

Molecular and genetic mechanisms of myocardial insulin resistance in humans and mice

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ABSTRACT

Patients with type 2 diabetes mellitus have a greatly increased risk of coronary artery disease and heart failure due to a number of factors such as insulin resistance, hyperglycaemia, arterial hypertension, and obesity. To study the changes seen in hearts of diabetic patients the aim of this project was to generate a clinically relevant diet-induced obese mouse model which could mimic these changes.

Male C57BL/6 mice were fed a 45% high fat diet for 8, 11, and 14 weeks. Metabolic and histological changes in animals that responded to the high fat diet (diet-induced obese, DIO) were investigated and the most appropriate feeding period was determined for further studies.

DIO animals showed significantly increased body weight gain, elevated fasting serum insulin levels and over time animals developed impaired glucose tolerance. Histological analysis of DIO hearts after 14 weeks of high fat diet showed significantly increased interstitial fibrosis and after all three time points a previously unrecognised coronary microvascular remodelling was observed in DIO mice. Molecular mechanisms involved in insulin resistance and coronary microvascular remodelling were investigated in DIO mice after 11 weeks of high fat diet. Animals showed alterations in coronary flow, endothelial dysfunction and myocardial gene expression analysis revealed differentially expressed genes involved in lipid and carbohydrate metabolism. Validation experiments showed that one of these genes, regulator of G-protein signalling (RGS2), had a significant reduction at both RNA and protein level in total heart lysates. Additionally, RGS2 expression in vascular smooth muscle cells of small vessels in the heart was significantly reduced in DIO mice. After a shift from high fat to a normal rodent chow a reversal of microvascular remodelling as well as a return of RGS2 expression levels back to baseline were observed.

In conclusion, this diet-induced mouse model shows features of cardiomyopathy and coronary microvascular remodelling which is closer to the phenotype seen in patients with arterial hypertension or hypertrophic cardiomyopathy compared to other mouse or rat models.

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DECLARATION

This thesis comprises results produced solely by myself except as noted otherwise. Some studies were carried out with the help of members from the laboratory or external collaborations and are indicated. It also contains illustrations from other laboratories which are stated in the figure legends. This work has been carried out at the Clinical Sciences Centre, Faculty of Medicine, Imperial College London.

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ABBREVIATIONS

ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
AGEs	Advanced glycation end products
Akt	Protein kinase B
AMPK	5'-AMP-activated protein kinase
ANOVA	Analysis of variance
APE1	Apurinic/aprimidinic endonuclease 1
APS	Ammonium persulfate
aPKC	Atypical protein kinase C
AT1	Angiotensin II type 1 receptor
ATP	Adenosine triphosphate
AUC	Area under the curve
BP	Blood pressure
BSA	Bovine serum albumine
CAD	Coronary artery disease
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
cm	Centimetre
CTRL	Control
CF	Coronary flow
CIRKO	Cardiac-specific insulin receptor knockout
CMRI	Cardiac magnetic resonance imaging
CST	Cell Signaling Technology
Ct	Threshold cycle
CTGF	Connective tissue growth factor
CVD	Cardiovascular disease
dH ₂ O	Sterile water
DAG	Diacylglycerol
db	Diabetic
DIO	Diet-induced obese

DIO-R	Diet-induced obese resistant
DIO-WL	Diet-induced obese – weight loss
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
EC	Excitation-contraction
EC70	70% of the maximal contractile force
ECG	Electrocardiography
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
Ehhadh	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial NO synthase
ET-1	Endothelin-1
FA	Fatty acids
FC	Fold change
Fdft1	Farnesyl diphosphate farnesyl transferase 1
FFA	Free fatty acids
FOXO	Forkhead box O transcription factor
FVB	Friend leukemia virus B
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine 5'-diphosphate
GK	Goto-Kakizaki
GLUT	Glucose transporter
GPCR	G protein-coupled receptor
Gpr22	G protein-coupled receptor 22
GSK1	Glycogen synthase kinase 1
GTP	Guanosine 5'-triphosphate
HCl	Hydrogen chloride
HCM	Hypertrophic cardiomyopathy
HFD	High fat diet

Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase
IDF	International Diabetes Federation
IHC	Immunohistochemistry
IL-6	Interleukin-6
IR	Insulin receptor
IRS	Insulin receptor substrate
JAK	Janus kinase
KCl	Potassium chloride
KO	Knockout
Lmcd1	LIM and cysteine-rich domain 1
L-NAME	N ^G -nitro-L-arginine methyl ester
L-NMMA	N ^G monomethyl-L-arginine
LV	Left ventricle
LVEDV	Left ventricular end diastolic volume
LVESV	Left ventricular end systolic volume
LVH	Left ventricular hypertrophy
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MHC	Myosin heavy chain
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MT2	Metallothionein 2
mTor	Mammalian target of rapamycin
NADPH	Nicotinamide-adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NO	Nitric oxide
NOS	Nitric oxide synthase
ns	Not significant
ob	Obesity
Ob-R	Leptin receptor

OD	Optical density
OGTT	Oral glucose tolerance test
OLETF	Otsuka Long-Evans Tokushima Fatty Rats
P	Phosphorylated
PAI1	Plasminogen-activator inhibitor 1
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline/Tween20
PCR	Polymerase chain reaction
PDK-1	3-phosphoinositide-dependent kinase-1
Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4
PE	Phenylephrine
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol-(4,5)-bisphosphate
PIP3	Phosphatidylinositol-(3,4,5)-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PMSF	Phenylmethylsulfonyl fluoride
PPAR α	Peroxisome proliferator-activated receptor α
PY	Phosphotyrosine
QC	Quality control
qRT-PCR	Quantitative real-time RT PCR
RAAS	Renin-angiotensin-aldosterone system
RGS	Regulator of G protein signalling
RIN	RNA index number
RMA	Robust multichip average
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
RT	Reverse transcriptase
RV	Right ventricle

Scd4	Stearoyl-coenzyme A desaturase 4
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sec	Second
SEM	Standard error of the mean
SH2	Src homology 2
Shc	SH2-domain and collagen-homologous region
SHR	Spontaneously hypertensive rat
siRNA	Small interfering RNA
SNP	Sodium nitroprusside
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin
Sv	Sample volume
SV	Stroke volume
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline/Tween20
TdT	Terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNF α	Tumor necrosis facotr α
UCP3	Uncoupling protein 3
UDG	Uracil DNA glycosylase
VSMC	Vascular smooth muscle cells
ZDF	Zucker diabetic fatty

Chapter One: Introduction

1.1 Diabetes mellitus

Diabetes mellitus is a chronic condition comprising a group of metabolic diseases, of which raised blood glucose (hyperglycaemia) is the most evident sign. The most common types of diabetes are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). The prevalence of diabetes mellitus has increased worldwide over the last 20 years. The World Health Organisation estimates that around 366 million people worldwide will be suffering from diabetes mellitus by the year 2030 due to population growth, aging, obesity and a lack of physical activity [1].

T1DM, also known as insulin-dependent diabetes mellitus, generally develops in children and adolescents. It is a disease, in which the pancreas fails to produce insulin due to an autoimmune-mediated destruction of pancreatic β -cells [2]. Patients with T1DM rely on exogenous insulin for survival and to prevent the development of diabetic coma.

Of the diabetic patients 90-95% suffer from T2DM, also known as non-insulin dependent diabetes mellitus [3]. Although T2DM is most common in middle age and later life, an increase in cases in adolescents and young adults has been reported in recent years due to an increase in child obesity and physical inactivity [4]. T2DM is a complex, multifactorial disease caused by environmental and genetic factors [5] complicated by microvascular and macrovascular disease [6]. Macrovascular complications such as heart attack and stroke constitute the primary cause of mortality in T2DM [7]. Risk factors for T2DM include genetic predisposition, age, obesity, lack of physical activity and cardiovascular disease (CVD) [8]. Phenotypically, it is characterised by impaired pancreatic β -cell function, reduced β -cell mass, glucagon hypersecretion, hyperglycaemia, insulin resistance and is associated with obesity in more than 80% of cases [9].

1.2 Physiological actions of insulin

Glucose is an important source of energy in the body. In the fasting state, glucose is produced endogenously via gluconeogenesis (formation of glucose primarily from lactate and amino acids) and glycogenolysis (breakdown of glycogen) through the conversion of glucose-6-phosphate to glucose by the enzyme glucose-6-phosphatase and released from the liver into the blood stream [10, 11]. Glucoregulatory hormones such as glucagon, insulin, amylin, cortisol, epinephrine and glucagon-like peptide-1 maintain circulating glucose concentrations in a relatively narrow range and both gluconeogenesis and glycogenolysis are partly under the control of glucagon, a hormone produced in the α -cells of the pancreas [10]. After a meal, glucose enters the circulation via intestinal absorption. Under normal physiological conditions, circulating glucose levels peak after food intake (postprandial stage), slowly decrease over the next several hours and return to fasting levels.

Insulin plays a central role in regulating carbohydrate and fat metabolism. It is produced by the pancreatic β -cells in response to increases in blood glucose levels and amino acids following the ingestion of a meal [10]. In order to control glucose concentrations after a meal, insulin firstly stimulates glucose uptake in insulin-sensitive tissues like heart, skeletal muscle, liver and adipose tissue through binding to specific receptors present on insulin-sensitive cells. This binding initiates a signalling cascade leading to the translocation of insulin-responsive glucose transporters to the cell membrane and thereby facilitating glucose uptake [10, 12]. Skeletal muscle represents the main target tissue with about 70% of blood glucose being taken up, whereas the heart is only a minor site of whole body glucose disposal [13] (Figure 1.1). Secondly, insulin suppresses the endogenous production of glucose by stimulating the synthesis of glycogen from glucose and inhibiting glycogenolysis in skeletal muscle and liver [10] and also decreases hepatic gluconeogenesis, thus preventing the influx of more glucose into the bloodstream (Figure 1.1). The net effect of all of these changes is to increase glucose uptake, reduce circulating glucose levels

and increase the conversion of glucose into the storage molecules, glycogen and fat [14] (Figure 1.1). In the kidneys insulin also stimulates several important physiological processes including sodium reabsorption, plasma volume, and reabsorption of phosphate and glucose [12, 15].

In the cardiovascular system insulin has many important roles. Among the most important cardiovascular actions of insulin is the modulation of blood flow via opposing effects of vasodilation and vasoconstriction of the vasculature. The arterial wall is composed of three layers: the intima containing the endothelium, the media, and the adventitia [16]. Each of these layers has individual roles like the regulation of vascular tone, haemostasis, and/or vascular permeability by vascular endothelium or regulation of blood delivery and generation of continuous blood flow by the media [17]. Insulin stimulates the increased production of the potent vasodilator nitric oxide (NO) from vascular endothelium [18] which relaxes the underlying vascular smooth muscle cells (VSMC) [19]. On the other hand, insulin can activate the sympathetic nervous system and stimulate the secretion of the vasoconstrictor endothelin-1 (ET-1) from vascular endothelium [12], which opposes the vasodilator actions of NO [20]. For example, in the postprandial state insulin stimulates dilation of terminal arterioles which increases the number of perfused capillaries (capillary recruitment) and elevates total blood flow by relaxation of larger resistance vessels leading to an increase in glucose uptake into insulin-sensitive tissues [21] (Figure 1.1).

In the heart, insulin plays a role in cardiac metabolism by modulating glucose uptake, glycolysis, lipid metabolism, protein synthesis, growth and apoptosis [22]. Additionally, insulin not only modulates cardiac contractility which results in increased myocardial work and oxygen consumption [23] but also augments myocardial perfusion by its vasodilatory action [12] (Figure 1.1). The above described biological functions of insulin which are related to the regulation of cardiovascular homeostasis are regulated by two major branches of the insulin signalling transduction pathway. The metabolic actions of insulin are generally

mediated via the phosphatidylinositol-3 kinase (PI3K)-dependent pathway whereas the non-metabolic, mitogenic and growth promoting effects of insulin are mediated via the mitogen-activated protein kinase (MAPK)-dependent pathway [14] (Figure 1.2).

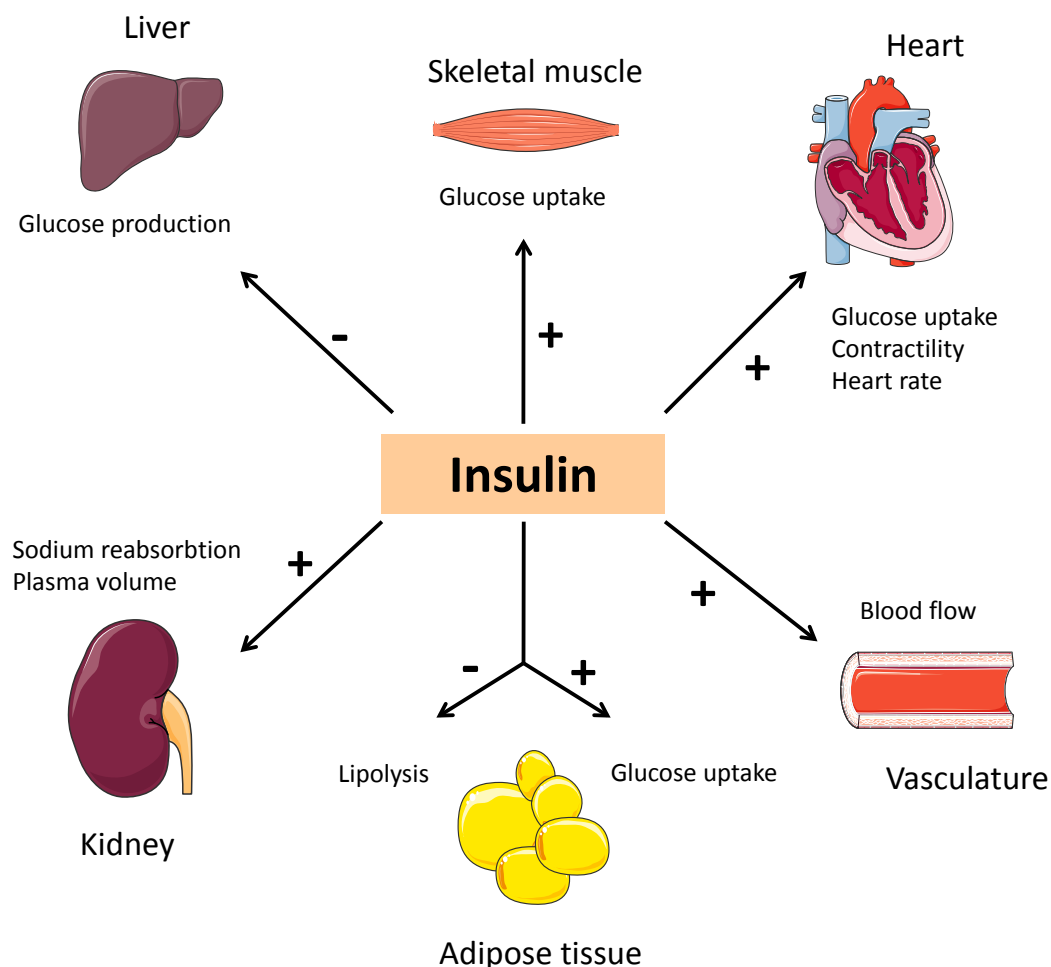


Figure 1.1: Physiological actions of insulin.

Insulin secretion promotes glucose uptake in insulin-sensitive tissues like skeletal muscle, heart and adipose tissue. Additionally, insulin regulates blood flow in the vasculature, heart rate and contractility of the heart but also sodium reabsorption and plasma volume in the kidney. At the same time insulin attenuates lipolysis in adipose tissue and glucose production in the liver. Adapted from [24]. Figure was produced using Servier Medical Art (www.servier.com).

1.3 Insulin signalling transduction pathway

1.3.1 General features of insulin signalling

Insulin elicits its biological actions by binding to the insulin receptor (IR) which is composed of an extracellular α -subunit and an intracellular β -subunit [25, 26]. As briefly mentioned before, this leads to the activation of two major branches of the insulin signalling transduction pathway, a PI3K-dependent pathway which is associated with the metabolic actions of insulin and a MAPK-dependent pathway which is associated with the regulation of mitogenesis, growth, and differentiation [12] (Figure 1.2).

Binding of insulin to IR results in tyrosine kinase activity of its β -subunit [27] leading to autophosphorylation as well as tyrosine phosphorylation of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) in the PI3K-dependent branch and the Src homology 2 (SH2)-domain and a collagen-homologous region containing (Shc) substrate in the MAPK-dependent branch [28-30].

Phosphorylated insulin receptor substrates interact with IRS-bound PI3K which is composed of a regulatory p85 subunit and a catalytic p110 subunit [31]. SH2-domains of the regulatory p85 subunit bind to tyrosine-phosphorylated motifs on IRS-1 which allosterically activates the p110 subunit to generate the lipid product phosphatidylinositol-(3,4,5)-triphosphate (PIP3) from the phosphatidylinositol-(4,5)-biphosphate (PIP2) substrate [32]. PIP3 binds to and phosphorylates 3-phosphoinositide-dependent kinase-1 (PDK-1) which in turn activates downstream protein serine-threonine kinases like protein kinase B (PKB, Akt) and atypical protein kinase C (aPKC) isoforms by phosphorylation [12] (Figure 1.2). This PI3K-dependent activation of the protein serine-threonine kinases leads to the phosphorylation of various downstream targets like endothelial NO synthase (eNOS), glycogen synthase kinase 1 (GSK1) or forkhead box O (FOXO) transcription factor that account for the metabolic effects of insulin such as glycogen synthesis, glucose uptake, or suppression of

gluconeogenesis in insulin-sensitive tissues like skeletal muscle, adipose tissue, liver or heart [12] (Figure 1.2).

In addition to the PI3K-dependent branch of insulin signalling pathways, tyrosine phosphorylation of IRS-1 or Shc also leads to the activation of the MAPK-dependent signalling branch leading to the binding of Shc to Grb-2 and thus activation of Sos, a guanosine triphosphate (GTP) exchange factor [33, 34] which in turn activates the small GTP binding protein Ras. This will initiate a kinase phosphorylation cascade which ultimately leads to phosphorylation of MAPK and thus to the activation of a variety of transcription factors that regulate gene transcription, to protein synthesis as well as cell growth and differentiation [12] (Figure 1.2 left).

1.3.2 Insulin signalling in the cardiovascular system

As described above, insulin plays an important physiological role in vascular endothelium by stimulating the production of NO [35]. NO is produced in vascular endothelium by activation of eNOS which catalyzes the conversion of the substrate L-arginine to NO and L-citrulline [36]. eNOS activity is regulated in a calcium-dependent and -independent manner as well as by other post-translational modifications like acylation and S-nitrosylation [37]. Calcium-dependent increases in enzymatic activity result through the activation of G protein-coupled receptors by vasodilators such as acetylcholine leading to a rise in intracellular calcium levels and thus interaction of calcium/calmodulin with the calmodulin binding site on eNOS [35]. The calcium-independent increase in eNOS activity occurs via the PI3K-dependent branch of the insulin signalling transduction pathway leading to the phosphorylation of eNOS by Akt and consequently to NO production in endothelial cells [37]. Subsequently, NO diffuses into VSMC leading to the activation of guanylate cyclase, an increase in cyclic guanosine monophosphate (cGMP) levels and thus to vasorelaxation [38]. Besides its role in vasorelaxation, NO also plays an important role in the inhibition of platelet adherence, leukocyte adhesion to endothelial cells and

smooth muscle cell proliferation and migration as well as the promotion of endothelial re-growth [39, 40].

In the heart, insulin is responsible for the regulation of cardiac metabolism by modulating glucose uptake, lipid metabolism, protein synthesis, growth, and contractility [41, 42]. Physiological growth of the heart is regulated through PI3K-dependent insulin signalling. Downstream targets of Akt are responsible for the promotion of cardiac growth (e.g. activation of mammalian target of rapamycin [mTor]) [43] or the regulation of cardiomyocyte size (e.g. suppression of GSK3 β or FOXO) [12]. Additionally, glucose uptake is modulated by insulin. Glucose is the major carbohydrate utilised by the heart and heart muscle glucose transport shares many similarities with that in skeletal muscle [44]. At the cellular level, insulin-mediated glucose uptake is dependent on the transmembrane glucose gradient as well as the recruitment of glucose transporters to the sarcolemma and transverse-tubule system via the PI3K-dependent insulin signalling branch [13, 45]. The glucose transporter GLUT1 is responsible for basal cardiac glucose uptake whereas GLUT4 is the dominant transporter in response to insulin stimulation in the adult heart, skeletal muscle and adipose tissue [46]. GLUT4 is stored in intracellular storage membrane vesicles from which it is recruited to the sarcolemma and t-tubules during insulin stimulation or exercise [47, 48] (Figure 1.2). In the fasting state, about 15% of total skeletal GLUT4 is located at the sarcolemma but within 30 min of insulin administration, about 80% will translocate to the sarcolemma [13].

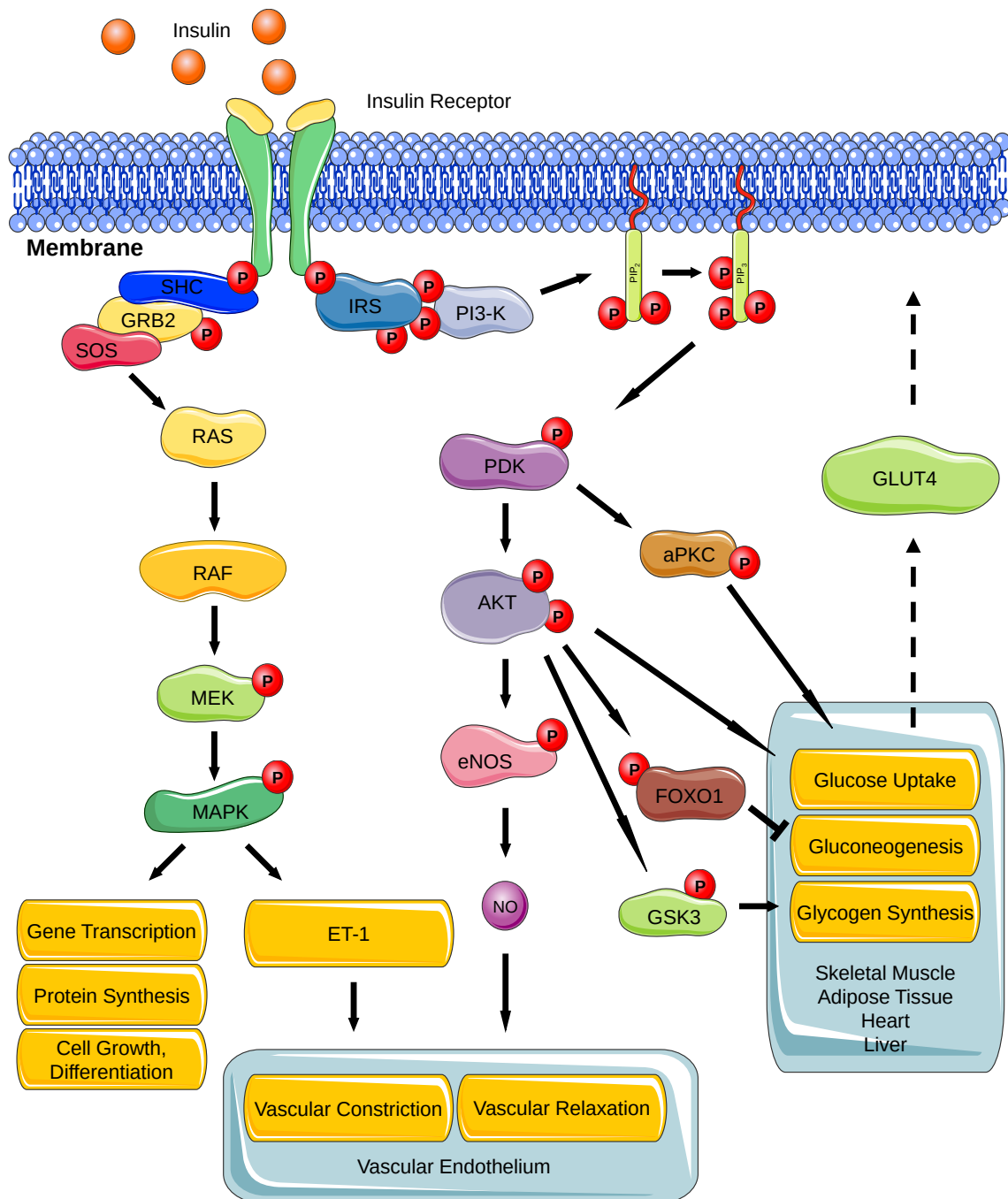


Figure 1.2: Insulin signalling transduction pathway.

Binding of insulin to the insulin receptors leads to the activation of two major branches of the insulin signalling transduction pathway, a PI3K-dependent pathway (right) which is associated with metabolic actions of insulin and a MAPK-dependent pathway (left) which is associated with the regulation of mitogenesis, growth, and differentiation. Adapted from [12]. Figure was produced using Servier Medical Art (www.servier.com).

1.4 Insulin resistance

Insulin resistance occurs when normal levels of insulin are less effective at lowering blood glucose concentrations as a maximal occupancy of the insulin receptor will not induce maximal stimulation of glucose uptake and utilisation in insulin-sensitive tissues [49]. Insulin resistant states are characterised by hyperinsulinaemia, hyperglycaemia and inappropriately high circulating levels of free fatty acids (FFA) and therefore constitute a major risk factor for the development of T2DM [50-52]. Studies have shown that insulin resistance is already present 10-20 years before the onset of T2DM [53, 54] and that insulin resistance is a consistent finding with T2DM [53, 55-57]. Thus, insulin resistance provides the necessary background predisposition to develop T2DM [58]. In people who develop T2DM, an increase in insulin secretion by the pancreas occurs to compensate for the insulin resistance and lower blood glucose levels [3]. A failure of this compensatory mechanism leads to attenuated glucose uptake and oxidation in skeletal muscle and adipose tissue and an increase in gluconeogenesis and glycogenolysis as well as a reduction in glycogen synthesis in the liver [59]. This will result in an increased release of glucose into the blood stream (hyperglycaemia) in the fasting state (impaired fasting glucose) [59] which is the basis of an impaired glucose tolerance in the postprandial stage. Although insulin resistance provides the necessary background predisposition, the progressive β -cell failure is responsible for the progression from insulin resistance to diabetes and its further worsening [58].

In adipose tissues, insulin resistance will lead to an increase in lipase activity resulting in increased breakdown of triglycerides and a reduction of FFA esterification and utilisation [59]. This will result in an increase in the FFA flux into the blood stream, an increase in blood triglyceride levels and thus reduce insulin secretion by the pancreatic β -cells and insulin sensitivity (Figure 1.3). Lipid deposition in liver or muscle will increase and result in ectopic fat accumulation which adds significantly to insulin resistance, atherogenic dyslipidaemia and hyperinsulinaemia [59] and thus promote the progression of

CVD and diseases like non-alcoholic fatty liver disease (NAFLD) [24, 59, 60]. Additionally, insulin resistance also promotes excessive adipogenesis leading to an increase in adipose tissue mass and obesity. Adipose tissue secretes a number of peptide hormones like leptin, adiponectin, tumor necrosis factor α (TNF α), interleukin-6 (IL-6) or plasminogen-activator inhibitor 1 (PAI1) that play an important role in metabolic and vascular homeostasis [12]. An increase in adipose tissue will stimulate the production of pro-inflammatory cytokines like TNF α or IL-6 and adipokines like leptin or PAI1 [61]. Also, low levels of the circulating anti-inflammatory adipokine adiponectin are associated with insulin resistance [62]. This will promote a pro-inflammatory and pro-thrombotic state which accelerates the development of endothelial dysfunction either indirectly through the metabolic risk factors or by direct action on the arterial wall leading to the development of atherosclerosis (Figure 1.3) [63].

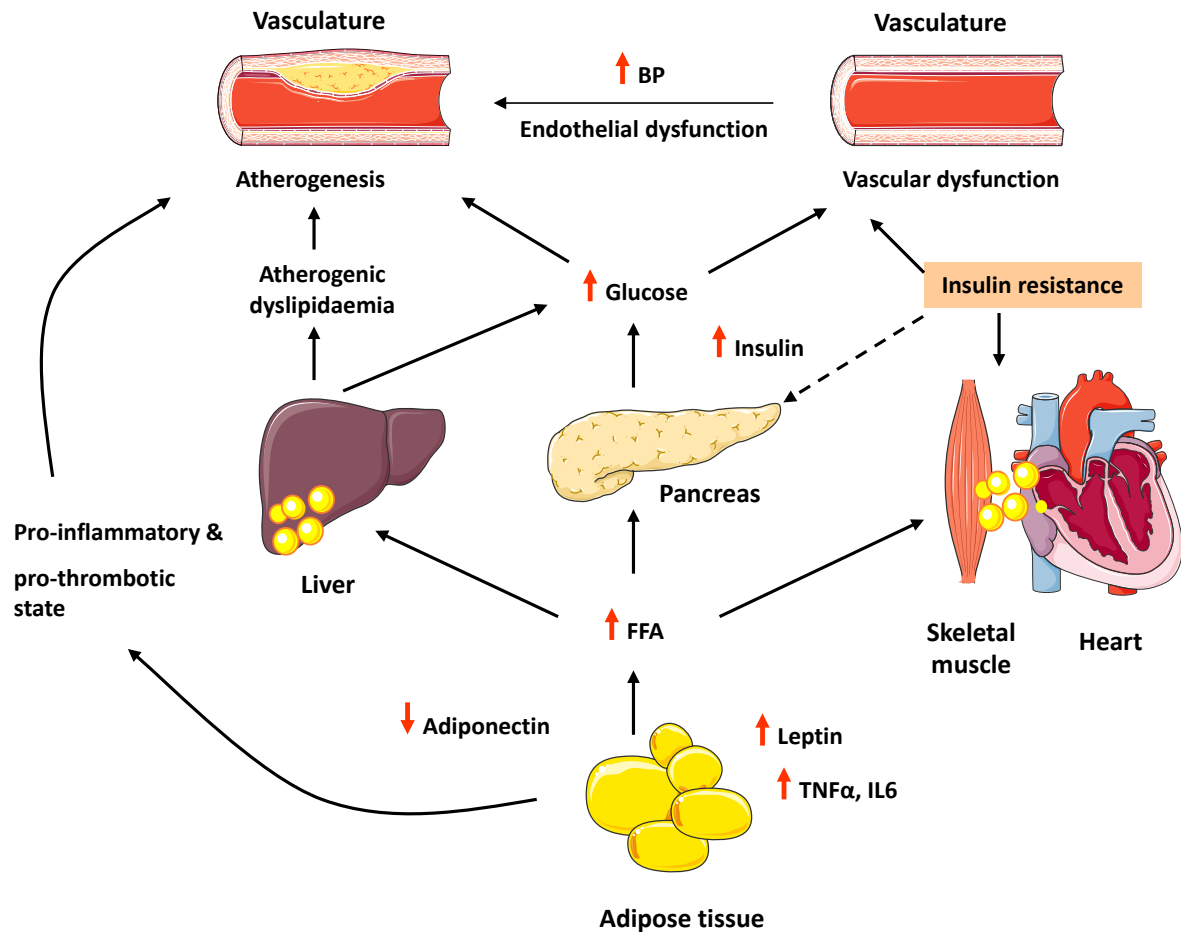


Figure 1.3: Role of insulin resistance in the development of metabolic abnormalities.

Insulin resistant states are characterised by hyperinsulinaemia and hyperglycaemia which affect cardiovascular function. Additionally, an increase in adipose tissue mass will lead to increased production of adipokines and excess release of free fatty acids (FFA). The excess release of FFA will lead to lipid accumulation in muscle, liver and the pancreas which adds to insulin resistance, atherogenic dyslipidaemia, and hyperinsulinaemia. The production of inflammatory cytokines like TNF α or IL-6 promotes a pro-thrombotic and pro-inflammatory state which is associated with vascular dysfunction and atherogenesis. BP, blood pressure; TNF α , tumor necrosis factor α ; IL-6, interleukin 6; $\uparrow$, increase. Adapted from [63]. Figure was produced using Servier Medical Art (www.servier.com).

The exposure of organs and the vasculature to high levels of FFA as well as to hyperglycaemia will lead to the formation of advanced glycation end products (AGEs), increased production of reactive oxygen species (ROS), diacylglycerol (DAG), and oxidative stress [64]. This will result in modulation of cellular processes in skeletal muscle, the myocardium, and vasculature and thus further promote an impairment of the insulin signalling transduction pathway (Figure 1.4), the development of endothelial dysfunction and atherosclerosis as well as structural and cellular damage in metabolic organs [13]. Insulin resistance has been proposed to be the result of disturbed insulin receptor binding, receptor defects or a disruption of insulin signalling transduction [24]. Impaired insulin signalling will lead to reduced GLUT4 translocation to the sarcolemma and thus to reduced glucose uptake and metabolism (Figure 1.4). Dysregulation of muscle-mediated glucose removal from the circulation is central to insulin resistance and T2DM [65]. In skeletal muscle, studies have shown that abnormalities already as early as at the level of IRS-1 tyrosine phosphorylation as well as IRS-1-associated PI3K activity and activation of PKC contribute to insulin resistance [13, 66]. The deficit of IRS-1 tyrosine phosphorylation in skeletal muscle might contribute significantly to the prognostically important, whole-body insulin resistance seen in diabetic patients [67, 68].

Although the myocardium is not a major site of glucose disposal, recent studies have revealed that reduced insulin sensitivity is a risk factor for mortality in individuals with ischaemic heart failure [68, 69]. Additionally, studies have shown that myocardial glucose utilisation during hyperinsulinaemia is reduced in diabetic patients [70, 71], which suggests that the diabetic heart is insulin resistant. At the molecular level, Cook *et al.* have demonstrated an increase in IRS1-PI3K activity and diminished levels of GLUT4 at the sarcolemma despite normal GLUT4 content in hearts of diabetic patients [72]. The reduced levels of GLUT4 at the sarcolemma due to decreased translocation is hypothesised to be a major mechanism for impaired glucose uptake in the diabetic heart [72]. Results from animal studies on the mechanisms of impaired myocardial GLUT4

translocation in insulin-resistant states vary depending on the model used. Mazumder *et al.* observed a complete failure of insulin to increase glucose uptake in isolated cardiomyocytes of *ob/ob* mice [73]. At the molecular level, an increase in basal tyrosine phosphorylation of the insulin receptor and a blunted response after insulin stimulation and lower levels of Akt phosphorylation were observed in these hearts. However, no changes in GLUT4 content were seen [73]. On the other hand, Cook *et al.* confirmed their findings of normal GLUT4 content but reduced GLUT4 at the sarcolemma in humans also using the *ob/ob* mouse [72]. Although both groups used the same model, Mazumder *et al.* used a working heart model whereas Cook *et al.* used non-stimulated baseline hearts [72, 73]. This might have contributed to the differences observed. Interestingly, another study has shown that a short duration of high-fat feeding as a model of T2DM causes an early reduction in glucose utilisation on the basis of reduced GLUT4 content and translocation [74] and a further study showed reduced total GLUT4 expression as well as Akt phosphorylation in the heart of C57BL/6 mice after 10 days of high-fat diet feeding [75].

In the vasculature, NOS is activated in a PI3K-dependent manner. Impaired insulin signalling transduction will lead to a reduction of eNOS activity and impairment of NO production (Figure 1.4). Additionally, enhanced ROS production in the vasculature via activation of nicotinamide-adenine dinucleotide phosphate (NADPH) will lead to an inactivation of NO and consequently to a reduction of NO bioavailability [35, 76]. All this will not only result in impaired insulin-mediated vasodilation but also to an enhanced production of pro-inflammatory cytokines, increased VSMC proliferation and increased platelet aggregation [77] and eventually contribute to endothelial dysfunction and the development of atherosclerosis.

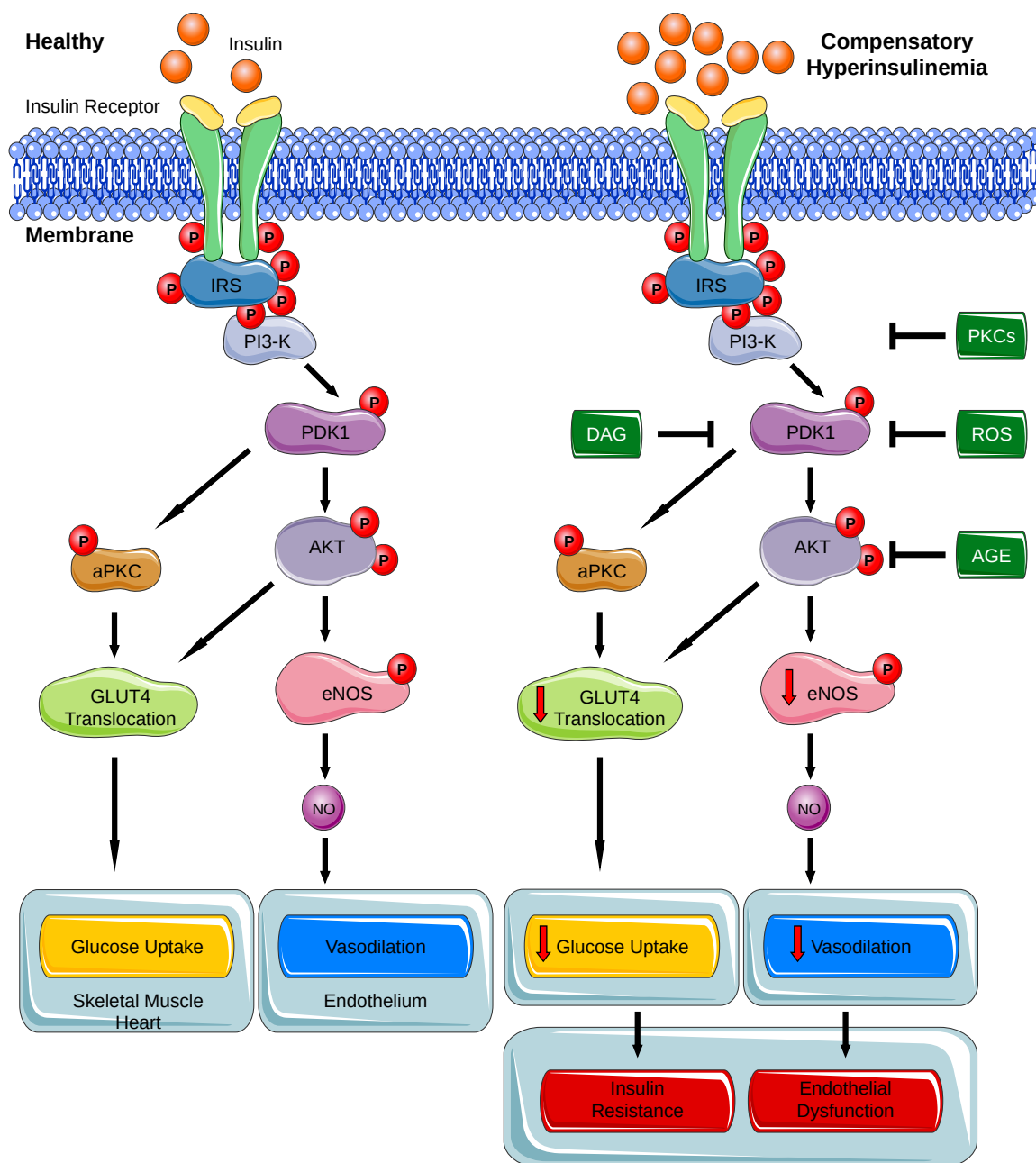


Figure 1.4: Impaired insulin signalling transduction pathway in compensatory hyperinsulinaemia.

Left: PI3K-dependent insulin signalling transduction pathway. Right: Impairment of PI3K-dependent insulin signalling pathway under pathological conditions contributes to insulin resistance and endothelial dysfunction. AGE, advanced glycated end products; DAG, diacylglycerol; ROS, reactive oxygen species; PKCs, protein kinase C isoforms; ↓, reduced. Adapted from [12]. Figure was produced using Servier Medical Art (www.servier.com).

1.5 The metabolic syndrome

Insulin resistance along with visceral adiposity, atherogenic dyslipidaemia, elevated blood pressure and a chronic subclinical inflammatory state were first clustered together into a group of metabolic abnormalities and disorders in 1988 and termed “Syndrome X” by Reaven *et al.* [50]. Later this cluster was termed “insulin-resistance syndrome” and now it is generally known as metabolic syndrome [78].

The metabolic syndrome is highly prevalent and is expected to rise due to the increase of obesity in the population. About 20-25% of healthy subjects and 80% of diabetic patients have the metabolic syndrome [79, 80] and patients with the metabolic syndrome have a 3-4 fold increased risk of developing T2DM [81, 82]. The metabolic syndrome allows identification of patients who are at high risk of developing T2DM, CVD and cardiovascular death [78] and helps to better understand the pathophysiological mechanisms that link the individual components with each other and with the increased risk of CVD [83].

Although there are variations in the definitions and criteria of the metabolic syndrome, the central features are insulin resistance, visceral obesity, atherogenic dyslipidaemia and endothelial dysfunction [83]. In an attempt to develop standardised criteria for the diagnosis, the current clinical approach focuses on waist circumference/obesity, dyslipidaemia and hypertension [83, 84]. A summary of the criteria of the metabolic syndrome defined by the International Diabetes Federation (IDF) can be found in Table 1.1 [84].

Table 1.1: Definition of the metabolic syndrome by the IDF

Obesity*	Waist circumference: ≥ 94 cm (male), ≥ 80 cm (female)
Hyperglycaemia	Fasting glucose ≥ 5.6 mmol/L and/or medication
Dyslipidaemia	Triglyceride levels ≥ 1.7 mmol/L or specific treatment
Dyslipidaemia (second, separate criteria)	HDL cholesterol: < 1.03 mmol/L (male), < 1.2 mmol/L female or specific treatment
Hypertension	$> 130/85$ mmHg and/or medication

* ≥ 3 of the above criteria necessary. Adapted from [84].

1.6 Diabetic complications

The sustained hyperglycaemia in T2DM causes damage to blood vessels and the organs they supply leading to the development of diabetes specific microvascular complications in the retina, renal glomerulus and peripheral nerve [85, 86]. Hyperglycaemia-induced pathophysiological changes include initial abnormalities in blood flow and increased vascular permeability but also microvascular cell loss, progressive capillary occlusion, or decreased production of trophic factors for endothelial and neuronal cells [86]. On the molecular level, increased AGE formation, the activation of PKC isoforms, as well as an increase in the polyol and hexosamine pathway flux have been implicated in these microvascular changes [86-89]. Eventually, these changes lead to oedema, ischaemia and hypoxia-induced neovascularisation in the retina, proteinuria, mesangial matrix expansion and glomerulosclerosis in the kidney, and multifocal axonal degeneration in peripheral nerves [86]. Thus, diabetes is a leading cause of blindness, end-stage renal disease and a variety of debilitating neuropathies, which all represent the main cause of morbidity in T2DM [86, 90].

Moreover, individuals with T2DM are also at a significantly higher risk of macrovascular diseases such as peripheral arterial disease, ischaemic stroke or coronary artery disease (CAD) [85] and have a greater likelihood of having hypertension, dyslipidaemia, and obesity [91]. The increased prevalence of CAD in diabetes may be accounted for by the presence of diabetes-associated risk factors like obesity, hypertension and dyslipidaemia although a substantial proportion remains unexplained [13, 92]. Diabetic patients suffer from a 2-4 fold increased risk of incident coronary heart disease or fatal CVD compared with patients without diabetes with heart attacks and stroke accounting for about 80% of all deaths [90].

1.6.1 Microvascular dysfunction

Dysregulation of vascular function and structure is common in patients with diabetes [93]. With complications such as stroke or acute coronary syndromes much attention has focused on diabetic macrovascular disease [94]. However, microvascular disease significantly contributes to morbidity associated with T2DM [90]. Microvascular dysfunction (in vessels smaller than 150 μm in diameter) may affect both peripheral vascular resistance and insulin-mediated glucose disposal, thereby contributing to hypertension and insulin resistance, respectively [95]. Additionally, disturbances in microvascular function may arise before overt hyperglycaemia and pathologic changes in large vessels are observed [96, 97]. It has been shown that the most consistent structural change in the microvasculature is thickening/remodelling of the vascular wall in arterioles in the glomeruli, retina, skin, muscle, and myocardium, resulting in the classic diabetic microangiopathy [94]. There are two types of vascular remodelling: eutrophic and hypertrophic remodelling. Eutrophic remodelling is the rearrangement of otherwise normal material around a narrow lumen and occurs in essential hypertension [98]. In contrast, hypertrophic remodelling is characterised by vascular smooth muscle hypertrophy or hyperplasia and occurs in renovascular hypertension [99]. Clinical and experimental studies of hypertension and diabetes have reported alterations in subcutaneous vessel morphology with an increase in the media-to-lumen diameter ratio in small arteries attributed to remodelling and hypertrophy of the vessel wall [93, 100]. Rizzoni *et al.* have shown that the effects of diabetes and hypertension on subcutaneous small artery morphology are quantitatively similar (despite the presence of different hemodynamic load), but qualitatively different, i.e. hypertrophic remodelling in diabetes vs. eutrophic remodelling in hypertension [100]. Moreover, they have shown that the addition of hypertension in diabetic patients has little additive effect on small artery remodelling [100]. Although the mechanisms of vascular remodelling are not fully understood, it has been suggested that neurohumoral factors, insulin and insulin-like growth factor 1 as

well as increased collagen deposition might play a role in vascular structural alterations, smooth muscle cell hypertrophy and cell growth [101].

In the heart, coronary microvascular dysfunction has been described in different diseases such as hypertrophic cardiomyopathy (HCM) [102] and arterial hypertension [103-105]. Similar to other vascular beds, coronary microvascular dysfunction is due to remodelling of intramural coronary arterioles (Figure 1.6). Pathogenic mechanisms of coronary microvascular dysfunction can be divided into structural, functional and extravascular [92]. Structural alterations are for example luminal obstruction, vascular remodelling or perivascular fibrosis whereas functional alterations include endothelial, smooth muscle, and autonomic dysfunction. A detailed description of the pathogenic mechanisms and their causes is summarised in Table 1.2.

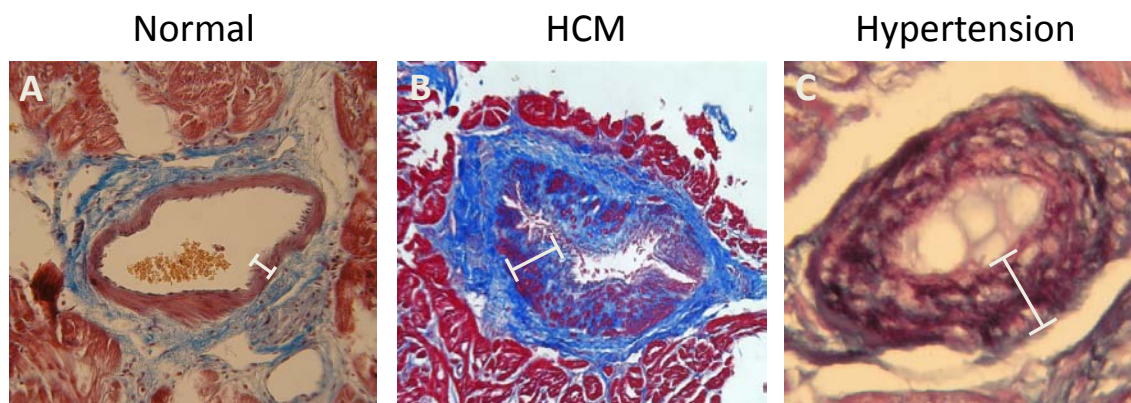


Figure 1.5: Coronary arteriolar remodelling in hypertrophic cardiomyopathy (HCM) and hypertension.

Histological sections of transmurals vessels of healthy subjects (A), and patients with HCM (B) or hypertension (C). Trichrome staining showing fibrosis in blue. Myocardium is shown in shades of red. Pathogenic alterations of coronary microvascular remodelling like thickening of the vessel wall (indicated by white bars), perivascular fibrosis (blue), or decreased luminal size are observed in HCM (B) and hypertension (C). Images provided by Prof P.G. Camici.

Table 1.2: Pathogenic mechanisms of coronary microvascular dysfunction

Alterations	Causes
<i>Structural</i> Luminal obstruction Vascular-wall infiltration Vascular remodelling Vascular rarefaction Perivascular fibrosis	Microembolisation in acute coronary syndromes or after recanalisation Infiltrative heart disease (e.g. Anderson-Fabry cardiomyopathy) HCM, arterial hypertension Aortic stenosis, arterial hypertension Aortic stenosis, arterial hypertension
<i>Functional</i> Endothelial dysfunction Dysfunction of smooth muscle cells Autonomic dysfunction	Smoking, hyperlipidaemia, diabetes HCM, arterial hypertension Coronary recanalisation
<i>Extravascular</i> Extramural compression Reduction in diastolic perfusion time	Aortic stenosis, HCM, arterial hypertension Aortic stenosis

HCM: hypertrophic cardiomyopathy. Adapted from [92].

Coronary microvascular dysfunction may also cause an impairment of coronary flow and coronary flow reserve thus contributing to myocardial ischaemia and fibrosis. Coronary flow reserve is a measurement of the ability of the coronary microvasculature to respond to a stimulus and is expressed as a ratio of peak (i.e. maximal hyperaemia) to baseline coronary flow [92]. Impaired coronary flow has not only been shown in patients with hypertension or HCM but also in diabetic patients [106]. Studies have shown that hyperaemic myocardial flow in patients with T2DM and macrovascular disease was decreased by 28% and even further reduced in diabetic patients with evidence of coronary microangiopathy [107, 108].

In the absence of CAD or myocardial disease, coronary microvascular dysfunction can be the functional counterpart of traditional coronary risk factors like smoking, hypertension, hyperlipidaemia, diabetes, or insulin-resistant states [109-111]. At this stage structural and functional alterations are, at least in part, reversible by lifestyle intervention or medical treatment [92]. In the presence of myocardial disease coronary microvascular dysfunction is sustained in most instances by adverse remodelling of intramural coronary arterioles [92]. This type of coronary microvascular dysfunction is found in primary (genetic) cardiomyopathies like HCM [102, 112, 113] or dilated cardiomyopathy [114-116] or secondary cardiomyopathies such as those due to arterial hypertension [117] or aortic stenosis [118, 119]. It may be severe enough to cause myocardial ischaemia and it is unclear whether medical treatment may reverse some cases [92].

1.6.2 Diabetic cardiomyopathy

Under normal physiological conditions, the heart utilises fatty acids (FA), carbohydrates, amino acids, ketones, and lactate. FFA and carbohydrates (especially glucose) are the major source from which the heart derives most of its energy [46]. In normal hearts, 60% to 90% of the energy is derived from FFA oxidation [120]. Depending on the physiological and pathophysiological conditions, the heart can rapidly switch substrate selection. The increase in circulating levels of insulin, glucose, and FFA in T2DM have been shown to affect myocardial energy metabolism leading to functional, biochemical and morphological abnormalities and increased vulnerability in the presence of stressors such as ischaemia and cardiac hypertrophy [121, 122]. Using euglycaemic clamps and positron emission tomography Izzo *et al.* have shown that the hearts of diabetic patients demonstrate reduced insulin-stimulated glucose uptake indicating that the diabetic heart is insulin resistant [70]. Myocardial insulin resistance leads to an increase in FFA uptake and the heart consequently adapts to using FA exclusively for adenosine triphosphate (ATP) generation [13]. High FFA uptake and metabolism augment the accumulation of FA intermediates and triglycerides [123] and increase oxygen demand and generation of ROS leading to cardiac damage [46]. Patients with diabetes also exhibit changes in cardiac structure and cardiomyocyte ultrastructure. This can be attributed to the diabetic milieu of hyperglycaemia, hyperinsulinaemia, increased cellular FFA uptake or hyperglycaemia-induced oxidative stress [13, 124]. At the macroscopic level left ventricular hypertrophy and increased heart mass with mildly reduced left ventricular systolic performance has been observed [125]. At the microscopic level morphological abnormalities like myocyte hypertrophy, perivascular fibrosis, increased quantities of matrix collagen, cellular triglyceride and cell membrane lipid have been demonstrated [126, 127]. Figure 1.5 summarises mechanisms that may lead to the development of diabetic cardiomyopathy.

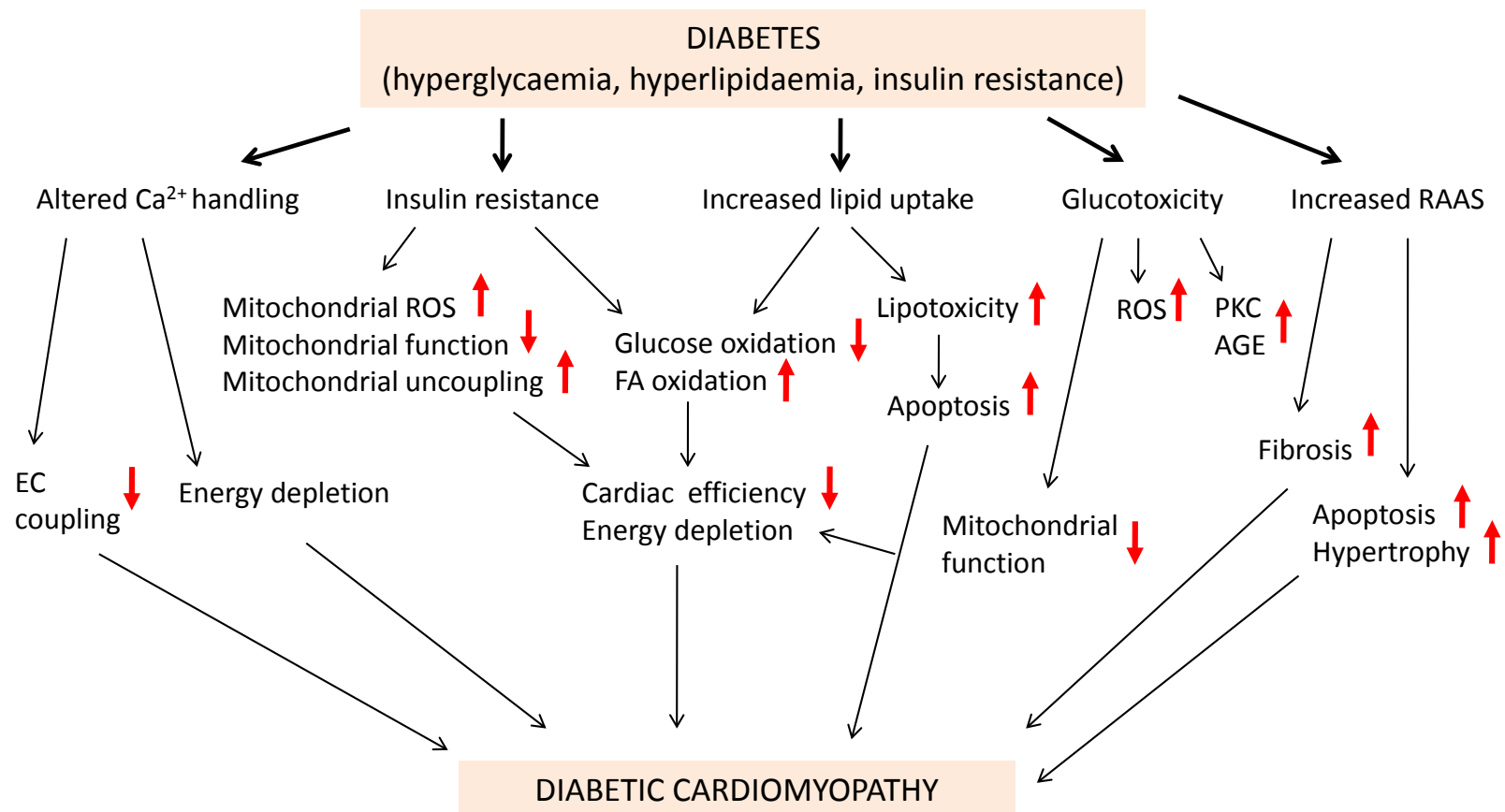


Figure 1.6: Summary of mechanisms that may lead to the development of diabetic cardiomyopathy.

EC: excitation-contraction; ROS: reactive oxygen species; FA: fatty acid; PKC: protein kinase C; AGE: advanced glycated end products; RAAS: renin-angiotensin-aldosterone system; Adapted from [128].

1.7 Animal models of insulin resistance and diabetic cardiomyopathy

It is important to understand the pathogenesis and underlying molecular mechanisms of insulin resistance and diabetic cardiomyopathy. However, research in humans is limited due to ethical considerations [8] and therefore appropriate models with a phenotype and pathogenesis resembling the human disease are crucial. Animal models are useful and advantageous in biomedical studies because they offer the promise of new insight into human disease. Both rodent and non-rodent (e.g. pig, dog, non-human primates) models have been developed, but most models are still based on rodents due to the expensiveness, practical difficulties, extreme care and ethical considerations of non-rodent models [8]. Mouse models have been a preferred model due to their easy maintenance, short generation time, and the ease of generating transgenic and knock-out models [128], but recently advances in generating rat knock-out models offer the possibility to complement existing transgenic mouse models [129]. Generally, rodent models have many traits in common with human diabetic cardiomyopathy and thus, several models of diabetes and insulin resistance have been extensively studied [130]. A selection of rodent models of diabetes is shown in Table 1.3.

A good model of diabetic cardiomyopathy should firstly show evidence of left ventricular (LV) dysfunction such as decreased ejection fraction and/or evidence of diastolic dysfunction [128]. Secondly, it should show evidence of myocardial injury such as interstitial or replacement fibrosis, and thirdly, LV hypertrophy. Several studies on rodent models of obesity, insulin resistance and T2DM have demonstrated LV hypertrophy, diastolic dysfunction, increased cardiac FFA uptake and utilisation, decreased cardiac efficiency, impaired mitochondrial energetics, increased myocardial lipid storage, and impaired Ca^{2+} handling [46, 128] which mirror similar observations that have been made in humans.

There are three main ways of creating animal models of insulin resistance, diabetic cardiomyopathy and either type 1- or type 2-like diabetes: (1) spontaneously diabetic, (2) genetically engineered and (3) artificially induced (Table 1.3). Models for both T1DM and T2DM have been used interchangeably due to a significant overlap in their cardiac phenotype characterised by increased FFA utilisation, decreased glucose utilisation and impaired calcium handling [128]. But despite this overlap, the overall physiology as well as the pathophysiological mechanisms of T1DM and T2DM are different and therefore the relative models should be considered separately.

Table 1.3: Selected Rodent Models of Diabetes

	Phenotypic alterations
Spontaneously Diabetic Rodents Otsuka Long-Evans Tokushima Fatty Rats (OLETF) Goto-Kakizaki Rats (GK) Zucker Diabetic Fatty Rats (ZDF) <i>ob/ob</i> mice <i>db/db</i> mice	Late onset hyperglycaemia (18 weeks of age), mild obesity, clinical onset of diabetes (mostly in males), hypertriglyceridaemia Mild type 2 diabetes, hyperglycaemia, hyperinsulinaemia, insulin resistance Obesity, hyperinsulinaemia, hyperglycaemia, hyperleptinaemia, increased serum fatty acids and triglyceride levels Transient hyperglycaemia, glucose intolerance, hyperinsulinaemia Hyperglycaemia, impaired insulin response to glucose
Genetically Engineered Diabetic Mice GLUT2-/- GLUT4-/- IR-/- IRS-/-	Moderate insulin resistance, glucose intolerance, decreased adipose mass Insulin resistance, diabetic hypertension, diabetic ketoacidosis, early postnatal death Growth retardation, mild insulin resistance, beta cell hyperplasia, hyperinsulinaemia Severe insulin resistance, beta cell hyperplasia, diabetes in 50% of adult mice
Artificially Induced Diabetic Rodents Neonatal STZ-induced Diabetes STZ-Spontaneously Hypertensive Rat Fat-fed/STZ-induced Diabetic Rodents High fat diet-fed Diabetic Rodents	Hyperglycaemia, impairment of glucose-stimulated insulin release Insulin resistance, hypertension Hyperglycaemia, hyperinsulinaemia, increased free fatty acid and triglyceride levels Hyperinsulinaemia, insulin resistance, glucose intolerance

GLUT: glucose transporter, IR: insulin receptor; IRS: insulin receptor substrate; STZ: Streptozotocin.
Adapted from [130].

1.7.1 Spontaneously diabetic models

The advantage of using spontaneously diabetic mice is that the development of T2DM is natural involving genetic factors and that animals develop characteristic features resembling human T2DM. However, although their homogeneous background keeps variability to a minimum and reduces sample size it also implies that the development of T2DM is highly genetically determined unlike the heterogeneity seen in humans. Furthermore, inbred animals are generally more expensive and their availability is limited.

1.7.1.1 Leptin and the leptin receptor

Examples of spontaneously diabetic mouse models are the obese, leptin-deficient *ob/ob* mouse and the diabetic, leptin receptor-deficient *db/db* mouse. Leptin is a key regulator of appetite, body weight, and energy balance in the central nervous system [12]. It is encoded by the obesity (*ob*) gene and expressed predominantly in adipose tissue and, at lower levels, in gastric epithelium and placenta [131]. Leptin interacts with the leptin receptor (Ob-R), which has six isoforms (Ob-Ra – Ob-Rf) [132] (Figure 1.7). Ob-Rb is the only isoform with a long cytoplasmic region containing all motifs required for activation of the major signalling pathway used by leptin, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathway [131, 132]. Besides the JAK/STAT signalling pathway, it has been shown that other signalling pathways like MAPK and 5'-AMP-activated protein kinase (AMPK) are also involved in leptin signalling [132] and that leptin interacts with insulin signalling [133]. Similarly to insulin, leptin acts on the vasculature by inducing endothelium-dependent vasodilation [134, 135] via a PI3K-Akt-eNOS pathway [136].

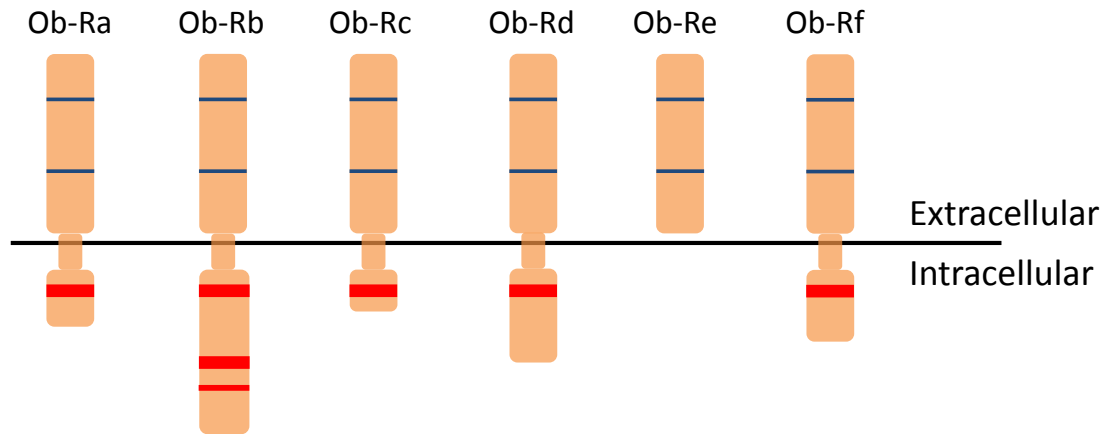


Figure 1.7: Leptin receptor (Ob-R) isoforms.

Ob-R isoforms share identical extracellular, ligand-binding domains but differ at the C terminus. Five isoforms have transmembrane domains but Ob-Rb is the only isoform that encodes all protein motifs for capable for activating the JAK/STAT signal transduction pathway. Ob-Re is truncated and secreted. The blue lines show the WSXWS motif, which indicates members of the cytokine receptor family and the red lines indicate JAK/STAT box motifs. Adapted from [131].

1.7.1.2 The leptin-deficient *ob/ob* mouse

Of the various mouse models available the leptin-deficient *ob/ob* mouse is one of the best characterised. Obese *ob/ob* mice are homozygous for the obese spontaneous mutation *Lep^{ob}* which originally occurred in an outbred multiple recessive stock prior to 1950 [137]. Animals with this mutation exhibit a significantly altered feeding behaviour, metabolism, and endocrine function due to the leptin-deficiency leading to reduced energy expenditure and increased food intake (hyperphagia) with body weights reaching up to three times the weight of normal animals. Additionally, *ob/ob* mice show a diabetes-like syndrome of hyperglycaemia, mild impaired glucose tolerance, and severe hyperinsulinaemia [138]. It is important to note that depending on the strain, *ob/ob* mice show phenotypic differences like transient hyperglycaemia (subsiding at around 14 to 16 weeks of age) on the C57BL/6 background and severe diabetes with regression of pancreatic islets and early death on the

C57BL/KS background [8]. At the myocardial level, dysfunction is associated with myocardial lipid accumulation, altered substrate utilisation (increased FFA oxidation, reduced glucose oxidation and increased myocardial oxygen consumption) and impaired myocardial insulin signalling [73]. Although the *ob/ob* mouse has many features of human T2DM, it has to be noted that severe obesity due to leptin-deficiency is extremely rare in humans. Congenital leptin deficiency with loss-of-function mutations in the leptin gene has been identified in only twelve subjects worldwide [139-141]. Furthermore, it has been shown that upon injection of leptin the phenotype of increased body weight, hyperphagia, reduced energy expenditure and insulin resistance is reversible [142, 143].

1.7.1.3 The leptin receptor-deficient *db/db* mouse

Mice homozygous for the spontaneous mutation in the leptin receptor (*Lepr^{db}*) were first identified in 1966 at the Jackson Laboratory in the C57BL/KsJ strain. The diabetic (*db*) gene in these animals encodes for a point mutation of the leptin receptor, leading to abnormal splicing and defective signalling of leptin [144]. The lack in leptin signalling results in hyperphagia, excessive thirst and excessive urine production as well as obesity which is identifiable after three to four weeks of age [145]. Depending on the background strain (C57BL/KsJ or C57BL/6) mice have differing levels of hyperglycaemia (less in C57BL/6) despite similar degrees of hyperphagia and weight gain which might be explained by the pancreatic islet cell hypertrophy in the C57BL/6 background [146]. With increasing age, progressive impairment of insulin response to glucose is observable in *db/db* mice [130]. Mice also exhibit ketosis and progressive body weight loss and do not survive longer than 8 to 10 months. In addition to the metabolic phenotype, *db/db* mice show abnormal cardiac muscle morphology, decreased cardiac muscle contractility and increased left ventricular diastolic pressure [124]. Unlike in the *ob/ob* mice, exogenous leptin administration in *db/db* mice fails to reverse the metabolic alterations due to the defect in the leptin receptor [8]. Similarly to leptin-deficient subjects with mutations in the

leptin gene, only one mutation in the leptin-receptor gene has been reported in three severely obese adults [147].

1.7.2 Genetically engineered diabetic mice

Genetic manipulations targeting members of the insulin signalling pathway (IR, IRS-1, PI3K, and GLUT4) have been developed to study the effect of single gene mutations on the development of insulin resistance and T2DM *in vivo*. These models are valuable in dissecting this complex disease on the genetic level [8] as they exhibit various phenotypic characteristics of T2DM. It is important to mention however, that the production of genetically modified animals requires specific knowledge for the generation and maintenance and thus make these models not suitable for high-throughput studies like drug testing.

1.7.2.1 Cardiac-specific insulin receptor knockout (CIRKO)

One way of ablating insulin signalling in the heart is the generation of mice with a cardiomyocyte-specific knockout of the insulin receptor via a cardiac-specific alpha-myosin heavy chain promoter. Compared to controls, hearts of these animals are up to 30% smaller in size due to reduced cardiomyocyte size [148]. Like the *ob/ob* and *db/db* mice, cardiac dysfunction in CIRKO mice is associated with impaired insulin signalling, altered substrate utilisation (increased FA oxidation, reduced glucose oxidation and increased myocardial oxygen consumption) and mitochondrial dysfunction [149]. Additionally, CIRKO hearts are more susceptible to ischaemic injury and increased premature mortality following coronary artery ligation was observed [148]. This is in line with human findings which have shown that adaptations of the diabetic heart to ischaemia are impaired and that sudden death after myocardial infarction is more likely in diabetic subjects [150, 151].

1.7.2.2 IRS-1 knockout

The IRS family is composed of four members (IRS-1 to 4) with distinctive patterns of tissue expression [152]. To clarify the physiological role of IRS-1 *in vivo* and its effects on peripheral insulin action, an IRS-1 knockout mouse model was constructed in 1994 [153]. Animals homozygous for this targeted disruption of the IRS-1 gene have lower birth weights than wild-type litter mates and maintain lower body weight proportional to their smaller size [154]. Additionally, these animals are insulin resistant and show abnormal glucose tolerance whereas no histological abnormalities in the heart, liver, spleen and kidney were found [153]. Together with knockout studies of other members of the IRS family, it appears that IRS-1 is the main mediator of insulin signalling in skeletal muscle, adipose tissue, and β -cell secretory function [152].

1.7.2.3 Cardiomyocyte-specific GLUT4 knockout

Mice with a cardiomyocyte-specific knockout of the glucose transporter GLUT4 via a cre-loxP system are normotensive, non-obese, and don't show any differences in their metabolic profile compared to wild-type mice [155]. GLUT4 deficiency in the heart leads to mild cardiac hypertrophy and under basal conditions GLUT4 deficient hearts are capable of maintaining normal function [155]. However, cardiac dysfunction occurs when the hearts are stressed with decreased contractile recovery following ischaemia/reperfusion [155, 156] and alterations in excitation-contraction coupling in isolated cardiomyocytes [157].

A detailed phenotypic description of mouse models of cardiomyopathy is shown in Table 1.4.

Table 1.4: Mouse models of cardiomyopathy

Cardiomyopathy Mouse Model	Type of Diabetes	Relevant Phenotype
<i>ob/ob</i>	Type 2	LVH, cardiac dysfunction, lipotoxicity, abnormal substrate metabolism, decreased cardiac efficiency, mitochondrial dysfunction, impaired insulin signalling, altered gene expression, oxidative stress
<i>db/db</i>	Type 2	Cardiac dysfunction, lipotoxicity, abnormal substrate metabolism, decreased cardiac efficiency, mitochondrial dysfunction, impaired insulin signalling, altered gene expression, oxidative stress
High-fat feeding	Type 2	Cardiac dysfunction, abnormal metabolism, impaired insulin signalling, decreased cardiac efficiency
CIRKO mouse (cardiomyocyte insulin receptor KO)	Type 2	Cardiac dysfunction, altered substrate metabolism, increased injury and dysfunction following LVH
IRS-1 KO mouse	Type 2	LVH, cardiac dysfunction and increased fibrosis following pressure overload hypertrophy
Cardiomyocyte GLUT4 KO	not defined	Cardiac hypertrophy, decreased cardiac glucose utilisation, decreased recovery from ischaemia

IRS-1: Insulin receptor substrate-1; GLUT4: Glucose transporter 4; KO: knockout; LVH: left ventricular hypertrophy. Adapted from [124].

1.7.3 Artificially induced diabetic rodents

1.7.3.1 Chemical manipulation

Chemical manipulation with streptozotocin for example will result in pancreatic β -cells toxicity causing DNA damage and ultimately leads to insulin deficiency [158]. Animals will develop hyperglycaemia within 7 to 14 days after the first injection [128] and also show increased serum FFA, cholesterol and triglyceride levels. However, animals treated with streptozotocin present progressively decreasing insulin levels with the duration of diabetes [159] and are generally used as models for T1DM.

1.7.3.2 Surgical manipulation

Surgical manipulation involves either a complete or partial pancreatectomy and is used for the induction of T1DM and T2DM. It has been reported mostly in dogs, pigs, rabbits but also been performed on rats and mice. A pancreatectomy of 70-90% does not cause a severe form of diabetes and is characterised by moderate hyperglycaemia [8]. Due to the reduced islet β -cell mass it resembles human T2DM; however removal of the pancreas also leads to dissection of glucagon-secreting α -cells which may lead to problems in counter regulatory response to hypoglycaemia [8].

1.7.3.3 Dietary manipulation

Many animal models of insulin resistance and diabetic cardiomyopathy have little clinical relevance for human diabetes as they are based on monogenic disorders or chemical destruction of pancreatic β -cells [160]. With changes in dietary habits in Western countries, diets rich in carbohydrates such as sucrose and fructose, as well as saturated fats [161-163] have been associated with many diet-induced complications including metabolic syndrome, CVD, and NAFLD [163, 164]. Mouse Western-style diets that mimic these risk factors in human Western populations have been used for many decades [163]. In 1988 Surwit *et al.* introduced the high-fat diet-fed C57BL/6 mouse model as a more

relevant model of early human diabetes [165]. High fat diets typically have 40-60% of their energy derived from fat with 60% diets often used for obesity studies, whereas diets with 45% of their calories derived from fat are considered to represent more the Western-style diet [161]. In general, animals fed a high-fat diet *ad libitum* gradually increase body weight and are characterised by hyperinsulinaemia, insulin resistance and glucose intolerance [165]. It is important to note that a number of different fat sources are available. Depending on the fat source (lard, soybean oil, milk fat, cocoa butter) and therefore also the fatty acid profile (saturated and unsaturated fatty acids) the metabolic outcome will be different. Animals fed a lard-derived high fat diet develop hyperglycaemia, hyperinsulinaemia and over time hypertension whereas a cocoa-derived high fat diet does not affect plasma glucose and insulin levels [166].

1.8 Aim

Insulin resistance plays an important role in the development of micro- and macrovascular complications in diabetic patients. Many rodent models have been extensively used to study the molecular mechanisms involved in the development and the effects of insulin resistance but drug-induced or genetically modified models do not fully mimic the pathophysiological changes seen in humans. Previous work in our group has shown decreased levels of GLUT4 at the sarcolemma in the heart of diabetic patients but not in patients with left ventricular dysfunction [72]. The results seen in T2DM were also confirmed in hearts of the leptin-deficient *ob/ob* mouse, one of the best studied mouse models of insulin resistance and diabetic cardiomyopathy [72]. Although these results mimic our findings in humans, the *ob/ob* mouse model is an extreme model with only a few cases occurring in the human population. Due to its extreme phenotype with leptin deficiency and overt obesity, results should be interpreted with care and questions whether this model is the most appropriate and representative for human diabetes should be asked. Therefore, the aim of this thesis was to characterise a diet-induced mouse model of insulin resistance which avoids the extreme features of *ob/ob* mice and mimics more closely human disease.

Chapter 3 describes the generation of a diet-induced obesity (DIO) mouse model of insulin resistance and T2DM which circumvents the problem of leptin deficiency and thus represents an alternative model to the *ob/ob* mouse. The characterisation of this model will be described by looking at whole body composition, metabolic parameters and histological analysis of target organs of insulin resistance, the liver and kidney. In addition, potential structural changes of the heart associated with insulin resistance will be investigated by histological analysis. Finally, we determine whether this model fulfils the general criteria necessary for an animal model of insulin resistance, and which length of feeding is most suitable to further investigate cardiovascular and molecular changes in the heart.

In **Chapter 4** the cardiovascular and metabolic changes seen in the heart will be further investigated. In particular we look at coronary microvascular dysfunction, mechanical properties of DIO hearts as well as potential effects on insulin signalling and cardiac gene expression in DIO hearts and compare the model to the leptin-deficient *ob/ob* mouse.

Finally, in **Chapter 5** the role of a potential novel candidate gene involved in coronary microvascular remodelling will be investigated by looking at its expression in vascular smooth muscle cells of control and DIO mice. Additionally, we describe the effects of a shift in diet from high fat diet to normal rodent chow on metabolic markers, coronary microvascular remodelling and the expression of this potential candidate.

Chapter Two: Materials & Methods

2.1 Experimental animals

Male C57BL/6 and *ob/ob* (B6.V-*Lep^{ob}*/OlaHsd) mice were obtained at 4-5 weeks of age from Harlan Laboratories, Inc (UK). Mice had access to a standard rodent chow and water *ad libitum* and were housed in temperature-controlled facilities with a 12 h light and 12 h dark cycle (5 animals per cage). C57BL/6 mice were randomised and put either on standard rodent chow (control group) or on a 45% high-fat, Western-style diet (SDS Diets, UK) for 8, 11 and 14 weeks (Table 2.1).

Body weight was measured weekly. After 6 weeks, animals were divided into two groups. Animals with the highest body weight gain were defined as “diet-induced obese” (DIO) mice. Animals with body weights ranging between the average $\pm$ 3 standard deviations of the control group were defined as “diet-induced obese resistant” (DIO-R) as described by Enriori *et al.* [167]. Only DIO mice were used in further experiments. Animal care and all experimental procedures were performed in accordance with the regulations of the UK Home Office.

Table 2.1: Composition of diets

	Normal rodent chow	High fat diet
Protein	27.3%	20%
Carbohydrate	61.2%	35%
Fat	11.5%	45%

% of calories consumed

2.2 Stock solutions and buffers

All chemicals were purchased from Sigma Aldrich, UK or VWR International Ltd, UK unless otherwise stated. Solutions were made from laboratory grade reagents and sterile water (dH₂O) and stored at room temperature unless otherwise stated. TAE buffer was purchased from the MRC CSC Media Kitchen. A detailed description of buffers and stock solutions used are listed in the Appendix (Appendix A – Buffers and stock solutions).

2.3 Tissue harvesting

2.3.1 Blood sampling

After terminal anaesthesia with isoflurane, blood was collected from the left carotid artery for serum and plasma preparation. Blood samples for serum preparation were incubated for 30 min at room temperature to allow clotting and centrifuged at 5,000 rpm for 10 min in a microcentrifuge. For plasma preparation, blood was collected in microcentrifuge tubes containing heparin (1000 IU/mL) and centrifuged immediately after collection. Serum and plasma samples were stored at -20 °C until further use.

2.3.2 Tissue harvesting for molecular studies

After sacrificing the animals by cervical dislocation, organs were quickly removed, rinsed in ice-cold sterile PBS and snap frozen in liquid nitrogen. For insulin stimulation intra-peritoneal injection of 0.5 U/kg human insulin or an equal volume of saline for controls was given and animals were sacrificed 30 min after injection. Organ weights and visceral fat pad weights as well as tibia length were recorded for each animal. For the preparation of RNA or protein, snap frozen tissue was placed in a custom made metal mortar which was cooled in liquid nitrogen. The pre-cooled custom made pestle was inserted and pushed with force to crush the tissue. The resulting powder was quickly transferred to a cryotube and stored at -80 °C until further use.

2.3.3 Tissue harvesting for histological studies

For histological studies, mice were sacrificed by cervical dislocation. Organs were excised, rinsed in ice-cold sterile PBS and fixed in 10% neutral buffered formalin solution (Sigma Aldrich, UK) for 24-48 h and embedded in paraffin.

2.4 Blood biochemistry

2.4.1 Oral glucose tolerance test

For the glucose tolerance test, mice were fasted over night for 12 h and a glucose dose of 4 g D-glucose/kg body weight, in a 50% aqueous solution, was administered by oral gavage. Glucose levels were measured in blood drops from the tail taken before and at 15, 30, 60 and 120 min after the administration using a commercially available blood glucose monitor (Boots, UK) with blood glucose test strips from Abbot Diabetes Care Ltd.

2.4.2 Determination of mouse serum insulin concentrations

Serum insulin concentrations in C57BL/6 and *ob/ob* mice were measured by enzyme-linked immunosorbent assay (ELISA) using the Rat/mouse Insulin ELISA kit (Linco Research, UK) according to manufacturer's instructions. Briefly, serum samples were applied to microtiter plate wells pre-coated with pre-titered amounts of monoclonal mouse anti-rat insulin antibodies to allow capture of insulin molecules present in the samples. After binding of biotinylated polyclonal antibodies to the captured insulin, streptavidin conjugated horseradish peroxidase enzyme was applied which allows the binding with the immobilized biotinylated immunocomplex. Quantification of the immobilised antibody-enzyme conjugates was performed by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5' tetramethylbenzidine. After acidification of formed products, the enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm, corrected for the absorbance at 590 nm to correct for optical imperfections in the plate. Rat insulin standards (0 – 10 ng/mL) were provided as well as two quality controls (one in the lower and the

other in the higher range of insulin concentration) as an estimate of the accuracy of the test. Unknown serum samples (10 μ L sample volume) were used in duplicates and absorbance was read in an Anthos 2001 plate reader within 5 min of adding the stop solution. To determine insulin concentrations, average absorbance of duplicates ($Abs_{450nm-590nm}$) was plotted on the y-axis and concentrations of insulin standards on the x-axis on a log scale graph. The concentrations of the unknown samples were derived from the standard curve by solving the equation of the standard curve trend line for “x” and entering the average absorbance values of unknown samples as “y”.

2.4.3 Determination of mouse serum leptin concentrations

Serum leptin concentrations were quantified using the Mouse Leptin ELISA kit from Millipore (Millipore Corporation, UK) according to manufacturer’s instructions with the same principles of the procedure as described in Section 2.4.2. Mouse leptin standards (0.2 – 30 ng/mL) were provided as well as two quality controls as an estimate of the accuracy of the test. Unknown serum samples (5 μ L sample volume for DIO mice and 10 μ L for control and *ob/ob* mice) were used in duplicates. To compensate the volume deficit of DIO samples, assay buffer was used to have a final sample volume of 10 μ L. Absorbance was read at 450 nm in an Anthos 2001 plate reader within 5 min of adding the stop solution. Absorbance at 590 nm was used as the reference wavelength to correct for optical imperfections in the plate. For the standard curve, average absorbance ($Abs_{450nm-590nm}$) of mouse leptin standards were plotted on the y-axis against the concentrations of mouse leptin standards on the x-axis on a log scale graph. Concentrations of unknown samples were derived from the standard curve by solving the equation of the standard curve trend line for “x” and entering the average absorbance values of unknown samples as “y”. For DIO samples, calculated data were multiplied by 2 to accommodate for the dilution factor.

2.4.4 Determination of mouse serum free fatty acid concentrations

Serum free fatty acid concentrations were quantified using the Free Fatty Acid kit by Abcam according to manufacturer's instructions. Palmitic Acid (0 – 10 nmol) was used as standards and Acyl-CoA synthetase used to convert fatty acids to their CoA derivatives, which were subsequently oxidized with the concomitant generation of colour. For testing serum samples of DIO and control mice, 10 µL serum was directly added to each well and sample volume adjusted with assay buffer for a final volume of 50 µL/well. Standards and test samples were run in duplicates. Optical density was measured at 570 nm on an Anthos 2001 plate reader and fatty acid concentration was calculated by subtracting the background value (the 0 Control) from all readings and then plotting the standard curve nmol/well vs. OD 570 nm. Sample readings were applied to the standard curve to obtain fatty acid amount in the sample wells. Fatty acid concentrations were calculated with the following equation:

$$\text{Fatty acid concentration} = Fa/Sv \text{ (nmol/}\mu\text{L or mM)}$$

Fa is the fatty acid amount (nmol) in the well obtained from standard curve
Sv is the sample volume (µL) added to the sample well

2.4.5 Determination of plasma asymmetric dimethylarginine (ADMA)

Plasma ADMA levels of DIO and control mice were determined using liquid chromatography/mass spectrometry by Dr James Leiper, MRC CSC Nitric Oxide Signalling Group as previously described [168].

2.5 Histological analysis and histomorphometry

Tissues for histological analysis were harvested as described in Section 2.3.3. Following paraffin embedding, multiple 4 µm thick sections were cut from each organ and stained with haematoxylin and eosin and picro-sirius red. Preparation of sections and staining was performed by Ms Martina Leopizzi, La Sapienza University Rome, Italy, or Ms Lorraine Lawrence, Faculty of Medicine, Imperial College London.

2.5.1 Haematoxylin and eosin staining

Sections were deparaffinised by incubating at 70 °C for 30 min in a dry oven followed by incubating twice in xylene for 10 min each. Samples were re-hydrated in a step of two times 5 min in 100% ethanol, 2 min in 95% ethanol and 2 min in 70% ethanol. Sections were washed briefly in distilled water and stained with Mayer haematoxylin solution for 5-8 min, rinsed in distilled water and washed in warm running tap water for 10-15 min and again rinsed in distilled water. Following haematoxylin staining sections were counterstained in eosin/Phloxine B solution for 15 sec and dehydrated again using 95% ethanol and two changes of 100% ethanol for 15 sec each. Samples were cleared in two changes of xylene for 5 min each.

2.5.2 Picro-sirius red staining

For picro-sirius red staining, paraffin sections were deparaffinised and hydrated as described in Section 2.5.1 followed by nuclei staining with Weigert's haematoxylin for 8 min and a 10 min wash in running tap water. After the wash, sections were incubated in picro-sirius red for 1 h and washed twice in acidified water followed by three incubations in 100% ethanol and clearing in xylene.

2.5.3 Histomorphometrical analysis

Analysis was performed by Dr Massimiliano Mancini, La Sapienza University Rome, Italy.

Histological and histomorphometrical studies were performed to evaluate size and structure of intramural arterioles. Images of arterioles with a diameter ≤ 150 μm were acquired at a 40x magnification and analysis was carried out using Metamorph 6.2 software (MDS Analytical Technologies Inc., Molecular Devices, USA). Total vessel area and lumen area were measured (Figure 2.1) and the following parameters calculated from this:

Medial area: Vessel area – Lumen area

Media-to-lumen ratio: Medial area / Lumen area



Figure 2.1: Histomorphometrical analysis of a mouse intramural arteriole.

Histological section of a mouse left ventricle stained with haematoxylin and eosin. Vessel area is indicated by the black circle and lumen area by the red circle.

2.6 Immunohistochemistry

Immunohistochemistry (IHC) was used to assess the expression levels and localisation of GLUT4 in mouse myocardium at baseline and after insulin stimulation. For this, heart sections were deparaffinised as described in Section 2.5.1 and blocked with goat serum for 30 min followed by incubation with primary antibodies against the carboxyterminal end of GLUT4 (1:250; Abcam, UK) at 4 °C over night. After incubation, sections were rinsed three times for 2 min in PBST and incubated in peroxidase blocking solution for 10 min at room temperature. Sections were subsequently incubated with biotinylated secondary antibody in PBS for 30 min at room temperature, rinsed three times in PBST and incubated in peroxidase-streptavidin solution for 30 min at room temperature. Following washes, sections were incubated in peroxidase substrate solution, rinsed as above and counterstained with Meyer haematoxylin. Sections were dehydrated by incubating in 95% ethanol for 1 min and 100% ethanol twice for 3 min, cleared in xylene for 5 min twice and mounted.

2.7 Immunofluorescence

Immunofluorescence was used to assess the expression level and localisation of RGS2 in vascular smooth muscle cells in left ventricle of DIO and control mice. Sections were deparaffinised as described in Section 2.5.1. Slides were rehydrated in sequential steps starting with 95% ethanol twice for 5 min, followed by 3 min each in 90%, 70% and 50% ethanol and 4 min in distilled water. Antigen retrieval was performed by microwave denaturing (5 min at 60% of 950 W) in citric buffer (pH 6.0) for 5 min followed by cooling down to room temperature for 30-45 min and washed first in distilled water for 2 min and then in PBS for 8 min with PBS changes every 2 min. Subsequently, sections were blocked in 5% BSA in PBS at room temperature for 1 h and mouse RGS2 primary antibody (1:50; Santa Cruz, UK) was added and incubated over night at 4 °C. The next day, slides were washed (5 x 2 min PBS) and then incubated with Goat Alexa 488 anti mouse secondary antibody (1:100; Invitrogen, UK) for

60 min in the dark at room temperature. To assess the expression and localisation of two proteins of interest on the same section, double staining was performed. For this, slides were subsequently washed (5 x 2 min PBS), blocked in 5% BSA in PBS for 1 h at room temperature and incubated with a second primary antibody, α smooth muscle actin (1:250; Abcam, UK) for 1 h at room temperature in the dark. Following primary antibody staining slides were washed as before and incubated with Goat Alexa 568 anti-rabbit secondary antibody (1:500) for 1 h at room temperature and washed again. The samples were then exposed to TO-PRO3 (1:200; Invitrogen, UK) for 20 min at room temperature to counterstain the nuclei and washed again for 6 x 2 min in PBS before mounting coverslips on slides for imaging using Vectashield (Vector Laboratories, Invitrogen, UK) and sealed using nail varnish. Slides were stored at 4 °C over night and imaged the next day.

2.7.1 Analysis of immunofluorescent staining of vascular smooth muscle cells by confocal microscopy

Myocardial vascular smooth muscle cells were imaged using an inverted laser scanning confocal microscope (LSM 510 Meta inverted; Zeiss, Germany) with a 40x magnification. Samples stained with secondary antibody only were used to optimise laser settings and microscope set up to avoid detection of background staining or autofluorescence. Vascular smooth muscle actin staining was used for identification and localisation of transmural small vessels ($\leq 120 \mu\text{m}$). Specialised software was used to quantify the fluorescent intensity (Zeiss LSM Meta software). Regions of interest were drawn around the vessels and mean fluorescence intensity was measured using this software.

2.8 Protein expression analysis

2.8.1 Protein extraction from tissue samples

Frozen tissue powder was homogenized in ice-cold protein lysis buffer (Cell Signaling Technology, UK) supplemented with 1 mM PMSF and 1 mM protease inhibitor cocktail (Sigma Aldrich, UK) immediately before use with a Tissue Tearor (BioSpec Products Inc., USA) and incubated on ice for 30 min. Cell lysates were centrifuged at 10,000 g (Eppendorf 5804R) for 10 min at 4 °C to remove cell debris and supernatants were stored at -20 °C until further use.

2.8.2 Total protein concentration estimation

Total protein concentration from lysates was estimated by the Bradford assay method [169] using protein assay reagent (BioRad Laboratories, CA, USA). Bovine serum albumin (BSA) was used as the standard ranging from 0 – 40 µg/µL. Absorbance was read at 595 nm on an Anthos 2001 plate reader and protein concentrations of the experimental samples were determined by the standard curve generated.

2.8.3 Phosphotyrosine (4G10) pulldown

For the anti-phosphotyrosine immunoprecipitation, cell lysates with a concentration of 1 µg/µL and a total concentration of 250 µg were used. 50 µL of a 50% protein agarose beads slurry were added to lysates and rotated for 3 h at 4 °C. After the incubation, samples were centrifuged for 5 min at 2,000 rpm at 4 °C and supernatant split into two new 1.5 mL microcentrifuge tubes. To one tube 50 µL anti-phosphotyrosine agarose conjugate (Millipore, clone 4G10) was added and to the other 50 µL protein A agarose beads. Samples were rotated over night at 4 °C. The next day, samples were centrifuged for 5 min at 2,000 rpm at 4 °C, the supernatant removed and beads washed three times with RIPA buffer. After the last wash the supernatant was removed and 50 µL 4 x SDS-PAGE sample buffer (NuPAGE LDS Sample Buffer, Invitrogen, UK) was added.

Samples were heated at 95 °C for 5 min to dissociate proteins from beads and 10 µL were loaded onto 7.5% agarose gels (Table 2.2).

2.8.4 Western blotting

Samples were incubated for 3 min at 95 °C in 4 x NuPAGE LDS Sample Buffer (Invitrogen, UK) and aliquots (20-25 µg) were loaded onto 7.5%, 10% or 12% acrylamide gels (Table 2.2) and subsequently separated by SDS-PAGE gel electrophoresis in 1x running buffer. Proteins were transferred onto polyvinylidene fluoride membrane (Immobilon-P, Milipore Corporation, USA) in transfer buffer and adequate transfer of proteins was confirmed by staining the membrane with Ponceau S Solution and the gel with Coomassie blue protein staining solution (Sigma-Aldrich Co.).

Table 2.2: Composition of agarose gels for western blotting

Substances	Running gel			Stacking gel
	7.5% (mL)	10% (mL)	12.5% (mL)	4.5% (mL)
Acrylamide	5	6.8	8.4	1.5
Tris-HCl, pH 8.8	5	5	5	-
Water	9.6	7.8	6.2	7.4
10 % SDS	0.2	0.2	0.2	0.1
10 % APS	0.1	0.1	0.1	0.1
10 % TEMED	0.1	0.1	0.1	0.1
Tris-HCl, pH 6.8	-	-	-	0.833

Acrylamide: 30% Acrylamide/0.8% Bis-acrylamide in H₂O (UltraPure Protogel, Fisher Scientific, UK); SDS: Sodium dodecyl sulphate (Sigma Aldrich, UK); APS: Ammonium persulfate (Sigma Aldrich, UK); TEMED: N,N,N',N'-Tetramethylethylenediamine (BDH Electran, VWR International Ltd, UK)

Following transfer, membranes were washed with Tris-buffered saline containing 0.1% Tween20 (TBST) and blocked for 45 to 60 min at room temperature with 5% skimmed milk or BSA in TBST. Blots were then probed with primary antibodies (Table 2.3) according to manufacturer's instructions at an optimised concentration which was derived from titration experiments. After incubation, blots were washed three times for 5 min with fresh TBST and incubated for 1 h at room temperature with either horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG secondary antibody at a 1:2500 dilution (Dako Cytomation, UK) in 1% skimmed milk or BSA in TBST. Proteins were visualized using the ECL Western Blotting Detection System (BioRad Laboratories, UK) followed by exposure to CL-XPosure Film (Pierce Biotechnology Inc., UK). Developed films were scanned and the relative densitometry was assessed using ImageJ software (NIH, Bethesda, MD).

Table 2.3: Primary antibodies

Protein	Species	Source	Dilution	Size (kDa)
Akt	rabbit	CST	1:1000	60
P-Akt	rabbit	CST	1:1000	60
CTGF	mouse	Abcam	1:1000	38
GAPDH	mouse	Abcam	1:2500	36
GLUT4	rabbit	Abcam	1:2500	45
IRS1	rabbit	CST	1:1000	180
MT2	mouse	Abcam	1:1000	15
RGS2	mouse	Santa Cruz	1:1000	32

CST: Cell Signaling Technology

2.9 RNA expression analysis

2.9.1 RNA extraction from frozen tissue

RNA was extracted using TRIzol reagent (Invitrogen, UK) according to manufacturer's instructions. 50 mg of heart powder was placed in a 7 mL Sterilin tube on ice and 1 mL TRIzol reagent was added. Samples were homogenized on ice with a Tissue Tearor (BioSpec Products Inc., USA) and incubated at room temperature for 5 min for complete dissociation of nucleoprotein complexes. After incubation, 200 μ L chlorophorm was added, samples were shaken by hand for 15 sec and incubated at room temperature for 3 min. To separate the aqueous phase containing the RNA, samples were centrifuged for 15 min at 12,000 g at 4 °C and the clear aqueous phase was transferred to a new tube containing 500 μ L isopropanol. Samples were mixed, incubated for 10 min at room temperature and centrifuged for 10 min at 12,000 g at 4 °C. Following centrifugation, the supernatant was removed and the pellet washed with 75% ethanol. Samples were mixed by hand and centrifuged at 8,000 g for 8 min at 4 °C. After washing, the supernatant was removed and the pellet air-dried for about 10 min. Isolated RNA was dissolved in 40 μ L RNase free water and incubated at 55 °C for 5 min. Centrifugations were carried out in a pre-cooled Eppendorf 5804R.

2.9.2 RNA sample cleanup

Extracted RNA was further purified using the RNeasy Mini kit (Qiagen, UK) according to manufacturer's description. The sample volume was adjusted to 100 μ L with RNase-free water, 350 μ L RLT buffer was added and samples were mixed by pipetting. 250 μ L ethanol (96-100%) was added to the diluted RNA, mixed by pipetting and directly transferred to a RNeasy Mini spin column. Columns were centrifuged at 8,000 g for 30 sec and the flow-through discarded. 500 μ L RPE buffer was added to the RNeasy spin column and again centrifuged at 8,000 g for 30 sec. This step was repeated once, centrifuging for 2 min followed by an optional centrifugation step without addition of RPE buffer for 1

min at maximum speed. RNeasy spin columns were placed to a new 1.5 mL collection tube and 30 μ L RNase-free water was directly added onto the membrane. Samples were centrifuged at 8,000 g and the elution repeated with 20 μ L RNase-free water.

Total RNA concentration was quantified by measuring optical density using the NanoDrop® ND-1000 spectrophotometer. Purity of the isolated RNA was assessed by calculating the ratio of the optical density at 260 nm and 280 nm and ratios between 1.8 and 2.1 were considered as pure. The total RNA was stored at -80 °C until further use.

2.9.3 cDNA preparation

cDNA was synthesised from 1 μ g of total RNA in reverse transcription reactions using SuperScript® III Reverse Transcriptase (RT) supplied with a vial of 5x First-Strand Buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$) and 0.1 M DTT (Invitrogen Ltd, UK). Total RNA was incubated together with dNTP mix (10 mM each), random hexamers (50 μ M, Applied Biosystems, UK) and RNase-free water for 5 min at 65 °C followed by a quick chill on ice. cDNA synthesis mix consisting of 4 μ L 5x First-Strand Buffer and 2 μ L of 0.1 M DTT per reaction was added and samples were incubated for 2 min at 25 °C. After incubation, 1 μ L SuperScript® III RT (200U/ μ L) was added and samples were incubated at 25 °C for 10 min and 42 °C for 50 min. The reaction was inactivated by incubating at 70 °C for 15 min. The resulting cDNA was stored at -20 °C until further use.

2.9.4 Quantitative real time RT PCR (qRT-PCR)

Transcript levels were quantified using the SYBR Green Jumpstart Taq Ready mix (Sigma Aldrich, UK) according to manufacturer's recommendations. cDNA was diluted to 5 ng/μL (10 ng final concentration) and gene specific primers to a final concentration of 200 mM (Table 2.4). Primer sequences were designed with GenScript Primer Design Tool [170].

To control for technical differences, triplicates were set up in an optical 96-well reaction plate (BioRad, UK) and real-time PCR was carried out using the 7900 HT Fast Real-Time system (Applied Biosystems, UK). Reactions were incubated at 94 °C for 2 min followed by 40 cycles at 94 °C for 15 sec, 60 °C for 1 min and 72 °C for 1 min. Relative gene expression was calculated using the comparative Ct method relative to control samples by comparing the number of thermal cycles that were required to generate threshold amounts of product. Cyclophilin A was used as a housekeeping control gene. All quantitations were normalised to Cyclophilin A to account for variability in the initial concentration and quality of total RNA. Ct was calculated for the genes of interest and for the housekeeping gene Cyclophilin A. For each cDNA sample, the Ct for Cyclophilin A was subtracted from the Ct for each gene of interest to give the parameter ΔCt , thus normalising the initial amount of total RNA used. The amount of each target was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is the difference between the ΔCt of the two cDNA samples to be compared.

Table 2.4: Primer sequences for qRT-PCR

Gene ID	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
<i>Acta1</i>	TATTCCTTCGTGACCACAGC	GGCCATCTCATTCTCGAAGT
<i>Arhgef19</i>	GGTCAAGAGCACCAGTGACA	TTGGTGACGTAAGGCAGGTA
<i>Cdkl1</i>	GATGAGGCTCGAGAGAGGTC	AGCCTTCTCCAATCTTTCCA
<i>Cirbp</i>	GGAGCAGGTCTTCTCCAAGT	GGTCTCCCTGTCCTTTACCA
<i>Ctgf</i>	CTGAGCATGTGTCCTCCACT	GCCACAAGCTGTCCAGTCTA
<i>Ehhadh</i>	TGGAGTTCCTGTTGCTCTTG	GATGGCTTCTTCAACTGGGT
<i>Fdft1</i>	TGACATGAGCATCAGTGTGG	CACTTGTCGGTCCTTCTCCT
<i>Gabarapl1</i>	AGGTGAAGACATCGGGAAAC	ACAGCCAATGCAACACAAGT
<i>Gpr22</i>	ACAGAAGAAGAAAGCCCGAA	CACCAAACACGACATTCTC
<i>Hist1h1c</i>	GCTGCTCAGGAAGTGCTATG	TTCAAATCACCTTGCAGTCC
<i>Hmgsc2</i>	GGAGGACATCAACTCCCTGT	TTGTCAATGATGGTCTCGGT
<i>Hrc</i>	CAGGGAGAGATTGCTCATCA	TGAGAGCTGTGGTCACCTTC
<i>Lmcd1</i>	CTAAGGACCTCAACCCTGGA	CAGACAAGCCACTCCTCTTG
<i>Mt2</i>	CTGCAAATGCAAACAATGC	ACTTGTCGGAAGCCTCTTTG
<i>Nes</i>	CCCTTGCCCTAATACCCTTGA	GCCTCAGACATAGGTGGGAT
<i>Nkiras1</i>	GTGGCAATCGTTGTGTTAGG	CCCACAGCTTGACTTTCTCA
<i>Pdk4</i>	CTGCTGGACTTTGGTTCAGA	GGCAGGATGTCAATCTCCTT
<i>Ppp1r3c</i>	TGTGGTAAGGGTGTGAGGAA	GTTCCCACCAATGGCTTAAT
<i>Rgs2</i>	GAGAAGGAAGCTCCCAAAGA	ACCCTCTTCTGAGCTGTGGT
<i>Scd4</i>	GCGGTATGCTGTGCTTCTAA	TTGAAACGAAGGGATTCTCC
<i>Tcp11l2</i>	GCTGAAAGCTACCACCAACA	TCCATTCTCATGAGGTCCAA
<i>Tfr</i>	GCATCTGCTAATGAGACCCA	ATTGTGGCATTCTTCCTTCC
<i>Tmem204</i>	TAGAAGCCGAGACAGGGAAT	CTGGATGTCCTGCTTCGTAA
<i>Tmem71</i>	TTCAAGAGCTGGTTTGATGC	GGCTAATTCTCCTCCCACAA
<i>Tmtc1</i>	CCTCCTGGCCTTCAATGTAT	TCCACCAGAGGGATACTTCC
<i>Ucp3</i>	CACGGCCTTCTACAAAGGAT	TAAGGCCCTCTTCAGTTGCT
<i>Cyclophilin A</i>	AATGCTGGACCAAACACAAA	CCTTCTTTCACCTTCCCAAA

2.10 Microarray set-up

GeneChip® Mouse Exon 1.0 ST Arrays (Affymetrix Inc, UK) were used to investigate the effects of high fat diet feeding on cardiac gene expression on the whole-genome scale. Unlike the classical 3' expression microarrays this system allows analysis of both gene expression and alternative splicing due to multiple probes per exon and approximately 40 probes per gene [171]. The protocol used has been validated by the MRC Genomics Laboratory and is based on protocols provided by Affymetrix with modifications made by the MRC Genomics Laboratory (Version 2, 14/02/2008).

Ribosomal RNA (rRNA) is removed from total RNA in the rRNA reduction step to maximise the sensitivity and specificity of the hybridisations. In the next step mRNA is copied into double-stranded DNA (cDNA synthesis kit) and amplified by *in vitro* transcription into cRNA. In three second cycle cDNA synthesis reactions deoxyuridine is incorporated into each cDNA strand and following incorporation double-stranded DNA is fragmented using apurinic/apyrimidinic endonuclease (APE1) and uracil DNA glycosylase (UDG) enzymes. Prior to hybridisation onto arrays, fragments are end-labelled using terminal deoxynucleotidyl transferase (TdT) and DNA labelling reagent.

A summary of the GeneChip® whole transcript assay is provided as a flow chart in Figure 2.2.

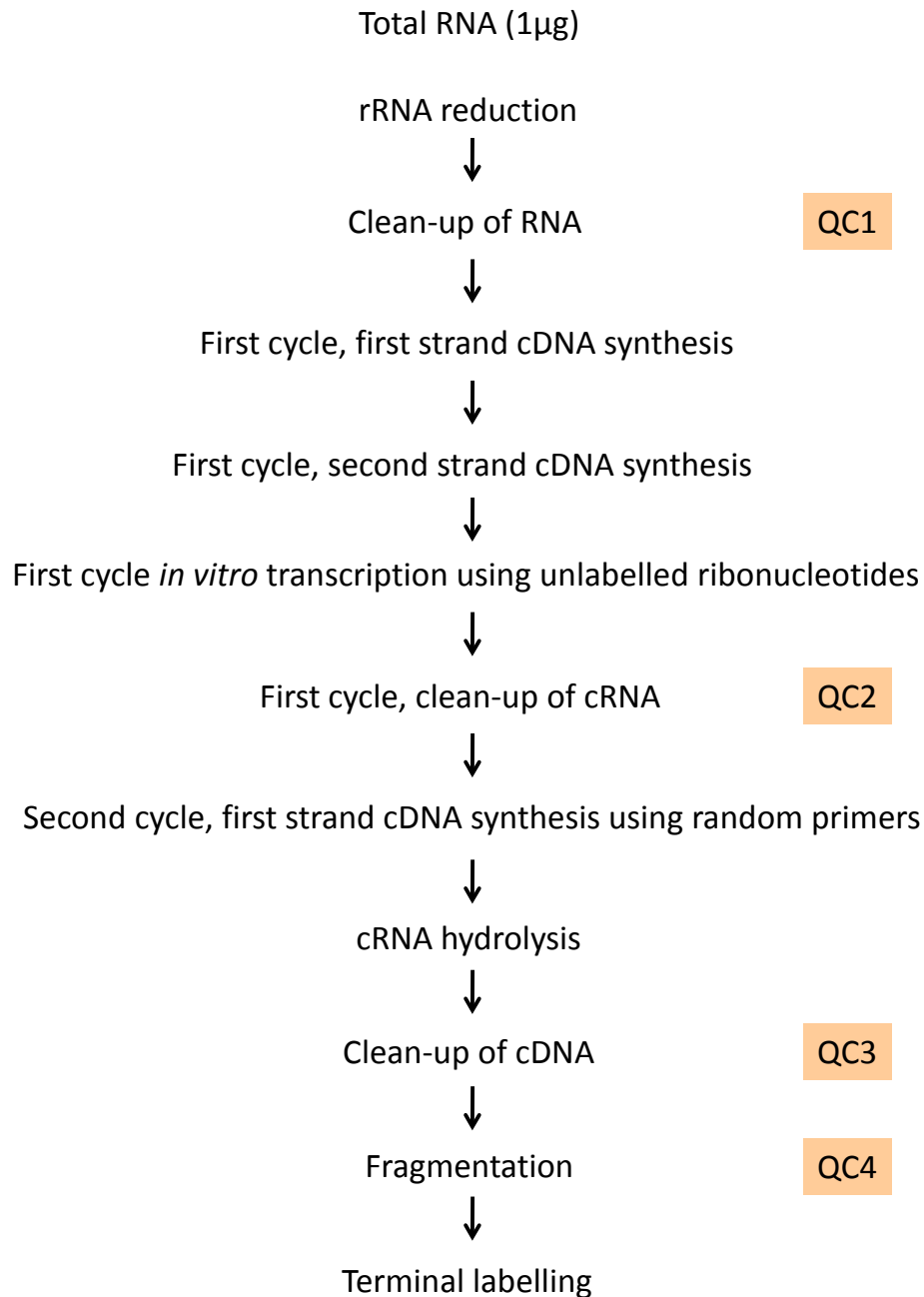


Figure 2.2: Flow chart of GeneChip® Whole Transcript Assay protocol.

Quality controls (QC) were: QC1 Bioanalyser trace and concentrations of total and reduced RNA. QC2 Concentrations, OD values (260/280 nm) and yield (µg) (at least 10 µg). QC3 Concentrations, OD values (260/280 nm) and yield (µg) (at least 5.5 µg). QC4 Bioanalyser trace before and after fragmentation

2.10.1 rRNA reduction

For the rRNA reduction step the RiboMinus Human/Mouse Transcriptome Isolation Kit (Human/Mouse) (Invitrogen, UK) was used. The Betaine Hybridisation Buffer was prepared by adding 54 μL of 5 M Betaine (Sigma Aldrich, UK) and 126 μL Invitrogen Hybridisation Buffer. In a 1.5 mL RNase free tube the total RNA/Poly A control mix was prepared by adding 1 μL of Poly A controls (provided by the MRC Genomics Laboratory) to 1 μg of total RNA per samples (maximum total RNA volume: 4.2 μL). RNase free water was added to a final volume of 5.2 μL . A mastermix of the Ribo Minus probe (100 pmol/ μL) plus the Betaine Hybridisation Buffer was prepared and 30.8 μL of this mastermix was added to each reaction tube. Samples were mixed, and incubated in a heated block at 70 $^{\circ}\text{C}$ for 5 min. Following incubation, samples were placed back on ice for at least 2 min and briefly spun and placed on ice until further use. The magnetic beads were prepared by transferring 55 μL of the bead suspension for each reaction into a 1.5 mL tube and placing the tubes on the magnetic stand for 1 min. Without disturbing the beads, the supernatant was removed and discarded and 55 μL of RNase free water added to each tube. Tubes were flicked to re-suspend the beads and spun. Beads were washed for a second time and for the third wash 55 μL of Betaine Hybridisation buffer was used instead of RNase free water. Tubes were returned to the magnetic stand, supernatant removed and 22 μL Betaine Hybridisation buffer added. 20 μL of the re-suspended beads were aliquoted into new 1.5 mL tubes, incubated for 2 min at 37 $^{\circ}\text{C}$ and the total RNA samples transferred to the prepared beads. Samples were mixed, incubated for 10 min at 37 $^{\circ}\text{C}$ in a heating block and placed again on the magnetic stand for 2 min. For each sample the supernatant was transferred to a new tube, 50 μL Betaine Hybridisation buffer added to the beads, incubated at 50 $^{\circ}\text{C}$ for 5 min and placed on the magnetic stand. The supernatant was transferred to the tubes already containing supernatant, total sample volume was determined and then placed on ice and immediately concentrated using the GeneChip IVT cRNA Clean-up Kit (Affymetrix Inc, UK) according to manufacturer's instructions.

Following this concentrating step the absorbance of the eluate was measured at 260 nm and 280 nm on a NanoDrop® ND-1000 spectrophotometer. The quality of the rRNA-reduced as well as the total RNA sample was assessed by visualizing an aliquot on the Agilent 2100 Bioanalyser using the RNA 6000 Nano Assay. The remaining sample was placed on dry ice and stored at -80 °C until further use. Absorbances and Bioanalyser traces of all samples can be found in the Appendix (Appendix C – Microarray Quality Controls).

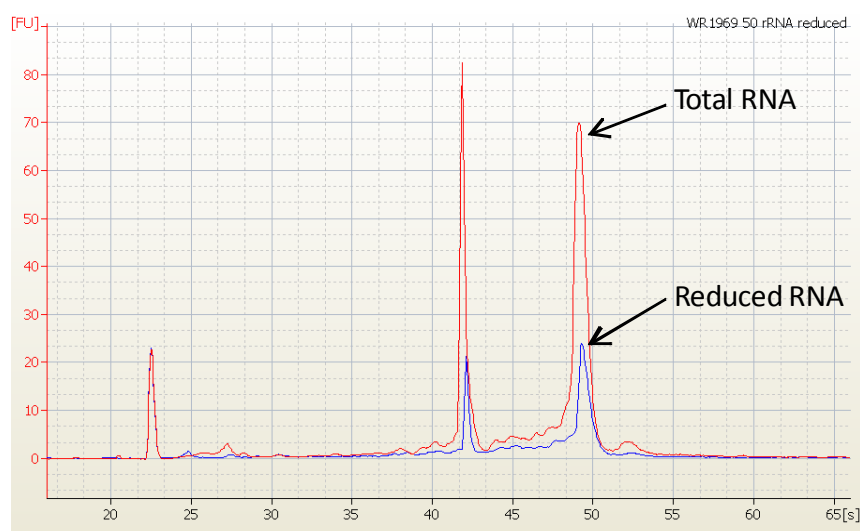


Figure 2.3: Bioanalyser trace of total RNA before and after rRNA reduction treatment

The red trace represents the total RNA and the blue trace the rRNA reduced total RNA. Samples were run by the MRC Genomics Laboratory and the picture was taken from the Whole Transcript 1 µg Labelling Assay manual.

2.10.2 cDNA Synthesis

For the cDNA synthesis the Affymetrix GeneChip WT cDNA Synthesis and Amplification Kit (Affymetrix Inc, UK) was used. First, a 1:5 dilution of the provided T7-(N)₆ primer in RNase free water was prepared. For each sample 4 µL of the rRNA-reduced Total RNA/PolyA mix and 1 µL of the diluted T7-(N)₆ primer was added to a 0.2 mL RNase free tube, mixed by vortexing and briefly spun. Reaction tubes were then placed in a thermal-cycler with heated lid option and incubated at 70 °C for 5 min, 4 °C for 2 min, held at 4 °C and then paced on ice. For each reaction, a mastermix consisting of 2 µL 5x First strand buffer, 1 µL 0.1 M DTT, 0.5 µL 10 mM dNTP Mix, 0.5 µL RNase inhibitor and 1 µL Superscript®II was prepared and 5 µL transferred to each of the reaction tubes. Samples were mixed by pipetting and placed in a thermal-cycler with heated lid option with the following programme: 25 °C for 10 min, 42 °C for 1 h, 70 °C for 10 min, 4 °C for 2 min and held at 4 °C. Following this, samples were briefly spun and immediately used for the second strand cDNA synthesis.

For the second strand cDNA synthesis a fresh dilution of 17.5 mM MgCl₂ was prepared by diluting 2 µL MgCl₂ (1 M) with 112 µL RNase free water. A mastermix was prepared (Table 2.5) and 10 µL added to each first strand reaction tube. Samples were mixed by pipetting and incubated at 16 °C in a thermal-cycler for 2 h without the heated lid option followed by an incubation at 75 °C for 10 min an a thermal-cycler with heated lid option and finally cooled for 2 min at 4 °C. After the second strand cDNA synthesis samples were immediately used for cRNA synthesis.

Table 2.5: Second strand cDNA synthesis master mix (for one reaction)

RNase free water	4.8 µL
diluted MgCl₂	4.0 µL
10 mM dNTP mix	0.4 µL
DNA Polymerase I	0.6 µL
RNase H	0.2 µL

First cycle of the cRNA synthesis was performed by preparing a mastermix for each sample consisting of 5 μ L 10x IVT Buffer, 20 μ L IVT NTP Mix and 5 μ L IVT Enzyme Mix. The mastermix was transferred to the second strand reaction tubes, mixed by pipetting and incubated in a thermal-cycler at 37 °C for 16 h with the heated lid option. After the incubation samples were put on hold until further processed using the Affymetrix GeneChip Sample Clean-up Module (Affymetrix Inc, UK) according to manufacturer's instructions. First cycle cRNA was eluted in RNase-free water and volume of eluate was measured. Absorbance at 260 nm and 280 nm of each eluate was measured on a NanoDrop® ND-1000 spectrophotometer and the first cycle cRNA yield was determined by multiplying the concentration of each sample with the eluate volume. For the second cycle cRNA synthesis, 10 μ L cRNA at a minimum concentration of 1.54 μ g/ μ L was needed.

In the second cycle, first strand cDNA synthesis was performed using the GeneChip WT cDNA Synthesis and Amplification Kit by Affymetrix (Affymetrix Inc, UK). For each reaction, 10 μ g cRNA from the previous step, 1.5 μ L random primers (3 μ g/ μ L) and RNase free water were added to a 0.2 mL tube to a final volume of 8 μ L. Samples were briefly vortexed and placed in a thermal-cycler with the heated lid option with the following programme: 70 °C for 5 min, 25 °C for 5 min, 4 °C for 2 min and held at 4 °C. After this incubation samples were put on ice and a mastermix was prepared for each sample (Table 2.6).

Table 2.6: Second cycle, first strand cDNA synthesis master mix (for one reaction)

5x First Strand Buffer	4.00 μ L
0.1 M DTT	2.00 μ L
10 mM dNTP	1.25 μ L
10 mM dUTP	1.25 μ L
Superscript®II	4.75 μ L

After mixing by pipetting, 12 μL of the mastermix was transferred to each of the reaction tubes and incubated in a thermal-cycler with the following programme: 25 $^{\circ}\text{C}$ for 10 min, 42 $^{\circ}\text{C}$ for 90 min, 70 $^{\circ}\text{C}$ for 10 min, 4 $^{\circ}\text{C}$ for 2 min and held at 4 $^{\circ}\text{C}$. To each single stranded cDNA tube 1 μL of RNase H was added, mixed by pipetting and put back in a thermal-cycler with heated lid option at 37 $^{\circ}\text{C}$ for 45 min, 95 $^{\circ}\text{C}$ for 5 min, 4 $^{\circ}\text{C}$ for 2 min and held at 4 $^{\circ}\text{C}$. Samples were immediately cleaned-up using the GeneChip Sample Clean-up Module according to manufacturer's instructions. At the end absorbance was measured on a NanoDrop® ND-1000 spectrophotometer and yield of the single stranded cDNA was calculated.

2.10.3 Fragmentation of single-stranded cDNA

For the fragmentation of single-stranded cDNA, the Affymetrix GeneChip WT Terminal Labelling Kit (Affymetrix Inc, UK) was used. For each sample, 5.5 μg single-stranded cDNA was transferred to a 0.2 mL tube and RNase free water was added to make a total volume of 31.2 μL . Additionally, 16.8 μL of the mastermix (Table 2.7) was added to each reaction tube, mixed and spun. The tubes were then placed in a thermal-cycler with the heated lid option and run the following programme: 37 $^{\circ}\text{C}$ for 60 min, 93 $^{\circ}\text{C}$ for 2 min, 4 $^{\circ}\text{C}$ for 2 min and held at 4 $^{\circ}\text{C}$ until further use. An aliquot of 1.5 μL of the fragmented cDNA sample was run on a Agilent 2100 Bioanalyser for size analysis using the RNA 6000 Nano Assay. Along-side the fragmented samples, an unfragmented sample was run for comparison (Figure 2.4). Bioanalyser traces can be found in the Appendix (Appendix C – Microarray Quality Controls).

Table 2.7: Fragmentation master mix (for one reaction)

RNase free water	10 μL
10x cDNA Fragmentation Buffer	4.8 μL
UDG (10 U/μL)	1.0 μL
APE1 (1,000 U/μL)	1.0 μL

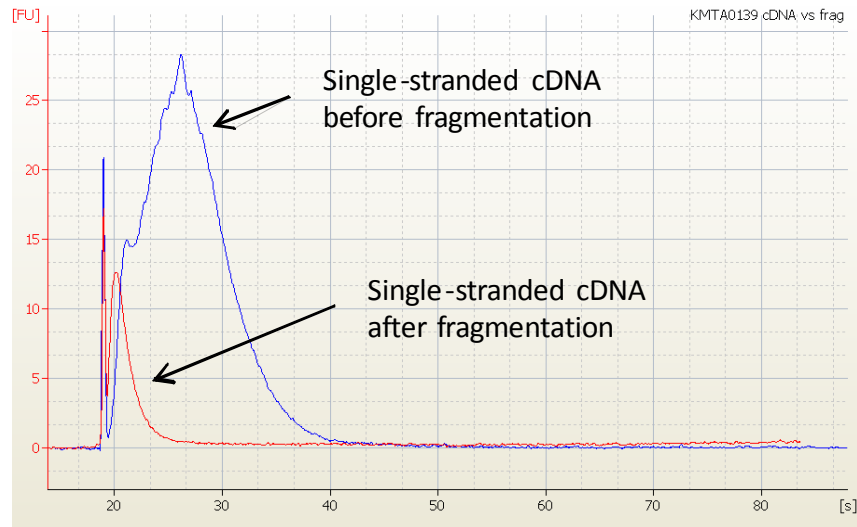


Figure 2.4: Bioanalyser traces comparing fragmented single-stranded cDNA with the unfragmented sample.

The blue trace represents the single-stranded cDNA before fragmentation and the red trace the single-stranded cDNA after fragmentation. Samples were processed at the MRC Genomics Laboratory and the picture was taken from the Whole Transcript 1 μ g Labelling Assay manual.

2.10.4 Labelling of fragmented single-stranded cDNA and hybridisation

For labelling of the fragmented single-stranded cDNA the GeneChip® WT Terminal Labelling Kit was used. 45 μ L of the fragmented cDNA was transferred to a new 0.2 mL RNase free tube and 15 μ L of a prepared mastermix consisting of 12 μ L 5x TdT Buffer, 2 μ L TdT and 1 μ L DNA Labelling Reagent (5 mM) was added to each sample. Tubes were placed in a thermal-cycler with heated lid option and the following programme was run: 37 °C for 60 min, 70 °C for 10 min, 4 °C for 2 min and held at 4 °C until further use. After the incubation, all of the labelled extract was transferred to a clean 1.5 mL RNase free tube and the volume was measured using a pipette. For each sample, sufficient RNase free water was added so that the final volume of the labelled extract was 60 μ L.

Labelled samples were stored on dry ice and hybridisation to GeneChip® Mouse Exon 1.0 ST Arrays (Affymetrix Inc, UK) and scanning was carried out at the MRC Genomics Laboratory by Ms Nathalie Lambie.

2.10.5 Data analysis

Gene expression summary values of the Affymetrix Gene Chip data were generated using the robust multichip average (RMA) algorithm [172]. The quality of the expression replicates was then determined using principal-components analysis, hierarchical clustering and hybridisation control plot using GeneSpring GX software (Agilent Technologies, UK) followed by a filtering step which included the removal of all values below the 20th percentile and the setting that 10 out of 10 samples should have probe values greater than the 20th percentile. After applying multiple corrections, no differentially expressed genes were found. An alternate approach of a fold change ≥ 1.5 and a p value of $p < 0.01$ was used as criteria for differential expression. Data analysis was performed at the Centre for Bioinformatics, Imperial College London with the great help of Mr Arshad Khan. Functional annotation was carried out using Ingenuity Pathway Analysis software (Ingenuity Systems Inc, USA).

2.11 Preparation of DNA samples for sequencing

PCR products for sequencing were generated from cDNA prepared as described in Section 2.9.3 by PCR amplification using *Pfu* DNA Polymerase according to manufacturer's instructions (Promega Corporation, USA). In a 50 μ L reaction 100 ng of cDNA was set up on ice together with dNTP mix (10 mM each), *Pfu* DNA Polymerase 10x Buffer (200 mM Tris-HCl (pH 8.8), 100 mM KCl, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM MgSO_4 , 1 mg/mL nuclease-free BSA, 1% Triton® X-100), forward and reverse primer (200 μ M each, Table 2.8), nuclease free water and *Pfu* DNA Polymerase. The reaction mix was placed in a thermal cycler with heated lid and denatured at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. Samples were incubated at 4 °C until further use.

Agarose gel electrophoresis of PCR products was carried out in wide Mini-Sub Cell GT tanks (Bio-Rad, UK). For 1.5% agarose gels, 1.5 g (w/v) low-melting agarose powder (Invitrogen, UK) was dissolved in 100 mL 1x TAE buffer (MRC CSC Media Kitchen) and boiled in a microwave. After the agarose was cooled down to approximately 55 °C, 4 μ L of 10 mg/mL ethidium bromide was added before pouring into the plastic mould and left to polymerise. DNA samples were mixed 1 in 10 with Orange G and electrophoresis was carried out in 1x TAE buffer at between 80 V for 30-90 min. The 1 kb DNA ladder (Invitrogen, UK) was used as a DNA size marker. DNA fragments were visualized using a UV transilluminator (BioRad, UK), photographed with the gel doc software (BioRad, UK) and excised from the agarose gel with a clean scalpel.

DNA was purified from the agarose gel using the QIAquick Gel Extraction Kit according to manufacturer's instructions (Qiagen, UK) and eluted from QIAquick columns with 60 μ L elution buffer (10 mM Tris-HCl, pH 8.5). DNA concentration was measured on a NanoDrop® ND-1000 spectrophotometer. 80 ng of purified DNA was submitted to the MRC Genomics Laboratory Sequencing Service with

3.2 picomoles of sequencing primer (Table 2.8) and adjusted with water to a total volume of 10 μ L. Cycle sequencing reactions were run on the 3730xl DNA Analyzer (Applied Biosystems, UK). Data were analysed in the BioEdit programme (Version 7.0.9) [173].

Table 2.8: Primer sequences for amplification and sequencing of the Rgs2 coding region

Amplification Primer		
Forward primer (5' -> 3')	CTGAGAATGCAAAGTGCCAT	
Reverse primer (5' -> 3')	GGCTGAATTCAAGGTCAGTTT	
Sequencing Primer		Start position
SeqFwd 1 (5' -> 3')	CTGAGAATGCAAAGTGCCAT	26
SeqFwd 2 (5' -> 3')	GGCAGAAGCATTGTGATGAAC	271
SeqRev 1 (5' -> 3')	GTCTTCACAAGCCAACCAGA	366
SeqRev 2 (5' -> 3')	GGCTGAATTCAAGGTCAGTTT	750

Primer sequences were designed with GenScript Primer Design Tool [170].

2.12 *In vivo* assessment of cardiac function using cardiac magnetic resonance imaging (CMRI)

Cardiac magnetic resonance imaging was performed at the MRC CSC Biological Imaging Centre with the help of Dr Marzena Wylezinska-Arridge, Dr Jordi Lopez Tremoleda and Mr Howard Parker.

Cardiac function was assessed using a high field Varian multinuclear, small-bore MRI system (4.7T). Animals were anaesthetised using 2.5% isoflurane in O₂ and maintained under anaesthesia with 1.5% isoflurane. Physiological parameters like heart rate, respiration and body temperature were monitored throughout the scan using a small animal imaging system integrated into the Varian scanner. Lead 1 ECG electrodes were secured to the chest of the animal and a pneumatic pillow was attached to the abdomen of the animal for monitoring respiration. Core body temperature was maintained at 37 °C using a warm air fan. The animal was placed in a coil in the supine position and the coil was then positioned in the magnet so that the heart was in the isocentre of the magnet. Long-axis scout images were acquired for localisation of the heart. Acquisition of dual-gated (ECG and respiration) dynamic cine FLASH MRI short axis images was performed with a repetition time TR of 4.35 ms, an echo time TE of 1.58 ms, a field of view of 40x40 mm and a matrix size of 192x192. Four averages of 8-10 consecutive 1.3 mm thick short-axis slices (6 frames per slice) were acquired to cover the entire left ventricle of the animal. (Figure 2.5)

Data analysis was carried out in a blinded manner using ImageJ software (NIH, Bethesda, MD) on reconstructed FDF files. Prior to analysis all FDF images per scan were converted to stacks and filtered using the byte swap plug-in in ImageJ. For all slices, both end-diastolic and end-systolic frames were visually determined by assessing filling size and wall thickening patterns of the LV. Endocardial contours were manually traced using ImageJ at end-systole and end-diastole for each slice to obtain the LV end systolic and end diastolic area (Figure 2.6).

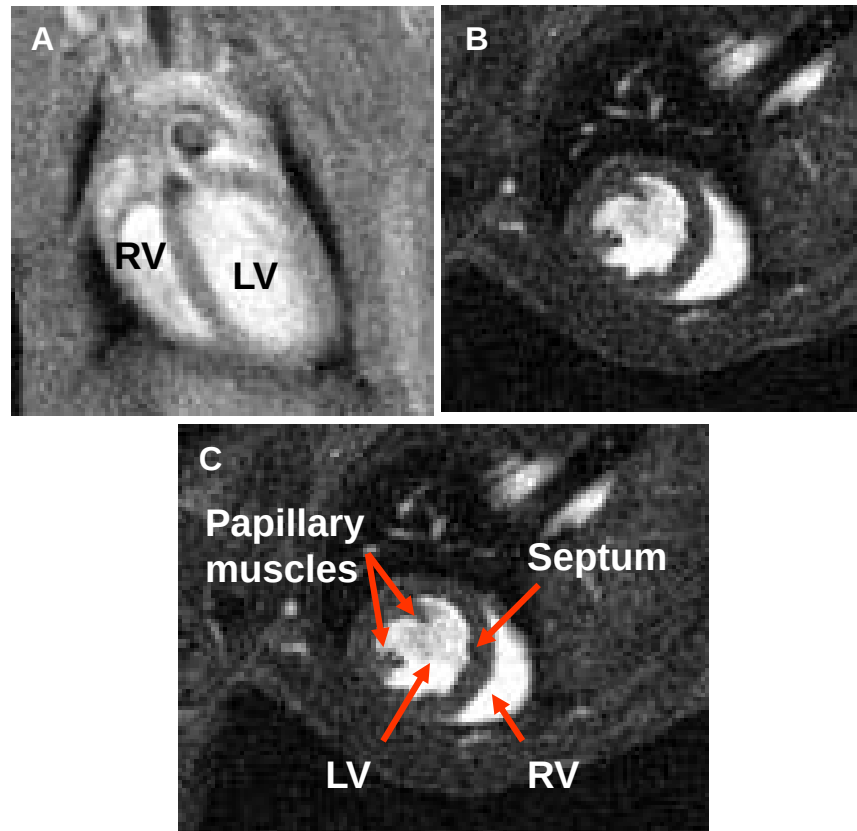


Figure 2.5: Long- and short-axis views of mouse heart.

(A) Long-axis scout image for localisation of the heart. (B) Short-axis cine FLASH image of left and right ventricles at end diastole. (C) Short-axis image shows left and right ventricles, septum and papillary muscles. LV, left ventricle; RV, right ventricle; Septum, interventricular septum.

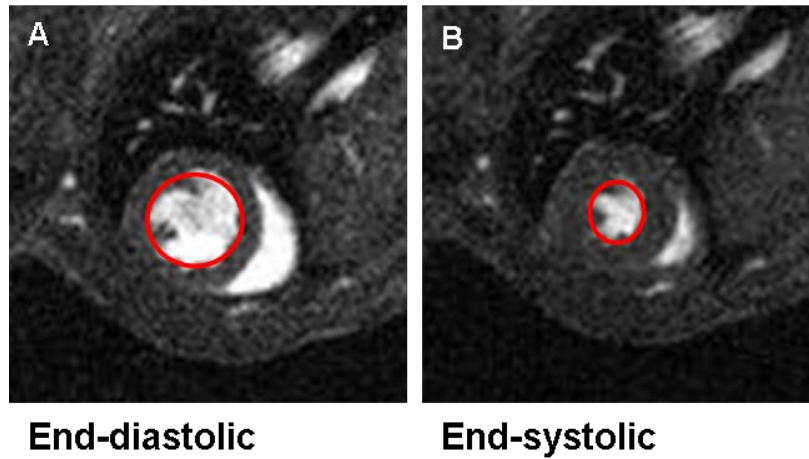


Figure 2.6: Manual planimetry of the mouse heart for functional analysis.

Functional cardiac parameters were determined by tracing the endocardial border of the left ventricle using ImageJ software. (A) Area of the blood pool (bright) in the left ventricle at the end of diastole (B) Area of the blood pool (bright) in the left ventricle at the end of systole. Papillary muscle was excluded from the area measurement.

Left ventricular end diastolic volume (LVEDV) and left ventricular end systolic (LVESV) volume was calculated as follows:

$$LVEDV = \sum_{\text{all slices}} (\text{end diastolic area}) \times \text{slice thickness}$$

$$LVESV = \sum_{\text{all slices}} (\text{end systolic area}) \times \text{slice thickness}$$

LV stroke volume (SV) and ejection fraction (EF in %) were calculated with the following formulae:

$$SV = LVEDV - LVESV$$

$$EF = (SV/LVEDV) \times 100$$

2.13 Invasive blood pressure measurements

For direct invasive measurements under terminal general anaesthesia animals were anaesthetised with 1.5% isoflurane in O₂ and placed on a heating pad to maintain the body temperature at 37 °C. Mean arterial pressure (MAP) was determined by cannulating the left carotid artery with a heparin-saline-filled polyethylene tube connected to a pressure transducer. MAP was recorded using CardioSOFT Pro (SonoMetrics Corporation, USA).

2.14 *Ex vivo* assessment of coronary flow using Langendorff perfusion

After invasive blood pressure measurement under general anaesthesia, the heart was removed and placed in a Petri dish with ice-cold heparinised Krebs-Henseleit buffer. Excess tissue was quickly removed under a dissection microscope, the aorta identified and attached to a mouse Langendorff cannula. Two sutures were tied around the aorta to ensure effective perfusion of the heart. The cannulated heart was attached to the Langendorff apparatus and allowed to equilibrate for at least 15 min at a perfusion pressure of 80 mmHg with oxygen-saturated Krebs-Henseleit buffer (5% CO₂ - 95% O₂ gas mixture). Perfusion was monitored via an integrated force transducer connected to an amplifier and PowerLab system (AD Instruments, UK) (Figure 2.7). Baseline coronary flow (CF) was measured using a Doppler flow probe (Transonic Systems Inc., USA) connected to a PowerLab amplifier. Hearts were subjected to a 1 min stop-flow (global ischaemia) and following reperfusion CF was measured and recorded at the peak of reactive hyperaemia. After returning to baseline, perfusion was stopped and hearts were weighed and fixed in 10 % neutral buffered formalin solution (Sigma Aldrich, UK). Baseline flow was calculated by averaging CF for one minute prior to ischaemia. Peak-to-baseline ratio of CF was calculated by dividing peak CF by baseline CF. Additionally, total peak area was calculated from CF traces and time to return to baseline after global ischaemia was recorded.

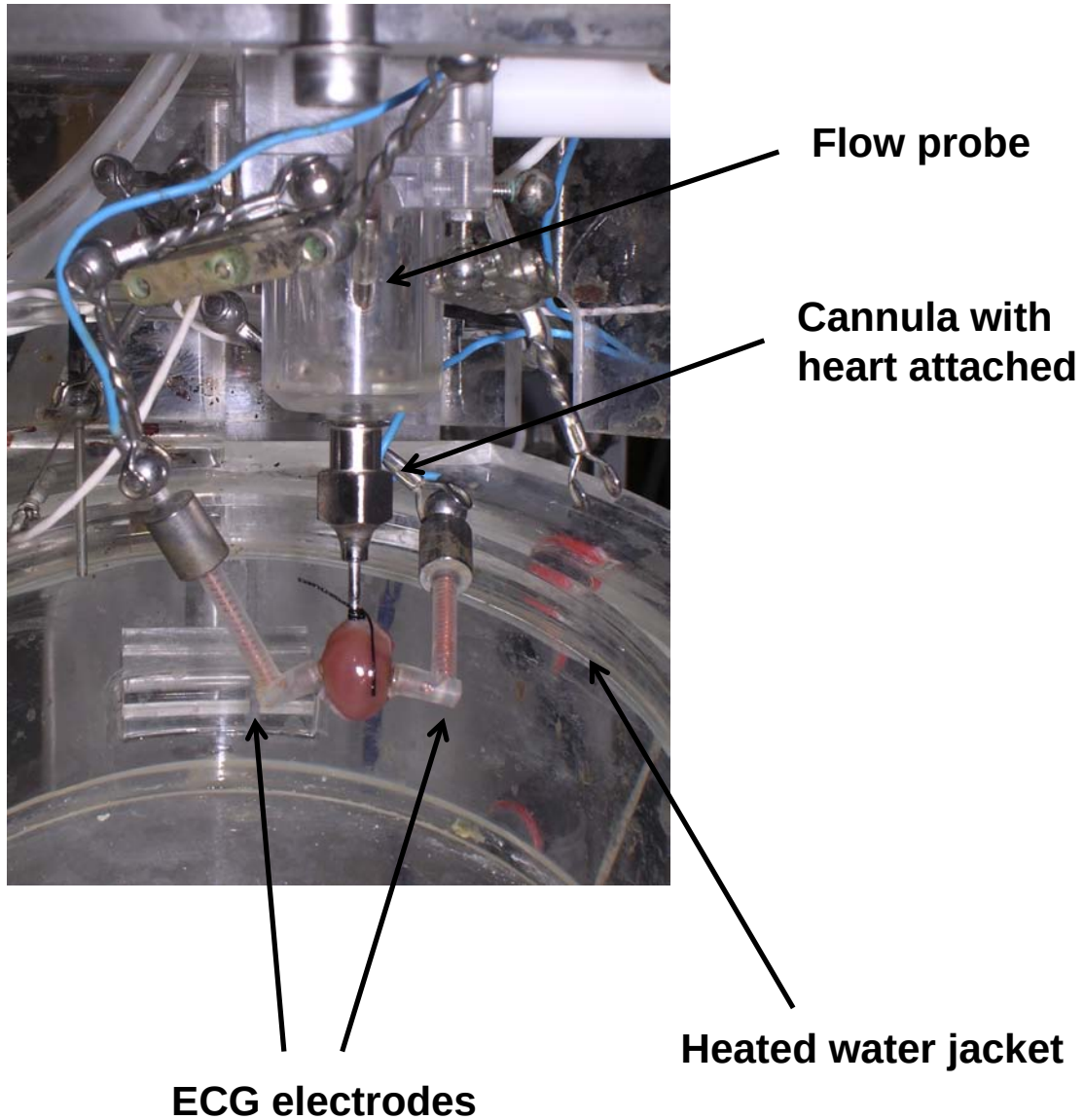


Figure 2.7: Example of a mouse heart attached to the Langendorff perfusion system.

The cannulated mouse heart is attached to a Langendorff apparatus and perfused with oxygen-saturated Krebs-Henseleit buffer. Coronary flow is measured using a Doppler flow probe.

2.15 Assessment of vascular function

2.15.1 *In vivo* assessment of vascular function

Vascular function was assessed *in vivo* during MAP measurement. As described in Section 2.13 animals were anaesthetised with 1.5% isoflurane in O₂ and placed on a heating pad to maintain the body temperature at 37 °C. Baseline MAP was determined by cannulating the left carotid artery with a heparin-saline-filled polyethylene tube connected to a pressure transducer and MAP was recorded using CardioSOFT Pro (SonoMetrics Corporation, USA). For the assessment of vascular function, acetylcholine (Sigma Aldrich, UK) was dissolved in physiological saline and a final concentration of 5 µmol was given by intravenous injection. Vasodilation was assessed by recording the immediate drop in MAP upon drug injection and the time needed to return to baseline MAP was recorded.

2.15.2 *Ex vivo* assessment of vascular function

For the *ex vivo* assessment of vascular function in DIO and control mice, wire tension myography on aortic rings was carried out by Dr Elizabeth Sujkovic, MRC CSC Nitric Oxide Signalling Group.

After sacrificing the animals by cervical dislocation, the thoracic aorta was dissected and placed in Krebs-Henseleit buffer. Connective tissue and fat were removed and the aorta cut into ring segments of 2-3 mm length. Special care was taken not to damage the endothelium during this procedure. Vascular rings were mounted in a wire tension myograph (Danish Myo Technology, Denmark), transferred to water-jacketed organ baths containing equilibrated Krebs-Henseleit buffer (95% O₂ - 5% CO₂ at 37°C, pH 7.4) and connected to force transducers. Resting tension was gradually increased to the optimal level as determined by the programme (PowerLab, AD Instruments, UK). Viability and maximal contractile capacity of the vessels were assessed by depolarizing the ring segments with potassium chloride (KCl, 70 mM). Viable segments were

then contracted with phenylephrine (PE) (dose response curve ranging from 10^{-9} to 10^{-5} M) and the integrity of the endothelium was determined by adding acetylcholine (ACh, 10^{-7} M). Endothelium-dependent vasodilation was assessed by precontracting arterial rings with PE (70% of KCl) and constructing cumulative relaxation curves of ACh (10^{-9} to 10^{-5} M). Endothelium-independent vasodilation was investigated using the NO donor sodium nitroprusside (SNP, 10^{-10} to 10^{-7} M).

2.16 Statistical analysis

All quantitative analyses display either the mean value $\pm$ standard deviation (SD) or $\pm$ standard error of the mean (SEM). Statistical analysis was performed using unpaired Student's t test when only two experimental groups were tested. One-way analysis of variance (ANOVA) (with Bonferroni's multiple comparison test) was used when more than two groups were tested. All statistical analysis was performed on Prism TM software (Version 5, GraphPad Software Inc., USA). Statistical significance was defined as $p < 0.05$.

Chapter Three: Generation and characterisation of a diet-induced obese mouse model

3.1 Introduction

The metabolic changes associated with insulin resistance manifest themselves in insulin-sensitive tissues like skeletal muscle, adipose tissue, or the liver and over years, chronic hyperglycaemia will lead to kidney damage, and other diabetic complications such as neuropathy, cardiomyopathy, atherosclerosis, and hypertension [85]. In addition, increased levels of FFA in the blood will overload muscle, liver and pancreatic β -cells with triglycerides and thus further add significantly to insulin resistance, dyslipidaemia and hyperinsulinaemia [46]. In the liver this ectopic lipid accumulation together with *de novo* lipogenesis will result in the development of nonalcoholic fatty liver disease, occurring in a wide spectrum of degrees ranging from the build-up of fat (hepatic steatosis) to inflammation often with fibrosis (non-alcoholic steatohepatitis) to cirrhosis and ultimately liver failure [174]. Furthermore, insulin resistance and the prolonged exposure to hyperglycaemia and dyslipidaemia will impair myocardial function leading to the development of diabetic cardiomyopathy and consequently increasing the risk of cardiovascular complications, which are the major cause of mortality in diabetic subjects [90].

Given the consequences of the metabolic dysfunction in diabetes, it is essential to understand the pathogenesis and underlying mechanisms of insulin resistance and diabetic complications like cardiomyopathy or nephropathy. The use of animal models allows detailed characterisation at the molecular, cellular and tissue level usually not feasible in humans and therefore offers promising new insights into human diabetes [8]. Models of insulin resistance and T2DM can be generated either by genetic, chemical, or nutritional manipulation or occur by spontaneous mutations. The expression and severity of metabolic, hormonal and pathologic abnormalities of diabetes can vary in different animal models according to genetic background, nutrition, age, sex, species and even strain. Additionally, due to the complex nature of the disease, monogenic or pharmacologically induced models must be interpreted with care. For example, the *ob/ob* mouse displays many pathological features of diabetes and diabetic

cardiomyopathy, however, the relevance of this model to human insulin resistance syndromes, with intact leptin signalling pathways and diabetic cardiomyopathy is less straightforward. As an alternative to genetically modified animals, the high-fat diet fed mouse was introduced in an attempt to generate a model more relevant to human pathology, and has become a clinically relevant tool to study insulin resistance and metabolic changes [175]. Animals on a high fat diet will gradually develop visceral adiposity, hyperglycaemia and insulin resistance as well as hepatic steatosis. The diet-induced obese model in male C57BL/6 mice is a commonly used experimental tool to investigate the pathophysiology of nonalcoholic fatty liver disease as it resembles closely the development of the metabolic syndrome in humans [176]. However, the multitude of different high fat diets ranging from 20% to 60% calories from fat makes it difficult to compare data from different studies and therefore make conclusive interpretations. Additionally, the high variety in fat components i.e. animal-derived fats like lard or beef tallow or plant oils like corn or safflower oil will result in various diets with different fatty acid compositions. Thus, it is important to standardise and validate certain metabolic parameters when conducting a study using a high fat diet model.

3.2 Aim

The aim of this chapter was to generate a diet-induced obesity mouse model by feeding male C57BL/6 mice a 45% lard-based high fat and characterise the resulting whole body, structural, and metabolic changes. Initially, animals were fed the high fat diet for 14 weeks and based on these results two further study periods with test time points at 8 and 11 weeks were introduced in order to gain information on the natural history of metabolic disturbances and pathological alterations of the diet-induced obesity model. Moreover, the most appropriate time point was determined to further study the molecular and genetic mechanisms of myocardial insulin resistance in humans and mice. The following points were investigated:

1. Whole body diet effects looking at both body weight and organ weight gain
2. Metabolic changes focusing on oral glucose tolerance, fasting glucose and insulin levels
3. Histological effects of high fat diet in heart, liver and kidneys

3.3 Results

3.3.1 Generation of a high fat diet-induced obesity model

Five week old male C57BL/6 mice were divided into two groups. The control group was fed a normal rodent chow whereas the other group was fed a 45% high fat, lard-based diet for 8, 11 and 14 weeks *ad libitum* (Table 3.1). After 6 weeks, the high fat diet group was further divided into mice that responded to the high fat diet by significantly gaining weight (DIO) and those that did not (diet-induced resistant, DIO-R). Body weight gain was recorded weekly and at the end of the feeding period, whole body and metabolic changes of diet-induced mice were investigated and compared to age-matched wild-type controls and *ob/ob* mice fed a normal rodent chow.

In order to show the development of metabolic disturbances and pathological alterations results are presented in a chronological way, starting with 8 weeks of high fat diet feeding.

Table 3.1: Composition of diets

	Normal rodent chow	High fat diet
Protein	27.3%	20%
Carbohydrate	61.2%	35%
Fat	11.5%	45%

% of calories consumed

3.3.2 Effects of 8 weeks of high fat diet feeding

3.3.2.1 Weight gain and body composition

At baseline, no significant differences were found in initial body weight or tibia length between controls and DIO mice. After 8 weeks of high fat diet feeding 70% of the mice were defined as DIO whereas 30% were defined as DIO-R. DIO mice had a significant increase in body weight gain of 19.86 ± 1.49 g compared to 7.7 ± 1.6 g body weight gain of age-matched wild-type controls ($p < 0.0005$). Accordingly, visceral fat pad masses were significantly heavier than in the control mice (2.97 ± 0.51 g vs. 0.58 ± 0.11 g, $p < 0.0005$) (Table 3.2). Body weight, heart weight and fat weight were corrected for differences in animal size using the tibia length of each animal. Both body weight-to-tibia ratio (1.511 ± 0.04403 g/mm vs. 2.179 ± 0.1180 g/mm, $p < 0.005$) and fat weight-to-tibia ratio (30.81 ± 5.354 mg/mm vs. 159.7 ± 25.86 mg/mm, $p < 0.005$) was significantly increased in DIO mice whereas no significant differences were observed in heart weight-to-tibia ratio (7.088 ± 0.6001 mg/mm vs. 7.997 ± 0.4751 mg/mm, $p = \text{ns}$) (Figure 3.1).

Table 3.2: Body composition after 8 weeks

	Control (n=5)	DIO (n=7)	DIO-R (n=3)
<i>Body weight (g)</i>			
Initial body weight	20.7 ± 1.25	20.57 ± 1.84	20.0 ± 0.50
End body weight	28.4 ± 1.14	$40.43 \pm 2.07^{***}$	$33.0 \pm 0.00^{**}$
Body weight gain	7.7 ± 1.60	$19.86 \pm 1.49^{***}$	$13.0 \pm 0.50^{**}$
<i>Fat pad masses (g)</i>	0.58 ± 0.11	$2.97 \pm 0.51^{***}$	$2.62 \pm 0.67^{***}$
<i>Tibia length (mm)</i>	18.80 ± 0.45	18.57 ± 0.79	18.83 ± 0.29

** $p < 0.005$; *** $p < 0.0005$

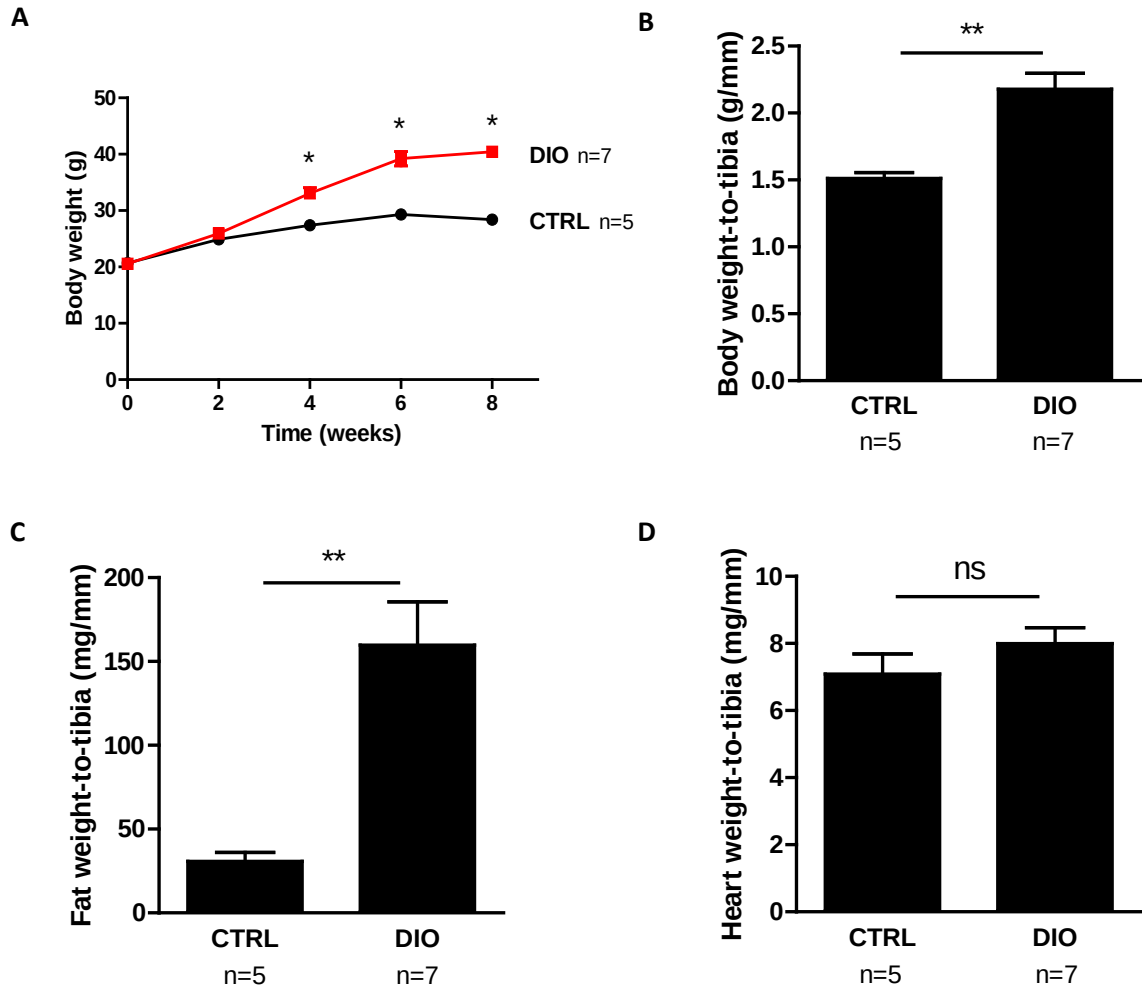


Figure 3.1: Body composition analysis after 8 weeks of high fat diet feeding.

(A) Body weight measured every two weeks in DIO (red) and control animals (black). (B) End body weight normalised to tibia length in g/mm. (C) Fat weight normalised to tibia length in mg/mm. (D) Heart weight normalised to tibia length in mg/mm. Data are expressed as mean±SD with n=5 for controls and n=7 for DIO mice. Data were analysed using Student's t-test. ns, not significant; * p<0.05, ** p<0.005.

3.3.2.2 Fasting serum insulin, glucose levels and oral glucose tolerance

Fasting serum insulin levels were measured in DIO after 8 weeks of high fat diet, age-matched *ob/ob* and control mice. DIO and *ob/ob* mice had increased fasting insulin levels compared to controls. However, a significant increase was only observed in *ob/ob* mice whereas only a trend was observed in DIO mice (2.67 ± 0.56 ng/mL vs. 0.42 ± 0.03 ng/mL for DIO vs. controls, $p = \text{ns}$ and 3.98 ± 0.68 ng/mL vs. 0.42 ± 0.03 ng/mL for *ob/ob* vs. controls, $p < 0.005$) (Figure 3.2).

Baseline fasting glucose levels in DIO after 8 weeks of high fat diet were slightly elevated compared to controls and age-matched *ob/ob* mice (10.80 ± 1.05 mmol/L vs. 7.40 ± 1.27 mmol/L, $p = \text{ns}$ for DIO vs. *ob/ob* and 10.80 ± 1.05 mmol/L vs. 6.88 ± 1.48 mmol/L, $p = \text{ns}$ for DIO vs. control) (Figure 3.3 A). After an oral glucose challenge, blood glucose levels peaked after 30 min for all three groups and returned back to baseline after 120 min in 8 weeks DIO and age-matched *ob/ob* mice. In DIO mice an increase in the area under the curve (AUC) of 53% and an increase of 28% was observed in age-matched *ob/ob* mice compared to controls ($p = \text{ns}$) (Figure 3.3 A and B).

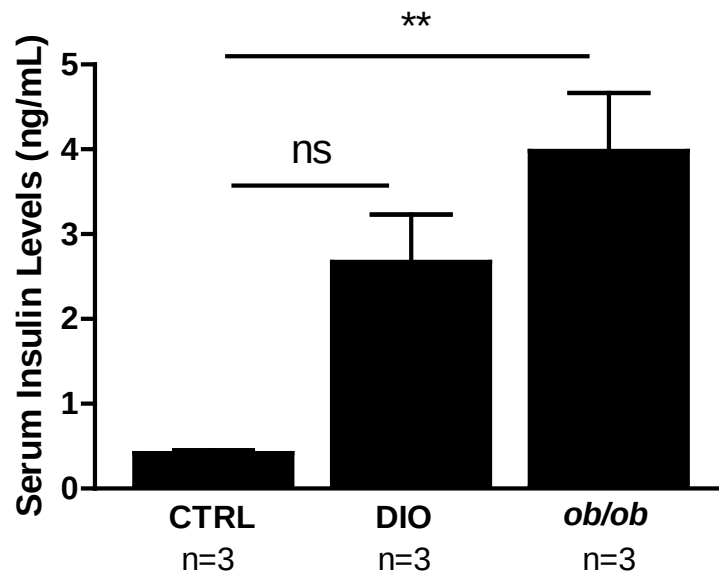


Figure 3.2: Effects of 8 weeks high fat diet on fasting serum insulin levels DIO mice.

Serum insulin levels in DIO after 8 weeks of high fat diet, and compared to control and *ob/ob* mice on normal rodent chow using an insulin ELISA. Insulin levels of each animal were analysed in duplicates. Data are expressed as mean $\pm$ SEM with n=3 for each group. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ns, not significant; ** p<0.005.

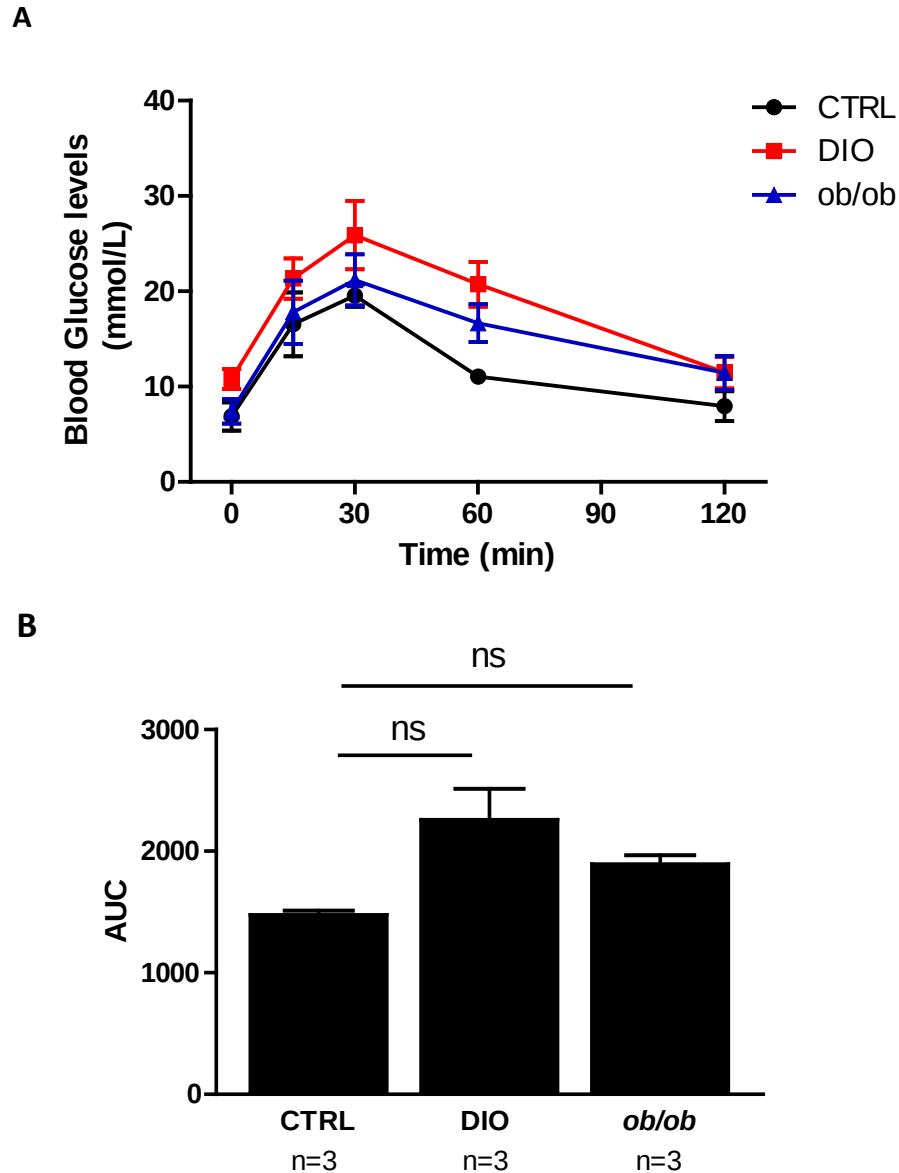


Figure 3.3: Oral glucose tolerance test (OGTT) in DIO animals after 8 weeks of high fat diet feeding, age-matched *ob/ob* and control mice.

(A) OGTT in DIO after 8 weeks of high fat diet (red) as well as age-matched *ob/ob* (blue) and control animals (black). Blood glucose levels at baseline, 30, 60 and 120 min after an oral glucose load. (B) Area under the curve (AUC) analysis of OGTT. Data are expressed as mean±SEM with n=3 for each group. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ns, not significant.

3.3.3 Effects of 11 weeks of high fat diet feeding

3.3.3.1 Weight gain and body composition

After 11 weeks of high fat diet, 75% of the animals were defined as DIO. Over the feeding period, DIO mice had a significant increase in body weight gain (Figure 3.4) and a total body weight gain of 22.56 ± 3.19 g compared to 9.04 ± 3.59 g of controls ($p < 0.0005$). As already observed after 8 weeks of high fat diet feeding, visceral fat pad masses were significantly heavier than in the control mice (2.99 ± 0.46 g vs. 0.70 ± 0.33 g, $p < 0.0005$) (Table 3.3). No significant differences were found in initial body weight or tibia length among the high fat diet and control groups. However, when examining the animals that did not respond to the high fat diet, lower initial body weight in this group was observed compared to DIO and control mice (17.52 ± 2.105 g vs. 20.33 ± 1.733 g, $p < 0.0005$ for DIO-R vs. control mice and 17.52 ± 2.105 g vs. 19.51 ± 1.629 g, $p < 0.005$ for DIO-R vs. DIO). Body weight, heart weight and fat weight were corrected for differences in animal size using the tibia length of each animal. As seen after 8 weeks, a significant increase in body weight-to-tibia (2.230 ± 0.1520 g/mm vs. 1.576 ± 0.1337 g/mm, $p < 0.0005$) and fat weight-to-tibia ratio (158.1 ± 22.80 mg/mm vs. 39.63 ± 16.06 mg/mm, $p < 0.0005$) was observed in DIO compared to age-matched controls. Additionally, significant changes in heart weight-to-tibia ratio were observed (7.492 ± 0.7744 mg/mm vs. 6.844 ± 0.6091 mg/mm, $p < 0.05$) (Figure 3.4).

Table 3.3: Body composition after 11 weeks

	Control (n=18)	DIO (n=30)	DIO-R (n=10)
Body weight (g)			
Initial body weight	20.33 ± 1.73	19.51 ± 1.63	$17.52 \pm 2.11^{***}$
End body weight	28.8 ± 2.73	$42.07 \pm 3.15^{***}$	$33.70 \pm 2.87^{***}$
Body weight gain	9.04 ± 3.59	$22.56 \pm 3.19^{***}$	$16.18 \pm 3.47^{***}$
Fat pad masses (g)	0.70 ± 0.33	$2.99 \pm 0.46^{***}$	$2.37 \pm 0.54^{***}$
Tibia length (mm)	18.95 ± 0.63	18.87 ± 0.70	18.50 ± 0.79

*** $p < 0.0005$

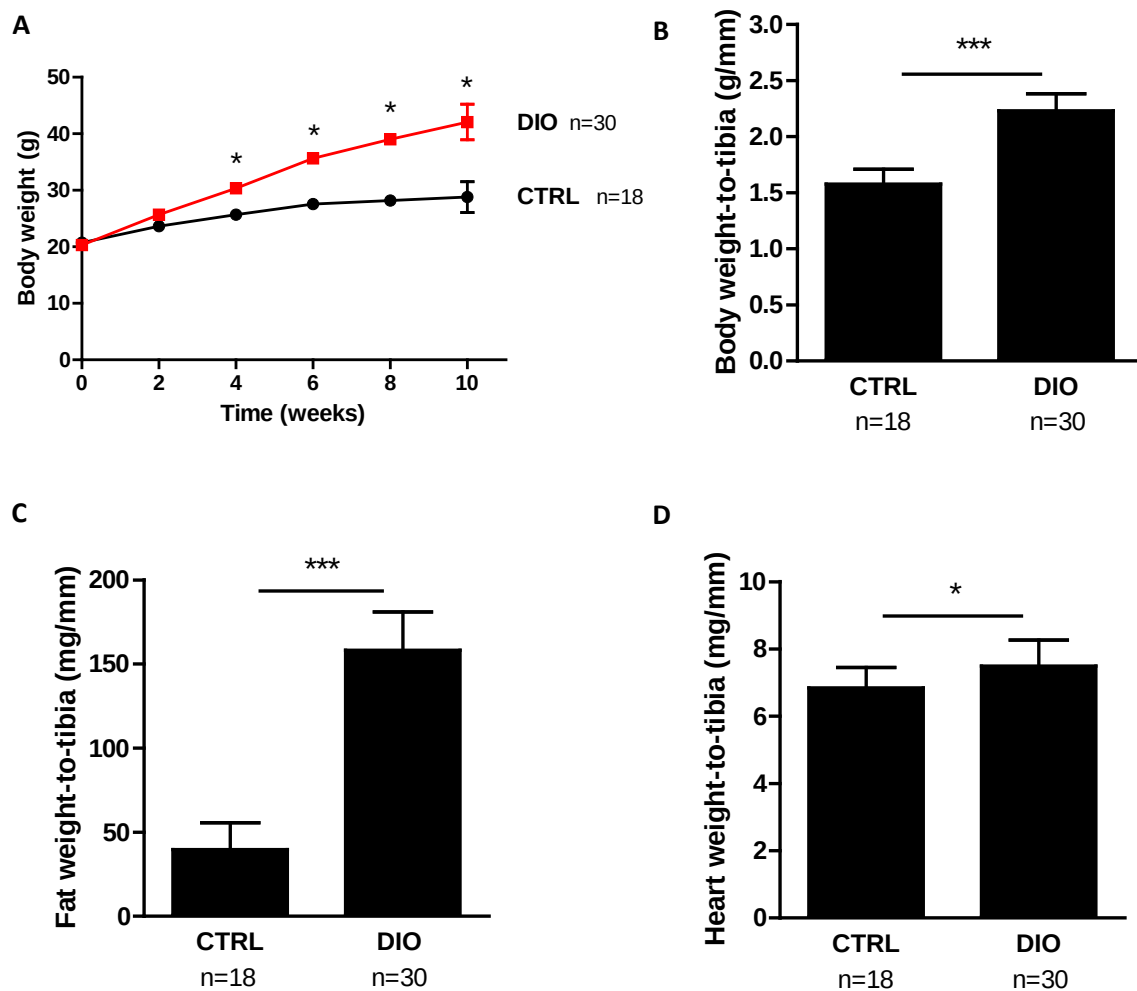


Figure 3.4: Body composition analysis after 11 weeks of high fat diet feeding.

(A) Body weight measured every two weeks in DIO (red) and control animals (black). (B) End body weight normalised to tibia length in g/mm. (C) Fat weight normalised to tibia length in mg/mm. (D) Heart weight normalised to tibia length in mg/mm. Data are expressed as mean $\pm$ SD with n=18 for controls and n=30 for DIO mice. Data were analysed using Student's t-test. * $p < 0.05$, *** $p < 0.0005$.

3.3.3.2 Fasting serum insulin, glucose levels and oral glucose tolerance

DIO mice after 11 weeks of high fat diet feeding and age-matched *ob/ob* mice had significantly increased serum insulin levels after a 12 h over night fast (2.27 ± 0.37 ng/mL vs. 0.59 ± 0.03 ng/mL, $p < 0.005$ for DIO vs. controls and 4.26 ± 0.23 ng/mL vs. 0.59 ± 0.03 ng/mL, $p < 0.0005$ for *ob/ob* vs. controls) (Figure 3.5).

Serum glucose levels were measured in DIO after 11 weeks of high fat diet feeding, age-matched *ob/ob* and control mice after a 12 h over night fast. Baseline glucose levels were significantly elevated in DIO but not in *ob/ob* mice compared to controls (10.42 ± 0.45 mmol/L vs. 7.17 ± 0.40 mmol/L for DIO vs. controls, $p < 0.0005$ and 7.95 ± 0.78 mmol/L vs. 7.17 ± 0.40 mmol/L for *ob/ob* vs. controls, $p = \text{ns}$). After an oral glucose challenge, impaired glucose tolerance in DIO was observed, evidenced by an increase in the AUC of 99% ($p < 0.0005$) compared to only 34% in age-matched *ob/ob* mice (Figure 3.6). End glucose levels tended to be higher after 120 min in both DIO and *ob/ob* mice.

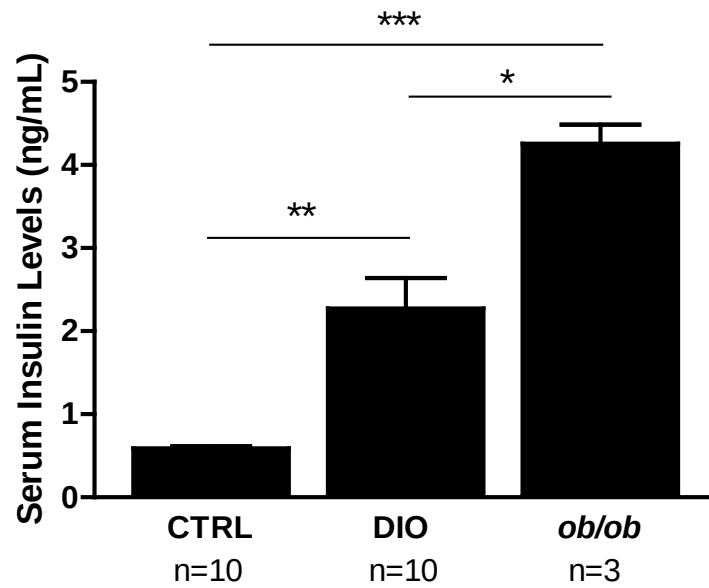


Figure 3.5: Effects of 11 weeks high fat diet on fasting serum insulin levels DIO mice.

Serum insulin levels in DIO after 11 weeks of high fat diet, and compared to control and *ob/ob* mice on normal rodent chow using an insulin ELISA. Insulin levels of each animal were analysed in duplicates. Data are expressed as mean $\pm$ SEM with n=3 for *ob/ob* and n=10 for DIO and controls. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison.

* p<0.05; ** p<0.005, *** p<0.0005.

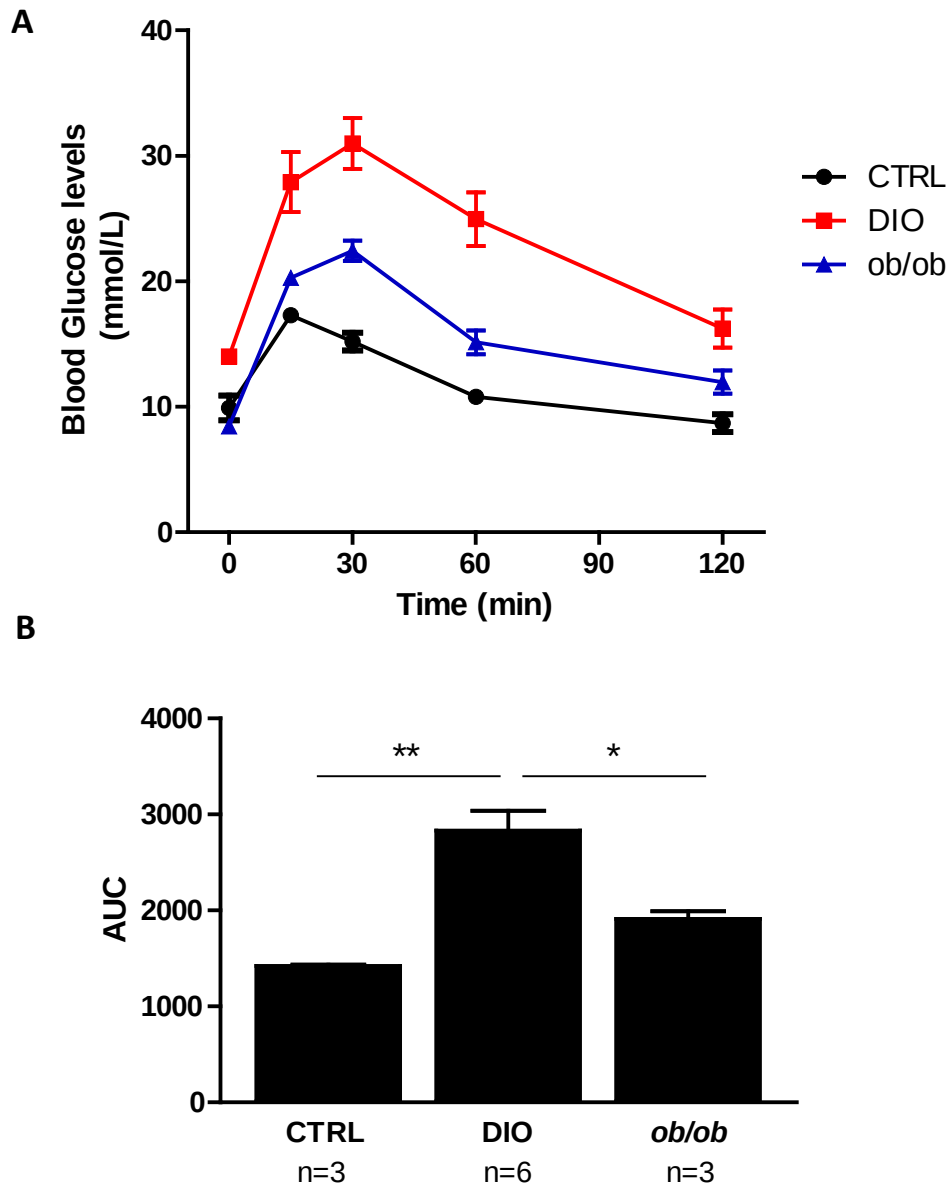


Figure 3.6: Oral glucose tolerance test (OGTT) in DIO animals after 11 weeks of high fat diet feeding, age-matched *ob/ob* and control mice.

(A) OGTT in DIO after 11 weeks of high fat diet (red) as well as age-matched *ob/ob* (blue) and control animals (black). Blood glucose levels at baseline, 30, 60 and 120 min after an oral glucose load. (B) AUC analysis of OGTT. Data are expressed as mean $\pm$ SEM with n=3 for controls and *ob/ob* mice and n=6 for DIO mice. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. * p<0.05, ** p<0.005.

3.3.4 Effects of 14 weeks of high fat diet feeding

3.3.4.1 Weight gain and body composition

After 14 weeks of high fat diet feeding, 70.6% of the mice were defined as DIO. Animals showed a significant increase in body weight over the 14 weeks with a total body weight gain of 25.41 ± 1.85 g compared to 12.22 ± 1.56 g of the age-matched control group ($p < 0.0005$) (Table 3.4). Accordingly, visceral fat-pad masses were significantly increased compared to age-matched controls (2.71 ± 0.31 g vs. 0.75 ± 0.28 g, $p < 0.0005$) (Figure 3.8). Upon visual examination of the whole body, a larger amount of white adipose tissue was observed around the heart of DIO mice compared to age-matched controls (Figure 3.7). No significant differences were found in initial body weight (20.80 ± 1.64 g vs. 20.74 ± 1.61 g, $p = \text{ns}$) or tibia length (19.23 ± 0.5250 mm vs. 18.88 ± 0.3769 mm, $p = \text{ns}$) between high fat diet and control groups. Body, fat and heart weight was corrected for possible size differences between groups using tibia length. For all three parameters a significant difference was observed between DIO and age-matched control mice.

Table 3.4: Body composition after 14 weeks

	Control (n=10)	DIO (n=12)	DIO-R (n=5)
<i>Body weight (g)</i>			
Initial body weight	20.74 ± 1.61	20.80 ± 1.64	18.90 ± 0.65
End body weight	32.66 ± 2.29	$46.21 \pm 1.41^{***}$	$37.00 \pm 2.92^{**}$
Body weight gain	12.22 ± 1.56	$25.41 \pm 1.85^{***}$	$18.10 \pm 2.68^{***}$
<i>Fat pad masses (g)</i>	0.75 ± 0.28	$2.71 \pm 0.31^{***}$	$2.46 \pm 0.61^{***}$
<i>Tibia length (mm)</i>	18.88 ± 0.38	19.23 ± 0.53	18.80 ± 0.27

** $p < 0.005$; *** $p < 0.0005$

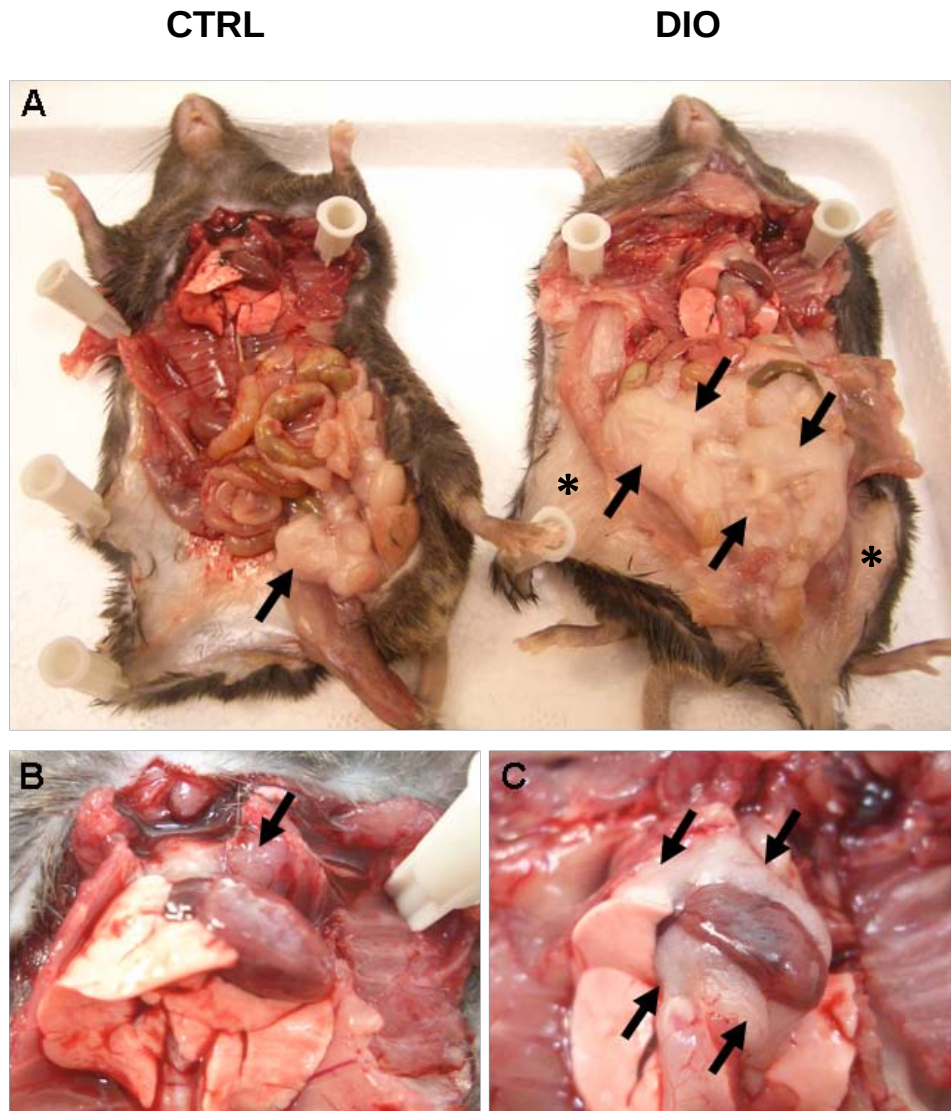


Figure 3.7: Visual comparison between DIO mouse after 14 weeks of high fat diet feeding and age-matched control mouse.

(A) Whole body view of control mouse (left) and DIO mouse (right). (B and C) Close-up image of representative control and DIO heart. A larger amount of visceral adipose tissue is noticeable around the heart of the DIO mouse (C) compared to the control heart (B). Black arrows indicate visceral adipose tissue whereas black asterisks indicate subcutaneous adipose tissue.

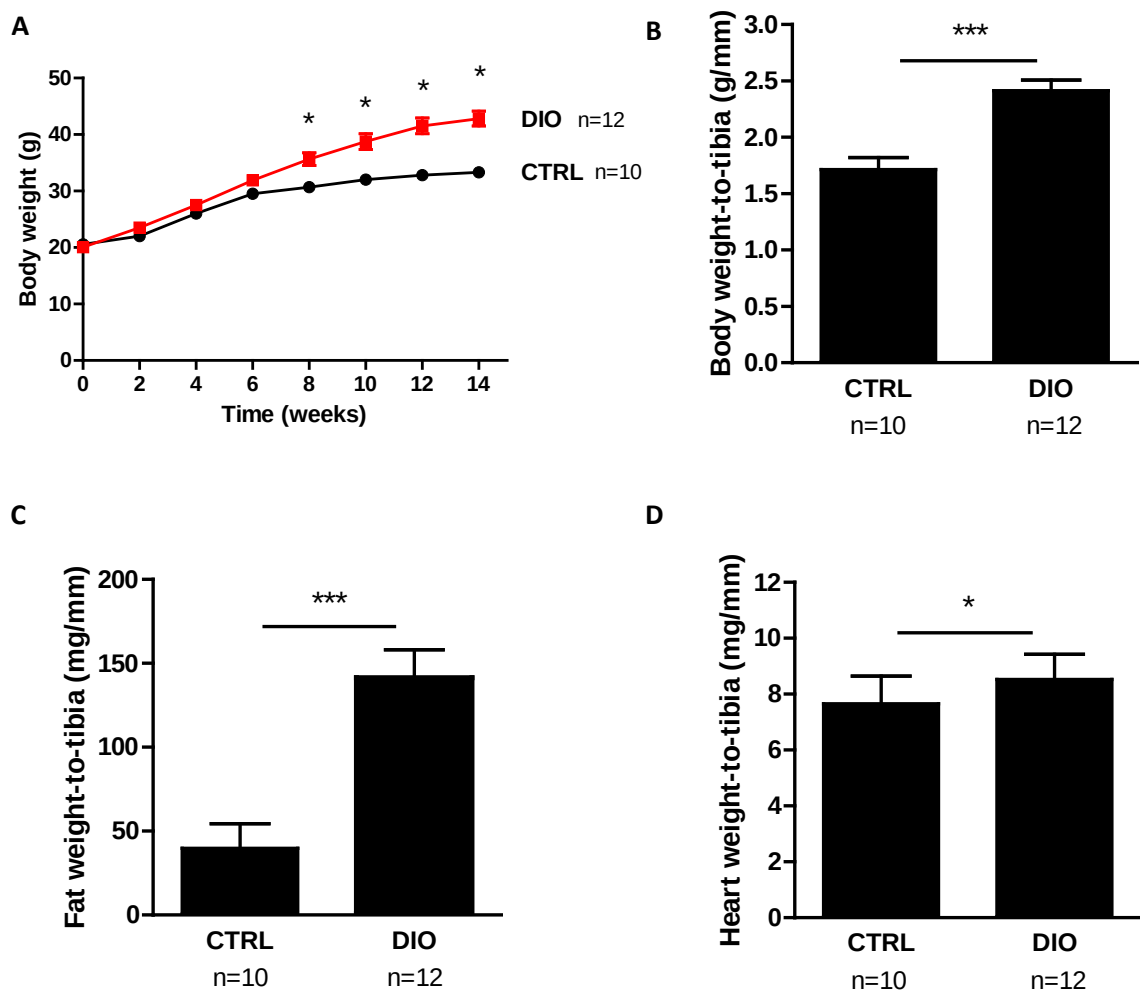


Figure 3.8: Body composition analysis after 14 weeks of high fat diet feeding.

(A) Body weight measured every two weeks in DIO (red) and control animals (black). (B) End body weight normalised to tibia length in g/mm. (C) Fat weight normalised to tibia length in mg/mm. (D) Heart weight normalised to tibia length in mg/mm. Data are expressed as mean±SD with n=10 for controls and n=12 for DIO mice. Data were analysed using Student's t-test. * p<0.05, *** p<0.0005.

3.3.4.2 Fasting serum insulin, glucose levels and oral glucose tolerance

After 14 weeks of high fat diet serum insulin levels of DIO mice were significantly elevated after a 12 h fast compared to normal controls (3.394 ± 0.7452 ng/mL vs. 0.6628 ± 0.1395 ng/mL, $p < 0.05$) (Figure 3.9). Age-matched *ob/ob* mice had a significant increase in fasting serum insulin levels compared to controls and DIO mice (7.76 ± 0.8492 ng/mL vs. 0.6628 ± 0.1395 ng/mL, $p < 0.0005$ for *ob/ob* vs. control) (Figure 3.9).

Baseline glucose levels were significantly elevated in DIO compared to age-matched controls (11.97 ± 1.162 mmol/L vs. 8.733 ± 0.4937 mmol/L, $p < 0.05$) but not in *ob/ob* mice (7.067 ± 0.2728 mmol/L vs. 8.733 ± 0.4937 mmol/L, $p = \text{ns}$). After an oral glucose challenge the AUC was significantly increased by 224% compared to only 72% in age-matched *ob/ob* mice ($p < 0.0005$ for DIO vs. controls and $p < 0.05$ for *ob/ob* vs. controls) (Figure 3.10). Two hours after administration of the oral glucose bolus plasma glucose levels in DIO had not yet returned to the fasting baseline value ($p < 0.0005$).

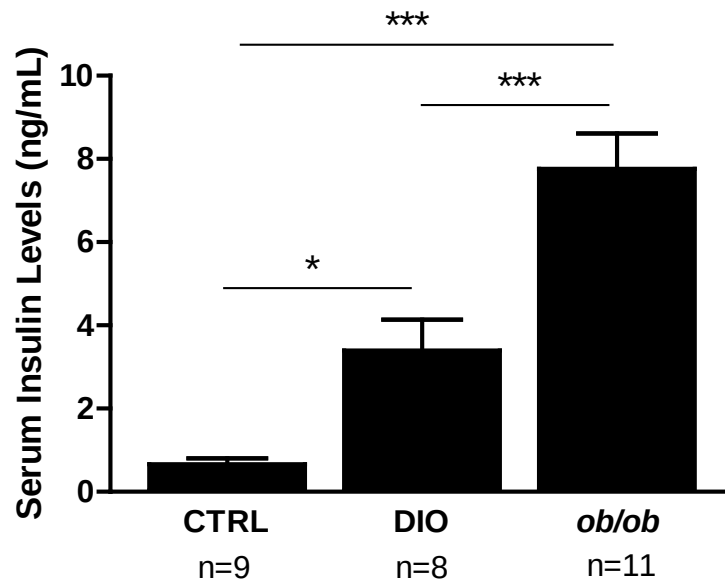
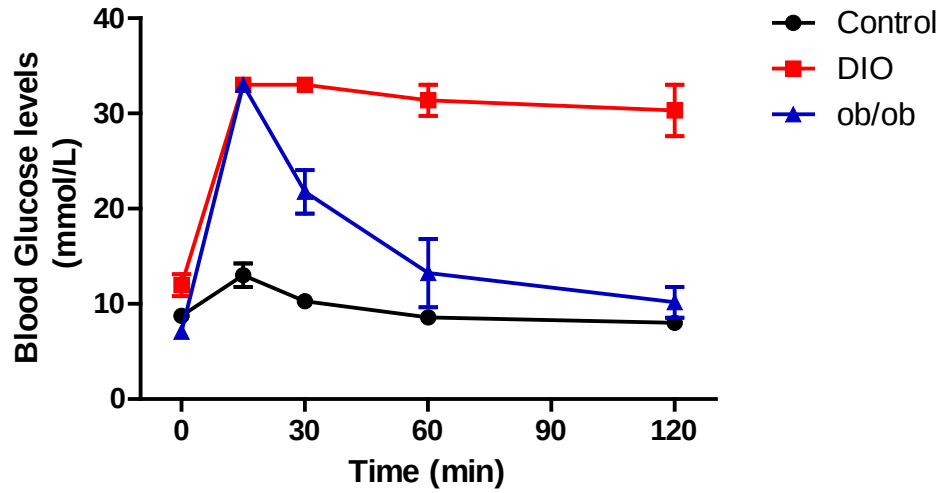


Figure 3.9: Effects of 14 weeks high fat diet on fasting serum insulin levels DIO mice.

Serum insulin levels in DIO after 14 weeks of high fat diet, and compared to control and *ob/ob* mice on normal rodent chow using an insulin ELISA. Insulin levels of each animal were analysed in duplicates. Data are expressed as mean $\pm$ SEM with n=9 for controls, n=8 for DIO and n=11 for *ob/ob* mice. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. * p<0.05; *** p<0.0005.

A



B

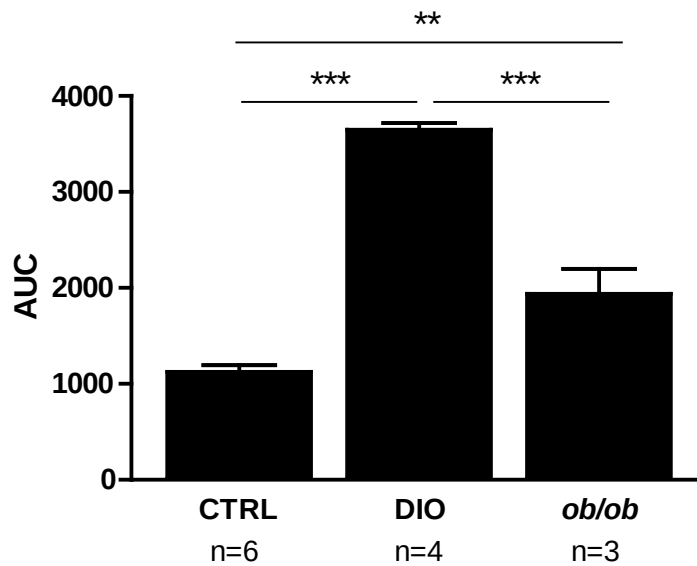


Figure 3.10: Oral glucose tolerance test (OGTT) in DIO animals after 14 weeks of high fat diet feeding, age-matched *ob/ob* and control mice.

(A) OGTT in DIO after 14 weeks of high fat diet (red) as well as age-matched *ob/ob* (blue) and control animals (black). Blood glucose levels at baseline, 30, 60 and 120 min after an oral glucose load. (B) Area under the curve (AUC) analysis of OGTT. Data are expressed as mean $\pm$ SEM with n=6 for controls, n=4 for DIO and n=3 for *ob/ob* mice. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ** p<0.005, *** p<0.0005.

3.3.4.3 Effects of high fat diet on of heart, liver and kidney after 14 weeks

The results described above clearly show that feeding a lard-based high fat diet will lead to a gradual increase in body weight, fasting insulin and glucose levels as well as to a gradual impairment of glucose tolerance with the most severe phenotype after 14 weeks of high fat diet. Since elevated blood glucose and insulin levels are associated with pathological changes in heart, liver and kidney of diabetic patients [85, 177, 178] the aim of this part of the study was to identify whether histopathological changes are associated with prolonged exposure to hyperglycaemia and hyperinsulinaemia. For this, histological sections from DIO animals fed the high fat diet for 14 weeks were examined.

3.3.4.3.1 Phenotypical analysis of the heart

In the development of diabetic cardiomyopathy structural changes such as cardiomyocyte hypertrophy, interstitial and perivascular fibrosis or increased matrix collagen have been described in the heart [13, 127]. To investigate whether high fat diet feeding lead to cardiomyocyte hypertrophy we looked at the heart weight as well as the nucleus-to-myocyte ratio in left ventricle of DIO mice after 14 weeks of high fat diet. Analysis of DIO hearts showed a significant increase in the heart weight-to-tibia ratio (8.520 ± 0.9088 mg/mm vs. 7.651 ± 0.9925 mg/mm, $p < 0.05$) compared to age-matched control hearts. Concomitantly to the increase in heart weight-to-tibia ratio a significant increase in interstitial fibrosis (measuring the total area of fibrosis) was observed in DIO mice compared to age-matched controls (7205 ± 921 μm^2 vs. 2891 ± 416.7 μm^2 , $p < 0.05$) (Figure 3.11). No significant differences in the nucleus-to-myocyte ratio were observed between DIO and control hearts.

Furthermore, investigation of intramyocardial coronary arterioles in left ventricles of DIO mice showed inward muscular remodelling with slight thickening of the intimal layer after 14 weeks of high fat diet feeding. Interestingly, no coronary microvascular remodelling was observed in age-matched controls as well as age-matched *ob/ob* mice (Figure 3.12).

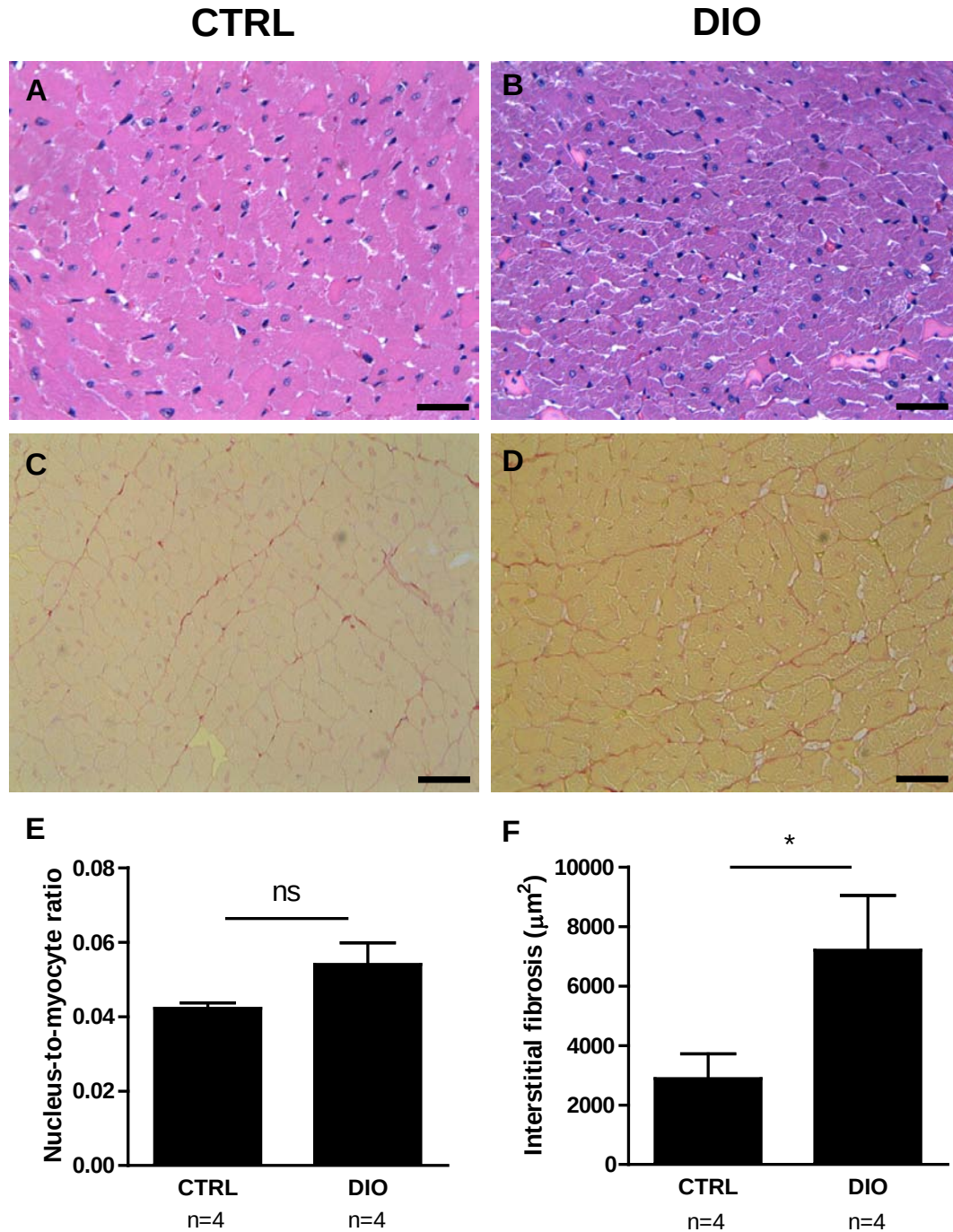


Figure 3.11: Histological analysis of the left ventricle in 14 weeks DIO and control animals.

Histological sections of left ventricles from control (A and C) and DIO (B and D) mice. (A and B) Haematoxylin and eosin staining. Nuclei are stained in dark blue whereas eosinophilic structures like cytoplasm or intra- and extracellular proteins are stained in shades of red. (C and D) Picro-sirius red staining of collagen (red) on a pale yellow background (cardiomyocytes). Images were

taken at 40x magnification and are representative for n=4 in each group. Black scale bars indicate 50 μm . (E) Nucleus-to-myocyte ratio of left ventricular sections in DIO and control animals. Data are expressed as mean $\pm$ SEM. (F) Quantification of interstitial fibrosis in μm^2 in DIO and controls. Data are expressed as mean $\pm$ SD. Data was analysed using Student's t-test. ns, not significant, * $p<0.05$. Histological analysis was carried out by Dr Massimiliano Mancini, La Sapienza University, Rome.

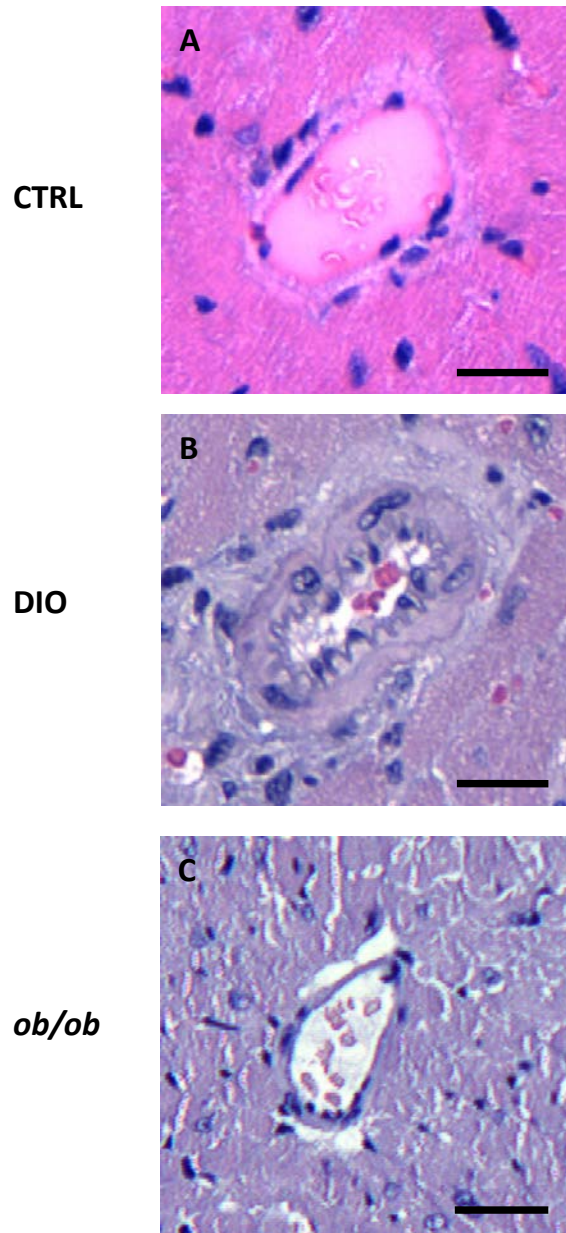


Figure 3.12: Histological changes in small intramyocardial coronary arterioles in left ventricles of 14 weeks DIO mice.

Representative histological sections of small arterioles in left ventricles of control (A) (n=4), DIO (B) (n=4) and *ob/ob* mice (C) (n=3) stained with haematoxylin and eosin using 40x magnification. Nuclei are stained in dark blue whereas eosinophilic structures like cytoplasm, intra- and extracellular proteins or red blood cells are stained in shades of red. Thickening of the medial area is present in DIO (B) but not in age-matched controls (A) and *ob/ob* mice (C). Black scale bars indicate 25 μm. Histological analysis was carried out by Dr Massimiliano Mancini, La Sapienza University, Rome.

3.3.4.3.2 Phenotypical analysis of the liver

In the liver, lipid accumulation has been associated with high fat diet feeding [59, 174, 176]. After 14 weeks of high fat diet livers of DIO mice were paler in colour and significantly increased in weight compared to age-matched controls (2364 ± 461.0 mg vs. 1330 ± 141.1 mg, $p < 0.005$). Additionally, when correcting for size differences between animals, a significant increase in the liver weight-to-tibia ratio was observed in DIO mice compared to controls (119 ± 23.20 mg/mm vs. 68.39 ± 7.384 mg/mm, $p < 0.005$).

Histological analysis of haematoxylin and eosin-stained liver sections revealed normal hepatic architecture in control animals whereas in DIO mice cytoplasmic lipid accumulation (microvescicular steatosis) but mostly distortion of the nucleus by lipid accumulation (macrovescicular hepatic steatosis) involving up to 99% of hepatocytes was observed. Micro- and macrovescicular steatosis were present predominantly in the periportal and midzone areas. No signs of inflammation (steatohepatitis) were found in either control or DIO group (Figure 3.13).

3.3.4.3.3 Phenotypical analysis of the kidney

Kidney weight in DIO animals was significantly increased compared to age-matched control mice (181.3 ± 11.17 mg vs. 144.0 ± 22.61 mg, $p < 0.05$). After correcting for size differences the kidney-to-tibia ratio in DIO mice was also significantly elevated (9.510 ± 0.4840 mg/mm vs. 7.351 ± 1.171 mg/mm, $p < 0.05$). Analysis of DIO kidneys showed no substantial histological changes in haematoxylin and eosin as well as picro-sirius red-stained sections at low magnification (see Appendix B – Kidney Histology). At a higher magnification, kidneys from DIO animals showed a significant increase in glomerulus area compared to controls (16699 ± 879.6 μm^2 vs. 12703 ± 261.0 μm^2 , $p < 0.05$, $n=3$) (Figure 3.14).

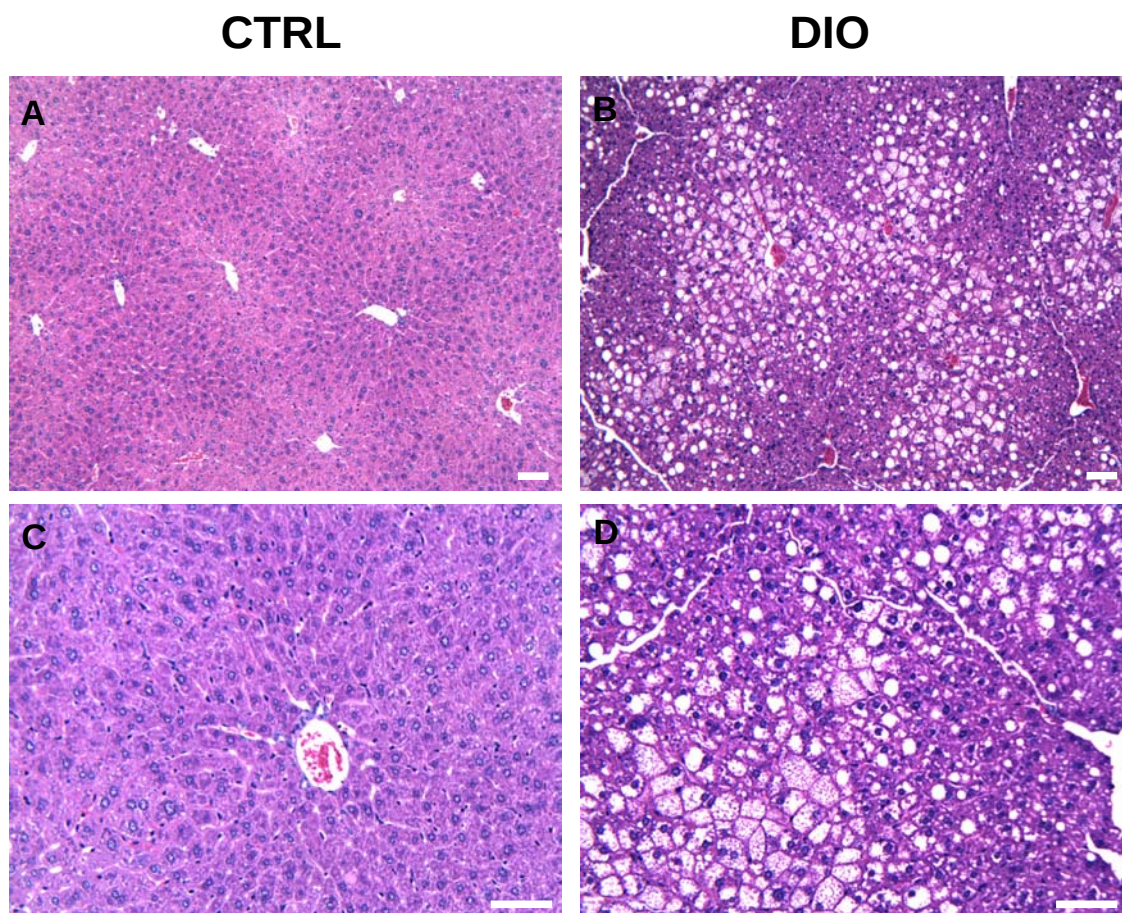


Figure 3.13: Histological changes in liver after 14 weeks of high fat diet feeding.

Histological sections of livers from control (A and C) and DIO mice (B and D) stained with haematoxylin and eosin. (A and B) Low magnification (10x) of liver sections from control animals (A) and DIO mice (B). White scale bars indicate 100 μ m. (C and D) High magnification (40x) of liver tissue from control animals (C) and DIO mice (D). White scale bars indicate 50 μ m. Nuclei are stained dark blue whereas eosinophilic structures like cytoplasm are stained in shades of red. White droplets in DIO sections (B and D) indicate lipid accumulation. Images are representative for n=4 in each group. Histological analysis was carried out by Dr Massimiliano Mancini, La Sapienza University, Rome.

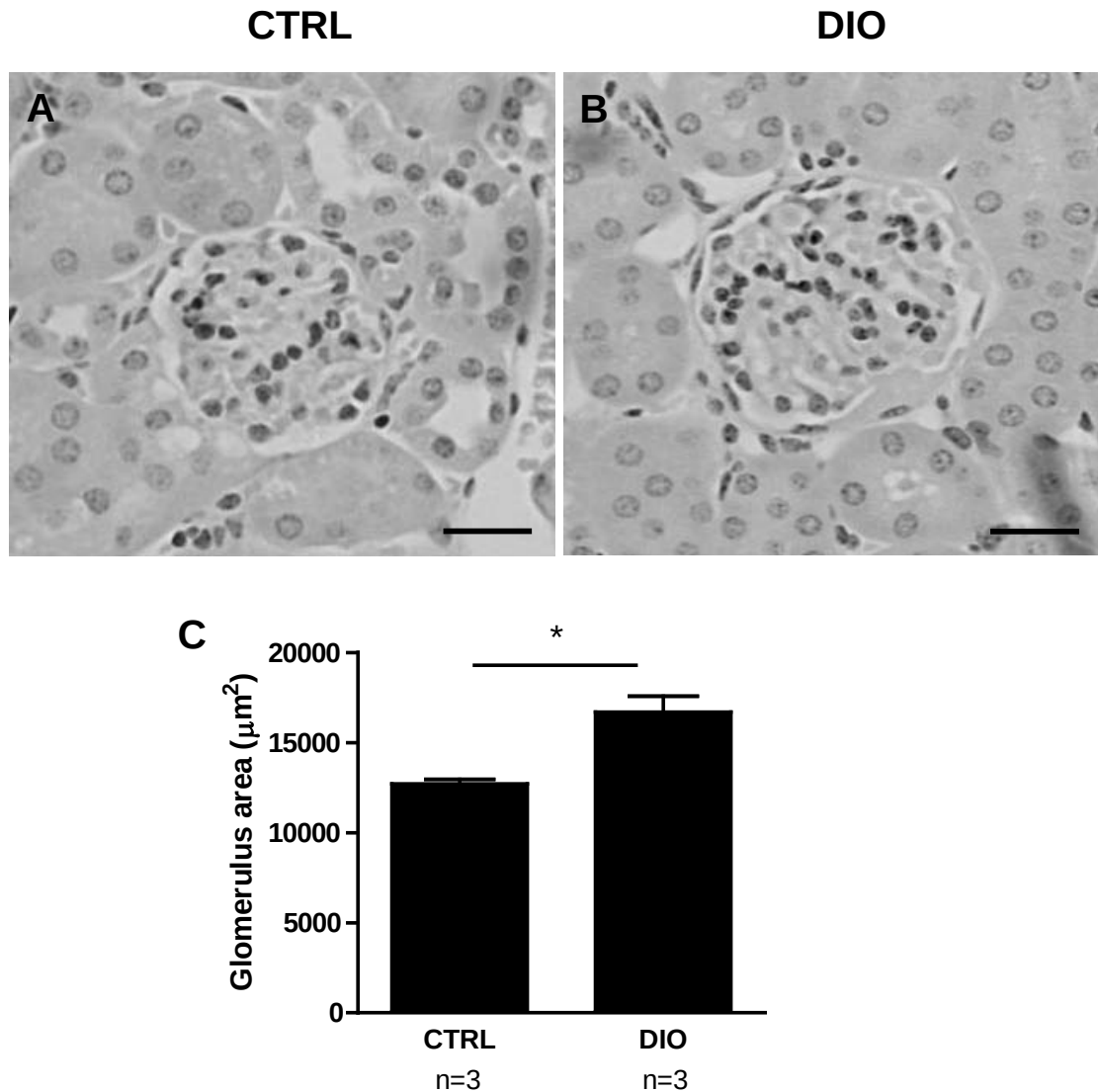


Figure 3.14: Histological changes of glomeruli in the kidney after 14 weeks of high fat diet feeding.

Representative histological sections of a glomerulus from control animals (A) and DIO (B) stained with haematoxylin and eosin (20x magnification). Nuclei are shown in black and eosinophilic structures in shades of grey. Black scale bars indicate 50 μm. Results are representative for n=3 in each group. (C) Quantification of glomerulus area in kidneys from control and DIO mice. Data are expressed as mean±SEM and representative for n=3 in each group. More than 20 glomeruli were measured in each kidney using ImageJ software. Data was analysed using Student's t-test. * p<0.05.

3.4 Discussion

The high fat diet fed C57BL/6 mouse is a recognised model of environmentally induced T2DM and obesity [160]. In our study animals were fed a high fat diet with 45% of its energy derived from fat for 8, 11 and 14 weeks to gain insight into the natural history of metabolic disturbances and pathological alterations of the DIO model but also to determine the most appropriate time point to study molecular and genetic mechanisms of myocardial insulin resistance. The main findings in the pilot study are that a lard-based high fat diet will gradually lead to an increase in the characteristic risk factors contributing to the development of insulin resistance and eventually T2DM. Microscopic changes in the liver and kidney exposed to hyperglycaemia and hyperinsulinaemia were observed and most importantly, previously not described coronary microvascular remodelling was observed in hearts of the diet-induced mouse models.

3.4.1 Effects of high fat diet feeding on metabolic parameters

The diet fed to our animals was a 45% fat diet with lard as the fat source. The term 'high fat diet' is used in the literature for various diets with different fatty acid compositions and this increases the variability of results and complicates comparisons between different studies [179]. Compared to the very high fat diet with 60% fat which is commonly used in obesity studies, the 45% fat diet has been suggested to mimic more a Western-style diet [161]. With 45% of calories deriving from fat, the relative caloric load of proteins and carbohydrates changes (20% from protein and 35% from carbohydrates) and therefore the macronutrient composition is different to the normal rodent chow used (11.5% from fat, 27.3% from protein and 61.2% from carbohydrates). With regards to the fatty acid profile, studies have shown that diets based on polyunsaturated omega-3 fatty acids have a beneficial effect on body composition and insulin action and therefore might decrease cardiovascular morbidity and mortality [180]. Diets based on lard have comparable quantities of saturated and monounsaturated fatty acids. Compared to other fat sources like coconut fat

(saturated fatty acids), olive oil (monounsaturated fatty acids), or fish oil (polyunsaturated fatty acids) lard is known to induce the typical high fat diet phenotype [179] and together with the altered macronutrient composition will lead to the development and progression of metabolic changes associated with insulin resistance and T2DM. However, as the human diet is more complex, a diet rich in carbohydrates together with fat might be closer to the human (Western) diet than a high fat diet alone [163].

Despite identical backgrounds, in our experiments on average only 73% of the mice significantly gained weight whereas the remaining 27% were able to maintain a normal body weight. This phenomenon has been previously reported in a study by Enriori *et al.* in which these animals were defined as having body weights ranging between the average ± 3 standard deviations of the control group and were termed diet-induced obese resistant (DIO-R) [167]. Enriori and co-workers studied leptin resistance in the brain by feeding C57BL/6 mice a 45% high fat diet for 20 weeks. They found that the only predictor of DIO-R versus DIO mice was the lower initial body weights at 6 weeks of age, which significantly correlated to final body weights [167]. In our study, a significant difference in initial body weight between DIO-R and DIO mice was observed in the 11 weeks high fat diet group which is in accordance with their finding. Mice which were defined as DIO-R in our study had a significantly lower initial body weight compared to both controls and DIO mice. Additionally, Enriori and co-workers showed that calorie intake in DIO-R animals decreased significantly from the fifth week until the end of the experiment [167]. It would be of interest to further investigate the underlying mechanisms of this phenomenon of resistance to high fat diet and therefore gain further insight into why some people are more prone to obesity than others and which genetic and environmental factors are associated with the development of obesity.

High fat diet feeding resulted in a gradual increase in the characteristic metabolic features associated with T2DM with the most prominent metabolic changes in DIO mice after 14 weeks of high fat diet (Table 3.5). After 14 weeks of high fat diet animals had significantly increased serum insulin levels and a severely impaired oral glucose tolerance compared to age-matched *ob/ob* mice and controls. Both DIO groups after 8 and 11 weeks of high fat diet showed elevated serum insulin levels but only in 11 weeks DIO mice insulin levels were significantly increased compared to age-matched controls. Although the insulin levels are similar between the 8 and 11 weeks groups, only a trend was observed in 8 weeks DIO mice which can be explained by the smaller sample size and larger variation between animals compared to the 11 weeks DIO group. Similarly, the response of 11 weeks DIO mice to an oral glucose load was an intermediate between the 8 and 14 weeks DIO mice and both 8 and 11 weeks DIO mice showed poor glucose disposal. Moreover, fasting serum glucose levels in 11 and 14 weeks DIO were significantly increased compared to age-matched *ob/ob* mice and controls.

Table 3.5: Summary of metabolic changes in DIO relative to age-matched controls

	8 weeks	11 weeks	14 weeks
Fasting insulin	↑ (ns)	↑ (**)	↑ (*)
Fasting glucose	↑ (ns)	↑ (***)	↑ (*)
OGTT (AUC, % increase)	53 (ns)	99 (**)	224 (***)

OGTT: oral glucose tolerance test; AUC: area under the curve;

↑: increase; ns, not significant; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$

Our results are in line with the metabolic data from other recently published studies [74, 75, 166]. Park *et al.* showed that high fat diet feeding (55% fat) resulted in a significant increase in body weight and whole-body fat mass already after 1.5 weeks and an increase in basal plasma insulin levels after 3 and 6 weeks of high fat diet feeding [75]. These results are further supported by a publication of Wright and co-workers in which no changes in fasting serum glucose and triglyceride levels but modestly increased insulin levels and a mildly impaired intraperitoneal glucose tolerance was observed after only two weeks on a 45% high fat/high sucrose diet [74]. The observed changes in fasting serum glucose levels in DIO mice represent the counterpart of impaired fasting glucose observed in the early phases of human T2DM. A study assessed the significance of glycaemic control for micro- and macrovascular complications in subjects with late-onset diabetes [181] and showed that hyperglycaemia is likely to be a stronger risk factor for microvascular complications than it is for cardiovascular disease [181]. However, numerous reports have also described the importance of hyperglycaemia in the development and progression of diabetic macrovascular disease in T2DM [182-187] and a correlation of both fasting and postchallenge hyperglycaemia with cardiovascular risk [186]. For example, subjects with impaired glucose tolerance have about a twofold increase in the risk of macrovascular disease including coronary heart disease and stroke compared to healthy subjects [188, 189] and higher CV mortality compared with individuals with impaired fasting glucose [190]. Moreover, in a study on Finnish patients with T2DM at baseline, high fasting plasma glucose predicted CHD events [191], and high fasting plasma glucose and HbA1c predicted fatal and nonfatal stroke [192]. This study, similar to the UKPDS study [193], also demonstrated that conventional CV risk factors like dyslipidaemia, blood pressure or smoking are important contributors to macroangiopathic complications in patients with T2DM.

3.4.2 Effects of high fat diet feeding on body composition and individual organs

At the whole body level the 14 weeks DIO group had an average body weight gain of about 25 g concomitant with a significant increase in visceral fat pad mass and organ weight. Although a gradual increase in body weight from 11 to 14 weeks of high fat diet was observed, visceral fat pad mass did not increase. This could be explained by the fact that not only visceral but also subcutaneous fat mass increased as shown in Figure 3.7 and thus further contributed to the increase in body weight. It is important to note that a large amount of ectopic fat around the kidneys and heart and within the liver was observed, which can impair the organ function by either physical compression or the secretion of locally acting substances by periorgan fat cells [194]. Also, accumulation of fat within the organ could lead to cell dysfunction and cell death (lipotoxicity) [194].

Our results show that together with the gradual development of visceral adiposity accumulation of fat droplets in the liver was observed. Livers of DIO animals after 14 weeks of high fat diet were paler in colour which suggests lipid accumulation and liver weight was significantly increased. Histological analysis revealed micro- and macrovesicular steatosis with up to 99% of the hepatocytes being affected which is the basis of NAFLD. Kirpich *et al.* showed that a high fat diet consumed for 8 weeks results in obesity with insulin resistance and NAFLD [174]. Similar to our findings, they observed a 2.4 fold increase in insulin levels but no differences in glucose concentrations [174]. Additionally, de Meijer and co-workers recently demonstrated that the development of hepatic steatosis in the C57BL/6 high fat diet mouse model directly results from an increased fat intake that is independent from the amount of calories consumed [176]. Similar to the diet consumed by obese and NAFLD patients, their experimental diet contained predominantly saturated fat and only small amounts of polyunsaturated fat. They hypothesised that saturated fatty acids may promote endoplasmatic reticulum stress and hepatocyte injury resulting in hepatic dysfunction in rodents [176].

Similarly, the kidneys of DIO animals were surrounded by large amounts of fat and showed a significant increase in weight compared to controls. It has been shown that obese rabbits also have larger kidneys as well as large fat deposits in the renal sinus but no fat accumulation in the kidney parenchyma [194]. Furthermore, structural renal changes like glomerulosclerosis, interstitial fibrosis and small vessel disease have been previously demonstrated in other mouse models of T2DM such as the FVB *db/db* mouse as well as in human diabetes [195-197]. After 14 weeks of high fat diet histological changes in DIO kidneys and signs of diabetic nephropathy were expected. Kidney sections from DIO mice showed a significant increase in glomerulus area compared to controls indicating glomerular hypertrophy. These results are supported by a study by Wei *et al.* which has shown glomerular hypertrophy and hyperfiltration in high fat diet fed C57BL/6 mice [198]. Glomerular hypertrophy is associated with diabetic nephropathy and is often observed along with hyperfiltration and hyperglycaemia in T1DM whereas in T2DM it is also possible that other factors like insulin, lipids or leptin may play a role [198]. Studies have shown that glomerular volume correlates with fasting insulin levels in non-diabetic patients with glomerulosclerosis [199] and mesangial expansion in obese Zucker rats [200] which suggests that hyperinsulinaemia in T2DM may evoke glomerular hypertrophy.

In human hearts, Szczepaniak *et al.* have shown a strong association between obesity and increased myocardial lipid accumulation, which correlated with left ventricular hypertrophy and systolic dysfunction [201]. This has been confirmed in models of obesity like the *ob/ob* mouse and Zucker diabetic fatty rat [202, 203]. In Zucker diabetic fatty rats a poor systolic function was observed whereas systolic function was only marginally affected in *ob/ob* mice. However, a clear association between intramyocardial lipid accumulation and diastolic dysfunction was observed in *ob/ob* mice [202]. Besides functional changes, characteristic features in the development of diabetic cardiomyopathy are also structural changes at the macroscopic and microscopic levels such as myocyte

hypertrophy, perivascular fibrosis, increased quantities of matrix collagen, or cellular triglyceride [13]. To investigate myocyte hypertrophy we examined the heart weight as well as the nucleus-to-myocyte ratio in DIO hearts. We observed a significant increase in the corrected heart weight in DIO mice after 11 and 14 weeks of high fat feeding but no significant changes in the nucleus-to-myocyte ratio after 14 weeks of high fat diet were observed compared to controls (a trend to a higher ratio was observed). However, picro-sirius red staining revealed a significant increase in interstitial fibrosis after 14 weeks of high fat diet compared to age-matched controls. Park *et al.* also support this finding showing a significant increase in heart weight of C57BL/6 mice fed a 55% high fat diet only after 20 weeks [75] whereas a significant increase in cardiomyocyte cross-sectional area was observed after 6 months of 45% high fat diet feeding in FVB mice [204]. The duration of high fat diet feeding could in part explain why we did not observe a significant change in nucleus-to-myocyte ratio. However, in genetically induced mouse models of diabetes such as the CIRKO or the dominant negative form of PI3K mouse increased replacement/interstitial fibrosis as well as the development of cardiac hypertrophy was observed [205].

A novel and previously unrecognised finding in our pilot study was the presence of inward muscular remodelling with slight intimal thickening of coronary arterioles after 14 weeks of high fat diet. Microvascular remodelling is defined as alterations in vessel morphology due to restructuring of VSMC and extracellular matrix components [93]. It has been extensively studied in patients with hypertension [104] and been reported in patients with diabetes [100, 206] as well as various animal models of hypertension and diabetes [207-209]. For T2DM, Rizzoni *et al.* showed an increased media-to-lumen ratio in subcutaneous small arteries in T2DM patients [100] and Schofield *et al.* demonstrated abnormalities in myogenic responsiveness in small arteries of diabetic patients which they suggest may explain the observed structural alterations [206]. In a drug-induced rat model of T1DM, Fukuda *et al.* demonstrated the presence of vascular hypertrophy and remodelling in aortas

[210]. In addition, medial thickening and increased media-to-lumen ratio has been observed in the mesenteric microvasculature of STZ-treated rats [211, 212] as well as the GK rat, a spontaneously diabetic, non-obese and normotensive rat model of T2DM [93]. Furthermore, it has been shown that coronary risk factors like diabetes, hypertension, smoking or hyperlipidaemia promote the development of structural and functional changes associated with coronary microvascular remodelling [92]. For example, the spontaneously hypertensive rat (SHR) is characterised by insulin resistance, hyperinsulinaemia, dyslipidaemia, and hypertension [213] and is therefore commonly used to study coronary microvascular remodelling in the context of hypertension [214]. Coronary microvascular remodelling has also been described in patients with HCM, a disease with increased susceptibility to cardiac arrhythmias and risk of sudden death caused by sarcomere protein gene mutations. Despite its clinical relevance, to the best of our knowledge there is no valid rodent model to study microvascular remodelling in the context of HCM, which fully mimics the histopathological and phenotypical characteristics of the disease. Attempts to generate a genetic mouse model of HCM, like the MHC^{403/+} have only been partially successful. The MHC^{403/+} mouse carries a missense mutation in codon 403 (Arg403Gln) of the myosin heavy chain (MHC) gene which is associated with a particularly severe HCM histopathology [215, 216]. Wolf *et al.* showed characteristic features of the disease like LV hypertrophy, myocyte fibrosis and disarray [217], however, in collaboration with our group it has been shown that despite the histopathological features of HCM, this mouse model does not show the characteristic microvascular remodelling described in humans (unpublished observations by Prof G. d'Amati, La Sapienza University, Rome). Despite the fact that the microvascular remodelling observed in our DIO model is most likely due to the effects of high fat diet, it is the first model that phenotypically clearly mimics the microvascular remodelling observed in the hearts of patients with arterial hypertension or HCM.

Interestingly, coronary microvascular remodelling was only observed in DIO hearts whereas it was completely absent in age-matched *ob/ob* hearts. It has been recently suggested that a linear relationship between hyperglycaemia and microvascular complications exists [94] and that glycaemic control might influence the degree of coronary microangiopathy [108]. Since hyperglycaemia is subsiding after 14 to 16 weeks of age in *ob/ob* mice [218], we also looked at coronary small vessel morphology in 12 week old, hyperglycaemic and hyperinsulinaemic *ob/ob* mice but again, no remodelling was observed. Since the development of metabolic changes and thus T2DM is highly genetically determined in *ob/ob* mice it is difficult to compare histomorphometrical results in DIO and *ob/ob* mice and thus be able to exclude hyperglycaemia and hyperinsulinaemia as contributing factors to coronary microvascular remodelling.

Furthermore, observations in our group have shown that the microvascular remodelling in DIO hearts is clearly distinguishable from the strong muscular outward remodelling of coronary small vessels seen in hearts of SHR. Although the structural characteristics suggest that microvascular remodelling in DIO hearts is independent of hypertension, this still needs to be confirmed and further studies are needed to ascertain whether the microvascular remodelling is also present after the two shorter periods of high fat diet feeding. With this novel finding our model provides the possibility to directly study potential links between metabolic and coronary microvascular changes.

In conclusion, our results demonstrate that a period of 14 weeks of high fat diet results in an extreme phenotype of clinical obesity whereas 11 weeks DIO mice represent a good model to study the early onset of T2DM. Our high fat diet model has clear advantages over the use of the leptin-deficient *ob/ob* mouse. Firstly, our high fat diet model allows the study of pathophysiological changes associated with both environmental and genetic risk factors of T2DM compared to the *ob/ob* mouse model in which the onset of symptoms is highly determined by genetic factors alone. Secondly, in contrast to normal basal blood glucose

levels in the *ob/ob* mouse model, our model exhibits both fasting and post-prandial hyperglycaemia as well as the majority of the characteristic features of patients with genetic predisposition to develop T2DM when they become obese. Finally, only our model shows the presence of coronary microvascular remodelling which allows one to study the link between metabolic and coronary microvascular changes.

Based on the results from this pilot study, DIO mice after 11 weeks of high fat diet feeding were chosen as the most appropriate model to further characterise and study genetic and molecular mechanisms of myocardial insulin resistance and microvascular remodelling.

Chapter Four: Myocardial insulin resistance and coronary microvascular remodelling in DIO

4.1 Introduction

Hyperglycaemia, hyperinsulinaemia and hyperlipidaemia promote the development of pathological changes in cardiac structure and cardiomyocyte ultrastructure and function [13]. It has been shown that the heart of diabetic patients is insulin resistant [70] resulting in a decrease in glucose utilisation possibly due to an impaired insulin signalling pathway [72]. Moreover, diabetes markedly affects the function of the cardiovascular system, both in the microcirculation as well as in large conduit arteries supplying vital organs such as the heart, brain, and kidney [219] leading to micro- and macrovascular complications. Despite different mechanisms contributing to the complex pathophysiology of chronic T2DM complications, dysregulation of vascular function and structure is a common finding [93]. Vascular and endothelial dysfunction play an important role in the development of atherosclerosis and other CVDs in T2DM. Additionally, vascular remodelling has been shown in small arteries of patients with diabetes or left ventricular hypertrophy [93, 220, 221] as well as in various animal models of hypertension and diabetes [207, 208, 210]. Vascular remodelling is associated with alterations in the vessel morphology leading to an increase in the medial area [93]. Although the phenomenon has been extensively researched in hypertension [104], the potential mechanisms of vascular remodelling in diabetes are not fully understood. Furthermore, the presence of vascular remodelling in the coronary microcirculation and the resulting coronary microvascular dysfunction has been observed in different diseases like T2DM, hypertension, or hypertrophic cardiomyopathy [92] and may lead to myocardial ischaemia and fibrosis as well as to an increased risk of myocardial infarction. In the previous chapter we have described the coronary microvascular remodelling occurring in our high fat diet induced model of insulin resistance which resembles microvascular remodelling seen in patients with HCM. However, no microvascular remodelling was observed in hearts of the leptin-deficient *ob/ob* mouse both in the presence (at 12 weeks of age, data not shown) or absence of hyperglycaemia (above 16 weeks of age; [218]). At this stage it is not clear whether the described coronary

microvascular remodelling in DIO hearts is due to structural and functional changes caused by hypertension, which is often present in diabetic patients [178].

4.2 Aims

Exposure to metabolic abnormalities like hyperglycaemia and hyperinsulinaemia due to diet-induced obesity leads to functional and structural changes in the cardiovascular system as well as the myocardium itself. To assess molecular and genetic mechanisms of myocardial insulin resistance and investigate our novel finding of microvascular remodelling in DIO hearts, the following aims were addressed:

1. Investigate coronary microvascular remodelling in DIO
2. Investigate cardiac and endothelial function in DIO
3. Examine gene expression profile in left ventricles of DIO and control mice by conducting a genome-wide microarray study
4. Examine myocardial insulin signalling in DIO mice

4.3 Results

4.3.1 Metabolic parameters after 11 weeks of high fat diet feeding

As described in Chapter 3, DIO mice after 11 weeks of high fat diet feeding show elevated fasting serum insulin and glucose levels as well as a significantly impaired tolerance to an oral glucose load (see Figure 3.5 and Figure 3.6). In addition, serum FFA concentrations as well as baseline serum leptin levels were investigated as additional markers of insulin resistance and T2DM. Leptin is produced by adipose tissue and blood leptin concentrations have been shown to correlate with body weight and the amount of adipose tissue [222]. An increase in adipose tissue mass results in increased leptin levels in serum. After 11 weeks of high fat diet, serum leptin levels quantified by ELISA were significantly increased in DIO mice compared to age-matched controls (59.86 ± 5.417 ng/mL vs. 4.528 ± 0.7614 ng/mL, $p < 0.0005$, $n = 11-13$) and positively correlated with visceral fat mass ($r = 0.91$, $p < 0.005$) (Figure 4.1 A and B). *Ob/ob* mice were used as additional controls due to their leptin-deficiency. No leptin levels were detected in serum samples of *ob/ob* mice (-0.02033 ± 0.01827 ng/mL vs. 4.528 ± 0.7614 ng/mL, $p < 0.0005$, $n = 6$) (Figure 4.1 A).

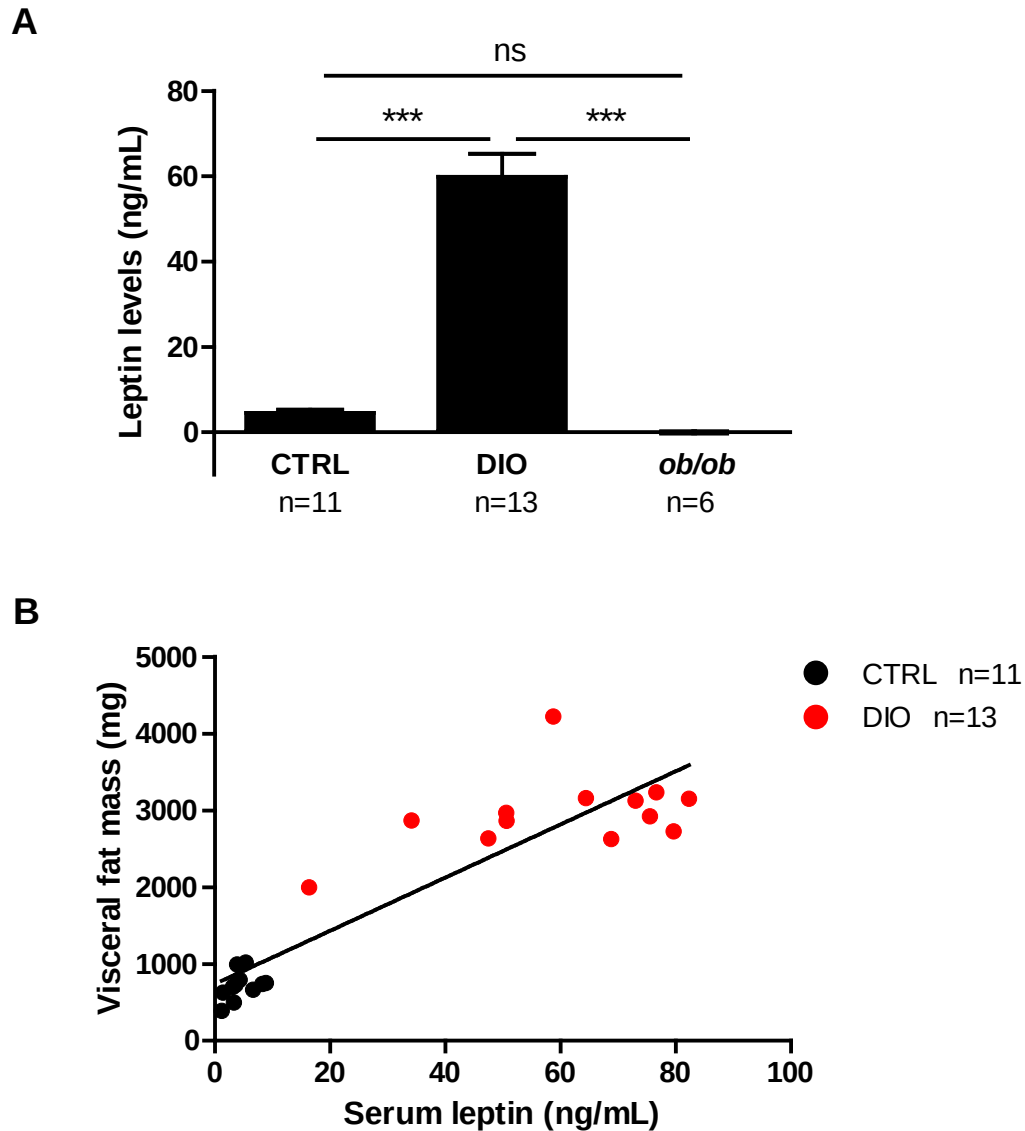


Figure 4.1: Serum leptin levels in DIO and age-matched *ob/ob* and control mice.

(A) Serum leptin levels in DIO, age-matched controls and *ob/ob* mice. (B) Correlation between serum leptin and visceral fat mass in DIO (red dots) and age-matched controls (black dots). Data are expressed as mean±SEM for three independent experiments with n=11 for controls, n=13 for DIO mice and n=6 for *ob/ob* mice. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison for serum leptin levels. To test for correlation linear regression analysis was performed. ns, not significant; *** p<0.0005.

To determine whether high fat diet feeding is associated with the previously described increase in FFA in the blood [223] FA concentrations in serum were measured in DIO after 11 weeks of high fat diet, compared to age-matched controls and *ob/ob* mice. FA concentrations in *ob/ob* mice were significantly increased compared to age-matched controls and DIO mice (1.342 ± 0.1848 nmol/ μ L vs. 0.5626 ± 0.06857 nmol/ μ L vs. 0.8437 ± 0.08455 nmol/ μ L; $p < 0.05$, $n = 15$ for *ob/ob* vs. controls and $p < 0.0005$, $n = 15-25$ for DIO vs. *ob/ob*) (Figure 4.2). In contrast, baseline levels in DIO mice were slightly lower compared to control mice but did not reach statistical significance (0.5626 ± 0.06857 nmol/ μ L vs. 0.8437 ± 0.08455 nmol/ μ L; $p = \text{ns}$, $n = 15-25$) (Figure 4.2). All metabolic parameters are summarised in Table 4.1.

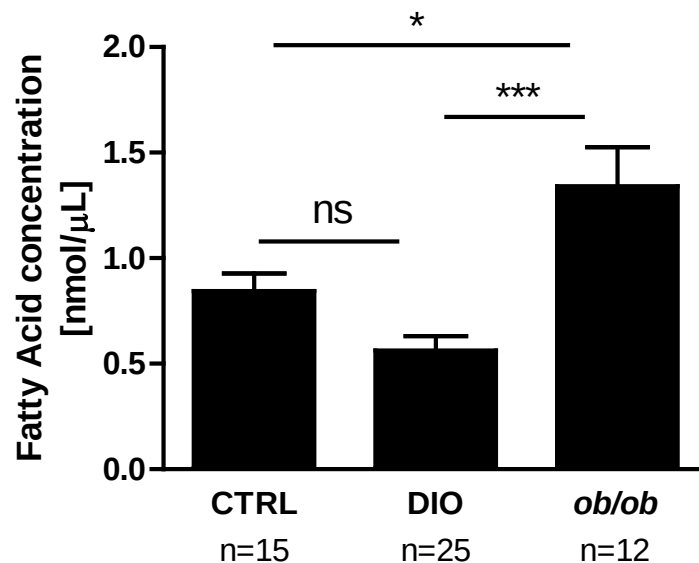


Figure 4.2: Fatty acid concentrations in serum of DIO mice, age-matched *ob/ob* and control mice at baseline.

Serum fatty acid concentrations in DIO, age-matched controls and *ob/ob* mice. Data are expressed as mean $\pm$ SEM for three independent experiments with $n = 15$ for control mice, $n = 25$ for DIO and $n = 12$ for *ob/ob* mice. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ns, not significant; * $p < 0.05$ and *** $p < 0.0005$.

Table 4.1: Summary of metabolic parameters in DIO mice after 11 weeks of high fat diet (HFD) relative to age-matched controls

	11 weeks HFD
Fasting Insulin	↑ (**)
Fasting Glucose	↑ (***)
OGTT (AUC, % increase)	99 (**)
Fasting Leptin	↑ (***)
Serum Fatty Acids	↓ (ns)

OGTT: oral glucose tolerance test; AUC: area under the curve

↑: increase; ↓: decrease; ns: not significant; ** $p < 0.005$, *** $p < 0.0005$

4.3.2 Coronary microvascular remodelling in DIO mice

As shown in Chapter 3 histological analysis of DIO mice hearts after 14 weeks of high fat diet showed a significant increase in interstitial fibrosis compared to age-matched controls (Figure 3.11). Additionally, a previously unrecognised inward muscular remodelling with intimal thickening was demonstrated in intramyocardial coronary arterioles of the left ventricles in DIO mice after 14 weeks of high fat diet feeding but not in age-matched controls or *ob/ob* mice with or without hyperglycaemia (Figure 3.12).

To investigate the onset of microvascular remodelling, small coronary arterioles and medial area were measured in DIO mice after 8 and 11 weeks of high fat diet feeding. At both time points inward muscular remodelling with a slightly thickened intima was observed. Histomorphometrical analysis showed a significant increase in medial area compared to control mice after both 8 and 11 weeks of high fat diet feeding ($3358 \pm 459.2 \mu\text{m}^2$ vs. $879.9 \pm 89.08 \mu\text{m}^2$ for 8 weeks, $p < 0.0005$) (Figure 4.3) ($2852 \pm 247.8 \mu\text{m}^2$ vs. $1499 \pm 221.4 \mu\text{m}^2$ for 11 weeks, $p < 0.0005$) (Figure 4.4). However, a significant increase in the media-to-lumen ratio was observed only in 11 weeks DIO compared to age-matched controls (3.966 ± 0.5309 vs. 2.771 ± 0.3469 , $p = \text{ns}$ for 8 weeks DIO vs. control and 2.791 ± 0.2530 vs. 1.617 ± 0.1648 , $p < 0.005$ for 11 weeks DIO vs. control) (Figure 4.3 and Figure 4.4).

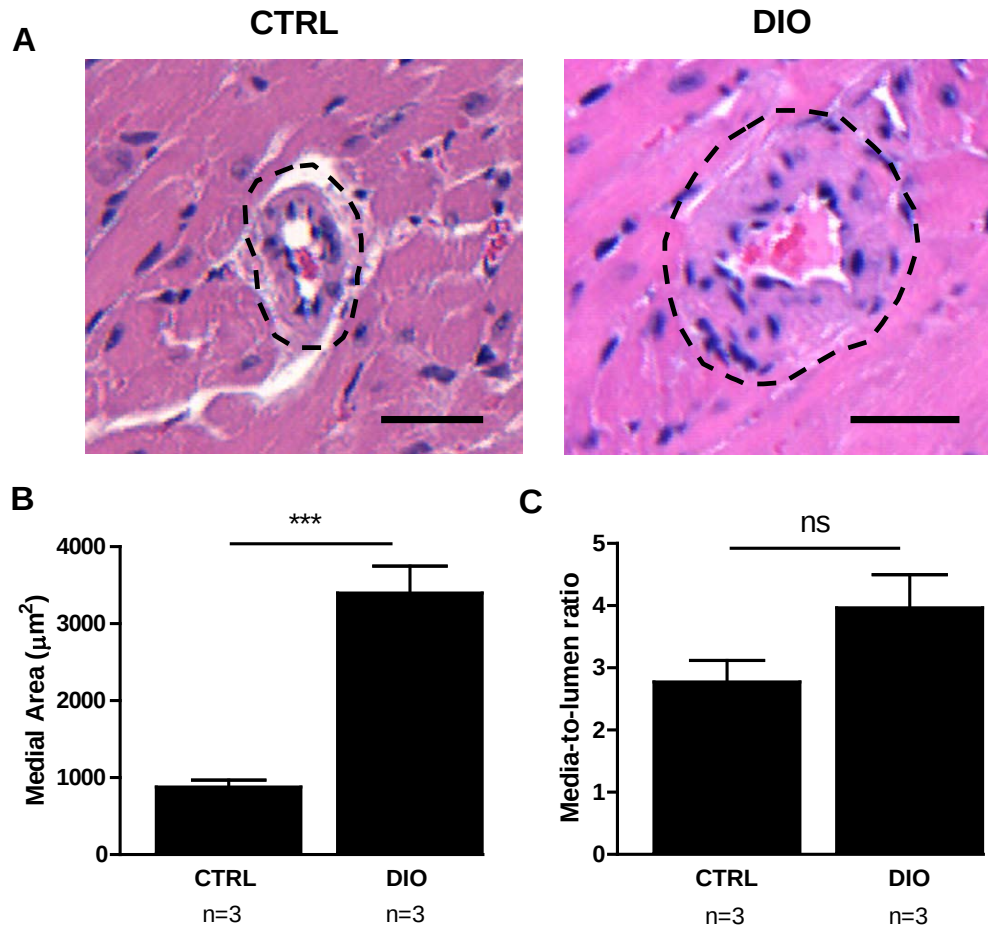


Figure 4.3: Effects of high fat diet on coronary small vessel morphology after 8 weeks of HFD.

(A) Histological sections of small arterioles (circled by dashed line) in left ventricles of control (left) and 8 weeks DIO mice (right) stained with haematoxylin and eosin. Nuclei are stained in dark blue whereas eosinophilic structures like cytoplasm, intra- and extracellular proteins or red blood cells are stained in shades of red. Images were taken using 40x magnification. Black scale bars indicate 25 μm . (B and C) Histomorphometrical analysis of intramyocardial arterioles. (B) Quantification of medial area in DIO and control mice. (C) Quantification of media-to-lumen area in DIO and control mice. Data are expressed as mean $\pm$ SEM for 3 animals per group and at least 6 vessels per heart. Data were analysed using Student's t-test. ns, not significant and *** $p < 0.0005$. Histomorphometrical analysis was carried out by Dr Massimiliano Mancini, La Sapienza University, Rome. Data analysis was performed by Christina Kleinert.

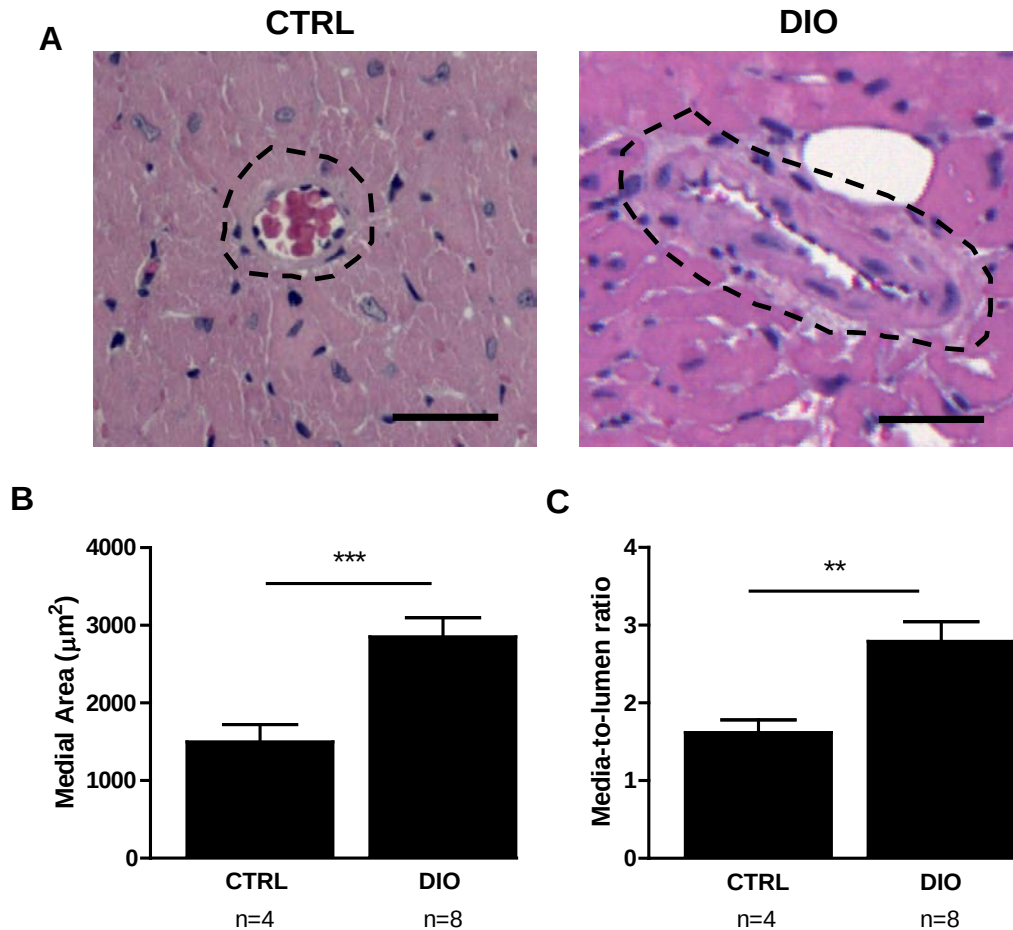


Figure 4.4 Effects of high fat diet on small vessel morphology after 11 weeks of HFD.

(A) Histological sections of small arterioles (circled by dashed line) in left ventricles of control (left) and 11 weeks DIO mice (right) stained with haematoxylin and eosin. Nuclei are stained in dark blue whereas eosinophilic structures like cytoplasm, intra- and extracellular proteins or red blood cells are stained in shades of red. Images were taken using 40x magnification. Black scale bars indicate 25 μm . (B and C) Histomorphometrical analysis of intramyocardial arterioles. (B) Quantification of medial area in DIO and control mice. (C) Quantification of media-to-lumen area in DIO and control mice. Data are expressed as mean $\pm$ SEM for n=4 for control and n=8 for DIO mice and at least 6 vessels per heart. Data were analysed using Student's t-test. ** p<0.005 and *** p<0.0005. Histomorphometrical analysis was carried out by Dr Massimiliano Mancini, La Sapienza University, Rome. Data analysis was performed by Christina Kleinert.

4.3.3 Cardiovascular function in DIO mice

4.3.3.1 Stroke volume and ejection fraction

To investigate the effect of high fat diet feeding on cardiac function in DIO mice we initially aimed to use echocardiography, a commonly used technique to investigate cardiac function in murine models [224-226]. However, due to the increased amount of pericardial fat in DIO mice (see Figure 3.7) we failed to obtain reliable data (data not shown). Thus, cardiac MRI was used as an alternative method to investigate stroke volume and ejection fraction in DIO mice after 11 weeks of high fat diet feeding and age-matched controls. Long-axis MRI images showed a change in axial orientation of the DIO hearts probably due to the increase in pericardial fat which might also explain why we failed obtain reliable data with echocardiography (Figure 4.5). Stroke volume was calculated by subtracting mean left ventricular end-systolic volume (LVESV) from mean left ventricular end-diastolic volume (LVEDV) and ejection fraction by dividing stroke volume by LVEDV. Analysis showed that there was no difference in stroke volume ($30.87 \pm 5.776 \mu\text{L}$ vs. $27.12 \pm 2.680 \mu\text{L}$, $p=\text{ns}$) (Figure 4.6 A) or ejection fraction ($70.70 \pm 7.501\%$ vs. $70.92 \pm 3.473\%$, $p=\text{ns}$) between DIO and age-matched controls (Figure 4.6 B).

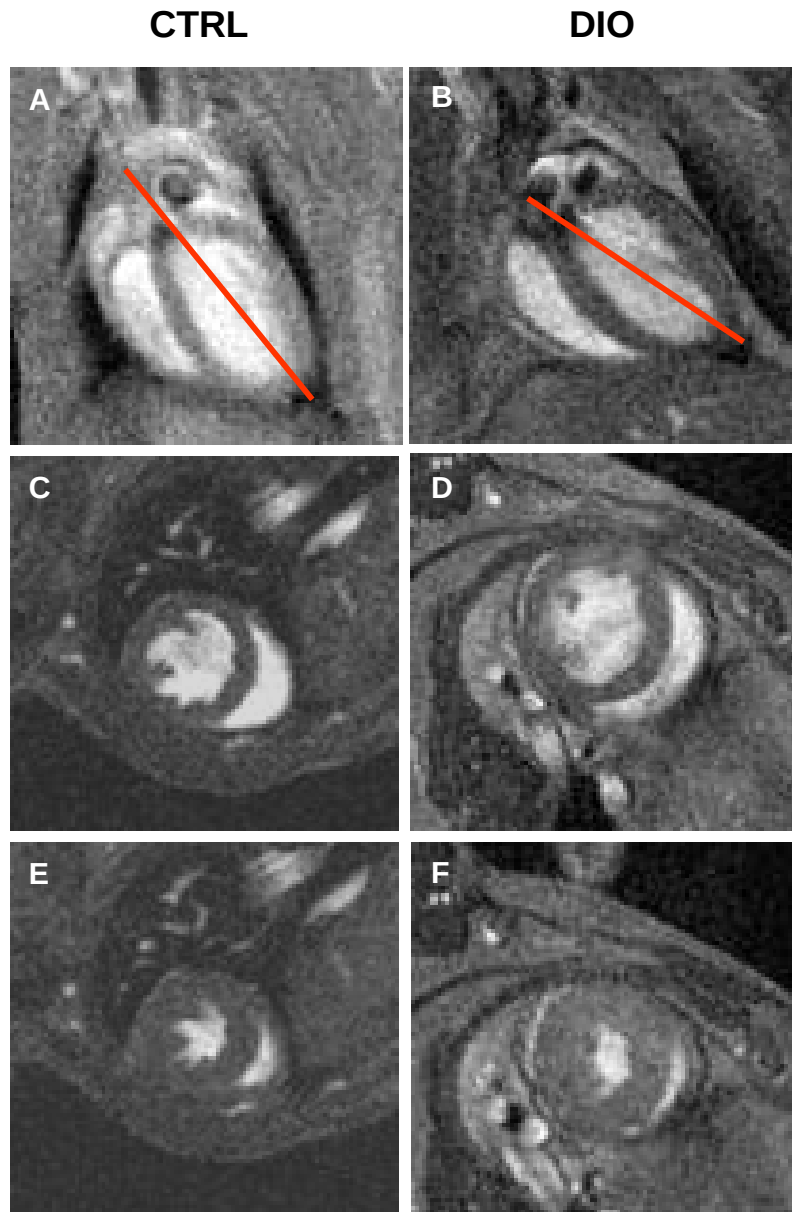


Figure 4.5: Cardiac function in DIO and controls using MRI.

(A and B) Long-axis scout images of control (A) and DIO (B) heart. Red line indicates axial orientation of the heart. DIO hearts had a different axial orientation compared to control hearts caused by increased deposits of white adipose tissue in the thorax. (C and D) Examples of short-axis MRI images of control (C) and DIO (D) heart at end diastole. (E and F) Examples of short-axis MRI images of control (E) and DIO (F) heart at end systole. Cardiac MRI was carried out by Dr Marzena Wylezinska-Arridge and Dr Jordi Lopez Tremoleda (MRC CSC Biological Imaging Centre). Data analysis was performed by Christina Kleinert.

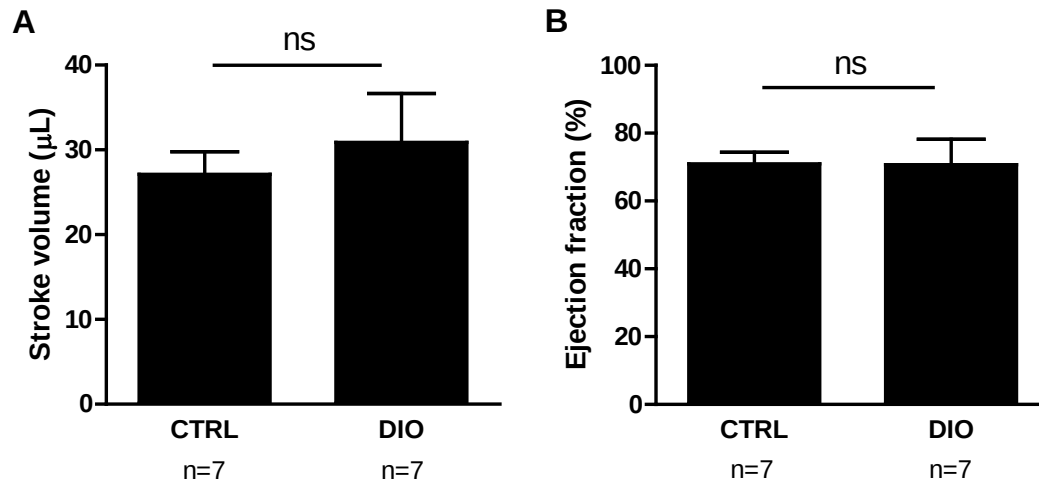


Figure 4.6: Stroke volume and ejection fraction in 11 weeks DIO and age-matched controls.

Cardiac MRI was used to measure left ventricular end systolic and end diastolic volumes. No significant changes were observed in stroke volume (A) as well as ejection fraction (B) in DIO animals compared to age-matched controls. Data are expressed as mean $\pm$ SD for 7 animals per group. Data were analysed using Student's t-test. ns, not significant.

4.3.3.2 Mean arterial pressure (MAP) and coronary flow

To determine whether DIO mice develop hypertension with high fat feeding, MAP pressure was measured in the left carotid artery using a heparin-saline-filled polyethylene tube connected to a pressure transducer. There was no difference in mean arterial pressure between DIO and control mice (88.00 ± 3.546 mmHg vs. 87.14 ± 4.914 mmHg, $p=ns$) (Figure 4.7).

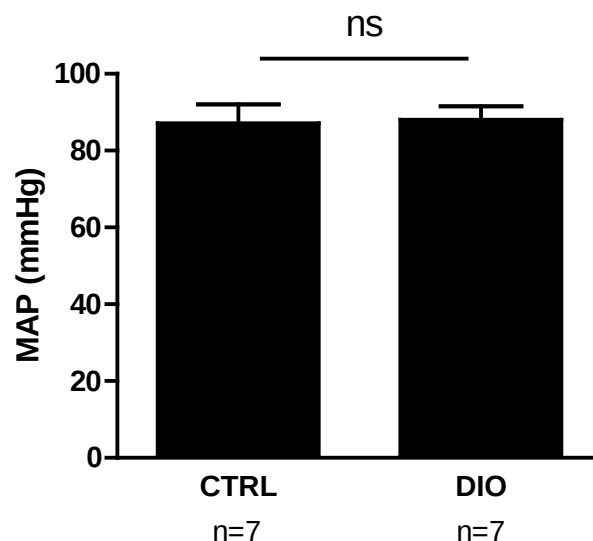


Figure 4.7: MAP in DIO and age-matched control mice.

MAP was measured invasively in the left carotid artery. No significant changes were observed between DIO and control mice. MAP is expressed as mean $\pm$ SEM for 7 animals per group. Data were analysed using Student's t-test. ns, not significant.

To further investigate the effect of high fat diet on cardiac function in DIO, coronary flow and coronary flow reserve after an ischaemic event were measured using *ex vivo* Langendorff perfusion. This technique is commonly used in physiological research and allows the examination of heart rate, contractile strength, and vascular effects *ex vivo*. Baseline and peak coronary flow as well as time to return to baseline flow were recorded (Figure 4.8 A). Analysis of Langendorff data resulted in no difference in peak-to-baseline ratio after a 60 seconds ischaemic event between DIO and control animals (4.126 ± 0.1491 vs. 4.207 ± 0.3681 , $p = \text{ns}$) (Figure 4.8 B). However, when looking at total peak area (area under the curve) (82.40 ± 12.15 vs. 48.45 ± 3.985 , $p < 0.05$) and time to return to baseline flow (144.5 ± 30.44 sec vs. 57.60 ± 10.49 sec, $p < 0.05$), a significant increase was observed in DIO hearts compared to controls (Figure 4.8 C and D) which suggests that high fat diet does not have an effect on the amount of ischaemia-induced vasodilation but on the duration.

A summary of functional and histological changes after 11 weeks of high fat diet feeding is shown in Table 4.2.

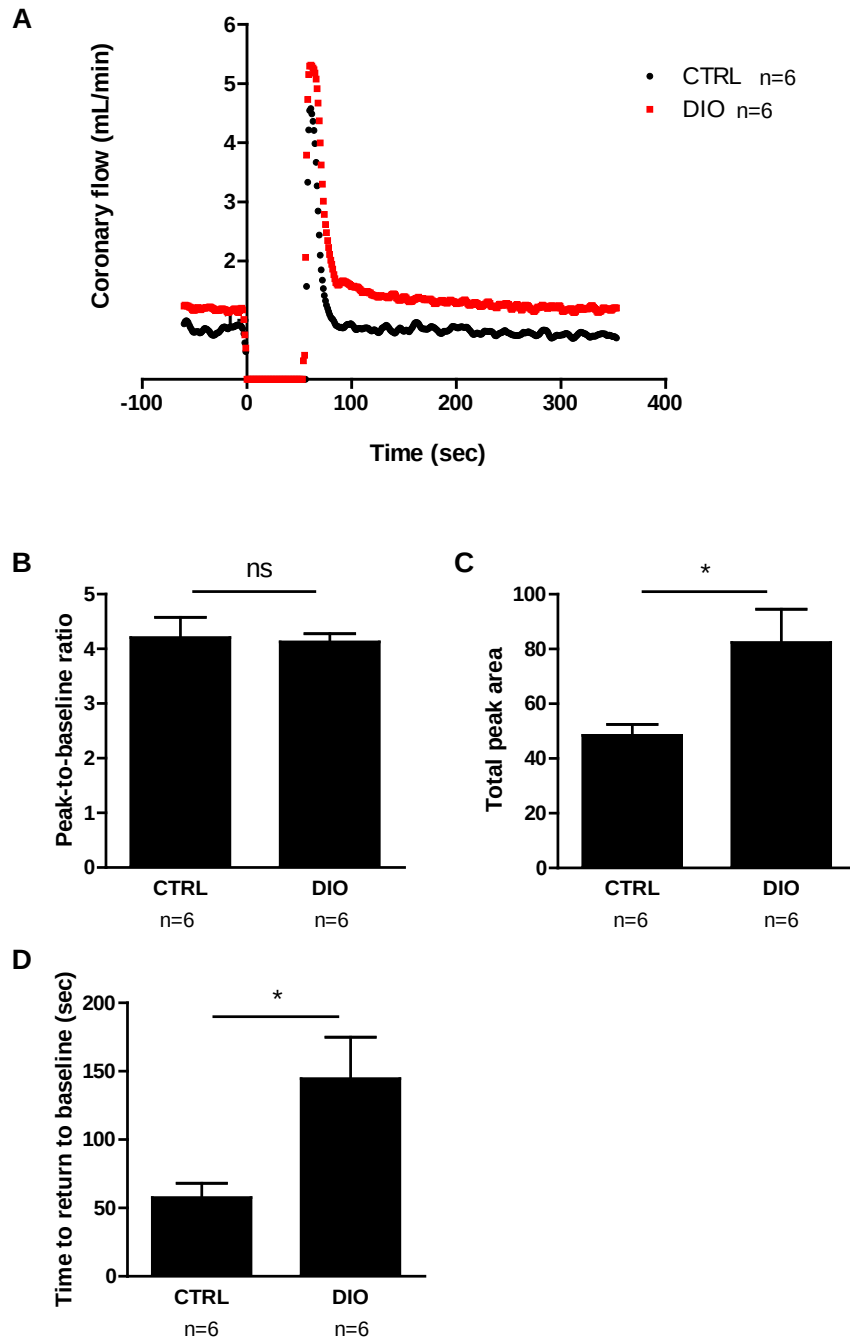


Figure 4.8: Effects of high fat diet on coronary flow.

(A) Representative trace of coronary flow in control (black) and DIO hearts (red). (B) Quantification of peak-to-baseline ratio of DIO and control hearts. (C) Total peak area (AUC) after a 1 min ischaemic event in DIO and control hearts. (D) Time to return to baseline coronary flow after a 1 min ischaemic event in DIO and control hearts. Data are expressed as mean $\pm$ SD for 6 animals in each group. Data were analysed using Student's t-test. ns, not significant; * $p < 0.05$.

Table 4.2: Summary of functional and histological changes after 11 weeks of high fat diet

	CTRL	DIO
MAP (mmHg)	87.14±4.914	88.00±3.546
Resting CF (mL/min)	1.177±0.2564	1.145±0.1778
Hyperaemic CF (mL/min)	4.763±0.3355	4.725±0.8060
Peak-to-baseline CF	4.207±0.3681	4.126±0.1491
Total peak area (AUC)	48.45±3.985	82.40±12.15 (*)
Time to return to baseline (sec)	57.60±10.49	144.5±30.44 (*)
Medial area (µm²)	1499±221.4	2852±247.8 (***)
Media-to-lumen ratio	1.617±0.1648	2.791±0.2530 (**)

* p<0.05, ** p<0.005 and *** p<0.0005

4.3.3.3 *In vivo* assessment of vascular reactivity

To ascertain whether microvascular remodelling was not only occurring in coronary arterioles but also in the periphery, vascular reactivity in the carotid artery was investigated. Intravenous injection of the endothelium-dependent vasodilator acetylcholine resulted in a significant difference in the drop of MAP between DIO and control mice (42.38 ± 7.981 mmHg vs. 30.60 ± 8.877 mmHg, $p < 0.05$) as well as a significant reduction of the recovery time back to baseline MAP after this challenge in DIO mice compared to controls (51.00 ± 10.10 sec vs. 123.0 ± 5.715 sec, $p < 0.0005$) (Figure 4.9).

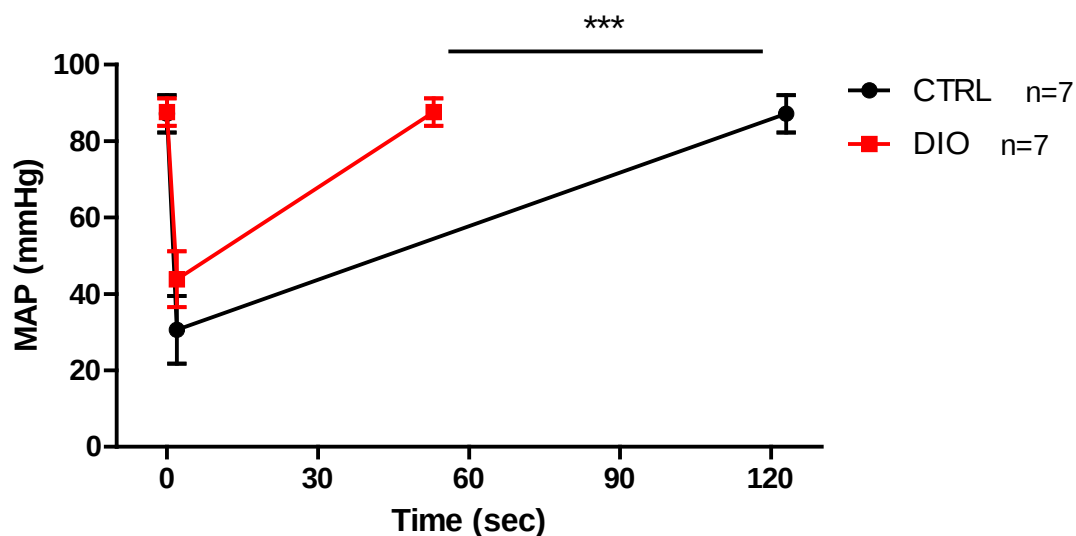


Figure 4.9: MAP changes in DIO and control mice after injection of acetylcholine.

A significant difference in the drop of MAP was observed between DIO and control mice. Additionally, the time to return to baseline MAP was significantly shorter in DIO mice compared to controls. Data are expressed as mean $\pm$ SD for 7 animals in each group. Data were analysed using Student's t-test. *** $p < 0.0005$.

4.3.3.4 *Ex vivo* assessment of vascular reactivity

Wire tension myography on aortic rings of DIO and control animals was chosen as an *ex vivo* functional measurement of vascular reactivity. First, maximal contractile capacity and viability of the vessel were determined by depolarising ring segments with KCl followed by dose-dependent contraction of segments with the vasoconstrictor phenylephrine (PE). Finally, the vasodilator acetylcholine (ACh) was added to determine the integrity of the endothelium. Once viability and maximal contractile capacity of the vessels was confirmed, endothelium-dependent and -independent relaxation in DIO and control aortic rings was measured. To study the endothelium-dependent relaxation, vessels were pre-contracted with PE to 70% of the maximal contractile force of the artery (EC70) and cumulative relaxation curves of ACh were constructed. Endothelium-independent relaxation was measured by cumulative addition of the nitric oxide donor sodium nitroprusside (SNP).

There was a trend to a lower maximal contraction of arterial smooth muscle to KCl in DIO segments compared to controls (4.961 ± 0.4495 for DIO vs. 6.046 ± 0.4548 for controls, $p=0.0641$) (Figure 4.10 A) whereas no differences in the amount of PE needed to elicit EC70 were observed between the two groups (Figure 4.10 B). Endothelium-dependent relaxation was investigated by constructing cumulative relaxation curves of ACh (10^{-9} to 10^{-5} M). Ring segments of DIO mice showed significantly decreased endothelium-derived relaxation to ACh compared to control mice ($p<0.05$ at all concentrations starting at 10^{-6} M) (Figure 4.11 A). Endothelium-independent relaxation was investigated using SNP. Arterial segments of both groups relaxed to SNP in a dose-dependent manner (Figure 4.11 B). At maximal SNP concentration (10^{-7} M) the control group relaxed by $94.75 \pm 2.136\%$ and the DIO group by $89.20 \pm 5.305\%$. There were no significant differences between the two groups which would suggest that high fat diet feeding does not affect endothelium-independent vasodilation (Figure 4.11 B).

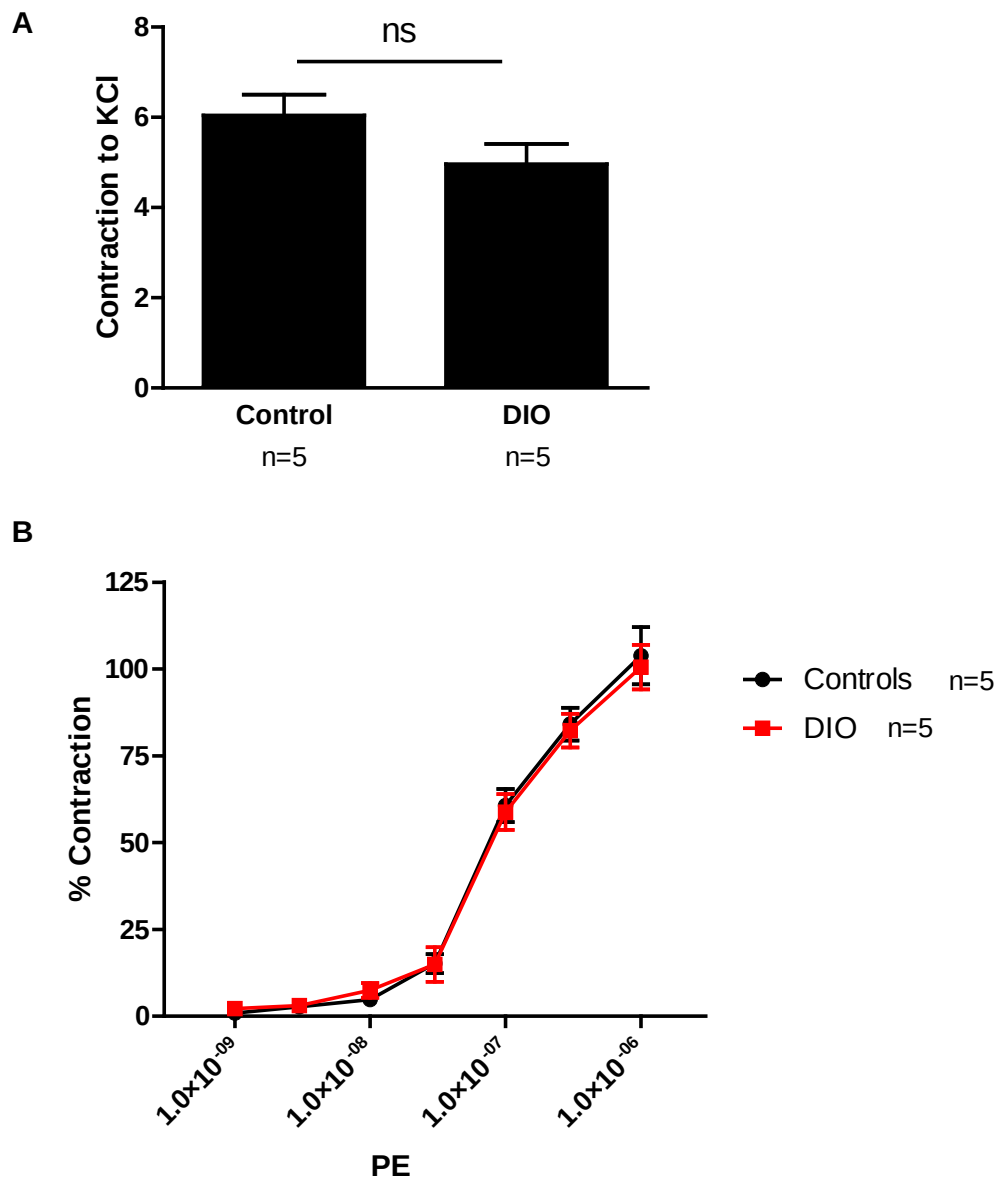


Figure 4.10: Effect of high fat diet on maximal contractile capacity and contraction to PE.

(A) Maximal contraction of ring segments in response to depolarization by KCl in control and DIO ring segments. (B) Cumulative concentration-response curves to PE in controls (black) and DIO mice (red). No significant differences in the percentage of contraction in response to PE were observed in DIO compared to controls. Data are expressed as mean ± SEM for 5 animals per group. Data were analysed using Student's t-test. ns, not significant. Myography experiments were carried out by Dr Elizabeth Sujkovic (MRC CSC Nitric Oxide Signalling Group).

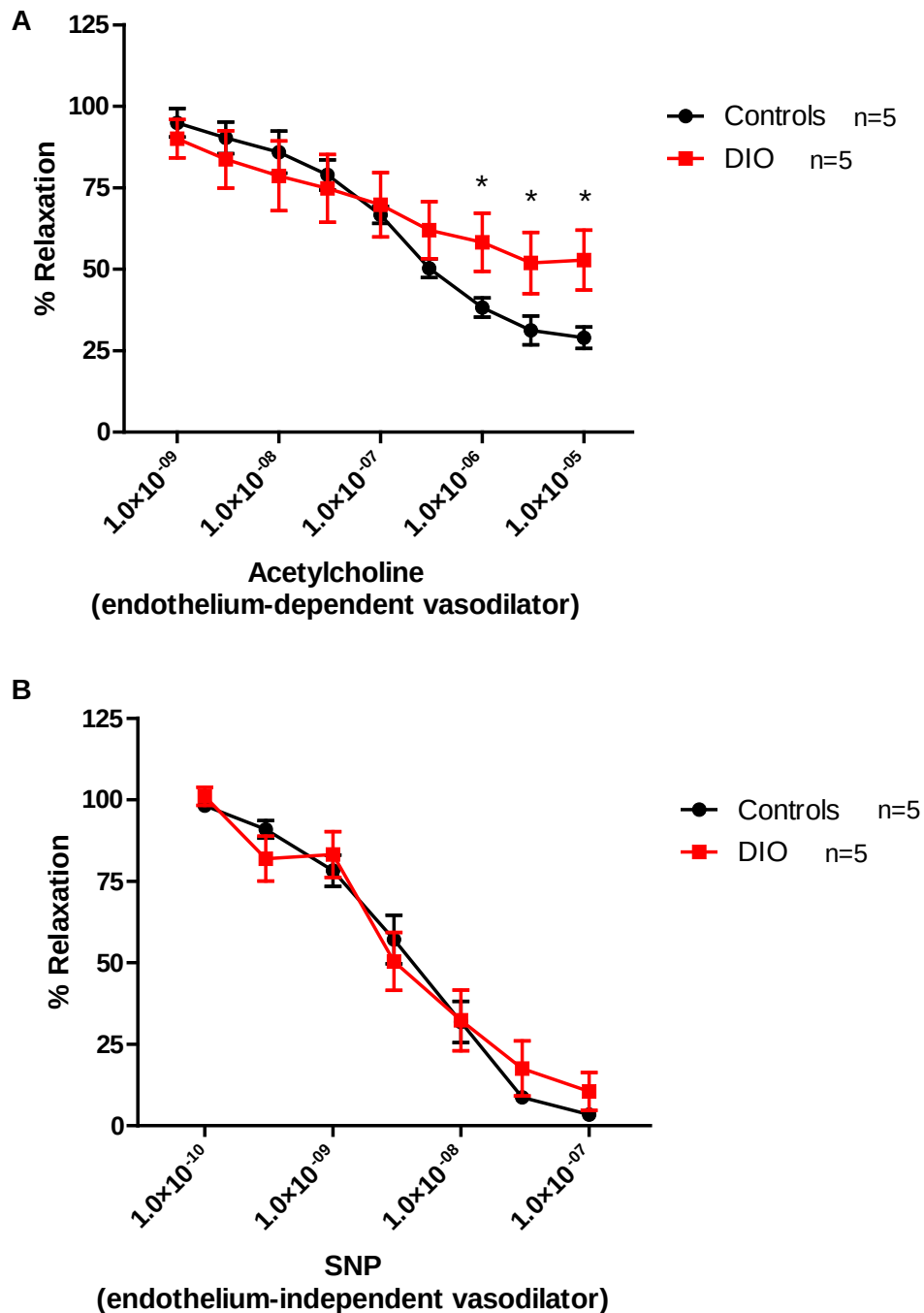


Figure 4.11: Effect of high fat diet on dose-dependent endothelium-dependent and endothelium-independent relaxation to ACh and SNP.

(A) Endothelium-dependent relaxation to ACh in DIO and control mice. (B) Endothelium-independent relaxation responses to SNP. Results are presented as means $\pm$ SEM from 5 animals in each group. Data were analysed using Student's t-test. * $p < 0.05$. Myography experiments were carried out by Dr Elizabeth Sujkovic (MRC CSC Nitric Oxide Signalling Group).

4.3.3.5 Measurement of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NOS

To further investigate the observed impairment in endothelium-dependent vasodilation in DIO aortas, the effect of high fat diet feeding on the endogenous inhibitor of NOS, ADMA, was determined. For this, ADMA levels were measured in plasma samples of DIO and control mice using liquid chromatography/ mass spectrometry. Plasma ADMA levels were not changed after 11 weeks of high fat diet (0.5365±0.1168 µM vs. 0.5161±0.1640 µM, p=ns, n=5 for each group).

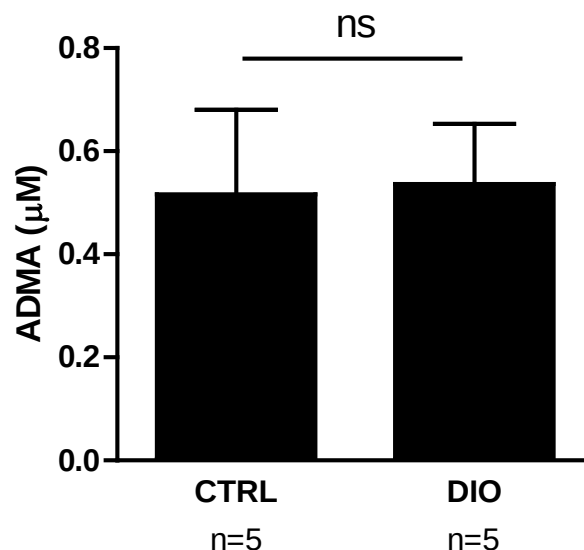


Figure 4.12: Plasma ADMA levels in DIO and control mice.

Plasma ADMA levels were measured using liquid chromatograph/mass spectrometry in DIO and age-matched control mice. Data are expressed as mean±SD for n=5 animals per group. Data were analysed using Student's t-test. ns, not significant. ADMA levels were provided by Dr James Leiper (MRC CSC Nitric Oxide Signalling Group).

4.3.4 Gene expression profiling in left ventricles of DIO mice

4.3.4.1 Microarray analysis

To identify gene expression changes associated with high fat diet-induced microvascular remodelling in the heart, cDNA microarray analysis was performed using mRNA isolated from left ventricles of 11 weeks DIO and age-matched control mice.

4.3.4.1.1 Quality assessment of RNA

For microarray analysis high quality RNA is required. Quality of isolated RNA samples was assessed by measuring the absorbance ratio A260/280 on a nanodrop as well as running the samples on a Bioanalyser to obtain the RNA index number (RIN) which is proportional to RNA quality. All absorbance ratios were in the recommended range of 1.9 to 2.1 indicating pure RNA (see Appendix C – Microarray Quality Controls) and RIN of all samples run on the Bioanalyser were within the recommended range of 7 to 10, with 10 indicating high quality RNA (Appendix C – Microarray Quality Controls). To ensure consistently good quality at the end of each stage quality assessments were performed after rRNA reduction, first and second cycle cDNA synthesis as well as before and after fragmentation of cDNA (Appendix C – Microarray Quality Controls).

4.3.4.1.2 Analysis of expression data

Gene expression summary values of the Affymetrix Gene Chip data were generated using the robust multichip average (RMA) algorithm [172]. The quality of the expression replicates was then determined using principal-components analysis, hierarchical clustering and hybridisation control plot using GeneSpring software followed by a filtering step which included the removal of all values below the 20th percentile and the setting that 10 out of 10 samples should have probe values greater than the 20th percentile.

Ideally, all replicates of one condition are expected to group together and all replicates of the other condition to group together. However, principal component analysis showed that there was variation between replicates in both groups. This variability between samples resulted in no differentially expressed genes after multiple corrections and setting the false discovery rate at 5%. Therefore, the fold change (FC) was lowered to $FC \geq 1.5$ and a p value of $p < 0.01$ was considered to be significant. Using this setting, analysis of the transcriptome showed that 29 genes were differentially expressed in response to high fat diet feeding (1.5-fold or greater, $p < 0.01$). Of these, 18 genes were down-regulated (Table 4.3) and 11 up-regulated (Table 4.4).

The high fat diet-induced changes in expression included genes which are involved in a variety of biological processes. Annotation of genes resulted in 26 genes being annotated and showed that the largest group of altered genes was associated with small molecule biochemistry, whereas lipid and carbohydrate metabolism were the next most commonly annotated groups. Using ingenuity system pathway analysis, we identified biologically relevant networks with top function in lipid metabolism associated with gene expression differences in response to high fat diet feeding (Figure 4.13 and Figure 4.14). At the individual gene level, genes involved in the biosynthesis of fatty acids (*Scd4*), oxidation of fatty acids (*Ehhadh*, *Ucp3*) as well as cholesterol biosynthesis (*Fdft1*) or the production of diacylglycerol (*Rgs2*) were down-regulated in response to high fat diet. Additionally, the gene encoding *Pdk4*, which plays an important role in glucose metabolism and whose expression is regulated by insulin was down-regulated. Of the down-regulated genes four were found to be located in the mitochondria (*Ucp3*, *Hmgcs2*, *Pdk4*, *Ehhadh*). Furthermore, genes encoding proteins which are associated with hypertrophy and cardiac fibrosis like *CTGF* and *LMCD1* were down-regulated. On the other hand, *Acta1*, the gene encoding the major actin in postnatal skeletal muscle was increased. The other up-regulated genes were found to play a role in phospholipase C signalling (*Arhgef19*), or G protein-coupled receptor mediated signalling (*Gpr22*).

Table 4.3: Down-regulated genes in left ventricles of DIO mice after 11 weeks of high fat diet

Gene	Symbol	NCBI Entrez Gene ID	FC	p value
Receptor (calcitonin) activity modifying protein 1	Ramp1	51801	-1.50	0.000816899
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2	15360	-1.50	0.005196151
Stearoyl-coenzyme A desaturase 4	Scd4	329065	-1.50	0.005372935
Cold inducible RNA binding protein	Cirbp	12696	-1.51	0.005479873
Farnesyl diphosphate farnesyl transferase 1	Fdft1	14137	-1.56	8.30242E-05
Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh	74147	-1.57	0.007041528
LIM and cysteine-rich domains 1	Lmcd1	30937	-1.58	0.002025513
Gamma-aminobutyric acid (GABA(A)) receptor-associated protein-like 1	Gabarapl1	57436	-1.58	0.003706662
Cyclin-dependent kinase-like 1 (CDC2-related kinase)	Cdkl1	71091	-1.59	1.43557E-05
Transmembrane protein 71	Tmem71	213068	-1.61	0.001730867
T-complex 11 (mouse) like 2	Tcp11l2	216198	-1.63	0.000164171
Pyruvate dehydrogenase kinase, isoenzyme 4	Pdk4	27273	-1.63	0.000503068
Transmembrane and tetratricopeptide repeat containing 1	Tmtc1	387314	-1.66	0.000327137
Uncoupling protein 3 (mitochondrial, proton carrier)	Ucp3	22229	-1.74	0.003806227
Regulator of G-protein signalling 2	Rgs2	19735	-1.76	0.005523294
Histone cluster 1, H1c	Hist1h1c	50708	-2.09	0.000474528
Connective tissue growth factor	Ctgf	14219	-2.13	0.000103499
Metallothionein 2	Mt2	17750	-2.17	0.008681912
Rank order by fold change (FC)				

Table 4.4: Up-regulated genes in left ventricles of DIO mice after 11 weeks of high fat diet

Gene	Symbol	NCBI Entrez Gene ID	FC	p value
Nestin	Nes	18008	1.52	0.001418669
Soluble carrier family 6 (neurotransmitter transporter, creatine), member 8	Slc6a8	102857	1.52	0.001909188
G protein-coupled receptor 22	Gpr22	73010	1.53	0.008851907
Amylase 1, salivary	Amy1	11722	1.55	0.008093013
Rho guanine nucleotide exchange factor (GEF) 19	Arhgef19	213649	1.56	0.000451041
Transmembrane protein 204	Tmem204	407831	1.57	0.001733081
Histidine rich calcium binding protein	Hrc	15464	1.63	0.005019038
Protein phosphatase 1, regulatory (inhibitor) subunit 3c	Ppp1r3c	53412	1.63	0.005030717
NF- κ B inhibitor interacting Ras-like protein 1	Nkiras1	69721	1.72	0.000266726
Actin, alpha 1, skeletal muscle	Acta1	11459	1.80	0.001347989
Transferrin receptor	Tfrc	22042	2.06	0.007599853
Rank order by fold change (FC)				

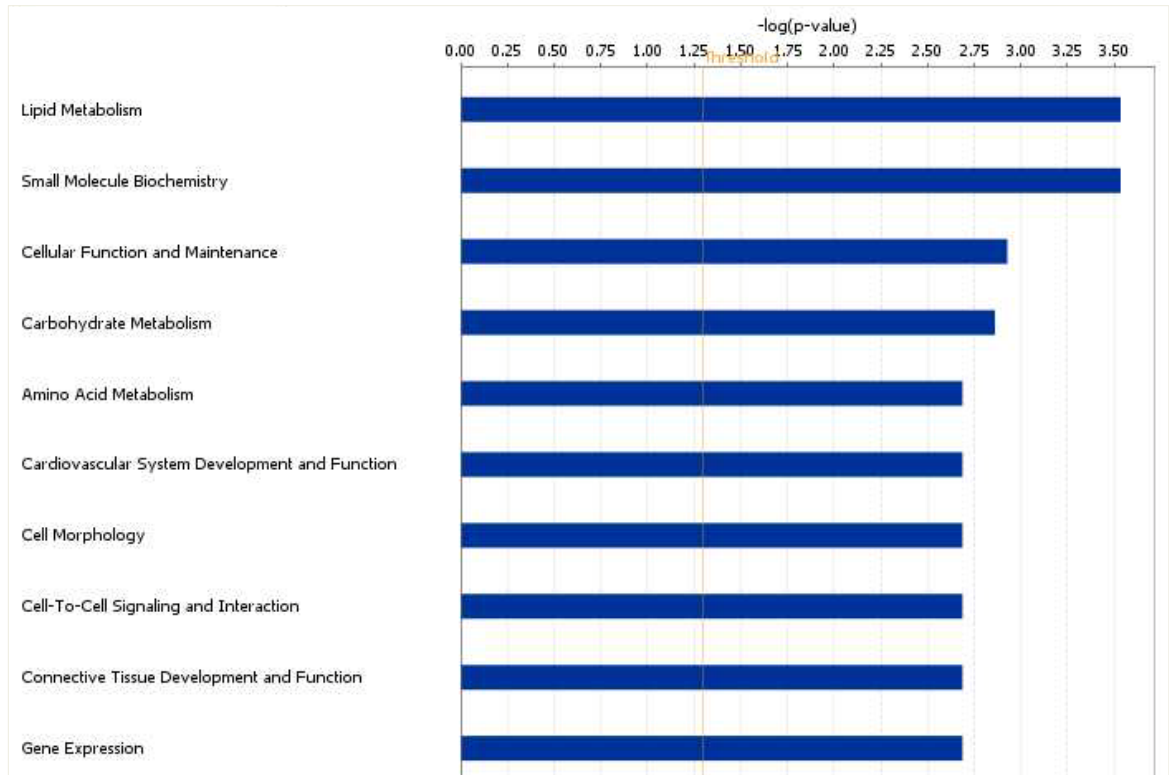


Figure 4.13: Ingenuity analysis of genes differentially expressed by high fat diet.

Functional annotation of 29 genes identified as being differentially expressed by high fat diet. The top ten groups are ranked according to p value significance, the threshold is indicated in yellow.

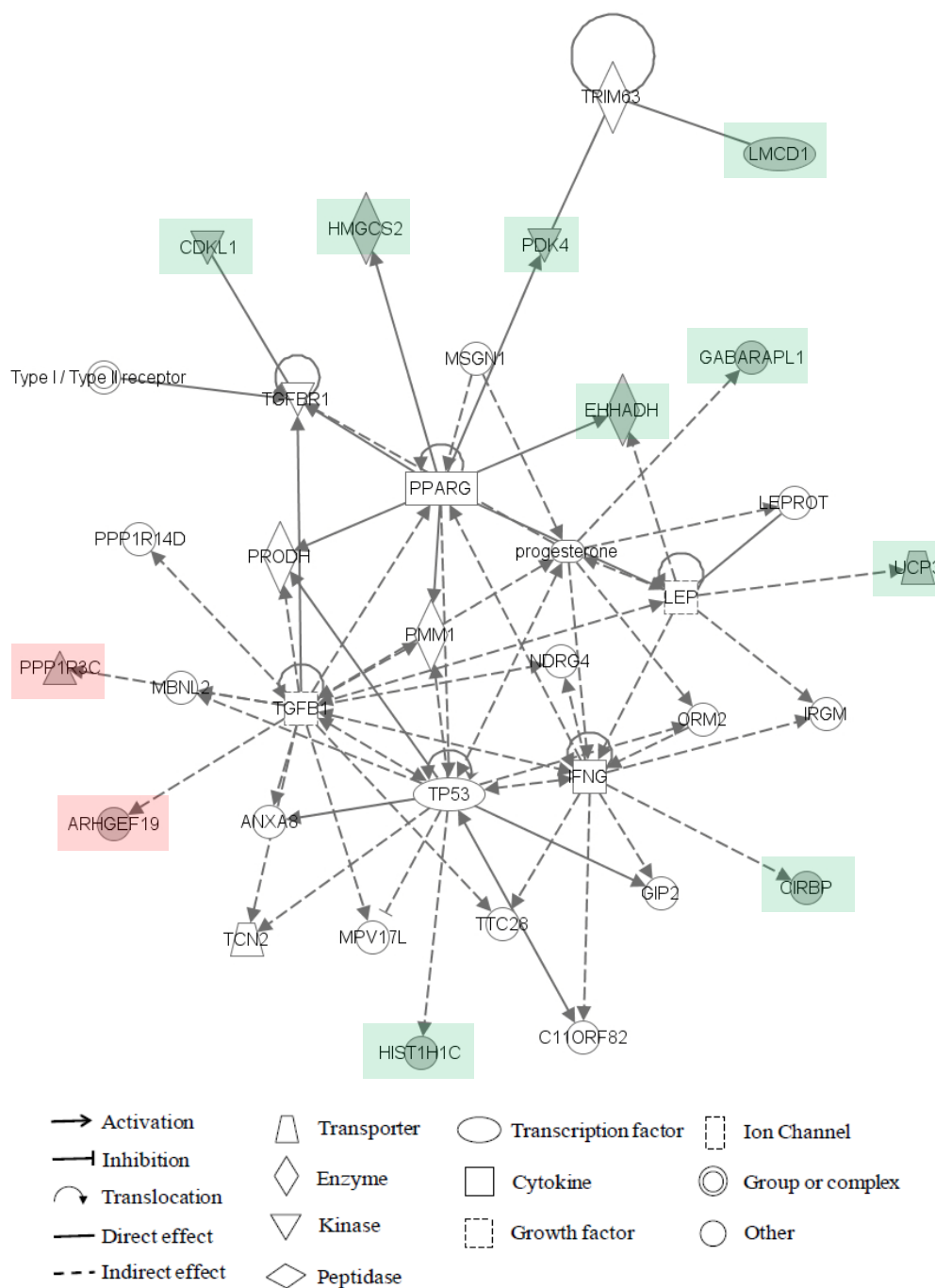


Figure 4.14: Regulatory network identified by Ingenuity network analysis.

Example of regulatory network (lipid metabolism) from the probe sets identified as being differentially expressed by high fat diet. Interactions are defined from Ingenuity database and comprise referenced published protein-protein interactions and transcriptional regulation. Up-regulated genes are highlighted in red and down-regulated genes are highlighted in green.

4.3.4.2 Validation of microarray findings

To validate the microarray findings, qRT-PCR was initially performed, followed by western blotting for a selection of differentially expressed genes and proteins.

4.3.4.2.1 Validation of microarray findings using qRT-PCR

To validate the microarray results, qRT-PCR was performed on differentially expressed genes. Primers were designed for the majority of annotated genes using GenScript Primer Design tool [170]. Gene expression levels were normalised to Cyclophilin A (*Ppia*), which has been shown not to change with diet [227, 228]. A comparison of gene expression levels measured by cDNA microarray and qRT-PCR demonstrated a good correlation in expression levels. Five of the nine up-regulated genes tested (Figure 4.15) and 12 of the 16 down-regulated genes were confirmed by qRT-PCR (Figure 4.16).

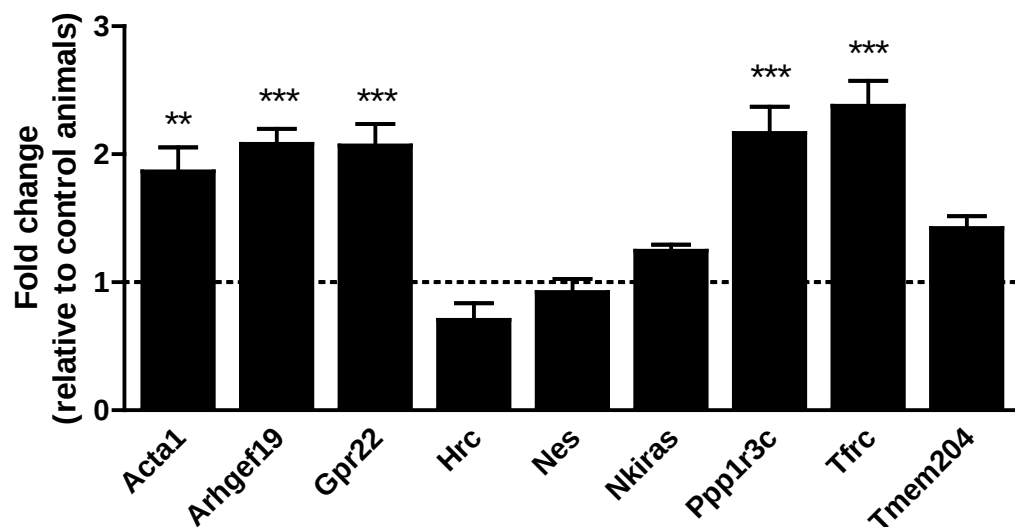


Figure 4.15: Validation of up-regulated genes using qRT-PCR.

Validation of up-regulated genes from microarray experiment using qRT-PCR. Fold change relative to normal chow controls expressed as mean $\pm$ SEM from three independent experiments normalised to Cyclophilin A. Dotted line indicates normal chow controls which was assigned as 1. Data were analysed using Student's t-test. ** $p < 0.005$ and *** $p < 0.0005$.

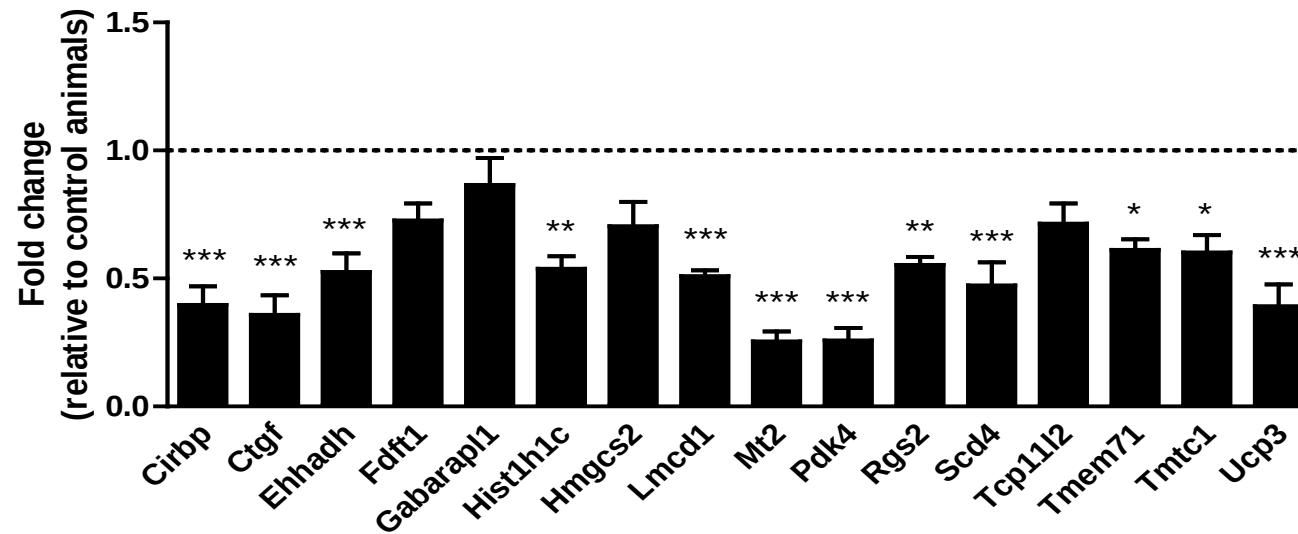


Figure 4.16: Validation of down-regulated genes using qRT-PCR.

Validation of down-regulated genes from microarray experiment using qRT-PCR. Fold change relative to normal chow controls expressed as mean $\pm$ SEM from three independent experiments normalised to Cyclophilin A. Dotted line indicates normal chow controls which was assigned as 1. Data were analysed using Student's t-test. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0005$.

4.3.4.2.2 Validation of microarray findings using protein expression analysis

Protein expression analysis was used to determine whether the differential expression at the mRNA level has an effect on the protein levels of a subset of validated candidates. To determine whether these candidates play a role in the observed microvascular remodelling in DIO left ventricles, we mainly focussed on candidate genes which have been shown to be expressed in the vasculature. CTGF protein levels in whole heart extracts were studied due to its role in vascular smooth muscle cell proliferation and migration as well as its expression in atherosclerotic lesions [229, 230]. RGS2 levels were investigated because of its role in blood pressure regulation and vasoconstrictor signalling [231, 232] and MT2 due to its role in coronary arterial relaxation [233].

Consistent to the microarray experiment, whole heart protein levels were investigated at baseline (without insulin stimulation). Left ventricles of age-matched C57BL/6 mice were used as controls. After correcting for equal loading using glyceraldehyde 3-phosphate dehydrogenase (GAPDH), densitometric analysis of CTGF and MT2 protein bands showed no significant fold changes between control and DIO mice (0.9293 ± 0.1145 vs. 1.000 ± 0.02582 , $p=ns$, $n=6$ for CTGF and 0.7746 ± 0.08105 vs. 0.9992 ± 0.04543 , $p=ns$, $n=3$ for MT2). However, a significant reduction of RGS2 protein expression was observed in DIO hearts compared to age-matched controls (0.5210 ± 0.06619 vs. 1.024 ± 0.05538 , $p<0.005$, $n=4$) (Figure 4.17).

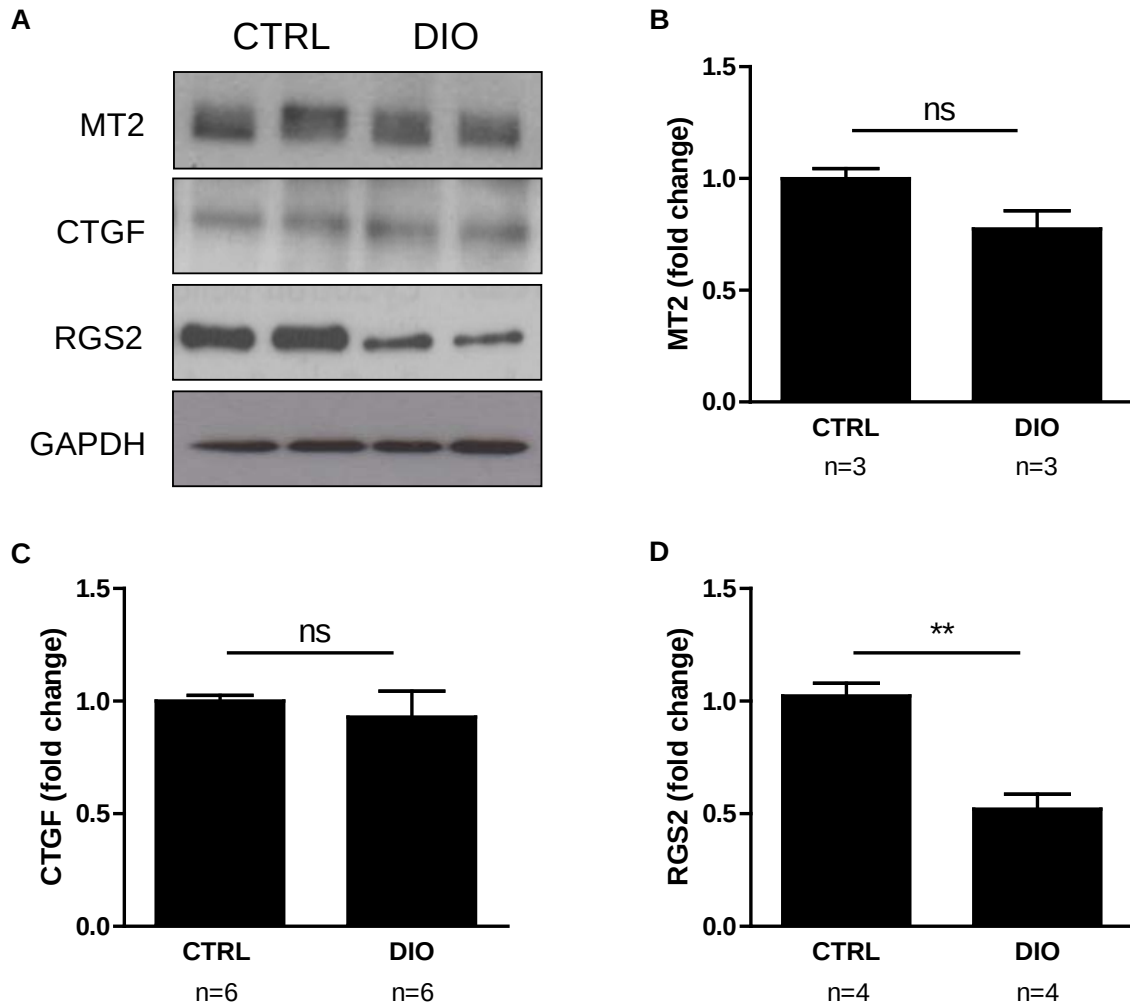


Figure 4.17: Effects of high fat diet protein expression of selected microarray candidates.

(A) Representative western blots of myocardial MT2, CTGF, RGS2 and GAPDH protein expression in control and DIO mice. (B-D) Densitometric analysis of western blots expressed as fold change after normalising to GAPDH to correct for loading differences. Data are expressed as mean $\pm$ SD for n=3 for MT2, n=6 for CTGF and n=4 for RGS2. Data were analysed using Student's t-test. ns, not significant and ** p<0.005.

4.3.5 Myocardial insulin signalling

The effects of high fat diet on myocardial insulin signalling was investigated in DIO animals after 11 weeks of high fat diet feeding at baseline and after insulin stimulation by looking at three members of the pathway, IRS1, Akt, and GLUT4.

Total IRS1 and tyrosine phosphorylation of IRS1 was measured to determine the effect of high fat diet on the initiation of the PI3K-dependent branch of the insulin signalling pathway. Western blot analysis showed no significant differences in total IRS1 expression between control and DIO mice at baseline and after insulin stimulation ($p=ns$, $n=3$ for all groups) (Figure 4.18 A and C). For tyrosine phosphorylation similar baseline expression was observed in DIO and control hearts (1.042 ± 0.05248 vs. 1.066 ± 0.1712 , $p=ns$, $n=6$). After insulin administration, tyrosine phosphorylation significantly increased by 59% in control hearts ($p < 0.05$, $n=4$ for insulin stimulation and $n=6$ for baseline) whereas only by 27% in DIO hearts ($p=ns$, $n=3$ or insulin stimulation and $n=6$ for baseline) (Figure 4.18 B and D).

Furthermore, expression levels of the downstream target Akt and its phosphorylation at the serine 473 residue (P-Akt) was investigated in DIO hearts and compared to *ob/ob* and control heart lysates. No significant changes were observed in baseline P-Akt-to-Akt ratio between DIO and controls (0.3436 ± 0.2486 vs. 0.1906 ± 0.1346 , $p=ns$, $n=3$). After *in vivo* insulin administration, a significant increase in P-Akt-to-Akt ratio was observed in both groups (1.343 ± 0.2840 vs. 0.1906 ± 0.1346 , $p < 0.05$ for CTRL + insulin vs. control; 2.370 ± 0.4877 vs. 0.3436 ± 0.2486 , $p < 0.0005$ for DIO + insulin vs. DIO) (Figure 4.19 A and C). When examining Akt expression in *ob/ob* mice, significantly higher baseline P-Akt-to-Akt ratio was observed compared to controls (0.6425 ± 0.2647 vs. 0.06908 ± 0.05993 , $p < 0.05$). After insulin administration, Akt phosphorylation significantly increased in control hearts (1.356 ± 0.1436 vs. 0.06908 ± 0.05993 , $p < 0.0005$) but was blunted in *ob/ob* hearts (1.061 ± 0.1449 vs. 0.6425 ± 0.2647 , $p=ns$) (Figure 4.19 B and D).

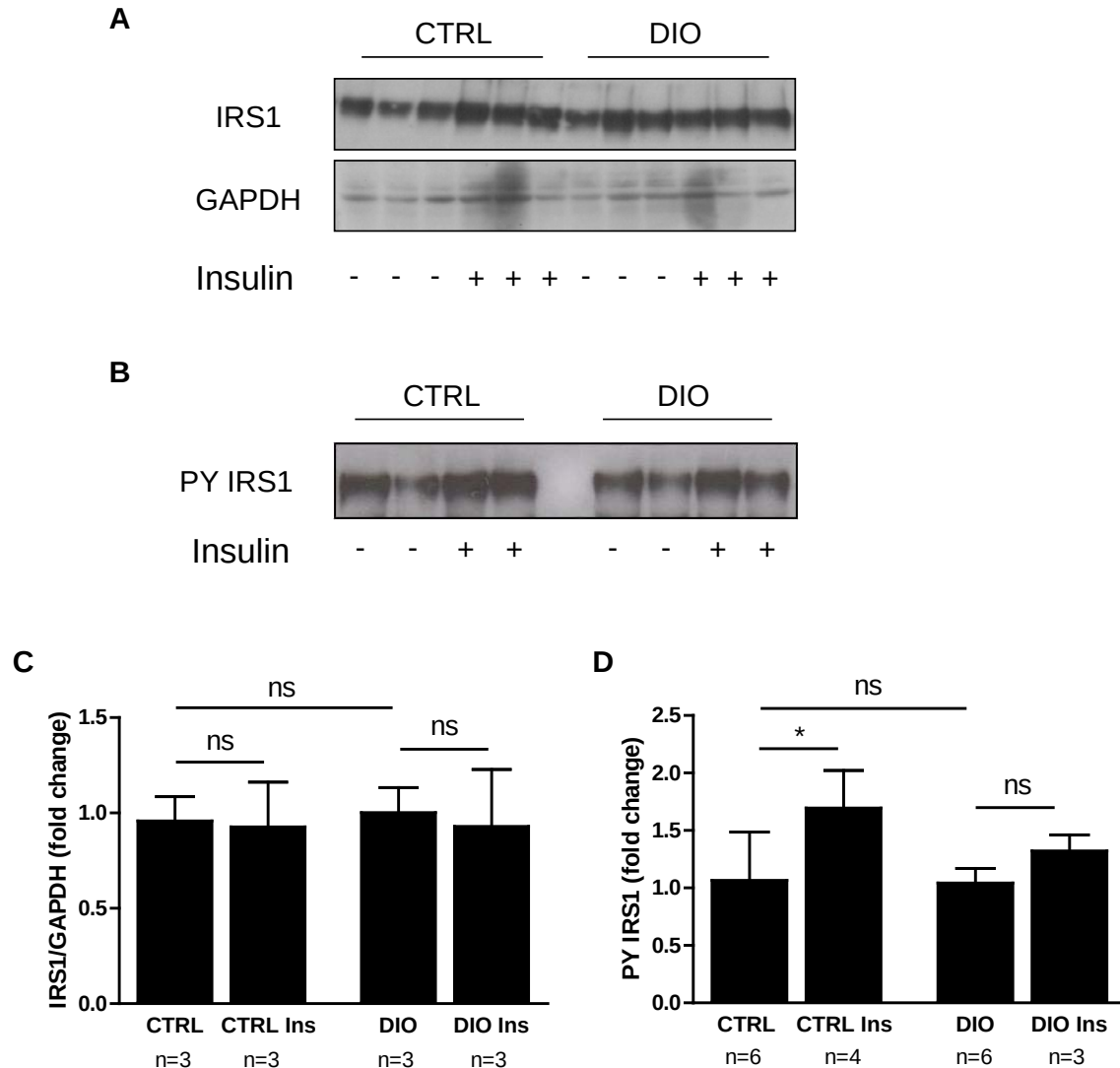


Figure 4.18: Effects of high fat diet on total IRS1 levels and IRS1 tyrosine phosphorylation.

(A) Representative western blot of total IRS1 (upper panel) and GAPDH (lower panel) expression at baseline and after insulin stimulation in DIO and control mice. (B) Representative western blot of IRS1 tyrosine phosphorylation (PY IRS1) at baseline and after insulin administration in DIO and control heart lysate. (C) Quantification of IRS1 expression normalised to GAPDH in DIO and control hearts. Data are expressed as mean±SD fold change relative to baseline control for n=3 animals per group. (D) Quantification of IRS1 tyrosine phosphorylation in DIO and control hearts. Data are expressed as mean±SD fold change relative to baseline control for n=6 animals in baseline groups and n=3-4 in insulin stimulated groups. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ns, not significant, * p<0.05.

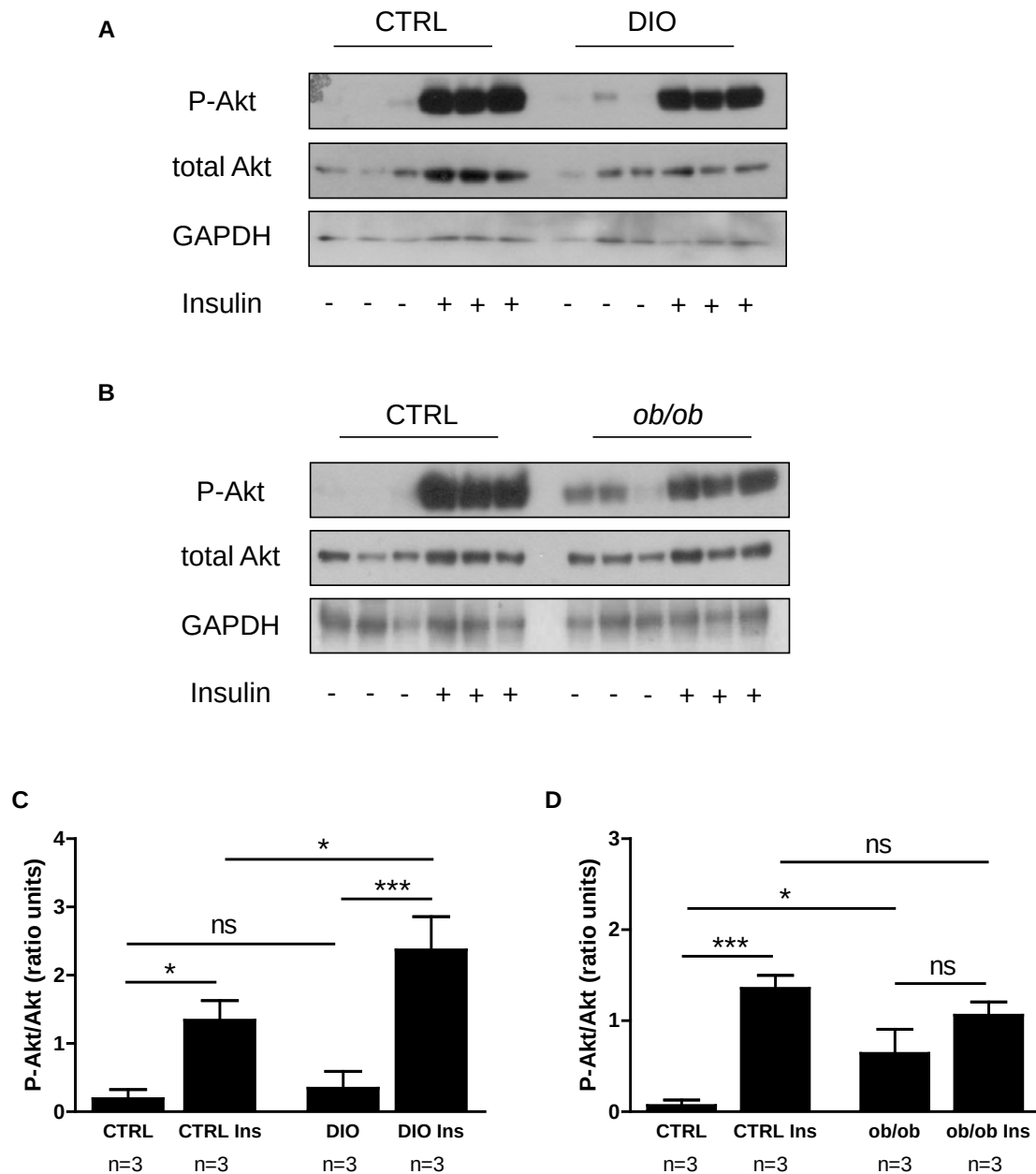


Figure 4.19: Effects of high fat diet on myocardial Akt expression.

(A) Representative western blot of myocardial phospho-Akt (top panel), total Akt (middle panel), and GAPDH (lower panel) in DIO and controls at baseline and after insulin stimulation. (B) Representative western blot of myocardial phospho-Akt (top panel), total Akt (middle panel), and GAPDH (lower panel) in *ob/ob* and control mice at baseline and after insulin stimulation. (C and D) Densitometric analysis of Akt expression normalised to GAPDH in DIO, *ob/ob* and control hearts. Phospho-Akt-to-Akt ratio is expressed as mean $\pm$ SD for n=3 animals per group. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ns, not significant; * p<0.05 and *** p<0.0005.

Moreover, the effect of high fat diet feeding on total GLUT4 content in the heart was investigated. Western blotting showed no significant changes in baseline expression of total GLUT4 in DIO whole heart lysates compared to age-matched controls (0.7593 ± 0.07705 vs. 0.9705 ± 0.05445 , $p=ns$) (Figure 4.20). After insulin stimulation, GLUT4 content significantly increased in controls (1.983 ± 0.5219 vs. 0.9705 ± 0.05445 , $p<0.005$) but not in DIO hearts (1.282 ± 0.2946 vs. 0.7593 ± 0.07705 , $p=ns$) (Figure 4.20).

Since GLUT4 content was determined in whole heart lysates we also determined GLUT4 expression specifically in cardiomyocytes using immunohistochemistry. GLUT4 immunohistochemistry showed strong and diffuse cytoplasmic staining of cardiomyocytes with membrane reinforcement in the controls at baseline. In the DIO hearts the expression was reduced, focal, and lacked membrane reinforcement (Figure 4.21 top panel). In contrast, after insulin stimulation GLUT4 expression increased in both groups with no obvious differences between them (Figure 4.21 bottom panel).

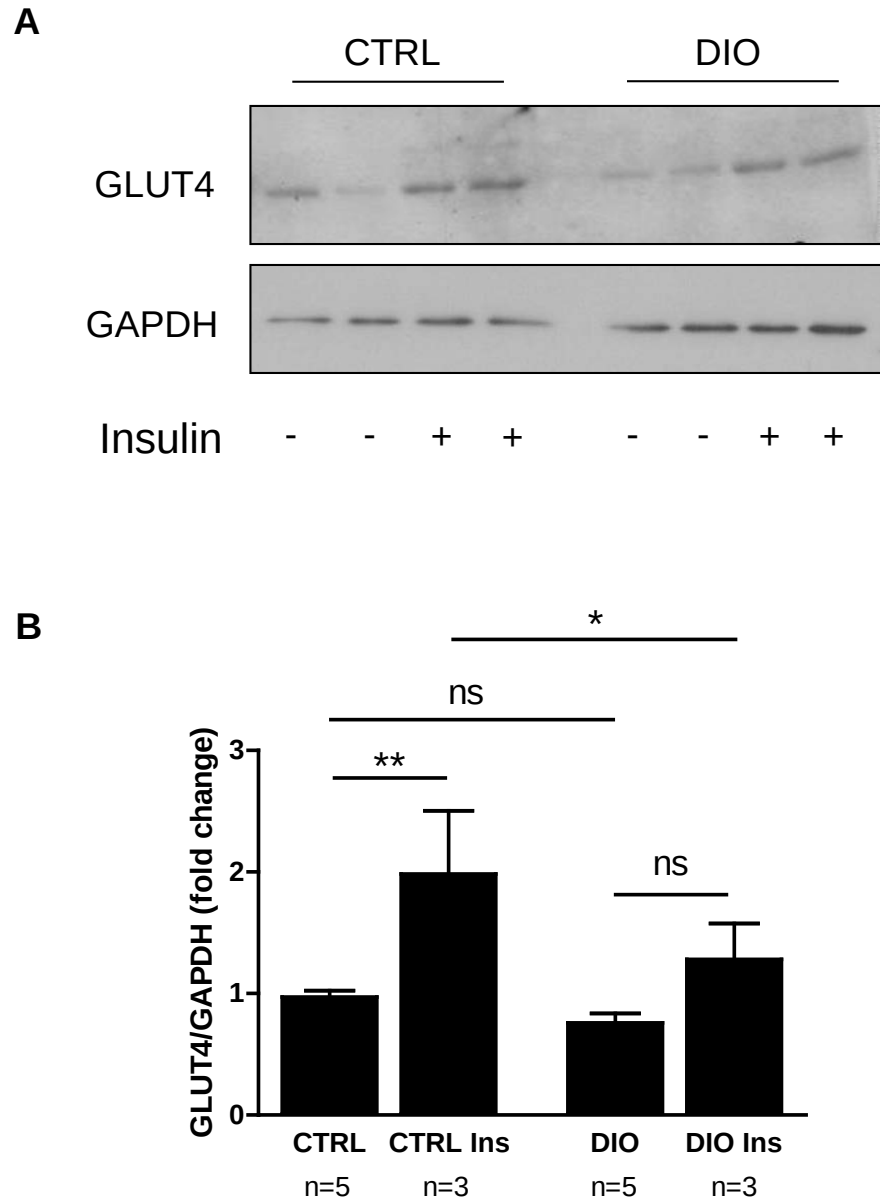


Figure 4.20: Effects of high fat diet on myocardial GLUT4 expression.

(A) Representative western blot of GLUT4 (upper panel) and GAPDH (lower panel) expression at baseline and after insulin administration in DIO and control heart lysate. (B) Densitometric analysis of GLUT4 expression in total heart lysate of DIO and control mice at baseline and after insulin stimulation normalised to GAPDH. Data are expressed as mean $\pm$ SD fold change relative to baseline control for n=5 animals for baseline DIO and control mice and n=3 for insulin stimulated DIO and control mice. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ns, not significant; * p<0.05; ** p<0.005.

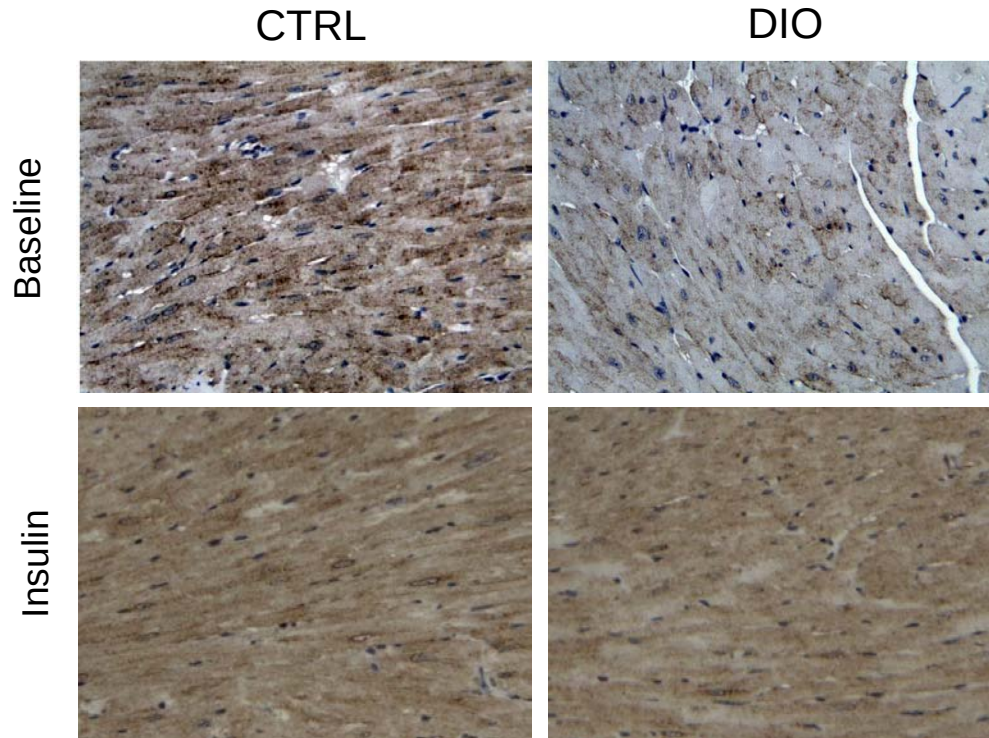


Figure 4.21: Effects of high fat diet on myocardial GLUT4 expression (IHC).

Representative GLUT4 immunohistochemistry image at baseline and after insulin administration in DIO and control heart sections. Sections were incubated in peroxidase-streptavidin solution (brown) and counterstained using Meyer haematoxylin. Images are representative of 4 animals per group. Immunohistochemistry experiments were carried out by Dr Massimiliano Mancini, La Sapienza University, Rome.

4.4 Discussion

In the first part of this chapter, our novel finding of coronary microvascular remodelling after high fat diet feeding was investigated. For this, the following questions were addressed: (1) Does coronary microvascular remodelling occur only after 14 weeks of high fat diet feeding or also at the two earlier time points investigated? (2) Is coronary microvascular remodelling associated with vascular functional changes as well as impairment of coronary flow? (3) Which genes/pathways might play a role in the development of coronary microvascular remodelling? Furthermore, in the second part of this chapter the effects of high fat diet feeding on members of the myocardial insulin signalling pathway were investigated.

The main findings in this chapter are that coronary microvascular remodelling is already present after 8 weeks of high fat diet feeding but progresses with increasing feeding duration. Also, coronary microvascular remodelling is accompanied by coronary microvascular dysfunction and endothelial dysfunction in resistance arteries but independent of hypertension or cardiac hypertrophy. High fat diet feeding is associated with down-regulation of genes involved in myocardial lipid and glucose metabolism, the development of hypertrophy and fibrosis as well as vascular reactivity. Moreover, when looking at members of the PI3K-dependent branch of the insulin signalling pathway we show that high fat diet feeding leads to a blunted phosphorylation of IRS1, a significantly higher P-Akt/Akt ratio, and no significant differences in GLUT4 expression in total heart lysates of DIO mice after insulin stimulation. When looking at cardiomyocytes of DIO mice, lower GLUT4 expression at baseline is observed compared to control mice. This is different to our findings in the myocardium of *ob/ob* mice, in which phosphorylation of Akt was attenuated and total GLUT4 unchanged at baseline.

4.4.1 Effects of high fat diet on coronary microvascular remodelling and metabolic markers

The observed coronary microvascular remodelling, which has never been described in a diet-induced mouse model before, provides the possibility to directly study potential links between metabolic and coronary microvascular changes. To further characterise this finding the first question we addressed was whether coronary microvascular remodelling was not only present after 14 weeks of high fat feeding but also after 8 and 11 weeks of high fat diet. We observed that thickening of the intimal layer was present after 8 weeks of high fat diet whereas the media-to-lumen ratio was not significantly increased. Concomitantly, fasting insulin and glucose were increased and glucose tolerance was mildly impaired after 8 weeks of high fat diet but not significantly changed. On the other hand, after 11 weeks of high fat diet both intimal layer thickening and media-to-lumen ratio as well as fasting insulin and glucose were significantly increased and glucose tolerance significantly impaired. Interestingly, in a study in female rats fed a 42% high fat diet for 8 weeks extensive perivascular fibrosis but no significant changes in the vessel wall or lumen area was observed in the hearts [234].

In addition to altered insulin and glucose levels we investigated leptin concentrations and FA concentrations in serum samples of DIO mice after 11 weeks of high fat diet as additional markers of insulin resistance and T2DM. Serum leptin levels in DIO mice after 11 weeks of high fat diet were significantly increased and positively correlated with visceral fat mass. It has been shown that fat deposition and adipose endocrine function are affected by dietary fat intake [222]. Moreover, depending on the degree and duration of exposure leptin can have both harmful and cardioprotective effects on the heart [235-237]. Myocardial lipid accumulation and cardiac pathology has been described in leptin-deficient mice suggesting that hyperleptinaemia might be cardioprotective [202, 238]. Also, studies in human and rodent cardiomyocytes show that leptin can directly stimulate hypertrophy and increase ROS generation [239-242].

Similar to the observations by Chess *et al.* we demonstrate in this study that high fat feeding significantly increases whole body adiposity and circulating leptin without obvious deleterious effects on the heart [222]. However, we cannot exclude that a combination of hyperinsulinaemia, hyperleptinaemia and hyperglycaemia contributes to the coronary microvascular remodelling we observed in DIO hearts. Besides increased leptin concentrations an increase of fatty acids in the blood of diabetic patients has been observed [50-52]. Several studies have shown that fatty acid levels were increased in genetic mouse models of T2DM due to the increased amount of lipolysis of stored triglycerides in adipose tissues [223, 243, 244]. However, publications on the effect of high fat diet on FA concentrations vary. In a study by Chess *et al.* a significant increase in plasma FFA was observed already after 6 weeks of high fat diet feeding (45% fat) [222] whereas Park *et al.* observed a significant increase in plasma FFA concentrations only after 15 weeks of high fat diet [75]. In our study FA concentrations in serum of 11 weeks DIO mice were slightly lower but not significantly changed compared to controls. Only in age-matched *ob/ob* mice a significant increase in serum FA concentrations was observed which is consistent with previous findings in *ob/ob* mice [243].

4.4.2 Effects of high fat diet on coronary blood flow and vascular reactivity

To determine whether hemodynamic load, i.e. blood pressure, contributed to the development of microvascular remodelling, MAP was investigated in DIO mice after 11 weeks of high fat diet and compared to age-matched controls. MAP did not differ between DIO mice and controls, indicating that hypertension did not contribute to changes observed in coronary vascular structure. Similarly, Roberts *et al.* have shown that hypertension in female Sprague Dawley rats developed only after 1 year of high fat, refined carbohydrate diet [245]. Additionally, we aimed to assess cardiac function of DIO hearts using echocardiography which is commonly used to investigate cardiac function in murine models [224-226]. Due to the increased amount of pericardial fat in DIO mice and the change in axial orientation of the heart we chose cardiac magnetic resonance imaging to investigate the effects of high fat diet on stroke volume and ejection fraction. Our results show no significant changes between DIO after 11 weeks of high fat diet and age-matched control mice. In other diabetic mouse models impairment of cardiac function has been observed. Using cardiac MRI, Yu and co-workers demonstrated ventricular wall thinning, increased end-systolic diameter and volume and diminished ejection fraction, stroke volume and cardiac output in STZ-induced T1DM mice at 12 weeks of age [246]. Additionally, increases in LV mass, wall thickness, and end-diastolic volume were observed in *db/db* mice between 5 and 13 weeks of age and decreased ejection fraction with no further changes at later stages [247]. Preliminary results by Wylezinska *et al.* in high fat diet fed mice show a trend to diminished ejection fraction which suggests that high fat diet may have implications on cardiac function [248]. Our results are in line with other studies that showed that short periods of high fat diet feeding does not affect baseline LV structure and function [166, 249]. However, the altered metabolic state due to the high fat diet was associated with increased LV remodelling and dysfunction after chronic pressure overload [166]. Park *et al.* showed that ventricular fractional shortening progressively declined after 10 to 15 weeks and was significantly reduced after

20 weeks of high fat diet feeding [75]. Accordingly, LV posterior wall thickness and heart weight increased only after 20 weeks [75]. Since cardiac function was not significantly blunted until after 20 weeks of high fat diet feeding, Park *et al.* suggested that cardiac insulin resistance *per se* does not mediate cardiac dysfunction in high fat-fed mice [75].

Since the microcirculation is responsible for the control of myocardial blood flow the effects of coronary microvascular remodelling on microvascular function were investigated by assessing both myocardial blood flow and reactive hyperaemia. Reactive hyperaemia is defined as the transient increase in blood flow following a brief period of ischaemia [250]. The shortage of oxygen will lead to a build-up of vasodilator metabolites resulting in the dilation of arterioles, the reduction in vascular resistance and consequently to an increase in blood flow. By calculating the peak-to-baseline ratio it is possible to determine coronary flow reserve, an indirect measure of coronary microvascular dysfunction in humans [92]. Using Langendorff perfusion of isolated hearts no significant changes in peak-to-baseline ratio between DIO and control hearts were observed. However, a significant increase in total peak area as well as a significant increase in the time needed to return to baseline flow was observed in DIO mice. This suggests that despite no obvious differences in vasodilation after the ischaemic event, recovery was impaired. The fact that no differences were observed in the peak-to-baseline ratio could be explained by the fact that the Langendorff set-up used is designed for the use of rat hearts and may thus be not sensitive enough to detect potential differences in mouse hearts.

Changes in coronary flow can generally be ascribed to either changes in microvascular structure or to functional changes. Thus, we aimed to assess the effects of high fat diet on endothelial function. Impairment of endothelial function is well established in the micro- and macrocirculation of patients with T2DM and is an early manifestation of atherosclerotic vascular damage [251, 252]. It is characterised by reduced NO bioavailability resulting from ROS overproduction,

lipid peroxidation, and increased generation of adhesion molecules, increased expression of pro-inflammatory and pro-thrombotic factors as well as abnormal vasoreactivity [253, 254]. In both clinical settings and animal models of diabetes like the *db/db* mouse reduced endothelium-dependent vasodilations are commonly observed [166, 245, 255, 256]. Additionally, leptin has been shown to contribute to endothelial dysfunction or damage with high concentrations of leptin leading to an impairment of acetylcholine-induced NO-dependent vasorelaxation both *in vitro* and *in vivo* [257]. Since previous studies in high fat diet fed animals reported endothelial dysfunction [166, 245, 255, 256] we investigated endothelial function *in vivo* by administering the vasodilator ACh and measuring the effect of this challenge on MAP as well as time required to return to baseline MAP. We hypothesised that the bigger the drop in MAP the higher the vasodilatory effect in response to ACh. Thus, in wild type mice we expected that MAP would drop upon ACh administration and slowly return back to baseline. On the other hand we hypothesised that high fat diet feeding would attenuate the response to ACh. Our results showed a significantly blunted drop in MAP after ACh administration in DIO mice and significantly reduced recovery time compared to age-matched controls which suggests an impaired vasodilation and greater resistance in DIO mice and thus indicates impairment of vascular reactivity and endothelial function in DIO mice *in vivo*. This result was further strengthened by *ex vivo* assessment of vascular reactivity in vessels of DIO mice. Wire tension myography on aortic rings of DIO and control animals showed that high fat diet consumption for 11 weeks induces endothelial dysfunction before the onset of hypertension and that endothelium-independent relaxation is not impaired in DIO mice. ACh was used to assess the effects of high fat diet on endothelium-dependent vasodilation. Our results are in line with previous publications demonstrating impaired endothelium-dependent vasodilation to ACh in aorta of mice fed a high fat diet for 15 and 30 weeks [258, 259], and in aorta of rats after 8 weeks [234, 260], or 7 month [245] of high fat diet treatment. To assess the effects of high fat diet on vascular smooth muscle function SNP was used. SNP-induced relaxation was similar in both DIO and

control mice, which is consistent with previous publications in aorta of mice and rats fed a high fat diet [245, 258]. This indicates that the ability of vascular smooth muscle to relax in response to exogenous NO was not impaired in DIO mice and that diet selectively impaired endothelium-dependent vasodilation.

To further investigate the observed endothelial dysfunction we looked at a possible underlying cause, an impairment of NO bioavailability. Impaired NO bioavailability could be due to an imbalance of NO production and/or increased NO inactivation [261-263]. As described in Chapter 1, NO is produced in vascular endothelium by activation of eNOS which catalyses the conversion of the substrate L-arginine to NO and L-citrulline [36]. Possible mechanisms for NO deficiency are reduced eNOS activity and/or reduced NO synthesis caused by asymmetrically methylated arginines (N^G monomethyl-L-arginine [L-NMMA] and asymmetric dimethylarginine [ADMA]) or production of ROS that inhibit NOS activity [36, 264, 265] (Figure 4.22). L-NMMA is the prototype inhibitor of NOS and is widely used as a pharmacological tool in cells and animal models [265]. ADMA exists normally in the body and has been found to be elevated to levels that can significantly inhibit NOS activity in individuals with hypercholesterolaemia, hypertension, tobacco exposure, and hyperglycaemia [168, 266, 267]. In DIO mice after 11 weeks of high fat diet no changes were observed in plasma ADMA levels compared to age-matched control mice which suggests that the observed endothelial dysfunction was not caused by increased NO inhibition by ADMA. However, it still needs to be determined whether L-NMMA, ROS generation or NOS activity are affected by high fat diet and thus are a potential underlying mechanism of impaired endothelium-dependent vasodilation observed in DIO mice.

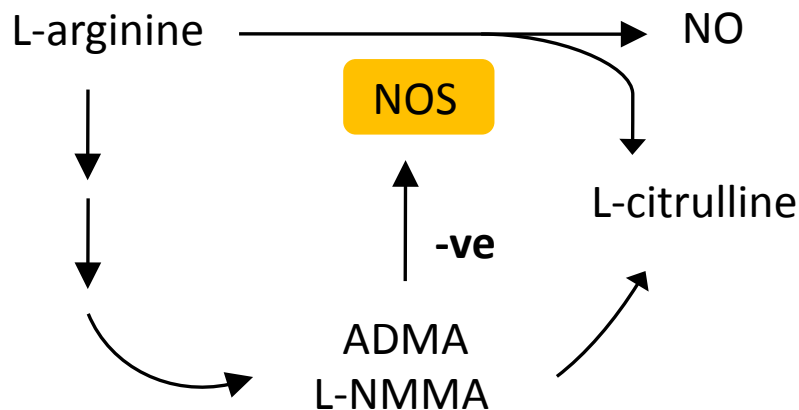


Figure 4.22: Simplified model for the regulation of NO synthesis.

L-arginine is metabolised to NO and L-citrulline by NOS. Free intracellular L-NMMA and ADMA inhibit the production of NO catalysed by NOS. ADMA, asymmetric dimethylarginine; NO, nitric oxide; NOS, nitric oxide synthase; -ve, negative. Adapted from [265].

Interestingly, a high variation in the ACh-induced endothelium-dependent vasodilation was observed in DIO aortic ring segments. In this context, it is important to note that the type of vessel as well as the origin of ring segment used plays an important role in the assessment of vascular reactivity. With regards to the origin of ring segments, a reason for variation in vascular reactivity could be the exposure of the vasculature to different blood flow patterns on the endothelial layer at distinct sites [252, 268]. It has been shown that the effect of blood flow contributes to the distinct physiological characteristics of the arterial wall which in some regions promote an anti-inflammatory, anti-thrombotic, anti-coagulative, profibrinolytic and anti-hypertrophic state [252, 268]. In contrast, other regions such as branches and arches of the arterial tree are susceptible to the development of atherosclerosis [252]. In our experiments ring segments were chosen randomly and thus we cannot exclude the possibility that both of the described regions were used and thereby might explain in part the high variation between samples.

Additionally, spontaneous contractions to ACh were observed in aortic ring segments which might have influenced the results. Studies in rat peripheral arteries have observed endothelium-dependent contraction to ACh under pathological conditions like hypertension and diabetes [269-271]. Faraci *et al.* demonstrated that ACh caused contraction in the carotid artery of eNOS knockout mice [272]. Since ACh has been shown to cause endothelium-independent contraction by activating muscarinic receptors located on VSMC in some vessels [273, 274] it is possible that the contraction was either mediated by an endothelial or smooth muscle action of ACh. Recently, Zhou *et al.* demonstrated for the first time that ACh causes endothelium-dependent contraction in wild-type and eNOS knockout mouse arteries [275]. They suggest that ACh initiates two competing responses in mouse arteries (endothelium-dependent relaxation mediated predominantly by NO and endothelium-dependent contraction mediated most likely by thromboxane A₂) and that contraction to ACh appears to be a normal physiological response of the blood vessels [275]. Although contraction of vessels in response to ACh occurs in pathological conditions in rats, the modulation of endothelium-dependent contraction still remains to be determined in mouse vascular pathologies [275]. Thus, it could be possible that the observed contractions in DIO ring segments may be due to a combinatory effect of the diet and high age of the animals since spontaneous contractions of aortic rings in older animals have been observed before [269]; unpublished observations by Dr Sujkovic).

Taken together, our results show that coronary microvascular remodelling in DIO mice is independent of hypertension and cardiac hypertrophy. Additionally, we observed an association between the development of muscular inward remodelling and the progressive worsening of metabolic parameters like insulin, glucose or leptin. The fact that no overt changes were observed in myocardial blood flow and cardiac function suggests that microvascular changes precede the impairment of cardiac function. Furthermore, high fat diet feeding impaired endothelium-dependent vasodilation in aortic rings.

4.4.3 Effect of high fat diet on myocardial gene expression and a potential role in vascular remodelling

To investigate which genes/pathways in the heart are affected by high fat diet feeding and which of those might play a role in the development of vascular remodelling, microarray analysis of left ventricles was chosen. This mixed tissues approach was chosen due to sample and technical limitations. Generally, laser capture microdissection of transmural arterioles with subsequent RNA isolation and microarray analysis would have been the preferred choice. However, due to the small size of the murine heart a large number of animals would have been necessary to obtain the required quantity of RNA.

Genes which were differentially expressed with high fat diet were divided into three groups depending on their function: (1) lipid metabolism, (2) hypertrophy and fibrosis and (3) vascular reactivity.

In our DIO mouse model, the majority of genes involved in lipid metabolism were down-regulated. Genes involved in lipid metabolism and their functions are listed in Table 4.5. Fatty acids play an important role in the healthy myocardium due to their role as fuels, mediators of signal transduction, or ligands for nuclear transcription factors like peroxisome proliferator-activated receptor α (PPAR α), a key player in transcriptional control of FA metabolism [276]. Generally, in lipid metabolism, FFA can enter the circulation and muscle fibres by diffusion once they are freed from glycerol. Beta-oxidation in mitochondria eventually splits long carbon-chains of FA into acetyl CoA, which can eventually enter the citric acid cycle. In T2DM elevation of plasma FFA levels leads to the activation of the transcription factor PPAR α which induces the expression of PPAR α -regulated genes like Pdk4 and Ucp3 [277]. Both Pdk4 and Ucp3 were down-regulated in DIO hearts. PDK4 is located in the matrix of mitochondria and contributes to the regulation of glucose metabolism by inhibiting the mitochondrial pyruvate dehydrogenase complex [278]. Its expression is regulated by insulin and studies

have shown that it is up-regulated in hearts of STZ-induced animal models of diabetes and down-regulated in animal models of hypertrophy (pressure overload-induced) [277]. In female albino Wistar rats fed a 43% high fat diet for 4 weeks PDK4 protein expression was significantly increased compared to controls on a low fat/high carbohydrate diet [279]. In transgenic mice with cardiac-specific overexpression of PDK4, hearts show low myocardial glucose oxidation and increased fatty acid oxidation (a fuel shift that has been shown to sensitise the heart to ischaemic insult and ventricular dysfunction) as well as protection against high fat diet-induced myocyte lipid accumulation [278]. UCP3 facilitates transfer of anions from the inner to outer mitochondrial membrane and the return transfer from outer to inner membrane and therefore regulates mitochondrial membrane potential. Like PDK4, UCP3 has been found to be up-regulated in diabetes and down-regulated in hypertrophy [277, 280]. This was confirmed in another study in FVB mice fed a 45% high fat diet for 20 weeks. Both PPAR α -regulated genes, PDK4 and UCP3, were upregulated in the hearts of animals fed the high fat diet [281]. Intriguingly, Schrauwen *et al.* have shown that UCP3 protein content was significantly lower in muscle biopsies of pre-diabetic subjects with impaired glucose tolerance as well as diabetic patients compared to healthy controls [282]. Additionally, UCP3 expression was found to be reduced following myocardial infarction in wild-type mice [148] and Young *et al.* have shown that progression of diabetes is associated with a dramatic decrease of PPAR α expression and the PPAR α -regulated UCP3 in the adult rat heart [280]. In DIO myocardium UCP3 mRNA levels were significantly lower expressed compared to controls, which is in line with the latter findings in diabetic rat hearts and muscle of diabetic patients and suggests that FA oxidation in DIO hearts is impaired.

Furthermore, the expression of Ehhadh, Hmgcs2, and Scd4 were significantly reduced in DIO hearts. Scd4 plays a role in the biosynthesis of monounsaturated FA. It is exclusively expressed in the heart and is induced by high carbohydrate diet [283]. In hearts of *ob/ob* mice Scd4 mRNA was increased

by 5-fold compared to wild type controls and decreased with leptin-treatment of *ob/ob* mice. Miyazaki *et al.* concluded that down-regulation of Scd4 expression by leptin may be one of the mechanisms by which leptin depletes lipid from the heart and exerts its anti-lipotoxic effects [283].

Ehhadh is a member of the peroxisomal enzymes of the classical peroxisomal β -oxidation cycle, which plays an essential role in the shortening of very long chain FA prior to complete oxidation in mitochondria [284] but little is known about its role in the diabetic heart. The lower expression of Ehhadh in DIO hearts suggests that peroxisomal β -oxidation cycle might be attenuated and thus potentially inducing accumulation of very long chain FA in the myocardium.

HMGCS2 is a mitochondrial enzyme that catalyses a rate-limiting reaction in ketogenesis, a mitochondrial process by which acetyl-CoA is converted into ketone bodies in states such as starvation, poorly controlled diabetes or low carbohydrate feeding [285]. Its transcription is induced by fatty acids both *in vivo* and *in vitro* and transcriptional activation seems to be mediated by PPAR [286]. In adult rat liver, a 40% saturated fat diet produces a 3-fold increase in mitochondrial Hmgcs2 mRNA and protein levels [287, 288] which is mediated by PPAR. Additionally, rats in which diabetes has been induced by STZ show increased mitochondrial Hmgcs2 mRNA levels compared to normal controls [288]. In the liver, insulin suppresses the expression of Hmgcs2 [289] and Wentz *et al.* have shown that in cultured cardiomyocytes Hmgcs2 induction is dependent on PPAR α and inhibited by insulin [285]. This would suggest that hyperinsulinaemia present in DIO animals might lead to the lower expression of Hmgcs2 at the transcriptional level.

Table 4.5: Genes involved in lipid metabolism and their protein function

Gene encoding for	Protein function
Stearoyl-coenzyme A desaturase 4 (Scd4)	Biosynthesis of monounsaturated FA
Enoyl-CoA hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (Ehhadh)	Peroxisomal β -oxidation
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (Hmgcs2)	Catalysation of ketogenesis
Farnesyl diphosphate farnesyl transferase 1 (Fdft1)	Cholesterol biosynthesis
Uncoupling protein 3 (Ucp3)	Regulator of mitochondrial membrane potential
Pyruvate dehydrogenase kinase 4 (Pdk4)	Phosphorylation and inhibition of pyruvate dehydrogenase

Besides lipid metabolism, differentially expressed genes involved in the development of hypertrophy and fibrosis were identified. Genes encoding connective tissue growth factor (Ctgf) and the protein LIM and cysteine-rich domains 1 (Lmcd1) were significantly lower expressed in DIO hearts compared to age-matched controls. LIM domain proteins interact with transcriptional regulators, kinases, and structural proteins, and have therefore been assigned roles in cell growth, differentiation, and cytoskeletal remodelling [290]. LMCD1 is expressed in most tissues but particularly high in skeletal and cardiac muscle [291]. Bian *et al.* have shown in primary cardiomyocytes and cardiac-specific Lmcd1 transgenic mice after a hypertrophic stimulus (transverse aortic constriction) that Lmcd1 overexpression increases the hypertrophic and fibrotic response via activation of the calcineurin/nuclear factor of activated T cells signalling pathway whereas in the absence of this stimulus overexpression alone does not induce cardiac hypertrophy [292]. Additionally, they have shown that hypertrophy can be blocked by inhibiting Lmcd1. One downstream target gene of Lmcd1, the cardiac hypertrophy marker Ctgf, was also significantly down-regulated in DIO myocardium at the transcriptional level. Ctgf is rapidly

up-regulated in cardiomyocytes exposed to prohypertrophic stimuli [292] and mediates high glucose and palmitate induced hypertrophy and apoptosis in cardiomyocytes of STZ-treated rats [293]. Similarly, in VSMC, overexpression promotes expression of more extracellular matrix protein collagen I and fibronectin, and is a mediator of angiotensin II-induced fibrosis *in vitro* [229]. Despite its lower mRNA expression in DIO hearts, protein expression was not significantly changed compared to control hearts.

Finally, differentially expressed genes encoding metallothionein 2 (Mt2), G protein-coupled receptor 22 (Gpr22) and regulator of G protein signalling 2 (Rgs2) were grouped together due to their role in vascular reactivity. Gpr22 is termed as an orphan G protein-coupled receptor due to the fact that its natural ligands have not been identified. Gpr22 mRNA and protein are highly expressed in cardiomyocytes and coronary arteries and Gpr22 knockout mice show increased susceptibility to heart failure under conditions of hemodynamic stress [294]. The enriched expression in coronary vascular cells suggests a possible role for this receptor in the regulation of coronary blood flow [294]. In contrast to these findings, Gpr22 mRNA expression was significantly up-regulated in DIO hearts compared to age-matched controls.

Another differentially expressed gene, Metallothionein 2 (Mt2), was significantly down-regulated in DIO hearts. Mt2 is an intracellular metal-binding and cysteine-rich protein, which mainly act as regulator of matrix metalloproteinases [233, 295]. It is a potent antioxidant and adaptive (or stress) protein to protect cells and tissues from oxidative stress [295]. It has been shown that enhanced Mt2 expression in heart and kidney provided significant protection from diabetes-induced organ dysfunction such as cardiomyopathy and nephropathy [295, 296]. Interestingly, both gene and protein expression was significantly lower in aortic tissue and cultured aortic smooth muscle cells from bicuspid aortic valve patients [233] which suggests a role of metallothionein in regulating extracellular matrix homeostasis within the media of the ascending aorta. The lower

expression of Mt2 mRNA in DIO hearts was accompanied by a reduction of MT2 protein expression of about 20%. However, due to the high variability between samples no statistical significance was observed. Despite this, the data suggest a possible impairment of the antioxidant action of MT2 in DIO hearts which may promote the development of oxidative stress induced cellular changes in myocardium or vascular tissue.

The gene encoding regulator of G protein signalling 2 (Rgs2) is another gene identified by microarray analysis which was differentially regulated by high fat diet. Regulators of G protein signalling accelerate the deactivation and inhibit signalling by acting as GTPase-accelerating proteins at active G α protein subunits [297]. RGS2 shows regulatory selectivity for the G α_q subclass of G proteins which is involved in signalling of many important cardiovascular hormones such as endothelin-1, angiotensin-II or norepinephrine [231]. Additionally, expression of RGS2 has been described in tissues that are important for blood pressure regulation like the kidney, vascular smooth muscle cells and the central nervous system [231]. Polymorphisms in Rgs2 have been observed in a Japanese hypertensive cohort [298] whereas hypotensive patients have increased expression of RGS2 at the RNA and protein level [299]. Moreover, animal studies have shown that homo- and heterozygous knockout mice exhibit a hypertensive phenotype [297]. In DIO hearts not only Rgs2 mRNA expression was significantly lower but also protein expression was significantly reduced by ~50% compared to control hearts. The *in vivo* role of RGS2 in regulating cardiac function and blood pressure together with the significant findings at both RNA and protein level after high fat diet feeding lead us to suggest that reduced expression of RGS2 could be of considerable pathophysiological significance and therefore might be a potential candidate involved in the development of microvascular remodelling in DIO hearts.

4.4.4 Effects of high fat diet on the myocardial insulin signalling pathway

Cellular insulin resistance involves a combination of factors and disruption of more than one signalling component [300, 301]. Despite the fact that the heart is only a minor site of whole body glucose disposal [13], a reduction in glucose uptake and utilisation as well as insulin resistance has been observed in the diabetic heart [45, 70]. Since insulin action on glucose transport in the heart appears to be primarily mediated by the PI3K-dependent branch [302], we focused on three members of this branch to investigate the effects of high fat diet feeding on insulin-mediated glucose uptake.

IRS1 is a critical point for the control of glucose uptake in heart and skeletal muscle and a deficit in IRS1 tyrosine phosphorylation has been observed in skeletal muscle of diabetic patients [300, 303]. After 11 weeks of high fat diet, no significant differences were observed in total IRS1 content between DIO and control mice at both baseline and after insulin stimulation. Moreover, similar baseline tyrosine phosphorylation levels of IRS1 were observed in DIO and control hearts. After insulin stimulation, phosphorylation significantly increased in control mice but not in DIO hearts. Similarly, no differences in baseline phosphorylation of Akt were observed between DIO and control hearts. When stimulated with insulin, phosphorylation significantly increased in both groups. In *ob/ob* mouse hearts, baseline P-Akt levels were significantly increased compared to controls and only in control hearts a significant increase in P-Akt after insulin stimulation was observed. Similarly to the results in *ob/ob* hearts, the observed results suggest that high fat diet feeding and the resulting compensatory hyperinsulinaemia activates the insulin receptor leading to tyrosine phosphorylation of IRS1. Although IRS1 phosphotyrosine levels are lower in DIO hearts it appears to be sufficient to activate the PI3K-dependent branch of the insulin signalling pathway resulting in a significant increase in phosphorylation of Akt. Akt is regarded as an important signalling node in the insulin signalling pathway. Activation of Akt results in modulation of various

downstream targets like eNOS activity, GLUT4 translocation, GSK1 or the transcription factor FOXO (described in Chapter 1). In contrast to our observations in high fat diet fed animals, Park *et al.* reported a reduction of insulin-stimulated Akt phosphorylation by 40% after 10 days of feeding a 55% high fat diet [75]. Similarly, no differences in basal P-Akt levels but a decrease in P-Akt after insulin stimulation was reported in *ob/ob* mice [73]. The differences in Akt phosphorylation between our study and the publication by Park and co-workers [75] could, in part, be explained by the differences in fat content of the diet (45% vs. 55%), the age of the animals as well as the feeding duration (11 weeks vs. 10 days and 3 weeks). It is possible that short-term high fat diet feeding results in a reduction of Akt activity whereas long-term feeding together with compensatory factors might have a different effect on Akt protein expression. Also, the possibility of cross talk between different signalling pathways should not be excluded. For example, up-regulation of the sympathetic nervous system has been described to play an important role in the pathogenesis of insulin resistance. Morisco *et al.* have observed interaction between beta-adrenergic and insulin receptors in neonatal cardiomyocytes [29]. Moreover, insulin and leptin signalling pathways are known to share certain downstream molecules such as IRS, PI3K, Akt, and MAPK [304] and *in vitro* data in skeletal muscle provide evidence that high leptin levels lead to the hallmarks of insulin resistance including inhibition of insulin-stimulated glucose uptake [305].

Reduced insulin-stimulated glucose uptake has been observed in different mouse models of insulin resistance and T2DM [73-75]. Wright *et al.* observed that a short duration of high-fat feeding (2 and 5 weeks of 45% high fat diet) causes an early reduction in glucose utilisation on the basis of reduced GLUT4 content and translocation [74]. Similarly, Park and co-workers demonstrate a reduction in total GLUT4 content in animals fed a high fat diet for 10 days as well as 3 weeks and conclude that diet-induced cardiac insulin resistance develops before alterations in whole-body glucose homeostasis and secondary

to defects in Akt-mediated insulin signalling and GLUT4 expression [75]. In contrast, we observed a slightly lower but not significant GLUT4 protein content in DIO mouse total heart lysates at baseline. When looking specifically at GLUT4 content in cardiomyocytes lower basal GLUT4 expression was clearly observed. Interestingly, insulin stimulation resulted in an increased expression of GLUT4 in control hearts but no significant differences were observed in DIO mice. Similarly, no changes in total GLUT4 content after insulin stimulation have also been observed in the leptin-deficient *ob/ob* mouse hearts whereas the ability of insulin to stimulate glucose uptake was completely blunted [73]. Since insulin-responsive glucose uptake in the heart is facilitated by the translocation of GLUT4 from intracellular storage vesicles to the sarcolemma [48] it is likely that blunted glucose uptake represents a defect in GLUT4 translocation to the sarcolemma. To determine whether a defect in GLUT4 translocation exists in DIO hearts after insulin stimulation we tried to investigate GLUT4 localisation at the sarcolemma as well as the major proteins involved in GLUT4 trafficking and docking like AS160, Syntaxin4, VAMP2/3, or Munc18c [306]. However, due to technical constraints we were unable to obtain the data and thus we cannot properly address this particular question.

**Chapter Five: Evaluating the role of
RGS2 in coronary microvascular
remodelling and blood pressure control
in DIO**

5.1 Introduction

Coronary microvascular remodelling is associated with functional changes as well as structural alterations in vessel morphology and has been observed in patients with hypertension, T2DM, or HCM [92]. As described in the previous chapters we have demonstrated the development of blood pressure-independent coronary microvascular remodelling in our DIO mouse model. Moreover, microarray analysis on DIO hearts resulted in differentially expressed genes involved in lipid metabolism, fibrosis and hypertrophy as well as regulation of cardiovascular function and disease (Chapter 4). Among the genes which were differentially expressed in DIO mice, we here focus on *Rgs2*, which was decreased at both RNA and protein level, and might play a significant role in the development of microvascular remodelling in DIO hearts.

5.1.1 Physiological role of RGS2

RGS2 expression has been described in tissues that are important for blood pressure regulation and vascular function like the kidney, VSMC and the central nervous system [231, 307]. Both clinical and basic studies have demonstrated that deregulation of RGS2 contributes to the pathogenesis of CVD [308, 309]. For example, studies have shown that RGS2 mRNA and protein levels are reduced in hypertensive patients [310, 311]. Yang *et al.* have identified two rare mutation of RGS2 in a Japanese hypertensive cohort (RGS2-Q2L and RGS2-Q2R) which were reported to affect protein stability [312]. In contrast to these results, in 2007 Bodenstein and co-workers showed that only RGS2-Q2L results in strong proteasomal degradation and lower expression in HEK293 cells [297]. Further studies have shown that another mutation, RGS2-R44H, disrupts the amphipathic α -helix which is crucial for proper plasma-membrane targeting and function [313] whereas the RGS2-C1114G variant has been shown to associate with hypertension and higher than normal body mass index which suggests a link between RGS2 and obesity and the metabolic syndrome [314]. Animal studies have further underlined the essential role of RGS2 in the regulation of

vascular tone. RGS2 knockout mice exhibit modest systemic hypertension and strikingly elevated mean arterial pressure [315, 316]. Additionally, Tang *et al.* have shown increased contraction and impaired cGMP-mediated relaxation in aortic rings from RGS2 knockout mice [315] and Sun *et al.* provided the first evidence indicating that RGS2 mediates the action of the NO-cGMP pathway on blood pressure by promoting relaxation of the resistance vasculature through its ability to attenuate vasoconstrictor-induced Ca^{2+} signalling [232]. Interestingly, a recently published study by Gurley *et al.* demonstrated that RGS2 expression in the kidney but not in other tissues including the central nervous system and peripheral vasculature is critical for the regulation of blood pressure [317]. Moreover, down-regulation of RGS2 has been described in animal models of hypertrophy [318] and occurs even before the onset of hypertrophy. Following pressure overload, RGS2 deficient mice display a marked dilation accompanied with significantly increased end diastolic and end systolic dimensions and decline in fractional shortening as well as impaired relaxation [319]. Furthermore, Zhang *et al.* have demonstrated that overexpression of RGS2 inhibits G α q/11-mediated cardiomyocyte hypertrophy [318].

5.1.2 G protein signalling regulation by RGS2

RGS2 is a member of more than 30 RGS domain-containing proteins that regulate G protein-coupled receptor (GPCR) signalling [232, 297, 317]. GPCRs like the angiotensin II type 1 (AT1) receptor are transmembrane receptors that are associated with heterotrimeric G proteins (consisting of an α , β , and γ subunit), which mediate a wide array of signalling processes [231, 297] (Figure 5.1 A). Binding of a hormone results in the activation of GPCRs, a conformational change of the receptor and thus in the activation of G proteins. G proteins exchange guanosine 5'-diphosphate (GDP) for GTP on the $G\alpha$ subunit leading to the dissociation of the $G\beta\gamma$ dimer from $G\alpha$ [309, 320]. This dissociation will activate other proteins further downstream in the signal transduction pathway leading for example to vasoconstriction and VSMC proliferation (Figure 5.1 B). G protein signalling is further regulated by proteins like RGS2. RGS2 accelerates the hydrolysis of $G\alpha$ -bound GTP to GDP and thus terminates the $G\alpha$ -effector interactions leading to the inhibition of vasoconstriction and VSMC proliferation in the example of the AT1 receptor [231, 297] (Figure 5.1 C). RGS2 shows regulatory selectivity for the $G\alpha_q/11$ subclass of G proteins where many important cardiovascular hormones such as ET-1, angiotensin II or norepinephrine activated receptors couple to [231].

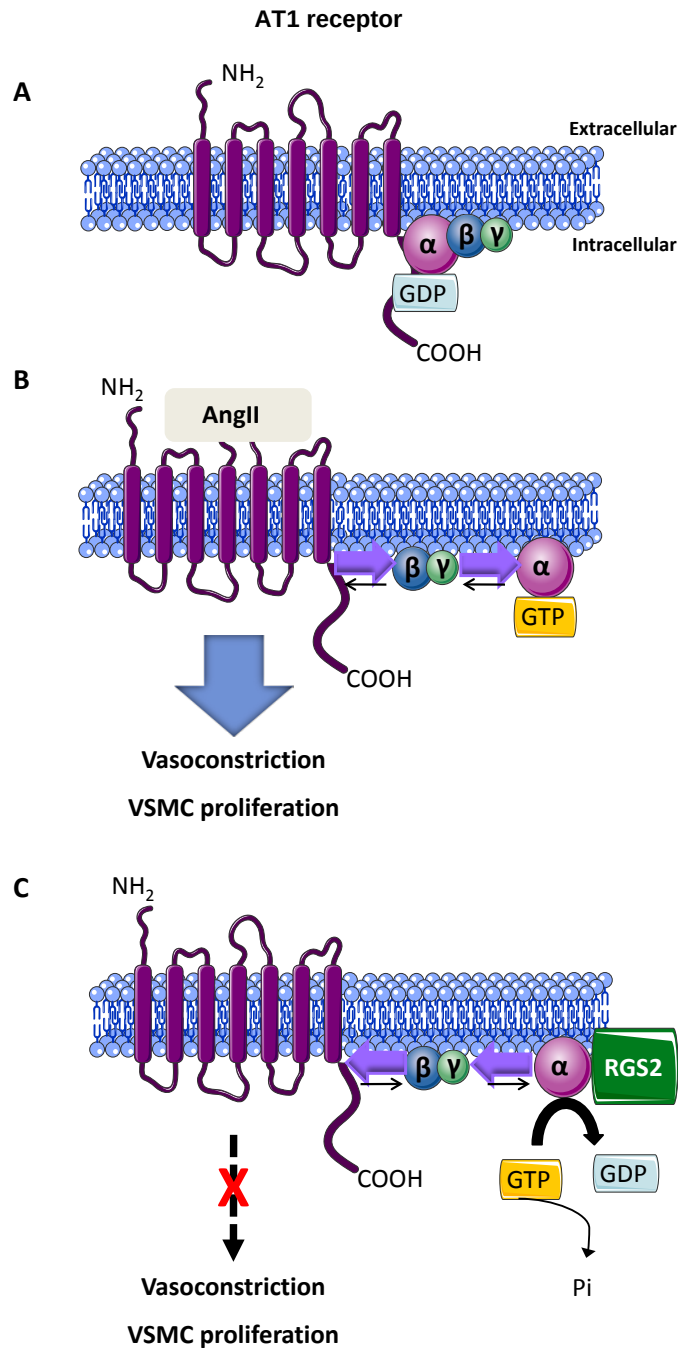


Figure 5.1: G protein signalling regulation by RGS2.

(A-C) Regulation of G protein signalling by RGS2 on the example of the AT1 receptor. (A) In the basal state the AT1 receptor is associated with a heterotrimeric G protein. (B) Binding of angiotensin II activates the G protein-coupled AT1 receptor, causing a conformational change and exchange of GDP for GTP on Gα_q. The Gα_q-GTP and Gβγ subunits dissociate leading to the activation of downstream proteins, which eventually results in physiological

actions such as vasoconstriction and VSMC hypertrophy. (C) In the presence of RGS2 the Gαq-catalysed hydrolysis of GTP to GDP is accelerated leading to a termination of the Gα-effector interactions. GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-triphosphate, VSMC, vascular smooth muscle cells. Adapted from [231]. Figure was produced using Servier Medical Art (www.servier.com).

5.2 Aims

We wanted to investigate the role of RGS2 in the development of coronary microvascular remodelling in DIO mice.

Furthermore, we aimed to assess the effects of a non-pharmacological lifestyle change due to a shift in diet from high fat back to normal rodent chow on coronary vascular remodelling and RGS2 expression in DIO mice.

To assess these questions the following aims were addressed:

1. Investigate the expression of RGS2 in VSMC and the kidney of DIO mice
2. Investigate effects of diet shift on metabolic parameters
3. Investigate effects of diet shift on RGS2 expression and vascular remodelling

5.3 Results

5.3.1 Evaluation of RGS2 expression in DIO

As described in Chapter 4, RGS2 mRNA and protein expression was significantly reduced in total heart lysates of DIO mice compared to control animals. To investigate the potential role of RGS2 in coronary microvascular remodelling RGS2 expression was measured in vascular smooth muscle cells of DIO and control mice. Heart sections of DIO and control mice were stained for RGS2 using immunohistochemistry. To assess RGS2 expression specifically in VSMC, they were detected using specific staining against α -smooth muscle actin. RGS2 expression was measured in vessels with a diameter smaller than 120 μm using digital morphometric analysis. Our results show a strong cytoplasmic expression of RGS2 in smooth muscle cells in control animals whereas mean RGS2 intensity in DIO mice was significantly reduced compared to controls (1.721 ± 0.8178 vs. 14.11 ± 2.193 for DIO vs. controls, $p < 0.005$, $n=4$) (Figure 5.2).

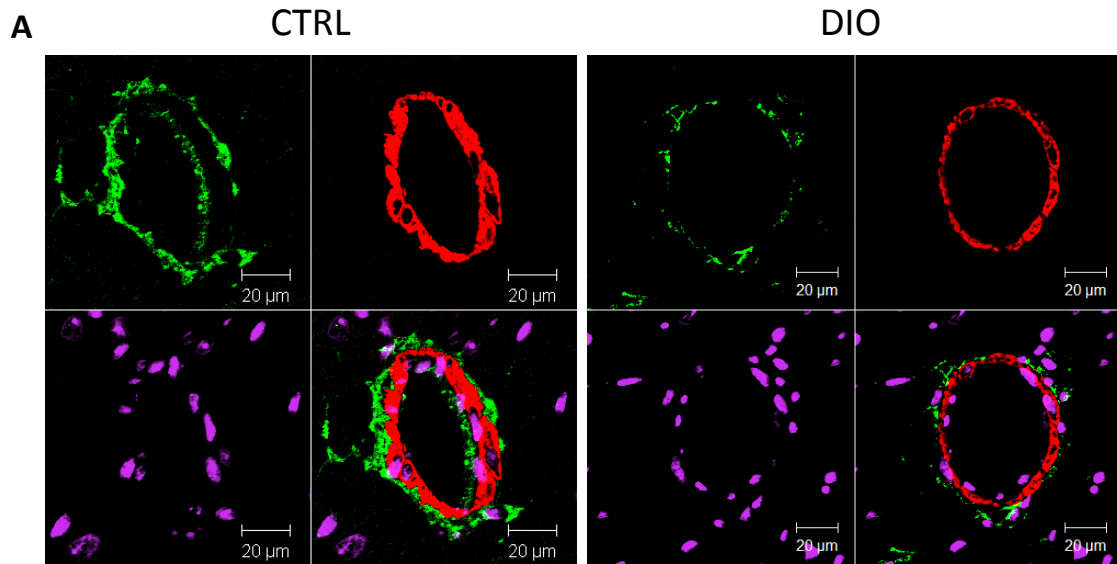
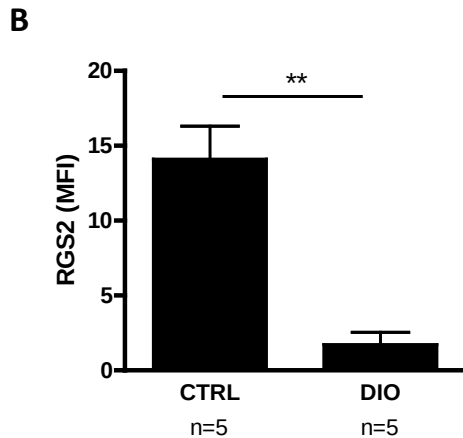


Figure 5.2: Effects of high fat diet on RGS2 expression in VSMC.

(A) Representative images of RGS2 expression in VSMC of control (left) and DIO (right) mice. RGS2 expression was detected by immunofluorescence staining using anti-RGS2 antibody and Alexa Fluor 488-conjugated secondary antibody (green). Identification of VSMC was achieved by staining with anti-vascular smooth muscle actin antibody and Alexa Fluor 568-conjugated secondary antibody (red). Nuclei were detected by TO-PRO3 staining (purple). Images were taken by confocal microscopy (Zeiss LSM 510) (40x magnification). White scale bars indicate 20 μm. (B) Quantification of RGS2 expression measuring mean fluorescence intensity (MFI) around multiple vessels (>10). Data are expressed as mean±SEM with 5 animals per group. Data were analysed using Student's t-test. ** p<0.005.

A study by Gurley and co-workers showed that the development of hypertension is solely determined by a reduced expression of RGS2 in the kidneys [317]. Since we did not observe hypertension in DIO mice we wanted to investigate whether RGS2 is differentially expressed in DIO kidneys compared to age-matched controls. For this, protein expression analysis using western blotting on total kidney lysates of DIO and control mice was performed. Mean protein expression of RGS2 in kidneys of DIO mice did not change compared to control animals (0.7733 ± 0.08388 vs. 0.8645 ± 0.09015 for DIO vs. controls, $p=ns$, $n=3$) (Figure 5.3).

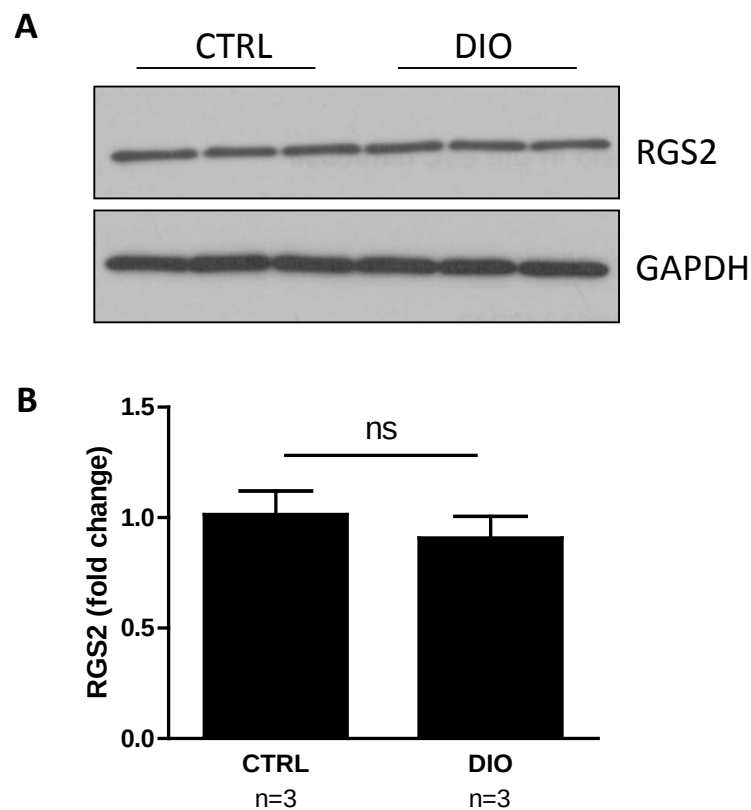


Figure 5.3: Effects of high fat diet on expression of RGS2 in kidneys.

(A) Representative western blot showing RGS2 (top) and GAPDH (bottom) expression in total kidney lysate in DIO and control animals ($n=3$). (B) Quantification of mean intensity of RGS2 protein expression after correcting for loading differences using GAPDH. Fold change is expressed as mean $\pm$ SD for 3 animals per group. Data were analysed using Student's t-test. ns, not significant.

5.3.2 Effects of diet shift on body composition and metabolic markers in DIO mice

In the previous chapter we have demonstrated that exposure to high fat diet leads to coronary microvascular remodelling. Therefore, we determined whether the observed microvascular remodelling is reversible in DIO mice animals. For this, animals were put back on the normal rodent chow for three weeks and the effects on body composition, metabolic markers, RGS2 expression in vascular smooth muscle cells, and coronary microvascular remodelling were investigated.

5.3.2.1 Whole body composition

Interestingly, after three weeks of normal rodent chow, body weight decreased in DIO mice back to the same weight as the age-matched controls (30.03 ± 1.915 g vs. 29.74 ± 2.349 g, $p = \text{ns}$, $n = 10$). These mice were labelled 'DIO-weight loss' (DIO-WL). This was also confirmed when correcting end body weight for differences in animal size using the tibia length. There was a significant difference body weight-to-tibia ratio between DIO mice and DIO-WL mice but not between DIO-WL and control mice (2.412 ± 0.09426 mg/mm vs. 1.719 ± 0.3729 mg/mm, $p < 0.0001$, $n = 10$ for DIO vs. DIO-WL and 1.719 ± 0.3729 mg/mm vs. 1.714 ± 0.1070 mg/mm, $p = \text{ns}$, $n = 10$ for DIO-WL vs. controls) (Figure 5.4 A).

Additionally, both fat weight-to-tibia ratio and liver weight-to-tibia ratio of DIO-WL animals went back to almost the same levels as in control animals (53.60 ± 20.78 mg/mm vs. 39.70 ± 14.76 mg/mm, $p = \text{ns}$, $n = 10$ fat weight-to-tibia ratio and 84.08 ± 14.04 mg/mm vs. 68.39 ± 7.384 mg/mm, $p = \text{ns}$, $n = 10$ for liver weight-to-tibia ratio) (Figure 5.4 B and E). No significant changes were observed in heart weight-to-tibia ratio and kidney weight-to-tibia ratio in DIO, DIO-WL and control mice. However, before correcting for tibia length, DIO mice showed a significant increase in kidney weight compared to control mice (177.0 ± 5.448 mg/mm vs. 150.1 ± 5.380 mg/mm, $p < 0.05$, $n = 10$) (Figure 5.4 C and D). Interestingly, after putting the mice back on the normal rodent chow the kidney weight in DIO-WL

mice did not go back to control levels but stayed similar to DIO levels (178.0±7.688 mg/mm vs. 177.0±5.448 mg/mm, p=ns, n=10 for DIO-WL vs. DIO).

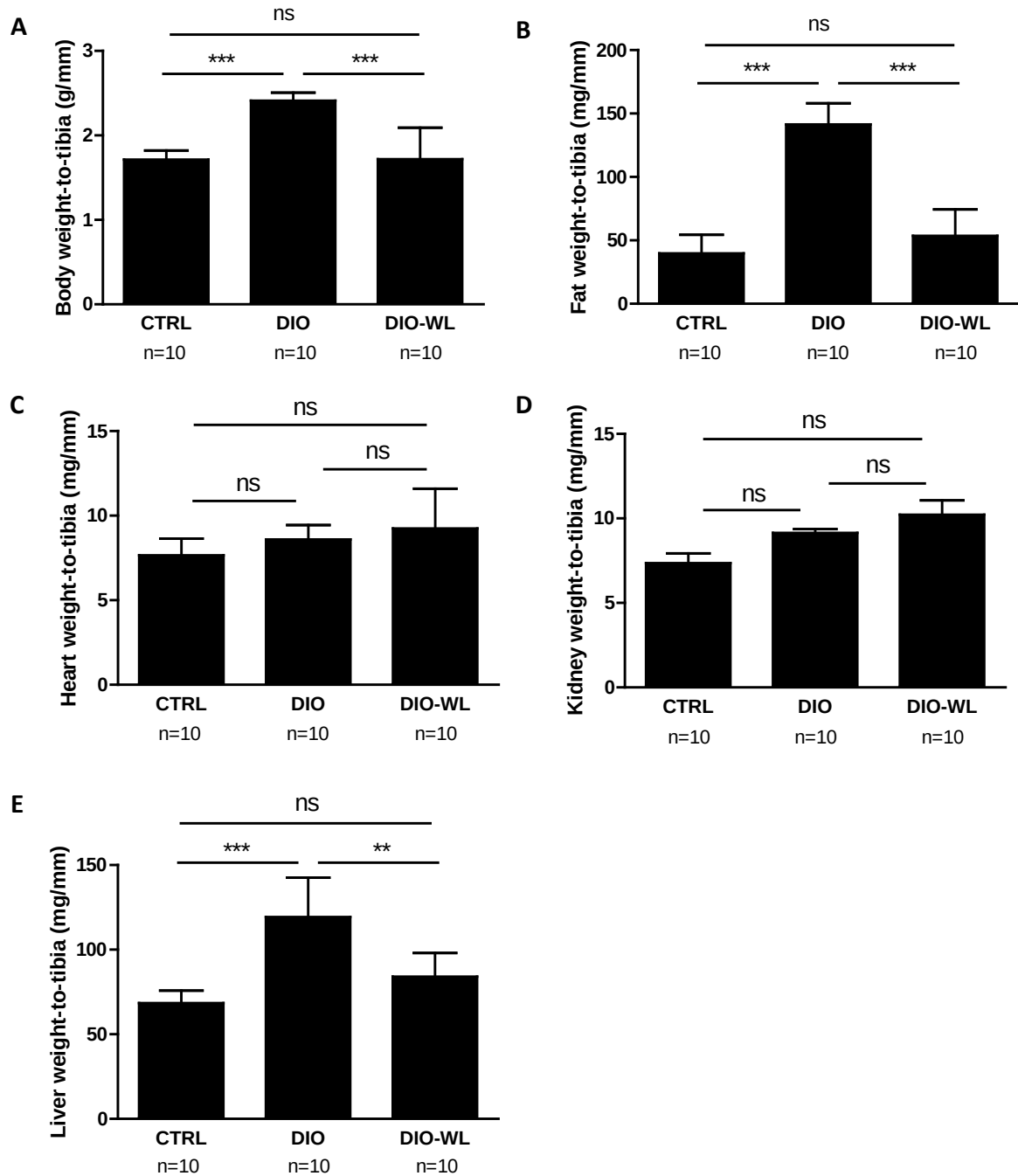


Figure 5.4: Effects of high fat diet and a shift in diet on body composition.

(A) End body weight normalised to tibia length in g/mm. (B) Fat weight normalised to tibia length in mg/mm. (C) Heart weight-to-tibia ratio in mg/mm. (D) Kidney weight-to-tibia ratio in mg/mm and (E) liver weight-to-tibia ratio in mg/mm. Data are expressed as mean±SD for 10 animals in each group. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ns, not significant; ** $p < 0.005$ and *** $p < 0.0005$.

5.3.2.2 Metabolic parameters

Concomitantly to the whole body weight loss, fasting serum insulin levels in DIO-WL mice improved and did not significantly differ from control levels (1.854 ± 0.5660 ng/mL vs. 0.5564 ± 0.08629 ng/mL, $p=ns$, $n=10$) (Figure 5.5). Similar to serum insulin levels, baseline fasting glucose levels improved with no significant differences between DIO-WL and control mice (9.950 ± 1.542 mmol/L vs. 9.900 ± 0.7000 mmol/L, $p=ns$, $n=4$) (Figure 5.6 A). After an oral glucose challenge, blood glucose levels peaked after 15 minutes in control mice and after 30 minutes in DIO and DIO-WL mice and returned back to baseline after 120 minutes in DIO-WL but not DIO mice (Figure 5.6 A). As described in Chapter 3, DIO mice had a significant increase in the AUC of 99% ($p<0.0001$) whereas only an increase of 8% in AUC was observed in DIO-WL mice compared to controls ($p=ns$) (Figure 5.6 B).

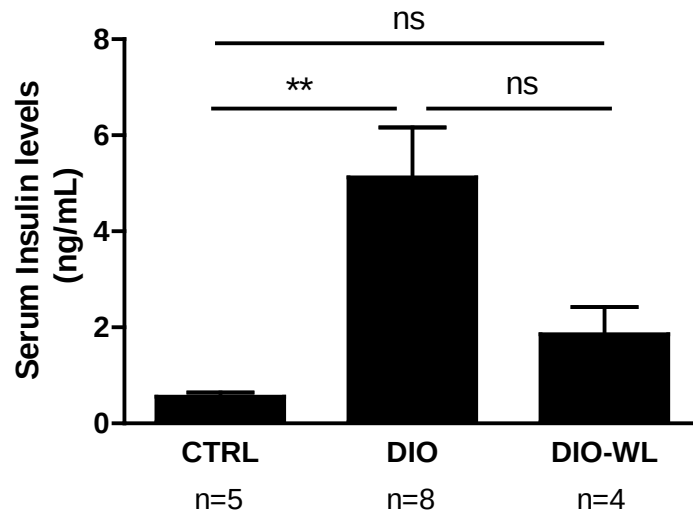


Figure 5.5: Fasting serum insulin levels in control, DIO and DIO-WL mice.

Serum insulin levels in DIO-WL after 3 weeks of normal rodent chow compared to control and DIO mice using an insulin ELISA. Insulin levels of each animal were analysed in duplicates. Data are expressed as mean $\pm$ SEM with $n=5$ for controls, $n=8$ for DIO and $n=4$ for DIO-WL mice. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ns, not significant; ** $p<0.005$.

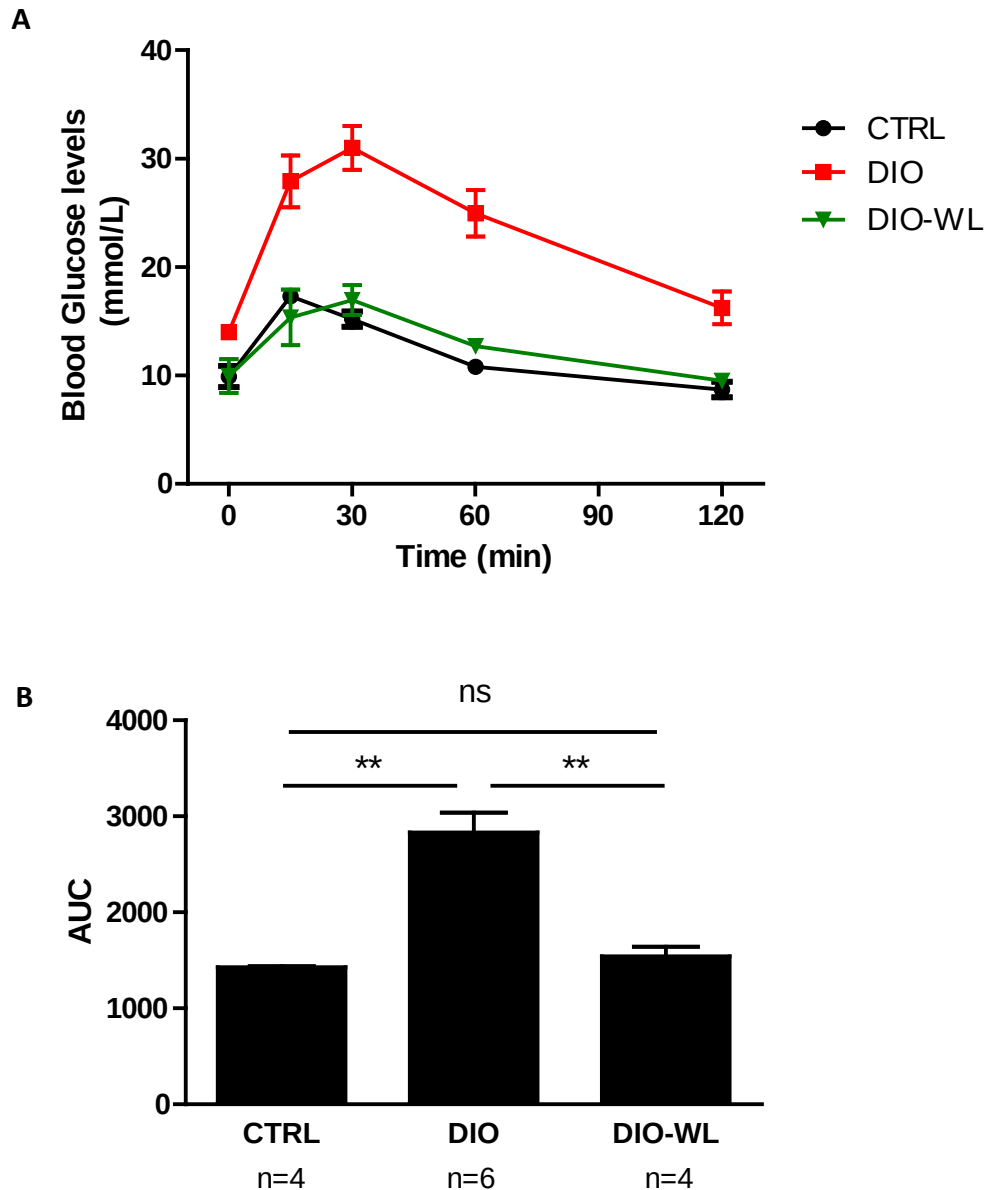


Figure 5.6: Oral glucose tolerance test (OGTT) in DIO-WL animals, age-matched DIO and control mice.

(A) OGTT in DIO-WL and age-matched DIO and control animals. Blood glucose levels at baseline, 30, 60 and 120 min after an oral glucose load. (B) AUC analysis of OGTT. Data are expressed as mean $\pm$ SEM with n=4 for control and DIO-WL mice and n=6 for DIO mice. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ns, not significant; ** p<0.005.

5.3.2.3 Effects of diet shift on coronary microvascular remodelling and RGS2 expression in vascular smooth muscle cells

To investigate whether the shift in diet has an effect on coronary microvascular remodelling, histomorphometrical analysis was performed. Three weeks of normal rodent chow resulted in a decrease in medial area ($1845 \pm 177.1 \mu\text{m}^2$ vs. $2687 \pm 207.3 \mu\text{m}^2$, $p=\text{ns}$ for DIO-WL vs. DIO) (Figure 5.7 A and B) and a significant decrease in media-to-lumen ratio (1.702 ± 0.2634 vs. 2.791 ± 0.2530 , $p<0.05$ for DIO-WL vs. DIO) in vessels smaller than $150 \mu\text{m}$ in the DIO-WL mice compared to DIO mice, returning to values similar to control mice (1.702 ± 0.2634 vs. 1.617 ± 0.1648 , $p=\text{ns}$ for DIO-WL vs. controls) (Figure 5.7 A and C).

In parallel to the reversal of coronary microvascular remodelling, mean intensity of RGS2 expression in vascular smooth muscle cells went back up to baseline control levels after 3 weeks of normal rodent chow (14.35 ± 2.742 vs. 14.11 ± 2.193 for DIO-WL vs. control, $p=\text{ns}$) (Figure 5.8).

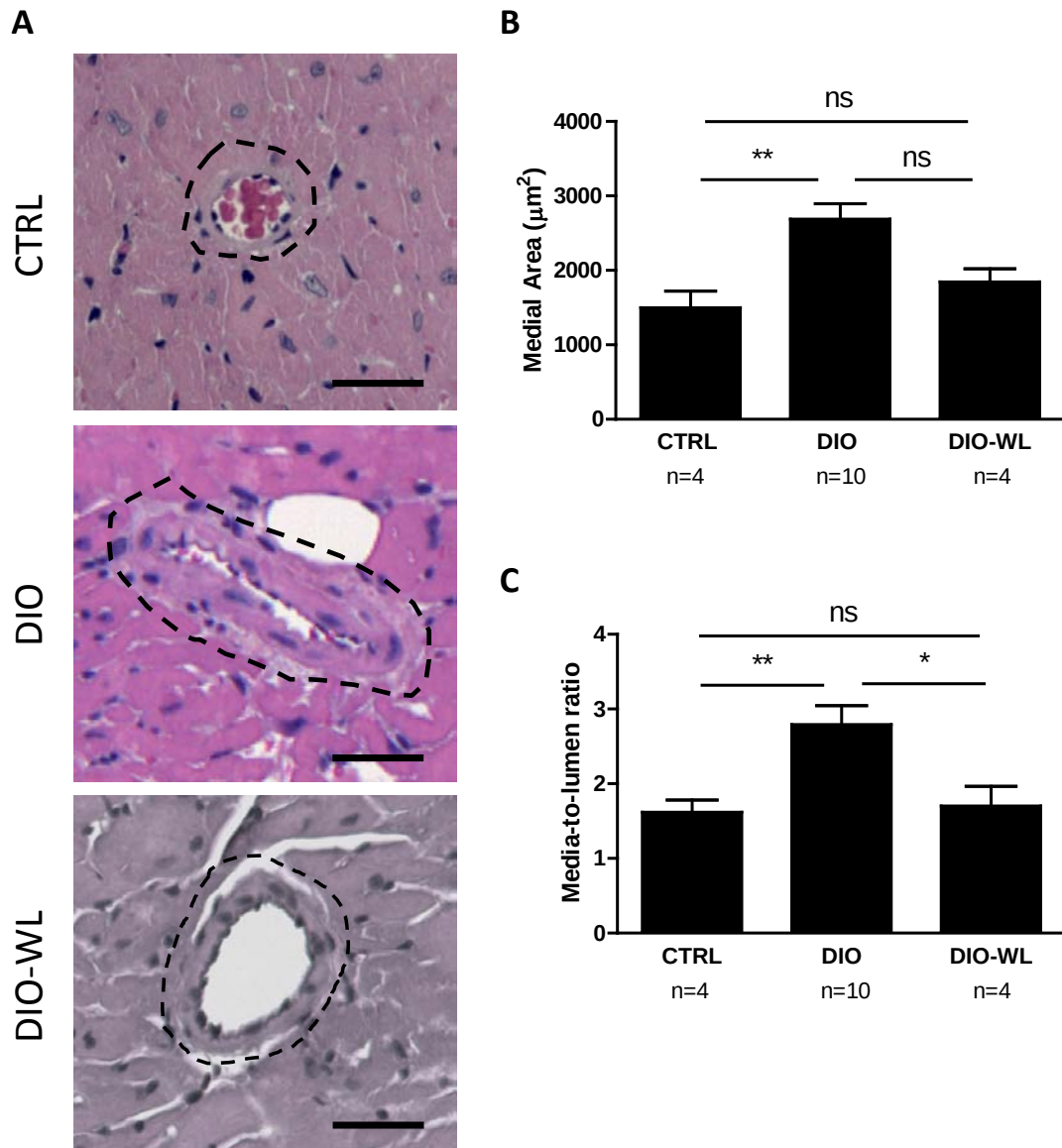


Figure 5.7: Effect of shift in diet on coronary microvascular remodelling.

(A) Histological sections of small arterioles (circled by dashed line) in left ventricles of control, DIO and DIO-WL mice. Sections were stained with haematoxylin and eosin. Nuclei are stained in dark blue whereas eosinophilic structures like cytoplasm, intra- and extracellular proteins or red blood cells are stained in shades of red. Images were taken using 40x magnification. Black scale bars indicate 25 μm . (B) Quantification of medial area of transmural vessels in DIO, DIO-WL and age-matched controls. (C) Quantification of media-to-lumen ratio of transmural vessels in DIO, DIO-WL and age-matched controls. Data are expressed as mean $\pm$ SEM for n=4 for control and DIO-WL mice and n=10 for DIO mice and at least 6 vessels per heart. Data were analysed using

one-way ANOVA with Bonferroni's multiple comparison. ns, not significant; * $p < 0.05$ and ** $p < 0.005$. Histomorphometrical analysis was carried out by Dr Massimiliano Mancini, La Sapienza University, Rome. Data analysis was performed by Christina Kleinert.

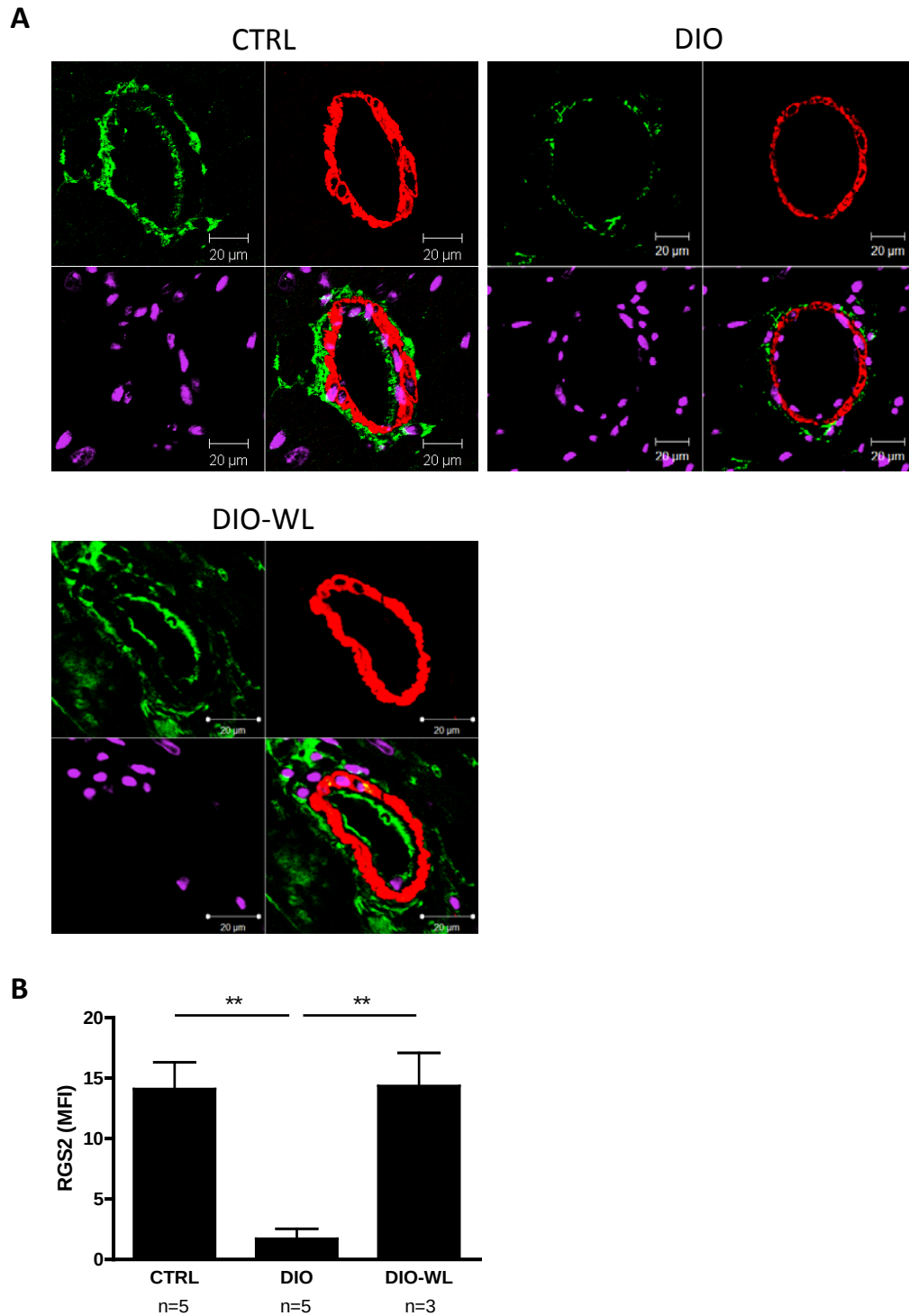


Figure 5.8: Effects of shift in diet on RGS2 expression in VSMC.

(A) Representative images of RGS2 expression in VSMC of control (top left), DIO (top right) and DIO-WL mice (bottom left). RGS2 expression was detected by immunofluorescence staining using anti-RGS2 antibody and Alexa Fluor 488-conjugated secondary antibody (green). Identification of VSMC was achieved by

staining with anti-vascular smooth muscle actin antibody and Alexa Fluor 568-conjugated secondary antibody (red). Nuclei were detected by TO-PRO3 staining (purple). Images were taken by confocal microscopy using a 40x magnification. White scale bars indicate 20 μ m. (B) Quantification of RGS2 expression measuring mean fluorescence intensity (MFI) around multiple vessels (>10). Data are expressed as mean $\pm$ SEM with n=5 for control and DIO mice and n=3 for DIO-WL mice. Data were analysed using one-way ANOVA with Bonferroni's multiple comparison. ** p<0.005.

5.4 Discussion

5.4.1 Effect of high fat diet on RGS2 expression in VSMC and kidney

In the previous chapter we have shown that high fat diet feeding is associated with alterations in metabolic markers, vascular structure and function as well as cardiac gene expression. In an attempt to identify a potential candidate involved in the observed microvascular remodelling in DIO hearts one differentially expressed gene, *Rgs2*, was of particular interest due to its role in blood pressure control, cardiac hypertrophy, vasoconstriction and VSMC proliferation [231, 309, 315]. We hypothesised that RGS2 protein levels are not only reduced in total hearts of DIO mice but also in VSMC and thus might contribute to the development of coronary microvascular remodelling in left ventricles of DIO mice. Our results show that RGS2 expression was significantly reduced in VSMC of DIO mice after 11 weeks of high fat diet feeding. The reduced expression of RGS2 in DIO hearts suggests that the inhibitory effect of RGS2 on G protein-coupled receptor signalling transduction is attenuated and thus may potentiate vasoconstriction in response to vasoconstrictors such as endothelin or angiotensin II [316], and lead to proliferation and hypertrophy of VSMC.

RGS2 expression and regulation has been extensively studied in hypertension. Down-regulation of RGS2 has been observed in cultured fibroblasts of hypertensive patients [310] as well as in animal models of hypertension [321]. It has been shown that RGS2 expression was reduced in saphenous arteries of SHR and adrenocorticotrophic hormone-induced hypertensive rats, in which vascular remodelling occurs [321]. A study by Sun *et al.* showed that RGS2 mediates the action of the NO-cGMP pathway on blood pressure and vasoconstrictor signalling in smooth muscle cells by promoting relaxation of the resistance vasculature through its ability to attenuate vasoconstrictor-induced Ca^{2+} signalling [232]. This was supported by an observation showing that RGS2 mRNA expression is down-regulated in aortic smooth muscle in a N^G -nitro-L-arginine methyl ester (L-NAME)-induced hypertension rat model which suggests

that decreased eNOS function may play a role in the down-regulation of RGS2 [322]. In the context of DIO mice it would be interesting to determine whether reduced eNOS function (possibly due to impaired insulin signalling transduction in VSMC or endothelial cells) may also be involved in down-regulation of RGS2 in DIO mice before the onset of hypertension.

Besides RGS2 expression in VSMC of DIO mice, we also investigated its expression in DIO kidneys after 11 weeks of high fat diet as a study by Gurley and co-workers showed that the development of hypertension is solely determined by a reduced expression of RGS2 in the kidneys [317]. As described in the previous chapter we did not observe any effects of high fat diet on mean arterial pressure (Figure 4.7). Concomitantly, no changes were observed in renal RGS2 expression after high fat diet feeding. This suggests that the reason why we did not observe hypertension in DIO mice might be due to the lack of differential/reduced expression of RGS2 in the kidneys.

5.4.2 Effects of shift in diet on whole body composition and vascular remodelling

Structural changes in the heart and vasculature can be attributed to the diabetic milieu of hyperglycaemia, hyperinsulinaemia, increased cellular FA uptake or hyperglycaemia-induced oxidative stress [13, 124]. It has been suggested that insulin or other related substances such as insulin-like growth factors may have a role in promoting vascular cell growth and thus promoting vascular remodelling [100, 323]. Additionally, high glucose concentrations can promote vascular complications by activating transcription factors that regulate pro-inflammatory and pro-atherosclerotic genes in endothelial cells and VSMC, stimulating oxidative stress, or VSMC proliferation, migration, and altered reactivity [94]. To the best of our knowledge no information is available about the regulation of RGS2 and its role in vascular remodelling in diabetes. To test whether RGS2 expression and coronary vascular remodelling is modulated by glycaemic and

insulinaemic control, we used a non-pharmacological approach. We hypothesised that a shift in diet from high fat back to normal rodent chow would beneficially influence RGS2 expression in DIO mice. Additionally, we hypothesised that improvement of insulin sensitivity and blood glucose levels would promote reverse remodelling in coronary small vessels. It has been shown that non-pharmacological lifestyle interventions such as diet, weight loss, or physical activity all increase insulin sensitivity, adiponectin levels and reduce endothelial dysfunction [12]. Our results show that a shift in diet back to normal rodent chow (11 weeks 35% carbohydrate and 45% lard-based high fat diet, then shift to 11.5% fat and 61.2% carbohydrates for three weeks) resulted in a significant reduction of body weight, fat weight and liver weight. In our study, food intake was not measured. Although a shift to a low fat/high carbohydrate diet resulted in body weight reduction, it is important to note that this could have been caused not only by the shift in diet but also by other factors like stress-dependent reduction in food intake.

Furthermore, glucose and insulin levels were positively affected by the shift in diet as well as insulin sensitivity. These results are in contrast with recently published work by Lee and co-workers in which they showed that a shift in diet from high fat to high carbohydrate (45% high fat diet for 9 weeks then shift to 70% carbohydrates for 3 weeks) in male C57BL/6 mice did not affect glucose and insulin levels as well as insulin sensitivity [324]. They concluded that a three weeks duration might not be sufficient to improve blood glucose and blood insulin levels even though body weight and body fat decreased significantly [324].

Concomitantly to the improvement of metabolic markers after the shift in diet, coronary vascular remodelling as well as RGS2 expression in VSMC was significantly improved. This suggests that a shift in diet and the resulting glycaemic and/or insulinaemic control was efficient enough to reverse coronary microvascular remodelling in DIO hearts and improve RGS2 expression

returning back to baseline control levels. Studies in T1DM and T2DM have shown that hyperglycaemia plays a key role in the pathogenesis of vascular dysfunction in diabetes [92]. A study in the spontaneously diabetic (T2DM), nonobese, and normotensive GK rat, Sachidanandam *et al.* investigated the effects of hyperglycaemia in mediating vascular complications [93]. They observed that glycaemic control with Metformin, which improves insulin sensitivity and endothelium-dependent vasodilation [12] shortly after the onset of diabetes can prevent activation of growth-promoting and pro-fibrotic vasoactive factors and thus prevent vascular remodelling and dysfunction in mesenteric arteries [93]. However, they did not investigate whether an intervention strategy with Metformin results in reverse remodelling or whether glucose regulation in established diabetes can prevent and/or reverse vascular remodelling and dysfunction [93]. Similarly, at this stage it is impossible to give a conclusive explanation as to whether the observed reverse remodelling observed in our study was due to the improvement of an individual factor (i.e. hyperglycaemia or insulin-resistance) or a synergistic effect. In other disease settings such as hypertension, non-pharmacological lifestyle interventions might not be sufficient to improve coronary microvascular remodelling. Therapies aimed at improving for example insulin resistance, inflammation, oxidative stress, or endothelial dysfunction are predicted to have simultaneous beneficial effects on both metabolic and cardiovascular function [12]. Work in our group has shown that pharmacological treatment with the ACE inhibitor Ramipril and/or the NO-independent soluble guanylate cyclase activator HMR1766 in SHR not only leads to a reduction in blood pressure but also to reverse remodelling of the coronary microcirculation and therefore also to functional improvement [325].

In addition to the improvement of metabolic markers, the shift in diet from a high fat to a high carbohydrate diet might also have contributed to a reversal of dietary fatty acid-induced changes in gene expression and therefore positively influenced RGS2 gene expression and regulation. Also, it has been shown that calorie restriction not only increases insulin sensitivity but also improves NO-

dependent vasodilation in obese or hypertensive individuals [326, 327]. Restoration of normal RGS2 expression levels in VSMC might have influenced vasoconstrictor signalling in smooth muscle cells and thus promotes relaxation of the resistance vasculature.

In summary, our results identify a previously unrecognised role of RGS2 in diet-induced coronary vascular remodelling before the onset of hypertension. A shift in diet from high fat to high carbohydrate diet resulted in significant reduction of body, fat, and liver weight but also in a substantial improvement of insulin sensitivity and blood glucose levels. Importantly, this diet shift was sufficient to reverse the observed coronary microvascular remodelling and restore RGS2 expression levels in VSMC.

Chapter Six: General Discussion

6.1 Summary of main study findings

Listed below is a summary of the main findings of this study:

1. The 11 weeks high fat diet-induced obese C57BL/6 mouse is a more appropriate model to study insulin resistance and T2DM than the *ob/ob* mouse model.
2. Coronary microvascular remodelling characterised by thickening of the media was present in DIO mice as early as 8 weeks of high fat diet but absent in both normoglycaemic and hyperglycaemic *ob/ob* mice.
3. Coronary microvascular remodelling in DIO mice was independent of hypertension and overt changes of the myocardium. A shift in diet from high fat to high carbohydrate diet resulted in reverse remodelling of the coronary microvasculature.
4. High fat diet feeding was not associated with overt changes in coronary blood flow but an impairment of endothelium-dependent vasodilation in the aorta.
5. Microarray analysis of the left ventricle revealed diet-induced changes in mRNA expression of genes involved in lipid metabolism, hypertrophy and fibrosis, and vascular reactivity. Moreover, high fat diet feeding was associated with diet-induced changes in protein expression levels of members of the insulin signalling pathway.
6. RGS2, a protein involved in blood pressure control and vasoconstrictor signalling, was differentially expressed in DIO mice. This effect was reversible by a dietary shift from high fat to normal rodent chow.

6.2 General discussion

One of the aims of this project was to generate a high fat diet induced mouse model of insulin resistance and T2DM and determine which of the different feeding periods studied would make the most suitable model to investigate the molecular and genetic mechanisms of myocardial insulin resistance. The results described in Chapter 3 show that high fat diet feeding gradually alters the characteristic risk factors contributing to the development of insulin resistance and eventually T2DM leading to macroscopic and microscopic changes in the liver, kidney, and heart. Our results together with numerous previous studies on diet-induced metabolic and pathological changes [74, 75, 166, 176, 198] underline the suitability of the high fat diet fed C57BL/6 mouse as a model of environmentally induced T2DM and obesity [160].

This thesis describes for the first time the progressive development of inward muscular remodelling of coronary arterioles in DIO mice which is not present in the heart of leptin-deficient *ob/ob* mice (Figure 6.1; Chapter 4). Microvascular remodelling and dysfunction has been studied in patients with hypertension [104, 328], diabetes [100, 206] or HCM [92, 102] and represents a predisposing factor for myocardial ischaemia in HCM [329, 330]. However, only few animal models of coronary microvascular remodelling exist [214]. We could demonstrate that the observed coronary microvascular remodelling in DIO mice is independent of hypertension or cardiac hypertrophy and that it is accompanied by mild coronary microvascular dysfunction and endothelial dysfunction. Moreover, a dietary shift from the high fat to a high carbohydrate diet lead to significant improvement of the metabolic markers of insulin resistance as well as to reverse remodelling of coronary arterioles (Figure 6.1). Although the observed remodelling is diet-induced, our work provides the first murine model that phenotypically mimics the microvascular remodelling observed in patients with HCM.

A key aim of this thesis was to identify gene expression changes associated with high fat diet-induced coronary microvascular remodelling. Microarray analysis identified several genes involved in myocardial lipid and glucose metabolism, the development of hypertrophy and fibrosis as well as vascular reactivity which were differentially expressed in left ventricles of DIO mice compared to age-matched controls (Chapter 4). Further analysis revealed one very interesting gene which might play a significant role in the development of microvascular remodelling in DIO hearts. RGS2, a member of the regulators of G protein signalling family, was differentially expressed at both the RNA and protein level in left ventricles and VSMC of DIO mice. Specifically, we demonstrated that RGS2 is down-regulated in VSMC of transmural arterioles in DIO hearts (Chapter 5). Expression of RGS2 has been described in tissues that are important for blood pressure regulation, vascular function, and vasoconstriction like the kidney, VSMC and the central nervous system [231, 307]. Previous clinical and basic studies have demonstrated that deregulation of RGS2 contributes to the pathogenesis of CVD [308, 309, 331]. Our work suggests a novel unrecognised role of RGS2 in diet-induced coronary vascular remodelling before the onset of hypertension. Moreover, the dietary shift back to a normal rodent chow (high carbohydrate diet) was sufficient to not only reverse the observed coronary microvascular remodelling but also restore RGS2 expression levels in VSMC (Figure 6.1).

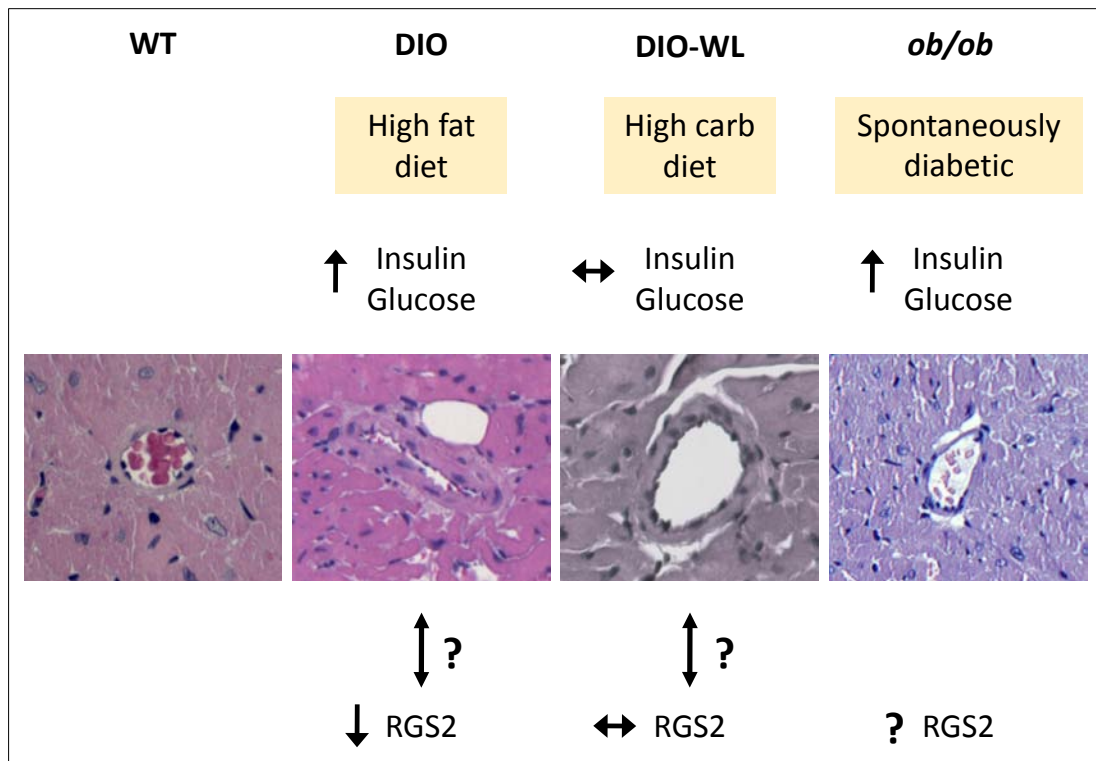


Figure 6.1: Effects of diet on coronary microvascular remodelling.

High fat diet feeding was associated with a significant increase in blood insulin and glucose levels, the development of coronary microvascular remodelling and down-regulation of RGS2 in VSMC. A shift in diet from high fat to a normal rodent chow (high carbohydrate diet) lead to reverse remodelling of coronary arterioles and the return of metabolic markers and RGS2 expression to baseline. In contrast, the spontaneously diabetic, leptin-deficient *ob/ob* mouse heart did not show any signs of coronary microvascular remodelling despite elevated blood glucose and insulin levels. WT, wild type; DIO, diet-induced obese; DIO-WL, diet-induced obese weight loss; RGS2, regulator of G protein signalling 2; ↑ increase; ↔ unchanged; ? unknown.

Furthermore, impairment of the PI3K-dependent branch of the insulin signalling transduction pathway has been observed in different mouse models of insulin resistance and T2DM [73-75]. We show that high fat diet feeding was associated with differential expression and activity of Akt, a down-stream target of the insulin signalling pathway as well as lower basal expression of GLUT4 in DIO cardiomyocytes but no significant differences in GLUT4 expression between

baseline and insulin-stimulated DIO hearts (see Chapter 4). Previous work from our group on human myocardial biopsy samples of diabetic patients has shown that impaired insulin signalling was associated with increased myocardial IRS1-PI3K activity, unchanged total GLUT4 content and a reduction of basal sarcolemmal GLUT4 [72]. Moreover, these results in human myocardium were confirmed in comparative studies in the leptin-deficient *ob/ob* mouse heart [72].

6.3 Future work

Our observations have opened up several new lines of enquiry which should now be addressed experimentally in future studies.

Firstly, it would be interesting to further study the phenomenon of why some animals did not respond to the high fat diet with significant body weight gain. Generally, only mice with the highest weight gain are used for further studies. However, on average 30% of the mice in this study did not increase their body weight when fed the high fat diet and were thus classified as diet-induced resistant. It has been suggested that a significantly lower initial body weight and lower calorie intake after 5 weeks of high fat diet [167] might be the reason for their diet resistance. Moreover, Burcelin and co-workers have started to classify high fat diet fed mice into lean non-diabetic, lean diabetic, obese diabetic, and intermediate phenotype and observed that the first three phenotypes are insulin resistant [332]. Nevertheless, it would be interesting to further investigate phenotypical and cellular differences between the diet-resistant and diet-induced obese mice and therefore gain further insight into the mechanisms involved in insulin resistance.

Secondly, with regards to the DIO mice further characterisation of metabolic changes in DIO mice might give a better insight into the pathophysiological changes associated with high fat diet feeding. The increase in adipose tissue due to the high fat diet will stimulate pro-inflammatory cytokine and adipokine

production [61] and suppress anti-inflammatory adipokines [62] and therefore promote a pro-inflammatory and pro-thrombotic state. It would be interesting to study inflammatory markers like TNF α or IL-6 as well as circulating levels of adiponectin, an anti-inflammatory adipokine, but also cholesterol and triglyceride levels, which might also be modulated by high fat diet feeding. Moreover, increased ROS production and oxidative stress have been implicated in the development of cardiovascular changes leading to endothelial dysfunction and the development of atherosclerosis [13]. Due to practical constraints, the effects of high fat diet on ROS production, protein oxidation or the free-radical scavenging capacity of DIO mice was not determined. Thus, it would be desirable to measure plasma hydrogen peroxide as an indicator for ROS generation, protein oxidation by protein carbonyl determination or the plasma antioxidant power to test the free-radical scavenging capacity [245].

Given our observations that high fat diet promotes impairment of endothelium-dependent vasodilation in the aorta we attempted to further investigate this. We demonstrated that high fat diet did not affect plasma levels of an endogenous inhibitor of eNOS, ADMA, which has been associated with a range of cardiovascular disease states [168, 266, 267]. To further investigate endothelial dysfunction and the underlying mechanisms in future experiments several approaches are possible. Firstly, NO availability could be evaluated by an ACh concentration-response curve after pre-incubation with the NOS inhibitor L-NAME. Secondly, to determine whether oxidative stress contributed to a decrease in NO bioavailability and thereby promoted endothelial dysfunction, ROS production could be evaluated by pre-incubating vessels with the antioxidant and $\cdot\text{O}_2^-$ dismutase mimetic Tempol and then constructing an ACh concentration-response curve similar to a report by Marchesi *et al.* [333] or by measuring ROS generation within the aorta *in situ* using dihydroethidium fluorescence [260]. Thirdly, eNOS activity in the vasculature could be investigated by measuring its expression, phosphorylation, and eNOS protein dimer formation which is necessary for eNOS activity [334] using western

blotting. Finally, proteins like Akt, Caveolin-1, soluble guanylate cyclase, or AMPK which have been shown to play vital roles in the regulation of NO production and activity [37, 334] could be investigated in the vasculature of DIO mice at baseline and after insulin stimulation to determine whether decreased insulin signalling was responsible for impaired NO release and vasodilation.

One of the key findings of this thesis was the presence of coronary microvascular remodelling in DIO hearts which we could not observe in the leptin-deficient *ob/ob* mouse heart. To further dissect which trigger might be responsible for the development of coronary microvascular remodelling, it would be desirable to investigate the earliest time point of high fat diet feeding after which coronary microvascular remodelling occurs. Moreover, due to time limitations the exact mechanism by which this remodelling occurs has not been fully studied. Our observations suggest a potential novel role of RGS2 in the development of coronary microvascular remodelling before the onset of hypertension due to its differential expression in coronary VSMC. To the best of our knowledge, no studies have investigated RGS2 expression and regulation in the context of diabetes. RGS2 expression has been shown to be regulated in a G protein-dependent and –independent way in the context of hypertension [309, 335]. It has been suggested that prolonged GPCR stimulation in response to chronic elevated catecholamines may induce down-regulation of RGS2 and thus contribute to the pathogenesis of CVD including hypertension and myocardial hypertrophy [309, 318]. Furthermore, phosphorylation of RGS2 has been shown to be regulated in a protein kinase G (PKG)-dependent way [309]. Mice lacking RGS2 have an impaired cGMP-mediated inhibition of Ca^{2+} transients elicited by vasoconstrictors [232, 316], and display decreased cGMP-mediated relaxation [232, 315]. Moreover, reduced eNOS-NO signalling is expected to cause a defect of the PKG-mediated RGS2 regulation, thus leading to an exacerbated G α_q signalling and contributing to the pathogenesis of CVD [309].

In our study it is important to mention that we cannot conclude that the differential expression of RGS2 promotes the observed remodelling or that the differential expression is a result of the remodelling. It would be very interesting to investigate whether the diet-resistant mice develop coronary microvascular remodelling and analyse the expression of RGS2 in VSMC of these mice. Moreover, further experiments are needed to investigate the function and regulation of RGS2 in VSMC of DIO mice. Since we only investigated RGS2 expression in the coronary microvasculature it would be important to determine whether RGS2 is generally differentially regulated in the DIO vasculature. If this is the case, further analysis of the regulation of RGS2 expression and activity could be performed. The involvement of the above mentioned signalling pathways should be further assessed in the context of vascular remodelling in DIO mice. For this, isolation of VSMC of DIO vessels with subsequent measurement of intracellular Ca^{2+} transients using a fluo-4 AM Ca^{2+} fluorescence assay [297] or *ex vivo* analysis of vascular remodelling in carotid arteries using adenoviral overexpression [336] or siRNA knock-down studies could be employed. Moreover, we only focussed on the effects of a shift in diet on RGS2 expression in VSMC using immunohistochemistry in DIO-WL hearts. However, we did not look at RGS2 RNA expression and protein content in whole hearts of DIO-WL mice which should be investigated in future experiments similar to the results described in Chapter 4.

The observed lower expression of RGS2 at the mRNA level could be due to epigenetic impairment by various factors such as dietary fatty acids, exposure of VSMC to hyperglycemia or angiotensin II with possible effects on chromatin domains, transcription factors, RNA transport, or mRNA degradation. It has been shown that the promoter region, introns and untranslated regions of RGS2 contain several single nucleotide polymorphisms and insertion/deletion polymorphisms [311, 313], with one resulting in enhanced calcium mobilisation in fibroblasts in response to angiotensin II [310], and another being linked to an increased risk of the metabolic syndrome in white Caucasian Europeans [337].

Additionally, it has been shown that the promoter region of RGS2 contains regulatory binding sites for transcription factors like STAT5, c-Jun, JunB, PPAR γ 1, or PPAR γ 2 [338, 339]. In this context, Yue *et al.* have shown that RGS2 is also a target gene of STAT3 in hearts of AT1 receptor transgenic mice [338]. Moreover, they have shown that regulation of RGS2 by AT1 receptor is critically dependent on the availability of higher levels of STAT3 and that binding of STAT3 to the promoter is essential for expression of RGS2. In an initial experiment we eliminated the possibility of pre-existing genetic differences in the RGS2 gene between DIO and control animals by looking at the RGS2 coding region in both DIO and control animals (Appendix D – RGS2 coding region). However, future experiments are needed to investigate the possibility of epigenetic changes leading to differential expression of RGS2. For example, methylation-specific PCR analysis and bisulphite sequencing could be used to further investigate whether methylation of the CpG island in the RGS2 promoter correlates with RGS2 expression [340]. Furthermore, the effects of high fat diet on the binding of transcription factors such as STAT3 to the RGS2 promoter should be investigated using chromatin immunoprecipitation assays [338].

With regards to the effects of high fat diet feeding on myocardial insulin signalling, further experiments are necessary to determine whether insulin-stimulated myocardial glucose uptake is impaired. Basal and insulin-stimulated glucose uptake could be measured *in vitro* in isolated cardiomyocytes using radioactively-labelled 2-deoxyglucose as described by Mazumder and co-workers [73] but also *in vivo* using positron-emission tomography with ^{18}F -labelled fluorodeoxyglucose. Furthermore, GLUT4 translocation to the sarcolemma and the major proteins involved in GLUT4 trafficking and docking like AS160, Syntaxin4, VAMP2/3, or Munc18c should be further investigated [44, 341-344]. For this, preparation of membranes from myocardial tissue by fractionation and sucrose-gradient centrifugation or isolation of cardiomyocytes and subsequent fluorescent imaging of proteins involved could be performed. Finally, only the PI3K-dependent branch of the insulin signalling pathway has

been investigated in this study. Further experiments are also needed to determine whether the MAPK-dependent branch is affected by high fat diet feeding.

6.4 Concluding remarks and relevance to human disease

The results described in this thesis provide the first direct evidence of coronary microvascular remodelling before the onset of hypertension or cardiomyopathy in a diet-induced mouse model of insulin resistance and T2DM. Moreover, our results suggest a potential role of a regulator of G protein signalling, RGS2, in microvascular remodelling. These findings support the use of a diet-induced mouse model to study the molecular and genetic mechanisms of myocardial insulin resistance in humans and mice. Understanding the pathophysiology and molecular mechanisms of coronary microvascular remodelling and how RGS2 can turn off maladaptive signals should lead to new insights into pathogenesis and reveal novel therapeutic approaches to treat microvascular disease and improve the structure and function of the vasculature which eventually may improve T2DM outcomes and reduce a large burden of disease.

APPENDICES

Appendix A – Buffers and stock solutions

Krebs-Henseleit Buffer	13.8 g NaCl, 0.70 g KCl, 0.44 g CaCl ₂ H ₂ O, 0.32 g MgSO ₄ H ₂ O, 4.18 g NaHCO ₃ , 0.32 g KH ₂ PO ₄ , 4.02 g Glucose in 2 L H ₂ O, filtered prior to use.
BSA	Bovine serum albumin to 10 mg/mL in H ₂ O, stored at -20 °C.
10% APS	1g Ammonium persulfate in 10 mL H ₂ O, stored at -20 °C.
10% SDS	100 g Sodium dodecyl sulphate in 1 L H ₂ O.
1 M Tris-HCl, pH 6.8	121.14 g Tris base in 900 mL H ₂ O, pH to 6.8 with HCl, made up to 1 L with H ₂ O.
1 M Tris-HCl, pH 8.8	121.14 g Tris base in 900 mL H ₂ O, pH to 8.8 with HCl, made up to 1 L with H ₂ O.
Acrylamide	30% Acrylamide/0.8% Bis-acrylamide in H ₂ O (UltraPure Protogel, Fisher Scientific)

Protein Lysis Buffer	10 mL 10x Cell Lysis Buffer (Cell Signaling Technology) made up to 100 mL with H ₂ O, stored at 4 °C. 1 mM PMSF and 1 mM Protease inhibitor cocktail prior to use.
Running Buffer	100 mL 10x Tris/Glycine/SDS Buffer (BioRad Laboratories) made up to 1 L with H ₂ O.
Loading Dye	4x NuPage® LDS Sample Buffer (Invitrogen)
Transfer Buffer	100 mL 10x Tris/Glycine/SDS Buffer (BioRad Laboratories), 200 mL methanol, made up to 1 L with H ₂ O.
TBS	100 mL 10x Tris-buffered saline (BioRad Laboratories) made up to 1 L with H ₂ O.
TBST	100 mL 10x Tris-buffered Saline (BioRad Laboratories), 10 mL 10% Tween20 Solution (BioRad Laboratories) made up to 1 L with H ₂ O.
RIPA Buffer	50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% Na Deoxycholate, 1% Triton® X-100 in H ₂ O, stored at 4 °C. 1 mM PMSF and 1 mM protease inhibitor cocktail prior to use.

Protease inhibitor cocktail	4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, Aprotinin, Bestatin hydrochloride, N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide, Leupeptin hemisulfate salt, Pepstatin A, stored at -20 °C.
PMSF (200 mM stock)	Phenylmethylsulfonyl fluoride to 35mg/mL in anhydrous isopropanol with heating, stored at -20 °C.
PBS	8 g NaCl, 0.2 g KCl, 1,44 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.24 g KH_2PO_4 , made up to 1 L with H_2O , pH 7.4
PBST	10 mL 10% Tween20 Solution (BioRad Laboratories) made up to 1 L with PBS.
Citric Buffer pH 6.0	0.35 g Citric Acid and 2.40 g Sodium Citrate tribase in 900 mL H_2O , pH to 6.0 with HCl, made up to 1 L with H_2O , stored at 4 °C.
dNTP mix	10 μL of 100 mM dATP, dCTP, dGTP and dTTP were mixed with 210 μL H_2O , stored at -20 °C.
Orange G	2.5 g Ficoll 400, 0.25 g Orange G made up to 10 mL in H_2O .

Appendix B – Kidney Histology

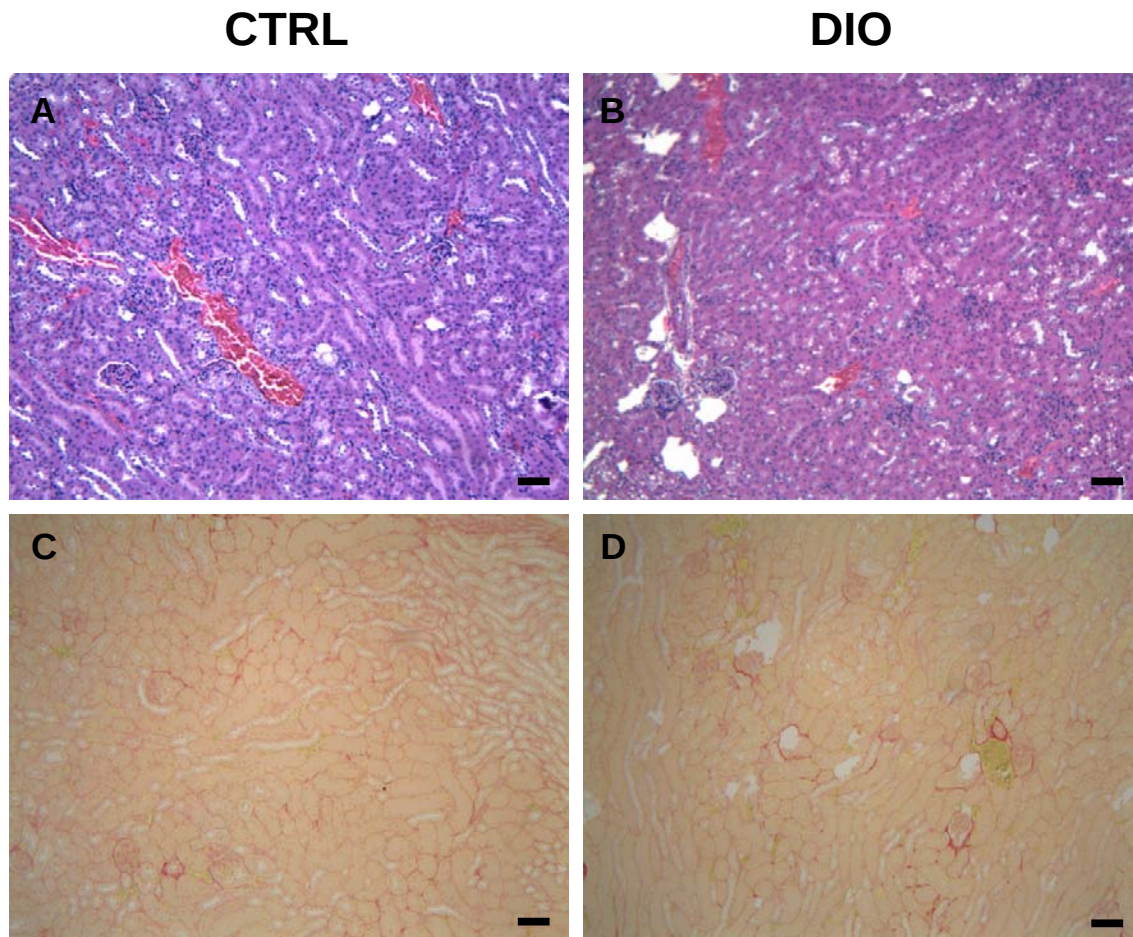


Figure B.1: Histological changes in kidney after 14 weeks of high fat diet feeding.

Histological sections of kidneys from control animals and DIO. (A and B) Haematoxylin and eosin staining of control and DIO kidney sections. Nuclei are stained in dark blue whereas eosinophilic structures like cytoplasm or intra- and extracellular proteins are stained in shades of red. (C and D) Picro-sirius red staining of collagen (red) on a pale yellow background (kidney parenchyma). Images were taken at 10x magnification and are representative for n=4 in each group. Black scale bars indicate 100 μ m. Histological analysis was carried out by Dr Massimiliano Mancini, La Sapienza University, Rome.

Appendix C – Microarray Quality Controls

Table C.1: QC1 – Concentrations and ODs of total and reduced RNA

Sample ID	total RNA (ng/μg)	OD (260/280 nm)	rRNA reduced (ng/μg)	OD (260/280 nm)
Control 1	357.0	2.01	45.6	2.01
Control 2	297.8	2.02	44.7	1.92
Control 3	404.4	2.10	37.5	2.07
Control 4	673.5	2.04	29.0	1.90
Control 5	415.4	2.09	49.0	1.96
DIO 1	343.1	2.11	33.6	1.95
DIO 2	668.9	2.04	32.9	2.10
DIO 3	510.5	2.10	42.7	2.05
DIO 4	358.3	2.08	33.9	1.98
DIO 5	495.8	2.02	28.1	2.02

OD: optical density

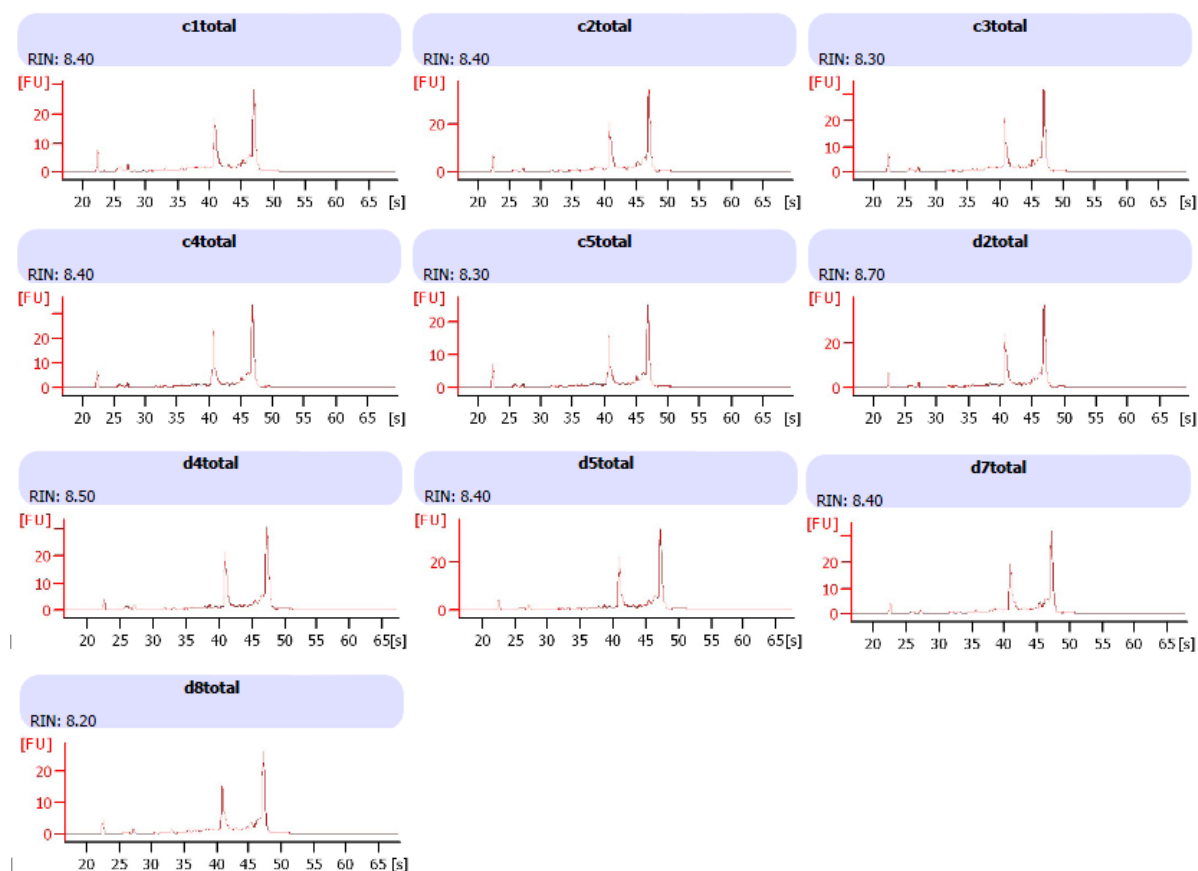


Figure C.1: QC1 - Bioanalyser trace of total RNA

Electropherogram of total RNA (1 µg) from control and DIO myocardium. Red trace indicates total RNA with peaks for the marker, 18S and 28S ribosomal RNA. Samples were run at the MRC Genomics Laboratory.

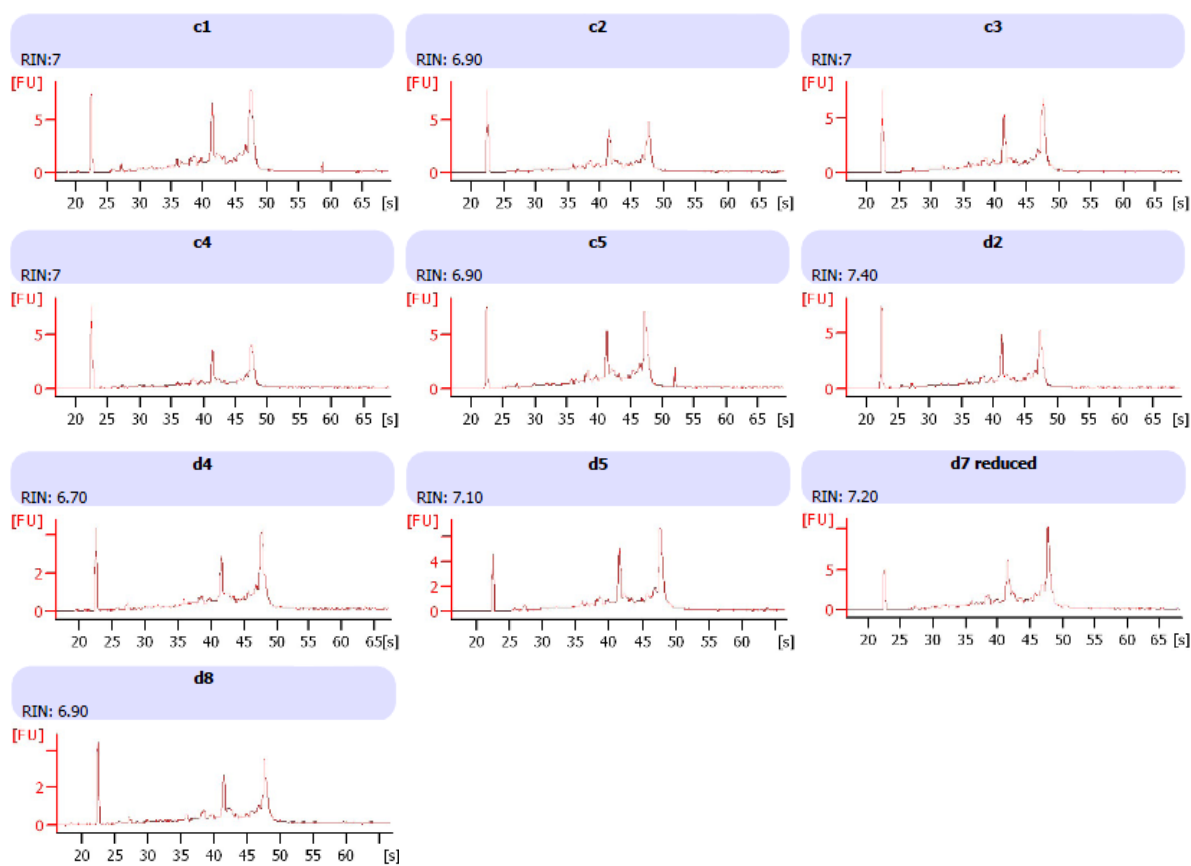


Figure C.2: QC1 - Bioanalyser trace of reduced RNA

Electropherogram of reduced RNA from control and DIO myocardium. Red trace indicates reduced RNA with peaks for the marker, 18S and 28S ribosomal RNA. Samples were run at the MRC Genomics Laboratory.

Table C.2: QC2 – Concentrations, OD values and yield

Sample ID	cRNA (ng/μg)	OD (260/280 nm)	Volume (μL)	Yield (μg)
Control 1	2747.8	2.05	13	35.721
Control 2	3266.6	2.08	14	45.732
Control 3	3037.8	2.10	16	48.605
Control 4	3256.8	2.08	17	55.366
Control 5	3189.1	2.09	19	60.593
DIO 1	3152.6	2.10	15	47.289
DIO 2	3185.4	2.11	18	57.337
DIO 3	2330.1	2.05	15	43.952
DIO 4	3044.3	2.03	19	57.842
DIO 5	1807.1	2.09	13	23.492

OD: optical density

Table C.3: QC3 – Concentrations, OD values and yield

Sample ID	cDNA (ng/μg)	OD (260/280 nm)	Volume (μL)	Yield (μg)
Control 1	284.0	2.03	26	7.384
Control 2	288.5	2.03	24	6.924
Control 3	297.4	2.04	25	7.435
Control 4	305.7	2.02	27	8.254
Control 5	311.3	2.01	22	6.849
DIO 1	290.1	2.02	24	6.962
DIO 2	349.6	2.02	20	6.992
DIO 3	277.9	2.03	24	6.670
DIO 4	289.1	2.01	25	7.228
DIO 5	391.8	2.02	24	9.403

OD: optical density

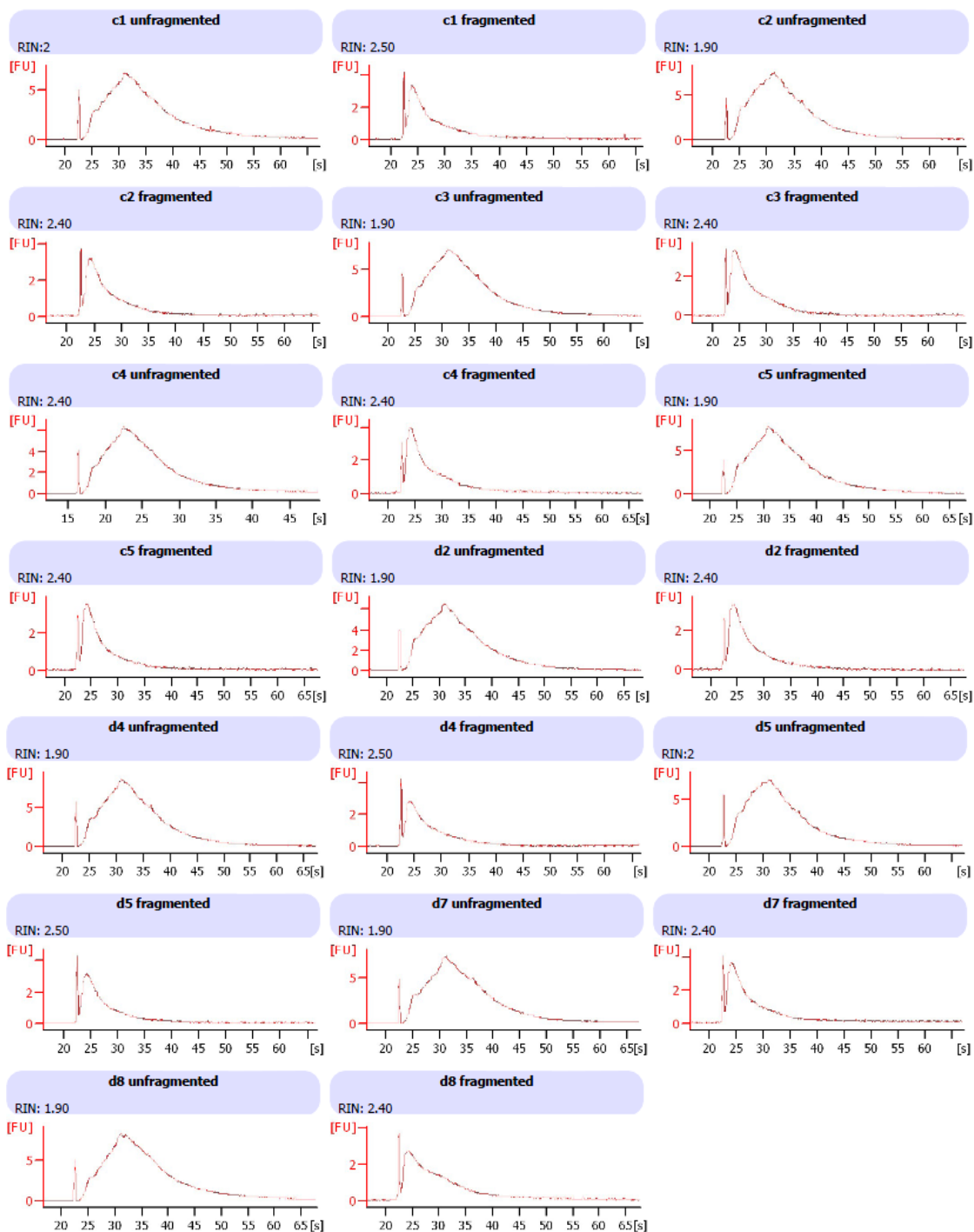


Figure C.3: QC4 – Bioanalyser trace before and after fragmentation

Electropherogram of single-stranded cDNA before fragmentation (unfragmented) and after fragmentation (fragmented) from control and DIO myocardium. Samples were run at the MRC Genomics Laboratory.

Appendix D – RGS2 coding region

Rgs2 mRNA	<div> <div> <div>..... </div> <div>5152535455</div> </div> <div> <div>ATGCAAAGTGCCATGTTCTGGCTGTCCAGCACGACTGCGTACCCATGGACAAGAGTGCA</div> <div>-M--Q--S--A--M--F--L--A--V--Q--H--D--C--V--P--M--D--K--S--A-</div> </div> </div>
DIO	<div> <div>ATGCAAAGTGCCATGTTCTGGCTGTCCAGCACGACTGCGTACCCATGGACAAGAGTGCA</div> <div>-M--Q--S--A--M--F--L--A--V--Q--H--D--C--V--P--M--D--K--S--A-</div> </div>
Rgs2 mRNA	<div> <div> <div>..... </div> <div>65758595105115</div> </div> <div> <div>GGCAACGGCCCCAAGGTCGAGGAGAAGCGGGAGAAAATGAAGCGGACACTCTTAAAGGAT</div> <div>-G--N--G--P--K--V--E--E--K--R--E--K--M--K--R--T--L--L--K--D-</div> </div> </div>
DIO	<div> <div>GGCAACGGCCCCAAGGTCGAGGAGAAGCGGGAGAAAATGAAGCGGACACTCTTAAAGGAT</div> <div>-G--N--G--P--K--V--E--E--K--R--E--K--M--K--R--T--L--L--K--D-</div> </div>
Rgs2 mRNA	<div> <div> <div>..... </div> <div>125135145155165175</div> </div> <div> <div>TGGAAGACCCGTTTGAGCTACTTCTTGCAGAATTCTCTGCTCCTGGGAAGCCCAAACT</div> <div>-W--K--R--T--L--S--Y--F--L--Q--N--S--S--A--P--G--K--P--K--T-</div> </div> </div>
DIO	<div> <div>TGGAAGACCCGTTTGAGCTACTTCTTGCAGAATTCTCTGCTCCTGGGAAGCCCAAACT</div> <div>-W--K--R--T--L--S--Y--F--L--Q--N--S--S--A--P--G--K--P--K--T-</div> </div>
Rgs2 mRNA	<div> <div> <div>..... </div> <div>185195205215225235</div> </div> <div> <div>GGCAAGAAAAGCAAACAGCAAACCTTTTATCAAGCCTTCTCCTGAGGAAGCGCAGCTCTGG</div> <div>-G--K--K--S--K--Q--Q--T--F--I--K--P--S--P--E--E--A--Q--L--W-</div> </div> </div>
DIO	<div> <div>GGCAAGAAAAGCAAACAGCAAACCTTTTATCAAGCCTTCTCCTGAGGAAGCGCAGCTCTGG</div> <div>-G--K--K--S--K--Q--Q--T--F--I--K--P--S--P--E--E--A--Q--L--W-</div> </div>
Rgs2 mRNA	<div> <div> <div>..... </div> <div>245255265275285295</div> </div> <div> <div>GCAGAAGCATTTGATGAACTGCTGGCCAGTAAATATGGGCTGGCTGCATTACAGGCGTTT</div> <div>-A--E--A--F--D--E--L--L--A--S--K--Y--G--L--A--A--F--R--A--F-</div> </div> </div>
DIO	<div> <div>GCAGAAGCATTTGATGAACTGCTGGCCAGTAAATAGGGGCTGGCTGCAAACAGGCGTTT</div> <div>-A--E--A--F--D--E--L--L--A--S--K--Y--G--L--A--A--F--R--A--F-</div> </div>

Figure D.1: RGS2 coding region in DIO and control mice.

First 300 bases of RGS2 coding region of DIO and control mice. No differences were observed over the entire RGS2 coding region between DIO and control mice. Sequences were analysed using the Bioedit programme [173].

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