017). Epidemiology suggests the dominant dietary factors include the types of fats consumed and/or excessive carbohydrates (Riserus, Willett et al. 2009, Malik, Popkin et al. 2010, Hu, Pan et al. 2012).

Carbohydrate digestion is governed by α-glycosidases throughout the gastrointestinal tract, producing monosaccharides which are then absorbed predominantly via sodium-dependent glucose transporter SGLT1 and glucose transporter GLUT2 (or GLUT5 for fructose) from the lumen of the small intestine into the blood in the hepatic portal vein (Williamson 2013). Glucose is taken up into the liver (primarily via GLUT2) and other peripheral tissues, such as skeletal muscle (via GLUT4), where any excess is stored as glycogen to minimise the post-prandial fluctuation in glycaemia (Cura and Carruthers 2012, Mueckler and Thorens 2013). In the fasting state, the liver plays a crucial role in producing glucose from glycogenolysis and gluconeogenesis to prevent a drop in glycaemia, controlled by glucagon; while in the post-prandial state, in response to increased glycaemia and insulinaemia, the liver shifts from net glucose output to net glucose uptake (Moore, Coate et al. 2012, Titchenell, Chu et al. 2015). Insulin lowers glucose synthesis and efflux in the liver and stimulates the translocation of GLUT4 to the plasma membrane for glucose uptake in muscle and adipose tissue. Insulin also increases glycolysis, promotes glycogenesis and lipogenesis, besides controlling a

variety of other cellular functions outside of glucose and lipid metabolism (Saltiel and Kahn 2001, Desvergne, Michalik et al. 2006, Huang and Czech 2007).

In a very simplified view, it can be said that persistent or excessive hyperglycaemia causes insulin resistance and this in turn exacerbates hyperglycaemia. However, it is debated in the literature whether a common cause in the aetiology of diabetes exists. Hyperglycaemia and hyperlipidaemia, resulting from chronic, excessive calorie intake, have a number of cellular consequences that start with a dysregulation in glucose and lipid fluxes and metabolism. Overactive lipogenesis results in lipid accumulation in the liver and adipose tissue, as well as in circulation, and obesity ensues (Taylor 2013, Zaccardi, Webb et al. 2016). Excessive levels of the lipid intermediates, diacylglycerol (DAG) and ceramide, can disrupt insulin signalling (Samuel, Petersen et al. 2010, Bruce, Risis et al. 2012) and surplus lipid storage (and glycogen too, as recently demonstrated (Ceperuelo-Mallafré, Ejarque et al. 2016)) in adipose tissue triggers the release of adipokines, inducing a pro-inflammatory state resulting in increased fatty acid release from adipocytes and leading to insulin resistance in myocytes and hepatocytes (Rabe, Lehrke et al. 2008, Harwood 2012, Kwon and Pessin 2013). Excessive consumption of fructose, while contributing less to hyperglycaemia and the associated adverse effects, has been linked with hepatic lipid accumulation and hyperlipidaemia (Bantle 2009). Insulin resistance leads to lowered glucose uptake and uncontrolled hepatic glucose metabolism and efflux, resulting in chronic hyperglycaemia, as well as dyslipidaemia. This in turn increases systemic oxidative stress, inflammation, and cardiovascular problems such as endothelial lipid disposition (Lumeng and Saltiel 2011, Tangvarasittichai 2015). The role of oxidative stress, as a result of high glucose specifically, in the onset of diabetes, will be the focus of the next section and further studies.

1.2.1 Oxidative stress in diabetes pathophysiology

High levels of glucose in the blood leads to intracellular oxidative stress and insulin resistance in tissues involved in glucose metabolism (Murphy 2009, Yan 2014, Gero, Torregrossa et al. 2016). A principal source of oxidative stress in the cell is the mitochondrion (Brand 2010). The catabolism of glucose by glycolysis, of free fatty acids (FFAs) by β-oxidation and of amino acids, generates NADH (nicotinamide adenine dinucleotide) via acetyl coenzyme A (acetyl-CoA)) and the tricarboxylic acid (TCA)

cycle. NADH is oxidised by complex I of the ETS (electron transfer system) to NAD^+, transferring electrons to downstream complexes and oxygen, and protons to the IMS (intermembrane space), generating the proton gradient for ATP (adenosine triphosphate) synthesis in order to meet cellular energy requirements, thus fulfilling the primary function of mitochondria. An inherent level of superoxide, the precursor to other ROS (reactive oxygen species), is produced by complex I but basal levels of ROS are required for cell signalling and are tightly controlled by the cell's antioxidant defence systems (Brandt 2006, Murphy 2009, Liemburg-Apers, Willems et al. 2015, Hirst and Roessler 2016). Overproduction of NADH from excessive glucose and lipid influx, particularly in hepatocytes and pancreatic β-cells where glucokinase of the glycolysis pathway is not inhibited by glucose-6-phosphate, results in a reductive stress. This puts pressure on complex I with the overflux of NADH increasing the inner mitochondrial membrane (IMM) potential, which stalls complex III and increases coenzyme Q_{10} (CoQ) half-life and electron leakage and superoxide production beyond basal. The accumulation of NADH, and the imbalanced NAD^+/NADH and redox status, creates a condition known as pseudohypoxia whereby oxygen cannot be effectively consumed by NAD^+, leaving more oxygen available for partial reduction by the leaked electrons. Oxidative stress ensues (Munoz and Costa 2013, Yan 2014).

The situation is worsened by the inhibition of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) by both ROS and NADH. This prevents carbon flux from glycolysis to pyruvate and the TCA cycle and induces alternative metabolic pathways branching from glycolysis to mediate the accumulation of glyceraldehyde 3-phosphate (G3P) (Yan 2014, Luo, Wu et al. 2016), all of which exacerbate oxidative stress (Fig. 1.1) and have implications in the onset of diabetes and associated complications.

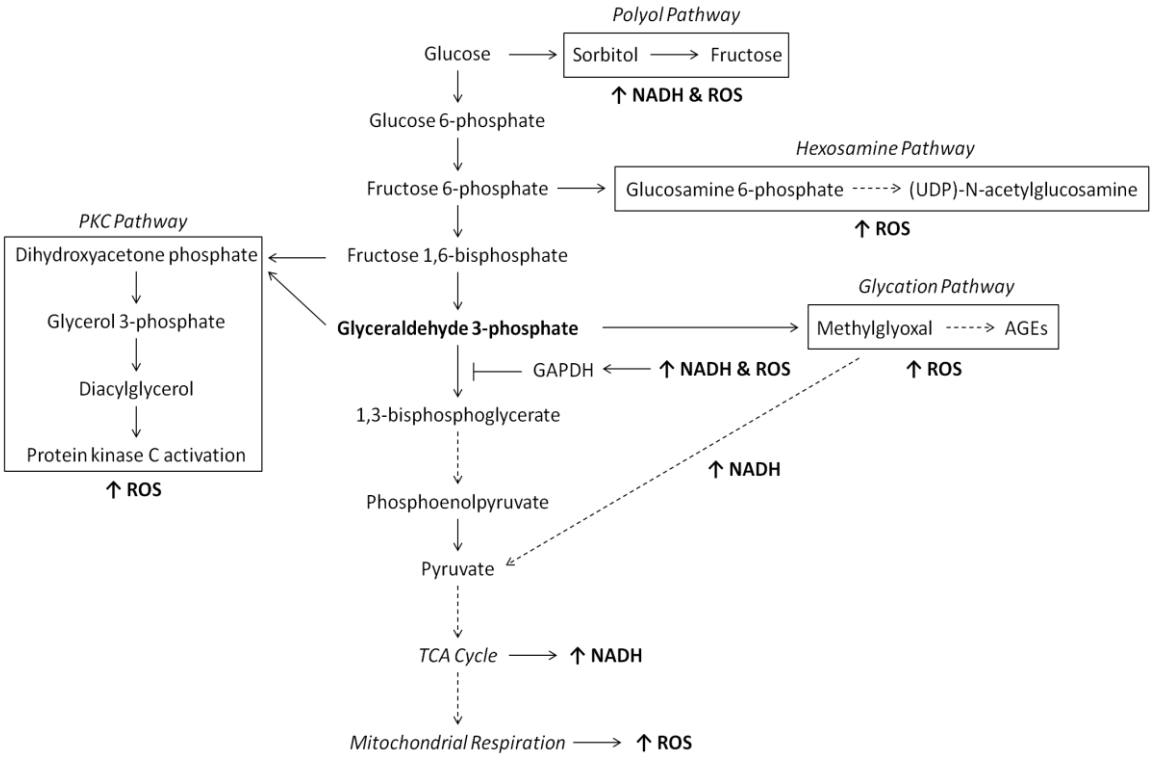


Figure 1.1: Excess intracellular glucose activates alternative metabolic pathways branching off glycolysis, which contribute to increased NADH and ROS. Increased NADH and ROS result in reductive and oxidative stresses respectively. Boxes indicate significant branching pathways; dashed lines indicate additional steps not shown. Adapted and added to from (Yan 2014).

In the polyol pathway glucose is converted to fructose via sorbitol and, in doing so, NADH is produced from nicotinamide adenine dinucleotide phosphate (NADPH), further contributing to the reductive stress. In addition, with less NADPH, cellular glutathione (GSH) decreases and compromises the antioxidant defence system (Tang, Martin et al. 2012, Yan 2014, Luo, Wu et al. 2016). The hexosamine pathway converts fructose 6-phosphate to uridine diphosphate (UDP)-N-acetylglucosamine via glucosamine 6-phosphate and this inhibits insulin receptor substrate (IRS)-1 (see 1.2.2), generates ROS (Issad, Masson et al. 2010) and instigates post-translational modifications in the pathogenesis of diabetic complications such as retinopathy (Gurel, Sieg et al. 2013). The accumulation of G3P increases DAG synthesis and this activates protein kinase C (PKC). The PKC activation pathway is also involved in diabetic complications; PKC-mediated expression and secretion of vascular endothelial growth factor (VEGF) in the mesangial cells of the kidney can lead to proteinuria and glomerular dysfunction

in diabetic nephropathy (Xia, Wang et al. 2007) and chronic PKC activation is involved in CVD comorbidities (Geraldes and King 2010). Furthermore, PKC activates NADPH oxidase (NOX), which generates superoxide from oxygen and can induce insulin resistance and endothelial dysfunction by inhibiting Akt phosphorylation and thus reducing Akt-dependent insulin signalling and nitric oxide synthase (NOS) activation (Naruse, Rask-Madsen et al. 2006). Another product of G3P is methylglyoxal and this forms advanced glycation end products (AGEs) via the glycation of proteins. One such AGE is glycated haemoglobin (HbA1c), used as a biomarker for diabetes. AGEs have implications in diabetic cardiac pathology (Mellor, Brimble et al. 2015) and Alzheimer's disease (Lovestone and Smith 2014), as well as inhibiting insulin signalling (Riboulet-Chavey, Pierron et al. 2006). The glycation pathway liberates ROS and leads to chronic inflammation via activation of AGE receptors and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). AGEs can also arise from the autoxidation of G3P, generating α-ketoaldehydes such as methylglyoxal (Wolff and Dean 1987, Brownlee 2001).

Enzymatic sources of ROS include oxidases, cyclooxygenase and nitric acid synthase (NOS) (Johansen, Harris et al. 2005). In a study comparing blood vessels from diabetic patients with coronary artery disease and nondiabetics it was evident that superoxide generation was enhanced in the former and this was predominantly mediated by increased activity and levels of NOX, and dysfunctional endothelial NOS (eNOS). The superoxide generation was diminished by a PKC inhibitor (Guzik, Mussa et al. 2002). The NOX family of enzymes are important in redox signalling and under controlled conditions generate ROS from NADPH at the plasma membrane in neutrophils to attack pathogens, but in a high glucose environment are over-activated by PKC and involved in the major diabetic complications (Gorin and Block 2013). However, the role of NOX signalling could be more complex as it was recently demonstrated in obese diabetic mice and hepatocytes *in vitro* that knockdown/inhibition of NOX4, and hence its dysfunction *in vivo*, alone can disrupt insulin signalling (Wu and Williams 2012). Regulation of NOS is another critical point. It requires cofactors and the substrate L-arginine to produce nitric oxide; if one of these is not present the oxidation of L-arginine and reduction of oxygen function of NOS is uncoupled and it can produce superoxide instead (Guzik, Mussa et al. 2002, Johansen, Harris et al. 2005). It has long been known that hyperglycaemia increases eNOS-dependent superoxide production (Cosentino,

Hishikawa et al. 1997). Xanthine oxidase and dehydrogenase are interconvertible enzymes involved in purine metabolism, converting hypoxanthine to xanthine and then uric acid, thereby generating NADH and superoxide, which under basal conditions is converted to hydrogen peroxide. Activity of these enzymes, and therefore reductive and oxidative stress, is increased in diabetics and associated with diabetic peripheral neuropathy and gout (Aliciguzel, Ozen et al. 2003, Maiuolo, Oppedisano et al. 2016, Miric, Kisic et al. 2016). The increased purine metabolism likely results from excessive nutrition, but is also related to vascular dysfunction, disrupted hepatic metabolism, increased adipose tissue and pro-inflammatory mediators (Miric, Kisic et al. 2016). In similar fashion, cyclooxygenase (COX) pathway is also involved in the pathogenesis of diabetic peripheral neuropathy, and other complications. Specifically, COX-2 is upregulated by high glucose and subsequently prostaglandins function is impaired, activating downstream inflammatory events (Kellogg, Cheng et al. 2008).

The conversion of superoxide to hydrogen peroxide under stress-free conditions is just one of the complex endogenous antioxidant defence mechanisms to keep ROS, normally required for basal redox and signalling functions, under control. Superoxide dismutase converts superoxide to hydrogen peroxide (H_2O_2) and then cytosolic/lysosomal catalase or mitochondrial glutathione peroxidase catalyses the detoxification of H_2O_2 to water and oxygen. The latter reaction uses glutathione regenerated by glutathione reductase forming the glutaredoxin system. Catalase also binds NADPH to prevent its own oxidation. Disposal of H_2O_2 and reactive compounds, and redox signalling, is additionally mediated by the peroxiredoxins, which also reduce organic hydroperoxides and peroxynitrite; the thioredoxin system, which includes thioredoxin reductase and NADPH regenerates active thioredoxin after reducing protein disulfides. Moreover, glutathione S-transferases catalyse the conjugation of glutathione to various xenobiotics for detoxification and haem oxygenase catalyses the degradation of haem. Carotenoids, vitamins, uric acid, α-lipoic acid, trace elements and glutathione itself all have roles as antioxidants too. Changes in the ratios of these redox systems, for example during high glucose-induced oxidative stress, can activate transcription factors such as NFκB, hypoxia-inducible factor 1 and activator protein 1, which induce expression of inflammatory and antioxidant genes, and regulate proliferation and apoptosis (Arner and Holmgren 2000, Maritim, Sanders et al. 2003, Birben, Sahiner et al. 2012, Hanschmann, Godoy et al. 2013). Nuclear respiratory factor (erythroid-derived 2)-like 2 (Nrf2) is a

central transcription factor to the antioxidant response element (ARE) of many genes, capable of inducing many of the antioxidant proteins. It is stimulated by several drugs and phytonutrients and recent findings that suggest a key role in regulating mitochondria and energy metabolism (Vomhof-Dekrey and Picklo 2012).

When oxidative stress exceeds the limits of the cellular antioxidant defence system, the enzymes and signalling pathways of the system themselves become targets of oxidation. Shortcomings and a lack of plausible evidence from clinical trials with antioxidant interventions suggests that further research into such therapies is required (Johansen, Harris et al. 2005, Matough, Budin et al. 2012), or, rather than targeting ROS directly, a better approach may be to modulate the systems that instigate the stress in the first place, such as promoting caloric restriction and increasing physical activity, or looking for compounds that help lower overproduction of ROS and restore the redox balance.

Excessive energy intake leads to obesity and metabolic diseases, with high glucose and lipid cellular influx both resulting in an oxidative stress caused by superoxide generation and a redox imbalance from the conglomeration of enzymatic, glycolysis-branching pathways and over-flux through mitochondria, which overrides the cellular antioxidant defence systems, as discussed. Disproportion between NADH and NAD^+ levels, referred to as a reductive stress, contributes in part to oxidative stress but the primary direct source of ROS is the mitochondria. Elevated cellular ROS can impact on a number of targets through (per)oxidation of proteins, lipids and modifications of deoxyribonucleic acid (DNA), as well as acting as a signalling molecule itself and influencing cellular redox status. Mechanisms affected include metabolic pathways, signalling cascades, gene expression, pro-inflammatory processes, proliferation and cell death and, as such, chronic oxidative stress has been implicated in a number of syndromes including cancer, neurodegeneration, CVD and metabolic disorders, besides playing a central role in the onset of diabetes. Specifically in diabetes, ROS impair insulin synthesis and secretion in the β-cells and induce insulin resistance in the peripheral tissues (Sakai, Matsumoto et al. 2003, Kim, Wei et al. 2008, Tangvarasittichai 2015). In support, oxidative stress biomarkers are increased in individuals with impaired β-cell function (Sakuraba, Mizukami et al. 2002, Butler, Janson et al. 2003) and/or insulin resistance (Urakawa, Katsuki et al. 2003, Katsuki, Sumida et al. 2004). And at the centre of these pathophysiological mechanisms is mitochondrial dysfunction.

1.2.2 Mitochondrial dysfunction and insulin resistance

Although mitochondria are a source of oxidative stress, they are also a principal target of attack by ROS themselves and by RNS (reactive nitrogen species) (Starkov 2008, Kang and Pervaiz 2012). The impairment of complex I, as a principal source of mitochondrial superoxide alongside CoQ_{10} and complex III, together accounting for approximately 60% of total superoxide generation in rat skeletal muscle mitochondria, (Brand 2010), is arguably the initiator of oxidative stress in diabetes. Overproduction of ROS by complex I and the damage on complex I by ROS results in dysfunction across the mitochondria that exacerbates the stress further in a downward spiral linked with the perturbations in cellular glucose and lipid metabolism, insulin resistance and impaired insulin secretion, and ultimately chronic hyperglycaemia and diabetes (Sivitz and Yorek 2010, Blake and Trounce 2014, Luo, Li et al. 2015). Cells try to prevent the increased ROS caused by the overflux of NADH by lowering insulin-stimulated glucose uptake and preventing substrate entry into the mitochondria, via the inhibition of fatty acid oxidation for example, which increases cellular FFAs and consequently lowers GLUT4 translocation, further inducing insulin resistance in muscle and adipose (Rudich, Tirosh et al. 1998, Tretter and Adam-Vizi 2000, Ceriello and Motz 2004). Mitochondrial dysfunction is also implicated in β-cell damage and endothelial dysfunction, relating to impaired insulin secretion and CVD, but as this work focuses on liver and muscle, only insulin resistance shall be discussed further in this context.

Increased ROS damages mitochondrial proteins and lipid membranes, lowers mitochondrial biogenesis and leads to mutations in both mitochondrial and nuclear DNA, leading to mitochondrial dysfunction. This in turn further increases ROS and lowers β-oxidation, aerobic respiration and ATP production, with increased compensatory glycolytic activity. Mitochondrial DNA (mtDNA) is particularly prone to mutagenic stress, given its proximity to the ROS source and the absence of protective histones, and it contains only coding sequences so most mutations will have a genotypic output (Kim, Wei et al. 2008). Mitochondrial capacity for oxidative phosphorylation (OXPHOS) is determined by expression and function of the ETS complexes (Ritz and Berrut 2005) and since some subunits of these complexes are encoded for by mitochondrial DNA, this is a potential mechanism for the mitochondrial dysfunction in insulin resistance. Fewer and smaller mitochondria are found in the skeletal muscle of obese and diabetic individuals (Ritov, Menshikova et al. 2005). Mitochondrial biogenesis is centrally regulated by

peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), the expression of which is increased with cellular ATP demand (Lehman, Barger et al. 2000, Finck and Kelly 2006) (Fig. 1.2) but generally decreased in insulin-resistant and diabetic humans (Patti, Butte et al. 2003). Adenosine monophosphate-activated protein kinase (AMPK) directly activates PGC-1α to increase mitochondrial proteins in coordination with NAD^+-dependent deacetylase sirtuins (especially SIRT1 and SIRT3) (Winder, Holmes et al. 2000, Jager, Handschin et al. 2007, Canto, Gerhart-Hines et al. 2009, Carling 2017). AMPK and SIRTs are activated by increases in AMP and NAD^+ and therefore also in line with ATP demand (Fig. 1.2). Decreased expression and activity of PGC-1α is likely the reason for the decrease in mitochondria in diabetes, and with excess nutrition the rise in reductive stress will account for the lowered activity. A study investigating respiratory output in skeletal muscle biopsies from healthy and diabetic subjects found no difference in mitochondrial function, but attributed the lower respiration to the lower mitochondrial content assessed by mtDNA and citrate synthase activity (Boushel, Gnaiger et al. 2007). However, Ritov *et al.* showed that ETS activity was in fact lower in diabetic muscle and only partly attributable to fewer mitochondria (Ritov, Menshikova et al. 2005), suggesting mitochondrial function and content are mutually important. Age and inherited genotype also play important roles in mitochondrial dysfunction (Kim, Wei et al. 2008) but the focus here shall remain on diet-induced stress and decline.

Mitochondrial dysfunction, caused by oxidative stress, fewer/injured mitochondria, depleted/mutated mtDNA, decreased oxidative function and OXPHOS protein expression, results in lower ATP synthesis, impaired β-oxidation, intracellular lipid accumulation and increased glycolysis in liver and muscle; in turn alterations in the insulin signalling cascade are induced and result in insulin resistance. Such changes are seen early in diabetes pathogenesis (Morino, Petersen et al. 2005). Insulin signalling pathways are initiated by insulin binding to the insulin receptor, causing a conformational change that activates the intrinsic tyrosine kinase activity of the receptor, resulting in autophosphorylation and the phosphorylation of insulin receptor substrates.

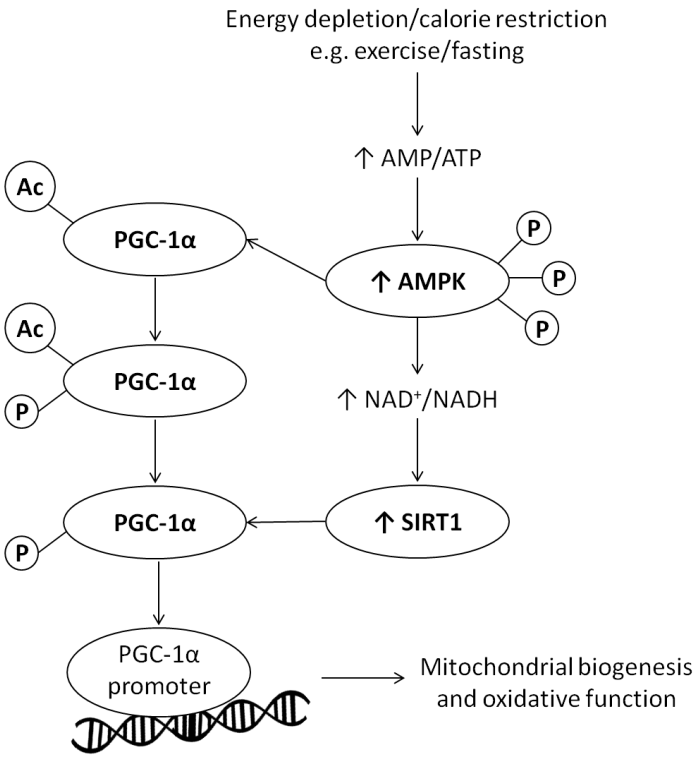


Figure 1.2: The PGC-1α/AMPK/SIRT axis. An increase in the intracellular AMP/ATP ratio activates AMPK, which phosphorylates PGC-1α. A corresponding increase in cellular NAD^+/NADH activates SIRT1, which deacetylates PGC-1α, a transcriptional coactivator regulating various genes involved in mitochondrial and cellular metabolism.

Phosphatidylinositol 3-kinase (PI3K) is activated and phosphorylates PI 4,5-bisphosphate to PI 3,4,5-triphosphate, stimulating a range of downstream serine kinases including PI-dependent kinase-1, Akt, atypical PKC (Boucher, Kleinridders et al. 2014). Key pleiotropic metabolic effects of this PI3K branch include GLUT4 translocation in muscle and adipose (via PKCδ); the activation of glycogen synthase (glycogen synthase kinase is phosphorylated by Akt and inactivated, preventing it from inhibiting glycogen synthase) and inhibition of gluconeogenesis (downstream of the forkhead box O (FOXO) proteins) in liver (Saltiel and Kahn 2001); stimulation of mitochondrial respiration and upregulation of mitochondrial proteins (FOXO) in liver and muscle; and vasodilation in the vascular system via eNOS (Stump, Short et al. 2003, Kim, Wei et al. 2008, Cheng, Tseng et al. 2010, Boucher, Kleinridders et al. 2014). FOXO and the mitogen-activated protein kinase (MAPK) branch of insulin signalling regulate cell survival, proliferation

and differentiation and, in addition, insulin stimulates ROS detoxification functions via NOS (Kim, Wei et al. 2008, Yu, Gao et al. 2011). There are numerous other physiological actions of insulin, and many other convergent signalling pathways with effects on glucose metabolism, which are beyond the scope of this review.

Various sites of the insulin signalling pathway have been implicated in the causes of insulin resistance. A principal mechanism is the increased serine phosphorylation of IRS proteins, which prevents them from interacting with the receptor and leads to their degradation. This can be triggered by endoplasmic reticulum stress, FFA-induced pro-inflammatory signals via c-Jun N-terminal kinase (JNK) and inhibitor of nuclear factor κB kinase-β (IKK-β), and by ROS (Kim, Wei et al. 2008, Boucher, Kleinridders et al. 2014). Accumulation of DAG activates PKCs to also increase serine phosphorylation of IRS (Itani, Ruderman et al. 2002) and ceramide inhibits Akt (Schmitz-Peiffer, Craig et al. 1999). Increased activity of phosphatases involved in the negative regulation of the pathway and decreased activation of molecules such as Akt may also play a role, but mitochondrial dysfunction and the associated lipid accumulation is a primary contender. Certainly interventions that improve mitochondrial function such as drugs, exercise and calorie restriction also restore insulin sensitivity (Kim, Wei et al. 2008). Furthermore, insulin regulates mitochondrial function through FOXO1 and FOXO3a, targets of SIRT1 and this puts insulin on the AMPK metabolic regulation axis (Canto, Gerhart-Hines et al. 2009). Likewise, under normal conditions ROS act as signalling molecules in the insulin pathway and enhance insulin sensitivity, so insulin signalling and mitochondria feedback to each other and regulate metabolism concurrently.

There are a plethora of recent reviews detailing the links between mitochondrial dysfunction and diabetes pathophysiology, and the exact cause-effect relationship between mitochondrial dysfunction and insulin resistance is still debated (Cheng, Tseng et al. 2010, Boucher, Kleinridders et al. 2014, Montgomery and Turner 2015, Tangvarasittichai 2015), but they clearly occur simultaneously as a result of the chronic high glucose and lipid stress. Regardless of the mechanism, the outcome is elevated oxidative stress and impaired insulin signalling, resulting in perturbed hepatic metabolism and impaired post-prandial glucose uptake into skeletal muscle. Dietary (poly)phenols have been shown to play a beneficial role in managing these and other features of diabetes development.

1.3 (Poly)phenols and their health benefits

A large group of plant-derived organic compounds, (poly)phenols have been the focus of many epidemiological, animal, human intervention and *in vitro* studies. This is due to the increasing evidence for beneficial effect to human health when consumed regularly in our diet. This group of naturally occurring phytochemicals exist in plants (Bravo 1998, Manach, Williamson et al. 2005), sharing the basic structure of an aromatic ring and attached hydroxyl groups. Based on further specific structural features, they have been grouped into a classification system (Tsao 2010). The most intensively studied compounds include isoflavones, phenolic acids, stilbenes and the flavonoids, a subgroup comprising flavonols, flavanones, anthocyanins and flavanols (see Fig. 1.1). An ongoing collaborative project has produced, and is continually updating, an online database, *Phenol-Explorer*, of more than 500 (poly)phenols (Rothwell, Perez-Jimenez et al. 2013). Foods and beverages rich in (poly)phenols include teas, coffee, cocoa, fruit, vegetables, wine, cereals, oils, herbs, spices, seeds, nuts, pulses and soy (Manach, Scalbert et al. 2004).

The proclaimed benefits of the bioactive compounds in these foods vary hugely and such claims led to the explosion of research interest in this area. Antioxidant activity has probably been the most publicised over the last few decades and therefore received the most interest, with around 10,000 hits currently on PubMed, which were reviewed as early as 1987 by Fraga *et al.* (Fraga, Martino et al. 1987) and by Rice-Evans *et al*. (Rice-Evans, Miller et al. 1996) and Landete more recently (Landete 2013). Nevertheless, there is now much evidence to suggest that these so-called antioxidant effects are not acting directly to remove free radicals and prevent oxidative stress *in vivo* (Hollman, Cassidy et al. 2011, Kerimi and Williamson 2016), and that actually it is the effects exerted on proteins, genes and metabolites, and even on the expression of micro ribonucleic acids (Milenkovic, Jude et al. 2013), having a functional impact through signalling pathways.

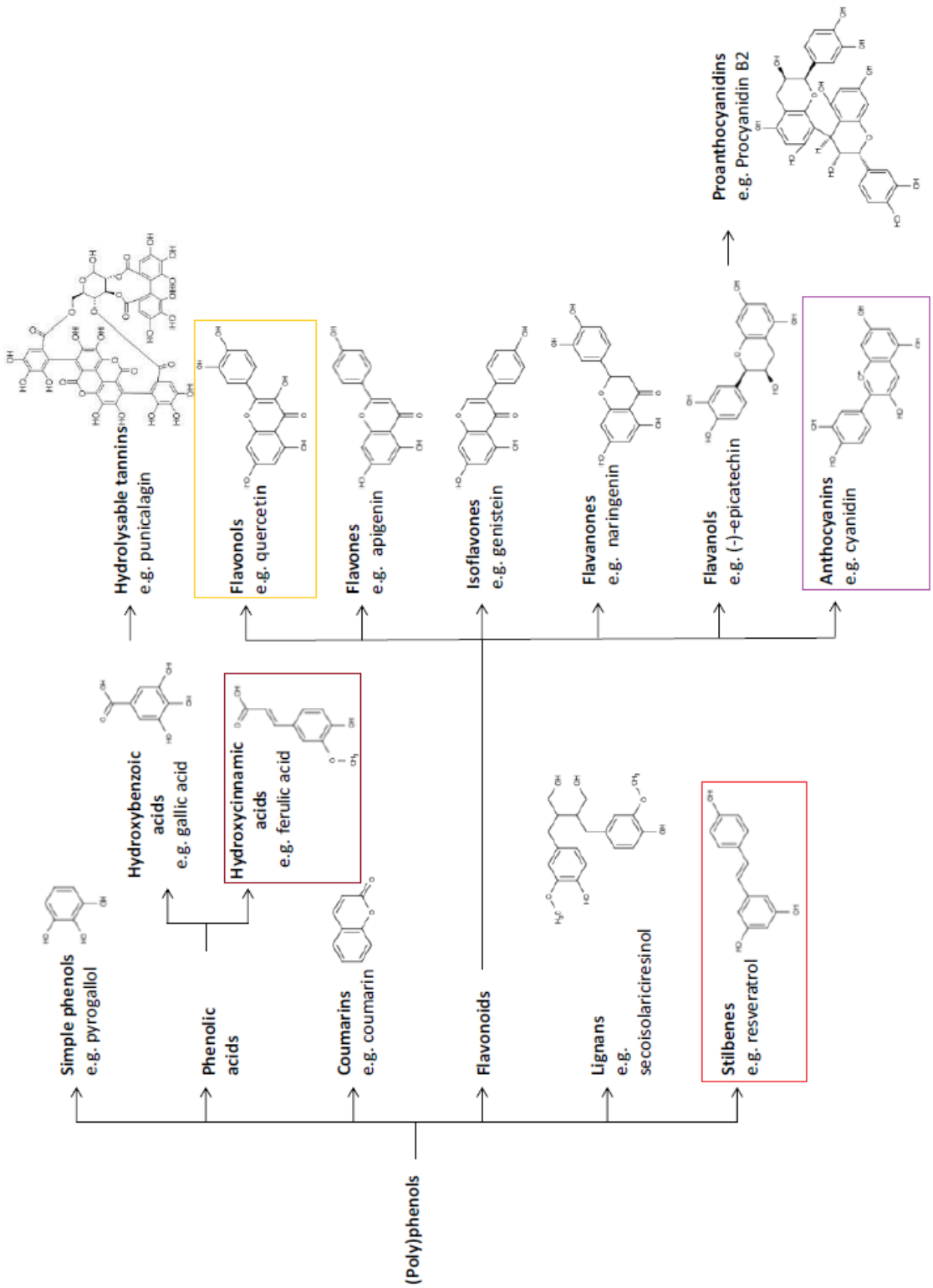


Figure 1.3: Classification of the main (poly)phenols.

Boxed classes and sub-classes comprise compounds investigated in the present study. Adapted from (Pimpão 2014).

Another significant property is their ability to affect enzymes. On a molecular basis, together with 'antioxidant' activity, this effectively accounts for almost all the other (poly)phenol biological effects. For example, green tea (poly)phenols (specifically (-)-epigallocatechin-3-gallate (EGCG)), curcumin and ellagic acid) are attributed as anticarcinogenic, with the proposed mechanisms of action discussed being primarily the enhancement of antioxidant and phase II metabolic enzymes (Stoner and Mukhtar 1995). Some of the earliest potential (poly)phenol benefits were seen in their effect on enzyme activity (Cochet, Feige et al. 1982), which ranges from enzymes on the physiological level, such as in the digestive tract (de la Garza, Milagro et al. 2011, Williamson 2013) to enzymes at the cellular level (Frezza, Schmitt et al. 2011). Other effects include neuroprotection (Spencer, Vauzour et al. 2009, LoPachin, Gavin et al. 2011), resistance against chronic diseases including cancer (Surh 2003, Ramos 2007, Arts 2008, Darvesh and Bishayee 2013, Gonzalez-Vallinas, Gonzalez-Castejon et al. 2013), CVDs (Arab, Liu et al. 2009, Kishimoto, Tani et al. 2013, Khan, Khymenets et al. 2014, Larsson 2014) and diabetes; there is even evidence for antimicrobial properties, for instance in an anticariogenic capacity (Ferrazzano, Amato et al. 2011, Goenka, Sarawgi et al. 2013).

Despite the mass of evidence for the many health claims, the exact mechanisms of action are still not proven. There are numerous epidemiological and human intervention studies that suggest positive roles for (poly)phenols in the diet, but the physiological and molecular 'truth' is much more complex and specific *in vitro* investigations are required to elucidate the mechanisms, and compounds, responsible. It is important to support *in vivo* data with appropriate *in vitro* data and vice versa. It was formerly thought that all (poly)phenols were beneficial for simply exerting a general antioxidant effect, however this is no longer the consensus (Sies 2007, Cooper, Donovan et al. 2008, Fraga, Galleano et al. 2010, Kim, Quon et al. 2014), as work focuses on the wider array of cellular effects and molecular interactions. Furthermore, researchers are starting to use physiologically relevant metabolites in the lower micromolar range, rather than high doses of the parent compounds, which is more representative of the *in vivo* scenario. Following ingestion of (poly)phenol aglycones and plant metabolites, compounds undergo extensive biotransformation before and after absorption into the circulation, with subsequent phase II metabolism in enterocytes, by the colonic microbiota and upon reaching the liver (Williamson and Clifford 2017).

1.3.1 (Poly)phenol metabolism and bioavailability

The biotransformation (i.e. conjugation, metabolism and degradation), cellular uptake and biological activity of many dietary (poly)phenols and their metabolites needs to be further ascertained (Manach, Scalbert et al. 2004, Yang, Sang et al. 2008). A range of possible modifications exists both in the foods and once absorbed, including conjugation with glucuronic acid, sulfation and methylation (O'Leary, Day et al. 2003, Williamson, Barron et al. 2005). Additionally, depending on chemical structure and any attached moiety such as sugars, many (poly)phenols will pass through the small intestine unabsorbed and are metabolised by the microflora in the colon, producing lower molecular weight catabolites, which are then absorbed here (Williamson and Clifford 2010). The link between *in vitro* data for potential benefits and realistic effects *in vivo* becomes harder to prove. Various flavonoids have shown promise in preventing pathways leading to cancer for example, but, without the knowledge on how to improve bioavailability, their full potential as therapeutic/'nutraceutic' agents will remain unknown (Nambiar and Singh 2013).

With such a broad range of dietary (poly)phenols available in the diet and huge complexities in their biotransformations and true bioavailabilities, it is reasonable at this point to focus just on those compounds that have been well characterized both *in vitro* and *in vivo* and form the focus of this project. The choice of compounds was centred on existing knowledge of their antidiabetic effects, and on them being sourced in frequently consumed (poly)phenol-rich foods (see Chapter 3, Table 3.1). Quercetin is a flavonol found in various foods, such as onions and apples (Rothwell, Perez-Jimenez et al. 2013), that may help modulate glucose digestion and metabolism (Nomura, Takahashi et al. 2008, Nyambe-Silavwe, Villa-Rodriguez et al. 2015). It is also known for its widespread effects on mitochondrial physiology (de Oliveira, Nabavi et al. 2016) and thus, with these pleiotropic effects, it has been well studied for its potential in managing the development of type 2 diabetes (Haddad and Eid 2016). Kaempferol is another flavonol and can stimulate glucose uptake in order to lower hyperglycaemia (Fang, Gao et al. 2008). Ferulic acid is a major hydroxycinnamic acid (phenolic acid) in wheat and its conjugates are also found in circulation following coffee consumption (Lempereur I 1997, Stalmach, Mullen et al. 2009). Ferulic acid reduces lipid-induced insulin resistance in skeletal muscle cells and rats (Gogoi, Chatterjee et al. 2014) and increases glucose uptake in myotubes (Bhattacharya, Christensen et al. 2013). The stilbene resveratrol, found in

grape seed, is well-documented for its anticancer properties, but also more recently its effects on diabetes and glucose uptake, which may in fact contribute to its anticancer properties (Leon, Uribe et al. 2017). Finally, anthocyanins such as cyanidin 3-*O*-glucoside are metabolised to form protocatechuic acid and other downstream (poly)phenols; and these, together with the metabolites of gallic acid (hydroxybenzoic acid, a phenolic acid), are all found in circulation following the consumption of berries (Pimpao, Ventura et al. 2015) [Pimpão PhD thesis, 2014], as well as other fruits, beans and cinnamon (Rothwell, Perez-Jimenez et al. 2013). Several recent human intervention studies have shown improved insulin responses and/or attenuated glycaemia following both acute and chronic consumption of berries (Stull, Cash et al. 2010, Torronen, Kolehmainen et al. 2013, Nyambe-Silavwe and Williamson 2016), and (poly)phenol compounds from berries were recently shown to improve glucose uptake and metabolism in liver and muscle cells *in vitro* (Nachar, Eid et al. 2017). The problem with the existing literature is that these studies have all been done using the parent compounds, or are interventions with whole foods without *in vitro* mechanistic follow-up; the metabolic profile of the plasma rarely includes the aglycone compounds, or does so in very small amounts, and thus the effects of circulating metabolites need assessing.

1.3.1.1 Quercetin

Quercetin present in food is typically attached to sugar moieties, such as quercetin 3-*O*-glucoside (isoquercetin), 3,4′-*O*-diglucoside, 3-*O*-rutinoside (rutin), 3-*O*-galactoside (hyperin) and 3-*O*-rhamnoside (quercitrin) (Rhodes and Price 1996, Lommen, Godejohann et al. 2000). After ingestion, the glucosides are rapidly absorbed in the small intestine following hydrolysis by the brush border enzyme lactase phloridzin hydrolase, though the rutinoside is only absorbed in the colon after hydrolysis by the microbiome. Quercetin aglycone passively diffuses into enterocytes and is metabolised by sulfotransferase (SULT), UDP glucuronosyltransferase (UGT) and catechol-*O*-methyl transferase (COMT) before entering the circulation, or returning to the intestinal lumen, via multidrug-resistant protein (MRP) transporters. In the colonocytes quercetin undergoes methylation or glucuronidation, but not sulfation, although most is subjected to bacterial ring fission to form hydroxyphenylacetic acid catabolites. Circulating metabolites are numerous and primarily include sulfates and glucuronides, with most having a short elimination half-life ($T_{½}$) before being removed from the bloodstream. Metabolites excreted in urine are more complex, indicative of phase II metabolism in the

liver and kidneys (Del Rio, Rodriguez-Mateos et al. 2013, Williamson and Clifford 2017).

Metabolites can enter cells and peripheral tissues, glucuronides passively and sulfates via organic anion transporters (OATs) (Wong, Akiyama et al. 2012), where they may be deconjugated and/or further metabolised (Kawai, Nishikawa et al. 2008, Ishisaka, Mukai et al. 2014). Kawai *et al.*, found the aglycone form was more bioactive intracellularly in macrophages (Kawai, Nishikawa et al. 2008), though it is important to note that this may not be the case in all cells, under all conditions, nor for all biological effects or all (poly)phenols. For example, there were comparatively different effects on intracellular ROS by quercetin and quercetin 3-*O*-glucuronide in the study by Shirai *et al*. (Shirai, Yamanishi et al. 2002). More research has been done on aglycones; treating cells with aglycone may result in bioactive derivatives intracellularly, just as the metabolites can be deconjugated to produce the aglycone.

1.3.1.2 Kaempferol

Kaempferol is also a flavonol and follows similar digestion, absorption and metabolic pathways (DuPont, Day et al. 2004, Serra, Macià et al. 2012). Kaempferol may be better absorbed than quercetin, or quercetin is more efficiently metabolised to other forms and/or excreted (de Vries, Hollman et al. 1998). Up to 80% of the circulating kaempferol following consumption of endive soup was identified as kaempferol 3-*O*-glucuronide (DuPont, Day et al. 2004). Plasma metabolites following ingestion of capers included kaempferol 3-*O*-glucuronide and 7-*O*-sulfate, of which the former accounted for 95% (Kerimi, Jailani et al. 2015).

1.3.1.3 Ferulic acid

Ferulic acid is a metabolite of the chlorogenic acids, such as caffeoylquinic acid, found at high levels in coffee. Small amounts of ferulic acid itself are also present in coffee, but it is found at much higher levels (though often bound to fibre) in wheat and other cereals, plus various fruits and vegetables (Zhao and Moghadasian 2008). Some hydrolysis by esterases in the small intestine removes the quinic acid moiety from the coffee chlorogenic acids, releasing the phenolic acids; free caffeic acid is then absorbed into the enterocytes and sulfated to form caffeic acid 3′-*O*-sulfate or, in the presence of methylation, ferulic acid-4′-*O*-sulfate, both rapidly appearing in the plasma within 30

minutes (Williamson and Clifford 2017). Most of the chlorogenic acid esters are not hydrolysed and so reach the colon, where the phenolic acids are released by the gut flora esterases (Plumb, Garcia-Conesa et al. 1999). Here they are further converted by the bacteria to dihydrocaffeic and dihydroferulic acids and these circulate as sulfates, or as a free form in the case of dihydroferulic acid. A recent study in our lab demonstrated that short-chain fatty acids derived from dietary fibre via the gut microbiome metabolism can modulate the transport and conjugation of ferulic acid, indicating that the microbiome has a complex role in the metabolism of ferulic acid, and other (poly)phenols (Van Rymenant, Abranko et al. 2017). These conjugates reach much higher concentrations in the plasma than ferulic acid sulfate after drinking coffee (Stalmach, Mullen et al. 2009).

When ferulic acid itself is ingested, it follows a different metabolic pathway. Ferulic acid is the most abundant phenolic compound in wheat, though its intestinal release from the indigestible polysaccharide matrix of the grain is low. This can be enhanced by bioprocessing, making the ferulic acid and other (poly)phenols more accessible (Mateo Anson, Aura et al. 2011). Following ingestion of 300 g of a control or bioprocessed (yeast fermentation and enzyme treatment) bread, the maximum plasma concentration (C_{max}) in humans increased to 2.7 µM in the latter, up from 0.9 µM in the control. For both, the time at which C_{max} was reached (T_{max}) was less than 2 hours, implying digestion and absorption of free ferulic acid occurs predominantly in the small intestine. The authors of this study suggested some of the absorbed ferulic acid was metabolised to vanillic acid and various benzoic acid conjugates by the liver, in agreement with other literature (Zhao and Moghadasian 2008). Most of the ferulic acid, though, was passed to the colon and entered the circulation mainly as 3-hydroxyphenylpropionic and phenylpropionic acids, the latter of which can be further metabolised to benzoic acid by β-oxidation and then to hippuric acid in the liver (Mateo Anson, Aura et al. 2011). Ferulic acid conjugates are also among the numerous metabolites present in plasma following ingestion of berries (Pimpao, Ventura et al. 2015) (see 1.3.1.5).

1.3.1.4 Resveratrol

Resveratrol is a stilbene that occurs as *cis* and *trans* isomers, as well as other derivatives in nature, including the 3-*O*-glucoside, *trans*-piceid. It is found in grape seed and best known for its presence in red wines, although it is usually present in much lower concentrations than other (poly)phenols (Crozier 2010). In fact, its presence in wine is so low that its credit for the health benefits of drinking red wine, such as in the review by Yang *et al.* (Yang, Li et al. 2014), could be completely misplaced. However, there may be benefits from circulating metabolites, especially over the course of a lifetime. Many studies tend to focus instead on its potential pharmaceutical capacity, rather than dietary. Bioavailability of free resveratrol is very low and this is because it is rapidly metabolised to its glucuronide and sulfate conjugates (Brown, Patel et al. 2010). This was demonstrated in two pharmacokinetic studies, where pharmacological doses of resveratrol were administered to humans either in a single dose or daily for a whole month, and the metabolites reached plasma concentrations in the micromolar range. The T_{max} was typically within 1-2 h, suggesting intestinal metabolism and absorption (Boocock, Faust et al. 2007, Brown, Patel et al. 2010). This is in line with other bioavailability studies, generally detecting the sulfates as predominant in plasma and urine (Del Rio, Rodriguez-Mateos et al. 2013). Some resveratrol is further metabolised by the gut microbiota to dihydroresveratrol and its phase II metabolites (Rotches-Ribalta, Urpi-Sarda et al. 2012).

Many of the bioavailability experiments on resveratrol have been done in rat models or cell models *in vitro* and thus may not be as relevant to the *in vivo* situation as performing pharmacokinetic interventions. It is well-known that the expression of transporters and enzymes, and in particular the gut microbiome composition, has large inter-individual variability (Tomas-Barberan, Selma et al. 2016), as well as inter-species variation, so it is unknown how useful such studies may be. Furthermore, there is some uncertainty over the metabolism of resveratrol in various peripheral tissues and the identity of compounds responsible for the biological effects. Resveratrol metabolites, specifically 3-*O*-sulfate, have been shown to inhibit the proliferation of colon cancer cells (Aires, Limagne et al. 2013), which may be due to enzymatic release of the aglycone intracellularly (Miksits, Wlcek et al. 2009). It was recently demonstrated that the metabolites lower lipid accumulation and adipokine expression, and enhance lipid metabolism in murine adipocytes (Lasa, Churruca et al. 2012, Eseberri, Lasa et al. 2013). In contrast, an earlier

study attributed the biological effects solely to the aglycone, with no impact reported for the metabolites (Kenealey, Subramanian et al. 2011), and also a recent study showed that only deconjugated resveratrol had an antilipolytic effect in human adipocytes (Gheldof, Moco et al. 2017). However, considering positive effects following resveratrol administration, resveratrol still holds its promise as a therapeutic, although it is important to determine the specific metabolites responsible in order to improve targeting and efficacy. The same applies to all (poly)phenols.

1.3.1.5 Berry (poly)phenol metabolism

The final group of compounds investigated in this project were chosen based on the results from a human intervention study done previously in our lab (Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015). A range of metabolites were detected and quantified in plasma following ingestion of a mixed fruit berry purée containing blueberry, blackberry, raspberry, strawberry and Portuguese crowberry. Phenolic sulfates, resulting mostly from colonic metabolism, reached high concentrations (up to 20 µM), though for many of them biological activities have not been reported. It was suggested that the metabolites, such as the conjugates of catechol and vanillic acid, were catabolites of protocatechuic acid and originated predominantly from the cyanidin 3-*O*-glucoside parent compound (Pimpao, Ventura et al. 2015) [Pimpão PhD thesis, 2014], though they have also been shown as derivatives of chlorogenic acids, B-type proanthocyanidins and other flavonoids such as quercetin glycosides (Xie, Lee et al. 2016). Other major phenolic sulfates present in the plasma after the berry meal were conjugates of gallic acid. Of these compounds that were investigated here, only 4-methylgallic 3-*O*-sulfate was not a colonic catabolite, as established by its lower T_{max} (Pimpao, Ventura et al. 2015).

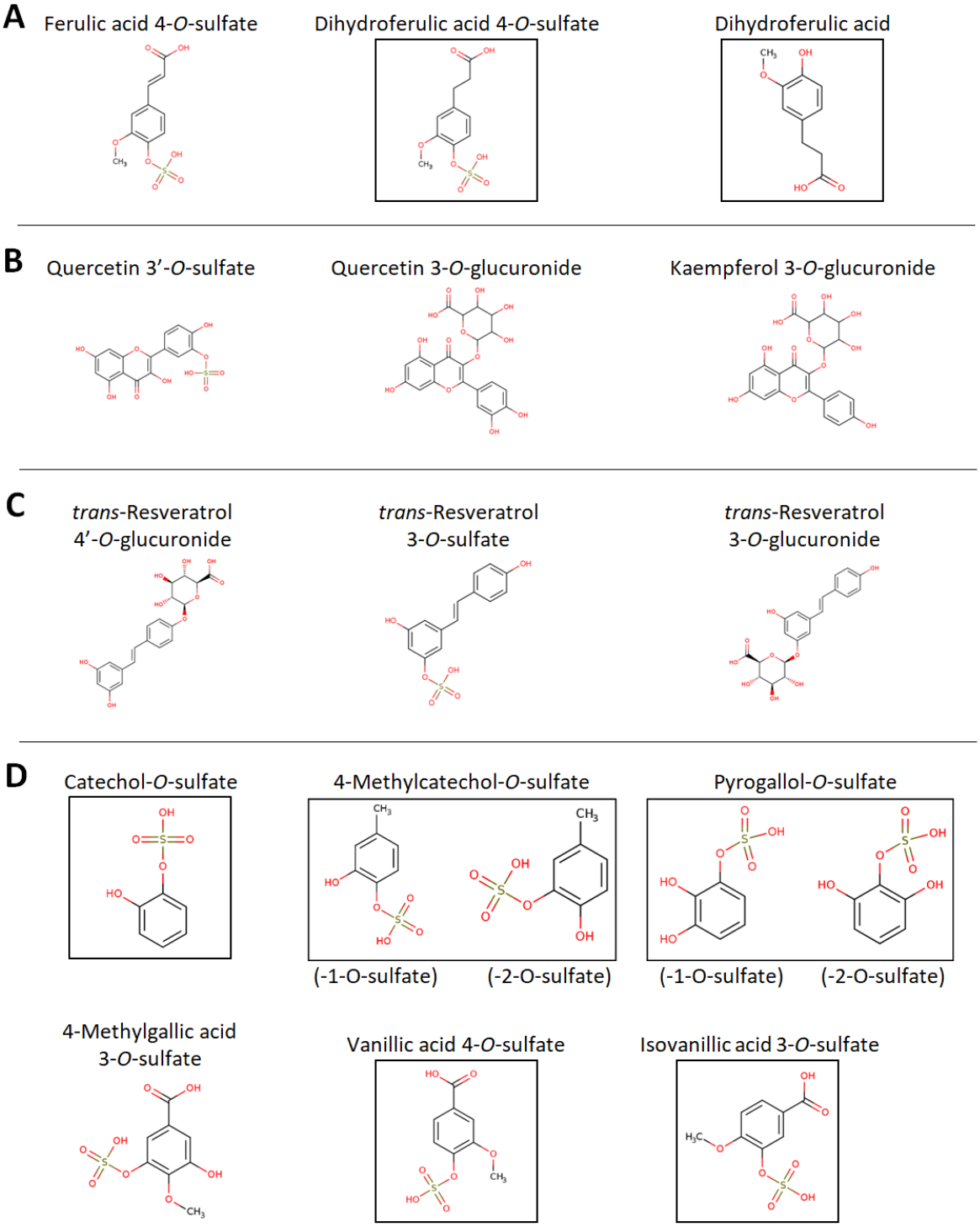


Figure 1.4: Chemical structures of the (poly)phenol metabolites investigated here for potential roles in modulating glucose uptake and metabolism in human skeletal muscle. Derivatives of ferulic acid (A); flavonol conjugates (B); resveratrol conjugates (C); phenolic sulfates in circulation after the consumption of berries (D). Boxed compounds originate from colonic microbiota metabolism, unboxed in the small intestine.

1.3.1.6 Pharmacokinetic data

The pharmacokinetic data suggests the body treats many (poly)phenols in a similar way to pharmaceutical drugs; intake, which in itself can be low to start with, is followed by rapid excretion due to the conjugation reactions. Therefore bioavailability is lower than that of the (poly)phenol doses employed in most *in vitro* investigations. On the other hand, as continuing research unfolds finer details of the metabolic transformations, absorption may be higher than first thought, due to some metabolites initially being missed in the detection. Understanding the distribution and accumulation of metabolites in tissues around the body can be limited by disproportionate reliance on *in vitro* cell models, which may express different levels of transporters and enzymes, and a different physiology in response to (poly)phenol treatments. Table 1.1 summarises some of the pharmacokinetic data for the (poly)phenols of interest, compiled from a comprehensive search of relevant human studies using *PubMed*, and helps to build a picture of the metabolite composition in the plasma following the consumption of (poly)phenol-rich foods or (poly)phenol supplements. It is worth noting that there are currently no pharmacokinetic data for isovanillic acid 3-*O*-sulfate exclusively, but data for vanillic acid 4-*O*-sulfate is included and so are data for total vanillic acid sulphates and the precursor protocatechuic acid. Furthermore, there is one study for the parent isovanillic acid compound, which is likely in circulation in lower concentrations than the sulfated compounds.

Table 1.1: Pharmacokinetics of (poly)phenol metabolites in humans.

Metabolite	Parent Compound/ Aglycone	Dose Administered in Human Study	T_{max} in plasma (h)	Mean C_{max} in plasma	Half Life (h)	AUC (µM*h) *	Cumulative Urine Excretion	Remaining in Plasma	Reference
Ferulic acid 4-*O*-sulfate	Ferulic acid	200 ml instant coffee (N = 11) (335 µM ferulic acids)	0.6	70 nM	4.9	467	11.1 µmol after 24 h	35 nM after 6 h, none after 24 h	(Stalmach, Mullen et al. 2009)
		350 ml PP-rich [a] drink (N = 10) (149 µM phenolic acids)	-	-	-	-	3.6 µmol after 24 h	-	(Borges, Mullen et al. 2010)
		500 ml fruit purée [c] (N = 9) (see (Pimpao, Dew et al. 2014) for quantification)	1	188 nM	-	-	Mostly 0-2 h but some still 8-24 h	S55 nM after 6 h	(Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015)
		350 ml coffee (N = 13)	1	226 nM	0.62	-	-	-	(Lang, Dieminger et al. 2013)
		300 g red raspberries (N = 9) (292 µmol anthocyanins)	1	47 nM	-	-	6.5 µmol after 48 h	None after ~12 h	(Ludwig, Mena et al. 2015)
		450 ml cranberry juice (N = 10)	3.6	2.27 µM	-	11.5	731 nmol 8 h, 1055 after 24 h	600 nM after 24 h	(Feliciano, Boeres et al. 2016)
Dihydro-ferulic acid 4-*O*-sulfate	Dihydroferulic acid	200 ml instant coffee as above	4.8	140 nM	4.7	1193	12.4 µmol after 24 h	130 nM after 6 h, none after 24 h	(Stalmach, Mullen et al. 2009)
		350 ml PP-rich drink as above	-	-	-	-	680 nmol after 24 h	-	(Borges, Mullen et al. 2010)
		500 ml fruit purée (see above)	< LOD after 6 h – likely present in plasma later		-	-	Almost all 8-24 h	-	(Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015)
		350 ml coffee (N = 13)	≥ 8	878 nM	-	-	-	-	(Lang, Dieminger et al. 2013)
		450 ml cranberry juice (N = 10)	6.8	197 nM	-	2.04	512 nmol 8 h, 1001 after 24 h	-	(Feliciano, Boeres et al. 2016)
		400 ml coffee (N = 9)	10	300 nM	-	-	-	-	(Redeuil, Smarrito-Menozzi et al. 2011)
Dihydro-ferulic acid	Dihydroferulic acid	4g instant coffee + hot water (N = 1) (900 µg chlorogenic acids)	8	1.2 µM	-	-	-	90 nM after 24 h	(Guy, Renouf et al. 2009)
		Two artichoke leaf extracts; A containing 107 mg caffeic acids, B containing 154 mg caffeic acids (N = 14)	6.34 (A) 6.21 (B)	140 nM (A) 202 nM (B)	2.91 (A) 2.48 (B)	147 (A) 207 (B)	-	~81 nM (12 h), ~54 nM (24 h)	(Wittemer, Ploch et al. 2005)

		350 ml coffee (N = 13)	≥ 8	94 nM (8 h)	-	-	-	-	(Lang, Dieminger et al. 2013)
		450 ml cranberry juice (N = 10)	6.1	304 nM	-	3.24	457 nmol 8 h, 524 after 24 h	-	(Feliciano, Boeres et al. 2016)
		400 ml coffee (N = 9)	10	820 nM	-	-	-	-	(Redeuil, Smarrito-Menozzi et al. 2011)
(Ferulic acid)	Ferulic acid	500 mg bolus of $^{13}C_5$-cyanidin-3-*O*-glucoside (N = 8)	11.3	940 nM	51.6	21.2 (0-48 h)	Max 1.5 µM after 48 h [b]	560 nM (24 h) [b]	(Czank, Cassidy et al. 2013)
		Two artichoke leaf extracts (as above)	0.77 (A) 0.98 (B)	46 nM (A) 79 nM (B)	6.35 (A) 5.23 (B)	355 (A) 544 (B)	-	~26 nM (12 h) and ~11 nM (24 h) (for both)	(Wittemer, Ploch et al. 2005)
Quercetin 3′-*O*-sulfate	Quercetin	270 g fried red onions (N = 6) (1.02 µmol/g flavonols)	0.75	660 nM	1.71	-	Trace in urine	425 nM after 3 h, 80 nM after 6 h	(Mullen, Edwards et al. 2006)
		Meal incl. pickled capers, blackcurrants and propolis supplements (N = 8)	1.9	62 nM	-	-	-	-	(Kerimi, Jailani et al. 2015)
Quercetin 3-*O*-glucuronide		270 g fried red onions (N = 6) (1.02 µmol/g flavonols)	0.6	350 nM	2.33	-	912 nmol after 9 h	110 nM after 3 h, 80 nM after 6 h	(Mullen, Edwards et al. 2006)
		300 ml enriched tomato juice (N = 6) (40 µM quercetin 3-*O*-rutinoside)	4.7	10 nM	5.7	-	250 nmol after 8 h, 271 nmol after 24 h	10 nM after 4 h, none by 8 h	(Jaganath, Mullen et al. 2006)
		450 ml cranberry juice (N = 10)	1.8	156 nM	-	0.91	79 nmol 8 h, 108 after 24 h	17 nM after 24 h	(Feliciano, Boeres et al. 2016)
Kaempferol 3-*O*-glucuronide	Kaempferol	450 ml cranberry juice (N = 10)	2.1	13 nM	-	0.118	145 nmol 8 h, 180 after 24 h	-	(Feliciano, Boeres et al. 2016)
		Capers, blackcurrants, propolis as above (N = 8)	3	360 nM	-	-	-	100 nM after 8 h	(Kerimi, Jailani et al. 2015)
		300 g endive soup (N = 8) (8.65 mg kaempferol)	0.9, 5.8	50, 100 nM	-	0.75	560 nmol after 24 h	20 nM after 24 h	(DuPont, Day et al. 2004)
(Total Quercetin)	Quercetin	1095 mg quercetin [d] aglycone (N=16)	6.1	1.43 µM	11.4	17.7	-	-	(Guo, Mah et al. 2014)
		Sautéed onion equiv. to 1 mg quercetin/kg bw	1.5	1 µM	-	-	-	-	(Nakamura, Murota et al. 2014)
		160 g stewed onion (100 mg quercetin) (N = 12)	0.7	7.6 µM	10.9	32.1	6.4 % after 48 h	Cleared within 24 h	(Graefe, Wittig et al. 2001)

trans-Resveratrol 4′-*O*-glucuronide	*trans*-Resveratrol	300-600 ml various red wines (N = 2-10) (8.8 µM total *trans*-resveratrol)	1	265 nM	-	-	-	None after 2 h	(Vitaglione, Sforza et al. 2005)
		85.5 mg piceid [e]/70 kg body weight + 500ml milk (N = 9)	6	190 nM	-	-	4.7 µmol after 12 h; no more	99 nM after 8 h, none after 10 h	(Burkon and Somoza 2008)
		Single doses of resveratrol caplets ranging from 0.5 g to 5 g (N = 10 for each dose); will look at 0.5 and 5 g data. Also, two glucuronides measured but not identified	0.5 g: (1) 2 (2) 1.5 5.0 g: (1) 2 (2) 2.5	0.5 g: (1) 1 µM (2) 915 nM 5.0 g: (1) 3.18 µM (2) 4.29 µM	0.5 g: (1) 2.85 (2) 3.09 5.0 g: (1) 7.90 (2) 5.83	0.5 g: (1) 4.75 (2) 3.19 5.0 g: (1) 24.5 (2) 21.1	All excreted after 12 h	Still present after 24 h with the 5.0 g dose	(Boocock, Faust et al. 2007)
		As above but daily doses for 29 consecutive days	0.5 g: 1.27 5.0 g: 1.5	0.5 g: 820 nM 5.0 g: 10.2 µM	0.5 g: 3.78 5.0 g: 7.55	0.5 g: 3.29 5.0 g: 49.4	-	All excreted by 12-24 h	(Brown, Patel et al. 2010)
trans-Resveratrol 3-*O*-sulfate		Single doses as above	0.5 g: 1.5 5.0 g: 2.05	0.5 g: 3.68 µM 5.0 g: 13.93 µM	0.5 g: 3.21 5.0 g: 7.71	0.5 g: 13.14 5.0 g: 100.2	All excreted after 12 h	Still present after 24 h with 0.5 g dose	(Boocock, Faust et al. 2007)
		As above but daily doses for 29 consecutive days	0.5 g: 1.04 5.0 g: 1.25	0.5 g: 2.47 µM 5.0 g 18.3 µM	0.5 g: 3.09 5.0 g: 7.98	0.5 g: 11.54 5.0 g: 126.2	-	All excreted by 12-24 h	(Brown, Patel et al. 2010)
trans-Resveratrol 3-*O*-glucuronide		Single doses as above (two glucuronides measured, not identified); given the pharmacokinetic data below it is likely that this was (2)							(Boocock, Faust et al. 2007)
		As above but daily doses for 29 consecutive days	0.5 g: 1.27 5.0 g: 1.5	0.5 g: 810 nM 5.0 g: 17.1 µM	0.5 g: 4.98 5.0 g: 5.19	0.5 g: 2.16 5.0 g: 54.6 µM	-	All excreted by 12-24 h	(Brown, Patel et al. 2010)
Catechol-*O*-sulfate	Catechol from protocatechuic acid [f]	350 ml coffee (N = 13)	0.75	2.469 µM	0.65	-	-	-	(Lang, Dieminger et al. 2013)
		500 ml fruit purée (above)	6	12.2 µM	-	-	Mostly 8-24 h	All 4-6 h	(Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015)
		450 ml cranberry (above)	7.1	24.6 µM	-	346	4.67 µmol 8 h, 10.5 after 24 h	6.4 µM after 24 h	(Feliciano, Boeres et al. 2016)

4-Methyl-catechol-*O*-sulfate		500 ml fruit purée (above)	6 (N = 7)	636 nM	-	-	All 8-24 h	Mostly 4-6 h, some participants earlier	(Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015)
		450 ml cranberry (above)	7.7	3.5 µM	-	54	564 nmol 8 h, 1625 after 24 h	-	(Feliciano, Boeres et al. 2016)
Pyrogallol *O*-sulfate [g]	Gallic acid, pyrogallic acid	500 ml fruit purée (above)	6 (1/2) (N = 7/8)	(1) 652 nM (2) 11.4 µM	-	-	Some 4-8 h, mostly 8-24 h	At 4 and 6 h, more in the latter esp for (2)	(Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015)
		450 ml cranberry (above)	(1) 8.7 (2) 6.2	(1) 199 nM (2) 339 nM	-	(1) 3.4 (2) 3.0	(1) 34, 146 nmol (2) 127, 302 nmol	-	(Feliciano, Boeres et al. 2016)
4-Methyl-gallic 3-*O*-sulfate	Gallic acid	500 ml fruit purée (above)	2	2 µM	-	-	Max 2-4 h; still 50% max during 8-24 h	Still ~800 nM after 6 h	(Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015)
		450 ml cranberry (above)	2.1	275 nM	-	2.2	206 nmol 8 h, 297 after 24 h	-	(Feliciano, Boeres et al. 2016)
Vanillic acid 4-*O*-sulfate	Vanillic acid/ Protocatechuic acid	500 ml fruit purée (above)	4 (N = 6)	1.3 µM	-	-	Some 2-4 h, mostly 4-24 h	All 4-6 h; peak at 4 h but statistically > baseline at 6 h	(Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015)
		30 ml virgin olive oil (N = 5) (400 mg/kg total polyphenols)	1	50 nM	-	-	-	40 nM after 2 h	(Suarez, Romero et al. 2009)
		450 ml cranberry (above)	2.1	1.05 µM	-	11.8	128 nmol 8 h, 288 after 24 h	200 nM after 24 h	(Feliciano, Boeres et al. 2016)
Total vanillic acid sulfates		500 mg bolus of $^{13}C_5$-cyanidin 3-*O*-glucoside (N = 8) (VA4S and IVA3S)	30.1	430 nM	NQ [h]	10.7	1.7 µM VA4S, 822 nM IVA3S after 3-4 h	Still present after 48 h	(de Ferrars, Czank et al. 2014)
(Protocat-echuic acid) [i]		500 mg bolus of $^{13}C_5$-cyanidin 3-*O*-glucoside (total protocatechuic acid metabolites, NOT just IVAS) (N = 8)	13.4	2.4 µM (cumulative 5 µM after 6 h, still there at 48 h	29.5	44 (0-48 h)	Max 6 µM after 24 h; 40 µM tracer after 48 h	Metabolites mainly in serum and urine in first 6 h, peaking at 24 h and still present in both after 48 h	(Czank, Cassidy et al. 2013)
(Isovanillic acid)		450 ml cranberry (above)	9.9	220 nM	-	3.1	481 nmol 8 h, 1.24 µl 24 h	-	(Feliciano, Boeres et al. 2016)

Bold font indicates colonic metabolites

* AUC – area under the curve

[a] PP-rich, (poly)phenol-rich

[b] Total phenylacetic/ phenylpropenoic acid metabolites

[c] fruit purée contained 100 g of each of the following Portuguese berries: blueberry, blackberry, raspberry, strawberry and crowberry and was consumed with a breakfast containing no other (poly)phenols, consisting of bread with ham or cheese, yogurt and biscuits

[d] Typical quercetin daily intake is only 16 mg, as stated in this paper

[e] piceid aka resveratrol 3-*O*-glucoside

[f] As suggested in (Pimpão 2014, Pimpao, Ventura et al. 2015)

[g] Roughly a 50/50 mix of -1-*O*-sulfate and -2-*O*-sulfate when synthesised by Rui

[h] Still present after 48 h so half-life not quantifiable from this experiment

[i] No studies with explicit isovanillic acid-3-*O*-sulfate data

1.3.2 The antidiabetic properties of (poly)phenols

(Poly)phenols, and their plant-derived metabolites or conjugates, may play a beneficial role in glucose homeostasis through mechanisms that slow digestion and absorption to modulate glycaemic index; once further metabolised and absorbed into the bloodstream, they may prevent the loss in number and function of pancreatic β-cells and enhance insulin synthesis and secretion; improve insulin sensitivity, glucose uptake and reduce inflammation in skeletal muscle and adipose tissue; modulate glucose and lipid metabolism in the liver and systemically; and alleviate cellular oxidative stress and the effects on associated signalling pathways. There are a plethora of recent reviews that detail the potential antidiabetic effects of (poly)phenols based on epidemiological, *in vitro*, animal model and human intervention data (Pandey and Rizvi 2009, Hanhineva, Torronen et al. 2010, de Bock, Derraik et al. 2012, Babu, Liu et al. 2013, Bahadoran, Mirmiran et al. 2013, Williamson 2013, Amiot, Riva et al. 2016, Kim, Keogh et al. 2016). The relevant knowledge on the (poly)phenols of interest shall be summarised briefly here, with more in-depth discussions in the corresponding Results chapters.

1.3.2.1 Quercetin and its conjugates

Quercetin is one of the most widely distributed flavonoids in the diet and unquestionably the most-studied. It has been reported to act on many targets in the gut, pancreas, liver, muscle and adipose tissue to control systemic glucose metabolism (Haddad and Eid 2016), as well as in endothelium in cardiometabolic pathologies (Kunasegaran, Mustafa et al. 2017). Quercetin is an inhibitor of α-amylase (Nyambe-Silavwe, Villa-Rodriguez et al. 2015) and GLUT2 (Kwon, Eck et al. 2007), suggesting it may reduce carbohydrate digestion and the absorption of glucose and fructose in the gut. Subsequently, a recent human study in our lab demonstrated that intervention with a fruit purée, rich in (poly)phenols including quercetin, attenuated postprandial glycaemic and insulinaemic responses (Nyambe-Silavwe and Williamson 2016). The Haddad research group have provided *in vitro* evidence that quercetin increases glucose uptake in murine C2C12 and in rat L6 myotubes via an AMPK-dependent pathway (Eid, Martineau et al. 2010, Eid, Nachar et al. 2015), however both studies use quercetin in excess of 25 μM, which would never be attainable from dietary sources. In the study by Anhê *et al.* the authors treated ob/ob obese mice with quercetin for 10 weeks and L6 myotubes with quercetin and palmitate or tumour necrosis factor-α (TNF-α). They reported that inflammatory

responses were lowered and insulin signalling and GLUT4 expression restored in both models, with an increase in glucose uptake in the muscle cells; this was also at 25 μM (Anhê, Okamoto et al. 2012). There are contrasting reports on the effects of quercetin on glucose uptake in adipocytes, as reviewed recently by Eid *et al.* (Haddad and Eid 2016). Although quercetin inhibited glucose uptake in normal murine 3T3-L1 adipocytes, when under inflammatory conditions induced by macrophage-derived conditioned medium, quercetin actually rescued the cells from insulin resistance, and it was demonstrated that these divergent effects were regulated via AMPK (Xu, Hu et al. 2014). In the same Eid *et al.* review, several studies are highlighted for demonstrating that quercetin has similar effects to the antidiabetic drug metformin on liver glucose metabolism, by inhibiting glucose 6-phosphatase-mediated gluconeogenesis, again involving AMPK activation, but without stimulating glycogenesis (Haddad and Eid 2016). Furthermore, in mice fed with a high fat diet, quercetin lowered lipid accumulation and steatosis-inducing gene expression in the liver, as well as circulating lipids and body weight (Hoek-van den Hil, van Schothorst et al. 2014).

The research on quercetin conjugates to date is mostly related to a potential benefit in CVD prevention. High glucose-induced apoptosis in human umbilical vein endothelial cells (HUVECs) was dose-dependently inhibited by sulfate and glucuronide conjugates prepared from the serum of rats administered with quercetin (Chao, Hou et al. 2009). In aortic rings isolated from rats, incubation with quercetin 3′-*O*-sulfate and 3-*O*-glucuronide partially recovered the oxidative stress-impaired nitric oxide response and at higher micromolar concentrations inhibited NADPH oxidase superoxide generation (Lodi, Jimenez et al. 2009). A more recent study revealed that both quercetin and its 3-*O*-glucuronide conjugate ameliorated palmitate-induced oxidative stress and inflammation in HUVECs (Guo, Zhang et al. 2013). Quercetin 3-*O*-glucuronide was also shown to accumulate in atherosclerotic lesions, but not normal aorta, with signs of preventing arteriosclerosis (Ishizawa, Yoshizumi et al. 2011). Other studies, however, demonstrate biological actions exerted by quercetin aglycone but not the conjugates; only quercetin had anti-inflammatory effects in rat pancreatic cells (Cho, Chang et al. 2012) and in human endothelial cells (Mochizuki, Kajiya et al. 2004, Winterbone, Tribolo et al. 2009). In a study on vascular smooth muscle cells from hypertensive or normal rats, quercetin 3-*O*-glucuronide inhibited NADPH oxidase to prevent superoxide generation, but the effect was absent in the presence of a β-glucuronidase inhibitor. This suggests

that the glucuronide is deconjugated to release the free, active quercetin (Jimenez, Lopez-Sepulveda et al. 2015), thus lack of deconjugation could explain why activity was only seen for the aglycone in the previous examples.

1.3.2.2 Effects of quercetin on mitochondria

Quercetin rapidly diffuses into cells and accumulates in mitochondria (Fiorani, Guidarelli et al. 2010), where it is able to modulate mitochondrial biogenesis, membrane potential, respiration, redox status and apoptosis, as has been recently reviewed (Sandoval-Acuna, Ferreira et al. 2014, de Oliveira, Nabavi et al. 2016). One of the key characteristics of quercetin is its redox capacity and, because of this, it has a hormetic effect and biphasic dose-responses (Vargas and Burd 2010). In the low micromolar range (< 50 µM) quercetin upregulates the Nrf2-mediated ARE, which promotes anti-oxidative stress response genes (Rotblat, Grunewald et al. 2013) and anti-inflammatory pathways and functions (Costa, Garrick et al. 2016). However, quercetin itself has a potent antioxidant ability and is able to reduce ROS, becoming a semi-quinone, and quinone in turn, in its oxidised form, which can react with reduced GSH to deplete cellular levels and result in a pro-oxidative state. In particular this occurs at the higher concentrations (≥ 100 µM) and in cancer cells, in which ROS are continually high due to the glycolytic phenotype; ROS is propagated by the pro-oxidant effect of quercetin and this triggers apoptosis (Gibellini, Pinti et al. 2010). It has been hypothesised that this pro-oxidant effect is what triggers antioxidant defence at the lower doses, the small increase in ROS activating the ARE of the defence system genes (Vargas and Burd 2010).

Studies on isolated mitochondria, usually from rat liver or brain, suggest that quercetin decreases membrane fluidity, respiration and ATPase/ATP synthase activities, while increasing calcium release, mitochondrial permeability transition pore (MPTP) activity and mitochondrial swelling (signs/triggers of autophagy and apoptosis); generally ROS and oxidative effects were lowered (Zheng and Ramirez 2000, Dorta, Pigoso et al. 2005, Dorta, Pigoso et al. 2008, De Marchi, Biasutto et al. 2009, Waseem and Parvez 2016). These effects were all with concentrations of ≥50 µM and with short incubation times. Indeed, lowering respiration would help lower the production of ROS, as would autophagy and mitochondria-induced apoptosis, as part of the cell's oxidative stress defence mechanism. In another study, 25 µM quercetin protected cells against oxygen/glucose deprivation-induced mitochondrial dysfunction by regulating

intracellular calcium, without affecting mitochondrial membrane potential (Panickar and Anderson 2011); Dorta *et al*. also showed no effect on membrane potential (Dorta, Pigoso et al. 2005).

Lagoa *et al*. investigated the potential effects of quercetin on the ETS and saw that quercetin dose-dependently prevented H_2O_2 production without interfering with oxygen consumption. This was also the case in the presence of rotenone and antimycin A, inhibitors of ETS complexes I and III respectively, which increases electron leakage and ROS, suggesting the action of quercetin was not a direct result of a direct interaction with ROS, but rather preventing ROS generation in the first place. The authors speculated this was due to inhibition of ROS generation at the ETS complexes and found this was the case specifically at complex I, without affecting its activity. Pre-treatment with CoQ_{10} decreased the effect, indicating that quercetin may competitively bind to the quinone site of complex I (Lagoa, Graziani et al. 2011). In contrast, Sandoval-Acuña *et al.* demonstrated that quercetin stimulated complexes I and IV, in a CoQ-mimicking fashion, in Caco-2 cells and isolated mitochondria. However, this was studied as preventing complex I inhibition (at the CoQ-binding site) by indomethacin and other non-steroidal anti-inflammatories in the absence of CoQ_{10} (Sandoval-Acuna, Lopez-Alarcon et al. 2012). Essentially, quercetin and CoQ_{10} compete for the CoQ-binding site and can work synergistically to block other inhibitors; when only CoQ_{10} and quercetin are both present, depending on their concentrations, quercetin may be seen to inhibit the action of CoQ_{10}. These two groups used quercetin at lower concentrations than those used by others, with some effects seen at 5 µM. Recently, Waseem and Parvez showed that quercetin increased complex I activity at 50 µM and this was without CoQ_{10} in the assay (Waseem and Parvez 2016).

In cells *in vitro* and in animal models *in vivo*, quercetin prevents or recovers stress-induced mitochondrial dysfunction. Mechanisms are similar to those in isolated mitochondria, including lowered ROS and restored membrane potential, complex I and ATP levels (Punithavathi and Stanely Mainzen Prince 2010, Carrasco-Pozo, Mizgier et al. 2012, Chakraborty, Stalin et al. 2012, Karuppagounder, Madathil et al. 2013, Sandhir and Mehrotra 2013, Bali, Ergin et al. 2014, Ben Salem, Boussabbeh et al. 2016). In cells, quercetin targets signalling pathways related to mitochondrial function in addition to affecting the mitochondria directly. Nrf2-ARE signalling pathways are upregulated to

prevent oxidative stress and hepatotoxicity (Ji, Sheng et al. 2015) and apoptotic pathways are regulated to prevent stress-induced cell death (Carrasco-Pozo, Pastene et al. 2012, Bali, Ergin et al. 2014). In diabetic pathophysiology, preventing oxidative stress and apoptosis in the pancreatic islets preserves insulin synthesis and secretion function (Haddad and Eid 2016), and in the kidneys and cardiovascular system this may protect against diabetic complications (Blake and Trounce 2014). There are some reports of quercetin inducing mitochondrial biogenesis in mammalian cells and tissues via the AMPK-SIRT-PGC-1α axis (Davis, Murphy et al. 2009, Rayamajhi, Kim et al. 2013, Kim, Kwon et al. 2015, Liu, Zou et al. 2015), though the evidence is limited, with others reporting lack of effect (Nieman, Williams et al. 2010, Nichols, Zhang et al. 2015). Rayamajhi *et al*. detected increased mitochondrial biogenesis in HepG2 (human hepatocellular carcinoma, immortalised) cells with 15 μM quercetin that was dependent on the heme oxygenase-1 (HO-1) and carbon monoxide system (Rayamajhi, Kim et al. 2013), and in primary murine hepatocytes , Kim *et al.* showed that PGC-1α was induced by quercetin through a Nrf-2/HO-1-dependent mechanism (Kim, Kwon et al. 2015). HO-1 is typically a stress-induced protein and also regulates autophagy and apoptosis, via AMPK (Dong, Zheng et al. 2015). This suggests that AMPK-mediated pathways play a role in mitophagy as well as biogenesis, basically regulating a mitochondrion-recycling system.

In general, evidence for the effects of quercetin on mitochondrial biogenesis, metabolic flux and the ETS is scarce and often conflicting. Effects are dose-dependent and reliant on cell type, situation and on co-treatment with a stressor and, moreover, are mostly limited to short treatment times or only applying quercetin to the mitochondria once isolated. It remains unclear how the pleiotropic effects of quercetin on mitochondria come together to impact cellular metabolism in the longer term, and there are no existing studies that have looked specifically at mitochondria in cells faced with a high glucose stress. Therefore the first half of this study shall focus on investigating the effects of quercetin on mitochondrial function in HepG2 cells in a high glucose environment, and whether it can alleviate the dysfunction associated with insulin resistance and diabetes. A secondary aim is to further elucidate the effects of quercetin in general, by combining assays for ETS function, metabolic flux, mitochondrial content and related gene expression, alongside intact cell respirometry to provide an overall picture of what a chronic exposure to quercetin may elicit.

1.3.2.3 Kaempferol and its conjugates

There has been much less published on kaempferol than on quercetin. Both flavonols increased glucose uptake in 3T3-L1 adipocytes (Fang, Gao et al. 2008) and kaempferol, obtained as a metabolite from elderflower, dose-dependently increased uptake in porcine myotubes (Bhattacharya, Christensen et al. 2013). In L6 myotubes it was only effective at 30 µM (Kawabata, Sawada et al. 2011). A plant metabolite from hops, kaempferol 3-*O*-neohesperidoside, acquired insulinomimetic status for its ability to increase glucose uptake and glycogen synthesis in rat soleus muscle via the PI3K-dependent pathway (Zanatta, Rosso et al. 2008, Cazarolli, Folador et al. 2009). However, there are no pharmacokinetic studies demonstrating presence of this metabolite in plasma following consumption, which would be a prerequisite for such effects on muscle. Thus, while the effects are clear, the claim regarding overall postprandial glycaemia benefits *in vivo* should be treated with caution. Similarly, Ho *et al*. recently demonstrated that kaempferol may have lasting effects on metabolism; longer incubation times with radiolabelled glucose in the presence of the compound lead to increases in uptake in primary human myotubes and HepG2 cells (Ho, Kase et al. 2017). However, the HepG2 data are not presented and the authors themselves highlight that it is unclear if these compounds are even relevant based on bioavailability *in vivo*, leading to scepticism in interpreting these data. Propolis extract, in which 4′-*O*-methylkaempferol (kaempferide) is a major compound, lowered postprandial glucose levels in mice after oral administration (Ueda, Hayashibara et al. 2013), which could be an indication that kaempferol metabolites in circulation may increase glucose disposal *in vivo*, but there are other flavonoids in propolis that could have been responsible. In a recent study in our lab, 20 µM kaempferol aglycone decreased deoxyglucose uptake in HepG2 cells, but increased glucose uptake (Kerimi, Jailani et al. 2015). The data on kaempferol are minimal and inconclusive and there are no published results for biological effects of the glucuronide or sulfate conjugates that have been identified in the few pharmacokinetic studies (Table 1.1).

1.3.2.4 Ferulic acid and its metabolites

Similar to quercetin, there is some evidence for ferulic acid displaying various antidiabetic effects, but very little on its metabolites. Earlier studies on ferulic acid demonstrated its ability to lower blood pressure, cholesterol and triglyceride levels in hypertensive rats, prevent oxidative stress in neuronal cells and induce anti-inflammatory effects (Pavlica and Gebhardt 2005, Ardiansyah, Ohsaki et al. 2008, Islam, Murata et al. 2008). A recent review by Martini *et al*. highlighted the effects of coffee consumption on lowering markers of oxidative stress in humans. However, despite the number of intervention studies, the authors called for more robust and well-controlled studies to gain more complete understanding (Martini, Del Bo et al. 2016). In recent primary studies, ferulic acid 4-*O*-sulfate, but not ferulic acid, elicited vasorelaxation and lowered blood pressure in mice and dihydroferulic acid exhibited a stronger inhibitory effect on *in vitro* platelet activation than its precursors; dihydroferulic acid, among other colonic catabolites, protected neuronal cells from oxidative stress; and both dihydroferulic acid and ferulic acid 4-*O*-sulfate were increased in plasma following prolonged orange juice consumption in correlation with lower markers of oxidative stress and inflammation (Verzelloni, Pellacani et al. 2011, Baeza, Bachmair et al. 2017, Rangel-Huerta, Aguilera et al. 2017, Van Rymenant, Van Camp et al. 2017). This suggests that the biological activities reported for ferulic acid may be accounted for by its metabolites.

In terms of potential effects in managing glucose metabolism, Prabhakar and Doble showed ferulic acid increased deoxyglucose uptake in 3T3-L1 adipocytes and L6 myotubes via a PI3K-dependent mechanism (Prabhakar and Doble 2009, Prabhakar and Doble 2011) and Jung *et al.* demonstrated that ferulic acid had hypoglycaemic effects in diabetic mice, decreasing blood glucose and cholesterol levels while increasing insulin and hepatic glycogen synthesis (Jung, Kim et al. 2007). Ferulic acid increased glucose uptake and insulin signalling also in other systems (Bhattacharya, Christensen et al. 2013, Gogoi, Chatterjee et al. 2014, Narasimhan, Chinnaiyan et al. 2015, Ho, Kase et al. 2017), but none of these groups tested ferulic acid metabolites. Again, there are no published data on the effects on glucose uptake or metabolism by circulating ferulic acid metabolites.

1.3.2.5 Resveratrol and its conjugates

For some time, resveratrol has been known for its anticancer properties (Aggarwal, Bhardwaj et al. 2004) and, more recently, its conjugates have shown such activity, notably in the colon by resveratrol 3-*O*-sulfate (Aires, Limagne et al. 2013). Much like quercetin, resveratrol has displayed pleiotropic activities, modulating various cellular targets, many of which hold promise as a potential antidiabetic therapeutic. Such effects on glucose uptake and cellular signalling and metabolism were reviewed earlier this year (Leon, Uribe et al. 2017). From *in vitro* and *in vivo* studies, in both animals and humans, it seems that resveratrol only improves insulin sensitivity in insulin-resistant models or individuals (Kang, Hong et al. 2012, Poulsen, Vestergaard et al. 2013, Liu, Zhou et al. 2014). On the other hand, an earlier study suggests that resveratrol may enhance insulin secretion from the pancreas, but only in normal rats and not in streptozotocin-diabetic rats (Chen, Chi et al. 2007). Mechanisms whereby resveratrol may improve insulin sensitivity are centred on AMPK-mediated pathways, rather than those that are PI3K-dependent. Breen *et al*. showed that deoxyglucose uptake was stimulated by resveratrol at 25-100 µM in L6 myotubes and that this was prevented by AMPK or sirtuin inhibition. Furthermore, while resveratrol did not significantly induce translocation of GLUT4 or GLUT1, the GLUT4 inhibitor indinavir also blocked the resveratrol-stimulated uptake (Breen, Sanli et al. 2008). Another study found that GLUT4 translocation was increased *in vivo* in rats and in C2C12 myotubes. GLUT4 expression was not increased but caveolin-3, a protein associated with GLUT4 translocation, was upregulated via activation of estrogen receptor (Tan, Zhou et al. 2012). In another study resveratrol upregulated GLUT4, SIRT1 and activated AMPK in the muscle of diabetics, resulting in improved energy expenditure similar to that with increased exercise (Goh, Lee et al. 2014).

Clinical studies, reviewed in (Leon, Uribe et al. 2017), demonstrated that resveratrol improved insulin resistance and lowered glycaemia, cholesterol, oxidative stress and markers of diabetes. In this review the authors draw on the evidence that the anticancer properties of resveratrol may be attributable, at least in part, to its effects on glucose uptake and metabolism (Leon, Uribe et al. 2017). In cancer cell lines, resveratrol inhibits GLUT1 (Salas, Obando et al. 2013, Gwak, Haegeman et al. 2015) and thus disrupts the glycolytic-dependent metabolism. Via activation of AMPK and SIRT, FOXO1 is another target of resveratrol (Kulkarni and Canto 2015, Sin, Yung et al. 2015), and so is the

insulin signalling pathway. FOXO1 negatively regulates insulin signalling to inhibit glucose uptake and glycolysis via pyruvate dehydrogenase lipoamide kinase 4 (PDK4), and, together with SIRT1 this stimulates mitochondrial function (Canto, Gerhart-Hines et al. 2009, Price, Gomes et al. 2012). Carbon flux is channelled away from the glycolytic pathways, which is another anticancer mechanism. Higher doses of resveratrol (100 μM) have been shown to inhibit insulin in human adipocytes (Gomez-Zorita, Treguer et al. 2013) and AMPK, β-oxidation and glucose metabolism in human myotubes (Skrobuk, von Kraemer et al. 2012), revealing its hormetic effects, akin to the dose-dependent effects of quercetin.

Resveratrol is also implicated in the management of diabetic complications, including CVDs (Huang, Huang et al. 2010). Multiple benefits of resveratrol have been highlighted, with further examples provided in Chapter 3. Although, once again, all reports are for the aglycone and not on resveratrol conjugates. However, resveratrol is that is rapidly metabolised *in vivo* and is found circulating almost entirely in conjugated forms (see 1.3.1.4). So while numerous studies demonstrate that resveratrol impacts glucose uptake and metabolism, with implications for diabetes, cancer, or CVDs, not one has examined the relevance of the sulfate or glucuronide conjugates to these effects.

1.3.2.6 Berries, anthocyanins and their phenolic sulfate metabolites

Berries are rich in anthocyanins among other (poly)phenols and ingestion results in a range of plasma metabolites. Human intervention studies have found antidiabetic benefits to consuming berries (Castro-Acosta, Lenihan-Geels et al. 2016) possibly linked to these metabolites. Supplementation with blueberries twice daily for 6 weeks was found to improve insulin sensitivity in obese, insulin-resistant individuals (Stull, Cash et al. 2010) and in two studies by Torronen *et al*. berries reduced postprandial insulin responses to ingestion of bread and postprandial glucose responses to sucrose, both in healthy subjects (Torronen, Sarkkinen et al. 2010, Torronen, Kolehmainen et al. 2013). A study in our lab found that for healthy subjects, consumption of a (poly)phenol-rich fruit purée containing apple and berries, along with green tea, also helped to attenuate postprandial glucose and insulin responses (Nyambe-Silavwe and Williamson 2016). Similar effects were seen for bilberry (Hoggard, Cruickshank et al. 2013) and strawberry lowered postprandial insulin and inflammatory markers (Edirisinghe, Banaszewski et al. 2011). The (poly)phenols found in cinnamon include caffeic acid and protocatechuic acid

(Rothwell, Perez-Jimenez et al. 2013) and, consequently, similar metabolites to those from berries may arise in the plasma following consumption. Several human studies have demonstrated benefits to eating cinnamon (Khan, Safdar et al. 2003, Hlebowicz, Hlebowicz et al. 2009, Lu, Sheng et al. 2012), for which phenolic sulfates may, at least in part, be accountable. Furthermore, as these metabolites have been shown to originate from chlorogenic acids (Xie, Lee et al. 2016), the same may apply for the beneficial effects of drinking coffee.

In vitro investigations suggest the primary mechanisms for the anthocyanin effects *in vivo* are inhibition of carbohydrate digestion and absorption in the gut (Adisakwattana, Ngamrojanavanich et al. 2004, McDougall, Shpiro et al. 2005, Iwai, Kim et al. 2006, Akkarachiyasit, Charoenlertkul et al. 2010, Ifie, Marshall et al. 2016) and increased glucose uptake into muscle and adipose. A range of studies established that a major parent anthocyanin conjugate, cyanidin 3-*O*-glucoside, and/or its metabolite, protocatechuic acid, elevated deoxyglucose uptake in L6 and C2C12 myotubes and in 3T3-L1 and human adipocytes via both insulin- and AMPK-dependent pathways (Yamamoto, Ueda et al. 2010, Scazzocchio, Vari et al. 2011, Chellan, Muller et al. 2012, Rojo, Ribnicky et al. 2012, Scazzocchio, Vari et al. 2015, Luna-Vital, Weiss et al. 2017). In the study by Rojo *et al*. the anthocyanin glucoside extract also lowered hepatic gluconeogenesis (Rojo, Ribnicky et al. 2012) and Luna-Vital *et al.* showed additional effects of cyanidin 3-*O*-glucoside in reducing adipocyte precursor differentiation, fatty acid synthase activity, inflammatory markers, leptin and ROS (Luna-Vital, Weiss et al. 2017). An earlier study generated similar data, as well as showing increased gene expression for mitochondrial function in muscle (Matsukawa, Inaguma et al. 2015) and an earlier review highlighted the potential benefits of anthocyanins for hepatic lipid metabolism (Valenti, Riso et al. 2013). While protocatechuic acid and cyanidin 3-*O*-glucoside are detected following anthocyanin ingestion, they comprise only a small percentage of the circulating metabolites (de Ferrars, Czank et al. 2014), so this could be another example of research focusing on potentially an irrelevant form of a compound and claiming it could account for antidiabetic effects of foods or extracts *in vivo*.

Phase II metabolites, such as vanillic acid and its sulfates, circulate in much greater proportions (de Ferrars, Czank et al. 2014, Ludwig, Mena et al. 2015). In another study by Ho *et al*., both protocatechuic and vanillic acids, among several other anthocyanins

and (poly)phenols, increased glucose uptake but this study has the same drawbacks as the one scrutinised in section 1.3.2.3 with regard to kaempferol (Ho, Kase et al. 2017). Almost no tests were performed for the phase II metabolites downstream of anthocyanins, protocatechuic acid and gallic acid absorbed from berries. The Haddad group took a small step in the right direction, with their two studies on fermented blueberry juice, which lowered hepatic gluconeogenesis and triglyceride content in adipocytes while increasing hepatic glycogen synthase activity and glucose uptake in muscle and adipose via AMPK (Vuong, Martineau et al. 2007, Nachar, Eid et al. 2017). However, in the subsequent experiments to elucidate the compounds responsible, the authors focused on the parent compounds identified in the juice, rather than potential metabolites.

The biological activities of phase II (poly)phenol metabolites from berries are practically unknown. Of the limited data available, most concern anti-inflammatory effects. Vanillic acid lowered cyclooxygenase-2 expression and NFκB activity, leading to lowered plasma interleukin (IL)-6 and suppressed ulcerative colitis in mice (Kim, Kim et al. 2010) and was shown to suppress elevated cytokine levels and improve sinusoid organisation in a murine liver injury model (Itoh, Isoda et al. 2009). Two recent studies by the Kay group highlighted the potential of anthocyanin and flavonoid metabolism to enhance vascular efficacy (Warner, Zhang et al. 2016, Warner, Smith et al. 2017). The metabolites, but not the parental compounds, inhibited vascular cellular adhesion molecules (VCAM) and inflammatory markers in human endothelial cells. Among the compounds tested, isovanillic acid and its 3-*O*-glucuronide metabolite lowered soluble VCAM-1 expression (Warner, Zhang et al. 2016) and pre-treating endothelial cells with anthocyanin metabolite signatures, which included (iso)vanillic acids and their glucuronide and sulfate metabolites, inhibited TNF-α stimulation of VCAM-1 and IL-6 secretions (Warner, Smith et al. 2017). Similarly, isovanillic acid and its 3-*O*-glucuronide conjugate have been shown to reduce lipopolysaccharide-induced TNF-α secretion in monocytes (di Gesso, Kerr et al. 2015) and a mixture of blueberry anthocyanin metabolites, which included vanillic acid 4-*O*-sulfate and isovanillic acid 3-*O*-sulfate, ameliorated palmitate-induced cell adhesion and inflammation in endothelial cells (Cavalcanti, Begaye et al. 2015). There are no published data for biological activities of other berry metabolites catechol-*O*-sulfate, 4-methylcatechol-*O*-sulfate, pyrogallol-*O*-sulfate or 4-methylgallic acid 3-*O*-sulfate.

Complementary to the antidiabetic effects, recent investigations have exhibited promising results for the consumption of anthocyanin-rich beverages or anthocyanin extract supplementation to aid skeletal muscle performance in athletes. A blackcurrant extract given to healthy men for 7 days, in a randomised, double-blind, crossover intervention, lowered blood pressure and muscle oxygen saturation while increasing cardiac output, femoral artery diameter and enhancing muscle activity (Cook, Myers et al. 2017). An açai beverage increased time to exhaustion in athletes running on a treadmill, while attenuating metabolic stress induced by the exercise (Carvalho-Peixoto, Moura et al. 2015). Moreover, six weeks of daily supplementation with 100 mg anthocyanin tablets significantly increased VO_2 max in athletes, though no changes in body composition or muscle damage were observed (Yarahmadi, Askari et al. 2014). A recent review of several studies that investigated the effects of cherry consumption on exercise-induced muscle damage found that typically the cherry juices prevented soreness and improved muscle recovery, while lowering or unchanging biomarkers of oxidative stress and inflammation (Coelho Rabello Lima, Oliveira Assumpcao et al. 2015). Further work is required, however, to elucidate specific mechanisms of action and compounds responsible and the anthocyanins do not always produce measureable effects, for example cherry juice had no effect on water polo players, though this could also indicate a dependence on the type of exercise involved and habitual diets of the participants (McCormick, Peeling et al. 2016). It would be interesting to investigate the physiological effects of anthocyanin metabolites on skeletal muscle both *in vivo* and *in vitro*.

1.3.2.7 Skeletal muscle cell models for studying glucose uptake and metabolism

The majority of *in vitro* analyses of the effects of parent (poly)phenol compounds on glucose uptake and metabolism have been done on rat or mice cell models, with some experiments in primary human cells. Primary cells from biopsies, ideally taken during the course of a disease, are important because they closely match the genotype of muscle *in vivo*, though only for the specific donor. However, this model relies on the availability of samples, there can be substantial variation in cells between cultures, even from the same donor, and they have a limited lifespan. The advantage of using an immortalised muscle cell line is that experiments can be easily seeded for with consistent preselected myoblast densities, multiple treatments across different dishes and allow for numerous biological (and technical) replicates of reproducible monolayer cultures. Use of an

immortalised clone enables a pure myogenic culture, free from contamination of other cells such as fibroblasts in an *ex vivo* or primary system, which is readily propagated to provide an unlimited source of cells of the same genetic background. Suitable markers of muscle differentiation are essential however, because proliferating myocytes eventually lose myogenic characteristics and become fibroblastic, and the degree of fusion and differentiation can vary (Miranda 2014).

While established rodent cell lines such as L6 or C2C12 for skeletal muscle, or 3T3-L1 in the case of adipose tissue, are useful tools for probing the effects of compounds on glucose uptake and metabolism, the mechanisms in play and the extent to which changes are induced may differ to those in human cells, thus caution should be taking when interpreting data from such models. In a recent study, cultured murine and human satellite cells were compared and divergence in the myogenic genetic programming were highlighted, indicating that the mechanisms regulating differentiation between the species are not all the same, and there were differences in inflammatory signalling in response to cytokine stimulation (Bareja, Holt et al. 2014). Differences between mouse and human at the transcriptome level are dependent on the muscle tissue type (Kho, Kang et al. 2006, Breschi, Gingeras et al. 2017). Expression of myosin heavy chain varies between muscle type and species (human, rat and mouse) at the tissue level (Bloemberg and Quadrilatero 2012) and glycosylation of proteins at the plasma membrane of differentiated myotubes *in vitro* also varies between mouse and human (McMorran 2017). Important to the context of this research project, an earlier review on the GLUT family highlighted the difference between rat and human glucose transport kinetics in response to insulin *in vitro* and the variation in GLUT4 expression between a diabetic mouse model and diabetic humans (Gould and Holman 1993).

Taking this information into account, in the present study, the immortalised human skeletal muscle cell line, LHCN-M2 (human skeletal myoblasts immortalised with lox-hygro-hTERT ("LH") and Cdk4-neo ("CN"), well-differentiating subclone M2) (Zhu, Mouly et al. 2007), is characterised as a model to explore the unknown effects of relevant (poly)phenol metabolites on glucose uptake and metabolism. Regardless of the pros and cons of different cell models, this is the first study on the effects of any (poly)phenol compounds on glucose in immortalised human myotubes.

1.4 Concluding remarks and project aims

Type 2 diabetes is a global endemic caused primarily by overnutrition and lack of physical activity. This results in a perturbation in glucose and lipid metabolism with increased systemic oxidative stress and mitochondrial dysfunction, elevated inflammation, and a loss of insulin sensitivity in liver, adipose tissue and skeletal muscle. (Poly)phenol-rich foods have been linked to the prevention and management of this disease and information from the literature suggests that this is, in part, via the prevention of oxidative stress and improvements in postprandial glucose uptake. However, most of the mechanisms remain to be ascertained or clarified in detail.

Quercetin in particular has shown promise in restoring mitochondrial function and yet there have been no studies investigating its effects in a high glucose environment. Furthermore, a greater understanding is required of its pleiotropic mechanisms of action and how its hormetic effects on the mitochondria may affect cellular metabolism in general, and glucose and lipid metabolism in the liver specifically. To this end, the well-established HepG2 cell line is utilised to investigate the mitochondrial dysfunction caused by high glucose and any effects that quercetin may have in ameliorating this. A combination of ETS activity assays and whole cell respirometry are used, together with markers for redox status and mitochondrial content and function, providing detail on numerous aspects of mitochondrial physiology.

A range of human intervention, animal and *in vitro* studies demonstrate that (poly)phenols may improve postprandial glycaemia and specifically uptake into skeletal muscle, but the effects of relevant metabolites that would be found in the plasma *in vivo* are unknown. Berries are one source of (poly)phenols for which trials have demonstrated the ability to affect postprandial glycaemia, insulinaemia and sensitivity and inflammation. The consumption of berries gives rise to a range of diverse plasma metabolites, but knowledge on the biological activity of specific compounds is unknown, in particular relating to their potential effects on glucose uptake. As such, the LHCN-M2 myoblast cell line shall be characterised and used as a model for human skeletal muscle to research the effects of glucose, insulin and (poly)phenol metabolites on glucose uptake and cellular metabolism.

Here is an outline of the specific aims for this project:

- Determine novel effects of high glucose and oxidative stress on mitochondrial function in HepG2 cells.
- Explore the acute and chronic effects of quercetin on mitochondrial physiology in HepG2 cells grown in a physiologically normal or high glucose concentration.
- Propose the mechanism(s) of action for the potential protective role of quercetin against high glucose-induced mitochondrial dysfunction.

- Characterise the LHCN-M2 cell line and establish appropriate biomarkers to assess its use as a model of human skeletal muscle specifically for metabolic studies.
- Investigate the effects of glucose and insulin on cellular metabolism and muscle characteristics such as myotube differentiation, metabolic phenotype and insulin-stimulated glucose uptake.
- Analyse the potential effects of relevant (poly)phenol metabolites, which can be found in circulation *in vivo* after the consumption of common parent compounds, on glucose uptake and metabolism in differentiated myotubes.
- Elucidate the mechanism(s) of action through which any effects may be elicited, such as expression of the GLUTs and interaction with insulin signalling.

Chapter 2

Quercetin preserves electron transfer system efficiency and redox status and stimulates mitochondrial function in high glucose-stressed HepG2 cells.

2.1 Abstract

Hyperglycaemia results in enhanced intracellular ROS, leading to mitochondrial damage and increased risk of developing insulin resistance and type 2 diabetes. Quercetin accumulates in mitochondria and by affecting various molecular targets it may beneficially impact cellular metabolism. The aim here was to investigate whether quercetin could reverse chronic high glucose-induced oxidative stress and mitochondrial dysfunction. HepG2 cells were cultured in normal (5.5 mM) or high (25 mM) glucose for 4 days and treated with various concentrations of quercetin. After culturing in high glucose, complex I activity in isolated mitochondria was significantly decreased, but this was concentration-dependently recovered by quercetin treatment. In addition, cellular ROS generation was lowered by quercetin in both high and low glucose. Respirometry data showed that quercetin reversed the detrimental increase in IMM proton leakage in high glucose. Oxidative respiration was also increased by quercetin, owing to elevated net mitochondrial respiration despite a decrease in ETS capacity, and lower non-ETS oxygen consumption. Furthermore, quercetin stimulated increases in cellular NAD^+/NADH and subsequently in PGC-1α mRNA (messenger ribonucleic acid), a well-established marker of mitochondrial biogenesis and function. These results suggest that quercetin may restrict high glucose-induced mitochondrial stress by increasing cellular NAD^+/NADH and activating PGC-1α-mediated pathways to increase mitochondrial oxidative function. The key outcome of this is lowered ROS generation and an improvement in complex I activity and ETS coupling efficiency after quercetin treatment, where the oxidative stress generated by high glucose is diminished and ultimately mitochondrial integrity is enhanced.

2.2 Introduction

Hyperglycaemia is a typical biomarker of obesity, type 2 diabetes and metabolic syndrome (Krentz and Hompesch 2016), since high levels of glucose in the blood lead to intracellular oxidative stress in tissues involved in glucose metabolism (Murphy 2009, Yan 2014, Gero, Torregrossa et al. 2016). Although mitochondria are a source of oxidative stress, they are also a principal target of attack by reactive oxygen and nitrogen species themselves (Starkov 2008, Kang and Pervaiz 2012). The resulting mitochondrial dysfunction exacerbates the stress further in a downward spiral linked with a perturbation in glucose and lipid metabolism, insulin resistance, and ultimately hyperglycaemia, and the onset of type 2 diabetes (Sivitz and Yorek 2010, Blake and Trounce 2014, Luo, Li et al. 2015, Chow, Rahman et al. 2016, Zaccardi, Webb et al. 2016). The cause-effect relationship between mitochondrial dysfunction and insulin resistance is still debated (Montgomery and Turner 2015), but it is likely they both occur as a result of the high glucose stress.

Quercetin is a flavonoid that accumulates in mitochondria (Fiorani, Guidarelli et al. 2010) and affects their function directly and indirectly through various signalling pathways, such as those associated with biogenesis, metabolic flux, respiration, mitochondrial membrane potential and apoptosis (Fiorani, Guidarelli et al. 2010, Sandoval-Acuna, Ferreira et al. 2014, de Oliveira, Nabavi et al. 2016). Quercetin and other flavonoids may have beneficial effects in modulating glucose digestion and absorption (Nomura, Takahashi et al. 2008, Williamson 2013, Nyambe-Silavwe, Villa-Rodriguez et al. 2015) and metabolism, through affecting expression of glycolysis-involved genes (Leiherer, Stoemmer et al. 2016) and the aforementioned effects on mitochondria. In streptozotocin-induced diabetic rats, quercetin dose-dependently decreased blood glucose, cholesterol and triglyceride levels, as well as increasing hepatic glucokinase activity (Vessal, Hemmati et al. 2003) and decreasing hepatic oxidative stress markers (Chis, Muresan et al. 2016). Activities on mitochondrial function and glucose homeostasis make quercetin a widely-studied compound for its potential uses in the prevention and management of type 2 diabetes (Akash, Rehman et al. 2014, Haddad and Eid 2016), however details of its pleiotropic mechanisms require further elicitation, not least that studies are lacking in considering these mechanisms in a high glucose environment.

The mechanisms of action of quercetin on mitochondria in hepatic cells, grown chronically in high or normal glucose (to model hyperglycaemia versus normoglycaemia), were explored in light of its potential to prevent oxidative stress, mitochondrial damage and subsequent diabetes pathophysiology at the cellular level. The majority of previous *in vitro* studies looking at the effects of quercetin on mitochondria have often employed acute treatments (several minutes or hours) directly to isolated organelles in a kinetic experiment setup (Lagoa, Graziani et al. 2011, Sandoval-Acuna, Lopez-Alarcon et al. 2012, Waseem and Parvez 2016), but in the present study it was assessed as to how intracellular mitochondrial function was affected by prolonged quercetin exposure. Complex I of the electron transfer system (ETS) was focussed on, as it is known to be a major source and target of mitochondrial superoxide generation (Hirst and Roessler 2016) and thus is central to high glucose-induced oxidative stress (Blake and Trounce 2014, Yan 2014), with the hypothesis that quercetin may have an alleviating role. Surplus glucose and thus NADH can overload complex I and cause excess superoxide generation and Luo et al. suggested if there was a compound that would allow complex I to be bypassed in the ETS it would dissipate the ROS generated (Luo, Li et al. 2015); quercetin may do just that. Lagoa et al. found that quercetin lowered hydrogen peroxide production and inhibited complex I in isolated rat brain mitochondria, with the latter being reversed by addition of CoQ_{10} (Lagoa, Graziani et al. 2011). Sandoval-Acuña et al. added quercetin to mitochondria isolated from rat duodenum and found it protected complex I from inhibition by non-steroidal anti-inflammatory drugs, suggesting a CoQ-like function for the flavonoid (Sandoval-Acuna, Lopez-Alarcon et al. 2012). This could be a chronic mechanism by which quercetin acts when complex I is faced with high glucose oxidative stress.

Likewise, the impact of quercetin on cellular and mitochondrial respiration in intact cells was investigated, and how respiration may be controlled via cellular oxidative status, drawing comparisons between high and normal glucose-treated cells. There is evidence of quercetin improving mitochondrial bioenergetics, increasing respiration coupling efficiency in rat pancreatic cells (Carrasco-Pozo, Tan et al. 2016) and increasing respiratory capacity and cell survival in primary cortical neuronal cells (Nichols, Zhang et al. 2015). Quercetin has also been shown to stimulate mitochondrial metabolic signalling pathways (Nichols, Zhang et al. 2015). Rayamajhi et al. and Kim et al. showed that quercetin increases PGC-1α, NRF-1 and mitochondrial transcription factor A

(TFAM) mRNA in HepG2 cells and mouse primary hepatocytes respectively (Rayamajhi, Kim et al. 2013, Kim, Kwon et al. 2015), but this has not yet been tested in a human *in vitro* system with a high glucose stress and chronic quercetin treatment. Presented here for the first time are the dose-dependent chronic (≥ 24 h) effects of quercetin on intracellular complex I activity and mitochondrial respiration in cells stressed by chronic high glucose, and with this a proposed mechanism by which quercetin exerts these effects, directly and via gene expression and oxidative signalling pathways.

2.3 Materials and Methods

2.3.1 Chemical compounds and reagents

All cell culture medium components and all other reagents were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. High purity (18.2 MΩ cm^{-1}) water supplied by a MilliQ system (Merck Millipore UK, Watford, UK) was used throughout this work.

2.3.2 Cell culture

HepG2 cells were derived originally from adolescent human hepatocellular carcinoma and is a well-established cell line (Knowles 1983) of the American Type Culture Collection (HB-8065, LGC Promochem, Teddington, UK). The cells were maintained in Eagle's minimum essential medium (MEM) containing 5.5 mM glucose and supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2% (v/v) non-essential amino acids, and 1% (v/v) sodium pyruvate, in a humidified atmosphere of 5% CO_2/95% air. Medium was changed at least every two days while sub-culturing and cells were used for experiments during passages 83-91, for which they were seeded in 75 cm^2 flasks, 6-well, 24-well or 48-well plates (all from Appleton Woods, Birmingham, UK), or 100 mm^2 dishes (Greiner Bio-One, Stonehouse, UK) at a density equivalent to 8 x 10^4 cells cm^{-2}. Medium was supplemented with additional glucose for cells grown in 25 mM glucose for experiments but was otherwise unchanged. Cells were monitored and images acquired using the Leica DM IL LED (Milton Keynes, UK) inverted microscope. For experiments, N/n values indicate

the number of independent biological passages (N) and the number of technical replicates (n) from which the data were acquired.

2.3.3 Trypan Blue exclusion cell viability assay

HepG2 cells were seeded into T75 flasks or 6-well plates and maintained in MEM containing 5.5 mM (NG) or 25 mM (HG) glucose for 96 h. Medium was changed 48 h after seeding and then again for the final 24 h but FBS was removed and 10 or 20 µM quercetin (Extrasynthese, Genay, France) (or DMSO controls) added. After the 24 h treatment cells were lifted with 0.25% trypsin-EDTA or TrypLE Express (Thermo Fisher Scientific, Warrington, UK), resuspended in 1 ml medium and an aliquot mixed 1:1 (v/v) with 0.4% Trypan Blue-PBS. Resuspended cells were counted and assessed for Trypan Blue exclusion as done previously (Strober 2001), but using a TC10 automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA). Viable cells excluding Trypan Blue were given as a percentage of total cells counted.

2.3.4 Isolation of mitochondria

An important aspect to this work was optimising the mitochondrial isolation process to gain viable and intact mitochondria. HepG2 cells were seeded into T75 flasks and grown in normal or high glucose medium for 96 h; for the final 24 h without FBS but with 2.5, 5, 10 or 20 µM quercetin (or DMSO controls) added. Cells were scraped into 1 ml PBS supplemented with 1% (v/v) protease inhibitor cocktail P8340, centrifuged at 210 g, 4^{o}C for 5 min and snap-frozen in ethanol over dry ice. Cell pellets were stored at -80^{o}C until required for mitochondrial isolation. Mitochondria were prepared from thawed cell pellets by homogenisation and differential centrifugation (see Fig. 2.1) using the reagents and Dounce homogeniser from the Mitochondria Isolation Kit for Cultured Cells (ab 110171, Abcam, Cambridge, UK). The manufacturer's protocol guidelines were followed, but adapted with an extra 1000 g, 5 min centrifugation step added at the end for a purer preparation.

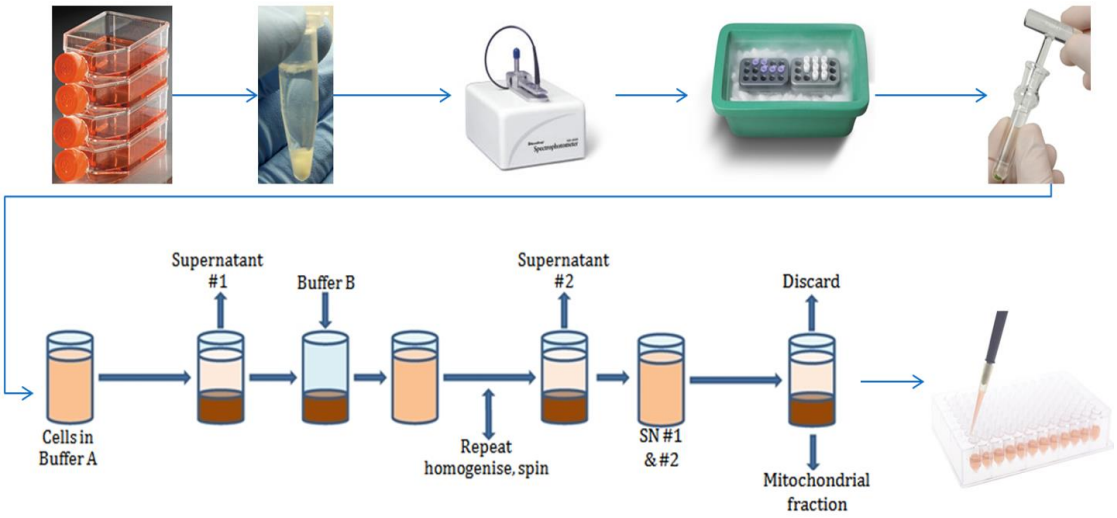


Figure 2.1: Isolation of mitochondria from HepG2 cells. Several homogenisation and differential centrifugation steps were used. Mitochondrial fraction was resuspended in 250 mM sucrose/10 mM HEPES/50 mM Tris buffer and spun again to remove any remaining debris.

Cell pellets were thawed then frozen-thawed twice more and whole cell pellet protein determined by absorbance at 280 nm on the NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA). Cells were resuspended at 5 mg/ml in the 'Reagent A' (containing triethanolamine, Tris, digitonin, EDTA) provided, incubated on ice for 10 min and homogenised in the pre-cooled 2 ml glass Dounce homogeniser, with 30 strokes of pestle B. The homogenate was centrifuged at 1000 g, 4^{o}C for 10 min (SPIN 1a) and the supernatant recovered. The pellet was resuspended in 'Reagent B' (containing Tris, digitonin, EDTA) in the same volume as used of Reagent A, and the incubation, homogenisation and centrifugation (SPIN 1b) repeated and the supernatant recovered, this time the pellet was discarded. The supernatants from SPINs 1a and 1b were combined and centrifuged at 12,000 g, 4^{o}C for 15 min (SPIN 2) and the pellet collected. The pellet, crude mitochondrial isolate, was resuspended in 0.5 ml 250mM sucrose/10 mM HEPES/50mM Tris buffer, pH 7.4 and centrifuged once more at 1000 g, 4^{o}C for 5 min (SPIN 3). The final mitochondrial pellets were resuspended in 250 mM sucrose/10 mM HEPES/50 mM Tris, pH 7.4, supplemented with 1% (v/v) protease inhibitor cocktail P8340, snap-frozen and stored at -80^{o}C until used for complex I and Western analyses.

2.3.5 Western blot assessment of mitochondrial isolation process

Aliquots from throughout the mitochondrial isolation process, of the initial cell lysate, supernatants, resuspended pellets and the final mitochondrial isolates, (see 2.3.4) were taken and snap frozen and stored in -80^{o}C to analyse by Western blot at a later date. Once thawed, total protein in each aliquot was determined by absorbance at 280 nm on the NanoDrop and 10µg of each were incubated with 4x Laemmli sample buffer (Bio-Rad Laboratories, Hemel Hempstead, UK) at 37^{o}C for 15 min. Standard sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) procedures were followed using a *Mini-PROTEAN TGX Stain-Free Precast Gel* (Bio-Rad Laboratories, UK) and then subsequent transfer to polyvinylidene fluoride (PVDF) membranes. After blocking, membranes were probed with mouse anti-NDUFB8 (ab110242, Abcam) (subunit of complex I) primary antibody followed by 1:10,000 horseradish peroxidase-conjugated (-HRP) secondary antibody (Bio-Rad Laboratories, UK). Immunoreactive proteins were visualised using *Clarity Western ECL Substrates* (Bio-Rad Laboratories, UK) luminol and peroxide (1:1 mix) and then developed and fixed onto photographic film (Fig 2.2).

2.3.6 Western analysis of mitochondrial fraction purity

Total protein in aliquots of the initial cell lysate and the isolated mitochondrial fraction of HepG2 cells was determined by absorbance at 280 nm on the NanoDrop and prepared for running on the ProteinSimple Wes automated western blot system (Bio-Techne, ProteinSimple, San Jose, CA, USA) using reagents supplied in the system kit according to the manufacturer's guidelines. Samples were denatured at 37^{o}C for 20 min and loaded onto the pre-filled assay plate at 4.8 mg/ml mitochondrial protein, with 1:100 primary mouse anti-NDUFB8 and the supplied anti-mouse secondary. Chemiluminescence was detected in high dynamic range (HDR) mode and the peaks seen at 27 kDa converted to traditional Western-like bands. This confirmed increased mitochondrial protein (NDUFB8) in the mitochondrial isolate, as seen with the traditional Western Blot (Fig. 2.2).

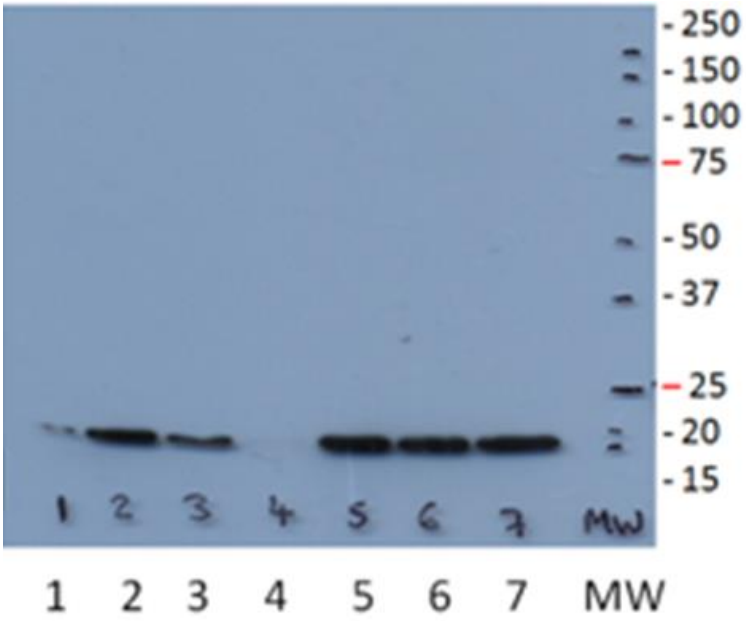


Figure 2.2: Western blot showing the expression of mitochondrial protein complex I subunit NDUFB8 in aliquots taken throughout mitochondrial isolation process. Protein samples were subjected to SDS-PAGE procedures with subsequent transfer to PVDF membranes. After blocking, membranes were probed with mouse anti-NDUFB8 primary antibody followed by HRP-conjugated secondary antibody. Immunoreactive proteins were visualised using luminol and peroxide and developed and fixed onto photographic film. 1 – whole HepG2 cell lysate; 2 – supernatant after first centrifugation; 3 – supernatant after second centrifugation; 4 – supernatant after third centrifugation, containing little to no mitochondrial material; 5-7 – resuspended mitochondrial isolates following high-speed centrifugation of combined first and second supernatants.

2.3.7 Complex I activity assay

Mitochondria from HepG2 cells grown in normal or high glucose for 96 h and treated with varying concentrations of quercetin (or DMSO controls) for the final 24 h were isolated in 250 mM sucrose/10 mM HEPES/50 mM Tris, pH 7.4, and assayed for complex I specific activity spectrophotometrically in 96-well plates on a PheraSTAR FS microplate reader (BMG LabTech, Ortenberg, Germany) at 37ºC. 10 µg isolated mitochondria (as determined by NanoDrop) were mixed with 150 µM NADH (VWR, Lutterworth, UK) and 50 µM coenzyme Q_1 (CoQ_1, analog of CoQ_{10}) and the decrease in absorbance at 340 nm followed for 60 min, as done previously (Ragan 1987, Janssen,

Trijbels et al. 2007, de Wit, Scholte et al. 2008, Usta, Kreydiyyeh et al. 2009, Oliveira, Kiyomoto et al. 2013) and optimised for sample mass and NADH/CoQ1 concentrations (see 2.3.7.1). Specific activity was calculated by converting rate of absorbance change into rate of NADH decrease per mg of total protein and then normalised to the normal glucose controls.

2.3.7.1 Optimising the complex I assay

Complex I enzyme activity was assayed spectrophotometrically using the isolated HepG2 mitochondria with various reaction conditions and protocol variants evaluated. After initial trials, the assay was tried with varied mitochondrial preparation amounts (enzyme concentration) and it was deemed that 10 µg mitochondria was sufficient to give a linear rate in NADH oxidation over 60 min, while allowing for plenty of material from each cell pellet. Varying NADH (substrate) concentrations of 150 µM, 500 µM, 750 µM and 1500 µM were assayed. ≥750 µM gave absorbance measurements of ≥2.0 and therefore could not be used and while 500 µM and 750 µM gave good rates, the original 150 µM NADH concentration gave the most reproducible and linear data. Furthermore, >150 µM NADH showed too much non-mitochondria-specific oxidation. Plotting complex I activity against NADH concentration gives a linear curve up to 500 µM and reaches V_{max} at >500 µM; complex I is saturated with substrate.

Linalool was added to the assay, as done by Usta *et al.* (Usta, Kreydiyyeh et al. 2009), at concentrations of 5 µM and 20 µM against an ethanol control in the hope of being able to use linalool as a negative control. However, across several assays it was shown to inhibit complex I activity in the initial rate of reaction but then the activity recovered, suggesting a reversible inhibition. By the end of the assay complex I activity with 20 µM was actually greater than that of the control; it may also be that the ethanol in the control is more detrimental than the linalool, despite the data published in the literature. Coenzyme Q_1 (CoQ_1) was added to the assay as an analog of coenzyme Q_{10} (ubiquinone), with and without linalool (1 µl of linalool was added neat, giving a final concentration of 27 mM, which was >1000x greater than previously used so any effect linalool was having would potentially be clearer). The CoQ_{10} was tried at 50 µM and 100 µM as has previously been published (Ragan 1987, Usta, Kreydiyyeh et al. 2009) and had a dramatic effect on the rate of NADH oxidation. There was a 500% increase in activity when 50 µM CoQ_1 was present in the assay and a ~900% increase with 100 µM CoQ_1,

compared to no CoQ_1 (with the volume of CoQ_1 replaced with buffer) (Fig 2.3). Furthermore, the linalool showed an inhibitory effect previously unseen when the CoQ_1 was present, and when the linalool was at lower concentrations. In the absence of CoQ_1 linalool had no effect on activity compared with the ethanol control, but with 50 µM CoQ_1 activity was decreased by 64% (Fig. 2.3). CoQ_1 acts as a final electron acceptor from the oxidation of NADH by complex I and therefore allows the reaction to progress at a quicker rate. The fact that inhibition is seen only with CoQ_1 present indicates that the linalool has an effect on the mechanism involved in complex I reducing the ubiquinone analog. Linalool could be used in future work as a negative control, however with such a high concentration required for complex I inhibition it may have other unseen detrimental effects and rotenone-inhibited controls are sufficient for the investigations here.

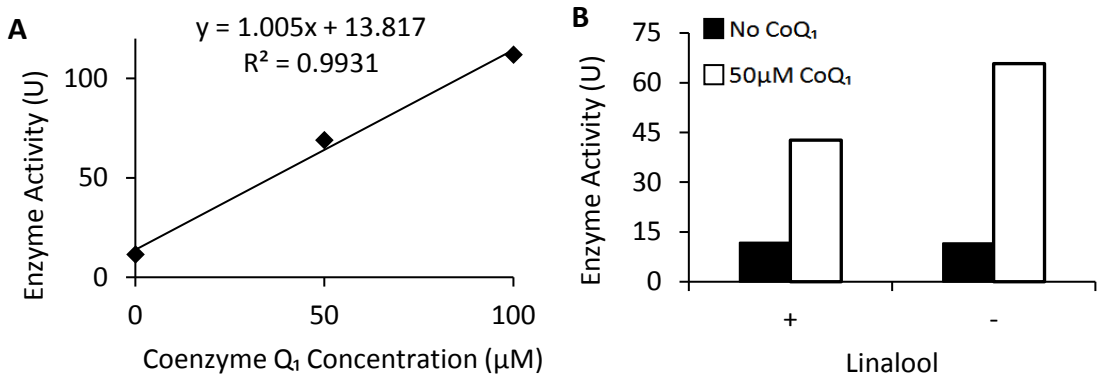


Figure 2.3: NADH oxidase activity of complex I in isolated HepG2 mitochondria. The effect of varying coenzyme Q_1 (CoQ_1) (A) and of CoQ_1 on linalool inhibition (B) on the complex I activity in 10 µg mitochondria, isolated from HepG2 cells grown for 96 h in 5.5 mM glucose, assessed with NADH (150 µM), corrected for non-mitochondrial oxidation by blank and rotenone-inhibited (2 µM) controls.

2.3.8 DCFH-DA ROS assay

HepG2 cells were grown in 6-well plates for 96 h in normal or high glucose and treated for the final 2, 12 or 24 h with 2.5, 5, 10 or 20 µM quercetin (or DMSO controls). The DCFH-DA assay was used for assessing oxidative stress as previously (Wang and Joseph 1999), but with modifications described as follows. Cells were washed with PBS, incubated with 10 µM DCFH-DA in PBS, 37^{o}C, 20 min in darkness, washed again and fluorescence of DCF was measured on a PheraSTAR FS (Ex/Em = 485/530 nm). Cells were then scraped into and lysed in CellLytic M supplemented with 1% (v/v) P8340 protease inhibitor cocktail and total protein measured using the Bradford assay (Pierce Coomassie (Bradford) Protein Assay Kit #23200, Thermo Fisher Scientific) as described previously (Bradford 1976, Ernst and Zor 2010). DCF fluorescence was corrected for protein and data normalised to a relative total ROS percentage of the controls.

2.3.9 *MitoOrange* fluorescence and confocal microscopy

The cytopainter 'MitoOrange' (ab176831, Abcam) was used to probe for mitochondria in live HepG2 cells grown on 48-well plates in normal or high glucose or galactose. The manufacturer's protocol was followed; the 500X MitoOrange was diluted to 1X in 20 mM HEPES in Hank's Balanced Salt buffer and added to the cells with medium 1:1 (v/v) for 30 min in the incubator. The MitoOrange-containing buffer/medium was aspirated, the cells washed with the buffer and medium only added. The labelled cells were subsequently viewed under the *Zeiss LSM 700* confocal laser scanning microscope (Fig. 2.4) as well as assayed for fluorescence intensity (Ex/Em 540/590) on the PheraSTAR FS plate reader. When the cells were treated with quercetin, it was found that the flavonoid interfered with the uptake of the MitoOrange antibody and thus this was not pursued further; the citrate synthase activity assay provided a more accurate, and quantitative, measurement of mitochondrial content.

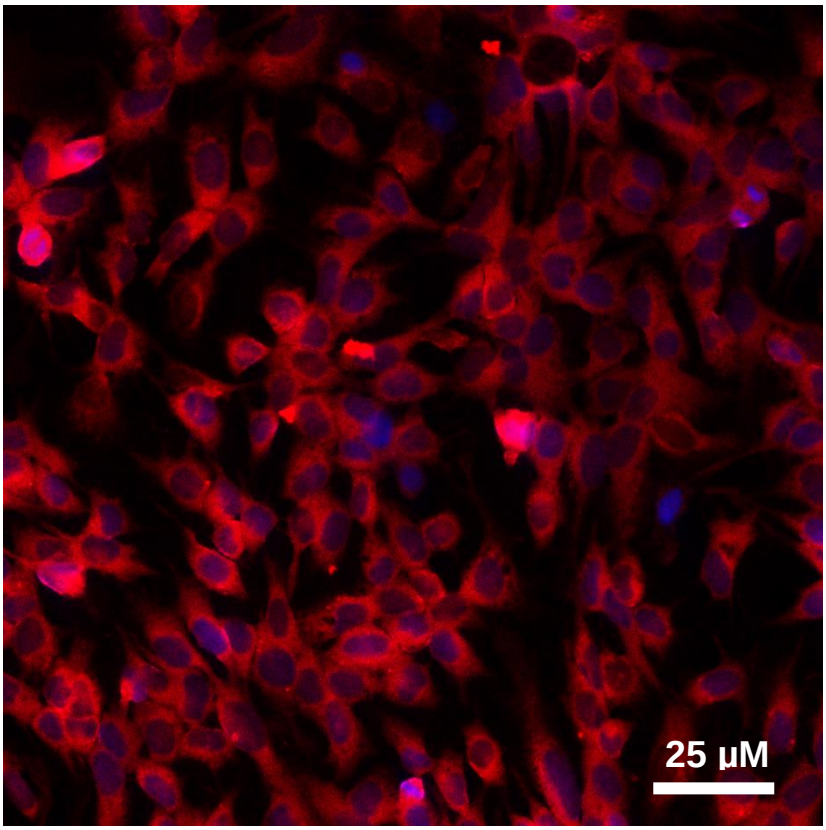


Figure 2.4: Confocal micrograph of HepG2 cells. HepG2 cells grown for 96 h in 5.5 mM glucose were stained with mitochondrial marker MitoOrange (red) and DAPI (nuclei, blue) and image acquired using the Zeiss LSM 700 confocal microscope, magnification 20X.

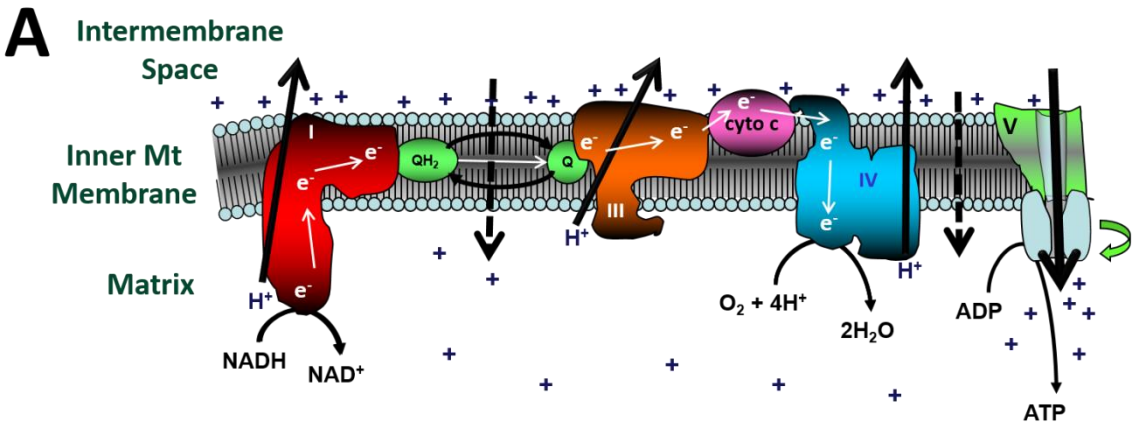
2.3.10 Citrate synthase activity assay

Cells were grown in T75 flasks in normal or high glucose for 96 h, with 10 or 20 µM quercetin (or DMSO controls) for the final 24 h. Cell pellets, prepared as for when isolating mitochondria, were lysed in CellLytic and assayed for citrate synthase activity, a recognised marker for total mitochondrial content (Larsen, Nielsen et al. 2012), using reagents from the Citrate Synthase Assay Kit (CS0720, Sigma-Aldrich) and following the manufacturer's instructions. Briefly, the formation of the 5-thio-2-nitrobenzoic acid colorimetric reaction product was measured on the PheraSTAR FS at 412 nm, 30ºC for 5 min and again following addition of oxaloacetic acid. Citrate synthase activity was calculated by correcting for the first reaction rate, a baseline of endogenous thiol/deacetylase levels, and for total protein, as measured optically at 280 nm (NanoDrop) prior to the assay.

2.3.11 High-resolution respirometry

HepG2 cells, treated as for the citrate synthase assay, were washed in PBS and lifted from T75 flasks using TrypLE Express and resuspended in serum-free medium at 1.5 x 10^6 cells/ml (after counting on the TC10 cell counter); this cell density was optimised for based on adequate material to provide distinguishable oxygen flux measurements in the various states and for oxygen in the sealed chamber to sustain the whole experiment. Oxygen concentration and flux was continuously measured as 2 ml of the cell suspensions were added to each chamber of the Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria), maintained at 37^{o}C, and a phosphorylation control protocol followed (Gnaiger 2014). Routine respiration was recorded first; Leak was measured following the addition of 250 nM oligomycin; ETS capacity was titrated by several additions of 5 µM FCCP (carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone); and 1.25 µM rotenone and 2.5 µM antimycin A were both added at the end of the experiment to measure residual oxygen consumption (ROX) (see Fig. 2.5). These inhibitor and uncoupler concentrations were also optimised in preliminary experiments.

The advantage of the O2k system is the precise measurement of oxygen flux in each respiratory state, but the disadvantage is the low throughput; one control and one treatment per respirometer. Each experiment took 96 h cell growth plus ~6 h experiment time to allow for 5.5. mM glucose versus 5.5. mM glucose + quercetin in one respirometer, 5.5 mM glucose versus 25 mM glucose in another, and 25 mM glucose versus 25 mM glucose + quercetin in a third experiment; the output from such an experiment is just four data points for each respiratory state. An extensive amount of time was spent optimising the protocol to account for cell density; too low and differences in flux owing to cell treatments are smaller, too high and the oxygen in the chamber is exhausted. HepG2 cells were very sensitive to oligomycin; adding too much resulted in the collapse of the ETS and effective cell death and again adding too little resulted in Leak not being achieved. Furthermore, the amount of FCCP required to uncouple ETS varied marginally in each experiment, hence the titration approach, and thus all of this combined lead to the need for a lot of experimental replicates to gain enough data for a realistic biological insight.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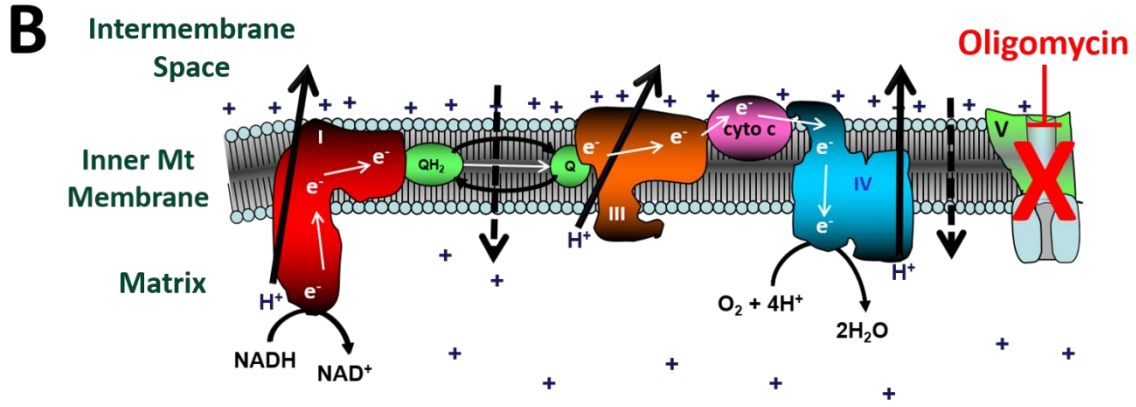
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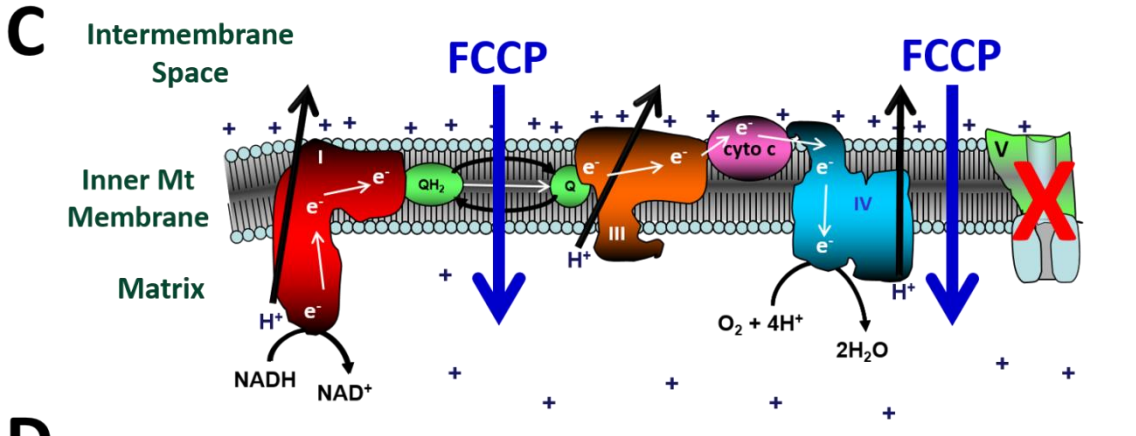
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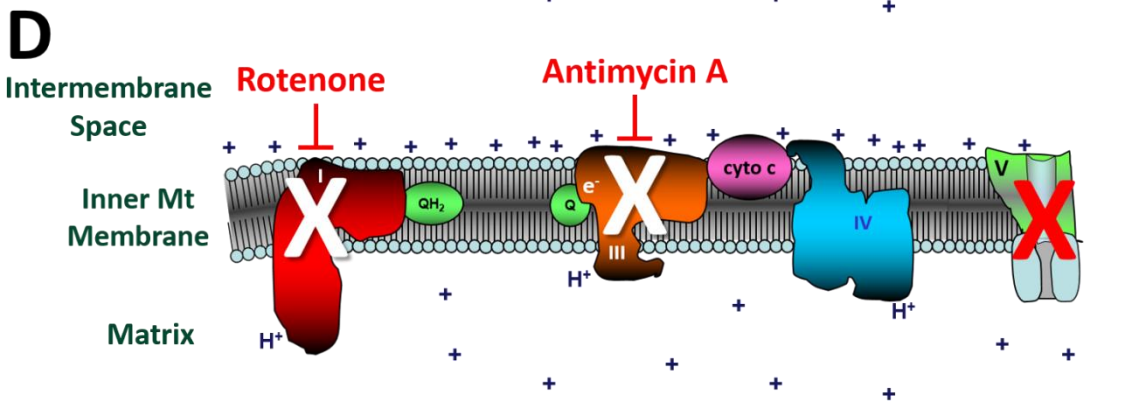
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Figure 2.5: The electron transfer system and respirometry protocol. NADH is oxidised by complex I at the matrix side of the IMM and the donated electrons are transferred in turn through the redox complex to CoQ_{10} (QH_2 and Q), complex III, cytochrome *c* (cyto c), complex IV and the final acceptor, O_2 (white arrows). Simultaneously, protons are transferred to the IMS by the complexes (solid black arrows ↑), contributing to the proton gradient that couples the ETS to OXPHOS of ADP to produce ATP at ATP synthase (solid black arrow ↓ at complex V). Some electrons leak back from the IMS to the matrix through the IMM (dashed black arrows ↓). This is known as state 3 respiration and is measured in the respirometer during Routine (A). When oligomycin is added to the respirometer, ATP synthase is inhibited and ETS may only continue by dissipating the proton gradient via the natural electron leak back across the IMM; the Leak measurement indicates how 'leaky' the IMM is and is indicative of mitochondrial dysfunction (B). FCCP uncouples the ETS completely by picking up protons in the IMS, diffusing across the IMM and releasing them in the matrix; effectively the unlimited reverse of the ETS complexes and thus electrons may flow without the rate-limiting step of proton transfer, providing the ETS maximal capacity measurement (C). Finally when rotenone and antimycin A, inhibitors of complexes I and III respectively, the ETS is completely inhibited and only non-ETS-specific residual oxygen consumption (ROX) occurs (D).

Additional experiments were conducted whereby quercetin (10-100 µM) was titrated directly into the respirometer chamber containing a HepG2 cell suspension, followed by an FCCP positive control, to assess if quercetin was acting as a direct ETS uncoupler. As a control for the effect of quercetin reacting directly with oxygen, quercetin (10-200 µM) was also titrated into cell-free medium.

2.3.12 Cellular NAD^+/NADH assay

Cells were grown in 24-well plates and treated as for the DCFH-DA assay. Cells were washed in PBS, lysed and collected, and NAD^+/NADH measured using the NAD/NADH Assay Kit (Fluorometric) (ab 176723, Abcam), in which enzymes specifically recognise NAD^+ or NADH in an enzyme recycling reaction. The manufacturer's instructions were followed; lysates were incubated with either a NADH or NAD^+ extraction solution at RT for 15 min in a black, clear-bottomed 96-well plate (Greiner Bio-One), then the opposite solution added and, after a final incubation at RT for 60 min, the signals were read on the PheraSTAR FS plate reader (Ex/Em = 540/590 nm). Each of the signals from the NAD^+-specific wells were divided by each signal of the NADH-specific wells to give the ratio.

2.3.13 RNA isolation and cDNA synthesis

HepG2 cells were grown in normal or HG in 100 mm^2 dishes for 60 h. At 60 h cells were treated with medium ± FBS for 12 h (72 h after seeding) before treatment with 20 µM quercetin (or DMSO controls) for up to 24 h. Samples were collected after 1, 3, 6, 12 and 24 h, with additional samples taken at 60 h (-12 h) and 72 h (time 0) in order to assess whether the FBS had an effect on mRNA expression. Total RNA was isolated using the Aurum Total RNA Mini Kit (#732-6820, Bio-Rad Laboratories, UK) and following the manufacturer's instructions. RNA content was determined spectrophotometrically at 260 nm (NanoDrop) and cDNA was synthesised by reverse transcription from 1 µg RNA using the High Capacity RNA-to-cDNA master mix kit (Applied Biosystems, Thermo Fisher Scientific), again following the manufacturer's protocol, and this was stored at -20^{o}C for later polymerase chain reaction (PCR) analyses.

2.3.14 Droplet digital PCR analysis

The QX100 Droplet Digital PCR system (Bio-Rad Laboratories, USA) was used to quantify changes in gene expression of PGC-1α and TBP (housekeeping gene) as previously described (Tumova, Kerimi et al. 2016). FAM-labelled PPARGC-1α-specific Taqman primer (Hs01016719_m1) was mixed with VIC-labelled TBP Taqman primer (Hs00427620_m1) and each 20 µl assay contained 1 µl of each primer, 10 µl of ddPCR

Supermix and 9 µl of cDNA diluted with MilliQ water corresponding to 19 ng of mRNA, assuming 1:1 reverse transcription efficiency. The mixtures were dispersed into oil droplets using the QX100 Droplet generator and then transferred to a C1000 Touch thermal cycler (Bio-Rad Laboratories, USA). Droplet-containing mixtures were incubated for 10 min at 95ºC, followed by 40 cycles of 30 sec at 94ºC and 1 min at 57.8ºC, and then finished with 10 min incubation at 98ºC. Products were maintained at 12ºC before analysis on the QX100 Droplet Reader. The QuantaSoft software (Kosiche, Slovakia) was used to analyse the data and determine concentrations of the target DNA in copies per µl from the fraction of positive reactions using Poisson distribution analysis. All reactions were performed in duplex mode with data collected independently for each target (PGC-1α and TBP) and presented as fold change of control treatment.

2.3.15 Statistical analyses

Data are expressed as means ± standard error of the mean. The significance of differences between groups of quercetin treatments was analysed by one-way ANOVA and post hoc Tukey's (for unequal sample sizes), Dunnett's (comparing multiple treatments to a single control) or two-tailed *t*-tests accordingly, with variance checks using Levene's test, and with Bonferroni correction where multiple treatments were compared, using SPSS 24 and presenting the data in Origin 2016. $p < 0.05$ was considered as statistically significant.

2.4 Results

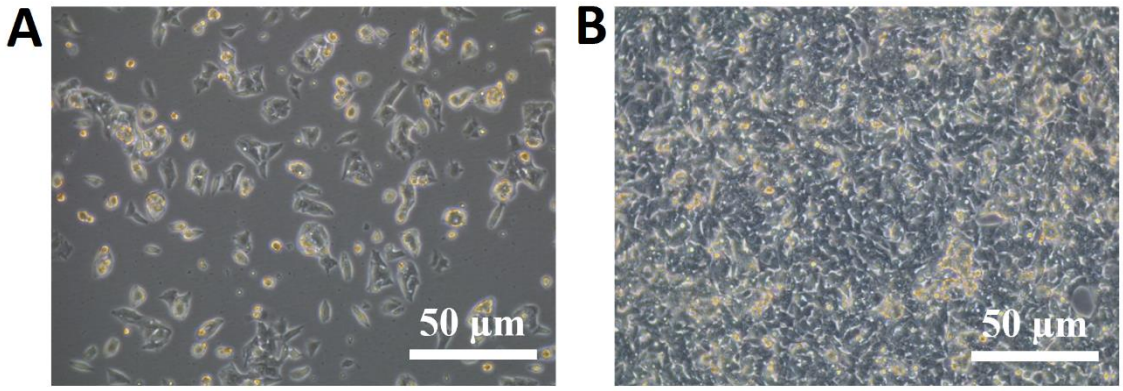

Figure 2.6: HepG2 cells in culture. Cells were maintained in Eagle's MEM supplemented with 10% FBS. Images were acquired using the Leica DM IL LED inverted microscope at 10X magnification 12 h (A) and 96 h (B) after seeding, at which point cells were confluent.

2.4.1 Mitochondrial isolation and complex I activity assay were optimised

The isolation process was optimised before preparing mitochondria for complex I assays on HepG2 cells grown in normal or high glucose and treated with quercetin. Initially, the resuspension of cells and pellets was improved, an extra centrifugation step was introduced at the end to improve mitochondrial purity and remove remaining cellular debris from the resuspended mitochondrial pellet and the whole procedure streamlined by removing an unnecessary protein assay step after SPIN 1a, instead keeping the volumes of reagent A and B consistent (see 2.3.4). Further optimisation was carried out by investigating the effect of variables in the protocol on the complex I activity mitochondrial protein yield/quality through protein assay and Western analysis. This included varying the number of homogenisation strokes, SPIN 2 speeds and the number of SPIN 2 centrifugations (trying three spins versus the one); reagent incubation times before both homogenisation steps, the speed of the final centrifugation, and checking the effect of freeze-thaw cycles on the mitochondrial isolate. The finalised protocol yielded the highest and purest yield i.e. highest complex I activity and cleanest peak signal on the ProteinSimple Wes system (Fig. 2.7A). It was clear that the isolation process had to be strictly adhered to for the lysate of each treatment to avoid unnecessary variation and risk masking minor changes in complex I activity.

Various conditions for the complex I activity assay were evaluated. 10 µg mitochondria was sufficient to give a steady rate of NADH oxidation over the 60 min assay and allowed for plenty of material from each cell pellet. Varying NADH concentrations were assayed (see 2.3.7.1) and 150 µM gave data with the highest reproducibility and linearity. CoQ_1 was added to the assay at 50 µM as previously been published (Ragan 1987, Usta, Kreydiyyeh et al. 2009) and had a dramatic effect on the rate of NADH oxidation, with a 500% increase in activity. Linalool was tested during preliminary assays, as investigated by Usta *et al.* (Usta, Kreydiyyeh et al. 2009). It inhibited complex I as expected, with a 64% decrease against an ethanol control, confirming the assay was valid and responsive to stimulation/inhibition. Linalool only showed an inhibitory effect in the presence of CoQ_1, with the higher basal activity. CoQ_1 accepts electrons from complex I as ubiquinone does *in vivo* and therefore was necessary to allow the reaction to progress with high linearity (see 2.3.71 and Figs. 2.3 and 2.7B). Blanks (no mitochondria) and controls for non-mitochondrial oxidation by rotenone inhibition were assayed alongside mitochondria from treated cells.

2.4.2 Quercetin recovers complex I activity lost with chronic high glucose

The complex I activity assay was put to use for assessing mitochondria in HepG2 cells grown under different conditions and with quercetin treatments. Complex I activity decreased with high glucose treatment by 11% ($p < 0.001$) after 24 h and by 19% ($p < 0.001$) after 96 h. Both hydrogen peroxide and serum removal also lowered the activity, whereas growing in galactose increased it (Fig. 2.7 C-D). In normal glucose, quercetin treatment caused a small decrease in complex I activity. However, in high glucose, quercetin up to 10 µM dose-dependently attenuated the high-glucose induced damage, up to 94% with 10 µM ($p < 0.001$), although at 20 µM the effect was less pronounced (Fig. 2.7E).

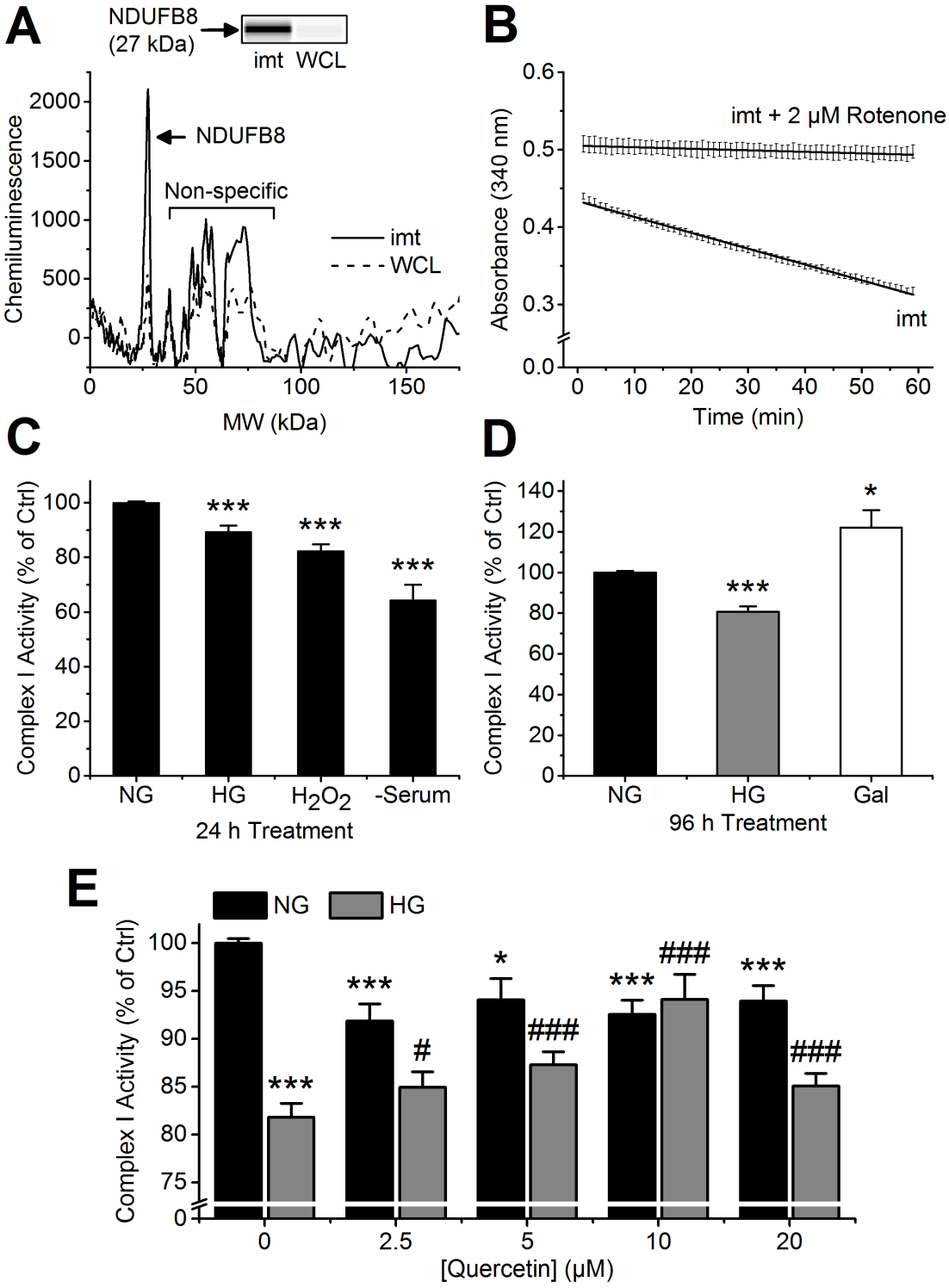
A
NDUFB8
(27 kDa)
imt WCL
Chemiluminescence
NDUFB8
Non-specific
imt
WCL
MW (kDa)
B
imt + 2 μM Rotenone
Absorbance (340 nm)
imt
Time (min)
C
Complex I Activity (% of Ctrl)
NG
HG
H2O2
-Serum
24 h Treatment
D
Complex I Activity (% of Ctrl)
NG
HG
Gal
96 h Treatment
E
NG
HG
Complex I Activity (% of Ctrl)
[Quercetin] (μM)

Figure 2.7: Quercetin dose-dependently recovered complex I activity decreased by high glucose in HepG2 cells. Purity of isolated mitochondrial fraction (imt). Protein expression of complex I subunit NDUFB8 increased when compared to the whole cell lysate (WCL), assessed by ProteinSimple automated Western, with traditional Western blot and pherogram views (A). Complex I activity in 10 µg imt assessed with NADH (150 µM) in the presence of coenzyme Q_1 (50 µM), and corrected for non-mitochondrial oxidation by blank and rotenone-inhibited (2 µM) controls. Lines of best fit for mean activity ± SEM (B). Complex I activity in cells grown in normal glucose (NG) medium supplemented with 10% serum for 72 h and then treated for 24 h with high glucose (HG), 0.5 mM hydrogen peroxide (H_2O_2) or serum-free medium. Complex I activity was normalised to the NG control (13.7 ± 0.8 nmol/min/mg) and data are expressed as mean percentages ± SEM (N/n = 3/9). ***$p < 0.001$ *vs* NG control (C). Complex I activity in cells grown for 96 h in NG, HG or 10 mM galactose (Gal); data are expressed as mean percentages of NG ± SEM (NG = 8.9 ± 0.9 nmol min^{-1} mg^{-1}) (N/n = 5/15). *$p < 0.05$, *** $p < 0.001$ *vs* NG control (D). Complex I activity in cells grown for 96 h in NG or HG and treated for the final 24 h (in the absence of serum) with various concentrations of quercetin (or DMSO controls). Data are mean percentages of the NG control ± SEM (NG = 8.6 ± 0.3 nmol/min/mg) (N/n = 6/18). *$p < 0.05$, ***$p < 0.001$ *vs* NG control; $^{\#}p < 0.05$, $^{\#\#\#} < 0.001$ *vs* HG control (E).

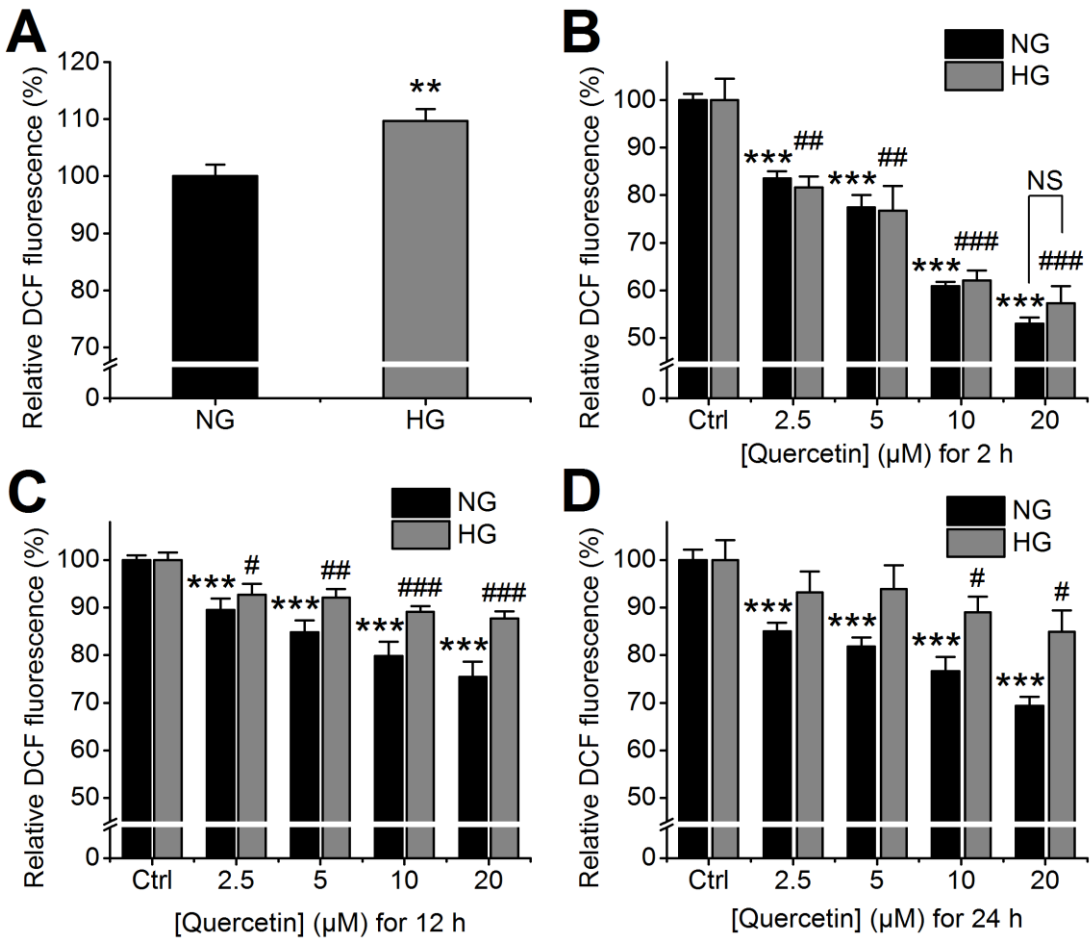

Figure 2.8: Quercetin decreased the relative DCF fluorescence in normal and high glucose cells. Cells were grown for 96 h in normal (NG) or high (HG) glucose and then incubated with 10 μM DCFH-DA for 20 min and DCF fluorescence measured and corrected for total protein (A). Cells grown in NG or HG for 96 h were treated with quercetin (or DMSO control), in the absence of serum, for the final 2 h (B), 12 h (C) or 24 h (D) and DCF fluorescence measured in the same way. All data are mean values expressed as (%) of the NG or HG controls accordingly ± SEM (N/n = 4/12). $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* NG controls; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* HG controls; NS = not significant.

2.4.3 Quercetin lowers ROS in HepG2 cells in normal and high glucose

DCF fluorescence, as an indicator of intracellular ROS, was increased by 10% ($p < 0.01$) when cells were grown in high glucose for 96 h (Fig. 2.8A). This increase in ROS was attenuated dose-dependently in both normal and high glucose following quercetin treatment at all time points up to 24 h (Fig. 2.8 B-D). The effect of quercetin was most pronounced at earlier time points, with relative ROS lowest in normal glucose (53%, $p < 0.001$) and high glucose (57%, $p < 0.001$) after treatment with 20 µM quercetin for 2 h (Fig. 2.8B). Quercetin was less effective in high glucose, notably after 12 and 24 h (Fig. 2.8 C-D).

2.4.4 Quercetin decreases mitochondria while increasing cell viability

Growing cells in normal or high glucose did not affect citrate synthase activity, a mitochondrial matrix marker, whereas quercetin significantly lowered citrate synthase activity after 24 h in normal glucose, and in high glucose with 20 µM, by 5-7% ($p < 0.05$) (Fig. 2.9A). Cell viability, assessed by Trypan Blue exclusion, was also not affected by glucose but concentration-dependently increased with quercetin, by up to 5% in high glucose ($p < 0.001$) (Fig. 2.9B).

2.4.5 Quercetin lowers high glucose-induced IMM proton leak and increases oxidative respiration

IMM proton 'Leak', as assessed by respirometry, was increased by high glucose treatment by 21% ($p < 0.05$), but with no significant changes in the basal 'Routine' respiration, the non-coupled 'ETS' capacity nor the non-ETS 'ROX' (Fig. 2.10B). Quercetin treatment concentration-dependently almost entirely reversed the high glucose-induced proton leak ($p < 0.01$). In high glucose, treatment with 10 µM quercetin also increased oxidative respiration; both the cellular Routine and net ETS respiration (calculated from (Routine-Leak)/ETS) were elevated ($p < 0.01$), though not with 20 µM (Fig. 2.10D). Respiration in normal glucose was unaffected by quercetin, but again Leak was lowered dose-dependently ($p < 0.01$) (Fig. 2.10C). In general with quercetin treatments ETS capacity was unchanged or lowered and ROX was dose-dependently decreased (Fig. 2.10 C-D).

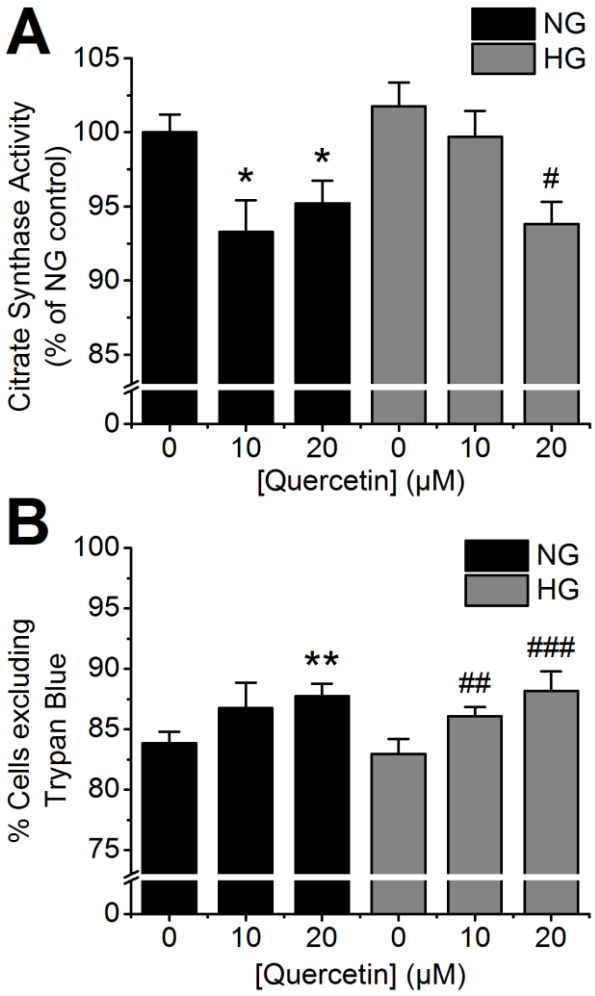

Figure 2.9: Quercetin decreased mitochondrial content and increased cell viability. Mitochondrial content (citrate synthase activity) in cells grown for 96 h in normal (NG) or high (HG) glucose and treated for the final 24 h with 10 or 20 μM quercetin (or DMSO control). All data are mean values expressed as (%) of the NG ± SEM (NG = 43.8 ± 0.5 nmol min^{-1} mg^{-1}) (n = 6 for 10 μM; 9 for 20 μM; 15 for controls). *p < 0.05 *vs* NG control; $^{\#}p$ < 0.05 *vs* HG control (A). Cells treated in the same way were assessed for plasma membrane integrity using the Trypan Blue exclusion test. Data are mean values of cells that excluded Trypan Blue expressed as (%) of total cells ± SEM (N = 6 for 10 μM; 13 for 20 μM; 19 for controls). **p < 0.01 *vs* NG control; $^{\#\#}p$ < 0.01 *vs* HG control; $^{\#\#\#}p$ < 0.001 *vs* HG control (B).

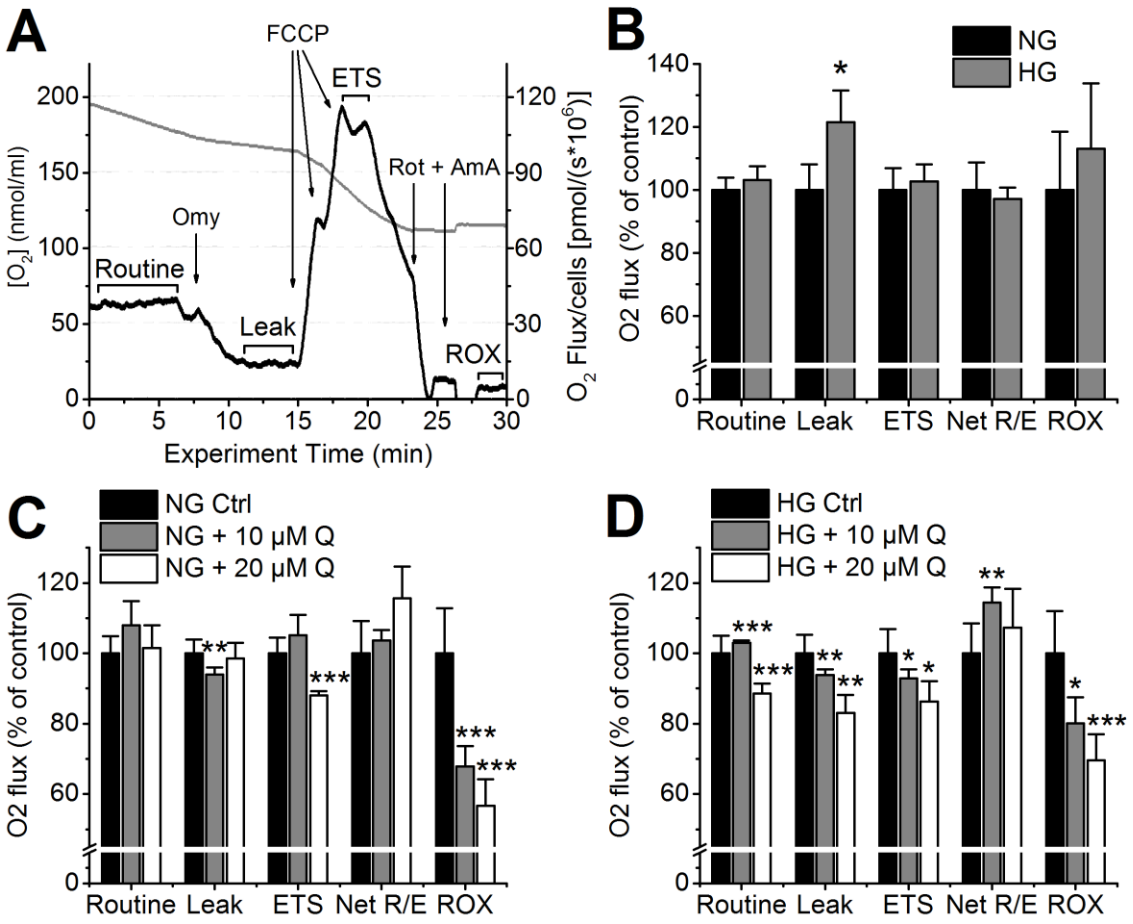

Figure 2.10: Quercetin decreases high glucose-induced IMM proton leak and increases oxidative respiration. Typical oxygen flux trace on the OROBOROS O2k high-resolution respirometer (grey line – $[O_2]$; black line – O_2 flux). Intact HepG2 cells were added to the respirometer and, in turn, the following states were measured: 'Routine' respiration after equilibration; IMM proton 'Leak' after addition of oligomycin (250 nM); electron transfer system capacity (ETS) in the non-coupled state induced by FCCP (5 µM) titration; and non-ETS residual oxygen consumption ('ROX') with the addition of rotenone (1.25 µM) and antimycin A (2.5 µM). Routine, Leak and ETS were all ROX-corrected (A). Respirometry data for cells grown in normal (NG) or high (HG) glucose for 96 h. Net R/E is the net routine flux control ratio, calculated as ((Routine-Leak)/ETS). Data shown are mean values and expressed as (%) of the NG control ± SEM (N/n = 9/16). *p < 0.01 *vs* NG (B). Cells grown in NG (C) or HG (D) for 96 h were treated with 10 or 20 µM quercetin (Q) (or DMSO control) for the final 24 h. Data are mean values and expressed as (%) of the NG or HG control accordingly ± SEM (N/n = 5/8 for 10 µM; 9/9 for 20 µM; 14/17 for controls). *p < 0.05, **p < 0.01, ***p < 0.001 *vs* respective controls. See Table 2.1 for control values.

Table 2.1: Control values for respirometry. O_2 flux values (pmol s^{-1} 10^{-6} cells) ± SEM. NG – normal glucose; HG – high glucose.

	Routine	Leak	ETS	Net R/E	ROX
Fig. 2.10B (NG Ctrl)	32.9 ± 1.2	8.1 ± 0.6	95.3 ± 6.5	0.25 ± 0.02	4.9 ± 0.9
Fig. 2.10C (NG Ctrl)	28.2 ± 1.5	10.3 ± 0.4	97.3 ± 4.2	0.19 ± 0.02	4.6 ± 0.6
Fig. 2.10D (HG Ctrl)	29.8 ± 1.5	11.2 ± 0.6	93.1 ± 6.4	0.21 ± 0.02	4.9 ± 0.6

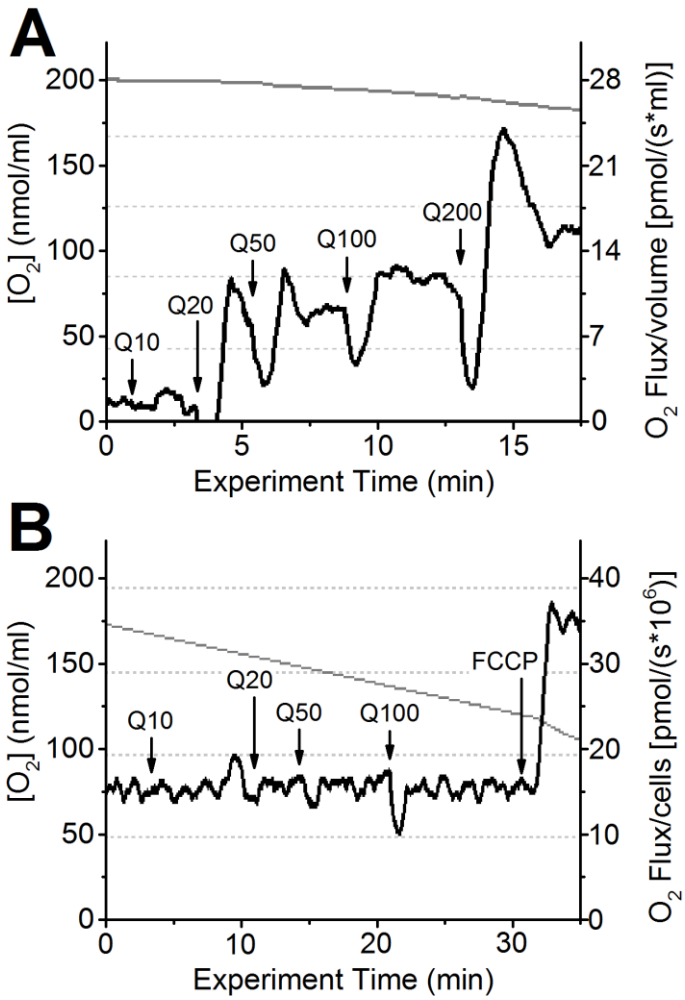

Fig 2.11: Quercetin reacts with oxygen and is rapidly taken up by HepG2 cells, without uncoupling ETS. Oxygen 'flux' trace for cell-free medium when titrated with quercetin (10-200 µM final) in the OROBOROS O2k (A). Intact HepG2 cells were added to the respirometer and quercetin (10-100 µM) was titrated in, followed by a single addition of FCCP (5 µM) as a positive control. Grey line – [O_2]; black line – O_2 flux; Q followed by number indicates cumulative quercetin concentration (µM) (B).

2.4.6 Quercetin is rapidly taken up by HepG2 cells without uncoupling mitochondrial respiration

When added directly to cell-free medium in the respirometer, quercetin dose-dependently reacted with oxygen (Fig. 2.11A). When added to HepG2 cells already in suspension and equilibrated in the respirometer chamber, the increase in oxygen consumption was no longer evident, indicating the quercetin was rapidly taken up by the cells quicker than it could react with oxygen in the medium. Quercetin did not act as a direct ETS uncoupler, unlike FCCP (Fig. 2.11B).

2.4.7 Quercetin reverses increased NADH in high glucose cells within 2 h

High glucose increased cellular NADH ($p < 0.001$) (Fig. 2.12A), but this effect had diminished at later time-points. Quercetin dose-dependently increased NAD^+/NADH in high glucose after 2 h ($p < 0.01$), but not in normal glucose, to reverse the increased NADH (Fig. 2.12A). NAD^+/NADH was elevated after 12 h with 10 µM quercetin in both normal and high glucose ($p < 0.001$) (Fig. 2.12B) and this effect was maintained in normal glucose up to 24 h ($p < 0.001$) (Fig. 2.12C). In high glucose, an effect was evident with 20 µM quercetin at 24 h ($p < 0.05$) (Fig. 2.12C).

2.4.8 Quercetin increases PGC-1α mRNA in normal and high glucose

Without serum PGC-1α gene expression was increased under both normal and high glucose conditions. Following 12 h in serum-free medium (t0) PGC-1α increased by 1.7-fold ($p < 0.001$) and remained stable for 12 h in normal glucose; in the presence of high glucose a further increase was evident at 6 h (2-fold, $p < 0.001$). At 24 h mRNA levels were significantly lower than in control cells (Fig. 2.13 A, C). Treatment with 20 µM quercetin increased PGC-1α gene expression within 3 h in normal glucose and within 6 h in high glucose (both $p < 0.001$). A maximal effect was reached at 6 h in both (Fig. 2.13 E, G). TBP (housekeeping reference) was mildly affected by the absence of serum and high glucose but these changes did not affect the PGC-1α measured gene expression (Fig. 2.13 B, D, F, H). The ddPCR determines the absolute number of mRNA molecules in the reaction, so the use of a stably expressed reference gene is not essential but was used to account for variations in the sample preparation.

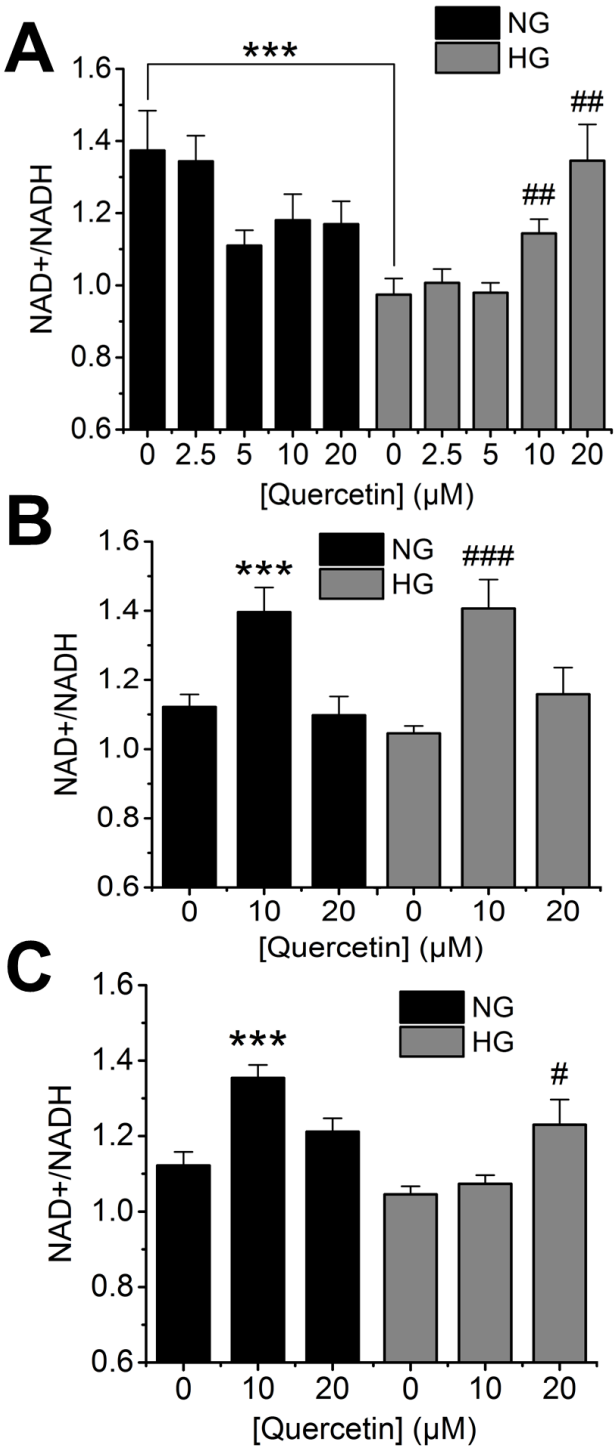

Figure 2.12: Quercetin reverses the high glucose-increased NADH. HepG2 cells grown in NG or HG for 96 h were treated with NG or HG plus various concentrations of quercetin (or DMSO controls) for the final 2 h (A). NG and HG cells were treated with 10 or 20 μM quercetin (or DMSO controls) for the final 12 h (B) or the final 24 h (C). All data are expressed as mean NAD^+/NADH ± SEM (N/n = 6/36). ***$p < 0.001$ *vs* NG control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* HG control.

2.5 Discussion

Type 2 diabetes and metabolic syndrome are manifested by high glucose-induced oxidative stress that leads to mitochondrial dysfunction, insulin resistance and continued hyperglycaemia (Yang, Jin et al. 2011). Quercetin is known for its potential health benefits, particularly in relation to mitochondrial dysfunction and metabolic diseases (Eid, Martineau et al. 2010, Kim, Kwon et al. 2015, Chis, Muresan et al. 2016, de Oliveira, Nabavi et al. 2016, Haddad and Eid 2016). Numerous studies have demonstrated that quercetin protects against oxidative stress in mitochondria, either through restriction of ROS generation locally or enhancement of oxidative defence mechanisms (Moskaug, Carlsen et al. 2005, Vargas and Burd 2010, Lagoa, Graziani et al. 2011, Ji, Sheng et al. 2015, Jimenez, Lopez-Sepulveda et al. 2015). It may also impact metabolism through interacting with proteins, regulating genes that control metabolic pathways and promoting mitochondrial biogenesis or mitophagy (Sandoval-Acuna, Ferreira et al. 2014, de Oliveira, Nabavi et al. 2016). Mechanisms by which quercetin affects mitochondrial function were reviewed by de Oliveira et al. (de Oliveira, Nabavi et al. 2016), but there is still a lack of understanding when it comes to the specifics involved with lowering mitochondrial dysfunction caused by oxidative stress, especially in cells grown in a high glucose environment. In the present study the latter was investigated against a normal glucose control. Beneficial effects were seen in HepG2 cells when exposed to quercetin in the low micromolar range, reversing the high glucose-induced stress in a dose-dependent manner, and higher concentrations of quercetin were used to probe the mechanisms of action further.

ETS complex I activity was lowered by H_2O_2 incubation or removal of serum, as means of inducing oxidative stress (Takeda, Akao et al. 2006, Jiang, Yu et al. 2014), and was increased when growing the cells in galactose, known for increasing oxidative phosphorylation fuelled by glutamine and fatty acids *in vitro* (Reitzer, Wice et al. 1979, Robinson, Petrova-Benedict et al. 1992, Marroquin, Hynes et al. 2007, Andrzejewski, Gravel et al. 2014). The decreased complex I activity in high glucose was likely a result of oxidative stress, supported by the increased DCF fluorescence; free radicals attack the ETS protein complexes, particularly prone as the principal sites of generation, and mitochondrial DNA, leading to additional defects in the complexes (Starkov 2008, Kang and Pervaiz 2012, Guo, Sun et al. 2013, Hirst and Roessler 2016).

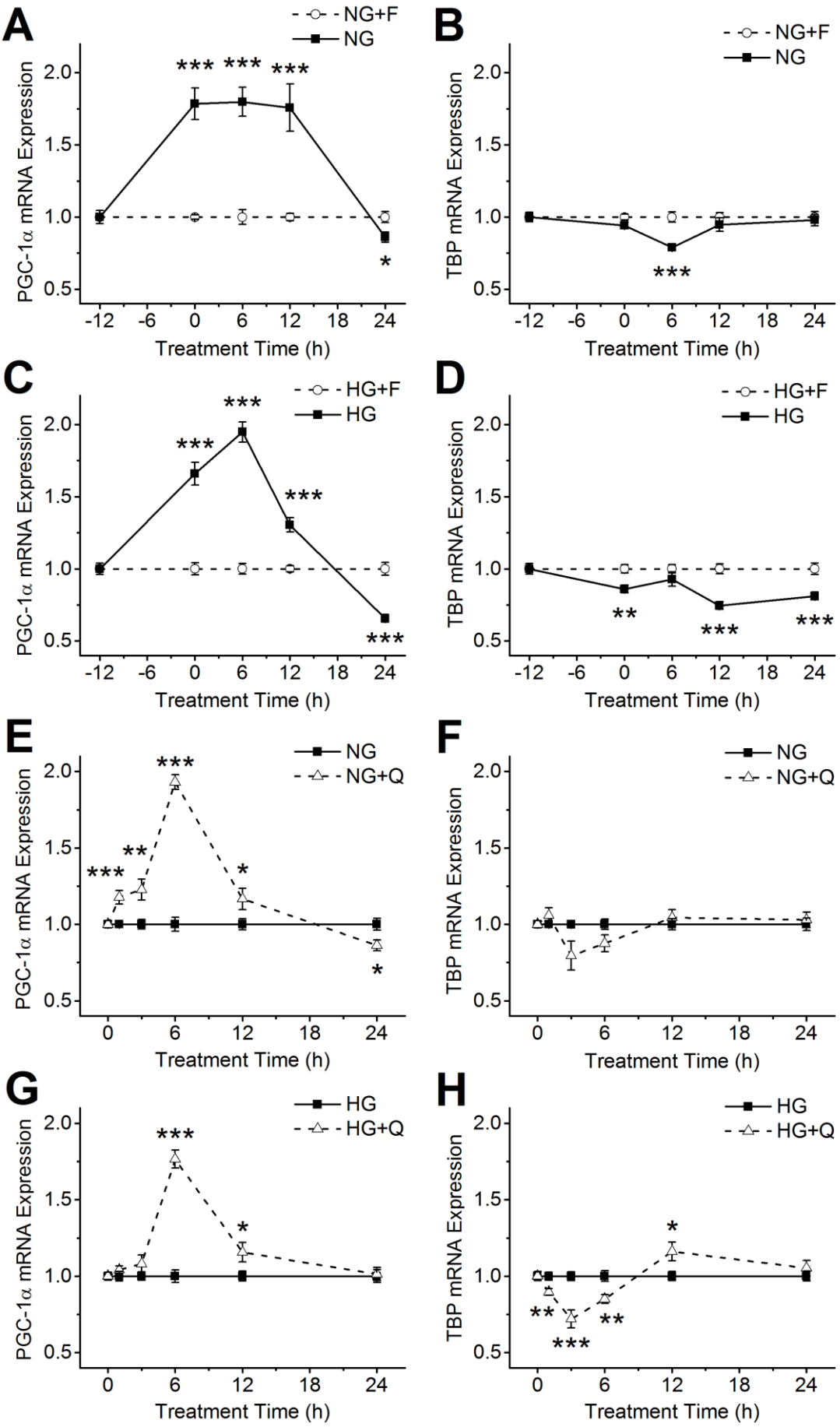
A
NG+F
NG
PGC-1α mRNA Expression
Treatment Time (h)
B
TBP mRNA Expression
C
HG+F
HG
D
E
NG+Q
F
G
HG+Q
H

Figure 2.13: Quercetin induced PGC-1α transcription in normal and high glucose. Cells were seeded in NG or HG media and after 60 h (-12 h) the medium was changed to medium ± serum (F – with FBS). Cells were treated again at 72 h (t = 0) ±FBS and samples were collected at 6, 12 and 24 h (A-D). Cells were treated with 20 μM quercetin (Q) at 72 h (T=0) (or DMSO controls) and samples were collected at 1, 3, 6, 12 and 24 h (E-H). RNA was extracted, cDNA synthesised and expression quantified by droplet digital PCR analysis. Data are mean values of fold-change of PGC-1α or TBP (housekeeping) ± SEM (n/N = 18/6). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ *vs* control accordingly. See Appendix 1 for PGC-1α data normalised to TBP housekeeping gene.

The diminished complex I activity in high glucose was dose-dependently recovered by quercetin to the level in the normal glucose mitochondria when also treated with quercetin. Cells grown in normal glucose had universally lowered complex I activity when treated with all concentrations of quercetin, suggesting quercetin has a potentially detrimental effect on complex I when not already faced with the stress of chronic high glucose, but a beneficial effect against when it is. Conversely, relative ROS levels were dose-dependently lowered in both normal and high glucose by quercetin, as seen previously (Bali, Ergin et al. 2014), suggesting the lower complex I activity in normal glucose cells was not a result of increased oxidative stress; quercetin can itself act as a pro-oxidant depending on the environment, but usually at higher concentrations (Laughton, Halliwell et al. 1989, de Oliveira, Nabavi et al. 2016). Previous studies on complex I suggest that quercetin may competitively inhibit the complex at the CoQ-binding site, preventing inhibition by other agents (Carrasco-Pozo, Mizgier et al. 2012), and/or act as a CoQ mimic, allowing electron transfer to continue from NADH to complex III (Hodnick, Bohmont et al. 1987, Lagoa, Graziani et al. 2011, Sandoval-Acuna, Lopez-Alarcon et al. 2012), two possible reasons as to why this phenomenon was seen. A third possibility is that quercetin oxidised NADH itself (Chan, Galati et al. 1999, Buss, Constantin et al. 2005), increasing the NAD^+/NADH ratio. This decreased pool of NADH led to consequently lower complex I activity (Niklas, Nonnenmacher et al. 2012, Santidrian, Matsuno-Yagi et al. 2013) in the normal glucose cells wherein the pool of reducing substrates will have been lower than in high glucose.

DCF fluorescence was generally lowered more in normal glucose as there were less ROS in those cells to begin with and the high glucose cells had more NADH to be oxidised by quercetin. Furthermore, these data show that the effect of quercetin in (directly and/or indirectly) lowering the relative ROS levels wore off with time during 24 h, an indication that the quercetin is consumed and/or metabolised, and more so in high glucose. Hashimoto et al. studied the effect of hyperglycaemia on quercetin metabolism and found that quercetin was almost fully metabolised within 4 h of treatment and that phase II metabolism was attenuated by high glucose in HepG2 cells, with less quercetin aglycone and metabolites combined in the cells and media (Hashimoto, Blumberg et al. 2016). This may help explain the time-dependence of the DCFH-DA data, but also the differing effects of quercetin in cells grown in normal or high glucose due to its differing metabolism. Likewise, previous work on HepG2 cells in our lab showed that 5 µM quercetin lowered glucose metabolism and uptake over 12 h (Kerimi, Jailani et al. 2015) (only tested in normal glucose) so quercetin may have lowered cellular NADH and lessened the high glucose burden in this manner. The DCF data are indicative that quercetin may push metabolism in a certain direction, with the shift in redox balance producing lesser ROS, which will have little relevance physiologically from one dose of quercetin, but continued chronic exposure, say even over a lifetime, may help maintain the redox shift.

Citrate synthase activity, a marker of mitochondrial content (Lanza and Nair 2009, Larsen, Nielsen et al. 2012), was lowered in the normal glucose cells akin to the complex I activity, but in high glucose too, a sign that the mitochondria that are present following quercetin treatment are more functional and their oxidative output more efficient. The Trypan Blue data suggest cells are more viable overall 24 h after adding quercetin, with improved plasma membrane integrity. Changes in citrate synthase activity and plasma membrane integrity were small and not as physiologically relevant as the changes observed in the respirometry data for example. Other studies have suggested a role for quercetin in membrane integrity (Modriansky and Gabrielova 2009, Margina, Gradinaru et al. 2013, Sanver, Murray et al. 2016) and as such the quercetin-attenuated proton Leak across the IMM was caused by an improvement in the IMM directly, as well as indirectly by the lowered oxidative stress. The lower Leak in high glucose cells further enhanced mitochondrial respiration by improving coupling efficiency, shown by the increased Routine respiration and net respiration/ETS flux control ratio (net R/E). The latter was

due to a combination of lowered Leak but also lowered ETS, in line with the citrate synthase activity. The increase in net R/E was only present in the cells treated with 10 μM quercetin; at 20 μM there was a decrease in routine respiration adding to the notion that at 20 μM complex I may have been inhibited. Previous studies have shown quercetin improves mitochondrial bioenergetics (Nichols, Zhang et al. 2015, Carrasco-Pozo, Tan et al. 2016) and this was shown here for the first time in an intact HepG2 system, and notably that these improvements are enhanced when in high glucose.

Another key finding from the respirometry data was in the non-ETS residual oxygen consumption (ROX) measurements. This was dose-dependently decreased by quercetin and is indicative of a lower dependence on glycolysis, NADH oxidation by quercetin and inhibition of ATPase enzymes such as those involved in trans-plasma membrane electron transport (tPMET); ROX is the amalgamated effect of oxygenase redox enzymes (Brand and Nicholls 2011, Banh, Iorio et al. 2016, Kerimi and Williamson 2016) and cell surface oxygen consumption via tPMET (Herst and Berridge 2007). The dependence on glycolysis is lowered because ATP is generated instead by quercetin-enhanced mitochondrial respiration and this includes substrates via glutamate and fatty acid metabolism (Gnaiger 2014). Furthermore, quercetin was shown previously to inhibit glucose-6-phosphatase in insulin-resistant HepG2 cells, effectively helping to reverse the effects of insulin resistance and allow glycolysis to continue towards pyruvate (Teng, Chen et al. 2016). Direct NADH oxidation by quercetin (Chan, Galati et al. 1999, Buss, Constantin et al. 2005, Niklas, Nonnenmacher et al. 2012) also allows glycolysis to continue without the need for tPMET (Herst and Berridge 2007), and quercetin has long been known to inhibit ATPases (Lang and Racker 1974, Davis, Middleton et al. 1983, Racker 1986, Gasparin, Salgueiro-Pagadigorria et al. 2003).

Previous studies suggested that quercetin (≥ 25 μM) uncoupled ETS and oxidative phosphorylation while inhibiting mitochondrial respiration in mitochondria isolated from rat kidney or liver (Dorta, Pigoso et al. 2005, Ortega and Garcia 2009). Quercetin also caused partial uncoupling in the low nanomolar range in rat heart mitochondria (Trumbeckaite, Bernatoniene et al. 2006). This was tested in intact cells, H9c2 rat cardiomyocytes, whereby quercetin increased Leak and partially protected complex I respiration, but only following hypoxia. Short-term incubation with quercetin (< 50 μM) did not induce uncoupling however, demonstrating that the effects seen in isolated

mitochondria are attenuated in whole cells (V. Zholobenko, Mouithys-Mickalad et al. 2017). Supporting evidence for this is provided here, as quercetin ≥ 100 µM did not act as an uncoupler in intact HepG2 cells. Quercetin reacted with oxygen when cells were not present, as shown previously (Morales, Günther et al. 2012), but it was taken up rapidly into the cells, also seen previously in Jurkat cells (Fiorani, Guidarelli et al. 2010), and oxygen consumption was not increased.

NADH oxidation by quercetin was also demonstrated by the general increases in NAD^+/NADH. Elevated NADH in high glucose cells is an indication of the reductive stress that leads to oxidative stress (Yan 2014). There was a decrease in this NADH pool following addition of quercetin, lasting up to 24 h in when treated 20 µM. The increase in NAD^+/NADH was seen too in cells grown in normal glucose but not when treated with 20 µM quercetin, nor for the dose-response at 2 h; further evidence of complex I inhibition, which was overridden by the high glucose-induced excess NADH. The increased NAD^+/NADH for 24 h is an effect that is temporally beyond direct oxidation alone and thus the lasting effect was due to the increased mitochondrial respiration in an environment with less oxidative stress resulting from the pleiotropic effects of quercetin combined. In accordance with this, Niklas et al. postulated that improved channelling of pyruvate into the mitochondria could result from a quercetin-induced increase in NAD^+/NADH ratio (Niklas, Nonnenmacher et al. 2012) and other studies have suggested that quercetin affects this (Buss, Constantin et al. 2005, Dorta, Pigoso et al. 2005, de Oliveira, Nabavi et al. 2016).

An increased pool of cellular NAD^+ activates NAD^+-dependent deacetylase sirtuins involved in the signalling network that governs central metabolism. The AMPK-SIRT-PGC-1α axis in particular regulates mitochondrial function and can increase mitochondrial biogenesis or ultimately lead to cell death (Canto, Gerhart-Hines et al. 2009, Austin and St-Pierre 2012, Tang 2016). Quercetin has been shown previously to activate these pathways (Ahn, Lee et al. 2008, Chung, Yao et al. 2010, Kim, Kwon et al. 2015, Haddad and Eid 2016, Leiherer, Stoemmer et al. 2016, Liu, Mei et al. 2016) and may be primarily via NADH oxidation (directly and/or indirectly). PGC-1α transcription was measured as a biomarker of mitochondrial biogenesis and oxidative function (Austin and St-Pierre 2012, Sugden and Holness 2012). PGC-1α mRNA was increased in response to transient starvation when FBS was removed from the media (Rodgers, Lerin

et al. 2005, Sen, Satija et al. 2011, Austin and St-Pierre 2012, Sugden and Holness 2012). Serum was removed 12 h earlier so any changes instigated by quercetin specifically were detectable. PGC-1α mRNA was not dependent on glucose concentration, however when quercetin was added to the serum-starved cells PGC-1α expression was increased further and in doing so may promote increased mitochondrial functionality. An increase in mitochondrial biogenesis was not seen according to the citrate synthase biomarker after 24 h, which is consistent with other reports on biogenesis; it is dependent upon cell type and quercetin concentration (Nichols, Zhang et al. 2015, de Oliveira, Nabavi et al. 2016). However, the increased PGC-1α mRNA and activation will have nonetheless affected the intrinsic oxidative nature of the cells, further boosting oxidative respiration (Austin and St-Pierre 2012, Rayamajhi, Kim et al. 2013). Autophagy is also induced by this and other signalling pathways regulated by quercetin (see 1.3.2.2) and thus damaged mitochondria may be disposed of and replaced with regenerated mitochondria more oxidative in function, enhancing the overall efficiency per mitochondrion. Increases in PGC-1α mRNA levels corroborates with the increased NAD^+ in cells treated with 10 μM quercetin; NAD^+-dependent SIRT1 activates PGC-1α, which in turn promotes its own transcription (Nelson, Valentine et al. 2012, Sugden and Holness 2012). The effects of quercetin are similar to those seen by metformin (Sugden and Holness 2012) and it has been shown previously that quercetin enhances oxidative metabolism via heme oxygenase-1 in obese mice (Kim, Kwon et al. 2015). Increased PGC-1α also helps to manage ROS (Baldelli, Aquilano et al. 2014) and suppresses glycolytic gene transcription (Rodgers, Lerin et al. 2005), shifting metabolic dependence towards the mitochondria.

Quercetin lowers local ROS generation and promotes antioxidant cellular defences to lower oxidative stress. It has been suggested that the principal mechanisms involve modulation of proteins and genes, such as those of the mitochondrial electron transfer system and involved in metabolic signalling pathways, as well as lipid membrane integrity, maintaining membrane-bound protein function and proton gradients. In the present study the data suggest that several such mechanisms are involved and have particular beneficial effects on reversing the high glucose-induced mitochondrial dysfunction in HepG2 cells (Fig. 2.14). Cellular ROS levels were lowered by quercetin as expected and this was both a cause and effect of other findings. It is proposed that quercetin forms a dimer quinone structure (which can be pro-oxidant and toxic itself at

higher concentrations) within the cell and mitochondrion (Metodiewa, Jaiswal et al. 1999, Buss, Constantin et al. 2005, Boots, Li et al. 2007, Lagoa, Graziani et al. 2011, Carrasco-Pozo, Mizgier et al. 2012, Sandoval-Acuna, Ferreira et al. 2014), accumulating in the latter (Fiorani, Guidarelli et al. 2010). Here it competitively inhibits complex I at the CoQ-binding site, but simultaneously oxidises NADH and transfers electrons to complex III (Sandoval-Acuna, Lopez-Alarcon et al. 2012) at the Q-junction, as well as those from $FADH_2$ and the electron transfer flavoprotein (Rosca, Vazquez et al. 2012, Gnaiger 2014), allowing OXPHOS to continue, while superoxide generation at complex I is impeded. Global oxidation of NADH lowers the substrate pool available to complex I and this adds to the overall effect on complex I and lowered ROS. The NAD^+/NADH ratio rises and leads to promotion of NAD^+-dependent enzymes and associated mitochondrial signalling pathways, seen by a transient increase in PGC-1α transcription to promote mitochondrial respiration and reprogrammed cellular metabolism in the longer term. Through direct incorporation of quercetin and/or indirect lowering of oxidative stress, the integrities of the inner mitochondrial and plasma membranes are enhanced and the heightened proton leak across the IMM in high glucose is attenuated, maintaining the proton gradient and efficient coupling, and mitochondrial respiration is increased. Non-ETS respiration is lowered through NADH oxidation in the cytosol and mitochondria and is indicative of a lesser dependence on glycolysis. The ultimate outcome of these effects is lowered oxidative stress induced by chronic high glucose and improved mitochondrial functionality, resulting in a net increase in complex I activity and respiration 24 h after a single dose of quercetin.

The positive effects of quercetin on mitochondrial bioenergetics in cells grown in high glucose have not been demonstrated previously and existing studies on the effects and mechanisms of quercetin on mitochondrial function have often employed an acute kinetic experiment setup on isolated mitochondria, but here the outcome following a chronic treatment to the cells has been presented, whereby the effects of a single dose of quercetin on metabolism, redox signalling and gene expression up to 24 hours later have been demonstrated concurrently. The differences between normal and high glucose helped to elucidate the specific mechanisms of action (Fig. 2.14). If quercetin is consumed on a daily basis, longer lasting pools may accumulate in the hepatic mitochondria and lead to a chronic effect to alleviate the stresses of hyperglycaemia and help to prevent insulin resistance *in vivo*. Proteomics studies have revealed that complex I is lowered in diabetic

patients (Peinado, Diaz-Ruiz et al. 2014), thus a greater understanding of these quercetin mechanisms of action in mitochondria and on cellular metabolism in a high glucose environment gives hope to the potential for nutraceutical or, with the use of nano-technology and optimised concentrations (de Oliveira, Nabavi et al. 2016), pharmaceutical applications to help absolve metabolic syndromes.

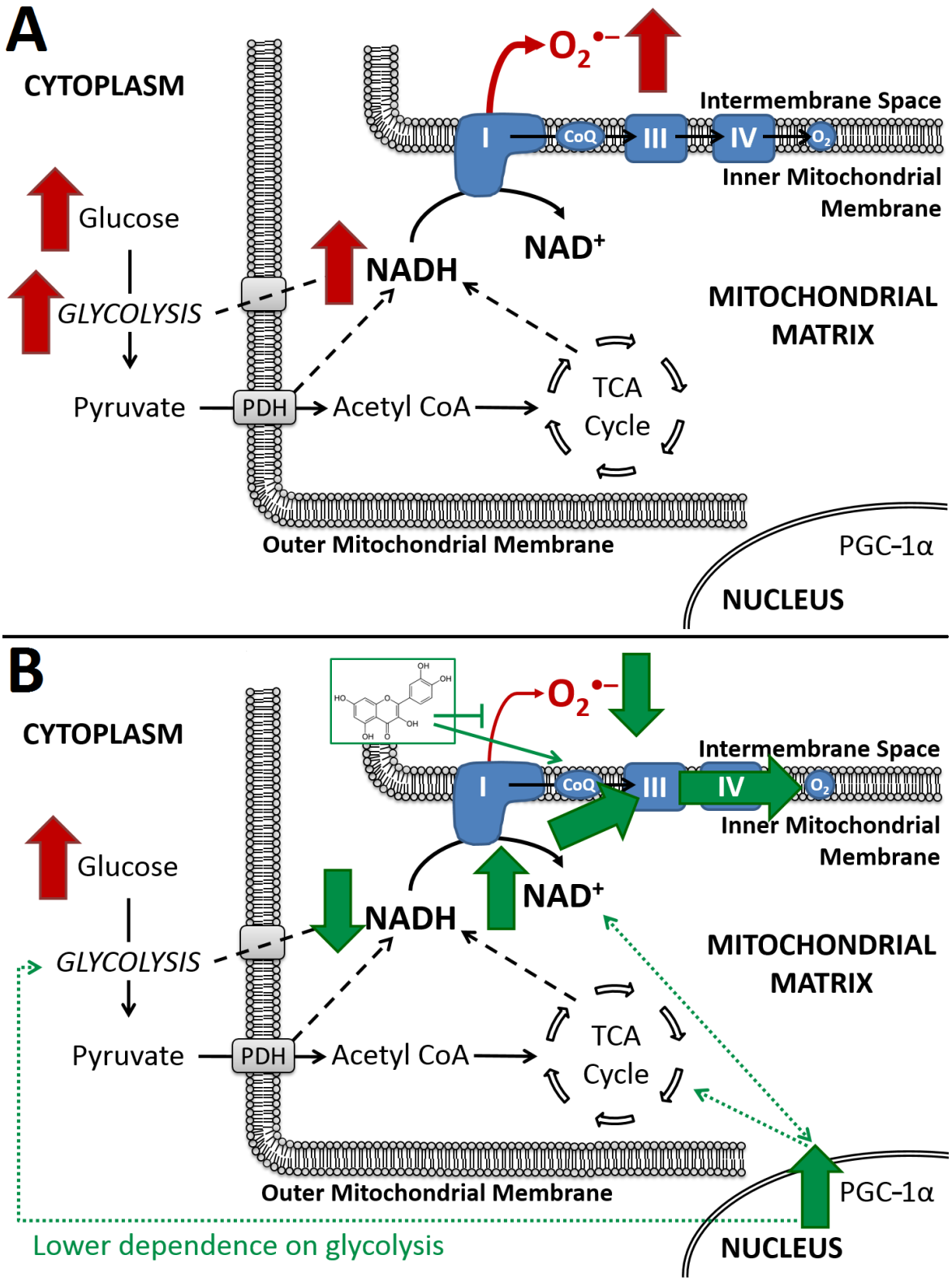
A
CYTOPLASM
$O_2^{\bullet-}$
Intermembrane Space
I
CoQ
III
IV
O_2
Inner Mitochondrial Membrane
Glucose
NAD+
NADH
GLYCOLYSIS
MITOCHONDRIAL MATRIX
Pyruvate
PDH
Acetyl CoA
TCA Cycle
Outer Mitochondrial Membrane
PGC-1α
NUCLEUS
B
CYTOPLASM
$O_2^{\bullet-}$
Intermembrane Space
I
CoQ
III
IV
O_2
Inner Mitochondrial Membrane
Glucose
NAD+
NADH
GLYCOLYSIS
MITOCHONDRIAL MATRIX
Pyruvate
PDH
Acetyl CoA
TCA Cycle
Outer Mitochondrial Membrane
PGC-1α
NUCLEUS
Lower dependence on glycolysis

Figure 2.14: Mechanisms of action of quercetin in mitochondria in HepG2 cells chronically exposed to high glucose. Elevated glucose leads to elevated NADH and overproduction of superoxide ($O_2^{\bullet-}$) at complex I of the electron transfer system (A). Quercetin inhibits complex I at the coenzyme Q_{10}-binding site (CoQ), suppressing superoxide generation while oxidising NADH and allowing electron transfer to continue to complex III, and attenuates the high glucose-induced proton leak across the inner mitochondrial membrane. This results in more efficient coupling with ATP synthesis. The NAD^+/NADH is increased in parallel with induction of PGC-1α gene expression by quercetin; mitochondrial function is promoted and metabolic flux shifted to mitochondrial respiration, leading to lower oxidative stress and attenuation of high glucose-induced mitochondrial dysfunction, with increased net respiration and recovered complex I activity. PDH – pyruvate dehydrogenase, TCA cycle – tricarboxylic cycle, Acetyl CoA – acetyl coenzyme A.

Chapter 3

(Poly)phenol metabolites increase glucose uptake in human skeletal muscle: gut microbiome metabolite isovanillic acid 3-*O*-sulfate upregulates GLUTs and activates insulin signalling.

3.1 Abstract

Epidemiological, animal and human intervention studies have revealed that consumption of (poly)phenols such as quercetin, ferulic acid and resveratrol has antidiabetic effects and this may be via increased postprandial glucose uptake in the skeletal muscle. Some *in vitro* studies have shown aglycone compounds can stimulate GLUT4 translocation, however these are limited to rat or mouse cells models and relevant (poly)phenol metabolites that are in circulation *in vivo* are yet to be tested. The immortalised myoblast line, LHCN-M2, was characterised as a human skeletal muscle model comprising myotubes differentiated in normal or high levels of glucose and insulin. This model was used to investigate the effects of a range of sulfated and glucuronidated (poly)phenol conjugates on glucose uptake and metabolism. Compounds were selected and grouped based on the known metabolite profiles following the consumption of parent compounds and (poly)phenol-rich foods. Derivatives of ferulic acid, resveratrol and anthocyanins increased glucose uptake and metabolism, notably in high glucose and insulin-treated myotubes, as evidenced by increased short-term intracellular transport of 2-[1-^{14}C(U)]-deoxy-D-glucose and decreased longer-term D-[^{14}C(U)]-glucose uptake respectively, assessed by liquid scintillation counting. When tested individually, isovanillic acid 3-*O*-sulfate (IVAS), a colonic microbiome catabolite of cyanidin 3-*O*-glucoside, stimulated a dose-dependent increase in 2-[1-^{14}C(U)]-deoxy-D-glucose transport and involved a PI3K-dependent mechanism, as confirmed by wortmannin and indinavir inhibition. Western analyses showed that IVAS upregulated GLUT1, GLUT4 and PI3K p85α, and increased phosphorylated Akt. This is the first time such metabolites have been shown to improve glucose uptake in human skeletal muscle, with promising effects on insulin signalling and GLUT expression, notably in myotubes exposed to a high glucose/insulin stress.

3.2 Introduction

Skeletal muscle accounts for at least 40% of the human body mass and is the primary site of postprandial glucose disposal (Tremblay, Dubois et al. 2003, Lauritzen and Schertzer 2010). Glucose uptake is facilitated by glucose transporters encoded by the SLC2 genes, with tissue-specific expression of different isoforms (Cura and Carruthers 2012, Mueckler and Thorens 2013). GLUT1 and GLUT4 are the major glucose transporters in skeletal muscle tissue and the latter is dominantly expressed in mature muscle *in vivo* and activated by translocation from internal vesicles to the plasma membrane, in response to insulin (Huang and Czech 2007, Cura and Carruthers 2012, Mueckler and Thorens 2013). In insulin-resistant tissues, insulin-stimulated GLUT4 glucose uptake is compromised (Kahn, Rosen et al. 1992, Saltiel and Kahn 2001, Deshmukh 2016). Insulin resistance therapies, including exercise, nutra-/pharmaceuticals and diet-related changes (Collier, Bruce et al. 2006, Oberg, Yassin et al. 2011, Stanford and Goodyear 2014, Alvim, Cheuhen et al. 2015, Kouzi, Yang et al. 2015, Cheng, Villani et al. 2017, Wu, Horowitz et al. 2017), lower postprandial glycaemia by restoring skeletal muscle uptake and enhancing metabolism. Glucose homeostasis is maintained without causing oxidative stress (Chapter 2), as glucose is converted to glycogen for storage (Saltiel and Kahn 2001), or some glucose may be metabolised aerobically through transduction of AMPK-mediated pathways (Jager, Handschin et al. 2007, Canto, Gerhart-Hines et al. 2009, Timmers, Konings et al. 2011). Conserving insulin sensitivity in skeletal muscle is imperative to reducing chronic hyperglycaemia and preventing or reversing the diabetic phenotype.

Diets high in (poly)phenols have been correlated with a lower incidence of type 2 diabetes (Kim, Keogh et al. 2016) and as such this has led to investigations *in vivo* and *in vitro* into how these compounds may help regulate glucose homeostasis. Mechanisms studied include the inhibition of carbohydrate digestion and absorption in the gut (Williamson 2013), stimulation of glucose uptake into muscle and adipose, improvement in pancreatic function and hepatic glucose metabolism, as well as lowering obesity-induced inflammation and oxidative stress (Hanhineva, Torronen et al. 2010, Babu, Liu et al. 2013, Williamson 2013, Kim, Keogh et al. 2016) (see 1.3.2). (Poly)phenols, or (poly)phenol-rich extracts or foods, have been shown to affect the expression and activity of the GLUTs. A cinnamon (poly)phenol extract was shown to increase GLUT1

expression in murine macrophages to help enhance their immune function (Cao, Urban et al. 2008), while phloretin, commonly found in apples, inhibits GLUT2 and induces cell cycle arrest in colorectal tumour cells (Lin, Tu et al. 2016). Recently, data were published from our lab that demonstrated green and chamomile teas inhibit GLUT2 in Caco-2 cells and *Xenopus* oocytes, and chamomile also inhibits GLUT5 (Villa-Rodriguez, Aydin et al. 2017). There are reports of (poly)phenols enhancing GLUT4-mediated transport into muscle via increased GLUT4 expression and/or via the insulin- or AMPK-mediated pathways. Citrus flavonoids hesperidin and naringin upregulated GLUT4 expression in the adipose of diabetic mice, as well as improving hepatic glucose metabolism, systemic lipid metabolism and lowering hyperlipidaemia (Jung, Lee et al. 2006). Grape seed procyanidin extract lowered hyperglycaemia in streptozotocin-induced diabetic rats and stimulated glucose uptake in L6 myotubes and 3T3-L1 adipocytes via increased GLUT4 translocation dependent on the insulin signalling pathways (Pinent, Blay et al. 2004). The same effects were elicited by an anthocyanin-rich bilberry extract in diabetic mice, but via stimulation of AMPK (Takikawa, Inoue et al. 2010).

However, the literature is lacking *in vitro* studies testing (poly)phenol metabolites that can be found in circulation *in vivo*. In the animal studies described, the effects on glycaemia and lipidaemia may have been caused by conjugates and catabolites of the (poly)phenols that were administered. More attention should be given to matching the compounds and their doses to the tissue in question i.e. gut cells *in vivo* are exposed to relatively higher concentrations of the compounds in their aglycone and plant metabolite forms, while hepatic, adipose and muscle cells will see mostly conjugates in the nanomolar to low micromolar range (Del Rio, Rodriguez-Mateos et al. 2013, Bohn, McDougall et al. 2015). Notably, there is less information on how catabolites of the colonic microbiome may play a role and yet the majority of (poly)phenols in circulation are metabolised in this way before absorption (Cardona, Andres-Lacueva et al. 2013, Williamson and Clifford 2017). Colonic metabolites are of particular interest because they can be present in the blood at high concentrations, similar to those tested *in vitro*, and remain so for long periods of time, increasing their bioavailability (Williamson and Clifford 2010).

Studies using combinations of metabolites would also be useful in mimicking the *in vivo* situation, because it is unlikely for tissues to be exposed to single compounds, and it has been shown previously that (poly)phenols may affect the metabolism and biological activity of others; several flavonoids are known to inhibit SULT for example and therefore will in turn affect the conjugation of other compounds (Huang, Chen et al. 2009). Even after taking a single supplement, ingestion of phytochemical(s) can yield tens of circulating metabolites; de Ferrars *et al*. reported the pharmacokinetic profiles of 17 metabolites in plasma, 31 in urine and 28 in faeces following ingestion of a single ^{13}C-labelled anthocyanin (de Ferrars, Czank et al. 2014). A recent study showed that resveratrol 3-*O*-sulfate inhibited colon cancer cell proliferation, which was more so than unconjugated resveratrol, and its activity was synergistically increased when in combination with resveratrol glucuronide conjugates despite they themselves exerting no effects. Moreover, the resveratrol metabolites, individually and in combination, synergised with chemotherapeutic drugs to induce cell death (Aires, Limagne et al. 2013). Further *in vitro* studies demonstrating the synergistic effects of natural compounds, including quercetin, and chemotherapeutics on cancer cells were reviewed recently (Aung, Qu et al. 2017).

Another challenge is the choice of model. There are scarcely any reported studies on (poly)phenols and glucose uptake in human skeletal muscle cells, either primary or immortalised. One recent study investigating the effects of epigallocatechin-3-gallate in increasing insulin sensitivity used both murine and primary human myotubes (Pournourmohammadi, Grimaldi et al. 2017) and a recent review looked at human studies with resveratrol intervention and subsequent muscle biopsy analyses (Sin, Yung et al. 2015). However, the rat L6 or murine C2C12 immortalised cell lines are favoured for their ease of culture, differentiation potential, accessibility (Cheng, El-Abd et al. 2014) and likely the abundant existing documentation to support new research, but it is important to acknowledge that mechanisms that occur in rodent muscle cells may not be the same as those in human. Such differences include the myogenic programming that regulates differentiation (Bareja, Holt et al. 2014), glucose transport kinetics in response to insulin and GLUT4 expression (Gould and Holman 1993).

The aim of this part of the project was to determine the LHCN-M2 myoblast line as a suitable human skeletal muscle cell model to study the effects of (poly)phenol

metabolites on glucose uptake and metabolism. A normal and diabetic-mimicking (hyperglycaemic and hyperinsulinaemic) model was established to compare metabolite activities between the two contexts. A recent study examining the effects of quercetin on glucose disposal in mice and C2C12 myotubes found that quercetin suppressed insulin-mediated glucose uptake under normal conditions, but rescued the impaired uptake under inflammatory conditions (Liu, Mei et al. 2016). Before testing the metabolites in the present study, the degree of differentiation, respiratory status, GLUT expression and insulin responses were characterised to confirm the muscle phenotype and evaluate the effects of differentiating the myoblasts in normal and high glucose/insulin. Hyperglycaemia and hyperinsulinaemia are classic biomarkers of diabetes (Wilcox 2005, Krentz and Hompesch 2016) and the effects of glucose and insulin on muscle glucose transport have been studied previously in human primary cells (Ciaraldi, Abrams et al. 1995), providing a reference for the model here.

(Poly)phenol metabolites were chosen based on their origins in frequently-consumed (poly)phenol-rich foods that have previously shown promise in regulating glycaemia *in vivo* and/or associated compounds or extracts have affected muscular glucose uptake *in vitro* (Table 3.1). Based on achievable plasma concentrations *in vivo*, ascertained from C_{max} values in pharmacokinetic studies, a dose of 2 µM was chosen for most metabolite compounds. This was slightly higher than that detected previously for dihydroferulic acid (DHFA) and its sulfate conjugate, and for the flavonol conjugates, but these values were recorded after a single dose, such as one cup of coffee or a portion of fried onions for example, so it is plausible that plasma concentrations could reach several micromolar from habitual consumption and following several meals in a day. The combination of phenolic sulfates was chosen on the back of a pharmacokinetic study done previously in our lab, with the identification (and subsequent synthesis) of these metabolites circulating in plasma following the consumption of a mixed berry purée (Pimpao, Ventura et al. 2015). Higher doses of 5 µM were chosen for these metabolites because they are mostly colonic microbiome catabolites with higher plasma concentrations achieved (Table 3.1) and generally have longer circulation times (Table 1.1). This is the first study to investigate the effects of such conjugates on glucose uptake and metabolism in human skeletal muscle, and with comparisons between myotubes differentiated in a normal or high glucose/insulin environment.

Table 3.1: (Poly)phenol metabolites selected for the present study, with reference to articles evidencing antidiabetic effects for parent/precursor compounds, or associated extracts or foods (more detail in 3.5).

(Poly)phenol Metabolite Profile (Metabolites)	**Food Sources** [1]	**[Plasma] Achieved in pharmacokinetic studies**	**Antidiabetic *in vivo* studies** [2]	**Antidiabetic *in vitro* studies** [3]
Ferulic Acid Ferulic acid 4-*O*-sulfate (FA4S) Dihydroferulic acid 4-*O*-sulfate (DHFA-4S) Dihydroferulic acid (DHFA)	Coffee, wheat, dark chocolate, dates, herbs, olive, berries, cinnamon	FA4S > 4.9 µM (Feliciano, Mills et al. 2017) DHFA-4S > 0.9 µM (Lang, Dieminger et al. 2013) DHFA > 1.2 µM (Guy, Renouf et al. 2009)	(Johnston, Clifford et al. 2003, Khan, Safdar et al. 2003, Thom 2007, Andersen, Koehler et al. 2008, Hlebowicz, Hlebowicz et al. 2009, van Dijk, Olthof et al. 2009, Lu, Sheng et al. 2012, Bozzetto, Annuzzi et al. 2015)	(Roffey, Atwal et al. 2006, Prabhakar and Doble 2009, Prabhakar and Doble 2011, Chellan, Muller et al. 2012, Tsuda, Egawa et al. 2012, Bhattacharya, Christensen et al. 2013, Gogoi, Chatterjee et al. 2014, Ho, Kase et al. 2017)
Flavonols Quercetin 3′-*O*-sulfate (Q3S) Quercetin 3-*O*-β-D-glucuronide (Q3G) Kaempferol 3-*O*-β-D-glucuronide (K3G)	Capers, onion, apple, tea, elderberry, endive, wine, beans, propolis	Q3S > 0.7 µM (Mullen, Edwards et al. 2006) Q3G ≥ 0.6 µM (Cialdella-Kam, Nieman et al. 2013) K3G > 0.4 µM (Kerimi, Jailani et al. 2015)	(Johnston, Clifford et al. 2002, Bozzetto, Annuzzi et al. 2015, Nyambe-Silavwe and Williamson 2016)	(Fang, Gao et al. 2008, Zanatta, Rosso et al. 2008, Eid, Martineau et al. 2010, Kawabata, Sawada et al. 2011, Bhattacharya, Christensen et al. 2013, Ueda, Hayashibara et al. 2013, Xu, Hu et al. 2014, Eid, Nachar et al. 2015, Liu, Mei et al. 2016, Ho, Kase et al. 2017)

Resveratrol *trans*-Resveratrol 4′-*O*-β-D-glucuronide (R4G) *trans*-Resveratrol 3-*O*-sulfate (R3S) *trans*-Resveratrol 3-*O*-β-D-glucuronide (R3G)	Wine, grape seed and various berries	R4G > 10.2 µM (Brown, Patel et al. 2010) R3S > 18.3 µM (Brown, Patel et al. 2010) R3G > 17.1 µM (Brown, Patel et al. 2010) [4]	(Brasnyo, Molnar et al. 2011, Timmers, Konings et al. 2011, Bhatt, Thomas et al. 2012, Crandall, Oram et al. 2012, Movahed, Nabipour et al. 2013, Goh, Lee et al. 2014, Zare Javid, Hormoznejad et al. 2017)	(Park, Kim et al. 2007, Breen, Sanli et al. 2008, Deng, Hsieh et al. 2008, Minakawa, Kawano et al. 2011)
Phenolic sulfates Catechol-*O*-sulfate (CS) 4-Methylcatechol-*O*-sulfate (MCS) Pyrogallol-*O*-sulfate (PGS) 4-Methylgallic 3-*O*-sulfate (MGS) Vanillic acid 4-*O*-sulfate (VAS) Isovanillic acid 3-*O*-sulfate (IVAS)	Berries, cherry, olive, plum, coffee, cinnamon, black soybeans	CS > 24.6 µM (Feliciano, Boeres et al. 2016) MCS > 5.0 µM (Feliciano, Mills et al. 2017) PGS > 12 µM (Pimpao, Ventura et al. 2015) [5] MGS > 2 µM (Pimpao, Ventura et al. 2015) VAS > 1.6 µM (Feliciano, Mills et al. 2017) IVAS – n/a (PCA [6] > 2.4 µM (Czank, Cassidy et al. 2013))	(Johnston, Clifford et al. 2003, Khan, Safdar et al. 2003, Thom 2007, Hlebowicz, Hlebowicz et al. 2009, van Dijk, Olthof et al. 2009, Stull, Cash et al. 2010, Torronen, Sarkkinen et al. 2010, Edirisinghe, Banaszewski et al. 2011, Lu, Sheng et al. 2012, Hoggard, Cruickshank et al. 2013, Torronen, Kolehmainen et al. 2013, Giacco, Costabile et al. 2014, Bozzetto, Annuzzi et al. 2015, Nyambe-Silavwe and Williamson 2016)	(Roffey, Atwal et al. 2006, Vuong, Martineau et al. 2007, Yamamoto, Ueda et al. 2010, Scazzocchio, Vari et al. 2011, Chellan, Muller et al. 2012, Rojo, Ribnicky et al. 2012, Tsuda, Egawa et al. 2012, Matsukawa, Inaguma et al. 2015, Scazzocchio, Vari et al. 2015, Boue, Daigle et al. 2016, Ho, Kase et al. 2017, Ho, Kase et al. 2017, Luna-Vital, Weiss et al. 2017, Nachar, Eid et al. 2017)

[1] from Phenol Explorer (Rothwell, Perez-Jimenez et al. 2013); foods not listed in any particular order

[2] where glycaemia was lowered, or similar antidiabetic effects exerted, in humans

[3] where glucose uptake in muscle, or adipocytes, was stimulated

[4] all resveratrol metabolite C_{max} values measured after 29 days of a daily 5 g supplement

[5] two isoforms of pyrogallol-*O*-sulfate combined

[6] PCA – protocatechuic acid, included as the precursor of (iso-)vanillic acid sulfates; IVAS is the methylated and sulfated metabolite of PCA

Note: lists are examples of relevant foods/studies and are not exhaustive

3.3 Materials and Methods

3.3.1 Chemical compounds and reagents

All reagents were purchased from Sigma-Aldrich (Merck) (Gillingham, UK) unless otherwise stated. High purity (18.2 MΩ cm^{-1}) water supplied by a MilliQ system (Merck Millipore UK, Watford, UK) was used throughout.

3.3.2 Cell culture

LHCN-M2 myoblasts, kindly provided by Vincent Mouly, are a cell line derived from a selected myogenic clone of the immortalised population of human satellite cells derived originally from the pectoralis major muscle of a 41-year old Caucasian heart-transplant donor (Zhu, Mouly et al. 2007). Myoblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM) (11966-025, Gibco (Life Technologies, Thermo Fisher), Paisley, UK) supplemented with 5.5 mM glucose, 10% (v/v) heat-inactivated foetal bovine serum, 10% (v/v) heat-inactivated newborn calf serum (Gibco), 0.01 µg ml^{-1} animal-free recombinant human epidermal growth factor (PeproTech, London, UK), 1 ng ml^{-1} animal-origin free recombinant human basic fibroblast growth factor (Gibco) and 0.4 µg ml^{-1} dexamethasone and then sterile-filtered. The composition of the growth medium was optimised in preliminary experiments (data not shown). Cells were passaged before reaching confluence to avoid terminal differentiation, at least every four days. For experiments, myoblasts, upon reaching ~80-90% confluence, were

differentiated to myotubes for 96 h after replacing growth medium with differentiation medium; DMEM supplemented with 5.5 or 25 mM glucose, 10 or 50 nM recombinant human insulin from *Saccharomyces cerevisiae* and 2% horse serum (Gibco) (myotubes differentiated in various concentrations of glucose and insulin were characterised – see Results) and this was changed every day. Cells were kept in a humidified atmosphere of 5% CO_2/95% air at 37ºC and used for experiments during passages 129-135. Sub-culturing was done in 75 cm^2 or 175 cm^2 flasks (Appleton Woods, Birmingham, UK) and for experiments cells were seeded on 6-well plates (Appleton Woods), 100 mm dishes or 150 mm dishes (Greiner CELLSTAR, Stonehouse, UK) at a density equivalent to ~1.8 x 10^4 cells cm^{-2}. For experiments, N/n values indicate the number of independent biological passages (N) and the number of technical replicates (n) from which the data were acquired.

HepG2 (American Type Culture Collection HB-8065, LGC Promochem, Teddington, UK), Caco-2/TC7 (kindly provided by Dr. Monique Rousset, Institut national de la santé et de la recherche médicale, Paris, France) and Human Umbilical Vein Endothelial Cells (HUVEC) (C2517A, Lonza, Visp, Switzerland) cells were cultured as done previously in our lab (HepG2 – see 2.3.2; Caco-2/TC7 – (Villa-Rodriguez, Aydin et al. 2017); HUVEC – (Tumova, Kerimi et al. 2016)) and their whole cell lysates used in the validation of the GLUT4 antibody for Western analyses.

3.3.3 Microscopy and cell viability checks

Viability was assessed when seeding LHCN-M2 myoblasts for experiments using the Trypan Blue exclusion assay as done previously (Strober 2001). Cells were lifted with 0.05% trypsin-EDTA, resuspended in medium and an aliquot mixed 1:1 (v/v) with 0.4% Trypan Blue-PBS and counted on a haemocytometer using a *Leica MD IL LED* inverted microscope; viable cells were those excluding Trypan Blue. During experiments non-viable cells would actually detach from the plate, due to cell death or over-differentiation causing auto-contractions in the myotubes. If this was extensive the experiment was abandoned and repeated with a new passage of viable myoblasts, and if any detached during experiments then only viable cells were collected for analyses at the end anyway. Differentiation progress was monitored daily by observing the formation of elongated and multinucleated myotubes, and images were acquired with the same microscope.

3.3.4 Cell treatments with (poly)phenol conjugates

LHCN-M2 myotubes, differentiated for 96 h in 5.5 mM glucose and 10 nM insulin or in 25 mM glucose and 50 nM insulin, were starved of glucose and serum in the presence of (poly)phenol metabolites in combination, an individual compound or DMSO control for 4 h before subsequent experiments for protein analyses or deoxy-/glucose uptake. Compounds were all reconstituted or diluted in DMSO. Comprising the ferulic acid metabolite profile were: ferulic acid 4-*O*-sulfate (FA4S), dihydroferulic acid 4-*O*-sulfate (DHFA-4S) (both synthesised by Dr. Nicolai Kraut in our lab previously; data unpublished but presented in his PhD thesis, 2015) and dihydroferulic acid (DHFA) (purchased from Alfa Aesar, Thermo Fisher Scientific, Warrington, UK), all at 2 µM when in combination or 6 µM individually. In the flavonol metabolite profile were: quercetin 3′-*O*-sulfate (Q3S) (synthesised by Dr. Dennis Wong in our lab previously (Wong, Botting et al. 2011)), quercetin 3-*O*-β-D-glucuronide (Q3G) and kaempferol 3-*O*-β-D-glucuronide (K3G) (both purchased from Sigma-Aldrich), all at 2 µM when in combination or 6 µM individually. Resveratrol metabolites comprised: *trans*-resveratrol 4′-*O*-β-D-glucuronide (R4G), *trans*-resveratrol 3-*O*-sulfate (R3S) and *trans*-resveratrol 3-*O*-β-D-glucuronide (R3G) (all purchased from Bertin Pharma, Montigny-le-Bretonneux, France), again at 2 µM in combination or 6 µM individually. The berry metabolite profile included the following phenolic sulfates: catechol-*O*-sulfate (CS), 4-methylcatechol-*O*-sulfate (MCS), pyrogallol-*O*-sulfate (PGS), 4-methylgallic 3-*O*-sulfate (MGS), vanillic acid 4-*O*-sulfate (VAS) and isovanillic acid 3-*O*-sulfate (IVAS) (all chemically/enzymically synthesised and characterised in our lab by Dr Rui Pimpão as described previously (Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015)), all at 5 µM in combination or 30 µM individually. IVAS was also tested further at various concentrations, always with the equivalent final DMSO (v/v). Isovanillic acid (Sigma-Aldrich) (IVA) was tested individually at 30 µM to compare to the effects of IVAS.

3.3.5 Protein extraction and BCA total protein assay

LHCN-M2 myotubes were differentiated for 96 h in 5.5 mM or 25 mM glucose with or without various concentrations of insulin and protein extracted at 96 h or at 0, 24, 48 and 72 h time-points during differentiation. Alternatively, myotubes were differentiated for 96 h in 5.5 mM glucose and 10 nM insulin or in 25 mM glucose and 50 nM insulin before being starved of glucose and serum in the presence of a (poly)phenol metabolite profile, individual compound or DMSO control for 4 h and then protein extracted. For analysis of Akt activation after incubation with IVAS, myotubes were incubated for a further 15 min with IVAS, insulin, IVAS and insulin together, insulin and 5 µM wortmannin (inhibitor of PI3K of the insulin signalling pathway (Clarke, Young et al. 1994, Elmendorf, Damrau-Abney et al. 1995, Roffey, Atwal et al. 2006), or DMSO control. Following treatment, protein was extracted by washing cells twice in PBS, scraping into Bicine/CHAPS lysis buffer (ProteinSimple, Bio-Techne, San Jose, CA, USA) supplemented with 1% (v/v) protease inhibitor cocktail (P8340) and phosphatase inhibitor cocktail (one tablet PhosSTOP/10 ml) (#04 906 845, Roche, Sigma-Aldrich), centrifuging at 500 g, 4ºC for 5 min, removing PBS and then snap-freezing in a dry ice/ethanol bath before storing pellets at -80ºC. Total protein in thawed pellets was assayed using the colorimetric detection of bicinchoninic acid (BCA) as done previously (Smith, Krohn et al. 1985), with quantification on a PheraSTAR FS microplate reader (BMG LabTech, Ortenberg, Germany). Whole cell lysates for HepG2, Caco-2/TC7 and HUVEC cells, used as negative controls in the GLUT4 analysis, were prepared and protein measured in the same way. A positive control for GLUT4, human skeletal muscle whole tissue lysate (#NB820-59253, Novus, BioTechne) was diluted in Bicine/CHAPS and protein measured in the same way.

3.3.6 Western protein analyses

Whole cell lysates were prepared for running on the *ProteinSimple Wes* automated Western system (ProteinSimple, Bio-Techne) using reagents supplied in the system kit according to the manufacturer's guidelines and were denatured at 37ºC for 20 min before being added to the pre-filled assay plate along with primary antibodies accordingly. Primary antibodies used were: mouse anti-troponin T (cardiac) (CT3, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) as a marker of differentiated muscle (Gardner, Anguiano et al. 2012); mouse anti-GLUT4 (1F8, Cell

Signalling Technology, Beverley, MA, USA); rabbit anti-GLUT1 (ab115730, Abcam, Cambridge, UK); mouse anti-PI3K (subunit p85α) (MAB2998, Novus, BioTechne) as a marker of the insulin signalling pathway proteins (Clarke, Young et al. 1994); rabbit anti-Akt (#9272, Cell Signalling Technology) and rabbit anti-phospho-Akt (Ser^{473}) ((D9E) XP #4060, Cell Signalling Technology) to determine the p-Akt/Akt ratio, a marker of insulin signalling pathway activation (Kohn, Barthel et al. 1998); and these were duplexed with mouse anti-α-actinin 1 (MAB8279, Novus, BioTechne) or rabbit anti-α-actinin ((D6F6) XP #6487, Cell Signalling Technology) (which recognises total endogenous α-actinin proteins) accordingly as loading controls, and run with anti-mouse or anti-rabbit secondary antibodies accordingly (supplied in the *Wes* kit). Lysate preps were loaded onto the *Wes* system at specific final protein concentrations, and chemiluminescence was detected after specific exposure times (or in the high dynamic range mode (HDR)), determined as optimal for each antibody combination: 0.4 mg/ml for troponin T/α-actinin 1 and after 16 s; 0.5 mg/ml for GLUT4/α-actinin 1 and after 512 s for the former and in HDR for the latter; 0.1 mg/ml for GLUT1/α-actinin and after 16 s; 0.4 mg/ml for PI3K p85α/α-actinin 1 and after 16 s; 0.1 mg/ml for (p-)Akt/α-actinin and after 512 s for the former and in HDR for the latter. See Figs. 3.2, 3.6, 3.7, 3.13, 3.14 for full setup and validation of antibodies. Chemiluminescent peak areas were converted into ratios of the target protein/loading control.

3.3.7 Uptake assays with radiolabelled 2-deoxy-D-glucose and glucose

LHCN-M2 myotubes were differentiated and treated as described previously for protein extraction. Following the 4 h starvation/incubation with the (poly)phenol metabolites or DMSO control, or insulin (for uptake experiments only), myotubes were further incubated with various insulin concentrations, (poly)phenol metabolites or DMSO controls and 0.075 µCi/ml 2-[1-^{14}C(U)]-deoxy-D-glucose (PerkinElmer, Beaconsfield, UK) in 0.55 mM 2-deoxy-D-glucose in DMEM for 30 min or 0.1 µCi/ml D-[^{14}C(U)]-glucose (PerkinElmer) in 0.55 mM D-glucose in DMEM for 4 h. Cells were then washed twice in the starvation medium (without glucose and serum), lysed in 1M NaOH, neutralised with 1M HCl and measured by liquid scintillation counting using a *Tri-Carb 1900 TR* Liquid Scintillation Analyzer (Canberra Packard, Harwell, Oxford, UK). Counts per minute were corrected for total protein, measured in aliquots of the lysates using the Bradford assay (Pierce Coomassie (Bradford) Protein Assay Kit #23200,

Thermo Fisher Scientific) as described previously (Bradford 1976, Ernst and Zor 2010) and extrapolated to total 2-deoxy-D-glucose and glucose uptake. Uptake of both in response to insulin in myoblasts (at the point of confluence where medium would be changed for differentiation) was also investigated in this way. Later experiments with IVAS on the myotubes also used 5 µM wortmannin and 20 µM indinavir, a known inhibitor of GLUT4 (Rudich, Konrad et al. 2003, Breen, Sanli et al. 2008).

3.3.8 High-resolution respirometry

LHCN-M2 myotubes differentiated for 96 h in 5.5 mM glucose were washed in PBS and lifted from 150 mm dishes in serum-free DMEM. Oxygen concentration and flux was continuously measured as 2 ml of the cell suspensions were added to each chamber of the *Oxygraph-2k (O2k)* (OROBOROS Instruments, Innsbruck, Austria), maintained at 37^{o}C, and a phosphorylation control protocol followed (see 2.3.10) (Brand and Nicholls 2011, Gnaiger 2014). 'Routine' basal respiration was recorded first, then 250 nM oligomycin added to measure IMM proton 'Leak'; 5 µM FCCP was titrated in to measure electron transfer system ('ETS') capacity, and 1.25 µM rotenone and 2.5 µM antimycin A both added for non-mitochondrial residual oxygen consumption, 'ROX', for which the previous measurements were corrected. Cell suspensions were collected after the respirometry experiment was complete, spun down and pellets lysed in Bicine/CHAPS and measured for total protein using the BCA assay as done for the Western lysates; respiration values were then corrected for total protein. To compare respiration in LHCN-M2 muscle cells to that in HepG2 hepatic cells, as part of the muscle model characterisation, the flux control ratios, which account for ETS capacity to enable comparison between cell types, were calculated from these respirometry values and compared to data collected for Chapter 2 (see 2.3.10 and 2.4.5). Flux control ratios were calculated from the measured oxygen fluxes as a factor of ETS; R/E – Routine/ETS, L/E – Leak/ETS, Net R/E – (Routine-Leak)/ETS, and ROX/E′ – ROX/non-ROX-corrected ETS.

3.3.9 DCFH-DA ROS Assay

LHCN-M2 myotubes differentiated in 6-well plates for 96 h in 5.5 mM or 25 mM glucose with 10 or 50 nM insulin or DMSO controls were assessed for relative total ROS levels using the DCFH-DA assay as done for the HepG2 cells (see 2.3.7) and elsewhere

previously (Wang and Joseph 1999). Briefly, cells were washed with PBS, incubated with 10 µM DCFH-DA in PBS at 37ºC for 20 min in darkness, washed again and DCF fluorescence measured on the *PheraSTAR FS* plate reader (Ex/Em = 485/530 nm). Cells were then scraped into and lysed in CellLytic supplemented with 1% (v/v) P8340 protease inhibitor cocktail and total protein measured using the Bradford assay. DCF fluorescence, in relative fluorescence units, was corrected for protein and normalised relative to total ROS in the 5.5 mM glucose control.

3.3.10 Statistical analyses

Data are expressed as means ± standard error of the mean. The significance of differences between groups of treatments was analysed by one-way ANOVA and post hoc Tukey's (for unequal sample sizes), Dunnett's (comparing multiple treatments to a single control) or two-tailed *t*-tests accordingly, with variance checks using Levene's test, and with Bonferroni correction where multiple treatments were compared, using SPSS 24 and presenting the data in Origin 2016 $p < 0.05$ was considered as statistically significant.

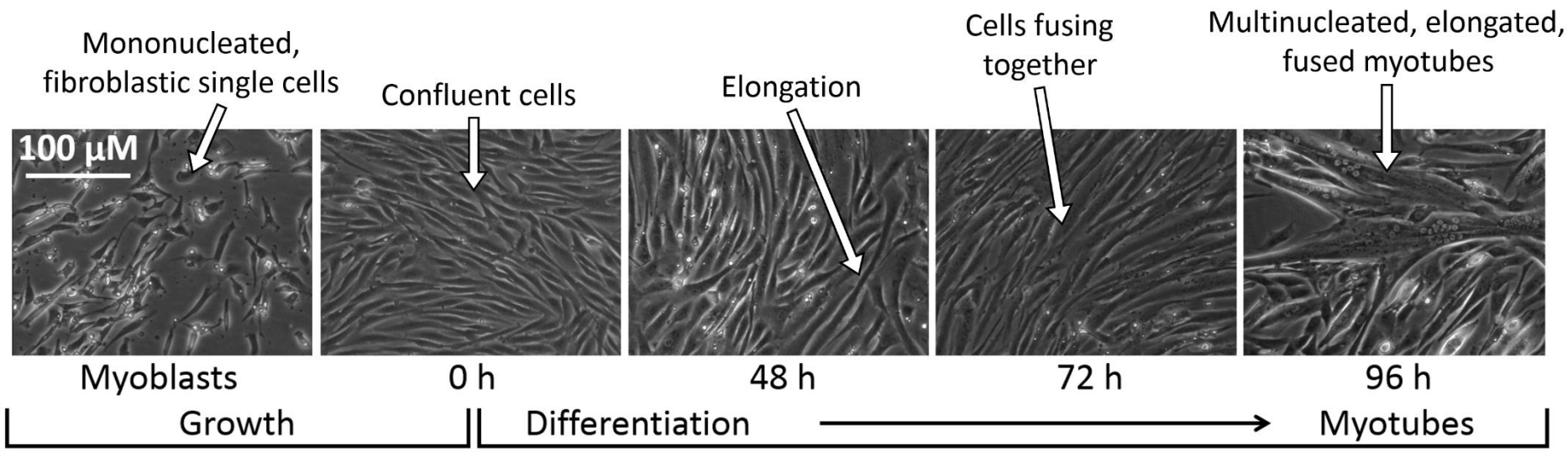

Figure 3.1: LHCN-M2 myoblasts differentiate to myotubes in 96 h. Myoblasts were grown in medium containing 5.5 mM glucose, 20% serum, dexamethasone and growth factors until confluent, at which point this was replaced with medium containing 5.5 mM glucose and 2% serum only and the myoblasts differentiated for 96 h. Images were taken daily using a Leica MD IL LED inverted microscope at 10X magnification.

3.4 Results

3.4.1 Characterising the human skeletal muscle cell model

3.4.1.1 Differentiation from myoblasts to myotubes

LHCN-M2 myoblasts were differentiated to myotubes, identified by multinucleated fibres formed by fused myoblasts, for 96 h in differentiation medium (Fig. 3.1). Protein analysis revealed that α-actinin levels remained constant, making it a suitable loading control for subsequent protein analyses, but troponin T increased by an average of 50% every 24 h (Fig. 3.3). As expected, myotubes were responsive to insulin and respired aerobically (Fig. 3.4). Insulin dose-dependently increased acute 2-[1-^{14}C(U)]-deoxy-D-glucose (2-[1-^{14}C(U)]-DG) uptake, up by 30% with 200 nM ($p < 0.001$), and D-[^{14}C(U)]-glucose (D-[^{14}C(U)]-Glc) (or ^{14}C in other metabolic forms downstream of glucose) retained in the cells after 4 h, up by 65% with 50 nM ($p < 0.001$) (Figs. 3.4A, B). Insulin did not stimulate 2-[1-^{14}C(U)]-DG uptake in myoblasts (0 h differentiation) but increased ^{14}C in the cells after longer-term uptake with D-[^{14}C(U)]-Glc at the precursor stage (Fig. 3.4C). Oxidative respiration was greater in myotubes than in hepatocytes grown in similar conditions, particularly net mitochondrial output by 70% ($p < 0.001$) (Fig. 3.4D).

3.4.1.2 Effects of glucose and insulin on myotube phenotype

Morphological differences in myotube differentiation caused by glucose or insulin could not be determined by eye, as seen in the micrographs in Fig. 3.5E, however respiration and differentiation were affected (Figs. 3.5A-D). Differentiating in high glucose increased IMM proton leak by 45% ($p < 0.05$) (Fig. 3.5A) and relative ROS levels in myotubes; 10 nM insulin reversed the latter but 50 nM insulin increased it further (Fig. 3.5B). The greatest difference in ROS was between 5.5 mM glucose with 10 nM insulin and 25 mM glucose with 50 nM insulin ($p < 0.001$). In normal glucose insulin dose-dependently increased the degree of differentiation after 96 h ($p < 0.05$), but in high glucose this was lowered with 50 nM ($p < 0.05$) (Fig. 3.5C). Interestingly, troponin T/α-actinin was the same in myotubes differentiated in 5.5 mM glucose with 10 nM insulin and 25 mM glucose with 50 nM insulin (Fig. 3.5C) and this was the case throughout the 96 h (Fig. 3.5D).

The glucose and insulin concentration of the differentiation medium also affected 2-[1-^{14}C(U)]-DG and D-[^{14}C(U)]-Glc uptake in myotubes. Acute 2-[1-^{14}C(U)]-DG uptake was greater in normal glucose myotubes (Fig. 3.8A) but high glucose myotubes were more responsive to insulin (Fig. 3.8B). This was the same for ^{14}C taken up and remaining in the myotubes after 4 h (Figs. 3.8C, D), and differentiating the cells in the presence of insulin generally lowered this uptake too (Figs. 3.8C, E). Increasing the differentiation medium insulin from 10 nM to 50 nM lowered basal D-[^{14}C(U)]-Glc uptake/retained ^{14}C in the normal glucose cells ($p < 0.05$) but did not affect the high glucose cells. The greatest difference in D-[^{14}C(U)]-Glc uptake was observed between the 5.5 mM glucose with 10 nM insulin myotubes and the 25 mM glucose with 50 nM insulin myotubes ($p < 0.05$ at each dose of insulin) (Fig. 3.8E). The insulin response, however, was not different between these two conditions and was instead heightened with increased insulin, particularly in the normal glucose cells ($p < 0.001$ across the curve for 5.5 mM glucose with 50 nM insulin). Adding 10 nM insulin to the cells differentiating in high glucose actually diminished the insulin dose-response (NS across the curve) (Fig. 3.8F).

Protein analyses for myotubes differentiated in 5.5 mM glucose with 10 nM insulin or 25 mM glucose with 50 nM insulin revealed that total GLUT4 was not different between the two (Fig. 3.8G), but GLUT1 was higher in the former ($p < 0.001$) (Fig. 3.8H). As the cells differentiated, GLUT4 protein increased (Fig. 3.8G), while GLUT1 decreased. GLUT1 was lower in the myotubes differentiated in 5.5 mM glucose with 10 nM insulin after 48 h ($p < 0.05$), but higher after 96 h ($p < 0.01$) (Fig. 3.8H). Subsequent experiments were performed on cells differentiated in one of these two conditions to compare a 'normal' glucose/insulin environment (5.5 mM glucose with 10 nM insulin) to 'high' glucose/insulin. For ease, these shall be referred to from here simply as 'normal' or 'high GI' myotubes.

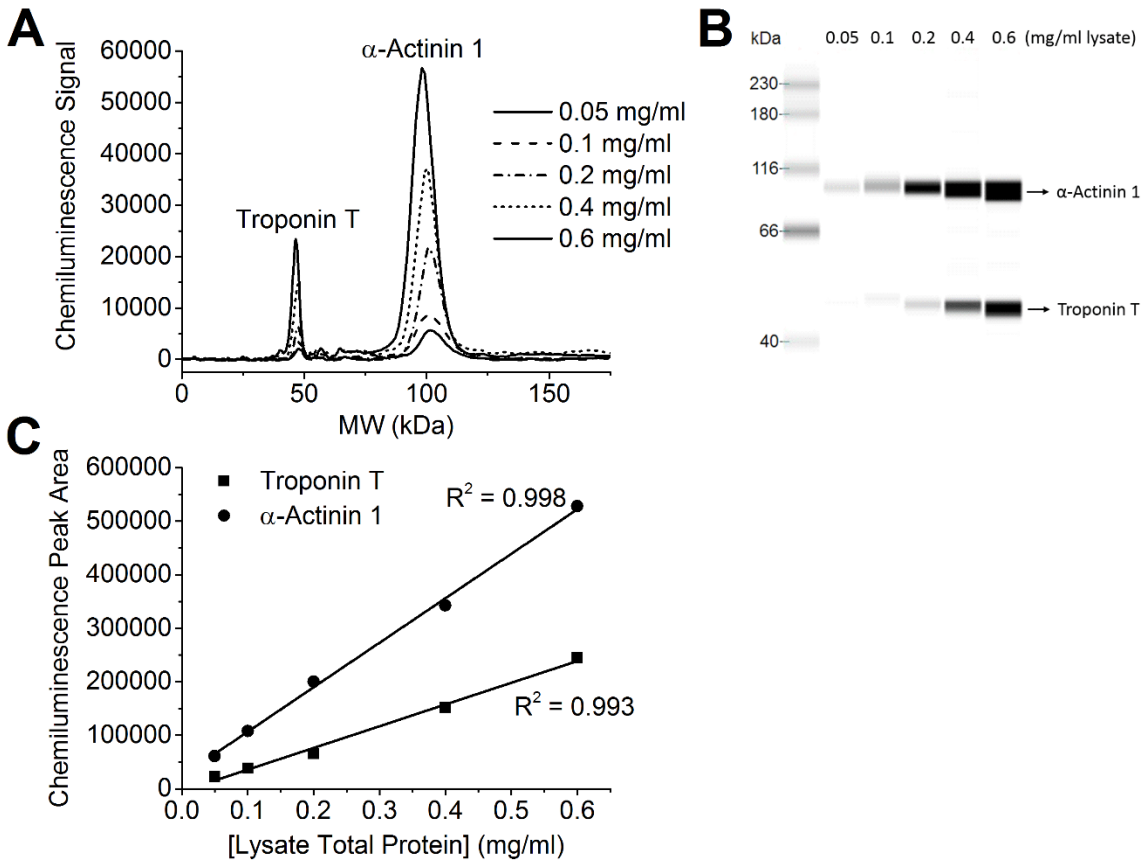

Figure 3.2: Antibody validation for Western analysis of troponin T and α-actinin 1. Various total protein concentrations of differentiated LHCN-M2 whole cell lysate were analysed with troponin T and α-actinin 1 antibodies and run in the same capillary on the ProteinSimple Wes automated Western system to establish standard curves, with electropherogram view (A) and gel-style lane view (B), for detection after a 16 s exposure. The peak areas taken from the pherogram were used to establish a standard curve for each antibody when duplexed (C).

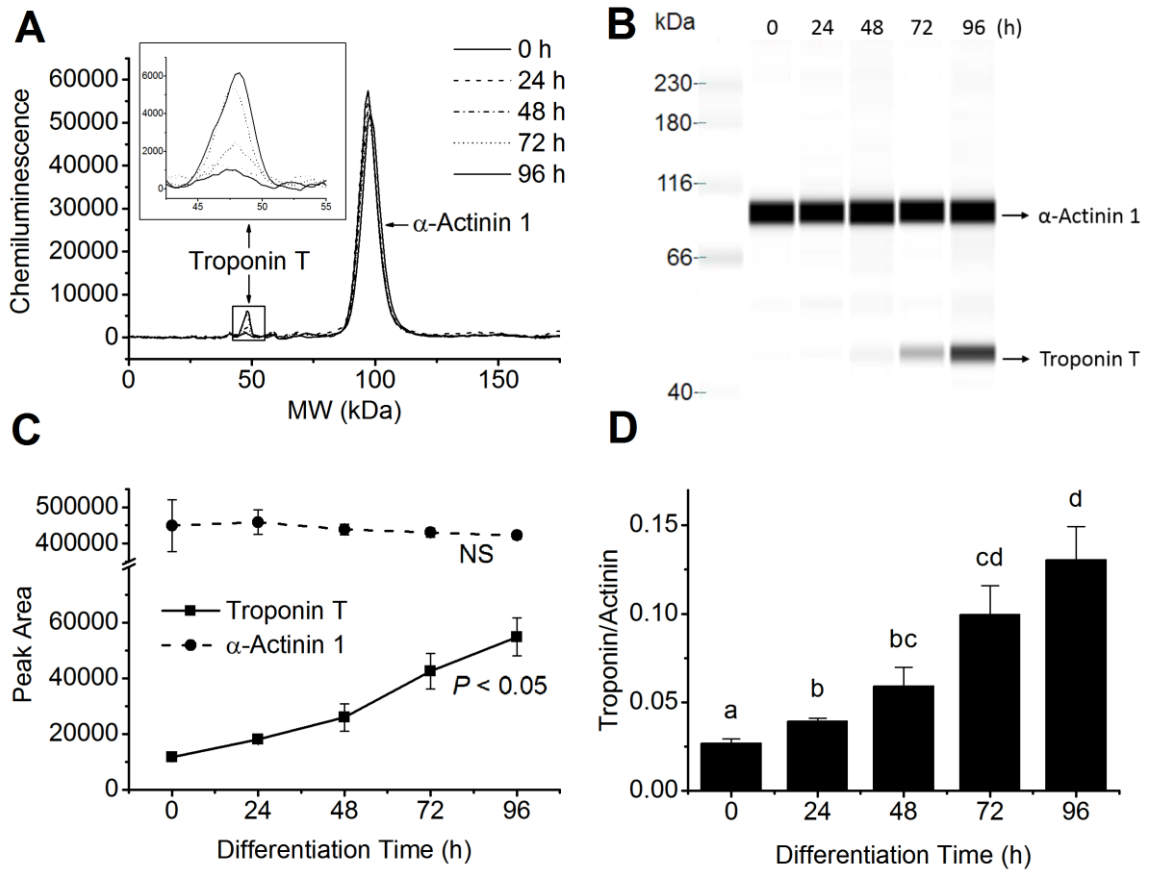

Figure 3.3: Troponin T is upregulated during myocyte differentiation. LHCN-M2 Myoblasts were grown and differentiated to myotubes over 96 h, as described in Fig. 3.1. Protein analysis of troponin T and α-actinin 1 was performed on the ProteinSimple Wes automated Western capillary system (see Fig. 3.2 for antibody validation and linear range of detection) using total cell lysates extracted each day during differentiation and loaded at 0.4 mg/ml. Chemiluminescence peaks after a 16 s exposure can be seen in the electropherogram (A) and gel image views (B). The areas under these peaks were plotted and data (C-D) are shown as mean ± SEM (N = 3). Letters above bars (D) denote where $p < 0.05$ between time-points.

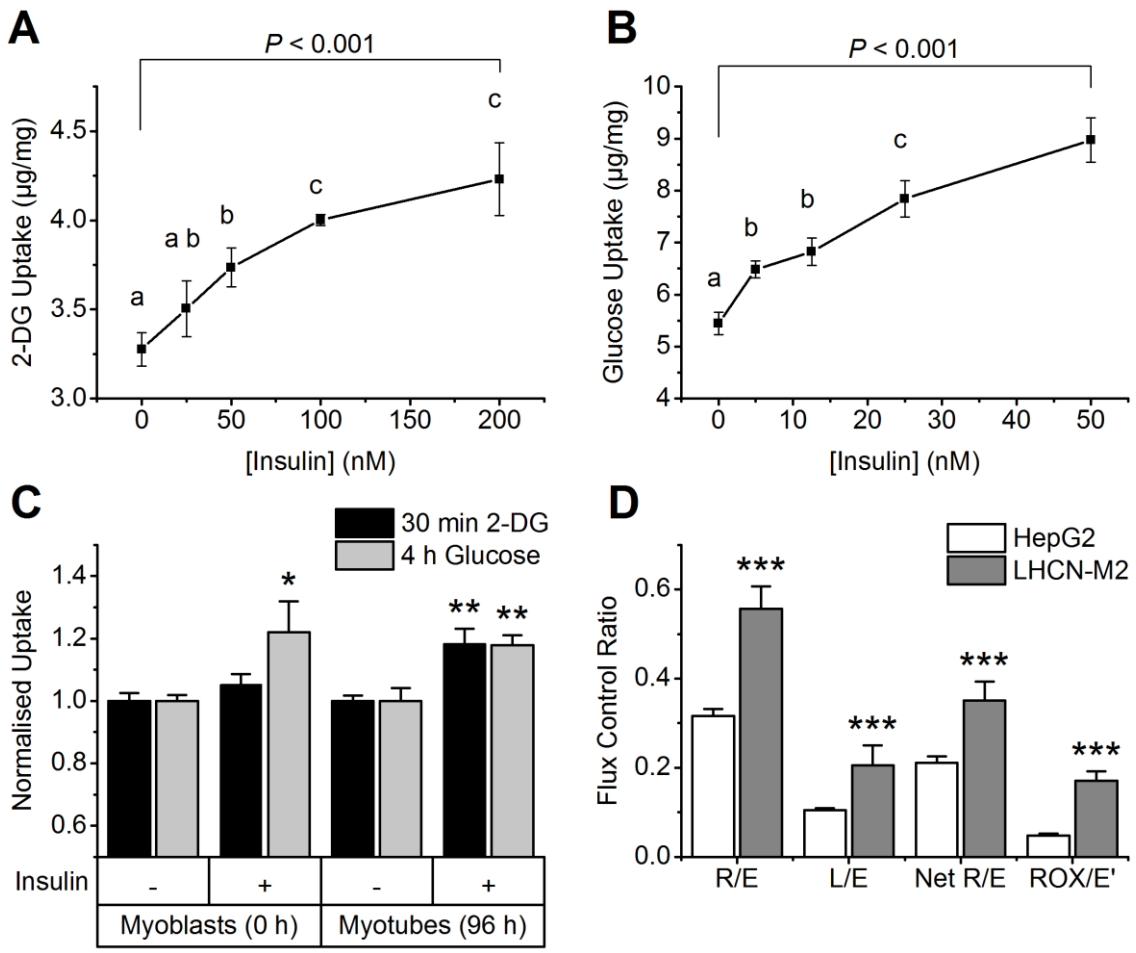

Figure 3.4: LHCN-M2 myotubes are insulin-responsive and more oxidative than hepatocytes. Acute uptake of 2-[1-^{14}C(U)]-deoxy-D-glucose (2-DG) (A) and longer-term D-[^{14}C(U)]-glucose uptake (or retention of ^{14}C metabolites) (B) in response to insulin in myotubes differentiated for 96 h was measured. Cells were starved of glucose and serum for 4 h then incubated with various insulin concentrations and 0.15 µCi/ml 2-[^{14}C(U)]-DG in 0.55 mM 2-DG for 30 min (A) or 0.2 µCi/ml D-[^{14}C(U)]-glucose in 0.55 mM D-glucose for 4 h (B), then washed, lysed and measured by liquid scintillation counting and corrected for total protein. The same was measured in myoblasts alongside myotubes for 30 min with 2-[1-^{14}C(U)]-deoxy-D-glucose (DG) ± 100 nM insulin or for 4 h with D-[^{14}C(U)]-glucose ± 25 nM insulin (C). Mitochondrial respiration was measured in myotubes and in HepG2 cells, grown in 5.5 mM glucose for 96 h, using the OROBOROS O2k respirometer and flux control ratios calculated as a factor of ETS (see Figs. 2.10A, 3.5A); R/E – Routine /ETS, L/E – Leak/ETS, Net R/E – (Routine-Leak)/ETS, and ROX/E′ – ROX as a factor of non-ROX-corrected ETS. All data are mean ± SEM (n/N = 12/4 (A); 24/5 (B); 12/4 (C); 25/17 (HepG2) and 5/5 (LHCN-M2) (D)). Letters denote $p < 0.05$ between doses (A-B). *$p < 0.05$ and **$p < 0.01$ *vs* 0 nM insulin controls (C). ***$p < 0.001$ *vs* HepG2 (D).

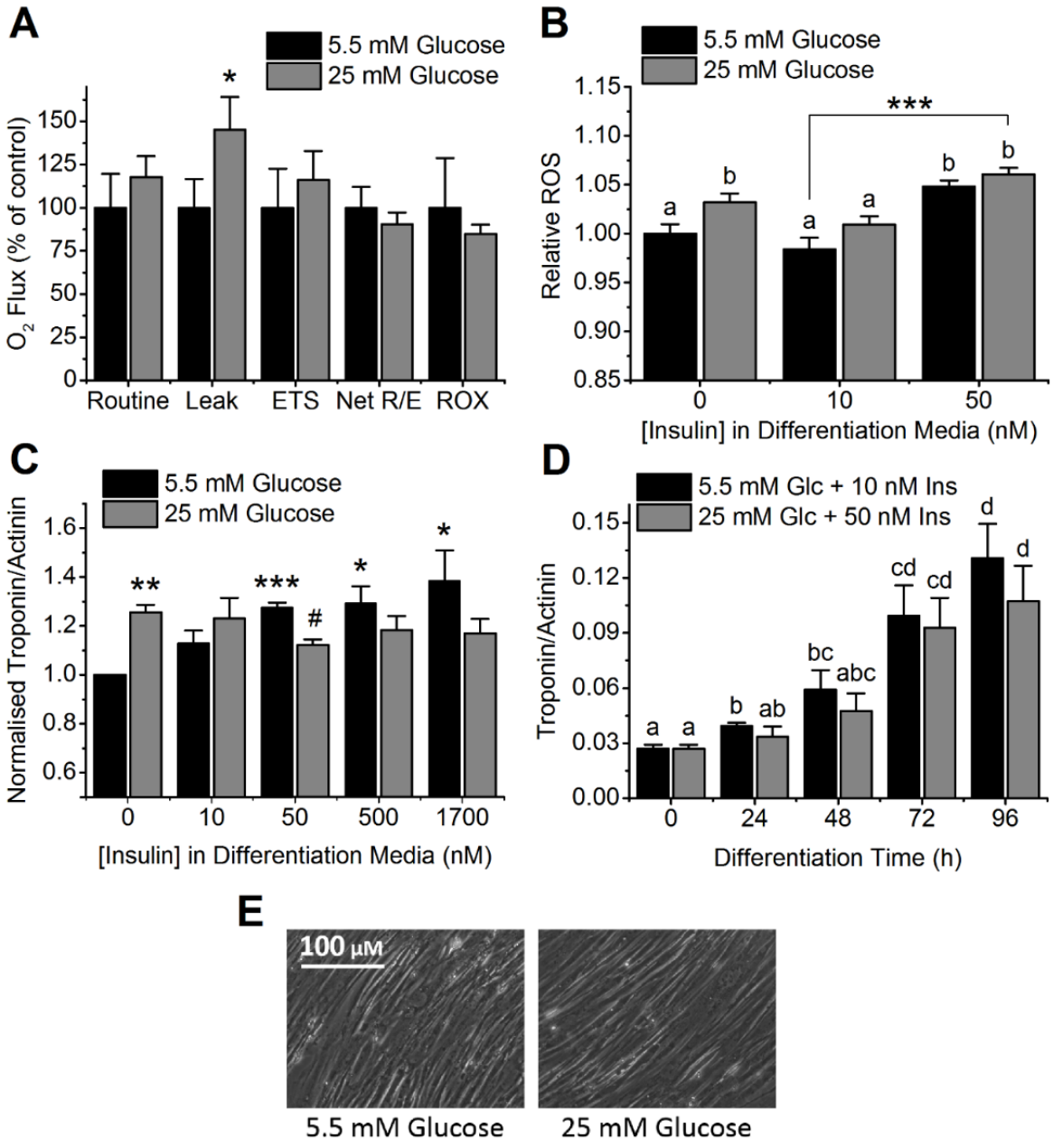
A
5.5 mM Glucose
25 mM Glucose
O_2 Flux (% of control)
Routine
Leak
ETS
Net R/E
ROX
B
5.5 mM Glucose
25 mM Glucose
Relative ROS
[Insulin] in Differentiation Media (nM)
C
5.5 mM Glucose
25 mM Glucose
Normalised Troponin/Actinin
[Insulin] in Differentiation Media (nM)
D
5.5 mM Glc + 10 nM Ins
25 mM Glc + 50 nM Ins
Troponin/Actinin
Differentiation Time (h)
E
100 µM
5.5 mM Glucose
25 mM Glucose

Figure 3.5: Glucose and insulin affect LHCN-M2 differentiation and myotube metabolism. Myotubes differentiated for 96 h in media containing 5.5 mM or 25 mM glucose and oxygen fluxes measured using the OROBOROS O2k high-resolution respirometer (A). Intact cells were added to the respirometer and a phosphorylation control protocol followed as for the HepG2 cells in Chapter 2 (see 2.3.10). Data were corrected for total protein and are expressed as mean percentages of the 5.5 mM glucose controls ± SEM (N = 5) (A). Relative ROS levels were assayed, by incubating with 10 µM DCFH-DA in PBS for 20 min and measuring DCF fluorescence, in myotubes differentiated in media with various glucose and insulin concentrations and data expressed as mean ROS relative to the 5.5 mM glucose + 0 nM insulin control ± SEM (n/N = 18/3) (B). Troponin T/α-actinin 1 protein in total cell lysates of myotubes differentiated in media with various glucose (Glc) and insulin (Ins) concentrations was measured on the ProteinSimple Wes automated Western capillary system (see Figs. 3.2, 3.3) and data are shown as mean ± SEM (N = 3) (C-D). Images of myotubes differentiated in various conditions were taken using the Leica MD IL LED inverted microscope at 10X magnification and an example of myotubes in 5.5 mM and 25 mM glucose is shown (E). *$p < 0.05$ *vs* 5.5 mM glucose (A). Letters above bars denote $p < 0.05$ (B). ***$p < 0.001$ for the biggest difference between two groups (B). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* 5.5 mM glucose + 0 nM insulin control (C). # $p < 0.05$ *vs* 5.5 mM glucose + 50 nM insulin (C). Letters above bars denote where $p < 0.05$ between time-points (D).

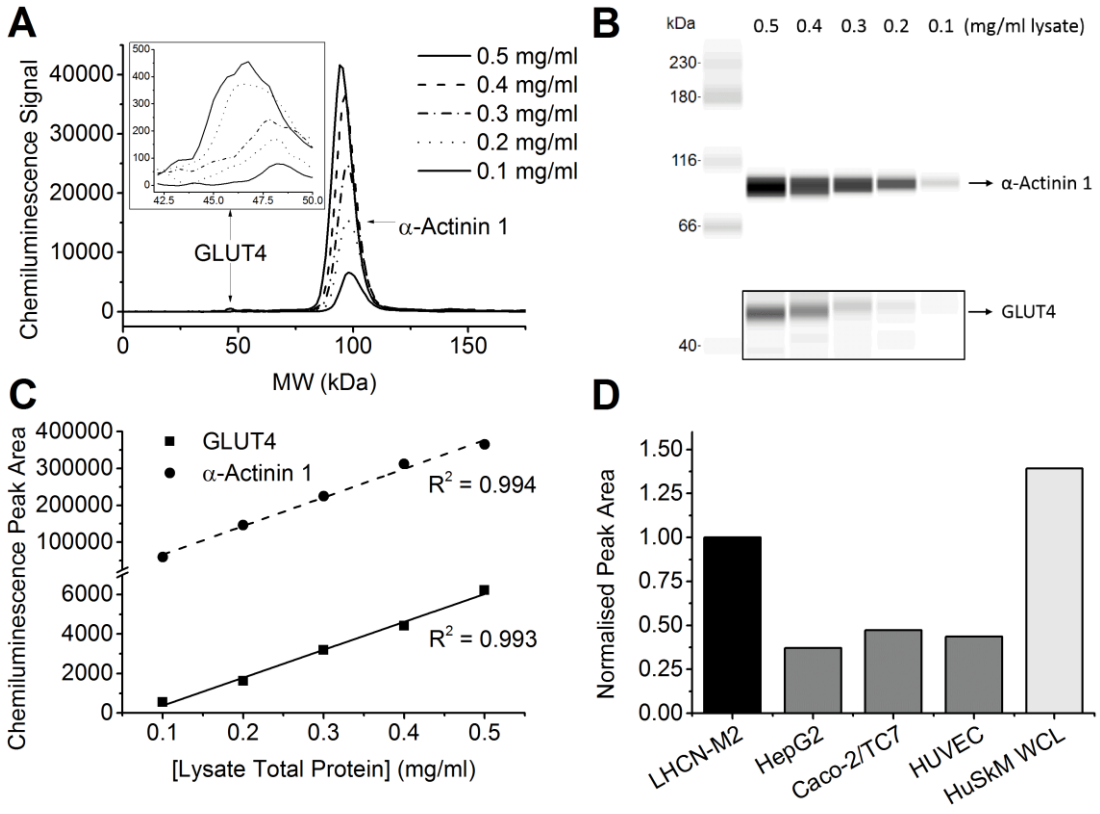

Figure 3.6: Antibody validation for Western analysis of GLUT4 and α-actinin 1. Various total protein concentrations of differentiated LHCN-M2 whole cell lysate were analysed with GLUT4 and α-actinin 1 antibodies and run in the same capillary on the ProteinSimple Wes automated Western system to establish standard curves, with electropherogram view (A) and gel-style lane view (B); α-actinin 1 was detected in high dynamic range mode while GLUT4 (boxed) was detected after a 512 s exposure. The peak areas taken from the pherograms were used to establish a standard curve for each antibody when duplexed (C). With relatively small peaks for GLUT4 and some non-target/non-specific noise, the specificity of the peak at ~47 kDa was supported by running the LHCN-M2 lysate alongside a human skeletal muscle whole cell lysate (HuSkM WCL) as a positive control and HepG2, Caco-2/TC7 and HUVEC total cell lysate negative controls (D).

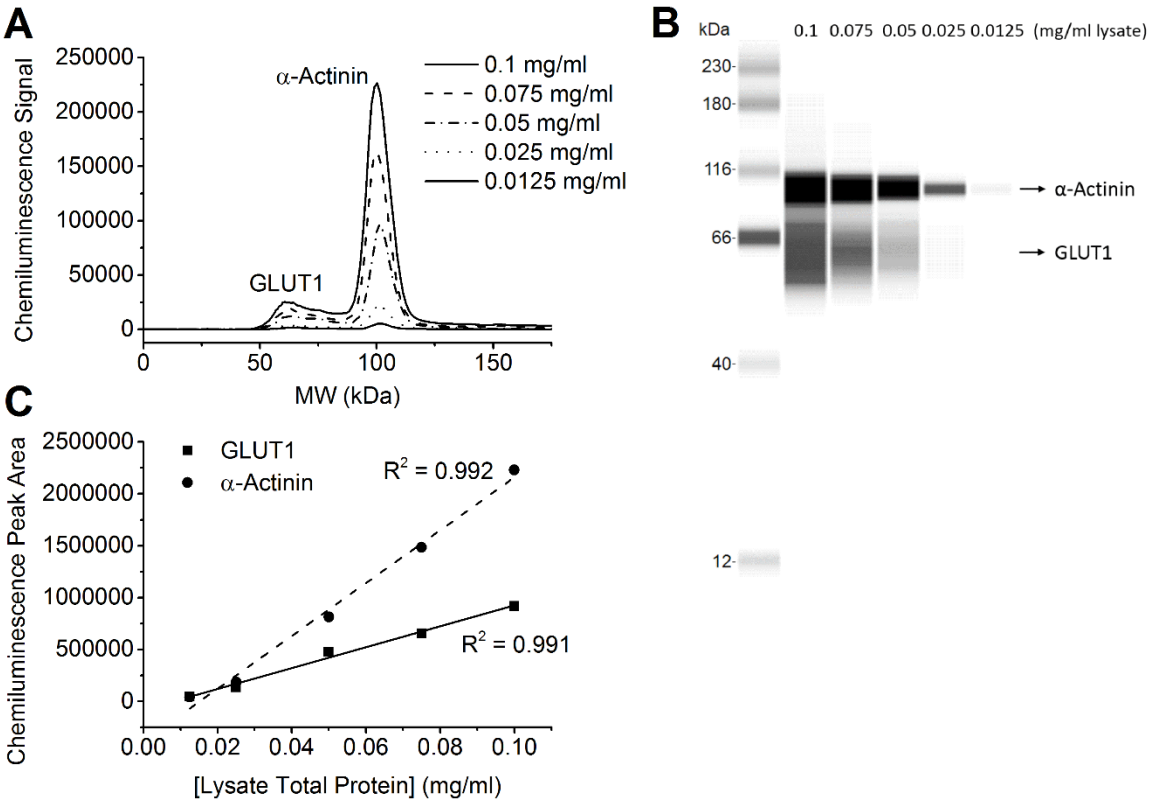

Figure 3.7: Antibody validation for Western analysis of GLUT1 and α-actinin. Various total protein concentrations of differentiated LHCN-M2 whole cell lysate were analysed with GLUT1 and α-actinin antibodies when run in the same capillary on the ProteinSimple Wes automated Western system to establish standard curves, with electropherogram view (A) and gel-style lane view (B) for detection after a 16 s exposure. The peak areas taken from the pherogram were used to establish a standard curve for each antibody when duplexed (C).

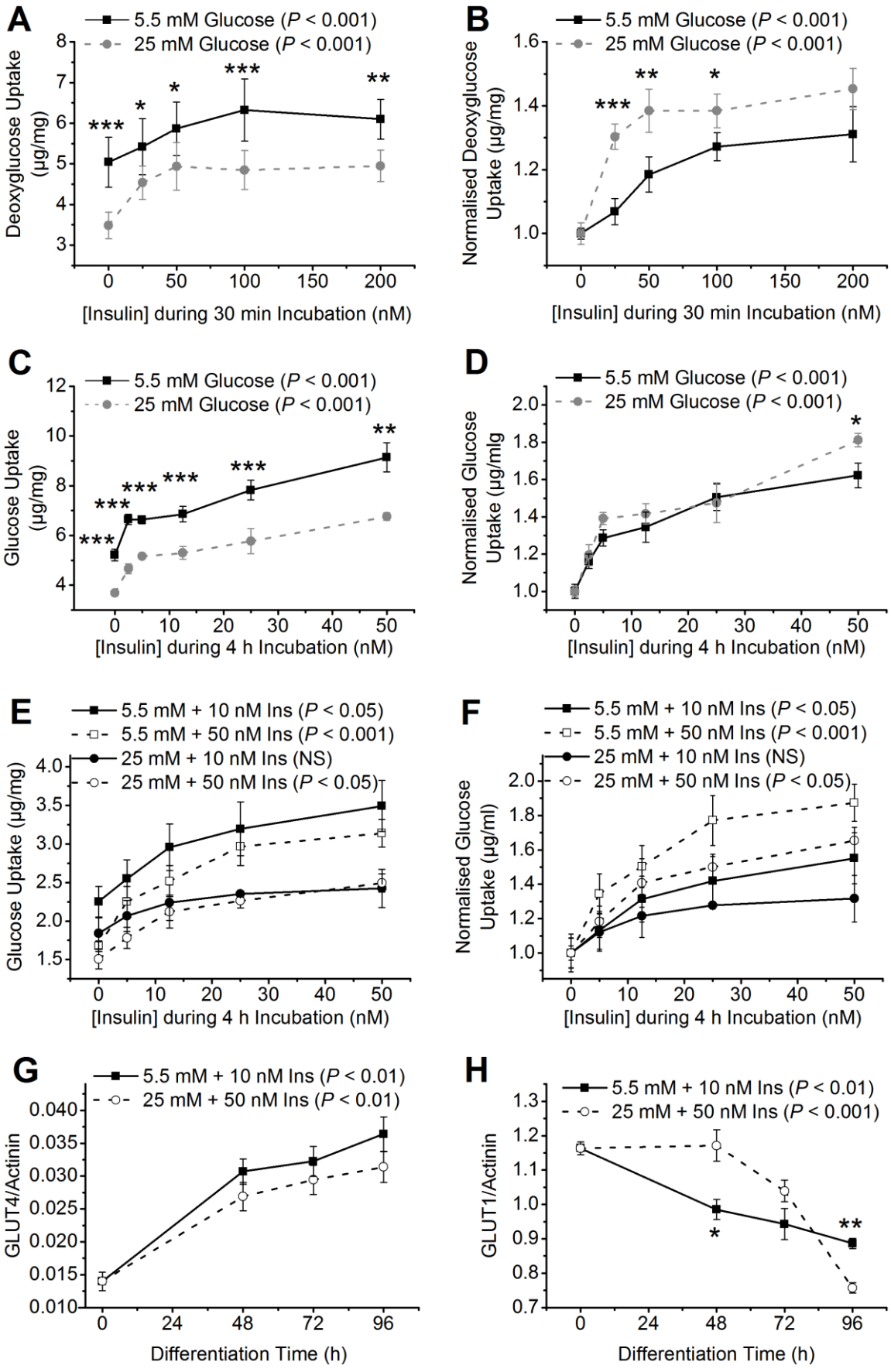
A
5.5 mM Glucose (P < 0.001)
25 mM Glucose (P < 0.001)
Deoxyglucose Uptake (µg/mg)
[Insulin] during 30 min Incubation (nM)
B
5.5 mM Glucose (P < 0.001)
25 mM Glucose (P < 0.001)
Normalised Deoxyglucose Uptake (µg/mg)
[Insulin] during 30 min Incubation (nM)
C
5.5 mM Glucose (P < 0.001)
25 mM Glucose (P < 0.001)
Glucose Uptake (µg/mg)
[Insulin] during 4 h Incubation (nM)
D
5.5 mM Glucose (P < 0.001)
25 mM Glucose (P < 0.001)
Normalised Glucose Uptake (µg/mlg
[Insulin] during 4 h Incubation (nM)
E
5.5 mM + 10 nM Ins (P < 0.05)
5.5 mM + 50 nM Ins (P < 0.001)
25 mM + 10 nM Ins (NS)
25 mM + 50 nM Ins (P < 0.05)
Glucose Uptake (µg/mg)
[Insulin] during 4 h Incubation (nM)
F
5.5 mM + 10 nM Ins (P < 0.05)
5.5 mM + 50 nM Ins (P < 0.001)
25 mM + 10 nM Ins (NS)
25 mM + 50 nM Ins (P < 0.05)
Normalised Glucose Uptake (µg/ml)
[Insulin] during 4 h Incubation (nM)
G
5.5 mM + 10 nM Ins (P < 0.01)
25 mM + 50 nM Ins (P < 0.01)
GLUT4/Actinin
Differentiation Time (h)
H
5.5 mM + 10 nM Ins (P < 0.01)
25 mM + 50 nM Ins (P < 0.001)
GLUT1/Actinin
Differentiation Time (h)

Figure 3.8: Glucose and insulin affect (deoxy-)glucose uptake and metabolism, insulin sensitivty and GLUT expression in LHCN-M2 myotubes. Acute uptake of 2-[1-^{14}C(U)]-deoxy-D-glucose (A-B) and longer-term D-[^{14}C(U)]-glucose uptake (or retention of downstream ^{14}C metabolites) (C-D) in response to insulin in myotubes differentiated for 96 h in 5.5 mM or 25 mM glucose was measured. Cells were starved of glucose and serum for 4 h then incubated with various concentrations of insulin and 0.15 µCi/ml 2-[^{14}C(U)]-deoxy-D-glucose in 0.55 mM 2-deoxy-D-glucose for 30 min (A-B) or 0.2 µCi/ml D-[^{14}C(U)]glucose in 0.55 mM D-glucose for 4 h (C-D), then washed, lysed and measured by liquid scintillation counting and corrected for total protein. In order to compare responses to insulin the data in A and C were normalised to the 0 nM insulin controls for both 5.5 and 25 mM glucose (B and D). Glucose (metabolised) in cells after 4 h was measured in the same way but in myotubes differentiated in 5.5 or 25 mM glucose with 10 or 50 nM insulin (E) and normalised to each 0 nM insulin control (F). Total GLUT4 (G) and GLUT1 (H) protein levels in whole cell lysates of myotubes differentiated in 5.5 mM glucose and 10 nM insulin or 25 mM glucose and 50 nM insulin were measured on the ProteinSimple Wes automated Western capillary system and corrected for α-actinin (see Figs. 3.6 and 3.7 respectively for antibody validation and linear range of detection). Lysates were extracted at time-points during differentiation and then for GLUT4 analysis loaded at 0.5 mg/ml and detected in high dynamic range mode for α-actinin 1 and after a 512 s exposure for GLUT 4 (G) and for GLUT1 analysis 0.1 mg/ml was loaded and both α-actinin and GLUT1 detected after a 16 s exposure. All data are shown as mean ± SEM (n/N = 12/4 (A-B); 16/4 (C-D); 9/3 (E-F); 3/3 (G-H)). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for differences between differentiation conditions at each incubation concentration or time-point.

3.4.2 (Poly)phenol metabolites increase glucose uptake and metabolism

3.4.2.1 Effects of metabolite profiles

2-[1-^{14}C(U)]-DG uptake in LHCN-M2 myotubes was stimulated by a range of (poly)phenol metabolites, including the ferulic acid conjugates, resveratrol conjugates and berry phenolic sulfates. The ferulic acid derived combination increased uptake, independent of insulin, in the high GI myotubes by 38% ($p < 0.05$) to match basal uptake in the normal, but had no effect on the latter. Insulin-stimulated uptake was slightly inhibited when the metabolites were also present (NS, FA *vs* FA + insulin) (Fig. 3.9A) ($p < 0.05$ *vs* Ins). This metabolite profile lowered the ^{14}C taken into/retained in the cells after a 4 h D-[^{14}C(U)]-Glc incubation, again only in the high GI myotubes and independent of insulin (down 12%, $p < 0.05$ *vs* control) (Fig. 3.10A).

The flavonols had no effect on 2-[1-^{14}C(U)]-DG uptake, although uptake in the high GI myotubes was no longer significantly different to that in the normal control (Fig. 3.9B). In the high GI myotubes the flavonols completely blunted the longer term effect of insulin (Fig. 3.10B), but otherwise again had no effects on basal uptake.

The combination of the resveratrol glucuronide conjugates and sulfate increased 2-[1-^{14}C(U)]-DG uptake in both normal and high GI myotubes, by almost 50% in the former ($p < 0.05$) and restored uptake in the latter to a similar level in the normal control (NS) (Fig. 3.9C). As with the ferulic acid derivatives, uptake was increased no further than that with insulin alone, nor was it increased further when incubated with both (Figs. 3.9A, C). The resveratrol metabolites lowered basal ^{14}C uptake/retention in the cells after 4 h in both normal and high GI, by as much as 25% in the latter ($p < 0.001$), as well as blunting the insulin effect in these cells too (Fig. 3.10C).

The phenolic sulfates increased basal 2-[1-^{14}C(U)]-DG uptake in the same manner in both normal and high GI myotubes, up by 60% in normal ($p < 0.05$) and nearly 50% in high ($p < 0.01$), and in the latter increased it further when incubated with insulin too, by 65% ($p < 0.001$) (Fig. 3.9D). They had no effects on the 4 h D-[^{14}C(U)]-Glc uptake however (Fig. 3.10D).

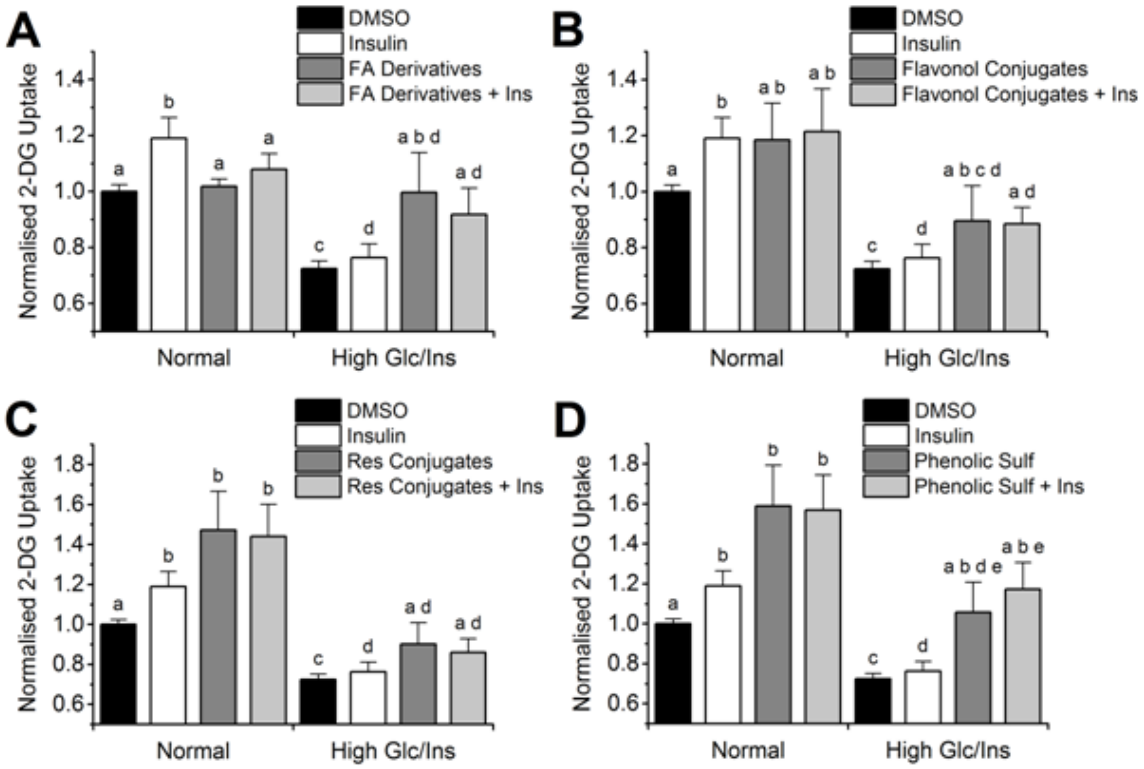

Figure 3.9: (Poly)phenol metabolite profiles stimulate 2-[1-^{14}C(U)]-deoxy-D-glucose uptake in LHCN-M2 myotubes. Myotubes, differentiated for 96 h in 5.5 mM glucose and 10 nM insulin ('Normal') or in 25 mM glucose and 50 nM insulin ('High Glc/Ins), were starved of glucose and serum in the presence of a selected combination of (poly)phenol metabolites (or DMSO control) for 4 h then incubated with a DMSO control, 100 nM insulin, the same metabolites or both metabolites and insulin, and 0.15 µCi/ml 2-[^{14}C(U)]-deoxy-D-glucose in 0.55 mM 2-deoxy-D-glucose for 30 min, then washed, lysed and measured by liquid scintillation counting and corrected for total protein. In order to compare responses to the various treatments and differentiation conditions the data were normalised to the DMSO controls for the Normal cells and shown as mean ± SEM (n/N = 12/4). Ferulic acid (FA) derivatives – FA4S, DHFA-4S and DHFA, all at 2 µM (A); flavonol conjugates – Q3S, Q3G and K3G, all at 2 µM (B); resveratrol (Res) conjugates – R4G, R3S and R3G, all at 2 µM (C); berry phenolic sulfates (Sulf) – CS, MCS, PS, MGS, VAS and IVAS, all at 5 µM (D). 2-DG – 2-deoxy-D-glucose; Ins - insulin. Letters above bars indicate $p < 0.05$ between groups.

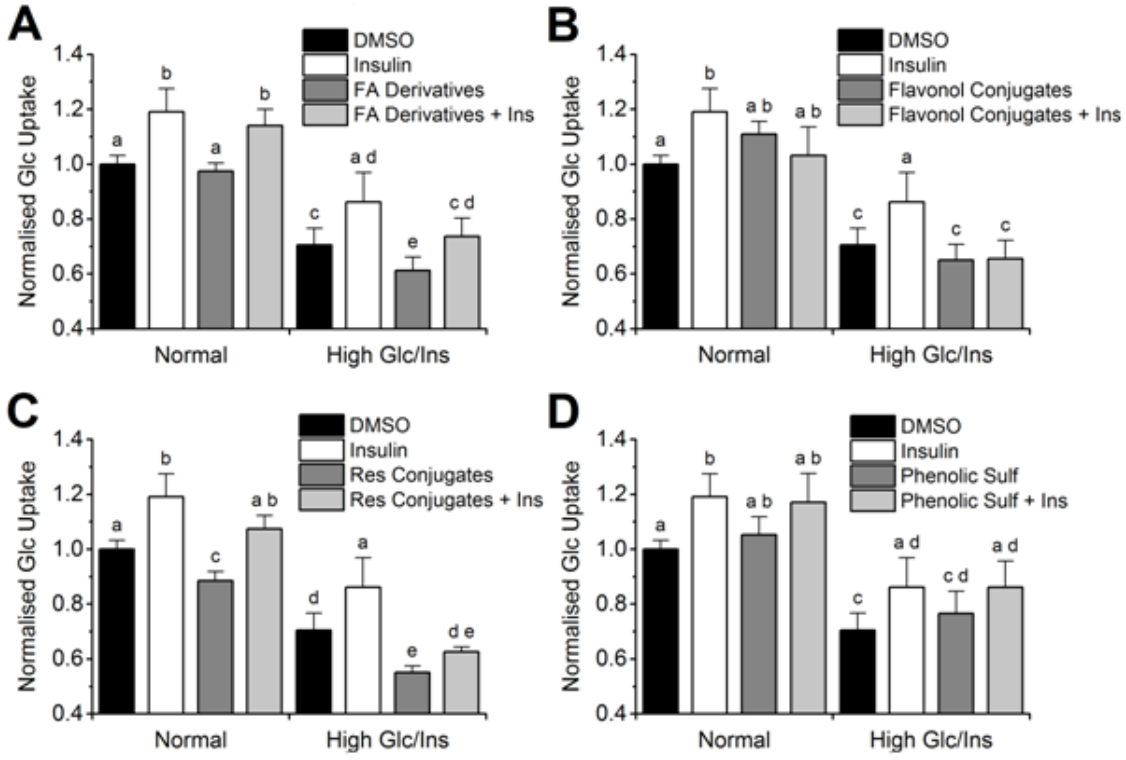

Figure 3.10: (Poly)phenol metabolite profiles stimulate glucose metabolism (less intracellular ^{14}C after 4 h) in LHCN-M2 myotubes. Myotubes, differentiated for 96 h in 5.5 mM glucose and 10 nM insulin ('Normal') or in 25 mM glucose and 50 nM insulin ('High Glc/Ins), were starved of glucose and serum in the presence of a selected combination of (poly)phenol metabolites (or DMSO control) for 4 h then incubated with a DMSO control, 25 nM insulin, the same metabolites or both metabolites and insulin, and 0.2 µCi/ml D-[^{14}C(U)]glucose in 0.55 mM D-glucose for 4 h, then washed, lysed and measured by liquid scintillation counting and corrected for total protein. In order to compare responses to the various treatments and differentiation conditions the data were normalised to the DMSO controls for the Normal cells and shown as mean ± SEM (n/N = 12/4). Ferulic acid (FA) derivatives – FA4S, DHFA-4S and DHFA, all at 2 µM (A); flavonol conjugates – Q3S, Q3G and K3G, all at 2 µM (B); resveratrol (Res) conjugates – R4G, R3S and R3G, all at 2 µM (C); berry phenolic sulfates (Sulf) – CS, MCS, PS, MGS, VAS and IVAS, all at 5 µM (D). Glc – glucose; Ins - insulin. Letters above bars indicate $p < 0.05$ between groups.

3.4.2.2 Individual metabolites

Individual metabolites were tested on high GI myotubes, where the greatest or most promising effects were seen, and with or without insulin according to whether previous effects were deemed insulin-dependent or not. The increases in 2-[1-^{14}C(U)]-DG uptake were primarily due to sulfated conjugates. FA4S and DHFA-4S increased uptake by around 15% ($p < 0.05$) (Fig. 3.11A) and R3S by 24% ($p < 0.001$) (Fig. 3.11C), but IVAS was most effective, with an increase of 41% ($p < 0.001$). This was the only phenolic sulfate in the berry profile to have an effect (Fig. 3.11D) and the effect was on top of an insulin-stimulated increase. The only glucuronide conjugate to exert an effect was R4G, with a 23% increase ($p < 0.01$). Despite having no significant effects on 2-[1-^{14}C(U)]-DG uptake when in combination, the flavonols were tested individually because the combined data looked as though there may be potential for one or two of the individual compounds to increase uptake, but this was not the case.

For the 4 h D-[^{14}C(U)]-Glc uptake the effects of the ferulic acid derived metabolite profile were exclusive to the high GI myotubes (Fig. 3.10A) and the effects of the resveratrol conjugates were most promising also on these cells (Fig. 3.10C), so these individual metabolites were only tested on high GI. Resveratrol conjugates were tested with and without insulin because effects were seen for both when the conjugates were combined (Fig. 3.10C). The insulin-blunting effect of the flavonol profile was explored for the individual compounds in normal and high GI myotubes because, although the insulin-blunting was only significant in the high, again the data looked as though there may have been differences between the two with individual conjugates. Originally the phenolic sulfates had shown no effect on 4 h D-[^{14}C(U)]-Glc uptake, but following the promising effects of IVAS alone on the 2-[1-^{14}C(U)]-DG uptake, this was tested in normal and high GI myotubes with and without insulin.

DHFA-4S and R4G were responsible for decreasing ^{14}C uptake/retention in the cells after 4 h by 27% ($p < 0.001$) and 11% ($p < 0.05$) respectively, and both independently of insulin (Figs. 3.11E, G). Despite having no effects on 2-[1-^{14}C(U)]-DG transport (Fig. 3.11B), Q3G lowered the ^{14}C after 4 h incubation with D-[^{14}C(U)]-Glc in normal myotubes by 22% ($p < 0.01$), and K3G did so in both normal and high GI, by 19% ($p < 0.01$) and 25% ($p < 0.05$) respectively (Fig. 3.11F). With the 2-[1-^{14}C(U)]-DG uptake

result for IVAS, it also lowered basal intracellular ^{14}C after 4 h by around 17% ($p < 0.05$) in both normal and high GI myotubes, and mildly inhibited the effect of insulin but only in the normal myotubes ($p = 0.08$ insulin *vs* IVAS + insulin; NS *vs* Control) (Fig. 3.11H).

3.4.3 Establishing a mechanism of action for isovanillic acid 3-*O*-sulfate

3.4.3.1 IVAS dose-dependently increases 2-deoxy-D-glucose uptake

For its superior capacity to increase 2-[1-$^{14}C(U)$]-DG uptake in myotubes, notably in high GI (Fig. 3.9D), and synergistically with insulin (Fig. 3.11D), the effects of IVAS were probed further to establish a mechanism of action. IVAS dose-dependently increased 2-[1-$^{14}C(U)$]-DG uptake in the low micromolar range; with 2.5 µM the transport was increased by 15% in normal ($p < 0.01$) and by 29% in high GI myotubes ($p < 0.001$) (Figs. 3.12A, B). Uptake plateaued with higher concentrations, likely that the cells were taking up 2-[1-$^{14}C(U)$]-DG at maximal rate; insulin had the same effect (Figs. 3.4A, 4A, B). Whilst actual uptake was higher in the normal myotubes, the high GI myotubes were more responsive to the IVAS than the normal ($p = 0.05$ at 10 µM and $p < 0.05$ at 30 µM) (Fig. 3.12B), again in the same manner as with insulin (Fig. 4B) and thus suggestive of a similar mechanism of action. 2-[1-$^{14}C(U)$]-DG uptake was increased dose-dependently synergistically when myotubes were treated with both insulin and isovanillic acid sulfate (Fig. 3.11D). The increase in 2-[1-$^{14}C(U)$]-DG uptake was not reliant on the sulfate group of the metabolite because isovanillic acid increased it to the same extent in normal and high GI myotubes (both NS *vs* IVA) (Fig. 3.12C).

3.4.3.2 IVAS Increases GLUT Expression and Activates Insulin Signalling

To test the insulinomimetic hypothesis, myotubes were incubated with insulin and IVAS in the presence of wortmannin, PI3K inhibitor, and indinavir, GLUT4 inhibitor. Both prevented insulin- and IVAS-stimulated 2-[1-$^{14}C(U)$]-DG uptake (both $p < 0.001$ *vs* insulin, both $p < 0.01$ *vs* IVAS), but neither had an effect on basal uptake (Fig. 3.12D). Furthermore, the acute effect (30 min incubation only) was tested and IVAS still increased 2-[1-$^{14}C(U)$]-DG uptake, though to a lesser extent than with the chronic effect (4.5 h total incubation) (19% *vs* 41%, $p < 0.05$) (Fig. 3.12E). This suggested both acute and chronic effects were in play and protein analyses revealed this to be true.

The degree of differentiation was unaffected by the 4 h treatment with IVAS (Fig. 3.15A), but GLUT4 expression was increased in both normal and high GI myotubes by a mean of 57% ($p < 0.001$) (Fig. 3.15B) and GLUT1 was increased by 23% in high GI ($p < 0.05$), to the same level as in the normal; in normal it was unchanged (Fig. 3.15C). PI3K was upregulated in both myotube models, but more so in the high (38% in normal, $p < 0.05$; 64% in high GI, $p < 0.001$) (Fig. 3.15D). It is also worth noting that PI3K was 16% higher in the high GI control than in the normal ($p < 0.05$) and 37% higher when comparing the two with IVAS treatment ($p < 0.01$). Incubation with IVAS for 15 min increased Akt phosphorylation by 54% ($p < 0.05$) in normal and high GI myotubes (Fig. 3.15E). As expected, Akt was phosphorylated with insulin and inhibited by wortmannin, and was synergistically activated more so with both insulin and IVAS (Fig. 3.15E).

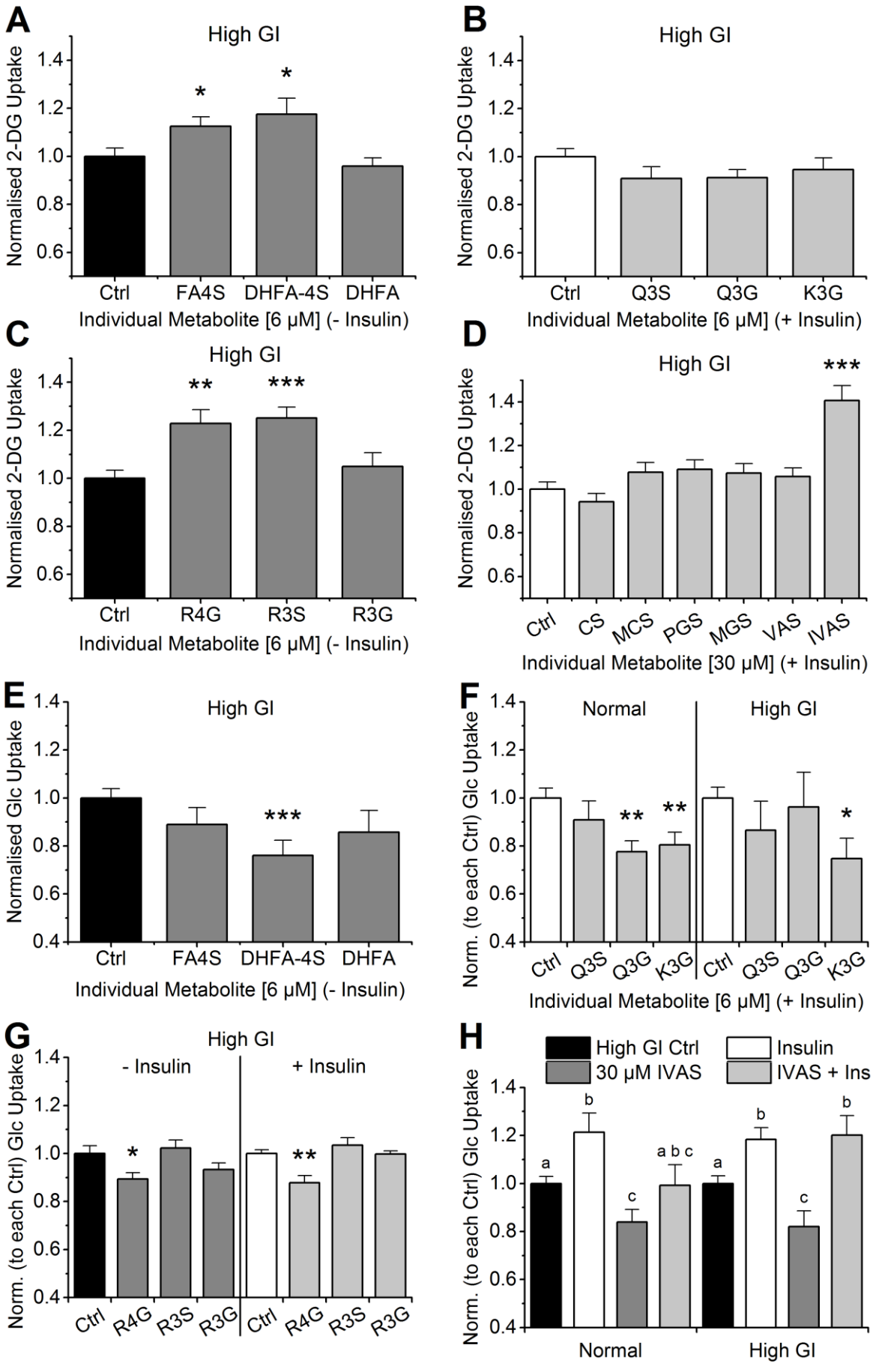
A
High GI
Normalised 2-DG Uptake
Ctrl
FA4S
DHFA-4S
DHFA
Individual Metabolite [6 µM] (- Insulin)
B
High GI
Normalised 2-DG Uptake
Ctrl
Q3S
Q3G
K3G
Individual Metabolite [6 µM] (+ Insulin)
C
High GI
Normalised 2-DG Uptake
Ctrl
R4G
R3S
R3G
Individual Metabolite [6 µM] (- Insulin)
D
High GI
Normalised 2-DG Uptake
Ctrl
CS
MCS
PGS
MGS
VAS
IVAS
Individual Metabolite [30 µM] (+ Insulin)
E
High GI
Normalised Glc Uptake
Ctrl
FA4S
DHFA-4S
DHFA
Individual Metabolite [6 µM] (- Insulin)
F
Normal
High GI
Norm. (to each Ctrl) Glc Uptake
Ctrl
Q3S
Q3G
K3G
Individual Metabolite [6 µM] (+ Insulin)
G
High GI
- Insulin
+ Insulin
Norm. (to each Ctrl) Glc Uptake
Ctrl
R4G
R3S
R3G
H
High GI Ctrl
30 µM IVAS
Insulin
IVAS + Ins
Norm. (to each Ctrl) Glc Uptake
Normal
High GI

Figure 3.11: Individual (poly)phenol metabolites also stimulate 2-[1-^{14}C(U)]-deoxy-D-glucose uptake and glucose metabolism. Myotubes, all differentiated for 96 h in 25 mM glucose and 50 nM insulin, were starved of glucose and serum in the presence of a metabolite or DMSO control for 4 h then incubated with the same metabolite (plus 100 nM insulin (B, D)) and 0.15 µCi/ml 2-[^{14}C(U)]-deoxy-D-glucose in 0.55 mM 2-deoxy-D-glucose for 30 min, then washed, lysed, measured by liquid scintillation counting, corrected for total protein and normalised to the control (A-D). Myotubes, differentiated for 96 h in 5.5 mM glucose and 10 nM insulin or in 25 mM glucose and 50 nM insulin, were starved of glucose and serum in the presence of a metabolite or DMSO control for 4 h then incubated with the same metabolite ± 25 nM insulin and 0.2 µCi/ml D-[^{14}C(U)]glucose in 0.55 mM D-glucose for 4 h, then washed, lysed, measured by liquid scintillation counting, corrected for total protein and normalised to the controls (E-H). All data are shown as mean ± SEM (n/N = 12/4). Ctrl – control; 2-DG – 2-deoxy-D-glucose; Glc – glucose; Norm. – normalised; Ins - insulin. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* the controls accordingly. Letters above bars (H) indicate $p < 0.05$ between groups.

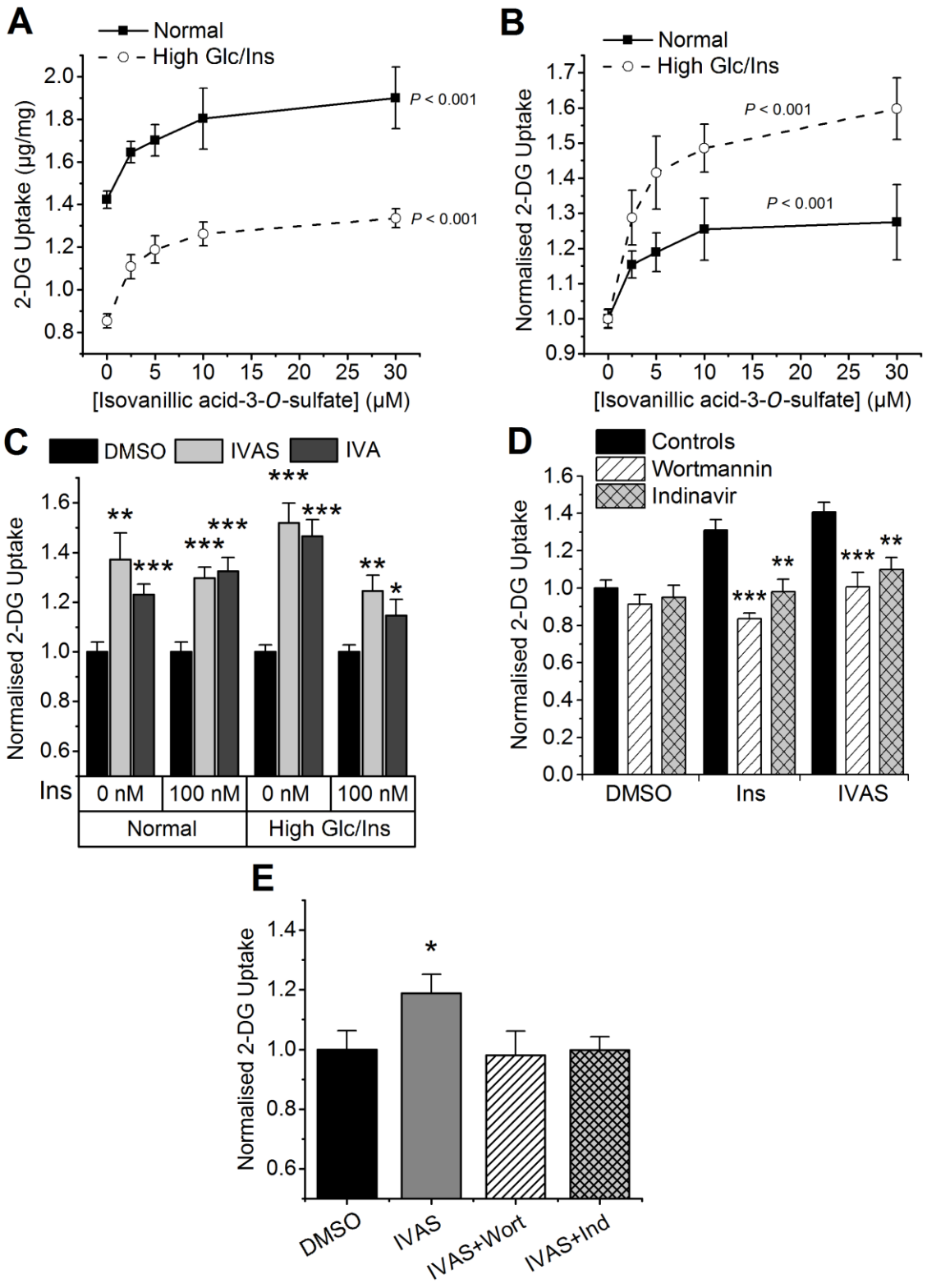
A
Normal
High Glc/Ins
2-DG Uptake (µg/mg)
P < 0.001
P < 0.001
[Isovanillic acid-3-O-sulfate] (µM)
B
Normal
High Glc/Ins
Normalised 2-DG Uptake
P < 0.001
P < 0.001
[Isovanillic acid-3-O-sulfate] (µM)
C
DMSO
IVAS
IVA
Normalised 2-DG Uptake
Ins
0 nM
100 nM
0 nM
100 nM
Normal
High Glc/Ins
D
Controls
Wortmannin
Indinavir
Normalised 2-DG Uptake
DMSO
Ins
IVAS
E
Normalised 2-DG Uptake
DMSO
IVAS
IVAS+Wort
IVAS+Ind

Figure 3.12: IVAS increases 2-[1-^{14}C(U)]-deoxy-D-glucose uptake in a dose-dependent manner via the PI3K-dependent pathway. Myotubes, differentiated for 96 h in 5.5 mM glucose and 10 nM insulin or in 25 mM glucose and 50 nM insulin, were starved of glucose and serum in the presence of various concentrations of IVAS or DMSO control for 4 h then incubated with the same plus 0.15 µCi/ml 2-[^{14}C(U)]-deoxy-D-glucose in 0.55 mM 2-deoxy-D-glucose for 30 min, then washed, lysed, measured by liquid scintillation counting and corrected for total protein (A-B). In order to compare responses to the IVAS, the data shown in A were normalised to the controls (B). The same was done with 30 µM IVAS or 30 µM isovanillic acid (IVA) (C) and with 100 nM insulin (during the 30 min incubation), 30 µM IVAS (during the 4 h starvation and 30 min incubation) or DMSO ± 5 µM wortmannin (inhibitor of PI3K) or 20 µM indinavir (inhibitor of GLUT4) (or DMSO controls) (D). Uptake was also measured in the same way but in myotubes treated acutely with IVAS for 30 min (starvation followed by 30 min incubation with 2-[1-^{14}C(U)]-deoxy-D-glucose), and with inhibitors added again, or DMSO control (E). All data are shown as mean ± SEM (n/N = 12/4). Ctrl(s) – control(s); 2-DG – 2-deoxy-D-glucose; Ins – insulin; Norm. – normalised; Wort – 5 µM wortmannin; Ind – 20 µM indinavir. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* controls accordingly. ## $p < 0.01$ *vs* 5.5+10 with 0 nM insulin + 30 µM IVA. † $p < 0.05$, †† $p < 0.001$ *vs* 25+10 with 0 nM insulin accordingly. NS not significantly different to the control.

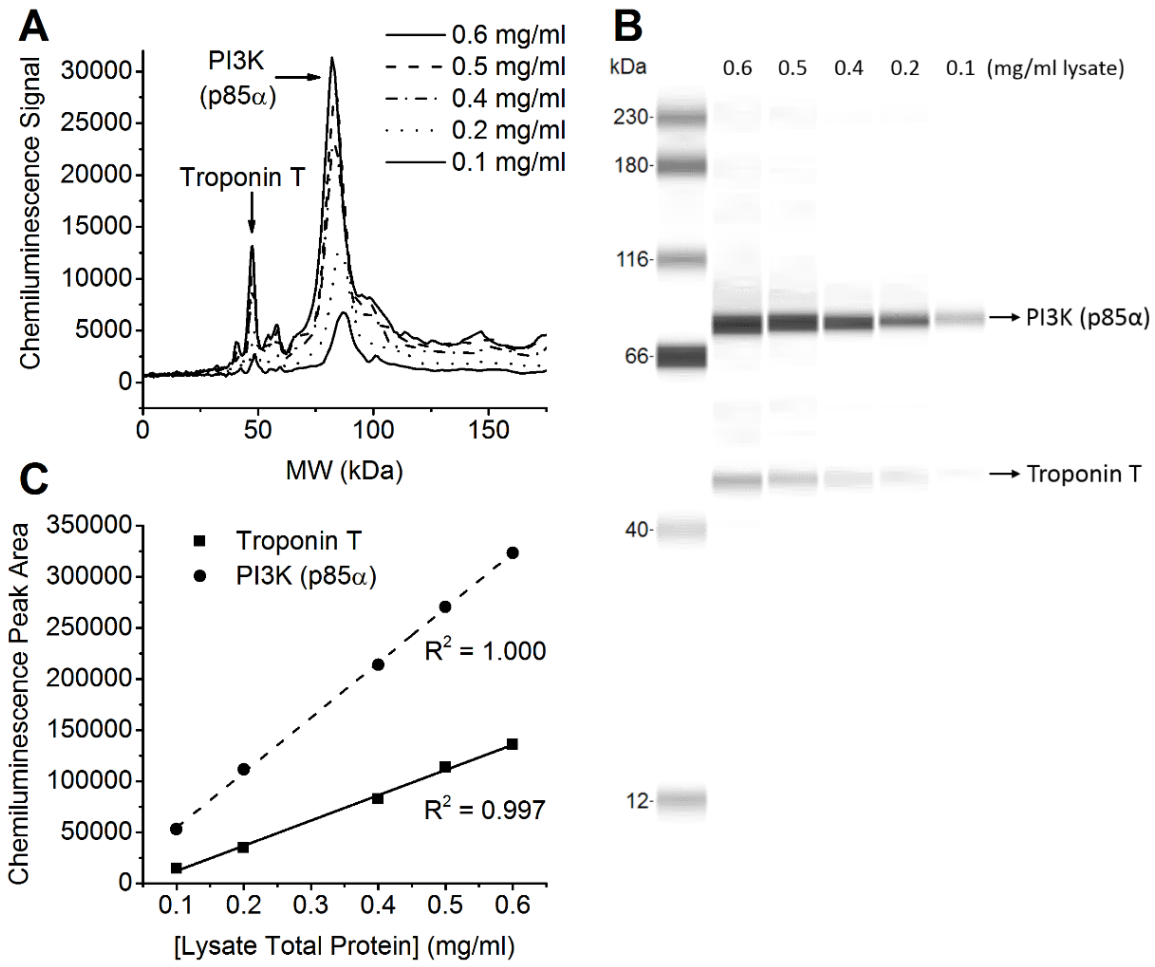

Figure 3.13: Antibody validation for Western analysis of regulatory unit p85α of PI3K and troponin T. Various total protein concentrations of differentiated LHCN-M2 whole cell lysate were analysed with PI3K p85α and troponin T antibodies and run in the same capillary on the ProteinSimple Wes automated Western system to establish standard curves, with electropherogram view (A) and gel-style lane view (B) for detection after a 16 s exposure. The peak areas taken from the pherogram were used to establish a standard curve for each antibody when duplexed (C). Note, troponin T was chosen as a loading control for PI3K p85α because the MW of the latter is too close to that of α-actinin.

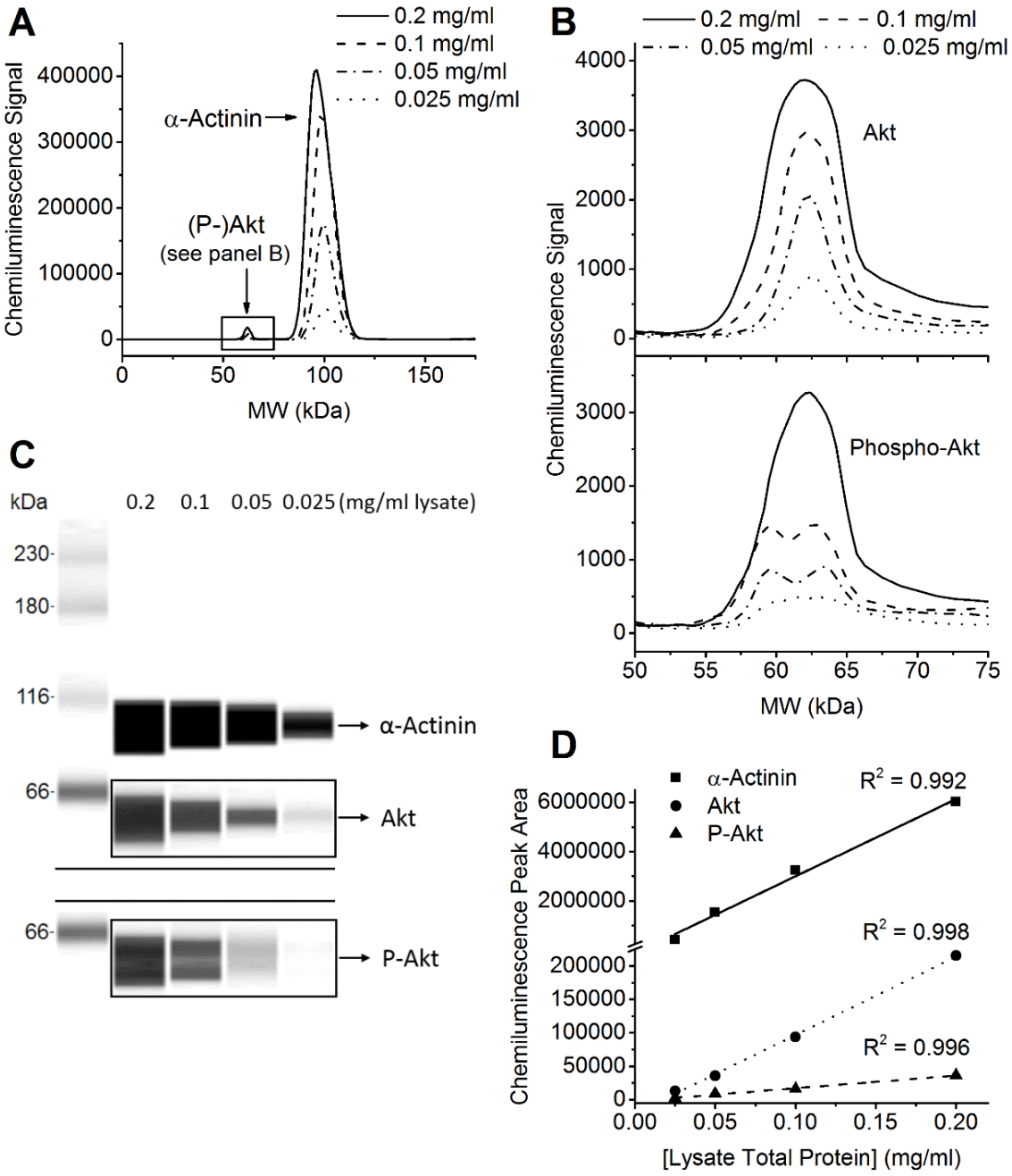

Figure 3.14: Antibody validation for Western analysis of total and phospho- Akt with α-actinin. Various total protein concentrations of differentiated LHCN-M2 whole cell lysate analysed with Akt or phospho-Akt (P-Akt) and α-actinin antibodies when run in the same capillary to establish standard curves, with electropherogram view (A-B) and gel-style lane view (C); α-actinin was detected in high dynamic range mode while Akt and P-Akt (boxed) were detected after a 512 s exposure. The peak areas taken from the pherograms were used to establish a standard curve for each antibody when duplexed. Note, the α-actinin curve consists of mean data of each of the curves run with Akt and P-Akt) (D).

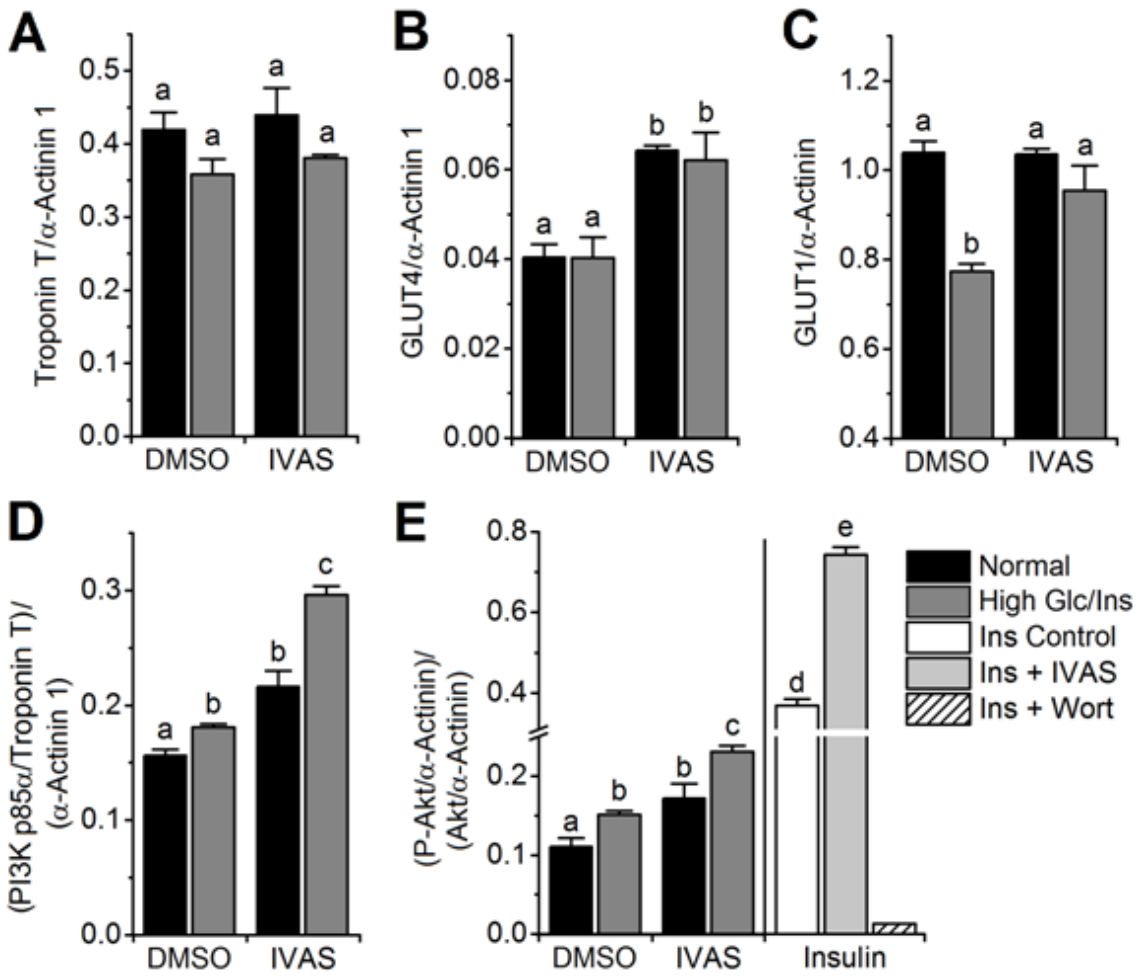
A
Troponin T/α-Actinin 1
0.0
0.1
0.2
0.3
0.4
0.5
a
a
a
a
DMSO
IVAS
B
GLUT4/α-Actinin 1
0.00
0.02
0.04
0.06
0.08
a
a
b
b
DMSO
IVAS
C
GLUT1/α-Actinin
0.4
0.6
0.8
1.0
1.2
a
b
a
a
DMSO
IVAS
D
(PI3K p85α/Troponin T)/
(α-Actinin 1)
0.0
0.1
0.2
0.3
a
b
b
c
DMSO
IVAS
E
(P-Akt/α-Actinin)/
(Akt/α-Actinin)
0.0
0.1
0.2
0.4
0.6
0.8
a
b
b
c
d
e
DMSO
IVAS
Insulin
Normal
High Glc/Ins
Ins Control
Ins + IVAS
Ins + Wort

Figure 3.15: IVAS upregulates GLUTs and PI3K, and activates Akt of the insulin pathway. Protein analyses were performed on the ProteinSimple Wes automated Western capillary system using total cell lysates of myotubes differentiated in 5.5 mM glucose and 10 nM insulin or 25 mM glucose and 50 nM insulin and then treated with 30 µM IVAS or DMSO control (Ctrl) for 4 h while starved of glucose and serum. Lysates were loaded onto the Wes system at 0.4 mg/ml, probed with troponin T and α-actinin 1 antibodies (see Figs. 3.2, 3.3) and chemiluminescence detected after a 16 s exposure (A); 0.5 mg/ml, probed with GLUT4 and α-actinin 1 antibodies (see Fig. 3.6) and detected in high dynamic range mode for α-actinin 1 and after a 512 s exposure for GLUT4 (B); 0.1 mg/ml, probed with GLUT1 and α-actinin antibodies (see Fig. 3.7) and detected after a 16 s exposure (C); 0.4 mg/ml, probed with PI3K p85α and troponin T antibodies (see Fig. 3.13) and detected after a 16 s exposure (D). After the 4 h starvation/incubation with IVAS or DMSO, myotubes were incubated with 0.55 mM glucose and 30 µM IVAS, 100 nM insulin (Ins, positive control), IVAS and insulin combined, insulin and 5 µM wortmannin (Wort) (as a negative control), or DMSO controls for 15 min then washed and lysed. Lysates were loaded onto the Wes system at 0.1 mg/ml, probed with Akt or phospho-Akt (Ser473) (P-Akt) and α-actinin antibodies (see Fig. 3.14) and detected in high dynamic range mode for α-actinin and after a 512 s exposure for Akt/P-Akt (E). All data are shown as mean ratios of the target protein/loading control (for PI3K the loading control was troponin T and this was corrected for the troponin T/α-actinin data (D)) ± SEM (N = 3). Letters above bars denote where $p < 0.05$ between treatments.

3.5 Discussion

Hyperglycaemia and insulin resistance are biomarkers of type 2 diabetes and overcoming these can help maintain healthy glucose homeostasis (Kahn, Rosen et al. 1992, Saltiel and Kahn 2001, Alvim, Cheuhen et al. 2015, Deshmukh 2016). With the skeletal muscle accounting for up to 75% of postprandial glucose disposal (Saltiel and Kahn 2001), improving insulin-stimulated glucose uptake in muscle cells is a promising therapy for diabetics. Epidemiological, animal, human intervention and *in vitro* cell studies have demonstrated that (poly)phenols can improve glucose transport into insulin-responsive muscle, but it is clear that there are a lack of studies investigating the effects of relevant conjugates that circulate in plasma *in vivo*, notably those metabolised by the gut microbiome. Furthermore, the majority of *in vitro* studies investigating glucose uptake and metabolism mechanisms in skeletal muscle use the murine C2C12 or rat L6 immortalised cells and not a human muscle model. It was demonstrated here for the first time that (poly)phenol metabolites affect glucose uptake and metabolism in immortalised human skeletal muscle cells, with different effects observed in myotubes differentiated in a normal or high glucose/insulin environment. In particular, isovanillic acid 3-*O*-sulfate, a catabolite of the gut microflora following the ingestion of berries (Pimpao, Ventura et al. 2015), has promising effects in restoring glucose transport in high GI myotubes through an acute activation of the insulin signalling pathway and a chronic upregulation of GLUTs 1 and 4 and of PI3K.

3.5.1 Skeletal muscle cell characterisation and model setup

LHCN-M2 myoblasts were differentiated to myotubes and consequently expression of troponin T increased as expected (Gardner, Anguiano et al. 2012). Total α-actinin and α-actinin 1 protein, recognised by the rabbit and mouse antibodies respectively, was unchanged during differentiation despite RNA-seq data showing a decrease in RNA from day 0 to 4 (Table 3.2). The myoblasts are in the growth phase on day 0 and thus require actinin synthesis, the day 4 myotubes are already established and the need for actinin synthesis has diminished, albeit the actinin protein remains. Protein expression of different actinin isoforms were previously reported to remain stable during differentiation (Goffart, Franko et al. 2006). Recognised traits of mature muscle, in addition to increased troponin, include insulin sensitivity (Iovino, Burkart et al. 2016)

and a greater aerobic metabolism than in other tissues, such as liver (Rolfe, Newman et al. 1999, Berg, Tymoczko et al. 2002). Here in the LHCN-M2 muscle, mitochondrial respiration was greater than in hepatocytes as expected, especially with HepG2 being a primarily glycolytic cell (DeBerardinis, Lum et al. 2008). The differentiated myotubes responded to insulin in a dose-dependent manner acutely, increasing 2-[1-^{14}C(U)]-deoxy-D-glucose (2-DG) uptake beyond basal, and chronically, increasing intracellular ^{14}C after 4 h with D-[^{14}C(U)]-glucose, stored predominantly as glycogen (Saltiel and Kahn 2001, Yeaman, Armstrong et al. 2001); the LHCN-M2 myoblasts express glycogen synthase (GYS1) and this is increased > 3-fold in the myotubes (Table 3.2).

Further support for the change in phenotype with differentiation was demonstrated by the lack of insulin-stimulated 2-DG transport in myoblasts; the response was limited to the longer term in the precursor cells. The insulin receptor, and its downstream proteins, is expressed (Table 3.2) and is active in myoblasts (Youngren, Goldfine et al. 1999), however GLUT4 is very low in LHCN-M2 myoblasts and increases with differentiation while GLUT1 decreases, shown here in Fig. 3.8 and Table 3.2, and previously (Al-Khalili, Chibalin et al. 2003). This leads to the insulin-stimulated increase seen in 2-DG uptake. GLUT1 is ubiquitously expressed (Mueckler and Thorens 2013) and is the primary glucose transporter in these cells, even after differentiation, and is generally increased in cultured cells compared to those *in vivo* (Al-Khalili, Chibalin et al. 2003). It is known primarily as the basal glucose transporter, though in rat it can also be stimulated by insulin (Klip, Volchuk et al. 1996, Rett, Wicklmayr et al. 1996). In LHCN-M2 undifferentiated myoblasts the cells responded to insulin in the longer term by increasing storage of glucose transported in through the basal GLUT1, but only when GLUT4 is upregulated was there an acute insulin-stimulated increase in 2-DG uptake.

High glucose heightened proton leak across the IMM and cellular ROS levels, as was seen in HepG2 cells (Chapter 2). ROS are generated by insulin signalling and this is enhanced by high glucose (Goldstein, Mahadev et al. 2005). Increasing insulin induced oxidative stress in the LHCN-M2 myotubes, similarly to a recent study on lymphatic muscle (Lee, Fluckey et al. 2017) and others (Aggeli, Theofilatos et al. 2011, Barazzoni, Zanetti et al. 2012), although 10 nM insulin was protective, suggesting a hormetic function. Insulin has been shown to protect against oxidative stress in neuronal cells (Ramalingam and Kim 2014) and is upstream of a nitric oxide-induced antioxidant

defence system (Yu, Gao et al. 2011). Increasing the concentration of insulin in the normal glucose medium increased the degree of myotube differentiation after 96 h, in line with insulin being a promoter of differentiation (Rochat, Fernandez et al. 2004). In high glucose, insulin had the opposite effect, notably with 50 nM, due to the increased oxidative stress. In the absence of insulin, high glucose increased differentiation, with higher troponin T at 96 h, likely through activation of the MLX (Max-like protein X) transcription factor, which promotes myogenesis (Hunt, Xu et al. 2015). Insulin concentrations of up to 1.7 µM were tested because this has been used in differentiation medium previously (Tzatsos and Kandror 2006). There were no significant differences between myotubes differentiated in 10, 50, 500 or 1700 nM and thus this demonstrates that such insulin concentrations in culture media are unnecessarily high and increasing insulin further is more likely to induce other, undesirable changes in signalling and metabolism, even death (Sampson, Bucris et al. 2010). Differentiation media with 5.5 mM glucose and 10 nM insulin or 25 mM glucose with 50 nM insulin resulted in the same rate of differentiation over 96 h and, coupled with the heightened stress in the latter versus the former, this was the model selected for comparing a normal to a high glucose/insulin phenotype.

Myotubes differentiated in normal glucose had greater (deoxy-)glucose uptake, but myotubes in high glucose were more sensitive to insulin stimulation. High glucose lowered GLUT1 content, accounting for the lower basal uptake and subsequent storage. Insulin was shown to upregulate GLUT1 previously in rat cardiac muscle (Laybutt, Thompson et al. 1997) and this was evident in the first 48 h in this study, but Laybutt *et al.* also showed that when high insulin was accompanied by high glucose this effect was blocked, and this was seen here by day 4. GLUT4 expression was not affected by glucose or insulin, consistent too with the rat cardiac muscle (Laybutt, Thompson et al. 1997) and with L6 myotubes (Huang, Somwar et al. 2002). However, it has been shown elsewhere that high glucose and insulin can increase basal GLUT4 activity, despite decreasing translocation and without affecting expression (Huang, Somwar et al. 2002). In adipocytes insulin increased GLUT4 expression (Valverde, Navarro et al. 1999) and V_{max} of glucose transport (Kobayashi and Olefsky 1978). Moreover, ROS was elevated in the high glucose and the high GI myotubes in the present study, and is known to enhance insulin sensitivity (Loh, Deng et al. 2009). These studies combined explain the increased insulin sensitivity with high glucose and/or high insulin that was seen here.

Despite enhanced sensitivity, the amount of ^{14}C stored in the cells after a 4 h incubation with D-[$^{14}C(U)$]-glucose was lower with increased insulin, and for increased glucose, in the differentiation medium. Sensitivity was lost in the high glucose myotubes when differentiated with the lower insulin. Insulin, at the lower concentrations, will increase metabolism (Nisr and Affourtit 2014), resulting in less glycogen storage and, in the LHCN-M2 cells, more glucose was metabolised to lactate or CO_2 and the ^{14}C expelled into the medium. In muscle tissue *in vivo*, insulin-dependent production of nitric oxide improves the action of insulin and glucose homeostasis via recruitment of capillaries and increased blood flow (Grassi, Desideri et al. 2015). While not relative to the *in vitro* model due to the lack of a capillary network, the outcome with increased insulin was the same, suggesting the system was a representative model. The increased insulin sensitivity in high GI myotubes was confirmed by the increased PI3K (p85α) protein expression and enhanced p-Akt/Akt.

3.5.2 Effects of (poly)phenol metabolites on glucose uptake and metabolism

A profile of ferulic acid derivatives, found in circulation after the consumption of coffee or cereals for example (Guy, Renouf et al. 2009, Fardet 2010) (Table 3.1), restored basal 2-DG transport and increased glucose metabolism in the high GI myotubes, but blunted the acute effect of insulin and did not affect metabolism in the normal myotubes. The sulfated conjugates, FA4S and DHFA-4S, were responsible for the stimulation of glucose transport and the latter alone for the increased metabolism. The thwarted acute insulin stimulation suggests a competitive mechanism, possibly at the insulin receptor, and the effects in high GI are independent of insulin. Unconjugated ferulic acid has been shown to increase (deoxy-) glucose uptake into muscle previously. Ferulic acid dose-dependently increased uptake in L6 myotubes, doing so synergistically with oral hypoglycaemic drugs, and increased expression of GLUT4 and PI3K (Prabhakar and Doble 2009). Ferulic acid also increased uptake in primary porcine myotubes and primary human myotubes (Bhattacharya, Christensen et al. 2013, Ho, Kase et al. 2017). A PI3K-dependent mechanism suggests ferulic acid operates as an insulinomimetic and the data from the present study demonstrate the same for its sulfated conjugates. Gogoi *et al*. recently found that ferulic acid recovered the saturated fatty acid-impaired expression of insulin receptor in L6 myotubes (Gogoi, Chatterjee et al. 2014), alluding to an additional chronic mechanism.

There were no effects on 2-DG uptake in myotubes treated with the flavonols when tested in combination or individually. The former lowered insulin-stimulated glycogen storage, while increasing glucose metabolism, with the glucuronides of quercetin and kaempferol eliciting this effect. The aglycone quercetin and kaempferol increased 2-DG uptake in L6 myotubes, but only with a 30 µM dose (Kawabata, Sawada et al. 2011). An 18 h treatment with 50 or 100 µM quercetin increased phosphorylation of acetyl-coA carboxylase, stimulating AMPK and increasing glucose uptake independent of insulin in C2C12 and L6 muscle cells (Eid, Martineau et al. 2010, Eid, Nachar et al. 2015). AMPK activation also increases carbon metabolism via fatty acid oxidation and mitochondria (Cederroth, Vinciguerra et al. 2008), and this is indicative of the conjugates here. It is possible that the conjugates had no effect on transport because the doses tested were much lower, and closer to a physiological relevance, than in these previous studies. With Q3S specifically the LHCN-M2 cells lack the OATs required for cellular uptake of sulfated compounds (Table 3.2); glucuronides can enter passively (Wong, Akiyama et al. 2012). Q3G was shown to activate AMPK in a recent study in which quercetin decreased insulin-stimulated glucose uptake in normal C2C12 cells, but under inflammatory conditions quercetin and Q3G (both at 10 µM) improved it; these opposing effects were regulated by AMPK (Liu, Mei et al. 2016). Again, this was still at concentrations higher than those tested in the present study. Various other studies report the effects of quercetin and/or kaempferol on glucose uptake. Primary human myotubes treated with 0.1-10 µM quercetin or kaempferol for 2 days showed increased glucose uptake over 4 h (Ho, Kase et al. 2017) and kaempferol was shown to reverse the high fat diet-induced decline in GLUT4 and AMPK expression in diabetic mice (Alkhalidy, Moore et al. 2015). A plant conjugate, kaempferol 3-neohesperidoside, displayed insulinomimetic properties in rat muscle (Zanatta, Rosso et al. 2008), though this is unlikely to be found in plasma following consumption. Based on the literature, the effects of Q3G and K3G seen in the present study are most likely attributable to increased metabolism via AMPK activation, but no effects were seen on transport either due to the use of lower doses than used in previous studies in which an effect was seen, and/or because Q3S specifically did not enter the cells in order to be deconjugated to active quercetin.

The resveratrol conjugates increased basal uptake and metabolism in both normal and high GI myotubes, restoring the lowered 2-DG transport and negating the longer term

effect of insulin in the latter. The increased transport was attributable to R4G and R3S, but only R4G was shown to increase metabolism, suggesting that more than one mechanism may be in play. Resveratrol aglycone has been shown previously to increase glucose uptake in muscle, with papers mostly citing an effect on the AMPK pathways. The studies by Breen *et al.* and by Park *et al.* showed that elevated uptake in myotubes, L6 and C2C12 respectively, was dependent on GLUT4 translocation through activation by sirtuins and AMPK (Park, Kim et al. 2007, Breen, Sanli et al. 2008), and in the latter it was shown that resveratrol potentiated the effect of insulin on uptake via AMPK activation, leading to PI3K/Akt pathway activation (Park, Kim et al. 2007). Other studies suggest both insulin-dependent and -independent pathways are involved (Deng, Hsieh et al. 2008, Frojdo, Durand et al. 2011, Minakawa, Kawano et al. 2011). Resveratrol enhances mitochondrial function, again through SIRT1 and AMPK activation (Price, Gomes et al. 2012), and, assuming R4G has the same effect, this will have contributed to the increased glucose metabolism. Furthermore, with the documented activity of resveratrol dominated by its anticancer properties, it is interesting that in the recent review by León *et al.* the authors link, at least in part, this asset to its effects on glucose uptake and metabolism (Leon, Uribe et al. 2017). Resveratrol is rapidly metabolised and circulates in plasma almost entirely in a conjugated form (Boocock, Faust et al. 2007) so, unless taken up and deconjugated in the muscle, it is more pertinent to test the effects on glucose uptake and metabolism using the circulating metabolites; this is the first study to do so.

The phenolic sulfates, predominantly colonic catabolites in circulation after berry consumption (Pimpao, Ventura et al. 2015), increased basal 2-DG uptake in normal and high GI myotubes and even potentiated the effect of insulin in the latter, the only combination of metabolites in this study to do so. When in combination, the metabolites had no effects on metabolism however. The increased transport was solely due to IVAS and thus this was tested alone for effects on metabolism and, without the other phenolic sulfates present, it increased basal metabolism in normal and high GI. Only several papers have been published on the biological activity of IVAS. It has been identified as a plasma metabolite following consumption of berries or cyanidin 3-*O*-glucoside (de Ferrars, Czank et al. 2014, Pimpao, Ventura et al. 2015), as a phase II conjugate of protocatechuic acid (Czank, Cassidy et al. 2013), but in these studies was undistinguishable from VAS. A recent study demonstrated that a mixture of anthocyanin

metabolites containing IVAS ameliorated the TNF-α-stimulated secretion of VCAM-1 and IL-6 in human endothelial cells (Warner, Smith et al. 2017), suggesting that such metabolites may have anti-inflammatory and cardiovascular benefits, otherwise this is the first study to investigate the effects of these metabolites on glucose uptake and the first to investigate the biological activity of IVAS individually. Several studies have demonstrated an increase in muscular glucose uptake when cells were incubated with extracts or mixtures that may have included IVAS; fermented blueberry juice for example, or phenolic compounds related to IVAS, such as its PCA precursor or upstream anthocyanins (Table 3.1). Fermented blueberry juice increased glucose uptake in C2C12 myotubes with and without insulin via AMPK activation (Vuong, Martineau et al. 2007, Nachar, Eid et al. 2017) and elderberry metabolites, including PCA, dose-dependently increased glucose uptake in primary human muscle cells (Ho, Kase et al. 2017). A recent study showed that PCA mimics insulin by activating the insulin receptor to increase glucose uptake in human adipocytes (Scazzocchio, Vari et al. 2015). It would seem PCA and its metabolite(s) may have an effect on both pathways that stimulate GLUT4 translocation, also shown by an *Artemisia princeps* extract on L6 myotubes (Yamamoto, Ueda et al. 2010). Here, the insulin-dependent mechanism was dominant, with no significant increase in metabolism when both IVAS and insulin were present.

IVAS-stimulated 2-DG uptake was dose-dependent and, much like insulin, had a greater effect in the high GI myotubes. Inhibition of IVAS-stimulated uptake by wortmannin and indinavir confirmed the mechanism was dependent on PI3K signalling and GLUT4 translocation. Furthermore, the effect was time-dependent, eluding to acute and chronic mechanisms. IVAS upregulated GLUT1, GLUT4 and PI3K p85α, as well as activating Akt in the short term. With a LogP of -0.73, IVAS would not enter the myotubes without facilitated transport. IVA, without sulfate conjugation, has a LogP of 1.81 (Agronomique. 2014) and thus would enter by passive diffusion. IVAS would not have entered the cells because the LHCN-M2 lack the appropriate transporters (Table 3.2) to facilitate the uptake of sulfated conjugates. It is therefore likely that IVAS, the effects of which were not dependent on the presence of the sulfate, acts as a substrate of the insulin receptor, initiating its autophosphorylation and downstream signalling events, including Akt phosphorylation and GLUT4 translocation and, in the longer term, upregulation of the GLUTs and insulin pathway itself. The activation of insulin signalling also led to glycogen storage being undeterred in the high GI myotubes, although the lower ^{14}C in

the normal myotubes after 4 h incubation with both insulin and IVAS indicates there could be a secondary mechanism involved, such as activation of AMPK; inhibition of insulin is less likely due to the synergistic effects on transport and Akt phosphorylation. Insulin, and maybe IVAS, could also have increased metabolism via the mitochondria (Nisr and Affourtit 2014), which would have been more effective in the normal myotubes lacking dysfunctional mitochondria (see 3.5A, B and Chapter 2). This was outweighed in the high GI by the increased storage via elevated PI3K expression, which was notably even more so when treated with IVAS.

In summary, the present study has demonstrated that an immortalised human skeletal muscle cell line, LHCN-M2, can be used as a model system to investigate effects on glucose uptake and metabolism in muscle. Differentiating these cells in various glucose and insulin concentrations highlighted that the LHCN-M2 myotubes had a phenotype typical of muscle seen in previous studies. Myotubes differentiated in 5.5 mM glucose with 10 nM insulin or in 25 mM glucose with 50 nM insulin differentiated at the same rate and this setup was used to draw comparisons between normal and high GI conditions when investigating the effects of (poly)phenol metabolites on glucose uptake and metabolism. Uptake was stimulated, notably in high GI myotubes, by sulfated conjugates derived from ferulic acid, resveratrol and cyanidin 3-*O*-glucoside. The only glucuronide to stimulate uptake was R4G, which also increased glucose metabolism, along with DHFA-4S, IVAS and the flavonol glucuronides. IVAS increased uptake and metabolism by upregulating GLUTs and PI3K and acutely activating the insulin pathway. (See Fig 3.16.)

This is the first time (poly)phenol metabolites have been investigated for effects on glucose uptake and metabolism in immortalised human myotubes, with comparisons drawn between normal and high glucose/insulin environments. It is also the first time that such compounds, notably colonic catabolites DHFA-4S and IVAS, have been reported to have beneficial effects on glucose transport into muscle and longer term metabolism. This highlights the importance of gut microbiome metabolites. In particular this is the first report of any biological activity for IVAS alone and should now be investigated further in animal and human intervention studies for its hypoglycaemic properties. Studies on foods from which IVAS is metabolised have already shown promise *in vivo*. Supplementation with blueberry bioactives for 6 weeks improved insulin

sensitivity in obese, insulin-resistant participants (Stull, Cash et al. 2010) and when bread was consumed with berries less insulin was needed to control the post-prandial glucose (Torronen, Kolehmainen et al. 2013). After following a (poly)phenol-rich diet insulin sensitivity was improved (Bozzetto, Annuzzi et al. 2015) and based on the foods consumed this could have been due in part to any of the metabolites presented in this study. It has been shown that berries and their anthocyanins also have effects on lowering post-prandial blood glucose by inhibiting carbohydrate digestion and glucose absorption in the gut (Castro-Acosta, Lenihan-Geels et al. 2016), suggesting pleiotropic effects on systemic glucose metabolism.

Table 3.2: Changes in the RNA* of selected genes during differentiation (from Day 0 to Day 4) support the changes in protein expression seen in the present study.

Gene #	**Protein**	**FPKM [1]**		**Ratio to Actinin**		**Differentiation fold-change**	
		Day 0	Day 4	Day 0	Day 4	FPKM	Ratio
181856	GLUT4	0.05	7.29	0.0003	0.11	146	323
117394	GLUT1	126	58.8	0.83	0.86	0.46	1.03
118194	Troponin T2 (cardiac)	74.5	1103	0.49	16.1	14.8	32.8
72110	α-Actinin 1	152	68.8	1.00	1.00	0.45	1.00
145675	PI3K α	7.85	9.93	0.05	0.14	1.26	2.80
105221	Akt2	45.1	33.3	0.30	0.48	0.74	1.63
171105	INSR	3.54	5.64	0.02	0.08	1.59	3.53
169047	IRS1	13.7	11.8	0.09	0.17	0.86	1.91
104812	GYS1	38.29	130.6	0.25	1.90	3.41	7.56
197901	OAT1 (SLC22A6)	0	0	-	-	-	-
137204	OAT2 (SLC22A7)	0	0	-	-	-	-
149452	OAT3 (SLC22A8)	0	0	-	-	-	-
168065	OAT4 (SLC22A11)	0	0	-	-	-	-

*Using total RNA-seq gene quantification data in files ENCFF581EE and ENCFF580JSN (LHCN-M2 myoblasts and myotubes respectively) taken from the *ENCODE* database (2012)

[1] Fragments Per Kilobase of transcript per Million mapped reads

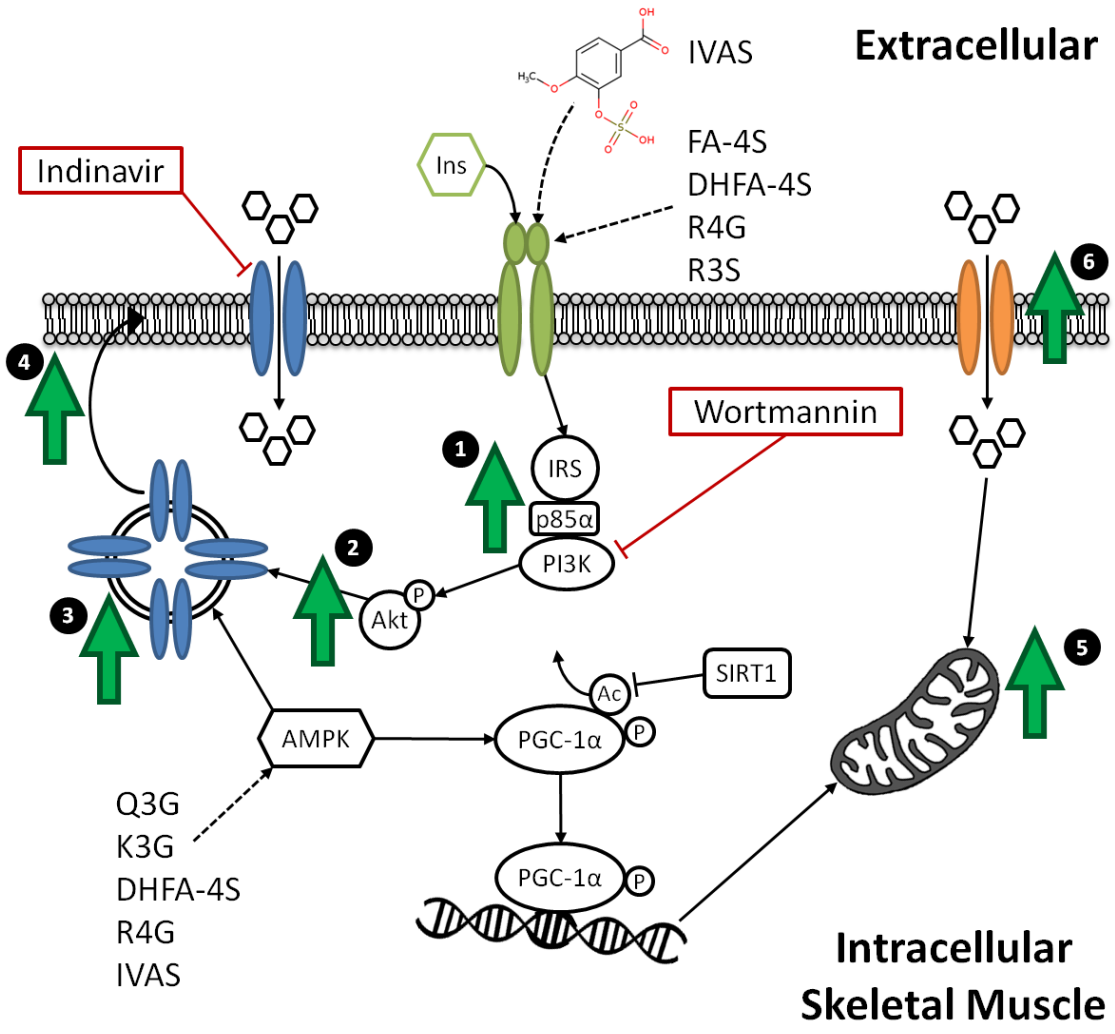

Figure 3.16: The mechanisms of action by which IVAS and other conjugates increase glucose uptake and metabolism in human skeletal muscle. Numbered green arrows are confirmed mechanisms for IVAS: 1) PI3K (p85α) is upregulated; 2) Akt phosphorylation is acutely increased; 3) total GLUT4 is upregulated; 4) GLUT4-mediated glucose uptake is increased; 5) glucose metabolism is increased, likely via an AMPK-dependent mechanism; 6) GLUT1 is also upregulated and this increases basal glucose uptake. Dashed arrows are proposed sites of action for other metabolites. Plasma membrane proteins: blue – GLUT4 (also shown stored in cytosolic vesicle); green – insulin receptor; orange – GLUT1. Ins – insulin; P – phosphate; Ac – acetylation; hexagons – glucose.

Chapter 4

Conclusions and Future Perspectives.

4.1 Summary, conclusions and impact of this research

4.1.1 Overview of the literature

The incidence of diabetes is growing at an alarming rate and being overweight or obese are the biggest risk factors for type 2, which accounts for at least 90% of cases (WHO 2016). An improved diet and increased physical activity can help lower risk (Chatterjee, Khunti et al. 2017). In addition to reducing carbohydrate and lipid consumption, and increasing dietary fibre, there is a substantial amount of evidence from epidemiological studies to suggest that a diet rich in (poly)phenols may prevent the development of metabolic diseases, and could help diabetics with the management of glucose homeostasis (Kim, Keogh et al. 2016). For example, the Nurses Health Study I and II, which investigated the urinary excretion of (poly)phenol metabolites in 1111 type 2 diabetes case-control pairs, found that (poly)phenols, including quercetin and caffeic acid, were associated with a ~45% lower type 2 diabetes risk during the first 4.6 years since urine collection ($p < 0.05$) (Sun, Wedick et al. 2015). Furthermore, two studies showed lower risk of type 2 diabetes associated with the consumption of apples and/or berries; in the Women's Health Study of 38,018 American women, the consumption of at least one apple per day showed a 28% lower risk compared with no apples ($p = 0.006$) (Song, Manson et al. 2005) and in a Finnish study of 10,054 men and women, in which there were 526 cases of type 2 diabetes, apples were associated with a 27% lower risk ($p = 0.003$) and berries a 26% lower risk ($p = 0.03$) (Knekt, Kumpulainen et al. 2002). In the latter study, a trend toward a lower risk of type 2 diabetes was associated specifically with higher quercetin intake ($p = 0.07$) (Knekt, Kumpulainen et al. 2002).

Type 2 diabetes is manifested by chronic hyperglycaemia and insulin resistance and is associated with excessive calorie intake (Taylor 2013, Krentz and Hompesch 2016, Zaccardi, Webb et al. 2016). This disrupts cellular and glucose metabolism and causes oxidative stress, in part via a reductive stress (Yan 2014). Lipid accumulation in the liver

and adipose not only precedes obesity, but can disrupt insulin signalling and induce systemic inflammation (Samuel, Petersen et al. 2010, Kwon and Pessin 2013). Insulin-secreting pancreatic β-cells become dysfunctional and insulin resistance in the peripheral tissues lowers postprandial glucose uptake and causes uncontrolled hepatic glucose metabolism and efflux. Chronic hyperglycaemia and dyslipidaemia ensues, which in turn propagate the oxidative stress and inflammation (Tangvarasittichai 2015) and cause associated complications such as CVDs, nephropathy, retinopathy, peripheral neuropathy and even neurodegenerative diseases such as Alzheimer's (Xia, Wang et al. 2007, Shenouda, Widlansky et al. 2011, Guo, Sun et al. 2013, Gurel, Sieg et al. 2013).

Oxidative stress is closely associated with hyperglycaemia and insulin resistance and causes dysfunctional mitochondria, as a primary source and target of ROS (Montgomery and Turner 2015). An increased intracellular pool of NADH, induced by excessive intake and metabolism of glucose and fatty acids, puts pressure on ETS complex I, increasing electron leakage and superoxide production and an imbalanced redox status (Brand 2010). ROS affect signalling and disrupt proteins, membranes and genetic expression in, and linked to, the mitochondria and this exacerbates the oxidative stress (Sivitz and Yorek 2010). The cell tries to protect itself from oxidative stress by lowering flux into mitochondria, but this increases free fatty acids, inhibiting GLUT4 translocation, and cellular dependence on glycolysis (Rudich, Tirosh et al. 1998, Ceriello and Motz 2004). Pathways branching off from glycolysis are induced to mediate the accumulation of G3P from excessive glycolysis, which worsens the situation by further augmenting NADH and ROS, and are regarded particularly important in the pathophysiology of various diabetic complications (Yan 2014). A principal mechanism in insulin resistance is increased phosphorylation of the IRS proteins, preventing their interaction with the insulin receptor. This may be caused by ROS, stress, inflammation and DAG accumulation (Itani, Ruderman et al. 2002, Kim, Wei et al. 2008, Boucher, Kleinridders et al. 2014). Insulin likewise regulates mitochondrial function, through the FOXO-SIRT axis, and ROS acts as a secondary messenger in the insulin signalling network under normal conditions, so the interplay between mitochondria and insulin signalling make it difficult to pinpoint the cause-effect relationship in the development of insulin resistance (Canto, Gerhart-Hines et al. 2009, Montgomery and Turner 2015).

A common factor in the onset of diabetes is hyperglycaemia and there is evidence from epidemiological, animal, human and *in vitro* studies eluding to various roles that dietary (poly)phenols may play in regulating glucose homeostasis, as well as attenuating oxidative stress and insulin resistance (Hanhineva, Torronen et al. 2010, Babu, Liu et al. 2013, Kim, Keogh et al. 2016). The potential benefits of (poly)phenols proposed by researchers may be difficult to accomplish *in vivo* due to the vast and varying combinations of compounds ingested on a daily basis and highly complex biotransformation of the aglycones and plant metabolites that are found in food. (Poly)phenols are metabolised in the small intestine and by the colonic microbiome before absorption, and in the liver and kidneys before excretion, and this often results in low bioavailability. Moreover, the intricate, and often pleiotropic, effects at the molecular and cellular levels remain to be fully understood (Del Rio, Rodriguez-Mateos et al. 2013, Williamson and Clifford 2017). Limitations in the literature generally include the testing of supra-physiological doses of (poly)phenols *in vitro* and a lack of regard for the metabolites that would be found in circulation *in vivo*.

From the range of (poly)phenols that may be ingested, or arise from metabolism, compounds were selected to focus on in this project based on their regular appearance in the diet and existing knowledge of the potential antidiabetic effects. The flavonols quercetin and kaempferol, found in onions, capers, endive, apples and berries, are rapidly metabolised in the small intestine and enter the blood mostly conjugated to a sulfate or glucuronide (Del Rio, Rodriguez-Mateos et al. 2013, Rothwell, Perez-Jimenez et al. 2013, Kerimi, Jailani et al. 2015, Williamson and Clifford 2017). Ferulic acid is a hydroxycinnamic acid found in cereals and is a derivative of the coffee chlorogenic acids. Some ferulic acid is absorbed and sulfated in the small intestine, but most passes to the colon where it is metabolised by the microbiome to dihydroferulic acid and others before absorption (Zhao and Moghadasian 2008, Stalmach, Mullen et al. 2009, Mateo Anson, Aura et al. 2011, Williamson and Clifford 2017). The stilbene resveratrol is found in grape seed and, like the flavonols, is rapidly metabolised to sulfate and glucuronide conjugates in the small intestine. It has been shown that bioavailability is particularly low, but the compound has shown promise as a pharmaceutical (Boocock, Faust et al. 2007, Brown, Patel et al. 2010, Del Rio, Rodriguez-Mateos et al. 2013). Consumption of berries gives rise to metabolites of anthocyanins and gallic acid, predominantly as phenolic sulfates from the colon after bacterial biotransformation (Williamson and

Clifford 2010, Pimpao, Ventura et al. 2015). Pharmacokinetic data has revealed that the bioavailability of the various (poly)phenol metabolites can vary depending on the compound in question, its source and the quantity ingested/administered, but generally metabolites, and some aglycones in smaller amounts, were found in the plasma in the nanomolar to low micromolar range.

4.1.2 Quercetin protects mitochondria from high glucose-induced stress

Quercetin sulfates and glucuronides are deconjugated in peripheral tissues (Kawai, Nishikawa et al. 2008, Hashimoto, Blumberg et al. 2016) and, in this regard, the aglycone has been shown to interact with various cellular targets, in particular accumulating in mitochondria and eliciting pleiotropic effects on mitochondrial function, including those in the context of diabetes (Fiorani, Guidarelli et al. 2010, de Oliveira, Nabavi et al. 2016, Haddad and Eid 2016). Quercetin was shown previously to affect mitochondrial biogenesis, metabolic flux and the ETS, however the evidence is scarce and conflicting, with mechanistic studies limited to an acute setup on isolated mitochondria. The aims of the first half of this project were to evaluate the effects of a chronic exposure to high glucose on mitochondrial function and explore the effects and mechanisms of quercetin in its potential protective role.

In Chapter 2 the mechanisms of action of quercetin on mitochondria were investigated in light of its ability to prevent oxidative stress. HepG2 cells were grown chronically in normal or high glucose and treated with quercetin for a prolonged period before mitochondria were isolated or cells collected to assay various aspects of mitochondrial function. Complex I activity was decreased in cells exposed to high glucose as a result of elevated NADH and ROS, however quercetin dose-dependently recovered this after 24 h. It was proposed, based on previous mechanistic work, that quercetin transiently inhibits complex I, and the associated superoxide generation, at the CoQ_{10}-binding site and transfers electrons from NADH to complex III. The pressure on complex I was relieved and the proton gradient restored, attenuating the high glucose-induced IMM proton leak and enhancing the mitochondrial respiration efficiency. Cellular NAD^+/NADH was increased within 2 h of quercetin treatment and induction of PGC-1α transcription followed, further increasing mitochondrial function and shifting metabolic output to mitochondrial respiration. The overall chronic result was lowered oxidative stress and enhanced mitochondrial integrity, with increased respiration, complex I

activity and improved cell viability. Furthermore, non-ETS oxygen consumption, together with the increased mitochondrial output, indicated the cells relied less on glycolysis for ATP synthesis and therefore the alternative glycolytic pathways were also likely mitigated.

The potential of quercetin to bind to PPARα and affect recruitment of its coactivator (PGC1-α) was tested in our lab by Dr Asimina Kerimi. A time-resolved fluorescence energy transfer (TR-FRET) assay was set up, able to detect binding interactions with high sensitivity and small variability, as shown by the control agonist GW7647 (Fig. 4.1A) and antagonist GW9662 (Fig, 4.1B) of PPARα. The LanthaScreen TR-FRET assay (#PV4684, Invitrogen, Thermo Fisher Scientific, UK) was used according to the supplier's guidelines. Briefly, PPARα ligand binding domain tagged with glutathione-S-transferase was added to either GW7647 (PPARα agonist) or GW9662 (antagonist), or quercetin, Q3S or Q3G, followed by the addition of pre-mixed fluorescein-PGC1-α coactivator peptide (in 50 mM HEPES, pH 7.5) and LanthaScreen terbium-labelled anti-GST antibody (in 137 mM NaCl, 2.7 mM KCl, 10 mM HEPES, pH 7.5). Following optimization by comparison of achieved Z′ values, calculated by using the "maximum agonist/antagonist" and "no agonist/antagonist" control data (Zhang, Chung et al. 1999), incubation in the agonist mode assays was set at 3 h and at 2 h for the antagonist mode (room temperature). The TR-FRET ratio was calculated by dividing E_m at 520 nm by E_m at 495 nm, measured on a PheraSTAR FS microplate reader with a LanthaScreen filter module. The EC_{50} and IC_{50} values were calculated by GraphPad Prism 6 after fitting the data on an equation for a sigmoidal dose response.

Quercetin was found to exert only minimal effects in the agonist mode, at ≥ 10 μM ($p < 0.01$) (Fig. 4.1C), while it had no effect as an antagonist (Fig. 4.1D). An EC_{50} was not calculated for quercetin because a plateau was not reached for the concentration range tested. Conjugates Q3S and Q3G were weaker agonists than quercetin aglycone; an increase in fluorescence emission was measured only at ≥ 100 μM ($p < 0.01$) (Fig. 4.1C). Direct effects of quercetin on PGC-1α activity were therefore minimal as quercetin was found to only weakly enhance PGC-1α binding to PPARα *in vitro* at concentrations ≥ 10 μM, although this could still occur in the cellular environment due to the rapid quercetin influx (Fiorani, Guidarelli et al. 2010). It can be concluded from this that the effects of quercetin on PGC-1α are most likely via indirect mechanisms as a result of the effects

seen on complex I, respiration and NAD^+/NADH. However, the involvement of other transcriptional factors in the regulation of PGC-1α by quercetin cannot be excluded.

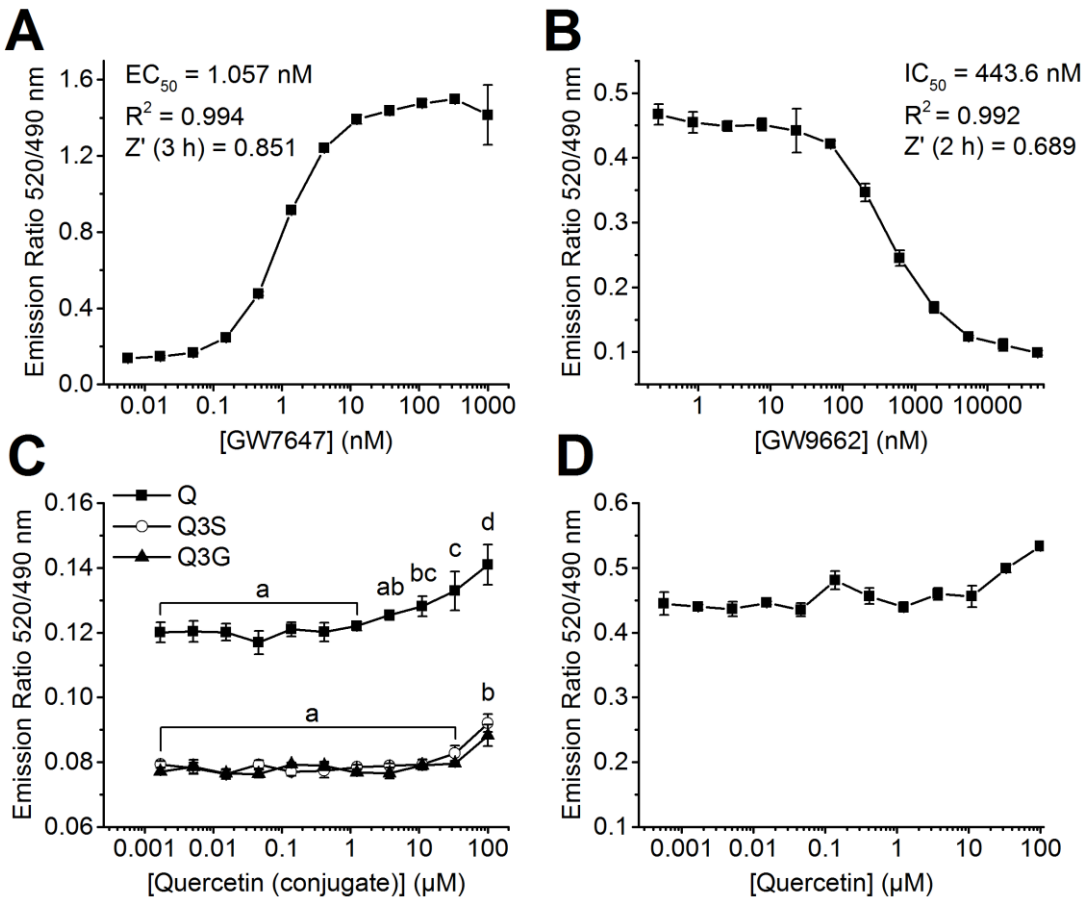

Figure 4.1: Quercetin has a minimal effect on PGC-1α recruitment to PPARα *in vitro*. A LanthaScreen TR-FRET assay was used to assess the potential of quercetin to bind to PPARα and affect recruitment of its coactivator (PGC1-α). PPARα ligand binding domain tagged with glutathione-S-transferase was added to either GW7647 (PPARα agonist) (A) or GW9662 (antagonist) (B), or quercetin (Q), Q3S or Q3G (C-D), followed by the addition of pre-mixed fluorescein-PGC1-α coactivator peptide and LanthaScreen terbium-labelled anti-GST antibody. Following optimization, agonist mode assays (A, C) were incubated for 3 h and antagonist mode assays (B, D) for 2 h. TR-FRET ratio was calculated by dividing E_m at 520 nm by E_m at 495 nm, measured on a PheraStar FS microplate reader with a LanthaScreen filter module and EC_{50} and IC_{50} values were calculated. Data are mean values ± SD (n = 18). Letters indicate significant differences between tested concentrations ($p < 0.01$).

This is the first study to bring these biomarkers together in this way, in particular the complex I activity assay with intact cell respirometry and redox flux in the way of NAD^+/NADH, to observe an overall lasting effect of quercetin on mitochondrial function. Furthermore, it is the first investigation into the effects of quercetin on mitochondria in cells in a high glucose environment. A novel mechanism of action for intracellular quercetin has been proposed. Future studies can utilise this experimental setup to elucidate further details of the mechanisms, and to investigate other promising compounds. If these effects are inferred to hepatocytes *in vivo* then preventing hepatic mitochondrial dysfunction would help alleviate insulin resistance and restore normal glucose and lipid metabolism. Concentrations of quercetin used to probe mechanisms here were higher than that generally found *in vivo*, although one report claims to have seen a plasma C_{max} of 7.6 µM for total quercetin and its metabolites (Graefe, Wittig et al. 2001). However, the effects were dose-dependent and quercetin accumulates in peripheral tissues, in mitochondria specifically. This implies that continued ingestion of quercetin-rich foods or supplements over a prolonged period, say a lifetime, could elicit the chronic mechanisms evidenced here and this may account for the benefits seen in epidemiological and animal studies (Kobori, Masumoto et al. 2009, Jacques, Cassidy et al. 2013).

The HepG2 model was chosen as a well-established and well-characterised cell line; there are close to 24,000 articles on HepG2 when searching on PubMed. The immortalised carcinoma-derived cell line is thought to retain some hepatic functions and is thus considered an adequate model for some areas of research, though a study that used microarray technology to compare the gene profiles of HepG2 and primary cells found ~2600 genes significantly downregulated and ~3600 upregulated in the former (Costantini, Di Bernardo et al. 2013). Among these changes were genes relating to their glycolytic character, and as such metabolic effects seen *in vitro* may not be translated to liver tissues *in vivo*. It may be that the effects of quercetin on mitochondria are heightened *in vivo*, because in the glycolytic HepG2 cells the changes in mitochondrial respiration may have been comparatively smaller. Growing the cells in galactose proved to increase complex I activity (Fig. 2.7D) and this could be used to prime the cells for quercetin-induced mitochondrial effects, by forcing them to rely more on OXPHOS and less on glycolysis as demonstrated previously, though this was not the case for all xenobiotics (Marroquin, Hynes et al. 2007). Furthermore, culturing conditions can cause significant

variation between experiments and between labs; even *ex vivo* tissue differs to *in vivo* (Kerimi and Williamson 2017), so consistent models to study (poly)phenols *in vitro*, *ex vivo* and *in vivo*, regardless of the context or cellular challenges imposed, should be universally employed.

Another key difference between HepG2 and primary hepatocytes/liver tissue is the down-regulation of the OATs, upon which sulfated flavonols rely for intracellular transport (Wong, Akiyama et al. 2012, Kerimi and Williamson 2017). Quercetin aglycone enters the cells rapidly by passive diffusion and was one reason why the aglycone was chosen for this study on mitochondria in HepG2. This was a reasonable model, since previous studies have shown quercetin 3′-*O*-sulfate is deconjugated to the more active form anyway, at least in macrophages (Kawai, Nishikawa et al. 2008), but it would be interesting compare how the effects of quercetin conjugates compare using OAT-transfected cells. Many cancer cells also have upregulated efflux transporters, so conjugated flavonoids and chemotherapeutic drugs may be rapidly transported out of the HepG2 cells (Kerimi and Williamson 2017), although it has been shown that quercetin and its metabolites were retained for at least 4 h (Hashimoto, Blumberg et al. 2016).

The protective effects of quercetin on high glucose-induced mitochondrial dysfunction holds promise for its future use as a nutraceutical or therapeutic against metabolic diseases. The so-called reductive stress may be a small aspect in the overall aetiology of type 2 diabetes, but if prolonged exposure to quercetin shifts the redox balance in hepatocytes to lower oxidative stress and subsequent mitochondrial dysfunction, this will help restore hepatic glucose homeostasis and prevent insulin resistance. Research into enhancing the bioavailability of quercetin, with nano-technology delivery systems for example, is useful. Nano-capsuled quercetin and high-dose free quercetin had anti-oxidative effects in rats subjected to ethanol-induced gastric inflammation, also restoring mitochondrial morphology and complex I and II functions (Chakraborty, Stalin et al. 2012). Further studies are understandably required, to ensure such administration would not result in side effects in humans; quercetin is hormetic and would be toxic at high doses.

4.1.3 (Poly)phenol metabolites increase glucose uptake in skeletal muscle

Some (poly)phenols were previously investigated *in vitro* for their potential in stimulating glucose uptake into skeletal muscle, in light of their capacity in regulating postprandial glycaemia *in vivo* (Kim, Keogh et al. 2016). Ferulic acid increases glucose uptake in L6 and primary porcine and human myotubes (Prabhakar and Doble 2009, Bhattacharya, Christensen et al. 2013, Ho, Kase et al. 2017) and it has been suggested that this is via an insulin-dependent mechanism (Prabhakar and Doble 2009, Gogoi, Chatterjee et al. 2014). Quercetin and kaempferol have been shown to increase uptake in L6 myotubes (Kawabata, Sawada et al. 2011) and for the former an AMPK-mediated increase in glucose metabolism was demonstrated (Cederroth, Vinciguerra et al. 2008, Eid, Martineau et al. 2010, Eid, Nachar et al. 2015). Quercetin 3-*O*-glucuronide is the only flavonol conjugate to have been explored on muscular glucose transport and, as with its aglycone, increased uptake in C2C12 myotubes under inflammatory conditions (Liu, Mei et al. 2016). For resveratrol, both insulin- and AMPK-stimulated glucose uptake has been reported (Deng, Hsieh et al. 2008, Frojdo, Durand et al. 2011, Minakawa, Kawano et al. 2011), accompanied with increases in metabolism through the SIRT1-AMPK pathway and enhanced mitochondrial function (Price, Gomes et al. 2012). To date, no studies have explored potential effects of berry-associated phenolic sulfate metabolites downstream of cyanidin 3-*O*-glucoside, via protocatechuic acid, and gallic acid. Berry extracts that may have included such metabolites have shown promising effects (Vuong, Martineau et al. 2007, Ho, Kase et al. 2017, Nachar, Eid et al. 2017) and, as with resveratrol, both insulin-dependent and -independent pathways may be in play (Yamamoto, Ueda et al. 2010).

However, the single study on Q3G aside, all of these studies were done with the parent aglycone compounds, or extracts or plant metabolites, and not on the bioavailable metabolites that would be found in circulation *in vivo*. Moreover, the majority of compounds had been tested only at supra-high concentrations and primarily in rodent myotube models. The key aims to this part of the project included establishing an appropriate model using an immortalised human skeletal muscle cell line, LHCN-M2, to investigate the potential effects of (poly)phenol metabolites, at concentrations closer to the physiologically achievable range (see Table 1.1 and Table 3.1), on glucose uptake and metabolism and elucidating the mechanism of action by which such effects may have occurred.

In Chapter 3 it was demonstrated that LHCN-M2 can be used as a model system in the context of investigating glucose uptake and metabolism in human skeletal muscle. Differentiating these cells to myotubes in high glucose led to elevated ROS and mitochondrial dysfunction, and increasing the chronic insulin led to increased differentiation of the normal myotubes, but impeded it in the high. Basal 2-deoxy-D-glucose and glucose uptake was higher in normal glucose but myotubes in high glucose were more sensitive to insulin. Myotubes differentiated in 5.5 mM glucose with 10 nM insulin or in 25 mM glucose with 50 nM insulin were differentiated by the same proportion after 96 h and this end model was used for comparisons of normal and high glycaemia/insulinaemia when investigating the effects of (poly)phenol metabolites.

FA4S and DHFA-4S caused the increase in basal uptake seen with a combination of ferulic acid derivatives in high GI myotubes and the latter increased glucose metabolism too. The flavonol conjugates had no effect on uptake but increased metabolism to supersede the insulin-stimulated glycogen storage. R4G and R3S increased glucose uptake in normal and high GI myotubes, and R4G increased metabolism in both. A berry metabolite profile consisting of phenolic sulfates was found to increase acute glucose transport synergistically with insulin, but had no effect on metabolism. When the compounds were tested alone only IVAS had an effect and this increased metabolism too, negating the longer-term effect of insulin in normal myotubes. IVAS increased uptake in a dose-dependent manner and this was inhibited by the addition of wortmannin and indinavir, indicating a PI3K- and GLUT4-dependent mechanism. Uptake and metabolism were increased by IVAS through upregulation of GLUTs 1 and 4 and PI3K, as well as acute activation of the insulin pathway, shown by increased Akt phosphorylation, and it was proposed that this was via stimulation at the insulin signalling receptor (see Fig. 4.2). This is the first time (poly)phenol metabolites have been reported to increase glucose uptake and metabolism in an immortalised human skeletal muscle model, with comparisons between normal and high GI conditions, and it is the first study to report biological activity specifically for IVAS.

It should be acknowledged that there are certain limitations to the cell model utilised in the present study, as there are with any model; especially with the low expression of GLUT4 in cultured cells and that the high GI myotubes were not insulin-resistant per se. GLUT1 expression outweighed that of GLUT4 and it was shown previously that GLUT1

may be upregulated by insulin in rat (Laybutt, Thompson et al. 1997), but the indinavir inhibition of insulin- and IVAS-stimulated uptake suggests translocation was by GLUT4 specifically. Indinavir is specifically a GLUT4 inhibitor at 20 µM (Rudich, Konrad et al. 2003) and it has been shown that GLUT1 translocation is not stimulated by the insulin pathway (Al-Khalili, Chibalin et al. 2003). The normal/high GI LHCN-M2 model suited its purpose in this study, as a useful tool to investigate glucose transport into muscle, for which other cultured human myotubes have been affirmed in this context (Al-Khalili, Chibalin et al. 2003), especially when investigating a compound that turns out to be insulinomimetic. Primary cell models have been used in uptake studies but this also carries drawbacks, namely availability and variability. Moreover, it was demonstrated recently that hyperglycaemia and hyperinsulinaemia are necessary to promote insulin resistance in lymphatic muscle, with reduced glucose uptake and mitochondrial dysfunction (Lee, Fluckey et al. 2017), so not dissimilar to the normal and high GI-differentiated cells used here. There is limited literature specifically on the LHCN-M2 cell line, so further characterisation may be necessary for its use as a model in other investigations.

Not only is the model suitable to study other compounds for effects on glucose uptake and metabolism in the context of diabetes, but so too are the biomarkers established with it. The use of the radiolabelled uptake assays with 2-[1-^{14}C(U)]-deoxy-D-glucose and D-[^{14}C(U)]-glucose is not a new technique (Kerimi, Jailani et al. 2015), but the benefit of distinguishing between effects on glucose transport and further metabolism, over a longer period than previously, in this system was highlighted. Emerging technologies such as high-performance anion-exchange chromatography can be used for carbohydrate analyses for the same purpose and it would be worthwhile comparing the two techniques for uptake and metabolism assays; downstream carbon metabolites of glucose can be measured and greater detail of effects on glycolysis, the TCA cycle, lactate production etc. may be revealed. This system has been used primarily for food analyses and recently for carbohydrate digestion assays (Rohrer, Basumallick et al. 2013, Pyner, Nyambe-Silavwe et al. 2017). Uptake assays coupled with precise protein expression measurements revealed details of the mechanisms involved. In particular, the use of an automated Western capillary system in this project has demonstrated that one should consider replacing traditional Western blotting and other immunoassays with this modern, quantitative and (most importantly) scientifically robust technique. The

ProteinSimple system used here is able to quantify proteins, and their post-translational modifications and phosphorylation events, accurately from ultralow sample input with high reproducibility and allows for multiplexing in the same capillary (Chen, Wakefield et al. 2015). The combination of both GLUTs 1 and 4, and insulin pathway analyses on the automated Western capillary system in the present study is the first of its kind in the context of glucose uptake in muscle and can be used for similar future studies.

4.1.4 Impact and implications of this research

The results from this project enhance our understanding of how the antidiabetic effects of dietary (poly)phenols are induced. Quercetin prevents oxidative stress and mitochondrial dysfunction, which improves hepatic glucose and lipid metabolism and prevents pancreatic β-cell damage *in vivo*, attenuating insulin resistance and enhancing insulin synthesis and secretion respectively. While quercetin is rapidly conjugated and absorbed in the small intestine, it has been shown that the aglycone accumulates in peripheral tissues and in particular in mitochondria. Quercetin has relatively low bioavailability but, with its reported high biological activity, habitual consumption may reduce the risk of chronic diseases such as type 2 diabetes and this has been demonstrated by several human intervention studies (Williamson 2017). Pure quercetin supplements are commercially available, but these are in aglycone form and less bioavailable than the quercetin in food (Shi and Williamson 2015).

The circulating metabolite profiles from commonly consumed (poly)phenol-rich foods (or resveratrol in tablet form) demonstrate promising effects on glucose uptake and metabolism in skeletal muscle, notably in a high glucose/insulin environment. Together with the inhibitory effects seen previously on carbohydrate digestion and absorption in the gut by food extracts and aglycones, this provides an insight into how (poly)phenols may regulate postprandial glycaemia and help to maintain healthy glucose homeostasis *in vivo*. Preventing or attenuating chronic hyperglycaemia would prevent systemic insulin resistance, oxidative stress and inflammation, especially if the effects are also seen in the insulin-stimulated adipocytes too.

Insulin and anti-hypoglycaemic drugs are currently prescribed to insulin-resistant and diabetic individuals to lower glycaemia and improve insulin sensitivity. There are several different classes, which are selected based on the individual's circumstances, and these

were reviewed recently (Chaudhury, Duvoor et al. 2017). Biguanides such as metformin enhance AMPK signalling pathways to lower hepatic glucose output, by preventing insulin resistance and gluconeogenesis. Thiazolidinediones (TZDs) reduce insulin resistance in muscle and adipose by activating PPAR-γ, which in muscle also lowers lipid uptake and storage and in adipose in particular this lowers inflammation. Sulfonylureas stimulate insulin release by pancreatic β-cells, and α-glucosidase inhibitors such as acarbose regulate glucose digestion in the gut (Chaudhury, Duvoor et al. 2017). The mechanisms of the compounds studied in the present study can be likened to those for prescribed therapeutics. Quercetin in particular, but the flavonol glucuronides, R4G and DHFA-4S too, all upregulated glucose metabolism as with metformin and the metabolites lowered carbon storage as with TZDs. The prevention of oxidative stress by quercetin in the pancreas would rescue insulin secretion, in similar fashion to sulfonylureas, though via a different mechanism. Furthermore, the sulfated conjugates and R4G that displayed insulinomimetic properties could lower the dependence on insulin injections.

Following successful clinical trials, (poly)phenols could be administered to replace, or work synergistically, with insulin and the other current therapeutics and at least until then the evidence is substantial enough for doctors to recommend a (poly)phenol-rich diet, although this is heavily dependent on patient cooperation. As bioavailability is improved with better supplementation and nanotechnology, administration of (poly)phenols may soon become a reality in the management of diabetes. There have already been clinical trials for resveratrol in cancer patients, where pharmacokinetics, efficacy and side effects were monitored (Brown, Patel et al. 2010). The same would need to be done with quercetin and others, as they often have multiple and hormetic roles, for example the dose-dependent effects of quercetin on mitochondria and its dual role in AMPK activation (de Oliveira, Nabavi et al. 2016, Liu, Mei et al. 2016). In the not-so-distant future, doctors and scientists envisage the possibility of personalised nutrition, based on individual genomics, metabolomics and gut microbiome fingerprints, becoming a reality and prescribed (poly)phenol intake could feature. The potential for this was eluded to in a review on nutritional genomics and (poly)phenols (Barnes 2008). The mechanisms highlighted in the present study hold promise for the beneficial roles of (poly)phenols against chronic diseases beyond diabetes too, such as CVDs, cancers and

neurodegeneration, the pathophysiology of which involve oxidative stress and glucose metabolism (Del Rio, Rodriguez-Mateos et al. 2013).

The present study will impact the field of (poly)phenols and health research, in providing new details of mechanisms of action and highlighting the importance of studying relevant metabolites, in addition to improved models and biomarkers. The study is part of the wider *POLYTRUE?* project (European Research Council Advanced Grant 322467), which set out to explore the true chronic effects and mechanisms of action of dietary (poly)phenols, with the hypothesis that the exposure of chronic, low levels of metabolites, relevant to *in vivo* nutrition, in an *in vitro* system will have significant and different effects to acute, high concentrations.

This study ties in with several of the objectives for *POLYTRUE?*, in that the chronic effects, such as longer-lasting changes to gene expression and metabolism, of metabolite mixtures were investigated in the presence and absence of chronic stress, which was high glucose and/or insulin in this instance. Metabolites were selected based on previous human intervention studies conducted, and some of them subsequently synthesised, as part of the *POLYTRUE?* project. New theories for the mechanisms of action of (poly)phenols on human health have been suggested; dietary quercetin prevents against high glucose-induced hepatic mitochondrial dysfunction through pleiotropic mechanisms resulting in improved redox status and enhanced mitochondrial respiration and gut microbiome catabolites, such as isovanillic acid 3-*O*-sulfate, restore glucose uptake and metabolism, via insulin-dependent pathways, in skeletal muscle cells.

Furthermore, novel *in vitro* cell systems and approaches were established and utilised for the study of such chronic effects. The immortalised human skeletal muscle cell model established here may be further used to investigate glucose uptake and metabolism in the diabetes context, and in particular the beneficial role (poly)phenol metabolites. Biomarkers for muscle and glucose metabolism were used in a novel way for Western protein analyses and previously unused combinations of systems and biomarkers, such as the chronic effects of glucose and quercetin in cells assayed for complex I activity, respiration and metabolite flux to ascertain an overall picture of mitochondrial function. The future direction of the project involves furthering the novel cell systems used here by expressing appropriate transporters (see 4.2) and establishing multi-cell models and

expressing appropriate transporters. This will enable an investigation into the effects of inter-cell interactions and the metabolic function of hepatocytes and/or myocytes in the presence of endothelial and colon cells when the system is exposed to chronic (poly)phenol treatments, to mimic the complexity of whole-body metabolism and physiology *in vivo*.

4.2 Future Perspectives

For the work on quercetin and mitochondria, a next step could be to analyse the effects in the muscle model to see if they are consistent or cell/tissue-specific. Mitochondrial assays were already tested on the LHCN-M2 myotubes and so this would be straightforward to set up. Furthermore, the metabolites for which effects on glucose metabolism were seen in the muscle could be tested for their potential roles in mitochondrial function too. The effects of quercetin were dose-dependent, with effects on complex I activity and ROS levels seen with 2.5 µM; any lower and the effects would likely not have been detected. To investigate effects even closer to the typical physiological range, a model could be established to try growing the HepG2 cells for a longer period (and/or through multiple passages) and treating with quercetin long-term i.e. daily treatments with 1 µM or even 0.1 µM, to see if changes are detectable in these assays, with comparisons to the 24 h treatments with 10 µM. Further investigations into the effects of quercetin on metabolic flux could be investigated using ion chromatography; analyses of lactate, pyruvate, succinate, citrate and so on would be interesting in the current HepG2 system and one would hypothesise seeing quercetin driving flux away from the glycolytic-dependent ATP synthesis towards the TCA cycle and mitochondrial respiration. Extracellular metabolites were measured by high-performance liquid chromatography recently (Niklas, Nonnenmacher et al. 2012) and this could be taken further by measuring intracellular metabolites in cell lysate extracts as well as the extracellular in the media, and comparing normal and high glucose. Furthermore, the effects on mitochondria will affect lipid metabolism as well as glucose, so analysis of the lipidome in HepG2 grown in normal and high glucose and treated with quercetin would be an interesting additional study; quercetin was already shown to exert lipid-lowering effects in the livers of diabetic mice via increased mitochondrial function (Kim, Kwon et al. 2015).

It would be challenging to investigate the effects of quercetin on hepatic mitochondria *in vivo* for obvious reasons; mitochondria could be isolated from rodent livers following quercetin intervention for example, but this certainly wouldn't be possible in humans. An *in vitro* setup could involve obtaining primary hepatocytes from normal and diabetic patients and treating these with quercetin to see how the effects compare, but this heavily relies on tissue availability and a large cohort to see beyond inter-individual variability. An easier *ex vivo* approach may be to take muscle biopsies following a long-term quercetin intervention and analyse the samples on the high-resolution respirometer and/or isolate mitochondria from the tissue.

The obvious studies to be carried out next for the effects of (poly)phenol metabolites on glucose uptake and metabolism are human interventions. With an *in vitro* mechanism of action established for IVAS, this would be a promising compound to start with. An intervention with the berry purée used in the studies by Pimpão *et al.* (Pimpao, Dew et al. 2014, Pimpao, Ventura et al. 2015), from which the phenolic sulfate profile in this study was selected, may lower glycaemia in participants, though it would be unclear if this was an effect on muscle uptake or more so on the inhibition of carbohydrate digestion and absorption seen with berries in previous interventions (Nyambe-Silavwe and Williamson 2016). Intervention with a cyanidin 3-*O*-glucoside tablet, such as that used in pharmacokinetic studies (Czank, Cassidy et al. 2013, de Ferrars, Czank et al. 2014), instead of berries may result in more specific metabolites at least, or encapsulating IVAS so a controlled dose reaches the bloodstream.

Finally, to expand on the beneficial roles of (poly)phenol metabolites, and to make the *in vitro* models closer to the *in vivo* situation, the HepG2 and/or LHCN-M2 cells could be transfected with OAT-expressing plasmids to upregulate the transporters associated with the facilitated uptake of the sulfated conjugates (Wong, Akiyama et al. 2012). In the muscle study here the sulfated conjugates were not taken up and their effects may differ if they were. It would also be interesting to compare the effects of quercetin aglycone and quercetin 3′-*O*-sulfate on the mitochondrial function and cellular metabolism in HepG2 cells. Likewise, LHCN-M2 transfected with GLUT4 to increase expression beyond the low endogenous level would further confirm the GLUT4 translocation-stimulated mechanism of IVAS and other metabolites.

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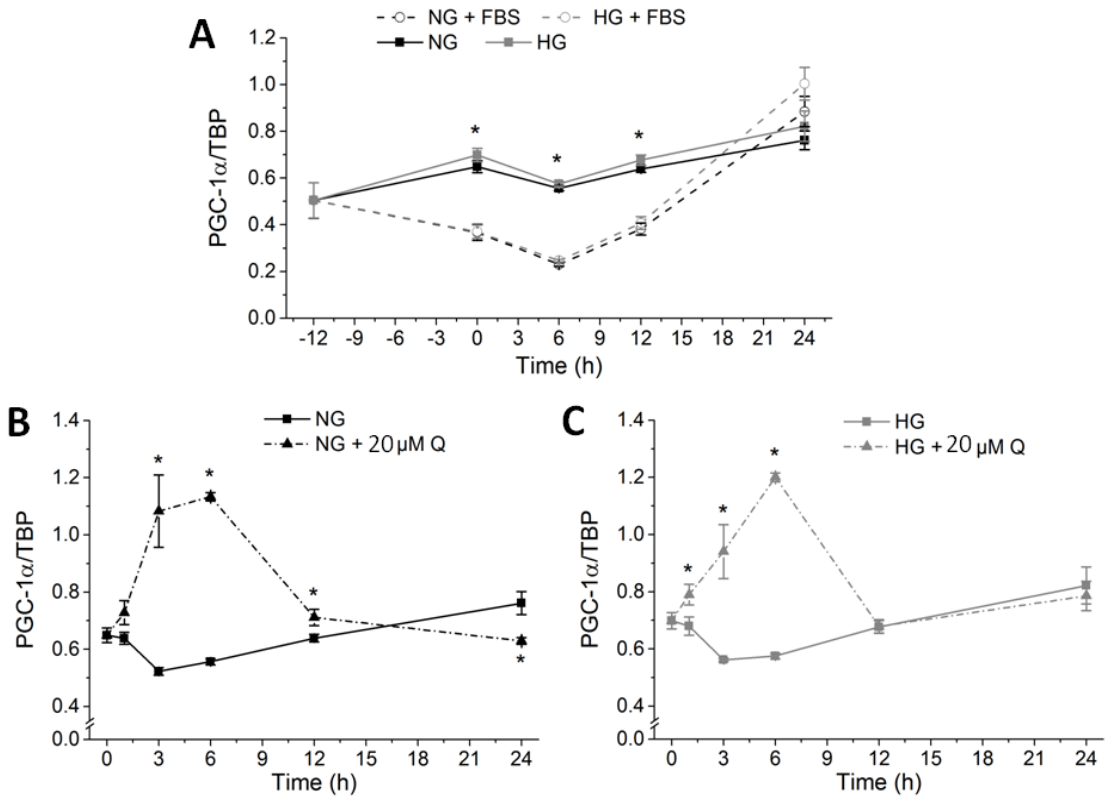
Note: Where journal volume/issue and page numbers are missing, these articles were only available as e-publications at the time of writing.

Appendix



Supplementary Figure 1: PGC-1α expression in HepG2 cells normalised to TBP housekeeping gene. Cells were seeded in NG or HG media and after 60 h (referred to as T = -12 h) serum (FBS) was removed from half. The medium was changed again at T = 0 h for the final 24 h of the experiment, still ± serum accordingly. Cells were lysed at various time-points, RNA extracted and PGC-1α measured alongside TBP control using droplet digital PCR (see Chapter 2). Data are expressed as mean ratios of PGC-1α/TBP ± SEM (n/N = 18/6). *$p < 0.05$ *vs* NG/HG +FBS controls (A). Cells were grown without FBS from 60 h after seeding but with 20 µM quercetin (Q) (or DMSO controls) added for varying times in the final 24 h. PGC-1α/TBP ratios were measured and expressed as means ± SEM (n/N = 18/6). *$p < 0.05$ *vs* NG or HG controls (B-C).