Regulation of Endoplasmic Reticulum Stress in Adipose Tissue Metabolism

By

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Abbreviations

%	Percent
% EBL	Percent excess body mass index lost
20x	20 times magnification
40x	40 times magnification
4μ8C	4-methyl umbelliferone 8-cabaldehyde
β-TrCP	Beta-transducin repeat containing E3 ubiquitin protein ligase
ΔCt	Delta threshold cycle
μCi/mL	Microcurie per millilitre
μg	Microgram
μg/mL	Microgram per millilitre
μL	Microlitre
μM	Micromolar
μm	Micrometre
μU/L	Microunits per litre
Abd SAT	Abdominal subcutaneous adipose tissue
acyl-CoA	Acyl-coenzyme A
Akt	Akt, also known as protein kinase B (PKB)
AMPK	AMP-activated protein kinase
AP-1	Activation protein 1
APC	Adenomatous polyposis coli
ARC	Arcuate nucleus
AT	Adipose tissue
ATF4/6	Activating transcription factor 4/6
ATGL	Adipose triglyceride lipase

ATP	Adenosine triphosphate
AU	Arbitrary units
AXIN2	Axis inhibitory protein 2
BAT	Brown adipose tissue
Bip	Binding immunoglobulin protein
BMI	Body mass index
BPD	Biliopancreatic diversion
Bq	Becquerel
Bq/mg	Becquerel per milligram
BSA	Bovine serum albumin
C	Celsius
C256Y	Cysteine to tyrosine mutation at residue 256
CCL2	C-C motif ligand 2
CD14/68	Cluster of differentiation 14/68
cDNA	Complimentary deoxyribonucleic acid
CEBPα/β/δ	CCAAT/enhancer-binding protein alpha/beta/delta
CHOP	C/EBP homologous protein
ChREBP	Carbohydrate response element binding protein
CKI	Casein kinase-1
cm	Centimetre
cm^3	Cubic centimetre
CVD	Cardiovascular disease
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
dH2O	Distilled and autoclaved water

DMEM/F12	Dulbecco's modified eagle medium/nutrient mixture F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNL	De novo lipogenesis
Dvl	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
eIF2α	Eukaryotic translation initiation factor 2 A
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated protein degradation
FA(s)	Fatty acid(s)
FABP	Fatty acid-binding protein
FFA(s)	Free fatty acid(s)
g/day	Grams per day
g/L	Grams per litre
GADD34	Growth arrest and deoxyribonucleic acid damage-inducible protein
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter type 4
GRP78	Glucose regulated protein 78 kilodalton
GRPs	Glucose regulated proteins
GSK-3	Glycogen synthase kinase-3
GWAS	Genome wide association studies
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein
HE	Hematoxylin and eosin
HMW	High molecular weight

HOMA-IR	Homeostatic model assessment – insulin resistance
HSL	Hormone sensitive lipase
IDF	International diabetes federation
IgG	Immunoglobulin G
IKK	I kappa B kinase
IR	Insulin receptor
IRE1α	Inositol requiring enzyme 1 alpha
IRS1/2	Insulin receptor substrate 1/2
IL-6/10	Interleukin - 6/10
JNK	c-Jun N-terminal kinase
kcal	Kilocalorie
kDa	Kilodalton
kg	Kilogram
kg/m^2	Kilogram per metre squared
KHB	Krebs-Henseleit buffer
L	Litre
LAGB	Laparoscopic adjustable gastric banding
LDL	Low-density lipoprotein
LD(s)	Lipid droplet(s)
LGCP	Laparoscopic greater curvature plication
LPS	Lipopolysaccharide
LRP5/6	Low-density-lipoprotein-related protein 5/6
LXR	Liver X receptor
MBTPS2	Membrane-bound transcription factor peptidase site 2
MEF	Mouse embryonic fibroblasts

mg	Milligram
mg/dL	Milligram per decilitre
mg/mL	Milligram per millilitre
MHO	Metabolically healthy but obese
miRNA	micro ribonucleic acid
mL	Millilitre
mm	Millimetre
mM	Millimolar
mmol/L	Millimoles per litre
mmol/mol	Millimoles per mole
MONW	Metabolically obese but normal weight
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NEFA	Nonesterified fatty acids
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
ng/L	Nanogram per litre
ng/mL	Nanogram per millilitre
NHS	National health service
NICE	National institute for health and care excellence
nm	Nanometre
nmol	Nanomole
NPY	neuropeptide Y
NT	Non-targeting
ORO	Oil Red-O

P.	Page
p	Phosphorylated
P	Probability
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline - tween
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
pH	Power of hydrogen
PKR	Protein kinase ribonucleic acid – activated
PM	Plasma membrane
PPARγ	Peroxisome proliferator-activated receptor gamma
qRT-PCR	Quantitative real time polymerase chain reaction
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RYGB	Roux-en-Y gastric bypass
SAT	Subcutaneous adipose tissue
siRNA	Small interfering ribonucleic acid
SNS	Sympathetic nervous system
SREBP2	Sterol regulatory element-binding protein 2
T2DM	Type II diabetes mellitus
T75	75 square centimetre growth area tissue culture flask
TAG	Triacylglycerol, also known as triglyceride

TCF7	T-cell factor 7
TCF/LEF	T-cell factor/lymphoid enhancer factor
TLR(s)	Toll like receptor(s)
TM	Tunicamycin
TNFα	Tumor necrosis factor alpha
TRAF2	Tumor necrosis factor receptor associated factor 2
TUDCA	Tauroursodeoxycholic acid
U/μL	Units per microlitre
Ub	Ubiquitin
UPR	Unfolded protein response
USA	United States of America
UV	Ultraviolet
V	Volt
VAT	Visceral adipose tissue
VLDL	Very low-density lipoprotein
WAT	White adipose tissue
WEF	World economic forum
WHO	World health organisation
Wnt (10b)	Wingless-type mouse mammary tumour virus integration site family protein (10b)
XBP1(u/s)	X-box binding protein 1 (unspliced/spliced)

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. I, Philip Demos Voyias, declare that it has been composed by myself and has not been submitted in any previous application for any degree and all the research has been undertaken in accordance with University safety policy and Guidelines on Ethical Practice.

The presented work, including data generation and data analysis, was carried out by the author except in the cases outlined below:

- The bariatric study: obtaining ethical approval, patient recruitment, obtaining patient consent, performing bariatric surgery and collecting and sending anthropometric data and SAT biopsies to Warwick Medical School were carried out by Jana Vrbikova, Vojtech Hainer and Olga Bradnova; Institute of Endocrinology, Prague, Czech Republic, Martin Fried, Petra Sramkova and Karin Dolezalova; OB Clinic, Prague, Czech Republic.

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Title: Bariatric surgery reduces endoplasmic reticulum stress in human adipose tissue and is associated with improvement in metabolic risk factors in obese type 2 diabetes patients

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Abstract

Obesity is the most significant risk factor for developing type II diabetes mellitus (T2DM). Obesity induces adipocyte endoplasmic reticulum (ER) stress, prior to onset of insulin resistance. A pathological inability of white adipose tissue (WAT) to expand to accommodate excess energy is predominantly due to impaired adipogenesis. The research hypothesis was that ER stress in human WAT is important in inducing WAT dysfunction and subsequent insulin resistance and T2DM. The aims of this study were to elucidate interactions of ER stress in human WAT and to characterise the role of ER stress in human adipogenesis. Abdominal SAT biopsies and anthropometry were collected from T2DM subjects before and after bariatric surgery and non-diabetic subjects. Preadipocytes were extracted from human WAT and differentiated into adipocytes. Lipogenesis, lipolysis, glucose uptake, insulin sensitivity, and ER stress and adipogenesis gene and protein expression were assessed in control cells and with ER stress inducers, inhibitors or siRNA. The results of this study found both restrictive and malabsorptive bariatric interventions are effective weight loss interventions for obese T2DM patients and result in significantly improved glucose and insulin levels six months after surgery. WAT health is better following restrictive procedures as shown by lower and better regulation of ER stress markers. Adipogenesis in primary human preadipocytes is influenced by adiposity and WAT depot and the IRE1α-XBP1s UPR is essential in human adipogenesis. XBP1s plays a vital role upstream of CEBPα and PPARγ in human adipogenesis and it is necessary for mediating the action of insulin. Wnt10b plays an inhibitory role in human adipogenesis and acts independently of XBP1s. Collectively these findings suggest that WAT function is key for metabolic health and can be impaired by ER stress; however regulated adipogenesis may serve to improve WAT function and therefore improve metabolic health.

Chapter 1: Introduction

Introduction

People with type II diabetes mellitus (T2DM) experience reduced quality and length of life. The disease presents a huge burden on global society through medical, social and financial implications. Current clinical interventions are failing to ensure patient glycaemic control. The initiation of insulin resistance in T2DM requires further elucidation. If the underlying cellular mechanisms that drive the initiation of insulin resistance can be characterised then a novel therapeutic window will be presented allowing early intervention and potentially reducing the encumbrance of the metabolic disease. This chapter reviews current literature about the importance of white adipose tissue (WAT) in metabolic health and reviews endoplasmic reticulum (ER) stress in adipocytes as a key factor in the initiation of insulin resistance. The review is human-focused, holistic, and organised in a reductionist way. It opens with the impact of T2DM on people and the global population. Then WAT multifunctional integrated physiology is considered because of its role in metabolism and because obesity, characterised by WAT expansion, is the most significant risk factor for developing T2DM. The importance of adipogenesis in WAT regulation is highlighted. ER stress as an initiator of T2DM through inhibiting insulin action and inducing WAT inflammation is reviewed. The chapter closes with a translation of findings to a clinical context before summerasing and ending with hypotheses, aims and objectives.

1.1 Type II diabetes mellitus

1.1.1 Definition of diabetes mellitus

Diabetes mellitus is defined in the Oxford Concise Medical Dictionary as ‘a disorder of carbohydrate metabolism in which sugars in the body are not oxidized to produce energy’ (Oxford, 2015). Type I diabetes mellitus is characterised by patients having little or no ability to produce insulin from birth or an early age. Type II diabetes mellitus (T2DM) is acquired and is characterised by patients where the pancreas retains some ability to produce insulin but this is inadequate for the needs of the body; or, the body becomes resistant to the effects of insulin (Oxford, 2015). The World Health Organisation (WHO) published its current diagnostic criteria for diabetes mellitus in 2006 as fasted plasma glucose above 7.0 mmol/L (126 mg/dL) or 2-hours post 75 g oral glucose challenge plasma glucose above 11.1 mmol/L (200 mg/dL) (WHO, 2006). In the United Kingdom, the National Institute for Health and Care Excellence (NICE) recommended the WHO diagnostic criteria in their ‘Type 2 diabetes: The management of type 2 diabetes’ guidelines published in 2009, updated in July 2014 (NICE, 2009) and they are currently being recommended for the National Health Service (NHS).

1.1.2 Clinical sequelae of type II diabetes mellitus

Patients with T2DM must balance their diet and clinical interventions to ensure glycaemic control and avoid hyperglycaemia or hypoglycaemia (plasma glucose below 4.0 mmol/L (DiabetesUK, 2015)) to prevent pathophysiology. People with T2DM without appropriate glycaemic control utilise fatty acids instead of glucose as an energy source. However, this disturbs the acid-base balance and ketones can

accumulate in the bloodstream (ketosis) and if untreated can cause diabetic coma (Umpierrez, *et al.*, 2006). Hypoglycaemia is associated with several pathologies including a higher risk of cardiovascular disease (CVD) as reviewed in a recent meta-analysis with almost one million T2DM participants (Goto, *et al.*, 2013). Chronic hyperglycaemia gives rise to a greater risk of microenvironmental defects including; retinopathy, neuropathy, nephropathy, microangiopathy and mobilopathy (impaired stem cell mobilisation) (Fadini, *et al.*, 2014; Gerstein and Werstuck, 2013), and macrovascular complications including; ischaemic heart disease, peripheral vascular disease and stroke (Zoungas, *et al.*, 2014). These hyperglycaemic and glucose toxicity induced pathologies arise via a myriad of cellular mechanisms including altered reduction and oxidation (redox) reactions, accumulation of advanced glycated end products, reviewed by Campos (Campos, 2012), and recently emerging epigenetic mechanisms (Kato and Natarajan, 2014). Lipid metabolism is also dysregulated in diabetes causing diabetic dyslipidemia, characterised by elevated postprandial triglyceride, also known as triacylglycerol (TAG), low levels of high-density lipoprotein (HDL) cholesterol, elevated low-density lipoprotein (LDL) cholesterol and the predominance of small, dense LDL particles, presenting a major cardiovascular risk (Wu and Parhofer, 2014). T2DM is therefore associated with reduced quality of life, significant morbidity and reduced life expectancy (Jia, *et al.*, 2013).

1.1.3 Epidemiology of type II diabetes mellitus

The prevalence of T2DM is increasing globally, over the last three decades it has more than doubled (Chen, *et al.*, 2012). The International Diabetes Federation (IDF) has estimated that worldwide diabetes prevalence will increase from 382

million in 2013 to 592 million in 2035 (IDF, 2014). The WHO predicts that deaths due to diabetes will double between 2005 and 2030 (WHO, 2011). There are variations in prevalence by geographical location, ethnicity, sex and age. Geographic variations have been attributed to differences in socio-economic status whilst ethnic variations seem to have more profound metabolic causes, possibly linked to adipose tissue (WAT) distribution and function (Chan*, et al.*, 2009). There is a small gender difference in the global numbers of people with diabetes, with about 14 million more men than women estimated to have diabetes in 2013, and the prevalence increases sharply with age in both sexes (Forouhi and Wareham, 2014).

T2DM costs individuals their quality and length of life whilst the clinical management of the chronic disease and reduced productivity has considerable financial implications. The excess lifetime medical spending of a person diagnosed with T2DM at 40 years old compared to without T2DM in the United States of America (USA) has been estimated to be $211,400 (Zhuo, *et al.*, 2014). People with diabetes require at least two to three times the health-care resources compared to people who do not have diabetes, and diabetes care may account for up to 15 % of national health care budgets (Zhang, *et al.*, 2010). An inexorable increase in the direct medical cost of diabetes and its detrimental economic impact through reduced productivity has been recorded (Herman, 2013). The World Economic Forum (WEF) have warned in their 2014 Global Risks Report that the projected increase in number of people with T2DM and the associated healthcare funding will pose ‘a defining challenge for all countries over the next 10 years’ (WEF, 2014). Therefore T2DM causes a huge burden on global society, both

medically and financially. Understanding the risk factors and causes of T2DM will allow development of suitable interventions to reduce this burden on global society.

1.1.4 Obesity and type II diabetes mellitus

There are several risk factors associated with developing T2DM, including smoking, adverse developmental origins, and genetic susceptibility (Chan, *et al.*, 2009). The main aetiological risk factors are age, obesity, family history and physical inactivity (Forouhi and Wareham, 2014). However the single most important risk factor in contributing to T2DM is obesity (Guh, *et al.*, 2009). Obesity is heterogeneous and alone it is not a definitive propensity for T2DM. For example, there is a defined subset of obese people who are metabolically healthy but obese (MHO) that do not exhibit an increased mortality, increased risk of CVD, or an increased risk of T2DM compared to normal weight controls (Seo and Rhee, 2014). In obesity there is an expansion of adipose tissue, and where the excess adipose tissue is deposited and how it functions influences T2DM risk. Excess abdominal adipose tissue, termed 'central obesity' poses a greater risk for developing T2DM than excess gluteal adipose tissue (Balkau, *et al.*, 2007). Paradoxically, obesity is not a prerequisite for T2DM. Indeed there are individuals who are not obese on the basis of height and weight, with a body mass index (BMI) of less than 30 kg/m^2, but are hyperinsulinemic, insulin-resistant and have T2DM (Ruderman, *et al.*, 1998). These individuals have been described as 'metabolically obese but normal weight' (MONW) (Ruderman, *et al.*, 1998). MHO and MONW populations have been investigated and WAT distribution, regulation and function hypothesised as a potential discriminant between the two

groups in risk of developing T2DM (Karelis, *et al.*, 2004). WAT dysfunction can lead to the accumulation of lipids in tissues not primarily intended for fat storage such as liver, pancreas, muscle and kidney. This ectopic fat has pathophysiological effects and may in part explain the difference in risk of developing T2DM in MHO and MONW (Hill, *et al.*, 2009; Strohle and Worm, 2014). WAT plays a vital role in systemic metabolism and since T2DM is a disease of metabolism, the initiation of insulin resistance and subsequent T2DM may arise due to adverse adipose tissue function.

1.2 Adipose tissue heterogeneity

1.2.1 Brown adipose tissue

Adipose tissue found in mammals can be broadly split into two categories; white adipose tissue (WAT) and brown adipose tissue (BAT) (Peirce, *et al.*, 2014). Investigations into BAT over thirty years ago in rats found the tissue has important thermogenesis functions that can be cold-induced and diet-induced (Rothwell and Stock, 1979). BAT is especially abundant in neonates and in hibernating mammals where it serves to generate body heat (Gesta, *et al.*, 2007). BAT contains a much higher number of mitochondria per cell and has a more extensive capillary network than WAT and therefore it can be distinguished from WAT by histology. BAT also has a characteristic protein, uncoupling protein-1 (UCP-1), which can be used as a marker of the tissue (Cannon and Nedergaard, 2004). Although BAT had been found in human neonates, evidence confirming the presence of BAT in adult humans remained sparse until recently. Paired computer tomography (CT) and positron-emission tomography (PET) scans have

indicated the presence of BAT in adult humans in the region of anterior neck to thorax and were confirmed by immunostaining and histology (Cypess, *et al.*, 2009). Furthermore, magnetic resonance (MR) imaging has been used to identify human BAT (Reddy, *et al.*, 2014). Not only has BAT been identified in adult humans, its activity has also been revealed. BAT thermogenesis activity has been demonstrated in healthy men when exposed to cold temperatures (16 C) and BAT activity was reduced in men who are overweight or obese (van Marken Lichtenbelt, *et al.*, 2009). BAT activity in humans has also been pharmacologically activated by an orally administered β3-adrenergic receptor agonist and shown to increase resting metabolic rate by about 200 kcal compared to control (Cypess, *et al.*, 2015). Cellular mechanistic studies have been conducted to compare properties of BAT and WAT, however these have been mostly restricted to rodent model systems (Rosell, *et al.*, 2014). BAT may play a role in adult human metabolism but WAT has a much larger role in metabolism, energy storage and energy regulation and WAT comprises the vast majority of human AT (Obregon, 2014). Furthermore, WAT is expanded in obesity, the most significant risk factor for developing T2DM. Therefore WAT will be considered further.

1.2.2 Distinct adipose tissue depots

WAT, like skeletal muscle, is distributed through the body in discrete depots (figure 1.2.2) (Shen, *et al.*, 2003). Subcutaneous adipose tissue (SAT) represents about 85 % of all body fat and is unequally distributed through the body, with larger depots in the abdomen (Abd SAT), thigh, gluteal, femoral and mammary region (Frayn and Karpe, 2014). Visceral adipose tissue (VAT) depots are deeper

in the abdomen than SAT and include omental that connects to the stomach. The functions and properties of each WAT depot overlap but there are distinct differences (White and Tchoukalova, 2014). Accumulation of adipose tissue in different depots has differential risk associated with development of T2DM. Early clinical observations by Vague suggested upper-body obesity, commonly referred to as central obesity, was associated with a much greater incidence of insulin resistance than lower-body obesity (Vague, 1956). In central obesity, different depths of abdominal SAT are functionally distinct and independently correlate with metabolic complications of obesity (Smith, *et al.*, 2001). These observations have been supported by several studies, reviewed by Frayn *et al.* (Frayn, *et al.*, 2003). The difference in risk of developing T2DM conferred by different WAT depots is likely to be due to a combination of WAT properties including lipid and carbohydrate metabolism (lipolysis, lipid accumulation and glucose uptake) and systemic signalling (protein and lipid secretion and innervation). Lipolysis, TAG synthesis and storage, and protein secretion varies between WAT depots as reviewed by Lee *et al.* (Lee, *et al.*, 2013). Hormone secretion varies between WAT depots, for example the hormone resistin is secreted from both preadipocytes and adipocytes and both gene and protein expression is higher in abdominal WAT compared to femoral or mammary WAT (McTernan, *et al.*, 2002). VAT has been found to secrete more pro-inflammatory molecules like interleukin 6 (IL-6) compared to SAT (Fried, *et al.*, 1998). However abdominal SAT can also secrete large quantities of pro-inflammatory proteins (Youssef-Elabd, *et al.*, 2012). WAT depots can have protective effects against T2DM, for example increased gluteal and femoral WAT in people with a lower waist hip ratio is associated with reduced metabolic risk (Manolopoulos, *et al.*, 2010). The

protective phenomenon of gluteal and femoral fat is credited to its beneficial secretion profile (Manolopoulos, *et al.*, 2010). WAT distribution therefore plays a role in relative risk of developing T2DM. Naturally occurring differences in WAT distribution may provide further insight into important depots for developing T2DM.

Figure 1.2.2 Major adipose tissue depots

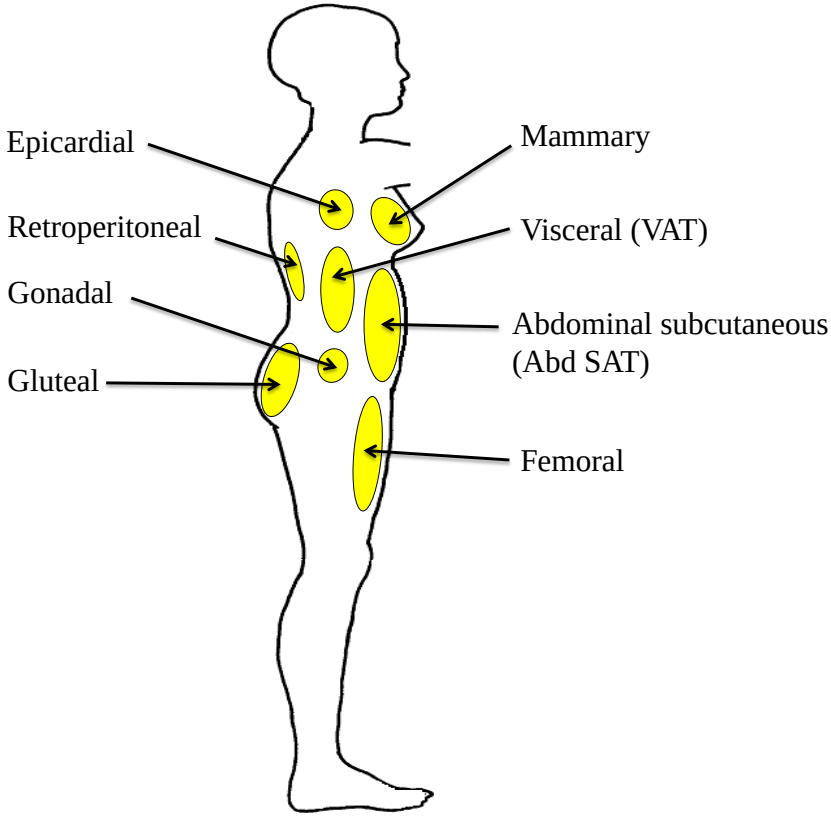


Figure 1.2.2 legend: Major adipose tissue depots. Adipose tissue is distributed throughout the body in discrete depots with functionally distinct properties.

1.2.3 Gender and race differences in adipose tissue distribution

There are sex specific differences in total WAT and its distribution (White and Tchoukalova, 2014). Women have generally higher total adiposity than men

(Blaak, 2001). Women accumulate more WAT in lower body depots like gluteal and femoral, while men accumulate more WAT in central areas, both Abd SAT and VAT (Geer and Shen, 2009). Perhaps this is partly why more men develop obesity linked T2DM than women. Mechanisms that underlie this sexual dimorphism are unclear. Sex steroid hormones androgen and estrogen have been investigated. Testosterone-treated men have less adiposity and selective loss of central WAT (Allan and McLachlan, 2010), however women with polycystic ovary syndrome (PCOS) with a hyperandrogenic state are prone to central obesity (Escobar-Morreale and San Millan, 2007). Sex specific differences in lipid and glucose metabolism have been investigated and may be an underlying explanation for the observed differences in WAT distribution (Varlamov, *et al.*, 2015). Sex hormone milieu has been linked to gender and race differences in obesity (Perry and Martin, 2014).

Evidence of race disparities in adiposity has been accumulating and these differences are becoming apparent from early childhood (Zilanawala, *et al.*, 2014). Obesity is the most significant risk factor for developing T2DM across different ethnicities, however the risk of developing T2DM varies by extent of obesity in different ethnicities. For example, a prospective study found that Europeans developed T2DM at a much higher obesity, measured by BMI and waist circumference, compared to South Asians and Arfican-Caribbeans (Tillin, *et al.*, 2015). These differences may be due to altered WAT function in different ethnicities. WAT was previously regarded as a dormant energy store, however in recent years a plethora of WAT functions has come to light. WAT dysregulation,

that is abnormal or impairment in the regulation of metabolic or physiological processes, may contribute to T2DM development.

1.2.4 Adipose tissue composition

WAT is comprised of many cell types that together orchestrate several important physiological processes (Trujillo and Scherer, 2006). WAT is a multifaceted tissue consisting of preadipocytes, adipocytes, endothelial cells, fibroblasts, vascular smooth muscle cells and resident immune cells such as monocytes, macrophages, T cells and B cells (Caspar-Bauguil, *et al.*, 2005; Nishimura, *et al.*, 2013). The precursor cells constitute the stromal vascular fraction of adipose tissue. Human WAT is a source of multipotent stem cells (Zuk, *et al.*, 2002). Adipocytes are the main cellular component of WAT and the key functional energy-storing unit at the interface of energy homeostasis dynamically influenced by a number of cues from surrounding cells and distant hormonal and neuronal stimuli. To facilitate systemic integration, WAT is supplied with a highly regulated and dynamic network of vasculature (Frayn and Karpe, 2014; Yilmaz and Hotamisligil, 2013), lymph nodes (Pond and Mattacks, 1995) and is innervated by the sympathetic nervous system (SNS) (Bartness and Song, 2007). The WAT as a whole is greater than the sum of the individual parts and the parts work together to accomplish a multitude of functions. Dysregulation of the WAT and consequent suboptimal functionality may contribute to the pathogenesis of T2DM.

1.3 Integrative adipose tissue functions

1.3.1 Immune functions

WAT plays such an extensive role in immunology it has been described as an 'immunological organ' by Grant and Dixit in their recent review (Grant and Dixit, 2015). WAT serves as a key site for the interaction of adipocytes with other specialised effectors of the immune system. This is particularly advantageous in SAT immediately below the skin as a first line of defence and may have arisen due to the primitive need to both acquire nutrients and defend against pathogens. Infiltration of inflamed WAT by immune cells such as eosinophils, macrophages and neutrophils bolsters the existing WAT resident immune cell repertoire and is an important feature of the immune system to provide host defence against pathogens (Exley, *et al.*, 2014). There are several similarities between adipocytes and a wide range of immune cells including macrophages, dendritic cells and T cells (Wellen and Hotamisligil, 2005). These features include pathogen sensing, phagocytic properties, complement activation and production of inflammatory mediators (Shoelson, *et al.*, 2006). In vitro studies on human adipocytes have shown antigenic stimuli increased the secretion of the adipokine resistin and that proinflammatory cytokine levels were increased in response to resistin (Kusminski, *et al.*, 2007). Toll like receptors (TLRs) are expressed on human adipocytes (Wang and Nakayama, 2010). The TLR family of receptors recognise specific components conserved among microorganisms but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). Examples of conserved features of pathogens include bacterial cell-surface lipopolysaccharide (LPS), lipoproteins and double stranded RNA of viruses. Adipocyte TLRs are thought to be activated by LPS of gram negative

circulating gut derived bacteria and therefore stimulate an innate immune response (Creely, *et al.*, 2007). Although WAT immune responses and the associated local inflammation assist the defence against pathogens they can be detrimental if the tissue remains inflamed. Resident WAT regulatory B cells play a role in reducing inflammation by secreting anti-inflammatory interleukin-10 (IL-10) (Nishimura, *et al.*, 2013). WAT inflammation must be regulated to prevent adipose tissue dysfunction and subsequent T2DM.

1.3.2 Endocrine functions

Cells within the WAT secrete several proteins and therefore to perform appropriately they require a functional protein secretion system. Given the resident cells of WAT secrete signalling proteins the WAT can itself be considered as an 'endocrine organ' (Galic, *et al.*, 2010). Cloning of the obese gene and the identification of its product, leptin (Zhang, *et al.*, 1994), was one of the first discoveries of an adipocyte derived signalling molecule and established an important role for adipose tissue as an endocrine organ. Since the discovery of leptin several other adipokines have been characterised, including chemerin, interleukin-6 (IL-6), apelin, visfatin, adiponectin, tumor necrosis factor-alpha (TNFα), plasminogen activator inhibitor-1 and retinol binding protein 4 (Ouchi, *et al.*, 2011). The term 'adipokinome' has been suggested to describe collectively the proteins and lipid moieties, such as prostaglandins and fatty acids, released by adipocytes (Trayhurn and Wood, 2004). The secreted molecules of the adipokinome have diverse roles in lipid metabolism, insulin sensitivity, vascular homoeostasis, the alternative complement system, blood pressure regulation, angiogenesis and the regulation of energy balance. Obesity-related adipokines and

their possible application as a treatment for obesity has been reviewed (Khan and Joseph, 2014).

Adiponectin is an adipokine synthesized almost exclusively by adipocytes and is the most abundant adipokine in circulation (3 to 30 µg/mL) (Ouchi, *et al.*, 2003). However adiponectin levels are decreased in obese individuals compared to lean individuals (Ryo, *et al.*, 2004). Consistent with this, the synthesis of adiponectin by adipocytes is inhibited by obesity related pro-inflammatory molecules such as IL-6 and TNFα (Berg and Scherer, 2005) as well as by oxidative stress and hypoxia (Hosogai, *et al.*, 2007). An association between adiponectin levels and obesity-linked metabolic dysfunction is supported by several clinical observations. Namely, high adiponectin levels are associated with a lower risk for developing type 2 diabetes, plasma adiponectin levels are decreased in patients with type 2 diabetes (Li, *et al.*, 2009), and plasma adiponectin levels negatively correlate with VAT accumulation (Ryo, *et al.*, 2004) therefore 'hypoadiponectinemia' is closely associated with the clinical phenotype of the metabolic syndrome. Experimental models indicate that adiponectin protects against obesity linked metabolic dysfunction. Diabetic mice have been shown to reduce hyperglycaemia following administration of adiponectin by enhancing insulin activity (Berg and Scherer, 2005), and when given to obese mice it reduced plasma levels of glucose, TAG and free fatty acids (FFAs) by increasing fatty acid oxidation in muscle tissue (Fruebis, *et al.*, 2001). Consistent with this, transgene mediated overexpression of adiponectin in *ob/ob* mice (leptin deficient) improves glucose metabolism independently of weight loss (Kim, *et al.*, 2007). Adiponectin seems to mediate its beneficial effects in part by activating AMP-

activated protein kinase (AMPK) in liver and skeletal muscle which leads to inhibition of gluconeogenesis in the liver and an increase in fatty acid oxidation and glucose uptake in muscle tissue (Tomas, *et al.*, 2002; Yamauchi, *et al.*, 2002). Adiponectin is synthesised as a 32 kDa monomeric protein and then assembled into low molecular weight trimers, medium molecular weight hexamers and high molecular weight (HMW) multimers (Galic, *et al.*, 2010). The HMW form has insulin sensitising effects (Liu and Liu, 2014).

Leptin is a 16 kDa pro-inflammatory adipokine produced mainly by adipocytes and is required for control of bodyweight and reproduction (Lord, 2006). Mice defective in leptin or the leptin receptor are hyperphagic, obese, insulin resistant and infertile (Lord, 2006). The leptin deficient (*ob/ob*) mouse has been used extensively as an *in vivo* model of obesity and T2DM (Drel, *et al.*, 2006). Mice injected with leptin on a daily basis experienced a fast reduction in food intake, body mass and percent body fat, but maintained lean muscle mass, and increased energy expenditure and restored reproductive function and euglycemia (Pelleymounter, *et al.*, 1995). Leptin is effective at improving metabolic dysfunction in patients with congenital leptin deficiency or lipodystrophy (Farooqi, *et al.*, 2002; Oral, *et al.*, 2002). Leptin resistance in obesity has been suggested since levels in the blood positively correlate with adipose mass and obese individuals have high leptin levels without the expected anorexic responses (Friedman and Halaas, 1998). Leptin interacts with both the neuronal and immune systems and the high levels in obesity may play a role in obese related brain diseases (Aguilar-Valles, *et al.*, 2015).

The diverse roles of the leptin and adiponectin and their altered expression in the obese state demonstrate the complexity of adipokine signalling. The two aforementioned adipokines influence the level of WAT inflammation; an important factor given increasing evidence indicates that obesity is causally linked to a chronic low-grade inflammatory state and T2DM ensues (Hotamisligil, 2006; Shoelson, *et al.*, 2006).

1.3.3 Energy homeostasis

Systemic energy homeostasis requires appropriate function of several physiological systems including the digestive, circulatory, musculoskeletal, endocrine, and nervous systems. WAT adipocytes play a vital integrative role between these systems and are the main energy store in the body (Rosen and Spiegelman, 2006). Energy homeostasis is governed by a neuroendocrine system that links WAT to the brain. An example of the WAT – hypothalamus axis is the reciprocal integration of WAT derived peripheral anorexic signal leptin and hypothalamic orexigenic peptide neuropeptide Y (NPY) (Zhang, *et al.*, 2014b). The arcuate nucleus (ARC) of the hypothalamus contains NPY neurons that sense and integrate peripheral energy signals, such as blood glucose concentration, leptin and insulin due to the unique anatomical structure of the ARC in lacking a blood brain barrier (Kohno and Yada, 2012). Energy deficiency and greater metabolic demand such as increased exercise induces the synthesis of ARC NPY (Leibowitz and Wortley, 2004). NPY regulates energy utilisation in WAT by promoting adipogenesis and lipid accumulation and inhibiting lipolysis, and affects food intake by innervating with other appetite regulatory factors in the central nervous system (Zhang, *et al.*, 2014b). WAT also regulates energy

homeostasis by influencing liver and muscle metabolism as reviewed by Saltiel and Kahn (Saltiel and Kahn, 2001). Adipocytes release FFAs into the circulation that reduce glucose uptake in muscle, insulin secretion from pancreatic β-cells and increase glucose production from the liver (Bergman and Ader, 2000). Adiposity influences muscle derived peptide irisin, which may have a central role in metabolism given it has been identified in human cerebrospinal fluid (Piya, *et al.*, 2014). WAT regulation is imperative for systemic energy homeostasis and if it becomes dysfunctional then diseases of energy homeostasis, such as T2DM, may arise.

1.4 Metabolism in human adipose tissue

The ability to take up and store energy during a fed state and break down and release energy in a fasted state provides an advantage through the ability to withstand fluctuations in energy supply. WAT adipocytes undertake anabolic and catabolic metabolic processes in response to the needs of the body and contain the biggest reserve of energy in the body in the form of lipid TAG; therefore WAT plays a vital role in systemic energy regulation. WAT adipocytes are able to take up energy from the blood, mostly in the form of carbohydrate (glucose) and lipid (nonesterified fatty acids – NEFA), store energy predominantly as lipid (TAG) and release energy when required predominantly as lipid (NEFA). WAT adipocytes have a very dynamic pattern of metabolism influenced by hormonal and neuronal stimuli and consequently rapidly respond to energy requirements. A dynamic crosstalk between systemic metabolic organs in which WAT plays a prominent role exists to ensure the energy needs of the body are supplied. During a fasted state glycogen stores in the liver become depleted triggering a liver-brain-

adipose neural axis that shifts the energy source from carbohydrate to TAG in adipocytes in order to maintain energy balance (Izumida, *et al.*, 2013). Conversely, in a fed state glycogen accumulates in the liver and lipogenesis is stimulated in adipocytes (Lu, *et al.*, 2014).

Proteins are essential at virtually every stage of systemic energy transfer; digestive enzymes, transport proteins in cellular uptake and circulation transfer, biochemical enzymes that catalyze anabolic and catabolic reactions, energy regulatory hormones, energy storage proteins such as those coating lipid droplets and many more function in systemic and WAT metabolism. Therefore functional protein metabolism, especially protein synthesis, is vital for systemic energy regulation. Thus the intracellular organelle ER that processes proteins plays a vital role in energy regulation and impaired ER function can cause several pathologies (Kim and Arvan, 1998; Ni and Lee, 2007). In this section about metabolism in human WAT, the roles of proteins are presented in an integrated way just as the roles of proteins are integrated in WAT metabolism. Excess WAT in obesity is the main risk factor for T2DM, a disease of impaired metabolism; therefore metabolic processes in WAT may play an important role in the initiation of T2DM.

1.4.1 Lipid metabolism

Dietary fat is absorbed by the gut and transported to the circulation in chylomicrons (Mansbach and Gorelick, 2007). Fatty acids from circulating lipids are provided to WAT by the albumin-bound NEFA pool, or either TAG incorporated in chylomicrons in the postprandial state, or TAG within very low-

density lipoprotein (VLDL) in the post-absorptive state. TAG must be hydrolyzed to fatty acids by extracellular enzyme lipoprotein lipase bound to the wall of capillaries before they can be taken up by adipocytes (Mead, *et al.*, 2002). The trafficking of fatty acids into and out of adipocytes is regulated by several proteins and enzymes and is under control by hormonal and metabolic factors (Thompson, *et al.*, 2010). Within adipocytes NEFAs are converted to TAG and stored in lipid droplets (LDs), the lipid storage organelles of all organisms. When energy is required in other tissues TAG is converted by lipases to NEFA and glycerol and both are released into the circulation (Walther and Farese, 2012). During fasting, plasma NEFA is almost entirely from hydrolysis of TAG stored in SAT LDs (Karpe, *et al.*, 2011). NEFA circulates predominantly bound to albumin and is taken up and oxidized in muscle, liver and other tissues. A proportion will be re-esterified to form new TAG in the liver, which is exported in VLDL and can be taken up by adipocytes after being broken down to NEFA by lipoprotein lipase (Frayn and Humphreys, 2012).

LDs and their associated proteins in adipocytes play a specific role in maintaining systemic energy homeostasis. The cytosolic LD is composed of a core of TAG surrounded by a monolayer of phospholipids and lipid droplet-associated proteins, including enzyme coactivators and structural proteins. Neutral lipids occupy the core of LDs and in human adipocytes are primarily TAG with few sterol esters. In mammalian cells TAG synthesis is catalyzed by acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) enzymes DGAT1 and DGAT2 (Buhman, *et al.*, 2001). DGAT1 and DGAT2 both reside within the ER, although DGAT2 is also found in relatively small quantities in mitochondrial-associated membranes (Stone, *et al.*,

2009). LD formation requires further clarification, however evidence suggests LDs form de novo in mammalian cells from the ER membrane. ER diacylglycerol (DAG) enrichment recruits perilipin 3, a LD coat protein (Skinner, *et al.*, 2009), LDs are arrested in the ER membrane by tight binding of lipidated apolipoprotein B-100 (ApoB) (Ohsaki, *et al.*, 2008), and ER localised fat storage-inducing transmembrane proteins 1 and 2 (FIT1 and FIT2) mediate partitioning of cellular TG into LDs (Gross, *et al.*, 2010). Lipid droplet formation is tightly regulated with cholesterol efflux to maintain intracellular free cholesterol level within a narrow range (Martin and Parton, 2006). Studies in macrophages have found that increases in ER free cholesterol can lead to the induction of the unfolded protein response via changes in the activity of the ER calcium pump sarco(endo)plasmic-reticulum calcium ATPase-2b (SERCA2b) that lead to the depletion of ER calcium stores (Feng, *et al.*, 2003; Li, *et al.*, 2004). Therefore the ER plays a vital role in the formation of lipid droplets and ER functional integrity is essential for appropriate LD formation and energy storage.

Lipolysis is the catabolism of TAG in LDs. In WAT lipolysis is a key process to regulate cellular and systemic energy homeostasis. Lipolytic pathways converge on either protein kinase A (PKA) or protein kinase G (PKG), which phosphorylate several proteins interacting with the LD, the site of action of lipases and cofactors (Arner and Langin, 2014). At the LD surface TAG are sequentially hydrolysed into DAG, monoacylglycerol (MAG) and glycerol, releasing one molecule of fatty acid (FA) at each step. Three lipases work in sequential fashion in human adipocytes to ensure complete hydrolysis of TAG: adipose triglyceride lipase (ATGL) (Zimmermann, *et al.*, 2004), hormone-sensitive lipase (HSL) and

monoglyceride lipase (Bezaire, *et al.*, 2009). Both ATGL and HSL require phosphorylation to become fully active lipases (Krintel, *et al.*, 2008; Pagnon, *et al.*, 2012). Key proteins, including perilipin (Brasaemle, 2007) and cell death-inducing DFFA-like effector A (CIDEA) (Puri, *et al.*, 2008), associate with the LD in human adipocytes and seem to play a role in TAG deposition and lipolysis. Lipolysis in WAT and the subsequent increase in plasma NEFA has been considered inevitable in obesity and was suggested as a mechanism for obesity induced T2DM. However many studies have shown that as WAT mass expands, NEFA release per kilogram WAT is downregulated. Furthermore, obese T2DM subjects can have normal NEFA concentrations and subjects with elevated NEFA concentrations do not necessarily have insulin resistance (Karpe, *et al.*, 2011). Lipolytic products including FAs and DAGs serve as signalling intermediates and may play a role in metabolic regulation (Masoodi, *et al.*, 2014; Zechner, *et al.*, 2012).

Adipose tissue is the body's biggest pool of cholesterol. In adipocytes nearly all cholesterol (>95 %) exists as free, non-esterified form and resides mostly in the LD (Murphy, *et al.*, 2009) and a lower amount in plasma membrane (PM) invaginations called caveolae (Pilch, *et al.*, 2011). Adipocyte cytoplasmic cholesterol derives mostly from lipoprotein uptake, whereas PM cholesterol is predominantly from de novo cholesterol biosynthesis (Musso, *et al.*, 2013). Caveolae are important signalling platforms, serving as a concentrating point for several signalling molecules, including glucose transporter type 4 (glut4) and insulin receptor, and their stability and functionality depends on high PM cholesterol content. Decreasing PM cholesterol, as is the case in hyperthrophied

adipocytes in dysregulated obesity, dilutes the PM cholesterol across an increased cell surface (Le Lay, *et al.*, 2001). Consequently, sterol regulatory element-binding protein 2 (SREBP2) detects cholesterol PM dilution as cholesterol depletion and activates transcription of cholesterol biosynthesis genes, further promoting cholesterol accumulation (Bauer, *et al.*, 2011). In addition, PM cholesterol depletion disrupts caveolae integrity and function and subsequently impairs intracellular insulin signalling (Breen, *et al.*, 2012). The regulation of adipocyte lipid metabolism is therefore paramount to ensure appropriate energy utilisation.

1.4.2 Carbohydrate metabolism

Metabolism of carbohydrates in WAT is essential for the transfer of energy to quickly available energy via the generation of adenosine triphosphate (ATP) and for long-term energy storage via integration with lipid synthesis. WAT stores the largest energy reserve in the body, however this is almost exclusively made up of TAG and relatively little carbohydrate is stored. The liver and skeletal muscle store most of the body's carbohydrate reserve as glycogen, and glycogen storage diseases primarily affect these tissues (Hicks, *et al.*, 2011). Glucose enters adipocytes through glut4 responsible for basal (unstimulated) and insulin-stimulated glucose uptake (Mueckler and Thorens, 2013). In WAT, glucose is mostly used for mitochondrial dependent aerobic respiration to generate ATP. Mitochondrial dysfunction in WAT has been linked to activation of ER stress (Rainbolt, *et al.*, 2014) and progression of T2DM (Kusminski and Scherer, 2012). Within adipocytes the interorganellar organisation between the mitochondria and ER is important to perform specialised tasks. Bidirectional communications

between these two compartments is supported by evidence suggesting the regulation of numerous physiological processes including mitochondrial metabolism, calcium signalling and cell death (de Brito and Scorrano, 2010). Therefore the functional integrity of the ER plays a vital role in cellular energy homeostasis and thus in turn systemic energy homeostasis.

In lipogenic tissues, such as WAT, the expression and activity of glycolytic and lipogenic pathways are linked. Carbohydrates are utilised in adipocytes both for the synthesis of TAG from existing fatty acids, and for the synthesis of new fatty acid molecules from non-lipid substrates in de novo lipogenesis (DNL). The synthesis of TAG requires glycerol-3-phoshate (G3P) for the initial step of fatty acid esterification. G3P is produced in adipocytes either from glucose through the first steps of glycolysis or from gluconeogenic precursors through glyceroneogenesis (Reshef, *et al.*, 2003). Human DNL contributions from liver (1.5 kg) and WAT (12 – 15 kg), on a whole body basis, are comparable between 1 – 2 g/day for each tissue (Diraison, *et al.*, 2003). DNL in human adipocytes is stimulated by glucose and a full stimulation requires the simultaneous presence of glucose and insulin (Foufelle, *et al.*, 1992). In the liver glucose acts by dephosphorylation of carbohydrate response element binding protein (ChREBP) allowing it to enter the nucleus where it binds to a specific response element in the promoter of glycolytic (liver pyruvate kinase, L-PK) and lipogenic (fatty acid synthase, FAS and acetyl-CoA carboxylase, ACC) genes. The roles of glucose and ChREBP in WAT are not as extensively studied as in the liver, however evidence suggests signalling is mediated through the activation of the classical ChREBP isoform ChREBP-α and the expression of a recently identified isoform,

ChREBP-β (Herman, *et al.*, 2012). This new isoform is a potent stimulator of the lipogenic pathway. Therefore carbohydrate metabolism in WAT is essential for systemic energy regulation and dysfunction in T2DM can have many adverse consequences.

1.4.3 Regulation of adipose metabolism

Adipocyte metabolism is subject to several levels of regulation, including endocrine, metabolic and neuronal that synergistically coordinate appropriate energy transfer for the varying needs of the body influenced by diet, physical activity and physiological processes. Endocrine and metabolic regulators are distributed to adipocytes via the blood flow, hence the regulation of blood flow itself can alter the metabolism of WAT (Frayn and Karpe, 2014). Insulin is the classical regulator of metabolism in WAT. The hormone is secreted by pancreatic β-cells and has systemic metabolic effects, altering liver, muscle and WAT metabolism. In adipocytes insulin activates several signalling cascades that include the activation by phosphorylation of Akt, also known as protein kinase B, that result in enhanced glucose uptake and inhibited lipolysis, as reviewed by Guo (Guo, 2014). Insulin secretion is stimulated in the post-prandial state by a class of gut hormones called incretins that include glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (Kim and Egan, 2008). Conversely, in the fasted state insulin secretion is inhibited by a recently identified hormone called limostatin which is expressed in β-cells and described as a ‘decretin’ (Alfa, *et al.*, 2015). Other important endocrine hormones also control adipose tissue physiology and metabolism, including growth hormone and thyroid hormone as reviewed by Carmean *et al.* (Carmean, *et al.*, 2014),

androgens (O'Reilly, *et al.*, 2014) and glucocorticoids (Lundgren, *et al.*, 2008). Metabolic nutrients including glucose, amino acids and fatty acids are capable of activating intracellular signalling cascades such as the hexosamine, mammalian target of rapamycin (mTOR) and adenosine monophosphate-activated protein kinase (AMPK) signalling pathways (Marshall, 2006). The aforementioned pathways are interconnected, coupled to insulin signalling and linked to the release of metabolic hormones from WAT (Holzer, *et al.*, 2011). Vitamin B12 deficiency has been linked to an adverse lipid profile in subjects with T2DM in multiple populations, therefore micronutrients including vitamin B12 may also influence WAT metabolism (Adaikalakoteswari, *et al.*, 2014). WAT is innervated by the SNS and adipocyte lipolysis can be stimulated by norepinephrine (NE) activating β-adrenoceptor and consequent downstream activation of HSL (Bartness, *et al.*, 2014). These endocrine, metabolic and neuronal factors interplay to coordinate adipocyte metabolism.

Adipocyte metabolism is also influenced by genetic makeup and epigenetic mechanisms of genome regulation. Genome wide association studies (GWAS) have identified candidate genes such as transcription factor 7-like 2 (TCF7L2) with specific alleles that confer an increased relative risk for developing T2DM through altered metabolism (Grant, *et al.*, 2006). Not only do certain genes confer an increased risk for developing T2DM, but also how genes are regulated. This is important during embryonic development, where several factors including the nutritional status of the mother influence the genetic regulation of the foetus by epigenetic mechanisms, such as DNA methylation, and predict metabolic health outcomes of the foetus (Kaijser, *et al.*, 2009). Epigenetic genome regulation is not

confined to development; indeed it is a dynamic process that occurs throughout life. DNA methylation profiles of several genes biologically relevant to the development of adiposity including lipase, hormone sensitive (LIPE) encoding HSL, in SAT of adult men and women are associated with extent of adiposity (Agha, *et al.*, 2014). Vitamin B12 insufficiency has been shown to induce cholesterol biosynthesis by limiting s-adenosylmethionine and modulating the methylation of SREBF1 and LDLR genes (Adaikalakoteswari, *et al.*, 2015). A further link between genome and metabolic profile has been hypothesised via telomere length. Enhanced biological aging in South Asian T2DM men with reduced telomere length tracked changes in lipids and BMI (Harte, *et al.*, 2012a). Therefore adipocyte metabolism plays a vital role in energy homeostasis and is regulated by several mechanisms. In T2DM these regulatory mechanisms fail and adipocyte metabolism becomes dysregulated causing considerable pathologies. Elucidating the initiation of adipocyte dysregulation will allow development of preventive measures and alleviate the burden of T2DM.

Figure 1.4.3 The importance of adipose tissue

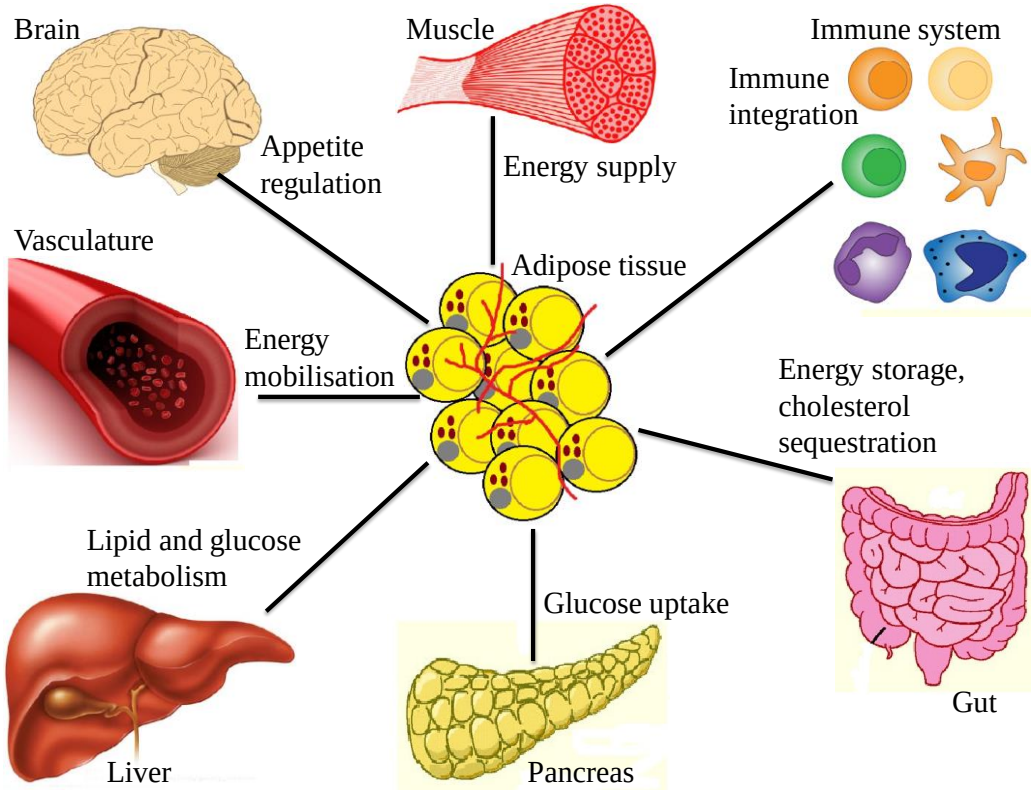


Figure 1.4.3 legend: The importance of adipose tissue. Adipose tissue is multifunctional and integrates several systems in the body to fulfill several vitally important roles.

1.5 Adipogenesis

Adipocytes are specialised cells that synthesise and store fat in WAT and play a major role in systemic energy homeostasis. In order to accommodate the energy requirements of the body WAT is able to expand and contract during adult life despite most development during prenatal and early postnatal life (Poulos, *et al.*, 2010). WAT expansion occurs primarily in two ways, by increasing adipocyte cell number (hyperplasia) or by increasing the size of existing adipocytes (hypertrophy). Evidence suggests obesity complications in humans result from the

inability of SAT to appropriately expand and store lipids, and consequently ectopic fat deposition and lipotoxicity contribute to insulin resistance (Virtue and Vidal-Puig, 2010). Impaired WAT expandability is mainly due to defects in progenitor cells to fail to supply specialised cells through their differentiation into adipocytes, a process termed adipogenesis (Isakson, *et al.*, 2009). Forced expansion of WAT via increased hyperplasia induced by transgenic overexpression of adiponectin in *ob/ob* mice normalized glucose and insulin levels and prevented insulin resistance despite massive obesity (Kim, *et al.*, 2007). Therefore adipogenesis is an important process to maintain WAT metabolic function. Adipogenesis is continually required throughout life since about 10 % of adipocytes in the body are regenerated annually (Spalding, *et al.*, 2008). In their 2013 *Nature Medicine* article, Scherer's group described understanding the underlying mechanisms of adipogenesis as 'more than an academic exercise' and 'of paramount importance with respect for clinical readouts for the metabolic syndrome in the clinic' (Wang, *et al.*, 2013). Furthermore Peirce *et al.* have proposed in their 2014 *Nature Review* article that 'the development of successful strategies to target adipose tissues will depend on investigations that elucidate their developmental origins and cell-type-specific functional regulators' (Peirce, *et al.*, 2014). Therefore characterising human adipogenesis is vital for gaining insight into the pathogenesis of metabolic diseases such as T2DM and for identifying potential targets for pharmacological intervention.

1.5.1 Progenitor origin and cell lineage

Several sites of adipocyte progenitor origin have been explored including ectodermal, mesenchymal, endothelial, and hematopoietic, however further

clarification is required as reviewed by Hyvonen and Spalding (Hyvonen and Spalding, 2014). Adipocytes are thought to derive mostly from mesodermal stem cells residing in the AT stroma. Human WAT is an established source if multipotent stem cells (Zuk, *et al.*, 2002). Furthermore, analysis of intact human SAT revealed adipose derived stem cells distributed in the stroma (Maumus, *et al.*, 2011). However, linear tracing studies in mice indicate that a subset of facial adipocytes originate from the neural crest and therefore derive from the ectoderm (Billon, *et al.*, 2007). Evidence suggests adipocyte precursors may have endothelial origin and exist in the AT vasculature, integrated in the walls of blood vessels in WAT (Sengenes, *et al.*, 2005; Tang, *et al.*, 2008). This finding is supported by reports indicating that angiogenesis and adipogenesis are tightly correlated (Cao, 2007). Bone marrow reconstitution studies found that bone marrow progenitor-derived adipocytes and AT progenitor-derived adipocytes both derive from hematopoietic cells via the myeloid lineage (Majka, *et al.*, 2010). These bone marrow progenitor-derived adipocytes accumulated more in women compared to men, and more in VAT compared to SAT and therefore may contribute to WAT depot heterogeneity.

Adipocyte progenitors in human AT have been found to be depot-specific (Tchkonia, *et al.*, 2007) and differences between adipocytes derived from SAT and VAT have been documented (Kovsan, *et al.*, 2009). Functional differences between mesenchymal stem cell populations are reflected by their transcriptome (Jansen, *et al.*, 2010). Adipocyte progenitors may contribute towards regional variation in WAT function and development (Baglioni, *et al.*, 2012). This may in part explain the depot-specific differences in metabolic function and association

with relative risk of developing T2DM (Karastergiou, *et al.*, 2013). With nutritional overload VAT expands predominantly by adipocyte hypertrophy whereas SAT by adipocyte hyperplasia (Joe, *et al.*, 2009). Hyperplasia allows a greater expansion compared to hypertrophy and therefore the difference in expansion method between SAT and WAT may partly explain why central obesity, characterised by expanded VAT and Abd SAT, confers a greater risk of metabolic dysfunction than gluteal and femoral SAT expansion.

1.5.2 Adipogenesis regulation

Adipogenesis describes the transition of fibroblast-like preadipocytes into differentiated, lipid-laden, insulin-responsive adipocytes, highly expressing adipocyte-specific genes such as adiponectin (Ouchi, *et al.*, 2003). Characterisation of adipogenesis regulatory processes have benefited from the discovery of key pathways and transcription factors that contribute to the adipogenic process, as reviewed by Lefterova and Lazar (Lefterova and Lazar, 2009). The transcription factors peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (CEBPs) are considered the crucial determinants of adipocyte fate. Although other factors including extracellular acting wingless-type mouse mammary tumour virus integration site family proteins (Wnts) and intracellular cell-cycle proteins also play roles in adipogenesis. There are several inhibitors of adipogenesis including CEBP homologous protein-10 (CHOP) upregulated in ER stress by the unfolded protein response (UPR) (Hou, *et al.*, 2013), and certain T-cell factor/lymphoid enhancer factor (TCF/LEF) proteins, activated by Wnt signalling (Farmer, 2006). Expression of CHOP in 3T3-L1 adipocytes attenuated adiponectin promoter

activity (Hosogai, *et al.*, 2007), therefore ER stress activation of CHOP during adipogenesis might suppress adipocyte gene expression. Recently the micro RNA (miRNA) miR-540 has been suggested to impair adipogenesis via suppression of PPARγ (Chen, *et al.*, 2015). Adipogenesis is associated with the UPR, and the UPR pathway IRE1α - XBP1s is indispensible for successful murine adipogenesis (Sha, *et al.*, 2009). Thus, ER stress activation of the UPR may disrupt adipogenesis and consequently limit the expansion of WAT by hyperplasia and increase the likelihood of developing insulin resistance. Therefore a network of transcription factors and cell-cycle regulators, in concert with transcriptional coactivators and corepressors, respond to extracellular stimuli to activate or repress adipogenesis.

CEBPα, -β and -δ belong to a family of highly conserved basic-leucine zipper proteins and were among the first transcription factors to be implicated in adipocyte differentiation (Otto and Lane, 2005). CEBPβ is induced early in adipogenesis to transactivate the expression of both CEBPα and PPARγ (Guo, *et al.*, 2015). CEBPα is induced late in adipogenesis and is most highly expressed in adipocytes where it is essential for insulin-dependent glucose uptake (Wu, *et al.*, 1999). CEBPα-knockout mice with liver abnormalities rescued to prevent death fail to accumulate WAT in all depots and have hyperlipidemia and hyperinsulinemia (Linhart, *et al.*, 2001). CEBPα has been shown to bind to several individual genes in adipocytes, however the extent of CEBPα binding to genomic DNA was unknown until the employment of GWAS. GWAS have revealed thousands of CEBPα binding sites, many of which have a considerable degree of colocalization with PPARγ (Lefterova, *et al.*, 2008). Therefore both

CEBPα and PPARγ may be required for the expression of genes at which the two transcription factors colocalize.

PPARγ induction during adipogenesis is both necessary and sufficient and no factor has been identified that can rescue adipogenesis in the absence of PPARγ (Tontonoz and Spiegelman, 2008). PPARγ belongs to the nuclear receptor superfamily of ligand-activated transcription factors (Lehrke and Lazar, 2005). The endogenous PPARγ ligand requires clarification, although lipid metabolites such as polyunsaturated fatty acids (PUFAs) and eicosanoids have been implicated as ligands (Fu, *et al.*, 2005). GWAS have shown PPARγ has approximately 5,300 DNA binding sites, several of which are also bound by retinoid X receptor (RXR), and related to the DR-1 consensus sequence (Nielsen, *et al.*, 2008). This is strengthened by evidence of PPARγ binding as a heterodimer with RXR on DNA at the DR-1 consensus sequence (Chandra, *et al.*, 2008). PPARγ can bind to target genes without a ligand bound. In this state several corepressors bind that collectively recruit histone deacetylases that repress transcription. When a ligand binds PPARγ a conformational change occurs that allows the binding of coactivators such as steroid receptor coactivators (SRCs) (Tontonoz and Spiegelman, 2008). In adipocytes some genes like fatty acid binding protein 2 (aP2) are constitutively associated with PPARγ and coactivators resulting in high transcription levels. However other genes such as glycerol kinase (Gyk) and oxidized LDL-receptor 1 (Olr1) require a ligand to bind to PPARγ to cause the exchange of corepressors for coactivators to activate gene transcription (Guan, *et al.*, 2005). Receptor-interacting protein 140 (RIP140) binds to the PPARγ liganded receptor but represses transcription (White, *et al.*, 2008). RIP140

antagonizes the effects of PPARγ coactivator-1α (PGC-1α), a coactivator that induces mitochondrial biogenesis and may play a role in systemic metabolism (Nautiyal, *et al.*, 2013). UPR is associated with adipogenesis and the UPR activated transcription factor X-box binding protein 1 (XBP1) is a novel key regulator of PPARγ in murine models (Cho, *et al.*, 2014). Elucidating the links between human UPR and adipogenesis will provide insight into the regulation of adipogenesis and may help derive pharmacological targets with clinical benefit for the treatment of obesity and T2DM.

1.5.3 Wnt signalling in adipogenesis

Wnt proteins comprise a family of highly conserved secreted proteins that exert autocrine and paracrine actions via binding to cell-surface receptors (Herr, *et al.*, 2012). Wnt ligands bind to the frizzled (FZD) and low-density-lipoprotein-related protein 5/6 (LRP5/6) coreceptor complex to activate the canonical Wnt signalling pathway (figure 1.5.3). The activated receptor complex disrupts or functionally inactivates a β-catenin destruction complex. The destruction complex consists of the scaffold protein Axin, adenomatous polyposis coli (APC), glycogen synthase kinase-3 (GSK-3) and casein kinase-1 (CKI). When Wnt is not bound to its surface receptors, the destruction complex is active and regulates the phosphorylation, ubiquitination and subsequent degradation of β-catenin. When Wnt is bound to its surface receptors, the destruction complex is inhibited, β-catenin accumulates in the cytoplasm and translocates to the nucleus where it engages the TCF/LEF transcription factors to activate the Wnt transcriptional program (Li, *et al.*, 2012). Several TCF/LEF targets are linked to cell differentiation. For example TCF7 has been shown to regulate a self-

renewal/differentiation switch that operates in the absence of Wnt signalling (Wu, *et al.*, 2012). Negative feedback regulation of the Wnt pathway is thought to be mediated through conductin, encoded by the *AXIN2* gene and also known as axin2 (Bernkopf, *et al.*, 2015).

Figure 1.5.3 Wnt signalling pathway

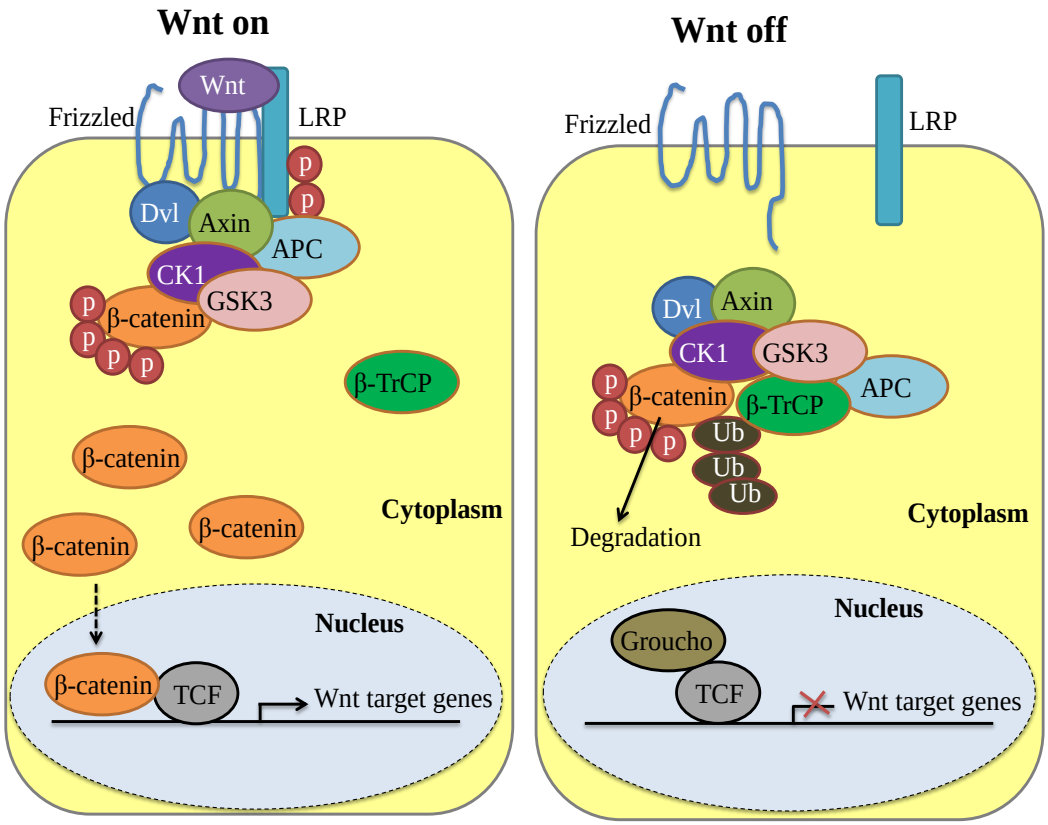


Figure 1.5.3 legend: Wnt signalling pathway. Wnt proteins are secreted and exert autocrine and paracrine actions via binding to cell-surface receptors and result in target cell β-catenin translocating to the nucleus to activate Wnt target genes. (Wnt; Wingless-type mouse mammary tumour virus integration site family protein, LRP; low-density-lipoprotein-related protein, Dvl; dishevelled, APC; adenomatous polyposis coli, GSK-3; glycogen synthase kinase-3, CKI; casein

kinase-1, β-TrCP; beta-transducin repeat containing E3 ubiquitin protein ligase, p; phosphorylated, Ub; ubiquitin, TCF; T-cell factor.)

The Wnt and β-catenin canonical pathway is well established in morphogenesis of bone, intestine and the hematopoietic system (Clevers and Nusse, 2012). The first report linking Wnt signalling to adipogenesis demonstrated that activation of the Wnt pathway impairs adipogenesis in 3T3-L1 preadipocytes (mouse derived preadipocytes with the capacity to undergo adipogenesis into adipocytes (Green and Kehinde, 1975)) (Ross, *et al.*, 2000). In a subsequent investigation, β-catenin was selectively deleted in uterine mesenchyme during early embriogenesis, which led to the replacement of uterine smooth muscle with adipocytes. The adipocytes were found to originate from the same precursors as smooth muscle cells, indicating Wnt proteins influence adipocyte cell fate *in vivo* (Arango, et al., 2005). It has been hypothesised that reciprocal repression might exist between Wnt signalling and CEBPα or PPARγ because activation of these factors can lead to a substantial reduction in β-catenin levels (Moldes, *et al.*, 2003). More recently it has been suggested that Wnt normally inhibits CEBPα as a negative feedback loop to reduce adipogenesis (Christodoulides, *et al.*, 2009).

Wnt10b has been extensively studied in an adipogenesis context (Rosen and MacDougald, 2006). Wnt10b has been shown to impair WAT and BAT formation *in vivo* when overexpressed specifically in mouse adipocytes (Longo, *et al.*, 2004). Furthermore, increasing Wnt10b levels in WAT leads to decreased susceptibility to obesity in both diet-induced (Longo, *et al.*, 2004) and genetic mouse models (Wright, *et al.*, 2007). Wnt10b also seems to play a key role in

human adipogenesis. Naturally occurring mutations in WNT10B gene are associated with early-onset obesity (Christodoulides, *et al.*, 2006). Furthermore, Wnt10b has been shown to impair human preadipocyte differentiation (Isakson, *et al.*, 2009). And abnormal expression of genes involved in Wnt signalling have been recorded in the adipose tissue of PCOS patients (Chazenbalk, *et al.*, 2012). Wnt10b may link the UPR with adipogenesis. XBP1s enhances adipogenic differentiation in murine cells through downregulation of Wnt10b (Cho, *et al.*, 2013). Clarifying the links between human UPR and adipogenesis will provide insight into the regulation of adipogenesis and may generate pharmacological targets with clinical benefit for the treatment of obesity and T2DM.

1.6 Adipocyte endoplasmic reticulum stress

WAT is comprised of adipocytes integrated in a connective tissue matrix with highly regulated vasculature and innervation. A principle function of WAT is to appropriately store and release energy. The endoplasmic reticulum is vital for adipocyte energy transfer coordination. To store energy, adipocytes synthesise TAG and then partition them into LDs, both processes take place at the ER membrane and require ER resident enzymes. To utilise energy, adipocytes depend on ER and mitochondrial coordination for suitable function. Adipocytes also regulate cellular function in distant tissues and organs including muscle, liver and the brain. This regulation is predominantly through endocrine mediated mechanisms that require functional ER for the synthesis, processing, and secretion of adipokines. Impairments in energy regulation and secretory function of WAT adipocytes in obesity are associated with the development of insulin resistance and T2DM. Although the cellular mechanisms leading to these impairments are

complex, accumulating evidence suggests dysregulated ER caused by ER stress may play a role.

1.6.1 Endoplasmic reticulum stress and the unfolded protein response

Virtually all secretory and membrane proteins are assembled into their secondary and tertiary structures within the extensive network of membranes that comprise the ER. This specialised organelle allows correct folding, maturation, storage and transport of these proteins. Misfolded or unfolded proteins are detected, removed from the ER and degraded by the 26S proteasome system (Marciniak and Ron, 2006; Schroder and Kaufman, 2005). The ER can experience perturbations and become stressed in a number of situations including the accumulation of unfolded proteins, nutrient and energy fluctuations, viral infections, hypoxia, toxins and increased demand on the synthetic machinery. When these conditions arise the ER activates a complex response system known as the unfolded protein response (UPR) (figure 1.6.1) to restore the functional integrity of the organelle (Marciniak and Ron, 2006; Schroder and Kaufman, 2005).

In mammals the UPR is comprised of three main branches, signalling through which is mediated by inositol-requiring enzyme 1α (IRE1α), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) (Harding, *et al.*, 2000b; Harding, *et al.*, 1999). In unstressed ER these three transmembrane proteins are bound by a chaperone, Bip/GRP78, in their intralumenal domains and rendered inactive (Bertolotti, *et al.*, 2000; Shen, *et al.*, 2002). Accumulation of improperly folded proteins and increased protein cargo in the ER results in the recruitment of Bip away from these UPR sensors. This and

potentially other yet to be discovered lumenal events result in oligomerization and activation of IRE1α and PERK that engage a complex signalling cascade (Ron and Walter, 2007). PERK is activated by homodimerization and transphosphorylation, which allows it to phosphorylate the α-subunit of the translation initiation factor eIF2 (p-eIF2α) and subsequently attenuate translation initiation. Paradoxically, p-eIF2α leads to selective translation of mRNAs containing open reading frames, such as activating transcription factor-4 (ATF4) (Schroder and Kaufman, 2005). ATF4 then upregulates the expression of several genes including DNA-damage-inducible transcript 3 (*DDIT3*) that encodes the effector protein CHOP (Woo, *et al.*, 2009). The third branch of the UPR requires translocation of ATF6 to the Golgi apparatus where it is processed by the serine protease site-1 protease (S1P) and the metalloprotease site-2 protease (S2P) to produce an active transcription factor (Chen, *et al.*, 2002). In response to ER stress ATF6 is reduced and only the reduced monomeric ATF6 can reach the Golgi apparatus (Nadanaka, *et al.*, 2007). In combination these three arms mitigate ER stress by reducing protein synthesis, facilitating protein degredation, and increasing production of chaperones that help proteins in the ER lumen to fold. Consequently ER stress is alleviated. If ER stress is not resolved the cell is functionally compromised and may undergo UPR mediated apoptosis.

Conserved from yeast to humans, IRE1α is a stress regulated kinase and ribonuclease that heads one of the UPR branches (Calfon, *et al.*, 2002). The endoribonuclease activity of IRE1α cleaves a 26 base-pair intron between nucleotides 531 – 556 from the mRNA of the unspliced X-box binding protein-1 (XBP1u), creating an alternative mRNA that is translated into the active (spliced)

form of the transcription factor (XBP1s) (Sidrauski and Walter, 1997; Uemura, *et al.*, 2009). XBP1s, alone or in conjunction with ATF6, can initiate transcription of chaperones like Bip, and proteins involved in phospholipid synthesis, ER biogenesis, ER-associated protein degradation (ERAD), and secretion. Therefore XBP1s plays a major role in alleviating ER stress. The adipocyte plays a central role in energy homeostasis and in the obese state hypertrophic adipocyte is challenged by several insults including inflammation, insulin resistance, surplus energy and considerable stress to various organelles. The ER is a vital organelle that demonstrates significant signs of stress and dysfunction in obesity and insulin resistance.

The ER within a functional adipocyte in a metabolically healthy organism must function under unique and testing conditions; adapting to meet the demands of energy storage by forming TAG droplets, increased protein synthesis and secretion and nutrient sensing. The ER can become overwhelmed when nutrient levels are in pathological excess in which case the UPR is activated (Ozcan, *et al.*, 2004).

Figure 1.6.1 Mammalian unfolded protein response pathways

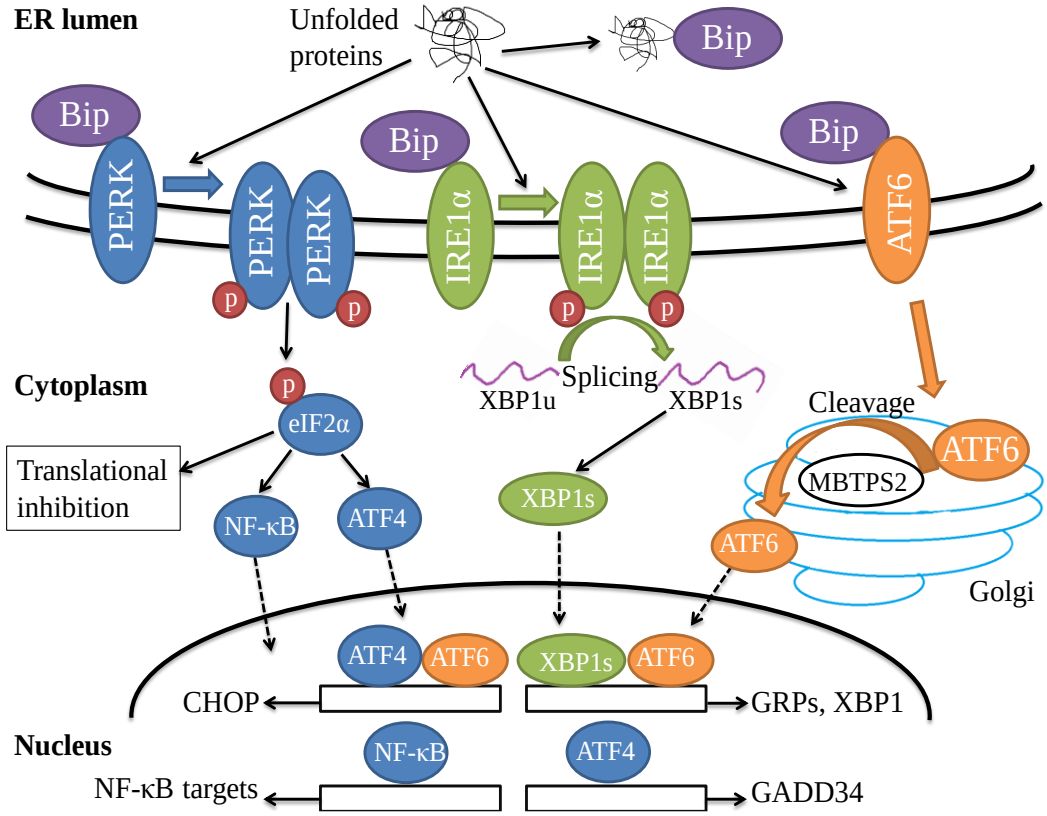


Figure 1.6.1 legend: Mammalian unfolded protein response pathways. Unfolded protein response signalling pathways originate in the ER lumen and transfer to the cytoplasm and nucleus to engage appropriate responses. (ER; endoplasmic reticulum, Bip; binding immunoglobulin protein – also known as GRP78; glucose regulated protein 78 kDa, PERK; protein kinase RNA-like endoplasmic reticulum kinase, p; phosphorylated, eIF2α; eukaryotic translation initiation factor 2 A, NF-κB; nuclear factor kappa-light-chain-enhancer of activated B cells, ATF4; activating transcription factor 4, CHOP; C/EBP homologous protein, GADD34; growth arrest and DNA damage-inducible protein, IRE1α; inositol requiring enzyme 1 alpha, XBP1u; X-box binding protein 1 unspliced, XBP1s; X-box

binding protein 1 spliced, GRPs; glucose regulated proteins, ATF6; activating transcription factor 6, MBTPS2; membrane-bound transcription factor peptidase site 2.)

1.6.2 Adipose tissue endoplasmic reticulum stress

Genetically inherited disorders of protein trafficking are almost exclusively attributed to mutations in the coding sequence of exportable proteins and result in retention of the affected proteins or subunits in the ER (Kim and Arvan, 1998). These diseases have revealed the importance of the ER in protein processing. Mouse models with specific genetic alterations to genes encoding ER chaperones have shown that resident ER chaperones are critical for quality control of proteins and for regulating ER signalling in response to ER stress (Ni and Lee, 2007). Growing evidence suggests that diseases involving the ER are not restricted to genetic disorders and can be acquired in obesity. SAT from the upper thigh in six obese insulin-resistant human subjects had significantly higher ER stress gene and protein expression compared to six lean insulin-sensitive subjects (Boden, *et al.*, 2008). In a different investigation WAT was collected from 78 healthy non-diabetic human subjects with a range of BMIs and UPR gene expression positively correlated with increasing BMI (Sharma, *et al.*, 2008). In a third study SAT ER stress expression reduced one year after undergoing gastric bypass surgery in 11 non-diabetic obese subjects with preoperative mean BMI of 51.3 ± 3.0 kg/m^2 and postoperative mean BMI of 31.4 ± 2.3 kg/m^2 (Gregor, *et al.*, 2009). Weight loss through calorie restriction in *ob/ob* mice also reduced ER stress expression in SAT (Tsutsumi, *et al.*, 2011).

1.6.3 Obesity induces adipocyte endoplasmic reticulum stress

Several obesity-related perturbations, including hyperglycaemia, endotoxemia, elevated circulating FFAs, overnutrition, increased ER demand and local tissue hypoxia may serve as initiating factors for ER stress in WAT (Gregor and Hotamisligil, 2007; Hotamisligil, 2010). This postulation is supported by murine *in vivo* and *in vitro* experimental models, for example in WAT of mice fed a high-fat diet for 16 weeks, indicators of ER stress such as PERK phosphorylation and c-Jun N-terminal kinase (JNK) activity are significantly increased compared with mice fed with a normal diet (Ozcan, *et al.*, 2004). Furthermore, WAT in *ob/ob* mice displayed signs of ER stress, including increased levels of phosphorylated PERK and IRE1α, compared to wild type mice. This was accompanied by an increase in JNK activity and XBP1 splicing (Ozcan, *et al.*, 2006). WAT of obese mice was characterised by hypoxia and ER stress and in an *in vitro* model hypoxia increased markers of ER stress in 3T3-L1 adipocytes (Hosogai, *et al.*, 2007). Thus, dysregulated WAT expansion in obesity may not be served by adequate vasculature and therefore the diffusion limit of oxygen may be exceeded, resulting in local hypoxia (Yilmaz and Hotamisligil, 2013). Mice with a heterozygous deletion of UPR chaperone Bip had an activation of UPR factors including CHOP and XBP1s and were protected against diet-induced obesity, hepatic steatosis and insulin resistance (Ye, *et al.*, 2010). A mixture of FFAs increased markers of ER stress in 3T3-L1 adipocytes (Jiao, *et al.*, 2011). Lipid perturbation-mediated UPR activation in mammalian cells revealed the ER lipid bilayer can directly modulate UPR signalling independently of changes to protein folding homeostasis in the ER lumen (Volmer, *et al.*, 2013).

Human adipocyte experimental models support murine investigations and indicate that obesity associated factors induce ER stress. High fat intake leads to acute postprandial exposure to circulating lipopolysaccharides in T2DM subjects (Harte, *et al*., 2012b). Obesity associated factors including LPS (100 mg/mL), high glucose (25 mM) and saturated FAs (2 mM) increased markers of ER stress in primary human adipocytes (Alhusaini, *et al*., 2010). Adipocytes increase the rate of secretion of several proteins in response to adipocyte expansion such as leptin, resistin and TNFα (Galic, *et al.*, 2010). Thus, the increased demand on the ER to process these secreted proteins may lead to accumulation of unfolded proteins and activation of the UPR. Therefore many physiological responses to obesity can influence adipocyte ER homeostasis and elicit activation of the UPR.

1.7 Endoplasmic reticulum stress induced insulin resistance in adipose tissue

The diversity of ER stress induced UPR signalling likely generates suitable outcomes that are specific to the stress imposed and the homeostatic needs of the cell. These outcomes include adaptation to restore ER functional integrity, alarm to induce an inflammatory response, and apoptosis if there is a failure to resolve ER stress (Kim, *et al*., 2008). Whilst these UPR outcomes may have evolved in nutrient scarce environments to improve cellular and tissue function, in obesity-linked nutrient excess the UPR is chronically activated and metabolic dysfunction via insulin resistance and subsequent T2DM can arise (Kammoun, *et al.*, 2014). Evidence suggests that ER stress is linked to insulin resistance (Flamment, *et al.*, 2012). For example, deletion of a key UPR transcription factor XBP1, results in the development of insulin resistance in mice (Ozcan, *et al.*, 2004). Further

experimental models have indicated that ER stress is essential to the initiation and integration of inflammation and insulin action in obesity and type 2 diabetes (Wellen and Hotamisligil, 2005). The consequences of UPR activation in WAT have been causally linked to the development of insulin resistance through several mechanisms including JNK activation, inflammation and oxidative stress (Hotamisligil, 2006; Samuel and Shulman, 2012). Hotamisligil has postulated that insulin is a crucial anabolic hormone and therefore transient insulin resistance in adipocytes may help the ER cope by downregulating metabolic synthetic pathways (Hotamisligil, 2010). This may be accurate and explain why chronic upregulation of the UPR in obesity is associated with insulin resistance and T2DM.

1.7.1 IRE1α activation of JNK and NFκB

ER stress master regulator IRE1α induces insulin resistance via JNK and activates inflammation via JNK and NF-κB. ER stress induced in rat pancreatic acinar AR52J cells by treatment with thapsigargin (Tg) (1 μM) that depletes the ER of luminal calcium stores, tunicamycin (TM) (2.5 μg/mL) that blocks ER protein glycosylation, and dithiothreitol (DTT) (10 mM) which interferes with disulfide bond formation, all exhibited increased JNK activity (Urano, *et al.*, 2000). The cytoplasmic part of UPR master regulator IRE1α was shown to activate JNK via TNF receptor associated factor 2 (TRAF2) (Urano, *et al.*, 2000). JNK promotes insulin resistance during association with insulin receptor substrate-1 and inhibitory phosphorylating key serine 307 (Aguirre, *et al.*, 2000), and induces the expression of inflammatory genes by phosphorylating the transcription factor activation protein 1 (AP-1) (Davis, 2000). JNK activity is abnormally high in

obesity and furthermore, an absence of JNK1 results in decreased adiposity, improved insulin sensitivity and enhanced insulin receptor signalling capacity in two models of mouse obesity (Hirosumi, *et al.*, 2002). The IRE1α-TRAF2 complex can recruit IκB kinase (IKK), which phosphorylates IκB, leading to the degradation of IκB and the nuclear translocation of NF-κB (Hu, *et al.*, 2006). NF-κB is a potent activator of inflammation in human adipose tissue (Harte, *et al.*, 2013). JNK and NF-κB influence visfatin expression in primary human SAT, therefore these factors can influence adipocyte hormone secretion and may have systemic consequences (McGee, *et al.*, 2011). Therefore obesity-induced ER stress forms IRE1α-TRAF2 complexes, which activate JNK and NF-κB leading to insulin resistance and inflammation (figure 1.7.1).

Figure 1.7.1 IRE1α activation of XBP1s and JNK

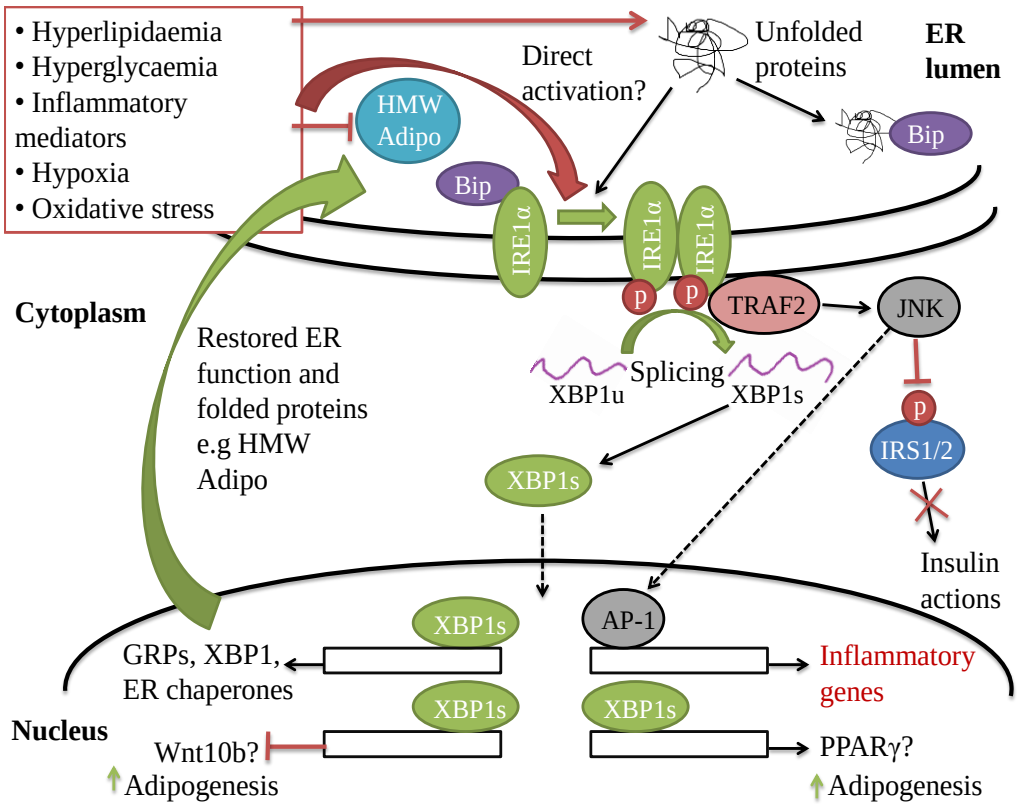


Figure 1.7.1 legend: IRE1α activation of XBP1s and JNK. IRE1α is activated by several factors (red box, top left), and splices XBP1u mRNA to XBP1s mRNA which is translated and the resulting protein transcription factor upregulates genes encoding proteins to help restore ER functional integrity. Simultaneously, IRE1α binds via its cytoplasmic domain to TRAF2 which activates JNK which inhibitory phosphorylates IRS1/2 thereby blocking insulin action. JNK also activates AP-1 to promote transcription of inflammatory genes. (HMW Adipo; high molecular weight adiponectin, ER; endoplasmic reticulum, Bip; binding immunoglobulin protein – also known as GRP78; glucose regulated protein 78 kDa, p; phosphorylated, IRE1α; inositol requiring enzyme 1 alpha, XBP1u; X-box binding protein 1 unspliced, XBP1s; X-box binding protein 1 spliced, TRAF2; TNF receptor associated factor 2, JNK; c-Jun N-terminal kinase, IRS1/2; insulin receptor substrate 1/2, AP-1 activation protein 1, GRPs; glucose regulated proteins, Wnt10b; Wingless-type mouse mammary tumour virus integration site family protein 10b, PPARγ; peroxisome proliferator-activated receptor gamma.)

1.7.2 ER stress induced inflammation and subsequent insulin resistance

The chronic nature of obesity produces tonic low-grade ER stress, inflammation and activation of the innate immune system in WAT (Lumeng and Saltiel, 2011). This physiological response to cell stress may have arisen due to the integrated immune role of WAT as a homeostatic mechanism to restore WAT function (Osborn and Olefsky, 2012). The adipocyte ER can be considered as an interface between the immune system and metabolism, both of which are incorporated in WAT (Unanue and Urano, 2014). The increased ER stress in obesity plays a key role in the inflammatory basis of metabolic disease, elegantly reviewed by

Hotamisligil (Hotamisligil, 2010). Inflammation is an immune response that serves to bolster the local tissue defence by attracting immune cells to prevent or engage with pathogens thereby protecting the body. Inflammation is predominantly controlled and directed by soluble mediators such as cytokines that are released by many cell types including adipocytes. Proinflammatory cytokines secreted by adipocytes such as TNFα and IL-6 are critically important to attract inflammatory leukocytes to WAT (Fantuzzi, 2005). However the secretion of these cytokines is increased in obesity-linked ER stress and they interfere with insulin action and glucose metabolism (Kammoun, *et al.*, 2014).

Obesity-induced ER stress in adipocytes activates inflammation via several mechanisms (Zhang and Kaufman, 2008; Zhang, *et al.*, 2006). IRE1α-TRAF2 complexes activate JNK and NF-κB leading to inflammation (section 1.7.1 above). IRE1α has also been linked to the activation of p38 mitogen-activated protein kinase and extracellular-regulated kinase (Hetz and Glimcher, 2009; Hu, *et al.*, 2006; Nguyen, *et al.*, 2004). These interactions suggest that IRE1α can activate diverse signalling pathways involved in activating inflammation. The PERK branch of the UPR can also activate inflammation. Saturated FAs activate ER stress and induce the PERK-eIF2α-ATF4 UPR pathway that plays a critical role in IL-6 production in macrophages, and the same mechanism may exist in adipocytes (Iwasaki, *et al.*, 2014). PERK also activates NFKβ mediated inflammation via reduction in the abundance of its inhibitor IKβ (Wu, *et al.*, 2004). Protein folding within the ER is an energy-consuming process and oxidising conditions are requires for the formation of disulphide bonds (Tu and Weissman, 2004). Molecular oxygen is used as the terminal electron recipient to

provide a driving force for disulphide bond formation, however this leads to the production of reactive oxygen species (ROS), exasperated with increased burden on ER in obesity (Tu and Weissman, 2002). ROS are small molecules that are highly reactive as the result of the presence of unpaired electrons and induce inflammation (Raha and Robinson, 2000). Therefore obesity-induced ER stress in adipocytes can initiate secretion of inflammatory cytokines resulting in WAT inflammation.

Obesity-induced inflammatory response signalling mediators including FFAs, cytokines, adipokines and lipokines interfere with insulin action. Adipocyte ER stress may be an important initiator of WAT inflammation, however the release of proinflammatory mediators from WAT in obesity is primarily due to immune cells (Fain, 2006). The proinflammatory cytokine TNFα is produced by adipocytes, is highly expressed in adipose tissue and is associated with obesity (Hotamisligil, *et al.*, 1993). In rodent cells TNFα elevated serine/threonine phosphorylation of IRS-1 and IRS-2 and inhibited their binding to the insulin receptor and impaired their ability to undergo insulin-induced tyrosine phosphorylation (Paz, *et al.*, 1997). Mice lacking TNFα or TNF receptors had improved insulin sensitivity in dietary and genetic (*ob/ob*) models of obesity (Uysal, *et al.*, 1997). TNFα has been shown to inhibit PPARγ in several experimental systems (Ye, 2008). Therefore TNFα may prevent adipogenesis and contribute towards WAT dysfunction. Obese human subjects demonstrated increased TNFα expression in WAT compared to non-obese, and following weight loss the cytokine expression reduced (Kern, *et al.*, 1995). Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both

in vivo and in vitro (Bastard, *et al.*, 2002). Furthermore, serum levels of IL-6 decrease in parallel with weight loss and improvement of insulin resistance in patients undergoing bariatric surgery (Kopp, *et al.*, 2003). Despite these findings, IL-6 has been associated with the resolution of inflammation in certain contexts and has versatile functions in metabolism (Mauer, *et al.*, 2015). Nonetheless, IL-6 expression is increased in obesity and may play a role in the development of insulin resistance and T2DM. Therefore WAT derived signalling molecules upregulated in response to adipocyte ER stress can interfere with insulin action and may contribute towards developing T2DM (figure 1.7.2).

Figure 1.7.2 Unfolded protein response, inflammation and insulin resistance

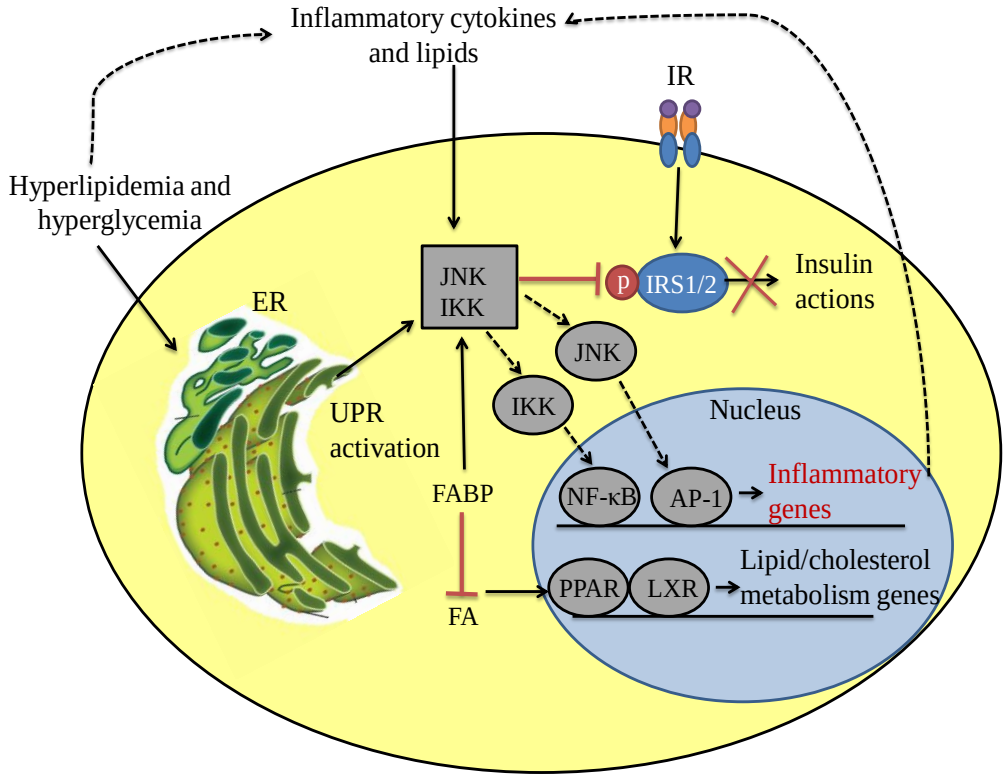


Figure 1.7.2 legend: Unfolded protein response, inflammation and insulin resistance. Adipose tissue derived signalling molecules upregulated in response to adipocyte ER stress can interfere with insulin action and may contribute towards

developing T2DM. (ER; endoplasmic reticulum, IR; insulin receptor, p; phosphorylated, JNK; c-Jun N-terminal kinase, IKK; I kappa B kinase, NF-κB; nuclear factor kappa-light-chain-enhancer of activated B cells, IRS1/2; insulin receptor substrate 1/2, AP-1 activation protein 1, PPAR; peroxisome proliferator-activated receptor, LXR; liver X receptor, FABP; fatty acid-binding protein, FA; fatty acid.)

1.7.3 ER stress integration with metabolism

Adipocytes play a key role in systemic metabolism and require functional ER to appropriately orchestrate metabolic processes (Unanue and Urano, 2014). Accumulating evidence suggests an integrated role between the UPR and glucose, lipid and protein metabolism in adipocytes. For example; ER stress regulates branched chain amino acid uptake and metabolism in adipocytes (Burrill, *et al*., 2015), the activity of key UPR chaperone Bip, also known as glucose-regulated protein 78 (Grp78), responds to metabolic stress (Lee, 2014), and asymmetrical dimethylarginine triggers lipolysis and inflammatory response via induction of ER stress in cultured adipocytes (Zhou, *et al*., 2009). ATF6 and XBP1s have been linked to lipid biosynthesis and ER membrane expansion via mechanisms that are partially distinct (Bommiasamy, *et al.*, 2009; Sriburi, *et al.*, 2004). ATF4 has been suggested to play a role in carbohydrate metabolism (Seo, *et al.*, 2009). Therefore obesity-induced chronic ER stress may dysregulate adipocyte metabolic processes and contribute to insulin resistance and T2DM (figure 1.7.3).

Models of UPR deficiency have shown that the UPR plays an important role in metabolism. An investigation into the regulation of eIF2α phosphorylation in the

liver revealed complex mechanisms that may be linked to liver glucose metabolism during chronic ER stress (Oyadomari, *et al*., 2008). A further study in liver hepatocytes found lipid metabolism is affected by protein misfolding and ER stress (Wang and Kaufman, 2014). PERK-deficient mice and animals with a homozygous mutation that eliminates eIF2α phosphorylation on serine 51 resulted in defective liver gluconeogenesis (Harding, *et al*., 2000a; Scheuner, *et al*., 2001). Protein kinase RNA-activated (PKR) responds to nutrient signals and ER stress and coordinates the activity of other critical inflammatory kinases such as JNK to regulate insulin action and metabolism. PKR also directly targets and modifies insulin receptor substrate and therefore integrates nutrients and insulin action (Nakamura, *et al*., 2010). The role of ER stress in glucose metabolism in other tissues and cells such as adipocytes remains unclear and Hotamisligil expressed 'future work should determine whether ER stress also affects glucose metabolism in other tissues' (Hotamisligil, 2010).

Adiponectin links adipocyte ER stress and the UPR to systemic insulin sensitivity. Adiponectin is almost exclusively produced by adipocytes (Ouchi, *et al*., 2003). An inverse correlation between adiponectin and insulin resistance has been established both in animals and humans (Turer and Scherer, 2012). Adiponectin improves insulin sensitivity through various mechanisms involving the liver and muscle (Kadowaki and Yamauchi, 2005; Turer and Scherer, 2012). HMW adiponectin is thought to confer insulin sensitising effects (Liu and Liu, 2014). Functional ER is essential for the synthesis, processing and secretion of adiponectin. ER resident proteins ER resident protein-44 (ERp44), ER oxidoreductin-1 (Ero1-1α) and disulphide bond A oxidoreductase-like protein

(DsbA-L) play important roles in adiponectin processing (Liu, *et al*., 2008; Qiang, *et al*., 2007; Wang, *et al*., 2007). ER stress has been implicated in diet-induced adiponectin downregulation (Liu and Liu, 2014). The treatment of genetically and diet induced mouse models of obesity with Tauroursodeoxycholic acid (TUDCA), a chemical chaperone that alleviates ER stress, resulted in increased cellular and serum levels of adiponectin (Zhou, *et al*., 2010). This observation is supported by another study in *ob/ob* mice where overexpression of UPR transcription factor XBP1s induced adiponectin multimerisation and improved glucose homeostasis (Sha, *et al*., 2014). Therefore, obesity-induced chronic ER dysfunction reduces adiponectin levels and may contribute towards decreased systemic insulin sensitivity and subsequent development of T2DM.

Figure 1.7.3 Balance between nutritional status, unfolded protein response activation and adipose tissue function

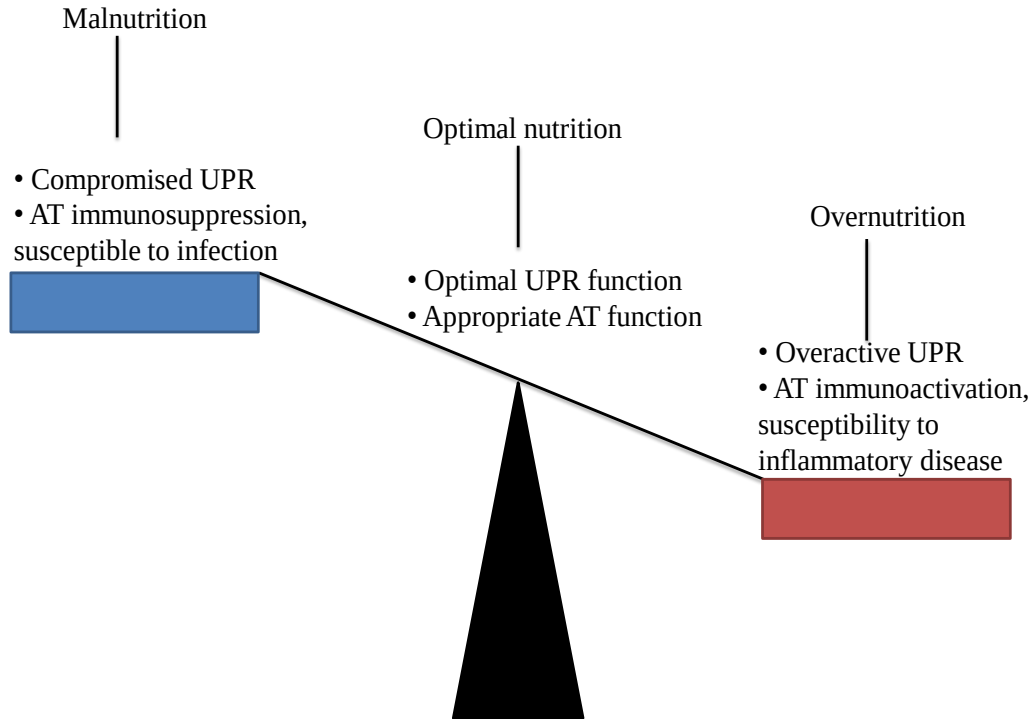


Figure 1.7.3 legend: Balance between nutritional status, unfolded protein response activation and adipose tissue function. A balance exists between the nutritional status and activation of the unfolded protein response (UPR) and influences adipose tissue (AT) function.

1.7.4 ER stress in adipogenesis

Obesity complications in humans result from the inability of SAT to appropriately expand and store lipids, and consequently ectopic fat deposition and lipotoxicity contribute to insulin resistance (Samuel and Shulman, 2012; Virtue and Vidal-Puig, 2010). Impaired WAT expandability is mainly due to defects in adipogenesis (Isakson, *et al.*, 2009). Forced expansion of WAT in *ob/ob* mice via increased adipogenesis normalized glucose and insulin levels and prevented insulin resistance despite massive obesity (Kim, *et al*., 2007). Adipogenesis is continually required throughout life since about 10 % of adipocytes in the body are regenerated annually (Spalding, *et al.*, 2008). Therefore adipogenesis is an important process to maintain WAT function. The UPR plays an essential role in adipogenesis. The UPR pathway IRE1α - XBP1s is indispensible for successful murine adipogenesis (Sha, *et al.*, 2009). Since this discovery, Cho *et al.* have explored the role of XBP1s in adipogenesis further. They revealed that XBP1s enhances adipogenesis in 3T3-L1 cells through the downregulation of Wnt10b (Cho, *et al.*, 2013). And XBP1s is a key regulator of adipogenic modulator PPARγ in 3T3-L1 cells (Cho, *et al.*, 2014). XBP1s has been shown to induce adiponectin multimerisation (Sha, *et al.*, 2014) and may activate this process during adipogenesis since adiponectin expression is almost exclusively from

adipocytes (Ouchi, *et al.*, 2003). Thus, obesity induced ER stress may disrupt adipogenesis and consequently limit the expansion of WAT and increase the likelihood of developing insulin resistance and T2DM.

1.8 Translation to the clinic

There are currently 14 classes of drugs available to treat T2DM in the USA, however only 36 % of patients with T2DM achieve glycaemic control with the available therapies (Miller, *et al.*, 2014). New treatment options may improve the rate of glycaemic control. Duration of T2DM is independently associated with the risk of macrovascular events, microvascular events and death (Zoungas, *et al.*, 2014). Therefore early intervention to restore glucose homeostasis will provide the greatest benefit. Since obesity is the biggest risk factor for T2DM and obesity-induced ER stress may be an initiating factor in development of insulin resistance and TD2M, preventing or treating obesity would reduce the burden of T2DM. Weight loss through diet modifications (Gregor, *et al.*, 2009; Seoane-Collazo, *et al.*, 2014) and through gastric bypass bariatric surgery in humans (Gregor, *et al.*, 2009) can reverse ER stress in adipose tissue and improve glucose homeostasis. Bariatric surgery has emerged as the most durably effective treatment of T2DM, however the mechanisms governing improvement in glucose homeostasis have not been fully elucidated (Nguyen and Korner, 2014). Some bariatric procedures improve glycaemic control in people with T2DM beyond that expected for weight loss alone (Dixon, *et al.*, 2012). The improved glycaemic control in such people may be due to improved adipocyte ER regulation and subsequent improved metabolic regulation. Targeting the UPR in disease has recently been reviewed by Hetz *et al.* (Hetz, *et al.*, 2013). Given that the adipocyte ER plays important roles

in systemic metabolism and energy homeostasis and that obesity-induced ER stress leads to insulin resistance, mechanisms for relieving ER stress and improving adipocyte ER regulation may improve metabolic regulation and reduce the impact of metabolic diseases such as T2DM.

ER stress may be alleviated through the action of metabolic hormones such as GLP-1. GLP-1 receptor (GLP-1R) agonist exendin-4 attenuated translational downregulation of insulin and improved cell survival in purified rat pancreatic β-cells following the induction of ER stress *in vitro* (Yusta, *et al.*, 2006). Furthermore, hyperglycaemia induced ER stress in human umbilical vein endothelial cells (HUVECs) was prevented by treatment with the GLP-1 analogue Liraglutide with maximum activity at 100 nM (Schisano, *et al.*, 2012). Investigations into the effects of GLP-1 analogues on adipocyte ER stress will provide valuable insight into metabolic regulation and warrant investigation. Hotamisligil suggested that 'a reciprocal relationship may exist wherein some hormones, which depend upon the ER for their translation, processing, and secretion, may act on target cells in ways that protect the biological function of that cell thus ensuring continued hormone action, thereby conferring a self-preserving activity' (Hotamisligil, 2010). GLP-1 may be one such hormone and there may be undiscovered hormones that have self-preserving and ER regulating mechanisms.

Studies have shown that chemicals can reduce ER stress and improve glucose regulation. Salicylate is an IκB kinase-β (IKKβ) inhibitor (Yuan, *et al.*, 2001) and it alleviated ER stress in human adipocytes (Alhusaini, *et al.*, 2010) and improved glucose regulation in T2DM subjects (Goldfine, *et al.*, 2010). In addition to kinase

inhibitors, salubrinal, a phosphatase inhibitor, might have therapeutic benefits. Salubrinal can protect cells against ER stress induced apoptosis by selectively inhibiting the dephosphorylation of eIF2α such that further protein synthesis and accumulation in the ER is inhibited (Boyce, *et al.*, 2005). Treatment with salubrinal leads to protection against ER stress induced cell death *in vitro* and *in vivo* (Sokka, *et al.*, 2007). Therefore modulating phosphorylation signalling in the context of the UPR may provide ER homeostatic benefits.

Chemical chaperones are small molecules that facilitate protein folding and can protect against ER stress. TUDCA and 4-phenylbutric acid are chemical chaperones, and both reduced phosphorylation of PERK and IRE1α and improved glucose tolerance and insulin sensitivity in insulin resistant obese mice (Ozcan, *et al.*, 2006). TUDCA also alleviated ER stress and improved glucose homeostasis in humans (Kars, *et al.*, 2010). The treatment of genetically and diet induced mouse models of obesity with TUDCA resulted in increased cellular and serum levels of adiponectin (Zhou, *et al.*, 2010). In addition, another chemical chaperone, the resveratrol tetramer vaticanol B, has been shown to inhibit both the UPR and the inflammatory response by reducing the protein folding load and maintaining ER membrane integrity, preventing ER stress induced apoptosis (Tabata, *et al.*, 2007). Therefore assisting protein folding in the ER with chemical chaperones may be a potential method for alleviating ER stress.

A recently identified inhibitor of the IRE1α-XBP1s UPR pathway may provide a suitable agent for modulating ER stress. 4-methyl umbelliferone 8-cabaldehyde (4μ8C) is a selective inhibitor of unconventional mRNA splicing by IRE1α

(Cross, *et al.*, 2012). 4μ8C forms a Schiff base with lysine 907 in the IRE1α endonuclease domain and blocks substrate access to the active site and selectively inactivates XBP1u mRNA splicing. Inhibition of IRE1α endonuclease activity does not sensitize cells to the consequences of acute ER stress, but rather interferes with the expansion of excretory capacity (Cross, *et al.*, 2012). 4μ8C has been used as a pharmacological agent; it attenuated joint inflammation in mice and was subsequently suggested as a potential therapeutic intervention for inflammatory arthritis (Qiu, *et al.*, 2013). In a cell culture model of ER stress, inhibiting IRE1α with 4μ8C blunted UPR gene expression output but did not affect ERAD or sensitize cells to apoptosis (Zhang, *et al.*, 2014a). Therefore, selectively inhibiting IRE1α with 4μ8C may blunt the expression of secretory facilitating genes and consequently reduce cell secretory output. In the context of insulin resistance, administering 4μ8C may have beneficial outcomes by reducing the secretion of proinflammatory mediators. However, detrimental consequences may arise since the secretion of beneficial adipokines such as adiponectin and leptin may be reduced and proteins intended for secretion may accumulate in the ER, further aggravating ER stress. Additionally, UPR gene output will be impaired thus potentially limiting the ability of cells to adapt to conditions of ER stress. 4μ8C as a potential pharmacological agent for T2DM requires further investigation, although it has been proposed as a potential therapy (Hetz, *et al.*, 2013). Nonetheless, 4μ8C is a useful tool for inhibiting IRE1α and can be used to investigate the UPR in model systems and may provide insight into the initiation of insulin resistance and subsequent T2DM.

1.9 Summary

People with T2DM suffer reduced quality and length of life. The chronic metabolic disease presents a huge burden on global society through medical, social and financial implications. Obesity, characterised by WAT expansion, is the most significant risk factor for developing T2DM. WAT is a multifunctional organ that plays a key role in metabolic health and regulating energy homeostasis. An inability of WAT to expand to accommodate excess nutrients is predominantly due to impaired adipogenesis and results in ectopic fat deposition, lipotoxicity and insulin resistance. Adipogenesis is influenced by several extracellular and intercellular factors and the IRE1α-XBP1s UPR pathway is indispensible for successful murine adipogenesis. Obesity associated factors such as hyperglycaemia, endotoxemia, elevated circulating FFAs, and local hypoxia induce adipocyte ER stress via several mechanisms including increased demand on the ER for protein synthesis and processing, lipid droplet formation and lipid partitioning, and elevated ROS production. ER stress is a key factor in the initiation of insulin resistance through inhibiting insulin action and inducing WAT inflammation and may interfere with adipogenesis. Interventions that alleviate ER stress may be suitable first-line treatments to restore ER function and metabolic regulation in metabolic diseases such as T2DM.

1.10 Research hypothesis, aims and objectives

1.10.1 Research hypothesis

The research hypothesis is that ER stress in human WAT is an important player in inducing WAT dysfunction, which can then lead to overall insulin resistance and T2DM.

1.10.2 Research aims

1. To elucidate interactions of ER stress in human WAT.
2. To characterise the role of ER stress in human adipogenesis.

1.10.3 Research objectives

1. Some bariatric procedures improve glycaemic control in people with T2DM beyond that expected for weight loss alone (Dixon, *et al.*, 2012). And ER stress in adipose tissue can be reversed with gastric bypass bariatric surgery in humans (Gregor, *et al.*, 2009). Therefore the WAT in T2DM subjects before and after different bariatric surgeries may provide useful information about interactions of ER stress. Thus, to elucidate the interactions of ER stress in human WAT, SAT biopsies and anthropometry will be collected before and six months after T2DM subjects undergo malabsorptive or restrictive bariatric surgeries. SAT ER stress and inflammation will be measured by western blotting and qRT-PCR and analysed with anthropometric data.

2. In order to accommodate the energy requirements of the body WAT is able to expand and contract during adult life despite most development during prenatal and early postnatal life (Poulos, *et al.*, 2010). Evidence suggests obesity complications in humans result from the inability of SAT to appropriately expand and store lipids, and consequently ectopic fat deposition and lipotoxicity contribute to insulin resistance (Virtue and Vidal-Puig, 2010). Impaired WAT expandability is mainly due to impaired adipogenesis (Isakson, *et al.*, 2009). The ER stress activated IRE1α - XBP1s pathway has been linked to adipogenesis in mouse studies (Sha, *et al.*, 2009). In order to characterise the role of ER stress in human adipogenesis WAT samples will be collected and primary human preadipocytes will be extracted and grown to confluence and then fully differentiated into adipocytes by standardised methods (Alhusaini, *et al.*, 2010). They will be assessed for lipogenesis, lipolysis, glucose uptake and insulin sensitivity, ER stress and adipogenesis markers in control cells without any treatment and with agents known to induce ER stress. ER stress pathways and adipogenesis markers will be investigated by western blotting and qRT-PCR and where required siRNA.

Chapter 2: Subjects, methods and materials

2.1 Ethical approval

2.1.1 Bariatric study

The Ethics Committee of the Institute of Endocrinology, Prague, Czech Republic provided ethical approval for the bariatric study. All participants provided written and informed consent in accordance with the Declaration of Helsinki and underwent bariatric surgery at the OB Clinic, Prague, Czech Republic. The European Foundation for the Study of Diabetes (EFSD) funded the study through the New Horizons Collaborative Research Initiative (EFSD New Horizons research grant no. 1113 09).

2.1.2 Human adipose tissue collection

The University Hospitals Coventry and Warwickshire NHS Trust Research and Development Department provided ethical approval for the collection of human adipose tissue and issued the following approval number: SK06/9309. All participants provided written and informed consent in accordance with the Declaration of Helsinki and had adipose tissue collected at University Hospitals Coventry and Warwickshire.

2.2 Subjects

2.2.1 Subjects who underwent bariatric surgery

For the bariatric study, a female cohort of thirty Caucasian adult women with morbid obesity (BMI $\geq$35 kg/m^2) and T2DM were recruited who underwent bariatric surgery (figure 2.2.1): laparoscopic adjustable gastric banding (LAGB; n=8) (Kormanova, *et al.*, 2004; Zinzindohoue, *et al.*, 2003) or laparoscopic greater

curvature plication (LGCP; n=13) (Bradnova, *et al.*, 2014; Fried, *et al.*, 2012) or biliopancreatic diversion (BPD; n=9) (Scopinaro, *et al.*, 2011) at the OB Clinic, Prague, Czech Republic. Abdominal SAT was collected by biopsy on the day of surgery and at six months post-surgery. For the purposes of this study all patients were prospectively investigated before the surgery (baseline) and 6 months following the procedure; both anthropometry and biochemical analysis were undertaken at both visits. Patients on incretin mimetics and/or insulin for T2DM treatment were excluded from this study (Bradnova, *et al.*, 2014). Fat mass was measured with bioimpedance method (Tanita TBF-300).

Figure 2.2.1 Bariatric surgery procedures under investigation

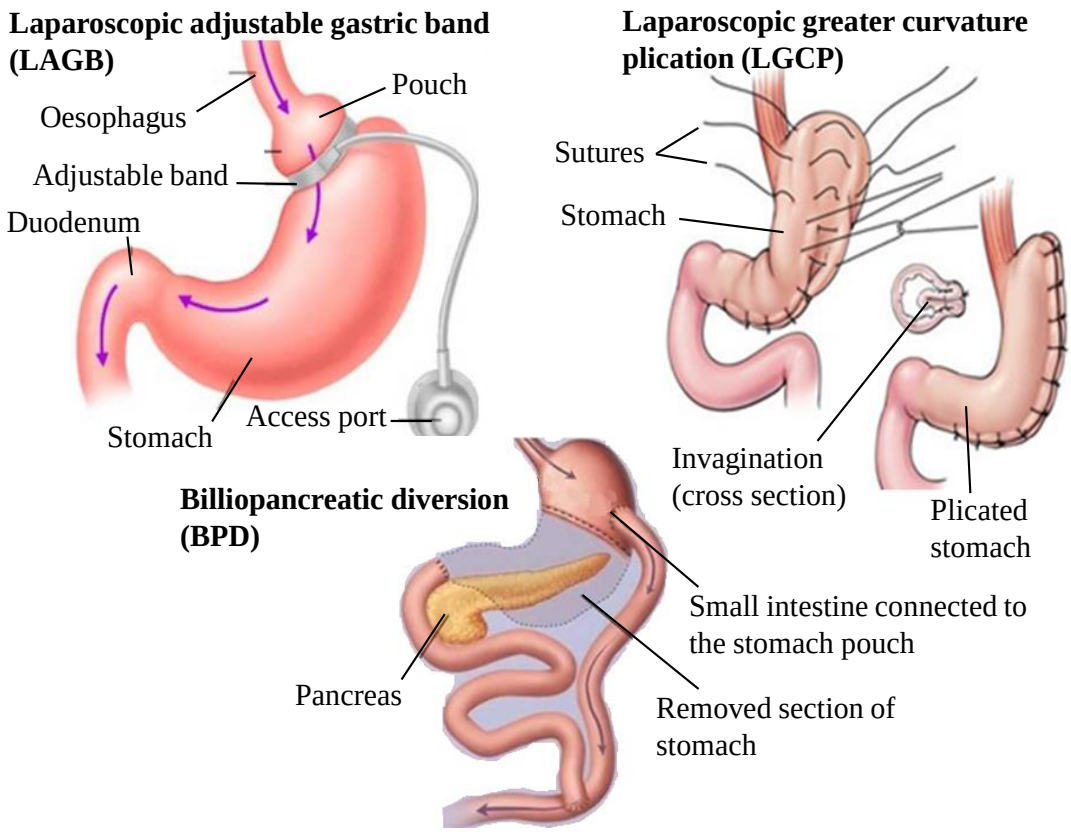


Figure 2.2.1 legend: Bariatric surgery procedures under investigation. Schematic diagram representing laparoscopic adjustable gastric banding (LAGB), laparoscopic greater curvature plication (LGCP) and biliopancreatic diversion (BPD) bariatric surgery procedures.

2.2.2 Adipose tissue collection

WAT was collected exclusively from non-smoking, non-diabetic, pre-menopausal, Caucasian women undergoing elective cesarean section at University Hospitals Coventry and Warwickshire (UHCW). Paired abdominal SAT and omental VAT was collected. Lean was considered pre-pregnant body mass index (BMI) of less than 25.0 kg/m^2 and obese was considered pre-pregnant BMI over 30.0 kg/m^2. The WNT10B C256Y subject consented to an abdominal subcutaneous adipose tissue biopsy and the biopsy was collected at the Warwickshire Institute for Diabetes, Endocrinology and Metabolism (WISDEM), at UHCW.

2.3 Tissue culture

2.3.1 Extracting primary human preadipocytes

Adipose tissue-derived stromal cell isolation was collected as previously reviewed (Schaffler and Buchler, 2007). Sterile conditions in a category 2 tissue culture room were used and to minimise lysis of fat the procedure was carried out as soon possible after collecting the adipose tissue (within 1 hour). Between 3 cm^3 and 10 cm^3 of WAT was placed in a 50 mL falcon containing 10 mL 2 mg/mL collagenase (Worthington, UK), pre-warmed to 37 C. The WAT was cut with

autoclaved and 70 % industrial methylated spirit cleaned scissors until the fat pieces were no bigger than 2 mm in diameter. The falcon was placed in a rack in a shaking water bath at 37 C for 30 minutes and shaken vigorously by hand at 10 minute intervals to form a smooth lipid and collagenase mixture. This mixture was filtered through sterile cotton mesh into a sterile 50 mL falcon and centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded then the preadipocyte pellet was resuspended in 5 mL lysis buffer, incubated at room temperature for 5 minutes and then centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and the remaining pellet was resuspended in 5 mL primary adipocyte growth media and transferred to a 75 cm^2 tissue culture flask (T75) (corning, UK) containing 15 mL primary adipocyte growth media that had been pre-incubated in a 37 C, 5 % CO^2 humidified incubator for 15 minutes. The flask was labelled and stored in a 37 C, 5 % CO^2 humidified incubator and the media was changed every 48 hours.

Collagenase:

- 50 mL Hank's balanced salt solution (HBSS)
- 445 mL sterile water
- 5 mL Penicillin/streptomycin

Pipetted 20 mL of the above solution into a bottle of 1 g collagenase then mixed up and down and put back into the solution and repeated three times and mixed until all collagenase was dissolved. Aliquoted 10 mL into 50 mL falcons, labelled with collagenase and the date and stored until use at -20 C.

Lysis Buffer:

- 1.001 g Potassium bi-carbonate (KHCO3)
- 8.29 g Ammonium chloride (NH3Cl)
- 0.0372 g Ethylenediaminetetraacetic acid (EDTA)
- Made up to 1 L with sterile deionised water

2.3.2 Human preadipocyte ChubS7 cell line

The ChubS7 cell line (Nestlé Research Centre, Lausanne, Switzerland) was derived from a preadipocyte extracted from Abd SAT of an obese female subject with a BMI of 54 kg/m^2 by coexpression of human telomerase reverse transcriptase (hTERT) and papillomavirus E7 oncoprotein (HPV-E7) genes (Darimont, *et al.*, 2003). ChubS7 retain the ability to undergo adipogenesis and have been used to study adipocyte metabolic function including glucose uptake (Gathercole, *et al.*, 2007) following a previously described glucose uptake assay method (Liu, *et al.*, 2001).

2.3.3 Cell culture media composition

Cell culture media was used as previously described (Alhusaini, *et al*., 2010).

ChubS7 growth media:

- DMEM/Ham's F-12 phenol-free medium 500 mL (Invitrogen #11039047)
- Penicillin/streptomycin/L-glutamine 100x, 5 mL (1 %) (Invitrogen #10378-016)
- Fetal bovine serum 50 mL (10 %) (Biosera #S1810)

Primary adipocyte growth media:

- DMEM/Ham's F-12 phenol-free medium 500 mL (Invitrogen #11039047)

- Penicillin/streptomycin/L-glutamine 100x, 5 mL (1 %) (Invitrogen #10378-016)
- Fetal bovine serum, 50 mL (10 %) (Biosera #S1810)
- Fibroblast growth factor-basic (FGF-basic), recombinant human 5 ng/mL (Fisher Scientific #VXPHG0026)
- Transferrin, human 5 µg/mL (Fisher Scientific #VX0030124SA)

Differentiation media (PromoCell Supplements):

- DMEM/Ham's F-12 phenol-free medium 500 mL (Invitrogen #11039047)
- Fetal bovine serum, 15 mL (3 %) (Biosera #S1810)
- Preadipocyte differentiation supplement pack x1 (Promocell #C39436)

The media contained the following supplements at their final concentration:

- Insulin, recombinant human 0.5 µg/mL
- Dexamethasone 400 ng/mL
- D-biotin 8 µg/mL
- Isobutylmethylxantine (IBMX) 44 µg/mL
- L-thyroxine 9 ng/mL
- Ciglitazone 3 µg/mL

Nutrition media (PromoCell Supplements):

- DMEM/Ham's F-12 phenol-free medium 500 mL (Invitrogen #11039047)
- Adipocyte nutrition supplement pack x1 (Promocell #C39439)

The media contained the following supplements at their final concentration:

- Insulin, recombinant human 0.5 µg/mL
- Dexamethasone 400 ng/mL
- D-biotin 8 µg/mL
- Fetal calf serum 0.03 mL/mL (3 %)

2.3.4 Propagation and differentiation of preadipocytes

The volume of media used in experiments was always the same in control and treatment flasks because adipocyte differentiation is affected by media height above the cell layer (Sheng, *et al.*, 2014). Primary human preadipocytes were isolated and cultured in T75 flasks as described above. When the T75 was 80 % confluent the cells were passaged into 3 further T75 flasks. Trypsinization and passage of cells: media was aspirated and cells were washed three times with sterile phosphate buffered saline (PBS) pre-warmed to 37 C, and incubated with 5 mL 0.05 % trypsin – EDTA (Life Technologies #25300-062) for 5 minutes. Cells were dislodged from the bottom of the flask by gentle tapping and trypsin was neutralized with 15 mL growth media, centrifuged at 1000 rpm for 5 minutes to form a pellet of cells. Supernatant was removed, cells resuspended in 5 mL growth media and transferred to 3 new T75s. Once 80 % confluent, preadipocytes were counted with a hemocytometer and seeded at a density of 4000 cells/cm^2 onto treated polystyrene six well culture plates (Corning) with 2 mL primary adipocyte growth media in each well. Growth media was changed every 48 hours, and once confluent, cells were incubated in growth media for a further 2 days. Differentiation was then initiated (day 0) by changing the media to differentiation media. During day 0 to day 6 of adipogenesis media was always changed every 48 hours with differentiation media. The media was then changed to nutrition media every 48 hours from day 6 to day 14.

2.3.5 Treatments

Prior to optimisation treatments, media were switched to detoxification media (DMEM/Ham's F-12 phenol-free medium containing only 3 % serum) for 24 hours to remove effects of growth factors and other components in nutrition media. The treatments including control were placed in the fresh detoxification media made on the day of treatment. Tunicamycin (TM) (Sigma #7765) treatment concentration was chosen based on previous reports investigating ER stress that used between 0.5 μg / mL and 2.0 μg / mL (Alhusaini, *et al.*, 2010; Ozcan, *et al.*, 2004). The concentration of 4μ8C was optimised based on the range of concentrations (1 μM to 64 μM) used by Cross *et al.* (Cross, *et al.*, 2012). TM and 4μ8C were diluted in dimethyl sulfoxide (DMSO) (Sigma #D8418). Where TM or 4μ8C treatments were used, the control always had the same volume of DMSO added as the volume needed to dissolve the treatment. All treatment media including control was filtered through a 0.22 μM filter before use.

2.3.6 Small interfering RNA transfection

ChubS7 cells were used for siRNA studies because of their consistency and reliability. Cells were grown to confluence on 12 well plates (Corning), media changed to detoxification media (DMEM/Ham's F-12 phenol-free medium containing only 3 % serum) for optimisation and differentiation media for adipogenesis experiment, for 30 minutes. SMARTpool: ON-TARGETplus Human XBP1 siRNA (Thermo Fisher Scientific Bioscience #L009552000005) and ON-TARGETplus non-targeting pool (control) (Thermo Fisher Scientific Bioscience #DZD18101020) were reconstituted with 5x siRNA buffer (Thermo Fisher Scientific Bioscience #B002000UB100) and vortexed for 30 minutes.

siRNA was diluted to a final concentration of 25 – 100 nmol in 1x Opti-MEM1 (Life Technologies #31985062), mixed with a final concentration of 0.5 % or 1.0 % DharmaFECT Duo (Thermo Fisher Scientific Bioscience #DZT201003), added to detoxification media or differentiation media as appropriate, mixed and then added to cells for 16 hours. Media was then aspirated and fresh appropriate media without siRNA was added.

2.3.7 Collection of conditioned media, protein and RNA

Conditioned media (1.5 mL), was collected from six separate wells for every sample time point and stored at -80 C until use for free glycerol assay. To collect protein, a lysis buffer was made with 5 mL 1x radioimmunoprecipitation (RIPA) (Millipore UK) with 100 μL of dissolved protease and phosphatase inhibitors (2 Roche Complete Mini protease inhibitor cocktail tablets and 8 mg sodium fluoride (NaF, Fisher Scientific) and 20 mg sodium vanadate (Na3VO4, Acros Organics) in 2 mL 1x RIPA). 250 μl of the protein lysis buffer was added to each well at 4 C, cells were scraped with sterile scrapers in each well for 60 seconds, the well contents was collected and stored at -80 C. The same method was used to harvest total RNA except 350 μL Buffer RLY (Bioline) and 3.5 μL β-mercaptoethanol (β-ME) (Sigma) were added per well instead of protein lysis buffer. Both protein and RNA were collected in triplicate for every time point.

2.4 Analysis of samples

2.4.1 Analysis of blood samples

All patients were examined in the morning after a 10 hour overnight fast and venous blood was sampled. All blood samples were collected into chilled ethylenediaminetetraacetic acid (EDTA) containing tubes with aprotinin as well as without for the measurements of glucose, insulin and C-peptide levels. The samples were aliquoted and stored at -80 C until assayed. Circulating glucose, insulin, HbA1c and lipids were measured using the Cobas 6000 analyzer. The homeostatic model assessment method was used to assess insulin resistance (HOMA-IR) as previously described, (Matthews, *et al.*, 1985), according to the following equation: HOMA-IR = [fasting glucose (mmol/L) x fasting insulin (μU/L)] / 22.5. LDL cholesterol was calculated according to the Friedewald formula (Friedewald, *et al.*, 1972).

2.4.2 RNA analysis

Total RNA was extracted from WAT samples using RNeasy lipid tissue kit (Qiagen, #74104) and from cell culture samples using Isolate II RNA Mini Kit (Bioline, #BIO-52073) according to the manufacturer's instructions. RNA was quantified using a spectrophotometer (Nanodrop ND-1000, Labtech, UK), measuring at an absorbance of 260 nm. The ratios between absorbances 260/280 nm and 260/230 nm were measured to give an estimate of RNA purity. A value between 1.8 and 2.1 for both ratios was accepted as suitable RNA purity for use. RNA integrity was assessed by ethidium bromide incorporated agarose gel

electrophoresis and two sharp distinct bands representing 18S and 28S were considered acceptable RNA integrity for use.

Complimentary DNA (cDNA) was synthesised as described elsewhere (Alhusaini, *et al.*, 2010). Briefly, a Bioline kit (#BIO-65026) was used. 200 ng RNA per sample, based on spectrophotometer (Nanodrop, Labtech) quantification, was used to make cDNA. RNA was pipetted into a sterile microcentrifuge tube (200 µL capacity), with 1 µL of random hexamers 50-250 ng (Bioline, UK) and 1 µL 10 mM dNTP mix (Invitrogen, UK), and mixed in RNase free water (Qiagen) to give a total volume of 10 µL. Samples were then vortexed, centrifuged briefly and heated to 70 C for 5 minutes and chilled on ice at 4 C for 2 minutes. Samples were then vortexed thoroughly and mixed with 10 µL reverse transcription mastermix (4µl of 5X reaction buffer, 1 µL RNase inhibitor, 0.5 µL reverse transcriptase (200 U/µL) made up to 10 µL by adding RNase free water) giving a final volume of 20 µL. Each sample was mixed thoroughly, briefly centrifuged and incubated at room temperature for 5 minutes and then transferred to a thermocycler (Biorad, UK). Samples were heated to 37 C for 5 minutes, 42 C for 55 minutes, and then 70 C for 15 minutes and stored at -20 C until use.

Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 standard Sequence Detection System (Applied Biosystems, UK). Reactions were carried out at 50 C for 2 minutes, 95 C for 10 minutes, and then 40 cycles of 95 C for 15 seconds then 60 C for 1 min. Reactions were prepared to 25 µL volumes in a 96 well plate. Pre-designed gene specific Taqman probes and primers were used (Applied Biosystems, UK, ATF4: Hs00909569_g1; CHOP (DDIT3):

Hs00358796_g1; IRE1α (ERN1): Hs00176385_m1; ATF6: Hs00232586_m1; IL10: Hs00961622_m1; CCL2: Hs00234140_m1; CD68: Hs02836816_g1, CD14: Hs02621496_s1, IL6: Hs00185926_m; CEBPα: Hs00269972_s1; PPARγ: Hs01115513_m1; Adiponectin (ADIPOQ): Hs00605917_m1; GLUT4 (SLC2A4): Hs00168966_m1; TCF7: Hs00175273_m1; AXIN2: Hs00610344_m1) in a reaction mix containing TaqMan universal PCR master mix (Applied Biosystems, UK). All reactions were multiplexed with the housekeeping gene 18S, provided as a pre-optimised control probe (Applied Biosystems, UK) enabling calculation of delta threshold cycle (ΔCt) values (where ΔCt = Ct of 18S subtracted from Ct of gene of interest). Data is expressed as 2 raised to the power of the difference between control ΔCt and test ΔCt (ΔΔCt method). For the bariatric study all gene expression was calculated for each individual relative to her pre-surgery gene expression. The inverse of ΔCt values were used for correlation analysis. Measurements were carried out in triplicate for each sample. A Bioline SensiMixTM SYBR Low-ROX Kit (#QT625-02) was used to measure mRNA expression using specific primers (Invitrogen UK) for XBP1s: Forward: 5'-GGTCTGCTGAGTCCGCAGCAGG-3', Reverse: 5'-GGGCTTGGTATATATGTGG-3', (Cho, *et al.*, 2014; Gregor, *et al.*, 2009; Lee, *et al.*, 2011); XBP1u: Forward: 5'-CAGACTACGTGCACCTCTGC-3', Reverse: 5'-GTTCATTAATGGCTTCCAGCT-3', designed such that the forward primer is complementary to nucleotides 549 – 569 of XBP1u and spans the 3' end of the 26 nucleotide intron located at 531 – 556 and therefore exclusively amplifies XBP1u mRNA (Uemura, *et al.*, 2009); 18S: Forward: 5'-GTAACCCGTTGAACCCCATT-3', Reverse: 5'-CCATCCAATCGGTAGTAGCG-3', (Gregor, *et al.*, 2009); WNT10B: Forward:

5’-CAGTCGGGCTCTAAGCAATGA-3’, Reverse: 5’-CTTGCTCAGGCCGGACAG-3’, (Isakson, *et al.*, 2009). The following were added per well: 12.5 µL SensiMixTM SYBR Low-ROX, 0.5 µL 10 µM forward primer, 0.5 µL 10 µM reverse primer, 10.5 µL nuclease free water and 1 µL cDNA template.

2.4.3 Protein analysis

Human WAT was homogenized and resuspended in 250 µL protein lysis buffer made with 5 mL 1x radioimmunoprecipitation (RIPA) (Millipore UK) with 100 µL of dissolved protease and phosphatase inhibitors (2 Roche Complete Mini protease inhibitor cocktail tablets and 8 mg sodium fluoride (NaF, Fisher Scientific) and 20 mg sodium vanadate (Na3VO4, Acros Organics) in 2 mL 1x RIPA). Protein concentrations were determined using the Bio-Rad detergent compatible protein assay kit (Bio-Rad Laboratories, CA) with standard concentrations of albumin from bovine serum (BSA) (Sigma #A9647) and quantified using a nanospectrophotometer (GeneFlow, UK). Western blot analysis was performed using a method previously described (Alhusaini, *et al.*, 2010). For Western blotting, in brief, 20 µg of protein samples were loaded onto a 10 % denaturing polyacrylamide gel (GeneFlow, UK), separated by electrophoresis and transferred at 4 C and 100 V for 60 minutes to membrane filter Immobilon-P transfer membranes 0.45 µm pore size (Fisher), blocked for 60 minutes in 0.2 % I-Block PBS-tween (PBST) and incubated in primary rabbit-derived antibody diluted in 0.2 % I-Block PBST (p-IRE1α 1:500, total IRE1α 1:200, Calnexin 1:1000, BiP/Grp78 1:750, β-actin 1:1000, Cell Signalling; XBP1s and XBP1u 1:250, glut4 1:80, Abcam; ATF6 1:250 Abnova; p-Akt 1:1000, total Akt 1:1000,

p-β-catenin 1:500, unphosphorylated β-catenin 1:500, Millipore, UK) at 4 C overnight. Equal protein loading was confirmed by examining β-actin protein expression. Membranes were washed three times for ten minutes in PBST and incubated in anti-rabbit IgG (whole molecule), horseradish peroxidase antibody produced in goat, IgG fraction of antiserum, buffered aqueous solution (Sigma #A9169). A chemiluminescent detection system, ECL/ECL+ (GE Healthcare, UK), enabled visualization of bands on hyperfilm MP (Fisher), and intensity was determined using densitometry (Genesnap, Syngene, UK).

2.4.4 Agarose gel electrophoresis

Agarose gel (2.5 % for resolving qRT-PCR products, 1 % for assessing extracted RNA integrity) was made by adding agarose (Fisher Scientific) to 1x TAE (Tris base, acetic acid and EDTA). The agarose was dissolved by heating for 60 seconds in a 900 Watt microwave. The solution was cooled before adding ethidium bromide to a final concentration of 1 ng/mL. After the gel had set, appropriate ladders and test samples were loaded. 1x TAE was used as a buffer and electrophoresis was carried out for 30 – 60 minutes at 100 V. The gel was viewed under UV light to show ethidium bromide incorporated DNA or RNA and digital images were taken (ChemiGenius).

2.4.5 Lipolysis assay

Lipolysis was measured by free glycerol in conditioned media using a free glycerol assay kit (Sigma) and following the manufacturers guidelines. Glycerol standard solution (Sigma #G7793) was serially diluted to make eight

concentrations ranging from 4 – 130 µg/mL. Free glycerol reagent (Sigma #F6428) was reconstituted in 40 mL sterile water. 25 µL of each calibration solution and test condition media were added to wells in a 96 microwell plate (Sterlin, UK) in triplicate. Then 200 µL reconstituted free glycerol reagent was added per well. Adhesive film was placed over the plate and incubated plate on shaker for 15 minutes. Absorbance was read at 540 nm using a spectrophotometer (Infinite 200 PRO NanoQuant, TECAN). Appropriate cell culture media was used as a blank because the media contains fetal bovine serum with lipids in (Boone, *et al.*, 1971).

2.4.6 Adipocyte staining

Adipocytes were grown on 6 well plates (corning), media was removed and the cells washed with PBS twice. The cells were fixed with 10 % formalin for 60 minutes at room temperature. Excess formalin was washed off with PBS and the wells were air dried completely for 30 minutes.

Hematoxylin and eosin staining: Stained in haematoxylin (Gill's haematoxylin number 3, Sigma #GHS380) for 45 seconds then washed 3 times in water. Stained in eosin (Eosin yellowish: 1 % solution in water, Gurr Certistain, BDH, VWR #341973R) for 1 minute and washed 3 times in water. Dehydrated in 50 %, 70 % and 90 % ethanol for 45 seconds in each, then 1 min in 100 % ethanol. Left to dry overnight lying upside down on tissue.

Oil Red-O staining: Stock Oil Red-O (ORO) solution was made by dissolving 0.5 g ORO powder (Sigma, UK) in 100 mL absolute propan-2-ol and stirring

overnight. Working ORO solution was made by diluting the stock ORO with distilled water 3:2, stirring for 10 minutes and then filtering through a 0.22 µm filter. Enough working ORO was made for the whole experiment to ensure all the dye used was exactly the same concentration. 500 µL working ORO was added to each well and incubated at room temperature for 60 minutes. All unbound ORO was washed off with distilled water before viewing under a light microscope to assess lipid accumulation. Digital photographs were taken. The wells were then air dried completely for 30 minutes. The bound ORO was eluted by adding 300 µL absolute propan-2-ol per well and shaking on an orbital shaker at 100 rpm for 20 minutes. To quantify the ORO in each well, 200 µL of eluted solution was transferred to individual wells of a 96 microwell plate (Sterlin, UK) and the absorbance at 520 nm was measured using a spectrophotometer (Infinite 200 PRO NanoQuant, TECAN). Two wells with only isopropanol 200 µL were used as blanks in the microwell plate.

2.4.7 Glucose uptake assay

Glucose uptake was carried out as previously described (Adaikalakoteswari, *et al.*, 2015; Gathercole, *et al.*, 2007; Liu, *et al.*, 2001). Measurement of 2-deoxy-D-[1-3H] glucose uptake: Cells were grown on 6 or 12 well plates (Corning), washed with PBS and incubated at 37 C for 3 hours in Krebs-Henseleit buffer (KHB) with 0.01 % BSA and 5 mmol/L glucose for insulin and serum starvation. Adipocytes were incubated for 30 minutes at 37 C with KRH buffer without glucose or BSA, then treated with DMSO (basal control) or 100 nm insulin (insulin solution, human, chemically defined, recombinant from Saccharomyces cerevisiae, sterile-filtered, (Sigma # I9278)). 1 µCi/mL of 2-deoxy-D-[1-3H]glucose (PerkinElmer)

was added and incubated for 10 minutes. Cells were washed three times with ice-cold PBS and harvested in 500 μL RIPA per well. 400 μL cell lysate was transferred to 4 mL scintillation fluid and radioactivity (becquerels, Bq) was counted using a scintillation counter. The remaining 100 μL cell lysate was used to quantify protein and glucose uptake was expressed as Bq/mg protein.

To make 1 L 5x KHB, the following reagents were added separately in the listed order to 900 mL distilled and autoclaved water (dH2O) in a 1 L beaker containing a sterile magnet stir bar. The final volume was adjusted to 1000 mL with dH2O, and stored at 4 C for later use.

Chemical	5X (mM)	5X (g/L)
NaCl	555	32.532
KCl	23.5	1.752
MgSO4	10	1.204
Na2HPO4	6	0.852

On the day before the assay, 500 mL of the following two reagents were prepared and filter sterilized:

1. 1x KHB, low glucose – 5 mM (450 mg), 0.01 % BSA (50 mg), 10 mM Hepes (1.1915 g), pH 7.4
2. 1x KHB, no glucose, no BSA, 10 mM Hepes (1.1915 g), pH 7.4

2.4.8 Cell viability assay

Cell viability assay measured the activity of living cells via the activity of mitochondrial dehydrogenases as previously described (Denizot and Lang, 1986; Sharma, *et al.*, 2014). An *in vitro* 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl

tetrazolium bromide (MTT) toxicology assay kit (Sigma # TOX1-1KT) was used according to the manufacturers protocol. Briefly, cells were grown to confluence on 96 well tissue culture plates (Corning) and treatments conducted. Following treatment, MTT was reconstituted and added to culture medium as described in the protocol and cells were returned to incubate for 4 hours. Following incubation, formazan crystals were dissolved by adding MTT solubilization solution and gentle mixing. Absorbance was measured at 570 nm and background absorbance at 690 nm was subtracted from the 570 nm measurement. Complete medium without cells was used as a blank.

2.5 Statistical Analysis

Statistical analysis was undertaken using Microsoft Excel, IBM SPSS Statistics 21.0 and online computer software (Preacher, 2002, May). Data were examined for normality using the Shapiro-Wilk test. Parametrically distributed data were analysed using the two-tailed paired sample t-test and non-parametrically distributed data were analysed with the Wilcoxon Signed Rank Test. Correlations between variables were tested using univariate analyses (Pearson's or Spearman's correlation where appropriate) then calculations to test the difference between two independent correlation coefficients were performed. All data is expressed as mean value ± standard error of the mean (SE). $P<0.05$ was considered statistically significant and significance levels are indicated as follows; *$P<0.05$, **$P<0.01$, ***$P<0.001$ and where more than one comparison was made: +$P<0.05$, ++$P<0.01$, +++$P<0.001$.

Chapter 3: Endoplasmic reticulum stress in human adipose tissue

3.1 Introduction

The endoplasmic reticulum (ER) is a multifunctional organelle that plays a key role in the folding, maturation, storage and transport of proteins. However, in conditions of obesity the ER functions can become dysregulated which manifests in misfolded and unfolded proteins, often referred to as 'ER stress' (Hotamisligil, 2006; Hotamisligil, 2010; Schroder and Kaufman, 2005). Several factors appear to heighten ER stress in obesity including hyperglycaemia and inflammation, as well as hypertrophic adipocyte conditions (Alhusaini, *et al.*, 2010; Gregor and Hotamisligil, 2007; Ozcan, *et al.*, 2004; Ozcan, *et al.*, 2006). Whilst the adipocyte continues to adapt to such stresses, often this leads to the point where the dysregulated ER initiates a complex response system known as the unfolded protein response (UPR) (figure 1.6.1) to restore the functional integrity to the organelle (Marciniak and Ron, 2006; Schroder and Kaufman, 2005). In mammals the UPR is comprised of three main branches, signalling through which is mediated by inositol-requiring enzyme 1α (IRE1α), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) (Harding, *et al.*, 2000b; Harding, *et al.*, 1999). In unstressed conditions, the UPR sensing transmembrane proteins are bound by a chaperone, BiP/Grp78, in their intraluminal domains and rendered inactive. In cases of accumulating unfolded proteins and increased protein cargo in the ER, BiP/Grp78 is recruited releasing the IRE1α, PERK and ATF6 (Bertolotti, *et al.*, 2000; Shen, *et al.*, 2002). Upon activation IRE1α with its endoribonuclease activity splices and excises a specific 26 nucleotide fragment from X-box binding protein-1 (XBP1) mRNA to form XBP1s (spliced) mRNA encoding a potent transcription factor. PERK phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) (Harding, *et*

al., 1999) and ATF6 is enzymatically processed into its active form (Chen, *et al.*, 2002; Nadanaka, *et al.*, 2007). Collectively, these factors attenuate protein synthesis and reduce ER protein overload to restore ER integrity (Calfon, *et al.*, 2002; Ruggiano, *et al.*, 2014; Sidrauski and Walter, 1997; Wek, *et al.*, 2006).

Obesity appears to have a particular effect on activating UPR, highlighted by mice fed a high fat diet or *ob/ob* mice, which initiates a raised UPR response in adipose tissue (Gregor and Hotamisligil, 2007; Ozcan, *et al.*, 2004; Ozcan, *et al.*, 2006). Further studies have also indicated that ER stress in adipose tissue can be reversed with diet induced weight loss (Gregor, *et al.*, 2009; Seoane-Collazo, *et al.*, 2014) as well as through gastric bypass bariatric surgery in humans (Gregor, *et al.*, 2009). Bariatric surgery has proven to be highly effective method for prevention as well as remission of type 2 diabetes (T2DM) in obese patients, however the rate of remission varies between different surgical procedures (Schauer, *et al.*, 2012). Each surgery type induces changes in metabolic profiles and anthropometry which may have differential impact on ER stress resolution in WAT although studies to date have not examined this (Madsbad, *et al.*, 2014). Bradley *et al.* (Bradley, *et al.*, 2012) compared malabsorptive, Roux-en-Y gastric bypass (RYGB), and laparoscopic adjustable gastric banding (LAGB) and concluded that marked weight loss itself was primarily responsible for increased insulin sensitivity, β-cell function and oral glucose tolerance in non-diabetic obese adults and not the surgery type. They also suggested that additional studies on obese T2DM subjects were required to confirm their observations. Therefore, the aim of this study was to investigate the effect of malabsorptive versus restrictive surgeries on the clinical parameters related to metabolic health of obese type 2

diabetics. For this study patients undergoing three types of bariatric surgeries were recruited: BPD (malabsorptive) (Scopinaro, *et al.*, 2011), LAGB (restrictive) (Kormanova, *et al.*, 2004) and laparoscopic greater curvature plication (LGCP; restrictive) (Bradnova, *et al.*, 2014; Fried, *et al.*, 2012). The effects of surgery induced weight loss on ER stress in abdominal SAT as well as the effects of each surgery type on this pathway were investigated. All measurements were carried out in obese T2DM subjects before undergoing LAGB or LGCP or BPD surgery and after 6 months, in order to understand the rate of improvements in each surgery type.

3.2 Results

3.2.1 Body composition, basal metabolic variables and sample analyses

There were significant changes observed in all subjects 6 months post-surgery independent of surgery type. Individual BMI ($P<0.001$), fasting blood glucose ($P<0.001$), insulin ($P<0.001$), HOMA-IR ($P<0.001$), TG ($P<0.05$), cholesterol ($P<0.001$) and LDL-cholesterol ($P<0.05$) were all significantly improved (Table 3.2.1). According to surgery types, body weight loss post-surgery varied from 10.5 ± 1.0 % observed for LGCP, 11.4 ± 1.6 % for LAGB to 16.8 ± 0.5 % for BPD. Additionally, BPD resulted in a significantly higher rate of weight loss measured as pre body weight (PBW) loss per 100 days compared to LAGB and LGCP. The PBW loss per 100 days for BPD was 11.2 ± 0.7 %, for LGCP it was 5.9 ± 0.7 % and for LAGB it was 7.3 ± 1.2 % (Table 3.2.1). BPD subjects had lower HbA1c and HDL cholesterol compared to LAGB subjects and lower total

cholesterol and HDL cholesterol compared to LGCP subjects. BPD subjects also had a higher TAG/HDL ratio compared to LAGB subjects.

Table 3.2.1 Body composition and metabolic characteristics before and after bariatric surgery (following page, whole page table)

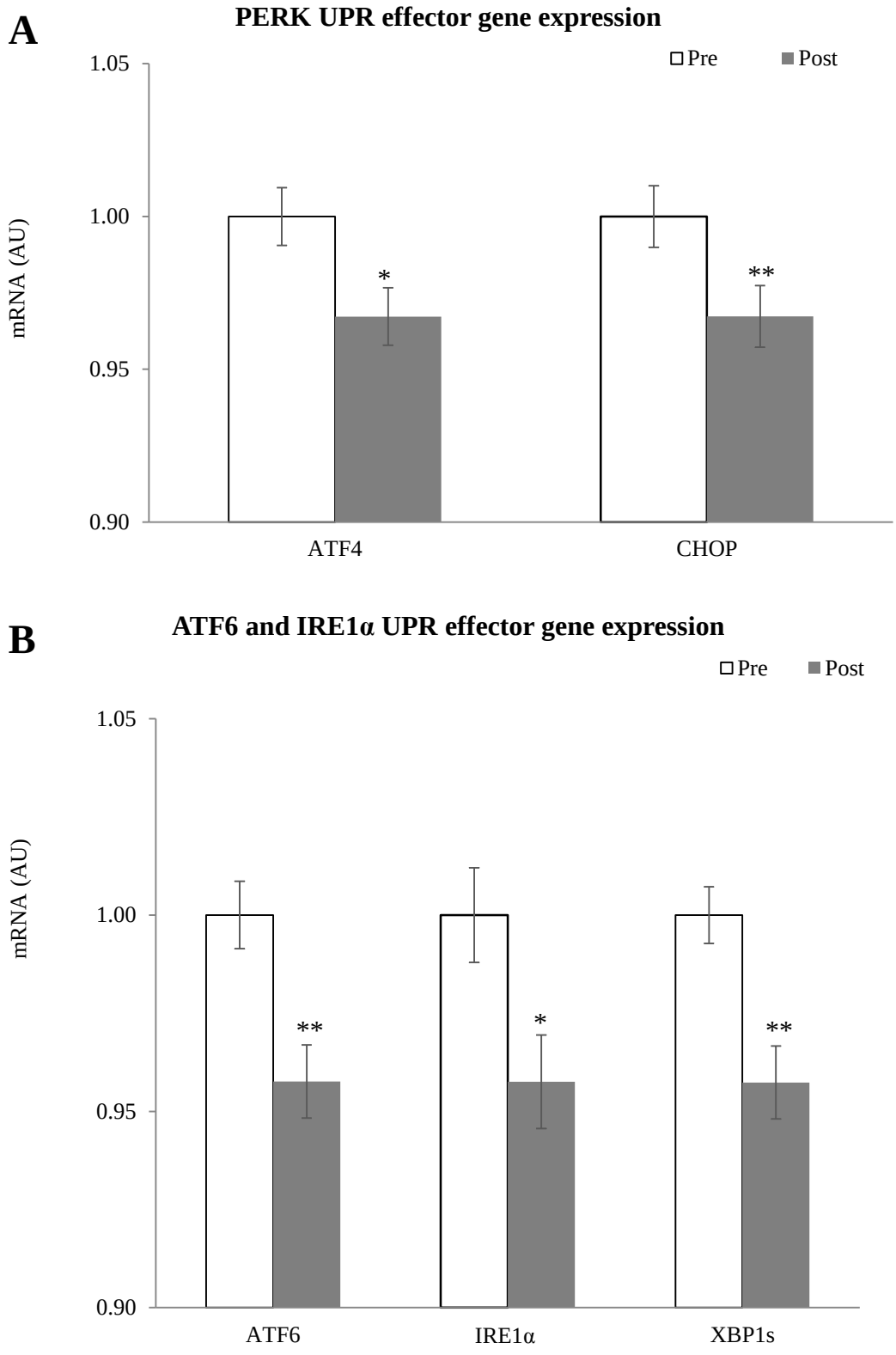
Table 3.2.1 legend: Body composition and metabolic characteristics before and after bariatric surgery. Anthropometric measurements were taken for 30 female subjects at the time of either laparoscopic adjustable gastric band (LAGB), laparoscopic greater curvature plication (LGCP) or biliopancreatic diversion (BPD) bariatric surgery (pre) and six months after surgery (post). Data is expressed as mean ± SE. Pre and post values were compared and $P<0.05$ was considered statistically significant and significance levels are indicated as follows; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 3.2.1	LAGB (n=9)		LGCP (n=13)		BPD (n=8)	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
Excess BMI Lost (% Excess BMI Lost)		31.85 ± 5.95		29.86 ± 3.18		40.96 ± 1.99
Time Between Biopsies (Days)		162.22 ± 6.83		184.62 ± 5.38		152.50 ± 8.18
Weight Loss Rate (% Lost per 100 Days)		7.30 ± 1.17		5.87 ± 0.70		11.24 ± 0.74
Age (Years)	53.89 ± 1.99	54.33*** ± 3.70	55.38 ± 1.07	55.89*** ± 1.65	52.38 ± 0.63	52.79*** ± 1.24
Body Weight (kg)	113.03 ± 6.63	100.52*** ± 6.98	106.23 ± 4.27	94.82*** ± 3.36	120.01 ± 4.90	99.95*** ± 4.32
BMI (kg/m^2)	41.92 ± 2.29	37.26*** ± 2.46	39.43 ± 1.44	35.16*** ± 1.14	43.30 ± 0.90	35.98*** ± 0.92
Fat Free Mass (kg)	57.16 ± 2.24	54.53* ± 3.50	54.62 ± 1.39	52.39** ± 1.51	59.93 ± 1.78	53.89** ± 1.90
Fat Mass (kg)	55.87 ± 4.57	46.00*** ± 3.93	51.61 ± 3.15	42.43*** ± 2.28	60.09 ± 3.80	46.06*** ± 3.48
Fat Mass (% Body Weight)	48.91 ± 1.31	45.34** ± 1.51	48.18 ± 1.10	44.51*** ± 1.06	49.80 ± 1.30	45.74** ± 1.80
Waist Circumference (cm)	118.19 ± 4.34	113.83* ± 5.18	113.81 ± 2.66	105.97** ± 2.48	122.88 ± 2.78	112.38* ± 5.06
Hip Circumference (cm)	131.13 ± 4.11	125.00** ± 4.45	128.65 ± 4.02	119.82** ± 2.82	135.44 ± 4.26	124.63* ± 3.79
Waist / Hip Ratio	0.90 ± 0.01	0.91 ± 0.02	0.89 ± 0.03	0.89 ± 0.02	0.91 ± 0.03	0.90 ± 0.02
HbA1c (mmol/mol)	53.44 ± 3.30	46.00* ± 2.26	56.77 ± 2.80	47.77** ± 2.95	58.63 ± 2.92	40.25** ± 0.86
Plasma Glucose (mmol/L)	9.60 ± 0.86	7.10*** ± 0.54	9.27 ± 0.60	7.38** ± 0.48	9.28 ± 1.09	7.23** ± 0.65
Plasma Insulin (mU/L)	26.69 ± 2.44	15.70** ± 1.88	27.19 ± 5.85	16.13** ± 3.27	35.26 ± 8.77	16.39* ± 3.10
HOMA-IR	10.70 ± 1.63	5.29** ± 1.02	11.40 ± 2.60	4.98** ± 1.01	13.68 ± 3.93	4.80* ± 1.05
Total Cholesterol (mmol/L)	4.83 ± 0.28	4.35 ± 0.22	4.89 ± 0.19	4.76 ± 0.23	5.15 ± 0.42	3.84*** ± 0.36
LDL Cholesterol (mmol/L)	2.98 ± 0.24	2.72 ± 0.18	2.81 ± 0.20	2.96 ± 0.23	3.41 ± 0.43	2.31** ± 0.27
HDL Cholesterol (mmol/L)	1.06 ± 0.09	1.08 ± 0.09	1.09 ± 0.08	1.15 ± 0.09	1.00 ± 0.08	0.76** ± 0.04
Triglyceride (TAG) (mmol/L)	1.72 ± 0.25	1.19* ± 0.16	2.15 ± 0.41	1.43 ± 0.21	1.60 ± 0.27	1.67 ± 0.26
HDL/LDL Ratio	0.37 ± 0.04	0.42 ± 0.05	0.42 ± 0.05	0.43 ± 0.06	0.33 ± 0.05	0.36 ± 0.04
TAG/HDL Ratio	1.78 ± 0.32	1.23* ± 0.23	2.32 ± 0.52	1.41 ± 0.25	1.75 ± 0.37	2.27* ± 0.43
C-Reactive Protein (ng/L)	4.65 ± 1.55	4.41 ± 1.47	8.61 ± 2.49	5.10 ± 1.47	4.47 ± 1.69	3.87 ± 1.46

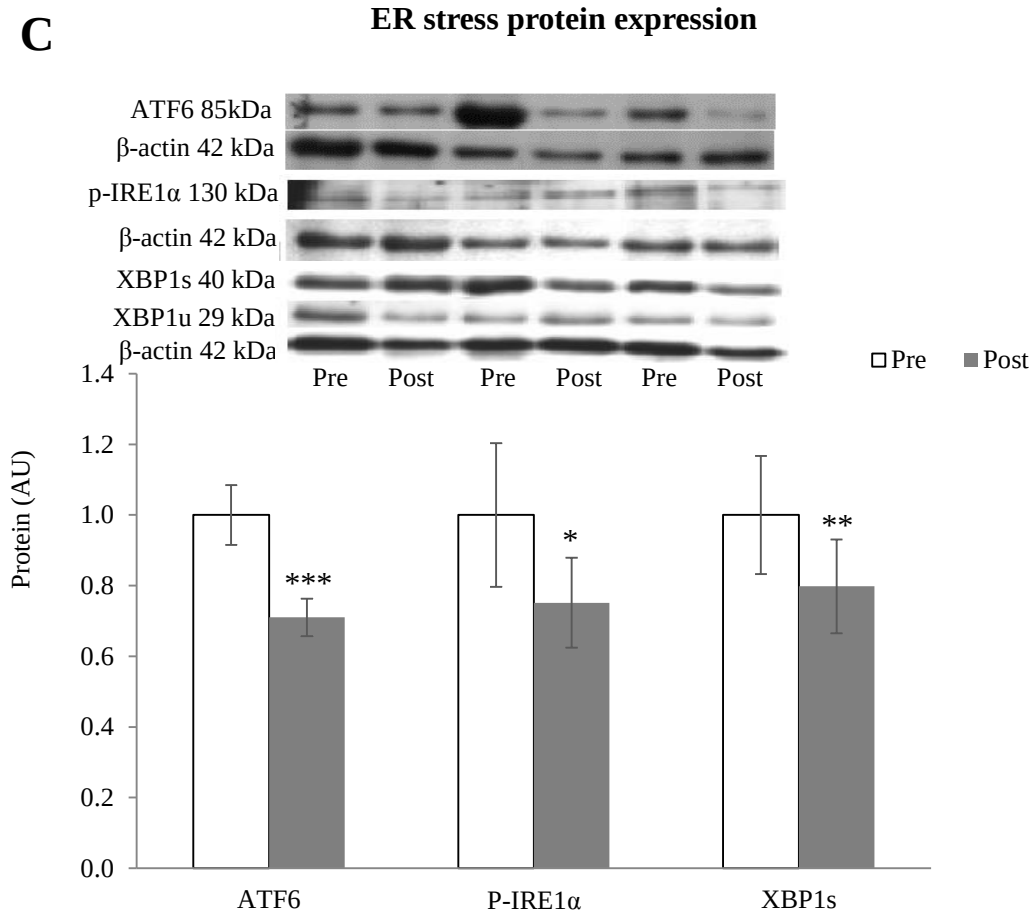
3.2.2 Adipose tissue expression of UPR related genes and proteins were reduced post bariatric surgery

Abdominal SAT mRNA and protein expressions of key UPR genes and proteins were evaluated in all 30 subjects before and 6 months after surgery. This was to assess the effect of weight loss alone. All the three UPR pathways; PERK, IRE-1α and ATF6 were assessed for changes in gene and protein expression. mRNA expression of downstream targets of PERK, ATF4 and CHOP, were both significantly reduced post-surgery ($P<0.01$; Figure 3.2.2 A). Both IRE-1α and ATF6 regulate expression of spliced XBP1 (XBP1s) (Guo, *et al.*, 2014). The IRE-1α mRNA and phospho-IRE-1α protein expressions were significantly reduced post-surgery (Figure 3.2.2 B and C). The mRNA and protein expressions of ATF6 and XBP1s were also significantly reduced 6 months after surgery ($P<0.05$; Figure 3.2.2 B and C). The protein expressions of important protein chaperones, BiP1/Grp78 and calnexin were also significantly reduced 6 months post-surgery (Figure 3.2.2D).

Figure 3.2.2 ER stress in abdominal subcutaneous adipose tissue was reduced six months after bariatric surgery in obese T2DM subjects



C


ER stress protein expression
ATF6 85kDa
β-actin 42 kDa
p-IRE1α 130 kDa
β-actin 42 kDa
XBP1s 40 kDa
XBP1u 29 kDa
β-actin 42 kDa
Pre
Post
Pre
Post
Pre
Post
Pre
Post
Protein (AU)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
*
**
ATF6
P-IRE1α
XBP1s

D

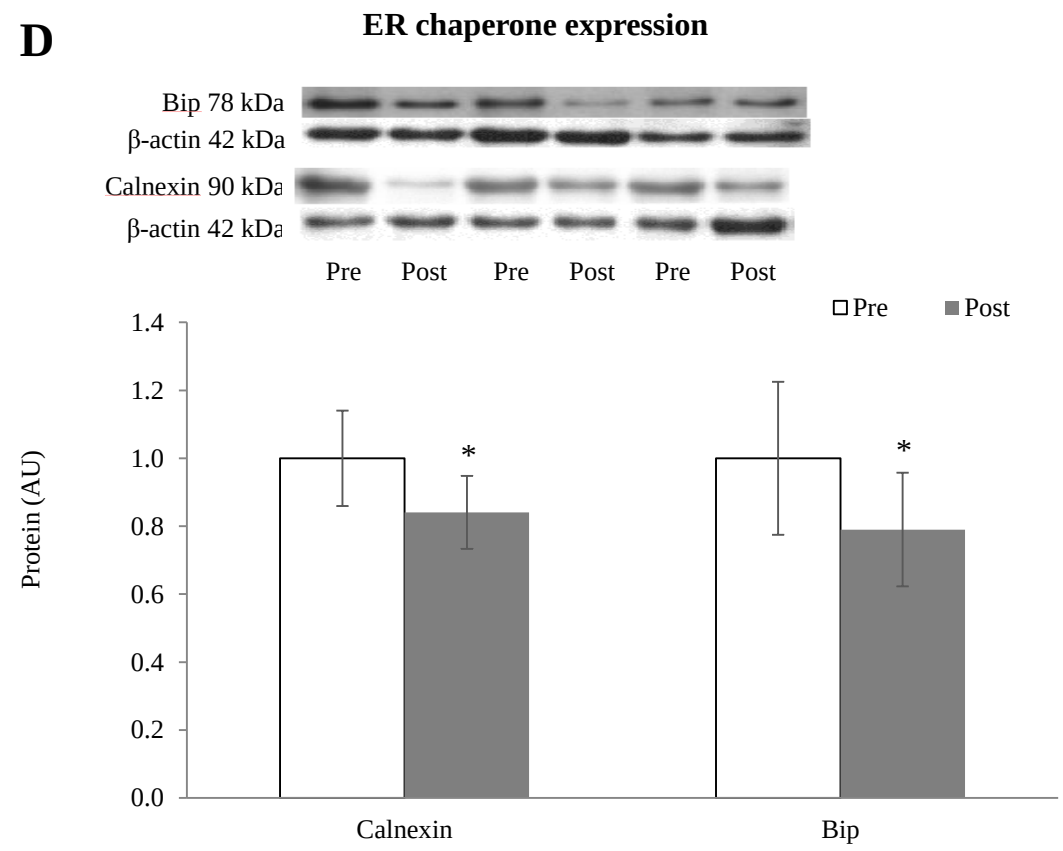
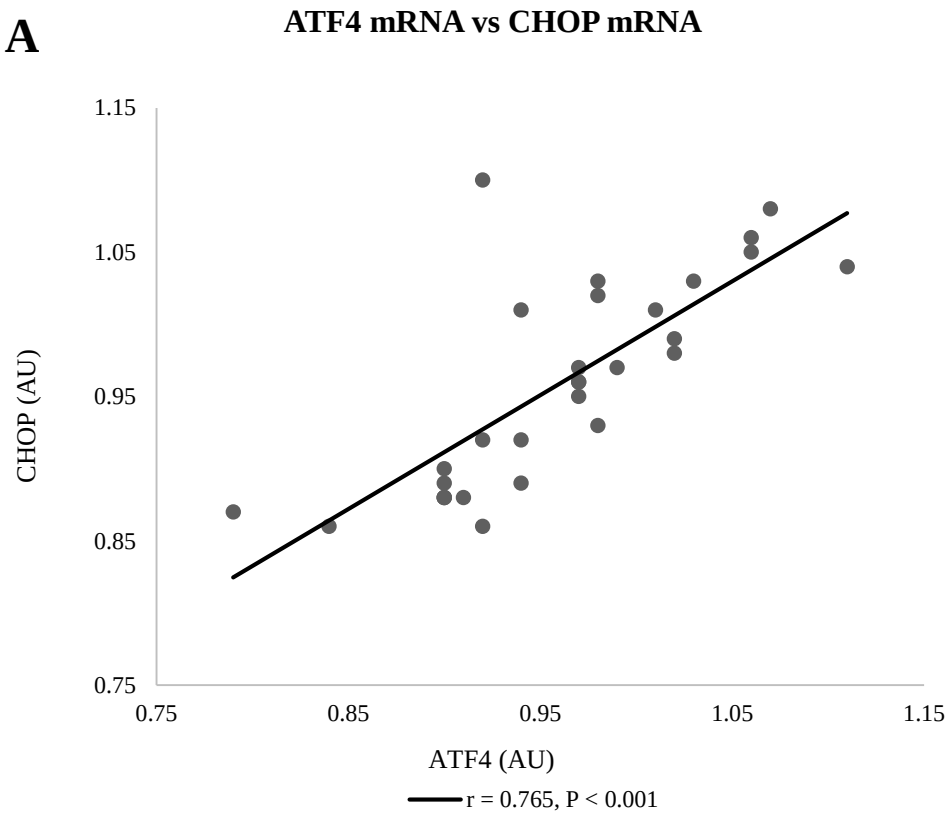

ER chaperone expression
Bip 78 kDa
β-actin 42 kDa
Calnexin 90 kDa
β-actin 42 kDa
Pre
Post
Pre
Post
Pre
Post
Pre
Post
Protein (AU)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Calnexin
Bip

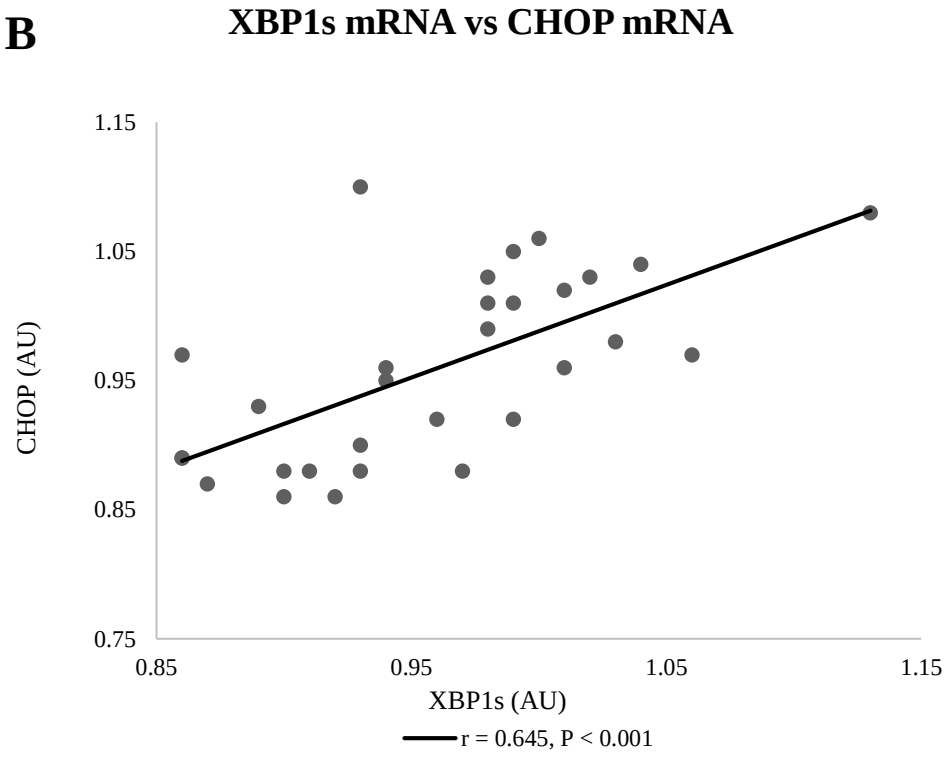
Figure 3.2.2 legend: ER stress in abdominal subcutaneous adipose tissue was reduced six months after bariatric surgery in obese T2DM subjects. ER stress markers from all three ER stress pathways and chaperones were measured in all subjects before (pre) and after (post) bariatric surgery. Gene expression of ATF4 and CHOP (A), ATF6, IRE1α and XBP1s (B). Protein expression of ATF6, pIRE1α, XBP1s (C), calnexin and bip (D). Data is expressed as mean ± SE. Pre and post values were compared and $P<0.05$ was considered statistically significant and significance levels are indicated as follows; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

3.2.3 Regulation of UPR markers was improved in adipose tissue 6 months post-surgery

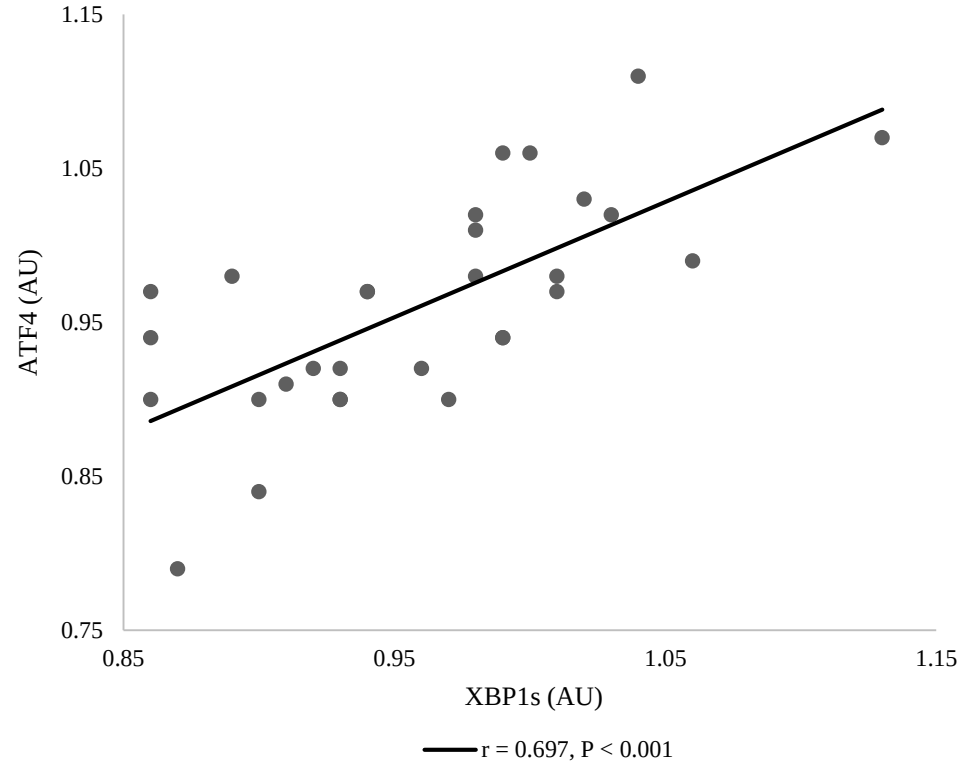
The UPR is activated in response to an accumulation of unfolded or misfolded proteins in the ER in order to restore normal cellular functions and this process is tightly regulated. In obesity this activation could be prolonged leading to increased inflammation or cellular dysfunction. PERK regulates expression of ATF4 and CHOP while IRE1α regulates synthesis of active form of XBP1, XBP1s by splicing inactive XBP1u (XBP1 unspliced) through its endoribonuclease activity. To investigate whether this regulation is improved after surgery, correlation studies were carried out. A significant correlation was observed between changes in ATF4 and CHOP mRNA expression post-surgery (Figure 3.2.3 A). There were significant correlations between the change in expression of XBP1s vs CHOP (Figure 3.2.3 B), XBP1s vs ATF4 (Figure 3.2.3 C) and pIRE1α vs calnexin (Figure 3.2.3 D; $P<0.001$). These association studies highlight the overall improved regulation of UPR pathways post-bariatric surgery.

Figure 3.2.3 ER stress signalling regulation in abdominal subcutaneous adipose tissue was improved six months after bariatric surgery





C **XBP1s mRNA vs ATF4 mRNA**



D **pIRE1α protein vs calnexin protein**

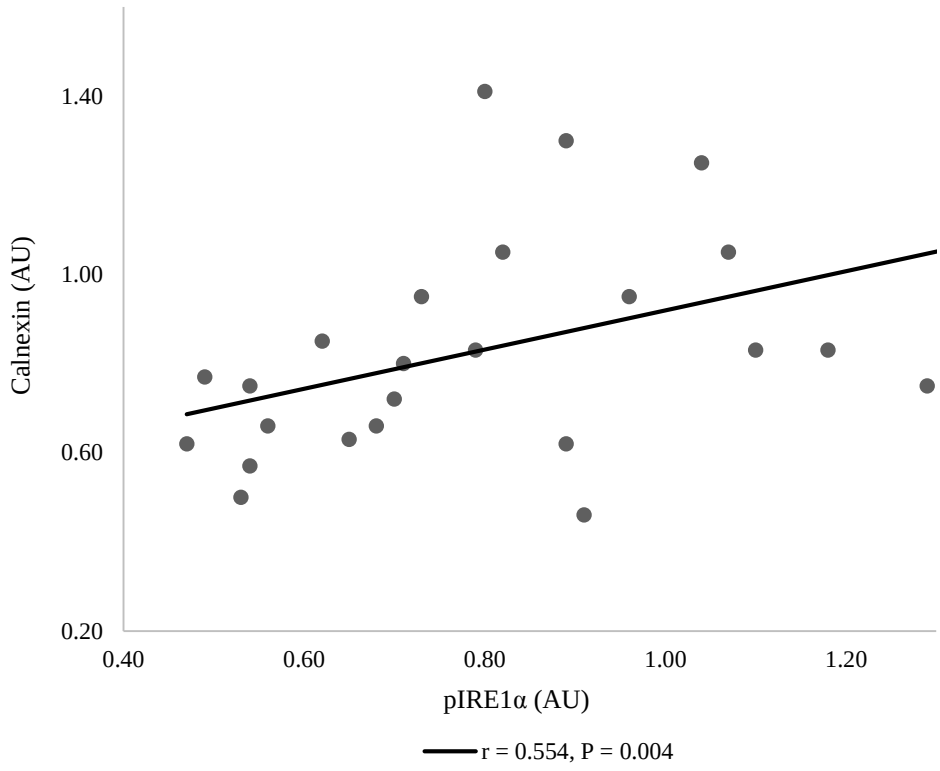
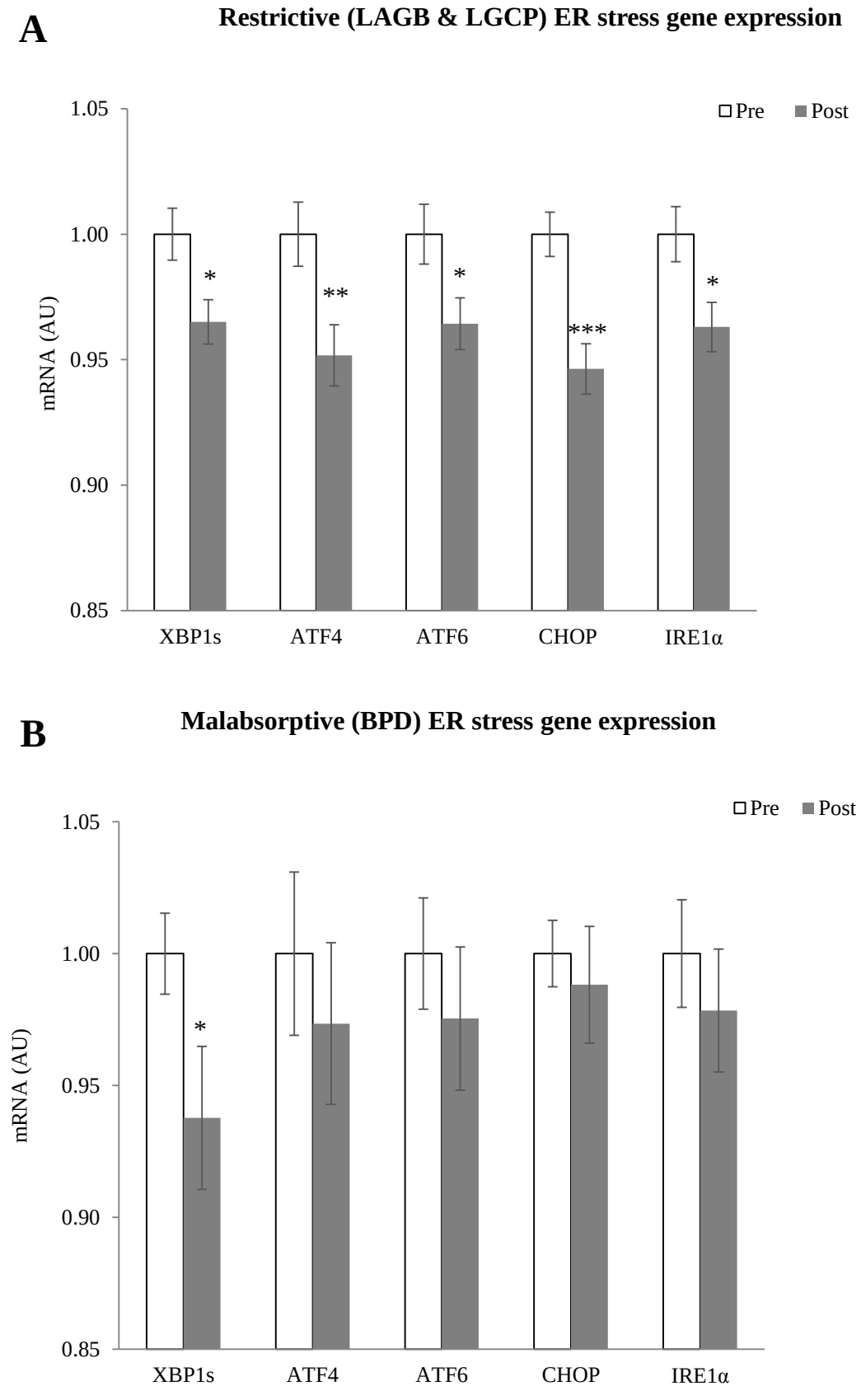


Figure 3.2.3 legend: ER stress signalling regulation in abdominal subcutaneous adipose tissue was improved six months after bariatric surgery. Correlation analysis for all subjects was undertaken between the change in ER stress signalling markers and chaperones at gene and protein expression level before and after surgery. ATF4 mRNA and CHOP mRNA (A), XBP1s mRNA and CHOP mRNA (B), XBP1s and ATF4 mRNA (C), pIRE1α protein and calnexin protein (D). $P<0.05$ was considered statistically significant and significance levels are indicated as follows; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

3.2.4 Effect of surgery type on ER stress (Restrictive versus Malabsorptive)

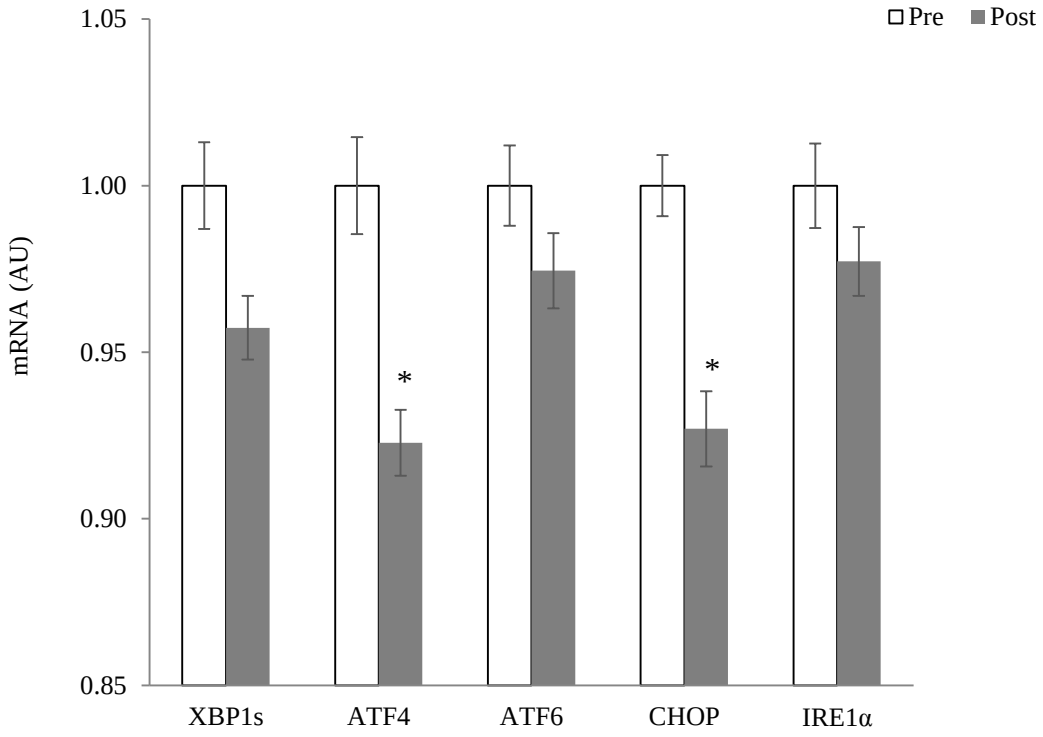
Bariatric surgery is highly effective for prevention as well as for resolving T2DM but the remission rates have been shown to vary between different surgical procedures. Therefore, the effect of each surgery type on ER stress markers in adipose tissue 6 months after each surgery was investigated. Overall the mRNA expression of XBP1s was significantly reduced across all surgery types. While all other markers studied were significantly reduced either in LAGB or LGCP surgery types but not in BPD, even though the overall trend was towards reduction for all the markers in all surgery types (Figure 3.2.4 A-D). It did not reach significance for some markers probably due to the smaller sample size for individual surgery types.

Figure 3.2.4 Post-surgery ER stress improvements in abdominal subcutaneous adipose tissue varied by bariatric surgery type



C

LAGB ER stress gene expression



D

LGCP ER stress gene expression

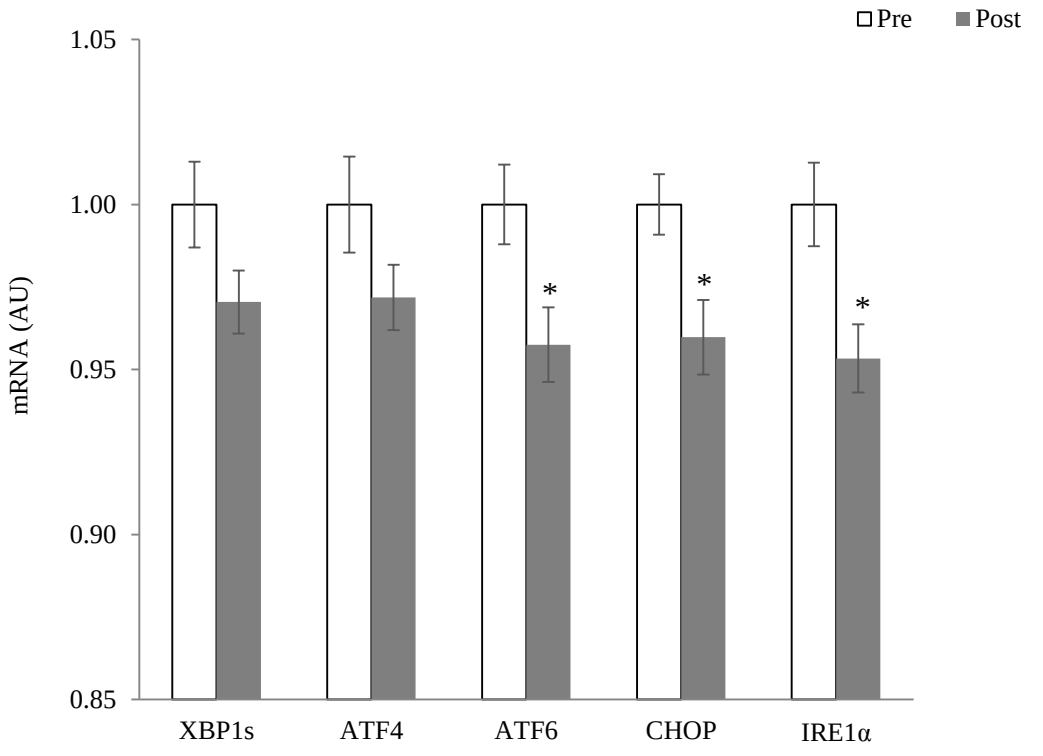


Figure 3.2.4 legend: Post-surgery ER stress improvements in abdominal subcutaneous adipose tissue varied by bariatric surgery type. XBP1s, ATF4, ATF6, CHOP, and IRE1α gene expressions were measured before (pre) and after (post) bariatric surgery, stratified by restrictive (LAGB and LGCP) (A), malabsorptive (BPD) (B), LAGB (C), and LGCP (D) surgery types. Data is expressed as mean ± SE. Pre and post values were compared and $P<0.05$ was considered statistically significant and significance levels are indicated as follows; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

3.2.5 Adipose tissue inflammation and macrophage infiltration

Adipose tissue inflammation and macrophage specific markers were assessed pre and post bariatric surgery. The pro-inflammatory markers IL-6 and CCL2 mRNA expressions were significantly reduced post-surgery (Figure 3.2.5; $P<0.05$) whilst there was no significant change in the anti-inflammatory IL-10 mRNA expression. The macrophage specific surface markers CD68 and CD14 mRNA expressions appeared to reduce post-surgery, although this did not reach significance. Further assessment of the potential link between IL-6 mRNA expression and macrophage markers showed there was no significant correlation with either CD68 or CD14.

Figure 3.2.5 Adipose tissue inflammation reduced after bariatric surgery independently of macrophage expression

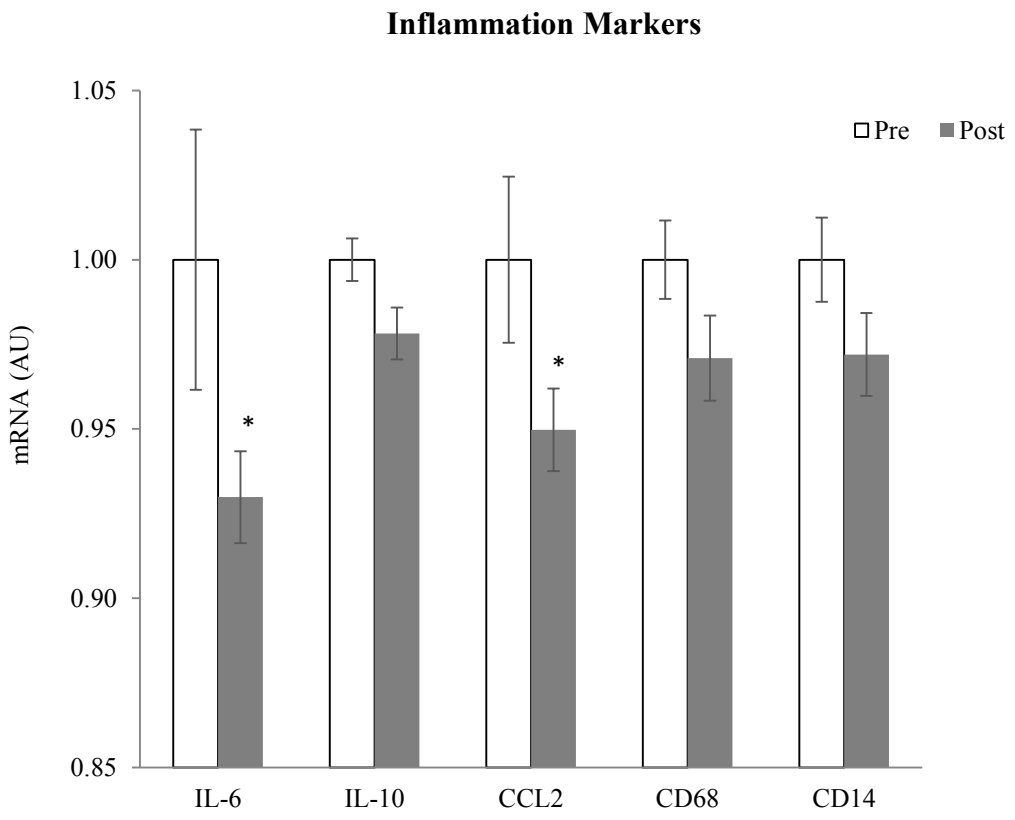


Figure 3.2.5 legend: Adipose tissue inflammation reduced after bariatric surgery independently of macrophage expression. Pro-inflammatory cytokine IL-6, anti-inflammatory cytokine IL-10, macrophage recruitment chemokine CCL2 and macrophage surface markers CD68 and CD14 gene expressions were measured for all subjects before (pre) and after (post) bariatric surgery. (IL-6; interleukin – 6, IL-10; interleukin – 10, CCL2; C-C motif ligand 2, CD68; cluster of differentiation 68, CD14; cluster of differentiation 14). Data is expressed as mean ± SE. Pre and post values were compared and $P<0.05$ was considered statistically significant and significance levels are indicated as follows; *$P<0.05$.

3.3 Discussion

This study was designed to investigate the effect of bariatric surgery types on metabolic health of obese T2DM patients. Both restrictive (LAGB and LGCP) as well as malabsorptive (BPD) surgery types were assessed for the outcome. Recent studies suggest an important role for ER stress in underlying mechanisms for insulin resistance and T2DM in obese subjects (Hummasti and Hotamisligil, 2010). The results of this study show that ER stress is activated in obese T2DM subjects and is significantly reduced after bariatric surgery. Surgery specific effects on ER stress and other metabolic parameters were observed.

The results of this study agree with previous findings in obese T2DM patients, showing that the significant weight loss six months post-bariatric surgery, comprising predominantly from reduced fat mass, resulted in marked improvements in both insulin and glucose homeostasis. More specifically, fasting insulin and glucose and HbA1c all significantly decreased suggesting a major role for excess adipose tissue in obesity-linked pathogenesis of T2DM (Bradnova, *et al.*, 2014; Nguyen and Korner, 2014). In addition, the significant weight loss post-surgery was also found to be associated with improvements in other obesity-related metabolic factors including significantly decreased LDL and TAG levels. Patients who underwent BPD had achieved higher percent excess BMI lost (% EBL) compared to the other bariatric interventions. BPD patients also had lower HbA1c, total cholesterol and HDL compared to restrictive surgeries, possibly due to their higher % EBL. There were no significant differences between surgery types in other metabolic parameters including body weight, BMI, fat mass, fasting plasma glucose, plasma insulin, or TAG.

The present study demonstrated that in obese T2DM subjects, UPR regulation in adipose tissue improved six months after bariatric surgery. In agreement with previous studies (Gregor, *et al.*, 2009) the expression of UPR markers at the level of master regulators (IRE1α and ATF6), downstream signalling effector molecules (XBP1s, ATF4 and CHOP) and key protein chaperones (Bip and Calnexin) were significantly decreased. UPR in WAT has been directly linked to insulin resistance via activation of JNK protein kinases by IRE1α (Urano, *et al.*, 2000). In this study expression of IRE1α in its active phosphorylated form and its immediate downstream target XBP1s were both significantly decreased. Furthermore, the correlation between their expressions was highly significant post-surgery ($P<0.001$), suggesting a tighter regulation.

In addition to the IRE1α pathway, the regulation of the PERK pathway was also improved following bariatric intervention. The association between the downstream targets of PERK, ATF4 and CHOP, significantly increased post-surgery suggesting improved regulation of this UPR pathway in addition to IRE1α-XBP1s pathway. The hypothesis that the ER in WAT is under a reduced demand post-surgery is supported by the finding in this study that the expression of protein chaperones BiP/Grp78 and calnexin are significantly reduced post-surgery compared to baseline and are significantly associated (Gulow, *et al.*, 2002). The expression of BiP/Grp78 protein coincides with the level of ER stress; therefore a reduction in BiP protein expression in the patients WAT post-surgery is indicative of a reduction in ER stress.

Gregor *et al.* (Gregor, *et al.*, 2009) have reported similar observations with regards to ER stress following malabsorptive bariatric surgery, i.e. gastric bypass, except for one notable difference. In their study, Gregor *et al.* observed no change to CHOP mRNA expression in spite of changes to upstream regulator p-eIF2α, in the PERK pathway and argued that CHOP expression was probably regulated by additional signals which do not respond to changes in body weight. On the contrary, this study documented significant reduction to CHOP as well as ATF4 mRNA expressions after weight loss at 6 months post-surgery. Upon further investigation CHOP expression was significantly reduced only in restrictive surgery samples (LAGB and LGCP) and not in malabsorptive surgery (BPD) (Figure 3.2.4). Therefore, our results indicate that CHOP expression is also probably affected by the type of the bariatric procedure and is regulated by yet another confounding factor that requires further investigation. In general almost all the ER stress markers were significantly reduced in restrictive compared to malabsorptive surgery.

Glucose homeostasis and WAT UPR regulation were both improved after bariatric induced weight loss and may be linked. This observation is supported by another study in *ob/ob* mice where overexpression of XBP1s has been shown to induce adiponectin multimerisation and improve glucose homeostasis (Sha, *et al.*, 2014). Another bariatric study using Roux-en-Y gastric bypass (RYGB) and LAGB samples by Bradley *et al.* (Bradley, *et al.*, 2012) has shown improved insulin sensitivity, oral glucose tolerance and beta cell function after surgery but does not provide a mechanism. The IRE1α-XBP1s pathway has now been linked to adipogenesis (Sha, *et al.*, 2009), differentiation of secretory cell types, such as

pancreatic and salivary exocrine cells and regulation of lactation (Gregor, *et al.*, 2013) in addition to regulation of glucose homeostasis.

Furthermore, since chronic inflammation of WAT has been linked to metabolic dysregulation (Wellen and Hotamisligil, 2003; Xu, *et al.*, 2003), the inflammatory status of patients' WAT was investigated. The expression of pro-inflammatory cytokine IL-6 was significantly reduced after bariatric surgery compared to baseline while no significant change was observed in mRNA expression of anti-inflammatory IL-10. IL-6 expression has been linked to macrophage infiltration in adipose tissue (Di Gregorio, *et al.*, 2005), therefore to establish whether macrophage played any role in the observed changes, macrophage surface markers (CD14 and CD68) and macrophage chemoattractant chemokine ligand 2 (CCL2), also referred to as monocyte chemotactic protein 1 (MCP1), were measured. There were no significant changes in CD14 or CD68 expression before and after surgery, although there was a significant reduction in CCL2 expression (figure 3.2.5). However, none correlated with IL-6 expression, therefore the IL-6 reduced expression was likely to be independent of macrophages.

3.4 Conclusions

This study has demonstrated that both restrictive and malabsorptive bariatric interventions are effective weight loss interventions for obese T2DM patients resulting in significantly improved glucose and insulin levels six months after surgery. While malabsorptive bariatric surgery involves more complicated procedures and requires long-term vitamin and other supplementation, the restrictive bariatric procedures are less complicated and induce almost equal benefits. In this study adipose tissue health was better following the two restrictive procedures (LAGB and LGCP) compared to BPD as shown by higher reduction of ER stress markers as well as improved regulation of CHOP. The data from Bradley *et al.* (Bradley, *et al.*, 2012) obtained from non-diabetic obese patients did not specifically support malabsorptive (RYGB) over restrictive surgery type (LAGB) and suggested that further studies were required which could apply to obese patients with T2DM. This study offers novel data in that direction indicating that while the clinical outcomes regarding parameters, such as % EBL and HbA1c, were better following BPD, the adipose tissue health was significantly improved following the restrictive procedures. The clinical implications of these findings in the longer term require further investigations. Overall, all the surgery types investigated significantly improved the metabolic health of obese diabetics included in this study irrespective of surgery type.

Chapter 4: Regulation of ER stress during adipogenesis

4.1 Introduction

Obesity, characterised by expansion of WAT, is the biggest risk factor for developing the metabolic disease T2DM (Guh, *et al.*, 2009). WAT is a multifunctional organ that plays a key role in energy regulation and metabolism. People with T2DM who lost weight predominantly through a reduction in WAT mass following bariatric surgery improved their glucose and lipid metabolic profile (table 3.2.1). Therefore excess WAT in obesity may lead to dysregulation and impaired metabolic health. However, not all WAT is equal. WAT, like skeletal muscle, is distributed through the body in discrete depots (Shen, *et al.*, 2003). SAT represents about 85 % of all body fat and is unequally distributed through the body, with larger depots in the abdomen, thigh, gluteal and mammary region (Frayn and Karpe, 2014). Abdominal VAT depots are deeper in the body than SAT and include omental that connects to the stomach. The functions and properties of each WAT depot overlap but there are distinct differences (White and Tchoukalova, 2014). Accumulation of adipose tissue in different depots has differential risk associated with development of T2DM. Accumulation of VAT and SAT in the abdomen is referred to as 'central obesity' and confers a higher risk of developing T2DM compared to lower body accumulation of fat. Therefore abdominal WAT was investigated. In central obesity, different depths of abdominal SAT are functionally distinct and independently correlate with metabolic complications of obesity (Smith, *et al.*, 2001). These observations have been supported by several studies, reviewed by Frayn *et al.* (Frayn, *et al.*, 2003). Lipolysis, TAG synthesis and storage, and protein secretion varies between WAT depots as reviewed by Lee *et al.* (Lee, *et al.*, 2013). The difference in risk of developing T2DM conferred by different WAT depots is likely to be due to a

combination of WAT properties including lipid and carbohydrate metabolism (lipolysis, lipid accumulation and glucose uptake) and systemic signalling (protein and lipid secretion and innervation).

In order to accommodate the energy requirements of the body WAT is able to expand and contract during adult life despite most development during prenatal and early postnatal life (Poulos, *et al.*, 2010). WAT expansion occurs primarily in two ways, by hyperplasia or hypertrophy. Evidence suggests obesity complications in humans result from the inability of SAT to appropriately expand and store lipids, and consequently ectopic fat deposition and lipotoxicity contribute to insulin resistance (Virtue and Vidal-Puig, 2010). Impaired WAT expandability is mainly due to impaired adipogenesis (Isakson, *et al.*, 2009). Adipocyte progenitors in human AT have been found to be depot-specific (Tchkonia, *et al.*, 2007) and differences between adipocytes derived from SAT and VAT have been documented (Kovsan, *et al.*, 2009). Adipocytes are central to metabolic regulation (Rosen and Spiegelman, 2006) and therefore differences in adipocyte progenitors may contribute towards regional variation in WAT function and development (Baglioni, *et al.*, 2012). This may in part explain the depot-specific differences in metabolic function and association with relative risk of developing T2DM (Karastergiou, *et al.*, 2013). However the underlying reasons why depot specific differences in adipocytes exist requires clarification.

Within adipocytes the ER plays a key role in metabolic regulation. ER stress can impair ER integrity and influence metabolism and function of adipocytes. Weight loss reduced ER stress and improved metabolic profile in humans (chapter 3). ER

stress might be directly linked to metabolism since abdominal SAT XBP1s correlated with plasma glucose after bariatric surgery induced WAT loss in humans (figure 3.2.3). Furthermore, the IRE1α-XBP1s UPR pathway is essential for adipogenesis in mouse cells (Sha, *et al.*, 2009). However XBP1s has not been studied in human adipogenesis. The underlying differences between WAT depots may be due to different levels of IRE1α and or XBP1s expression in adipogenesis. Scherer's group have expressed an urgency for understanding the underlying mechanisms of adipogenesis and highlighted that it will bring potential clinical benefit for combating metabolic disease (Wang, *et al.*, 2013).

Therefore the aims of this study were to investigate the extent of adipogenesis and expression of IRE1α and XBP1s during adipogenesis in preadipocytes from paired human abdominal SAT and VAT depots and whether the IRE1α-XBP1s UPR pathway is essential in human adipogenesis. To assess depot specific differences in adipogenesis paired primary human abdominal SAT and VAT from lean and obese non-diabetic subjects were collected. Preadipocytes were extracted from WAT depots, cultured and adipogenesis initiated. To assess the effect of ER stress on adipogenesis, TM was administered during early adipogenesis. To assess whether the IRE1α-XBP1s pathway is essential during human adipogenesis, IRE1α endonuclease activity was selectively blocked with the small molecule inhibitor 4μ8C during early adipogenesis. In order to further assess the role of ER stress on adipogenesis a human adipocyte cell line Chub-S7 was used (Darimont, *et al.*, 2003). Functional differences between mesenchymal stem cell populations are reflected by their transcriptome (Jansen, *et al.*, 2010). Therefore IRE1α and

XBP1s mRNA expressions were measured during adipogenesis in control conditions and following ER stress induction and XBP1s inhibition.

4.2 Results

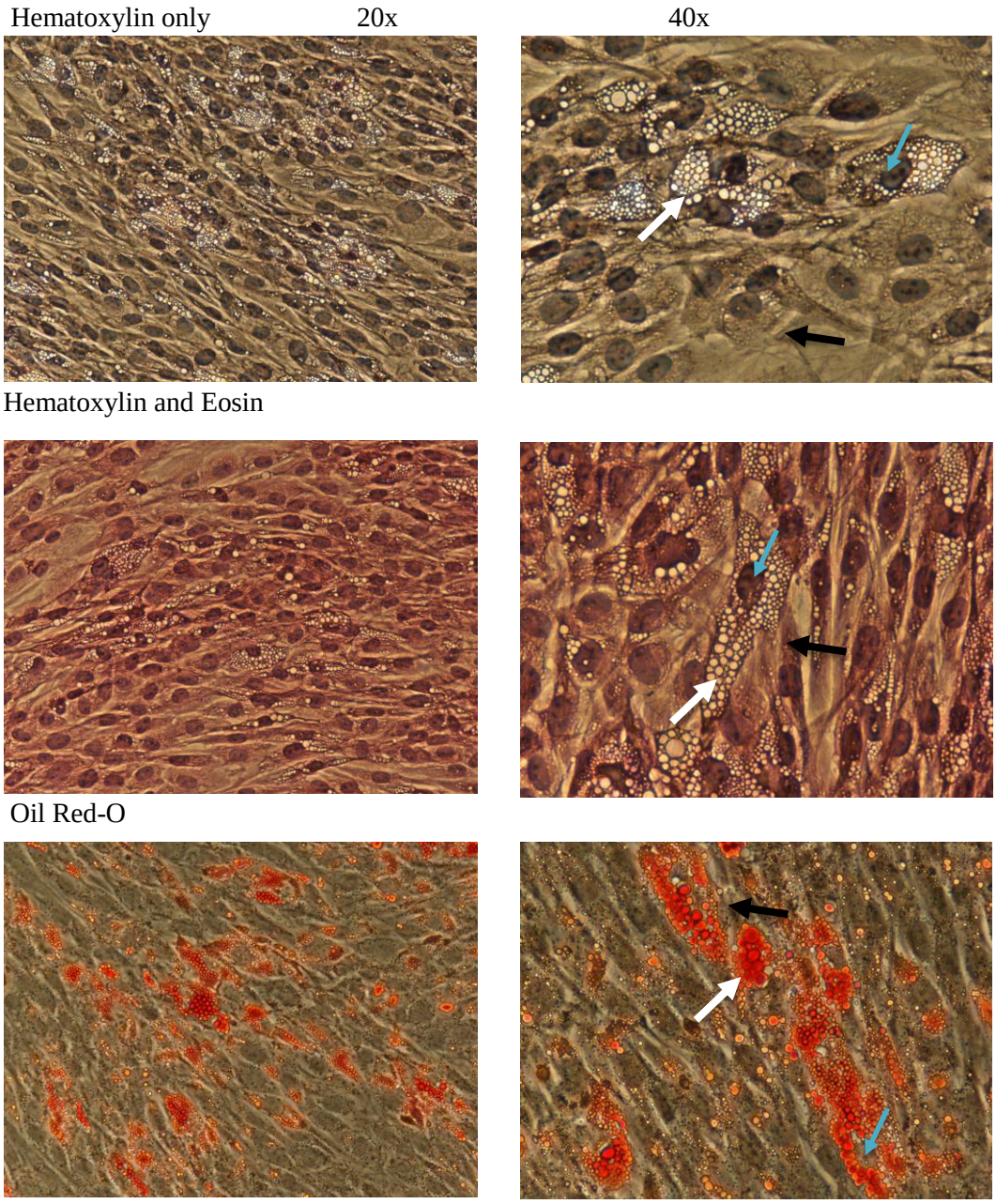
4.2.1 Cultured primary human preadipocytes had depot and BMI specific differences in lipid metabolism during adipogenesis

Preadipocytes were extracted from primary human tissue, cultured and adipogenesis stimulated. On day 14 after induction of adipogenesis staining was performed (figure 4.2.1 A). Hematoxylin staining dyed the basophilic nucleus dark blue (blue arrows in figure 1.4.1 A) and indicated that lipid droplets (white arrows in figure 1.4.1 A), brightly reflecting circular shapes, formed in the cytoplasm (black arrows in figure 1.4.1 A) around the nucleus. Eosin staining dyed the acidophilic cytoplasm pink and in combination with hematoxylin staining it showed that lipid droplets can occupy virtually the entire adipocyte cytoplasm. ORO staining dyed TAG and lipids red and provided a clearer indication of lipid accumulation compared to HE staining. Therefore ORO staining was used as an indicator of adipogenesis during subsequent adipogenesis experiments. Preadipocytes derived from lean and obese Abd SAT and VAT depots had adipogenesis induced and ORO staining was performed every 48 hours up to 14 days (figure 4.2.1 B – D). Preadipocytes from obese subjects accumulated more lipids during adipogenesis than from lean. Preadipocytes derived from VAT accumulated more lipids than derived from SAT. Lipolysis, an indicator of adipocyte function, was assessed during adipogenesis every 48 hours by measuring free glycerol in conditioned media (figure 4.2.1 E). In preadipocytes

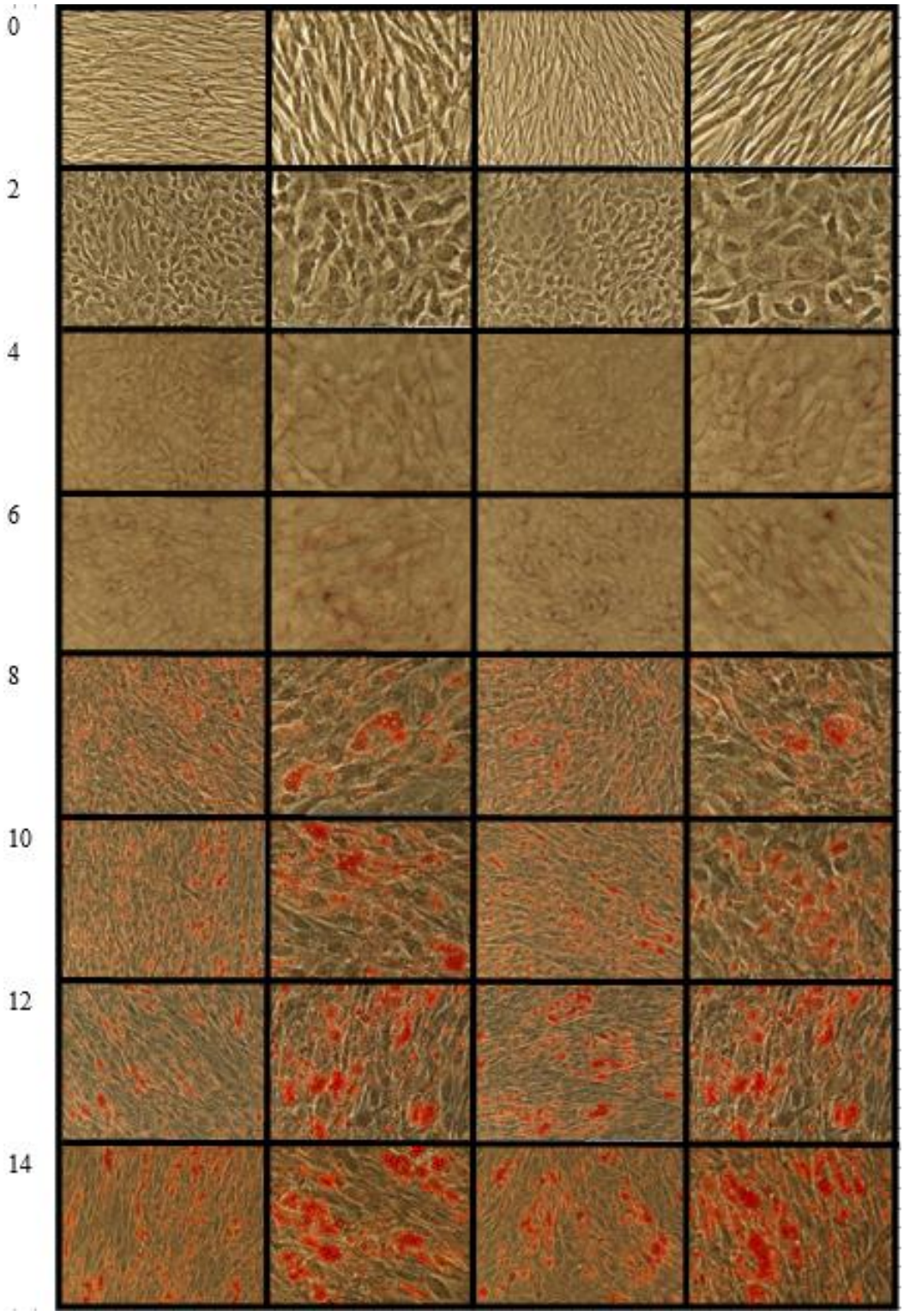
derived from SAT, those derived from obese subjects underwent more lipolysis than from lean. However in VAT derived preadipocytes, those derived from lean subjects underwent more lipolysis than from obese.

Figure 4.2.1 Primary human adipocytes had depot and BMI specific differences in lipid accumulation and lipolysis during adipogenesis

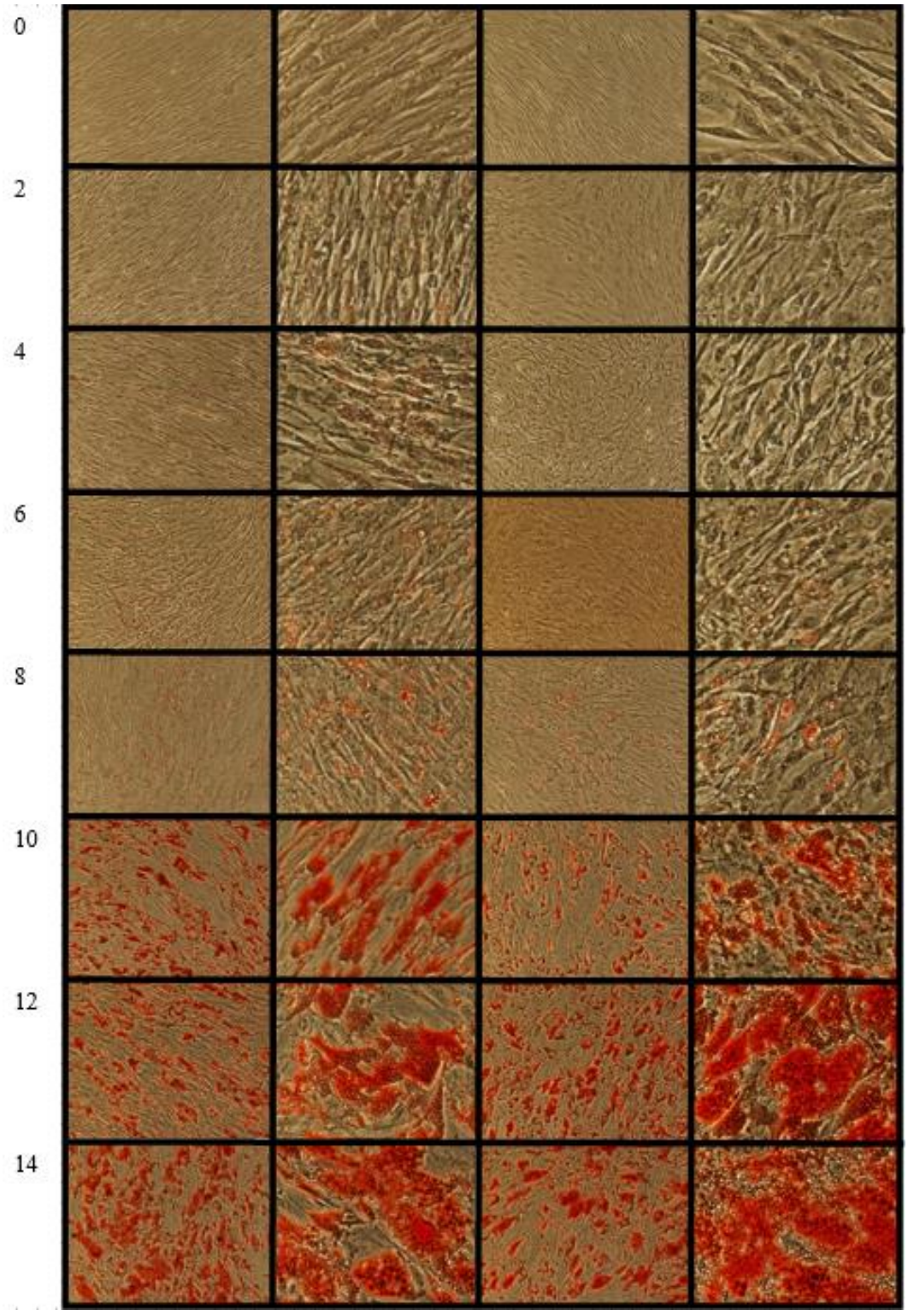
A **Adipocyte staining**



B **Lipid accumulation during lean and obese abdominal subcutaneous adipocyte adipogenesis**

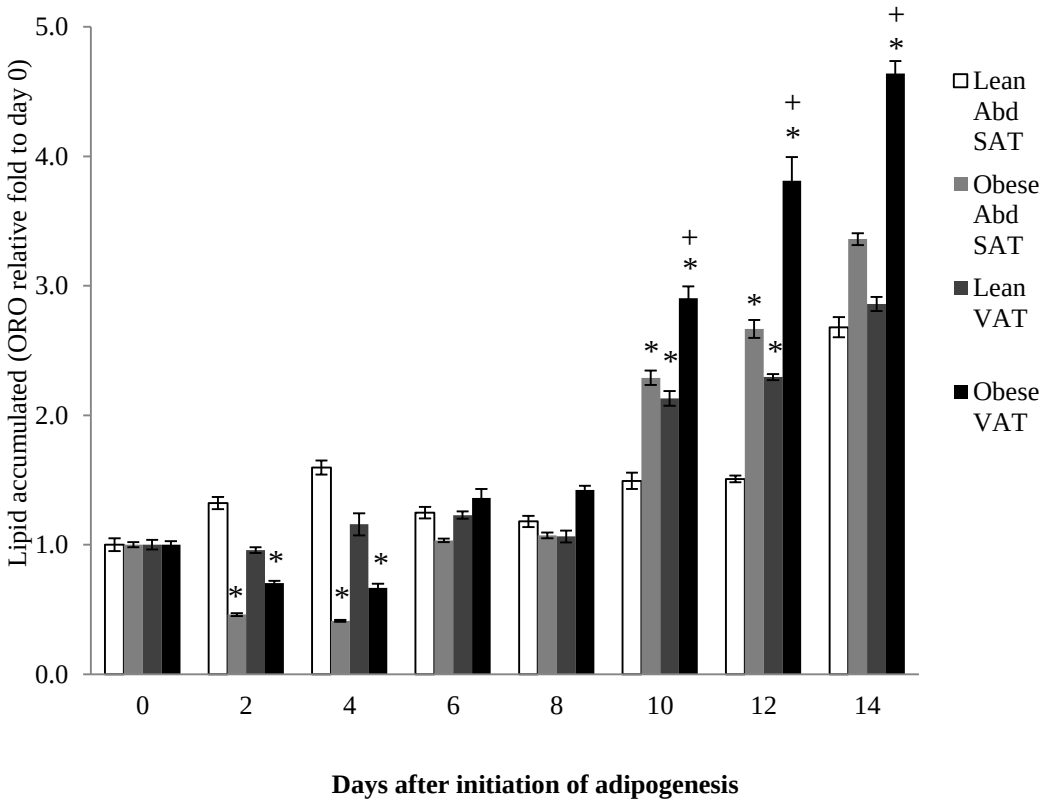


C **Lipid accumulation during lean and obese visceral adipocyte adipogenesis**



D

Adipocyte lipid accumulation during adipogenesis



E

Adipocyte lipolysis during adipogenesis

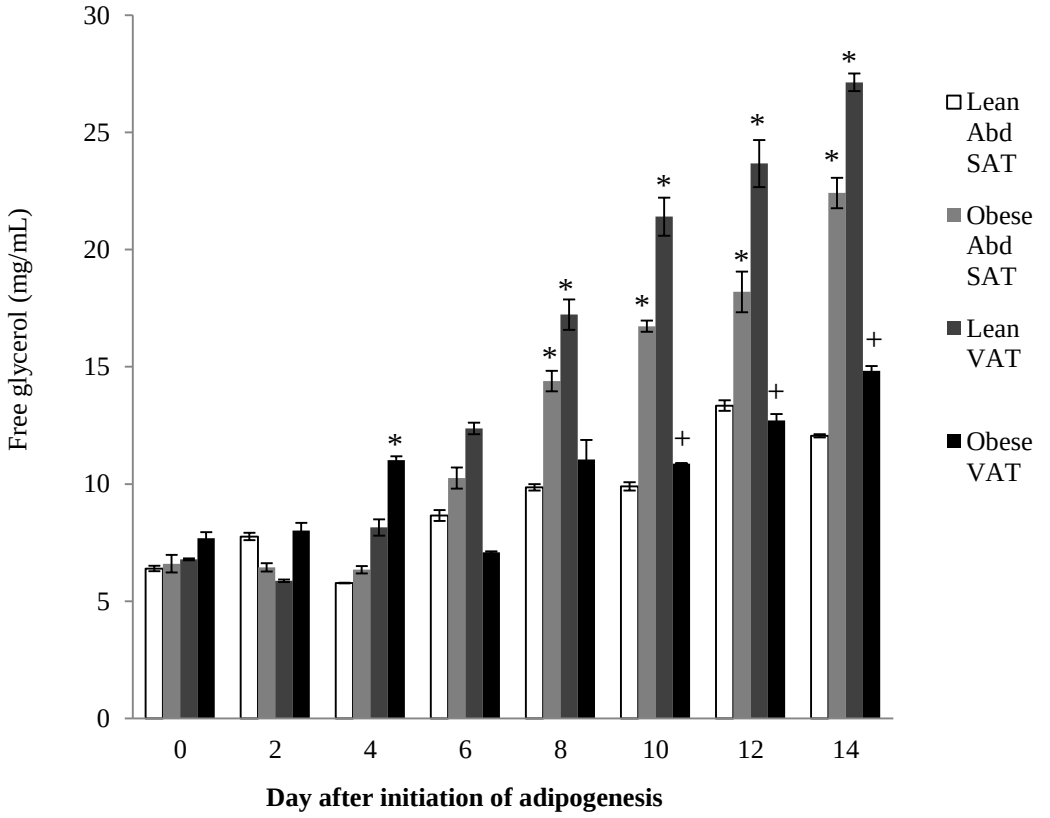


Figure 4.2.1 legend: Primary human adipocytes had depot and BMI specific differences in lipid accumulation and lipolysis during adipogenesis. Preadipocytes from paired abdominal subcutaneous (Abd SAT) and visceral (VAT) depots from lean and obese subjects were cultured and had adipogenesis induced for 14 days. Hematoxylin and eosin (HE) and oil red-O (ORO) staining were compared in Abd SAT obese adipocytes on day 14 after induction of adipogenesis (A), nucleus - blue arrows, lipid droplets - white arrows, cytoplasm - black arrows. Images of ORO stained adipocytes from lean and obese Abd SAT (B) and VAT (C) were compared and lipid accumulation (D) and lipolysis (E) was measured every 48 hours. Data is expressed as mean ± SE. Significantly different values at each time point are indicated as follows: * $P<0.05$ compared to lean Abd SAT, + $P<0.05$ compared to lean VAT.

4.2.2 Cultured primary human preadipocytes had depot and BMI specific differences in glucose uptake during adipogenesis: VAT is insulin resistant

To further characterise Abd SAT and VAT lean and obese adipogenesis, functional glucose uptake and insulin sensitivity was assessed 14 days after induction of adipogenesis (figure 4.2.2). SAT adipocytes from lean and obese subjects significantly increased glucose uptake after insulin stimulation ($P<0.05$ - $P<0.001$), however the adipocytes from lean had a greater increase in insulin stimulated glucose uptake compared to obese. VAT adipocytes from lean and obese subjects increased glucose uptake after insulin stimulation but did not reach significance. Their basal glucose uptake was significantly higher ($P<0.05$) than from SAT. The basal glucose uptake was significantly higher in obese subjects

compared to lean in both Abd SAT and VAT depots (P<0.05 and P<0.01 respectively).

Figure 4.2.2 Glucose uptake and insulin sensitivity in primary human adipocytes varied depending on adipose tissue depot and BMI

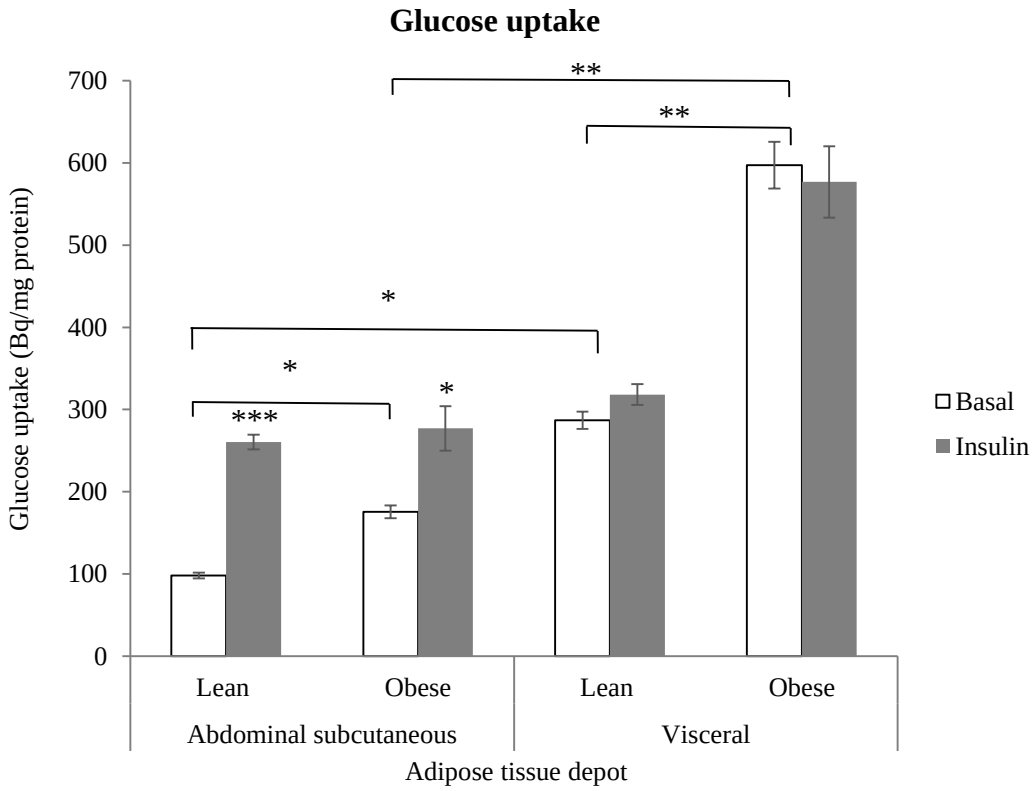


Figure 4.2.2 legend: Glucose uptake and insulin sensitivity in primary human adipocytes varied depending on adipose tissue depot and BMI. Paired Abd SAT and VAT preadipocytes from lean and obese subjects were cultured and differentiated for 14 days. On day 14, adipocytes were subjected to basal and insulin stimulated glucose uptake assays. Data is expressed as mean ± SE. Significantly different values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

4.2.3 The IRE1α - XBP1s UPR pathway was elevated during adipogenesis in obese subjects compared to lean

ER stress in WAT is elevated in obese compared to lean subjects and ER stress was reduced in obese subjects after bariatric surgery (chapter 3). To compare ER stress during adipogenesis in lean and obese SAT and VAT derived preadipocytes, IRE1α and XBP1s mRNA expression was measured every 48 hours during adipogenesis (figure 4.2.3 A – B). IRE1α and XBP1s mRNA expression in the lean SAT and VAT derived adipocytes decreased after the induction of adipogenesis. In contrast, IRE1α and XBP1s mRNA expression in the obese SAT and VAT derived adipocytes increased after the onset of adipogenesis in two phases. XBP1s mRNA expression was higher than day 0 for the entire duration of adipogenesis in obese SAT and VAT derived adipocytes.

Figure 4.2.3 The IRE1α - XBP1s pathway was elevated during adipogenesis in adipocytes derived from obese subjects compared to lean

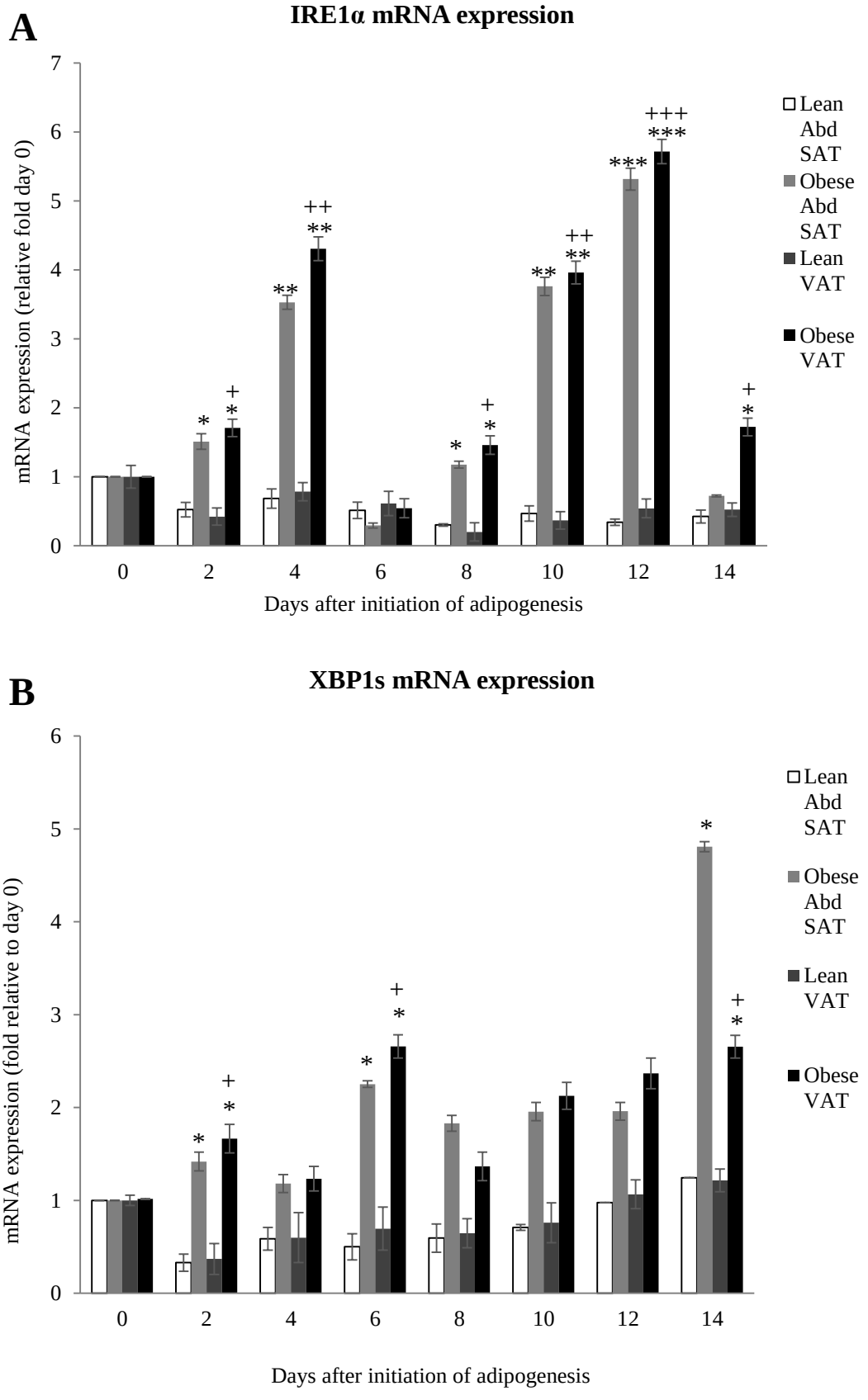


Figure 4.2.3 legend: The IRE1α - XBP1s pathway was elevated during adipogenesis in adipocytes derived from obese subjects compared to lean. Preadipocytes from paired Abd SAT and VAT depots in lean and obese subjects were differentiated for 14 days. IRE1α (A) and XBP1s (B) mRNA expression was measured every 48 hours. Data is expressed as mean ± SE. Significantly different values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

4.2.4 Chronic disruption of the UPR after induction of adipogenesis inhibited adipogenic capacity

The expression of UPR markers IRE1α and XBP1s varied between lean and obese derived preadipocytes during adipogenesis. To assess whether ER stress influences adipogenesis, ER stress was induced during early adipogenesis with TM. Lipid accumulation was considered a marker of adipogenic capacity and measured by ORO staining 14 days after the initiation of adipogenesis (figure 4.2.4 A – B). Lipid accumulation in adipocytes from both SAT and VAT depots was significantly reduced (P<0.001) following TM treatment compared to control. The IRE1α - XBP1s UPR pathway was differentially expressed during adipogenesis in lean and obese derived preadipocytes. To assess whether the UPR pathway is necessary during adipogenesis IRE1α endonuclease activity was inhibited during early adipogenesis with 4μ8C at two concentrations; 16 μM and 32 μM. Lipid accumulation was considered a marker of adipogenic capacity and measured by ORO staining 14 days after the initiation of adipogenesis (figure 4.2.4 C – D). Lipid accumulation was significantly reduced (P<0.001) in adipocytes derived from both SAT and VAT following 4μ8C addition. The higher concentration of inhibitor resulted in lower lipid accumulation.

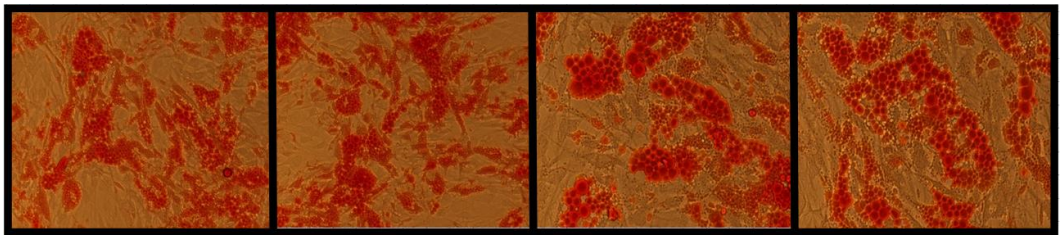
Figure 4.2.4 Chronic disruption of the UPR after induction of adipogenesis inhibited adipogenic capacity

A

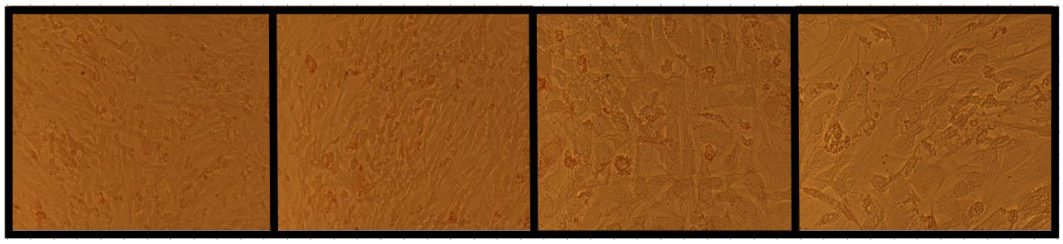
Tunicamycin (TM) induced ER stress during early adipogenesis blocked lipid accumulation

20x 20x 40x 40x

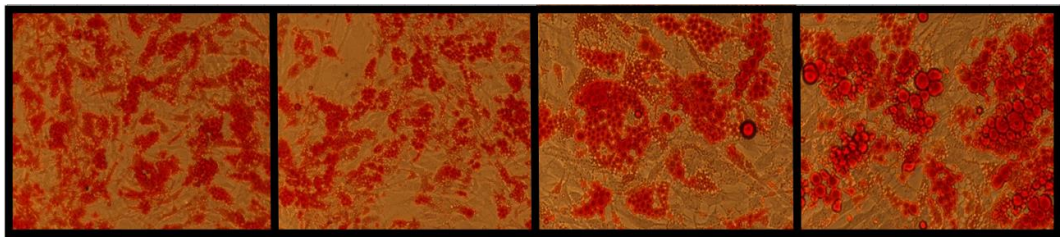
Abd SAT day 14 control



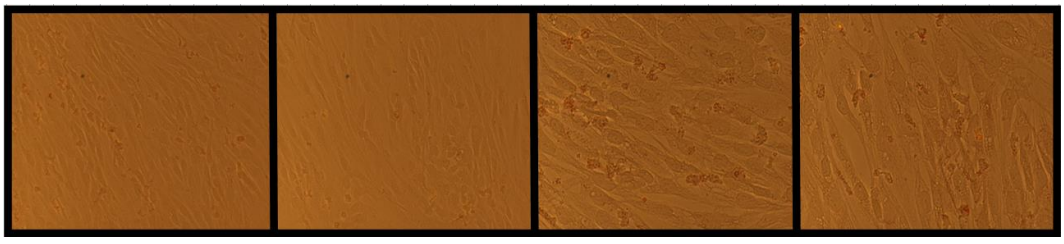
Abd SAT day 14 after TM 1.0 µg/mL day 0 – 4



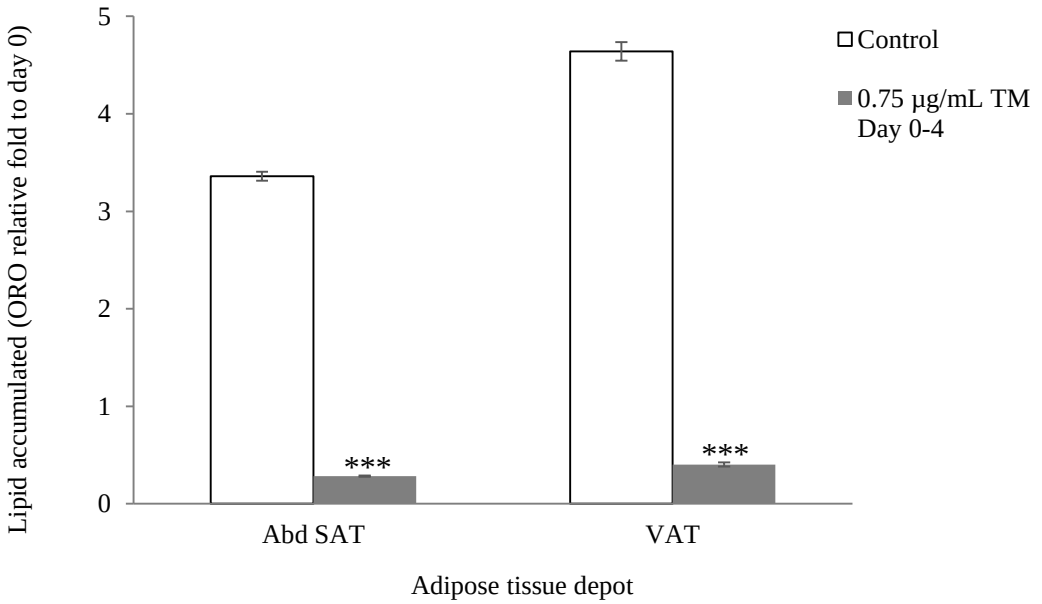
VAT day 14 control



VAT day 14 after TM 1.0 µg/mL day 0 – 4

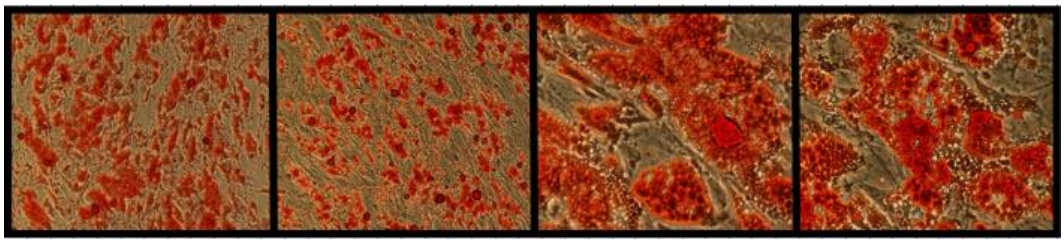


B **Tunicamycin (TM) induced ER stress during adipogenesis blocked lipid accumulation**

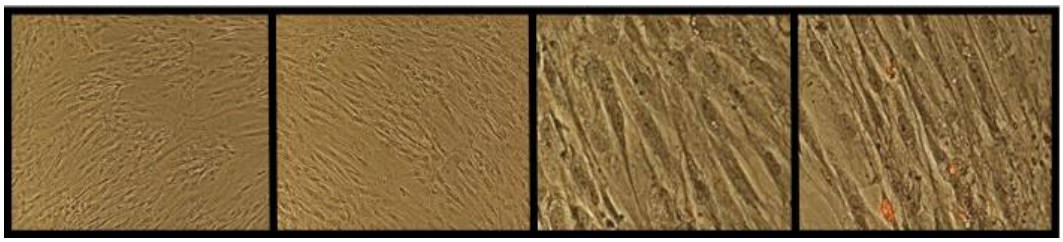


C **4µ8C blocked lipid accumulation**

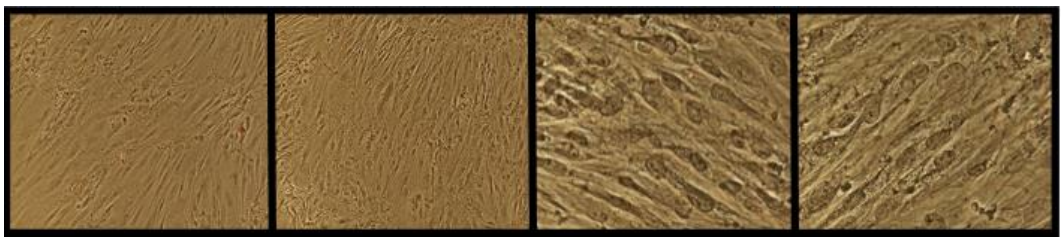
Abd SAT day 14 control



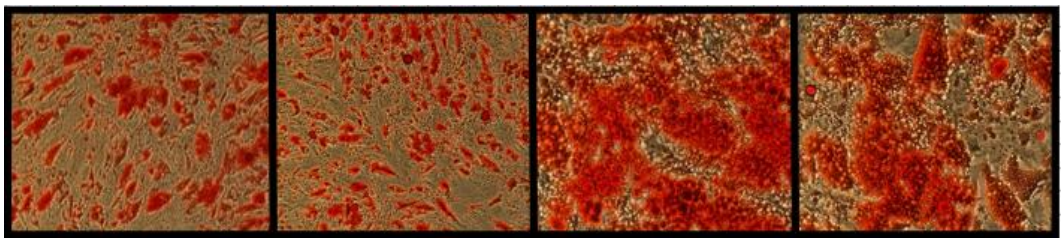
Abd SAT day 14 following 16 µM 4µ8c day 0 – 4



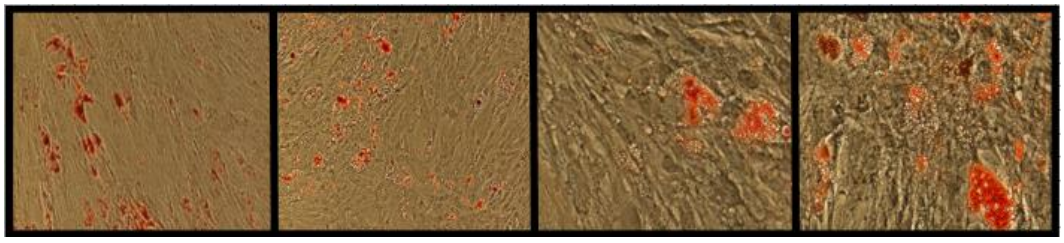
Abd SAT day 14 following 32 µM 4µ8c day 0 – 4



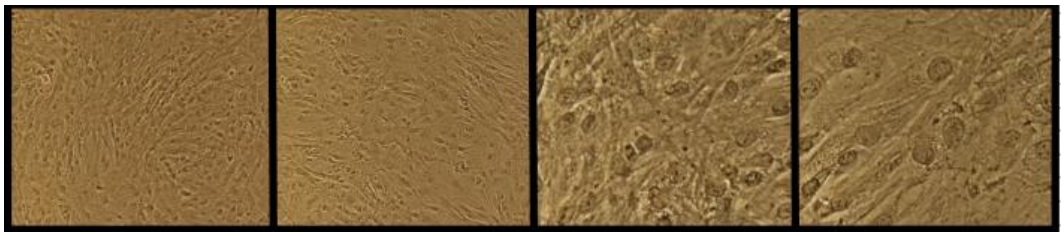
VAT day 14 control



VAT day 14 following 16 µM 4µ8c day 0 – 4



VAT day 14 following 32 µM 4µ8c day 0 – 4



D

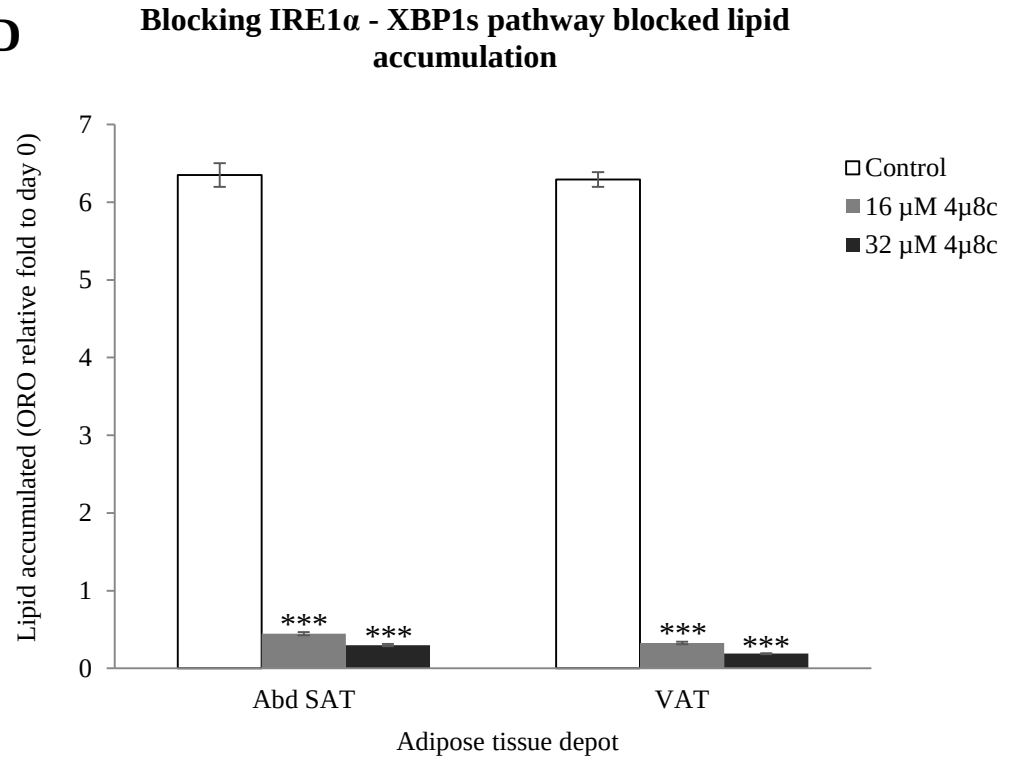
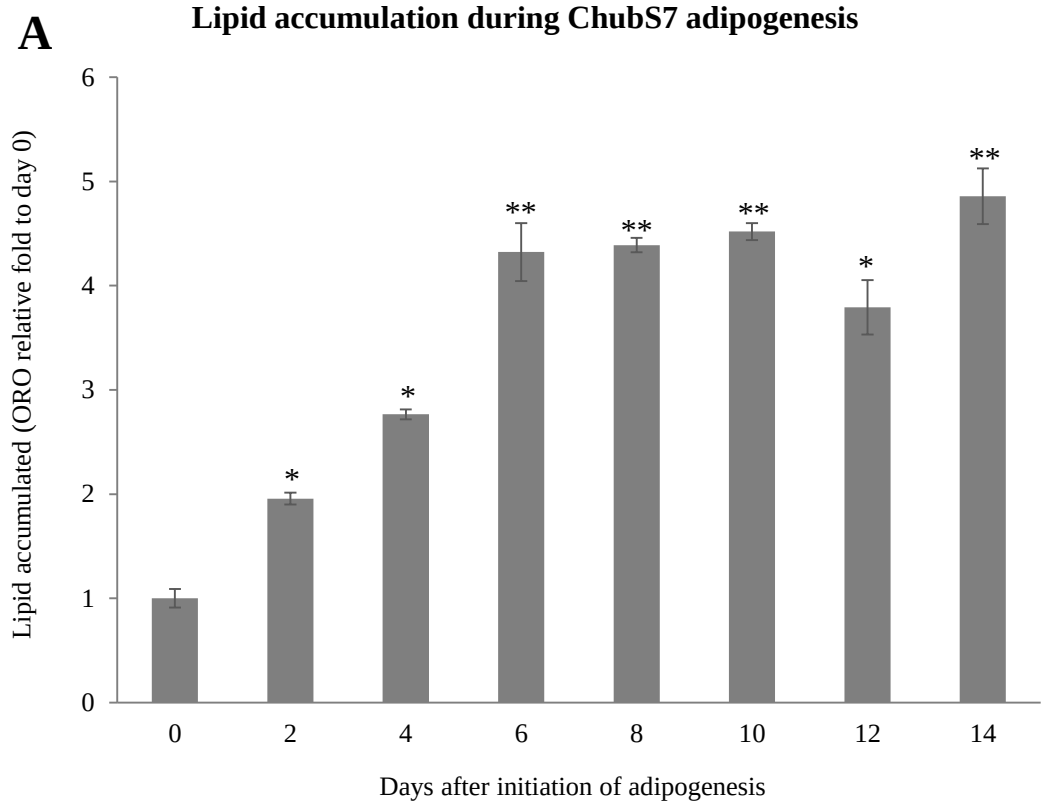


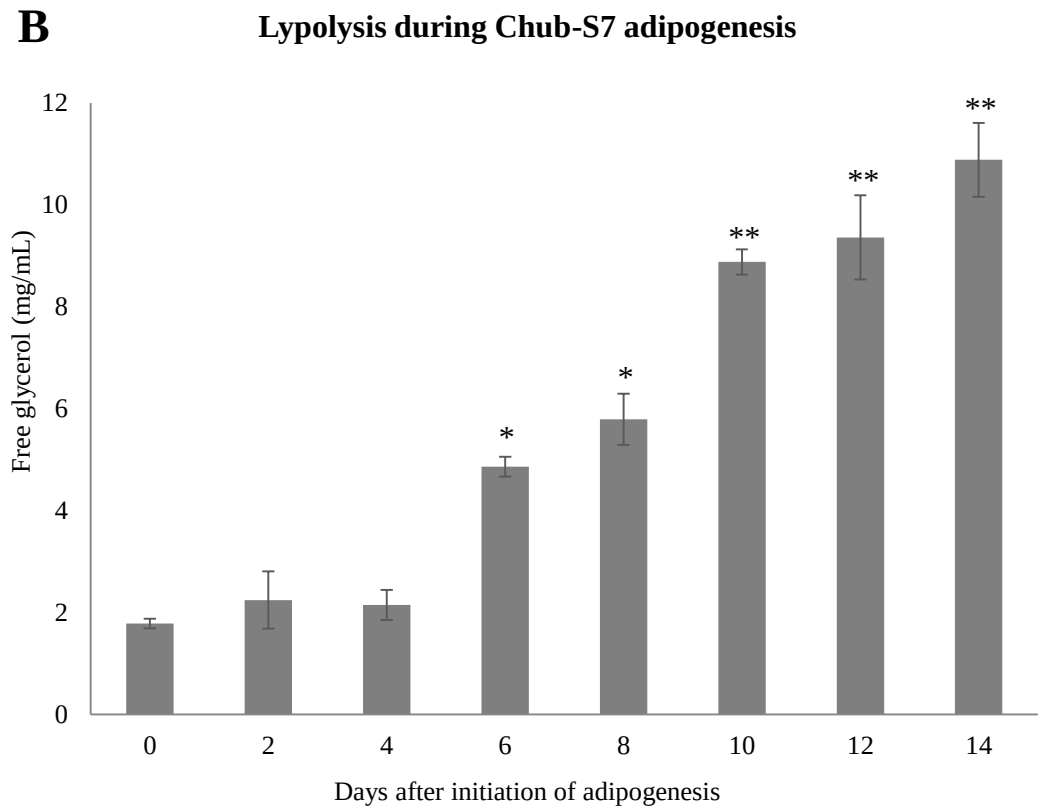
Figure 4.2.4 legend: Chronic disruption of the UPR after induction of adipogenesis inhibited adipogenic capacity. Paired Abd SAT and VAT preadipocytes from lean subjects were cultured and differentiated for 14 days. Adipocytes were subjected to DMSO (control) or 1.0 μg/mL TM (A - B), or 16 μM or 32 μM 4μ8C (C - D) from induction of adipogenesis for 96 hours (day 0 to 4) and then treatments were withdrawn. On day 14 after induction of adipogenesis, adipocytes were subjected to ORO staining and images captured. Data is expressed as mean ± SE. Significantly different values are indicated as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

4.2.5 To investigate the IRE1α - XBP1s pathway further a reproducible model system, human preadipocyte cell line ChubS7, was used

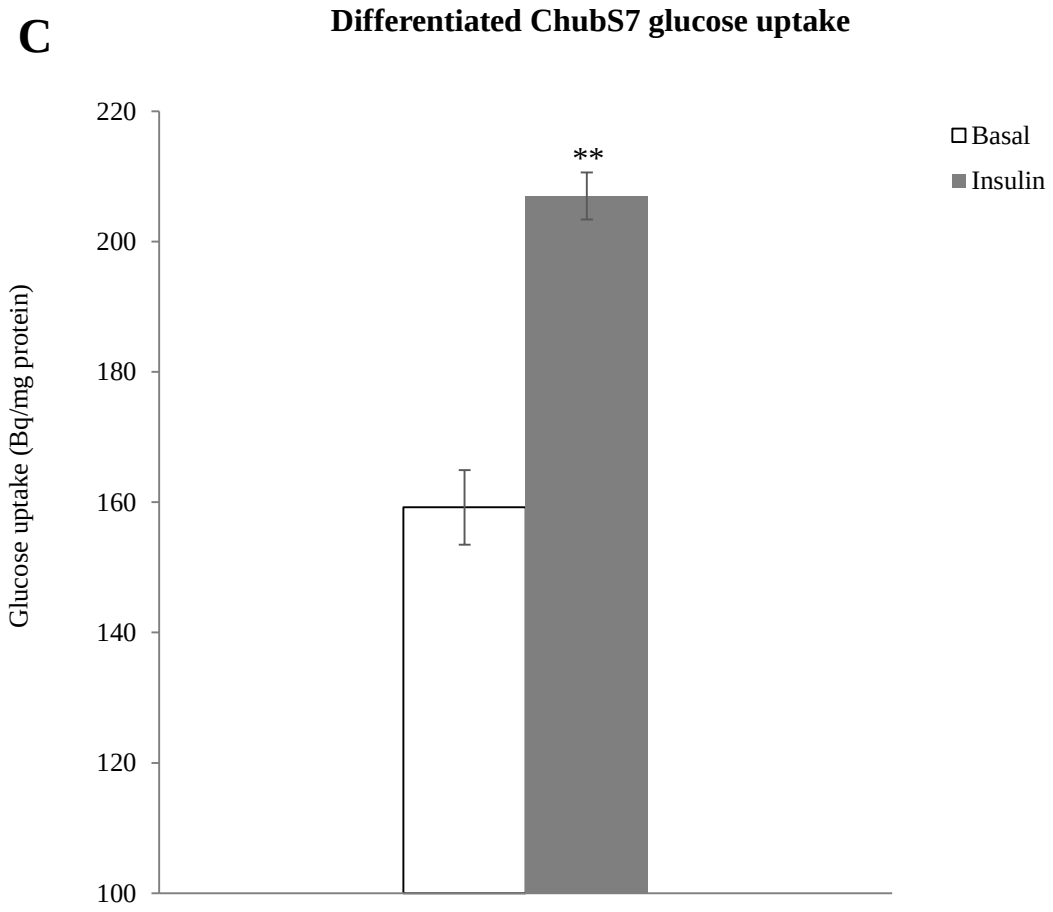
In order to further assess the role of IRE1α - XBP1s during adipogenesis a suitable, reliable and consistent human preadipocyte model system was required. To assess ChubS7 adipogenic capacity and ER stress expression, adipogenesis was induced in ChubS7 preadipocytes for 14 days. Successful adipogenesis was confirmed by increased lipid accumulation, lipolysis, insulin stimulated glucose uptake and increased gene expression of adipogenesis regulators (figure 4.2.5 A – D). IRE1α and XBP1s mRNA expression was assessed every 48 hours (figure 4.2.5 E). IRE1α expression initially decreased, then gradually increased to the same level as day 0. XBP1s expression followed the same pattern of expression as IRE1α although remained below the day 0 level.

Figure 4.2.5 To investigate the IRE1α - XBP1s pathway further, a reproducible model system, human preadipocyte cell line ChubS7, was used

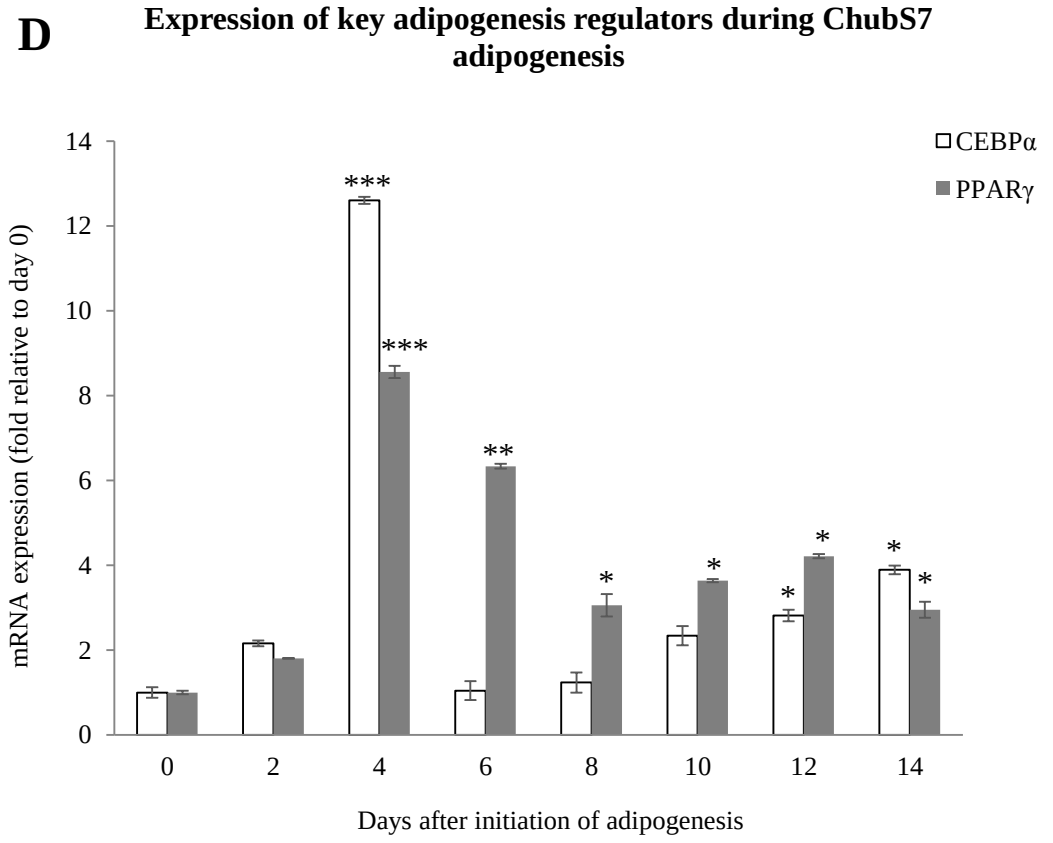




C


Differentiated ChubS7 glucose uptake
Glucose uptake (Bq/mg protein)
220
200
180
160
140
120
100
**
Basal
Insulin

D


Expression of key adipogenesis regulators during ChubS7 adipogenesis
mRNA expression (fold relative to day 0)
14
12
10
8
6
4
2
0
***
***
**
*
*
*
*
*
0
2
4
6
8
10
12
14
Days after initiation of adipogenesis
CEBPα
PPARγ

E **IRE1α and XBP1s during chub-S7 adipogenesis**

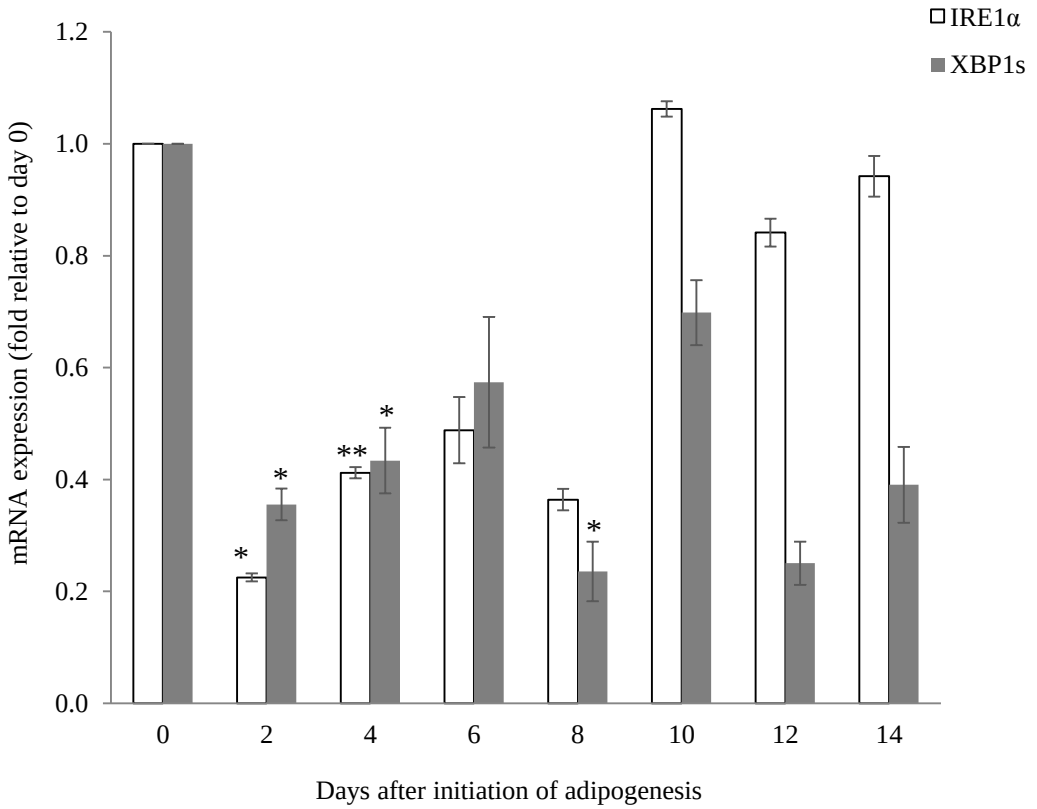


Figure 4.2.5 legend: To investigate the IRE1α - XBP1s pathway further, a reproducible model system, human preadipocyte cell line ChubS7, was used. Adipogenesis was induced in ChubS7 preadipocytes and the cells cultured for 14 days. Lipid accumulation (A) and lipolysis (B) were measured every 48 hours. Glucose uptake without and with insulin stimulation was measured on day 14 (C). mRNA expression of adipogenic markers CEBPα and PPARγ (D) and UPR pathway IRE1α and XBP1s were measured every 48 hours (E). Data is expressed as mean ± SE. Significantly different values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

4.3 Discussion

This study was designed to investigate adipogenesis in preadipocytes derived from different primary human WAT depots. The role of IRE1α - XBP1s UPR pathway in adipogenesis in preadipocytes from abdominal SAT and VAT depots from lean and obese subjects was studied. Accumulation of WAT in different depots has been reported to associate with different risk of developing T2DM (White and Tchoukalova, 2014). ER stress has been suggested to play an important role in the underlying mechanisms of insulin resistance and T2DM in obese subjects (Hummasti and Hotamisligil, 2010). And the ER stress pathway IRE1α-XBP1s has been reported to be essential in murine adipogenesis (Sha, *et al.*, 2009). The results of this study show that the extent of human adipogenesis depends on subject adiposity and specific WAT depot. Furthermore, ER stress significantly disrupts human adipogenesis and the IRE1α-XBP1s pathway is essential in human adipogenesis.

Human abdominal adipogenesis is influenced by subject adiposity (Isakson, *et al.*, 2009; Ma, *et al.*, 2015; Maumus, *et al.*, 2008). The results from this study support this theory. Preadipocytes from obese SAT and VAT depots accumulated more lipids during adipogenesis than matched depots from lean subjects. This finding contradicts van Harmelen *et al*. who reported no regional difference between human abdominal SAT and VAT differentiation, however they used adipocyte morphology and determination of glycerol-3-phosphate dehydrogenase (GPDH) to assess differentiation and therefore may not have resolved differences in lipid accumulation and lipolysis (van Harmelen, *et al.*, 2004). Preadipocytes derived from obese SAT underwent more lipolysis during adipogenesis than lean SAT;

however conversely, lean VAT underwent more lipolysis than obese VAT. Furthermore in lean derived preadipocytes, those from VAT had higher levels lipolysis compared to SAT whereas in obese derived adipocytes SAT had higher levels of lipolysis than VAT. These findings are consistent with the hypothesis of Karpe *et al.* that NEFA concentration is largely unrelated to body fat mass (Karpe, *et al.*, 2011). The observed differences in lipid accumulation and lipolysis may be due to different physiological functions of abdominal SAT and VAT, in accordance with existing dogma (Kissebah, *et al.*, 1982). SAT may primarily serve as a long-term energy store whereas VAT may be more plastic and therefore release more NEFA. In lean subjects the higher VAT lipolysis may serve to supply the nearby liver with NEFA to be oxidized to transfer energy in a controlled way.

This study indicated differences during adipogenesis in preadipocytes from different origin and importantly also showed differences in adipocyte function after adipogenesis. Adipocyte function was assessed by insulin stimulated glucose uptake. Lean Abd SAT adipocytes had the lowest basal glucose uptake and the biggest fold increase following insulin stimulation, over twice the basal rate. This reflects regulated metabolism and suggests the lean subjects Abd SAT was metabolically healthy and highly sensitive to insulin. Lean VAT adipocytes had a greater basal glucose uptake rate than Abd SAT, however there was no change after insulin stimulation. This further supports the hypothesis that WAT depot function is distinct (Kovacova, *et al.*, 2012; McLaughlin, *et al.*, 2011). Obese Abd SAT adipocytes had a higher basal glucose uptake and a lower fold increase upon insulin addition compared to lean but still had a significant increase. This suggests

obese Abd SAT adipocytes metabolism is compromised and less sensitive to insulin action. Obese VAT adipocytes had the greatest basal glucose uptake and it did not increase following insulin stimulation. This suggests that the obese VAT adipocytes are dysregulated and insulin resistant. Collectively these findings support human investigations that suggest Abd SAT is protective for insulin resistance whereas VAT mass has the opposite effect (McLaughlin, *et al.*, 2011).

The IRE1α - XBP1s UPR pathway is indispensible in murine adipogenesis (Sha, *et al.*, 2009), therefore IRE1α and XBP1s mRNA expressions were measured during human adipogenesis. Abd SAT derived preadipocytes from both lean and obese subjects decreased IRE1α and XBP1s expression during adipogenesis. Conversely VAT derived preadipocytes from both lean and obese subjects increased IRE1α expression in a biphasic way peaking on days 4 and 12, and increased XBP1s expression throughout adipogenesis. Therefore there were clear depot specific differences in gene expression irrespective of adiposity. This study indicated adiposity and WAT region influence adipocyte metabolic function through altered lipid accumulation, lipolysis and glucose uptake and it also showed adiposity and WAT region influence gene expression during adipogenesis. These findings support Tchkonia *et al.* that WAT depot-specific characteristics are retained in strains derived from human preadipocytes (Tchkonia, *et al.*, 2006). Since functional differences between mesenchymal stem cell populations are reflected by their transcriptome (Jansen, *et al.*, 2010), the observed differences in gene expression likely reflect different adipocyte functions and further support the hypothesis that regional WAT depots have different functions (White and Tchoukalova, 2014).

This study showed that chronic inhibition or activation of the UPR during adipogenesis inhibits adipogenic capacity. The IRE1α - XBP1s pathway was differentially expressed in SAT and VAT adipogenesis and activation of the UPR has been linked to metabolic dysfunction (Hummasti and Hotamisligil, 2010). Therefore the UPR was hypothesised to play a key role in adipogenesis. Induction of ER stress with TM or blocking IRE1α endonuclease activity with 4μ8C and depleting cellular XBP1s during the initial 4 days of adipogenesis prevented cell morphological changes and lipid accumulation, collectively blocking adipogenesis. Therefore functional ER integrity and XBP1s are required for successful adipogenesis in humans. The finding that XBP1s is essential for human adipogenesis supports murine studies (Sha, *et al.*, 2009) and therefore provides a translation from mouse to man. The importance of ER integrity in adipogenesis supports the postulation that the ER is tightly connected to metabolic regulation (Unanue and Urano, 2014). Bariatric surgery induced WAT loss led to a reduction in ER stress and an improved metabolic profile (chapter 3). Therefore the reduced ER stress following WAT loss may have improved WAT metabolic function partly through improved adipogenesis regulation.

SAT was investigated further because SAT represents the vast majority of AT (about 85 % of all body fat (Frayn and Karpe, 2014)) and the inability of SAT to expand has been causally related to obesity complications such as T2DM (Virtue and Vidal-Puig, 2010). In order to investigate SAT adipocyte signalling pathways in more detail a suitable human adipocyte cell line, ChubS7, was used for reproducibility. ChubS7 cells have previously been characterised in detail and

shown to express typical white adipocyte markers upon adipogenesis (Bujalska, *et al.*, 2008; Darimont, *et al.*, 2003; Gathercole, *et al.*, 2007). In this study during adipogenesis ChubS7 preadipocytes accumulated lipids, had increased lipolysis, and increased adipogenic regulator gene expression, and adipocytes had significantly increased glucose uptake. Therefore they successfully differentiated in agreement with literature. IRE1α and XBP1s mRNA expression levels in ChubS7 cells significantly reduced upon activation of adipogenesis and then oscillated during the subsequent 14 days and therefore may play an important role in regulating adipogenesis. However ChubS7 cells were derived from an obese female subject with a BMI of 54 kg/m^2 and ChubS7 cells coexpress human telomerase reverse transcriptase (hTERT) and papillomavirus E7 oncoprotein (HPV-E7) (Darimont, *et al.*, 2003), therefore caution must be taken when extrapolating results from ChubS7 cells to adipocytes. Nonetheless, ChubS7 cells serve as a useful tool to optimise adipocyte treatment conditions due to their consistency. Thus in order to investigate the IRE1α - XBP1s pathway in further detail, ChubS7 cells were used for optimisation and pilot studies before key experiments were repeated in primary human adipocytes.

4.4 Conclusions

In summary, this study showed that adipogenesis in primary human preadipocytes is influenced by both adiposity and WAT depot of preadipocyte source and that the IRE1α - XBP1s UPR pathway plays an integral role in human adipogenesis. Adipogenesis is an important process within WAT to allow normal turnover of adipocytes (Spalding, *et al.*, 2008) and facilitate appropriate WAT expansion to respond to the energy needs of the body (Wang, *et al.*, 2013). This investigation indicated that during adipogenesis obese derived preadipocytes accumulated more lipid than lean irrespective of WAT depot, however lipolysis and insulin sensitivity were both influenced by adiposity and WAT depot. Collectively these findings support human investigations that suggest Abd SAT is protective for insulin resistance whereas VAT mass has the opposite effect (McLaughlin, *et al.*, 2011). The expressions of IRE1α and XBP1s during adipogenesis differed between depots and may in part explain the observed functional differences in adipocytes. ER stress induction prevented adipogenesis irrespective of preadipocyte origin and may in part explain why WAT ER stress is associated with metabolic dysfunction (Hotamisligil, 2010). The novel finding that XBP1s is a prerequisite for human adipogenesis strengthens the link between UPR and adipogenesis, although further investigation is required to elucidate the role of XBP1s in human adipogenesis. These findings provide insight into adipocyte functional differences and their integration with the UPR and therefore this information will benefit development of translational clinical interventions to improve metabolic health.

Chapter 5: The role of the IRE1α - XBP1s UPR pathway in human adipogenesis and metabolism

5.1 Introduction

WAT is able to expand and contract during adult life to accommodate the energy requirements of the body despite most development during prenatal and early postnatal life (Poulos, *et al*., 2010). Evidence suggests obesity complications in humans such as T2DM result from the inability of SAT to appropriately expand and store lipids, and consequently ectopic fat deposition and lipotoxicity contribute to insulin resistance (Hill, *et al.*, 2009; Virtue and Vidal-Puig, 2010). Impaired WAT expandability is mainly due to defects in adipogenesis (Isakson, *et al.*, 2009). Therefore adipogenesis is an important process to maintain metabolic function. Further understanding of adipogenesis may present targets to improve metabolic regulation. Preadipocytes derived from SAT and VAT were both effected by ER stress and blocking XBP1s expression during adipogenesis to the same extent (figure 4.2.4). SAT will be investigated further because SAT represents the vast majority of WAT (about 85 % of all body fat (Frayn and Karpe, 2014)) and the inability of SAT to expand has been causally related to obesity complications such as T2DM (Virtue and Vidal-Puig, 2010).

Within adipocytes the ER plays a key role in metabolic regulation. ER stress can lead to activation of the UPR (figure 1.6.1) and influence metabolism and function of adipocytes (figures 1.7.1 and 1.7.2). Inducing ER stress with TM during induction of differentiation prevented adipogenesis (chapter 4). Therefore ER function is a prerequisite for successful adipogenesis. The IRE1α-XBP1s UPR pathway is essential for adipogenesis in mouse cells (Sha, *et al*., 2009) and this study has confirmed this in human cells for the first time (chapter 4). However the role of XBP1s in human adipogenesis is not known. Understanding the role of

XBP1s in adipogenesis will provide insight into the regulation of adipogenesis and may reveal targets to boost adipogenesis. Forced expansion of WAT via increased hyperplasia induced by transgenic overexpression of adiponectin in *ob/ob* mice normalized glucose and insulin levels and prevented insulin resistance (Kim, *et al*., 2007). Therefore upregulating adipogenesis may have protective effects for metabolic health. Since XBP1s is essential for adipogenesis, then targeting XBP1s or its downstream targets may provide a mechanism to boost adipogenesis and subsequently help WAT expansion and prevent ectopic fat deposition and lipotoxicity, and therefore help metabolic regulation.

Understanding how XBP1s fits into the complex system of adipogenesis regulation may provide pharmacological targets and therefore have clinical benefit. Characterisation of adipogenesis regulatory processes have benefited from the discovery of key pathways and transcription factors that contribute to the adipogenic process, as reviewed by Lefterova and Lazar (Lefterova and Lazar, 2009). The transcription factors PPARγ and CEBPs are the crucial determinants of adipocyte fate although other factors including extracellular acting Wnts and intracellular cell-cycle proteins also play roles in adipogenesis. There are several inhibitors of adipogenesis including CHOP, which is upregulated in ER stress (Hou, *et al*., 2013), and certain TCF/LEF proteins, activated by Wnt signalling (Farmer, 2006). Recently miRNA-540 has been suggested to impair adipogenesis via suppression of PPARγ (Chen, *et al*., 2015). Therefore a network of transcription factors and cell-cycle regulators, in concert with transcriptional coactivators and corepressors, respond to extracellular stimuli to activate or repress adipogenesis. Cho *et al.* have shown in mouse 3T3-L1 cells that XBP1s

enhances adipogenesis through the downregulation of Wnt10b (Cho, *et al*., 2013). And in mouse 3T3-L1 cells that XBP1s is a key regulator of adipogenic modulator PPARγ (Cho, *et al.*, 2014). However the role of XBP1s in human cells during adipogenesis has not been studied.

Therefore the aim of this study was to investigate the role of XBP1s in human adipogenesis. Human adipocytes were used to optimise inhibition conditions with IRE1α endonuclease inhibitor 4μ8C and XBP1 siRNA. Human preadipocytes were subjected to various XBP1s inhibition studies via inhibitor or siRNA during adipogenesis. Adipogenesis and adipocyte function were assessed by lipid accumulation, lipolysis and glucose uptake. Expression of key adipogenic and ER stress proteins and mRNAs were measured during adipogenesis and following XBP1s inhibition.

5.2 Results

5.2.1 IRE1α endonuclease activity was specifically inhibited with the small molecule inhibitor 4μ8C in adipocytes without effecting cell viability

To investigate the IRE1α - XBP1s pathway in adipogenesis a method of blocking the signalling pathway was sought. The small molecule inhibitor 4μ8C has been shown to block IRE1α endonuclease activity in mouse embryonic fibroblasts (MEF cells) (Cross, *et al.*, 2012), but not human adipocytes. In order to assess the effects of 4μ8C in human adipocytes on IRE1α inhibition, adipocytes were treated with DMSO (control) or subjected to ER stress by treatment with 0.75 μg/mL TM for 24 hours in the presence of 4μ8C concentrations 0 – 64 μM and

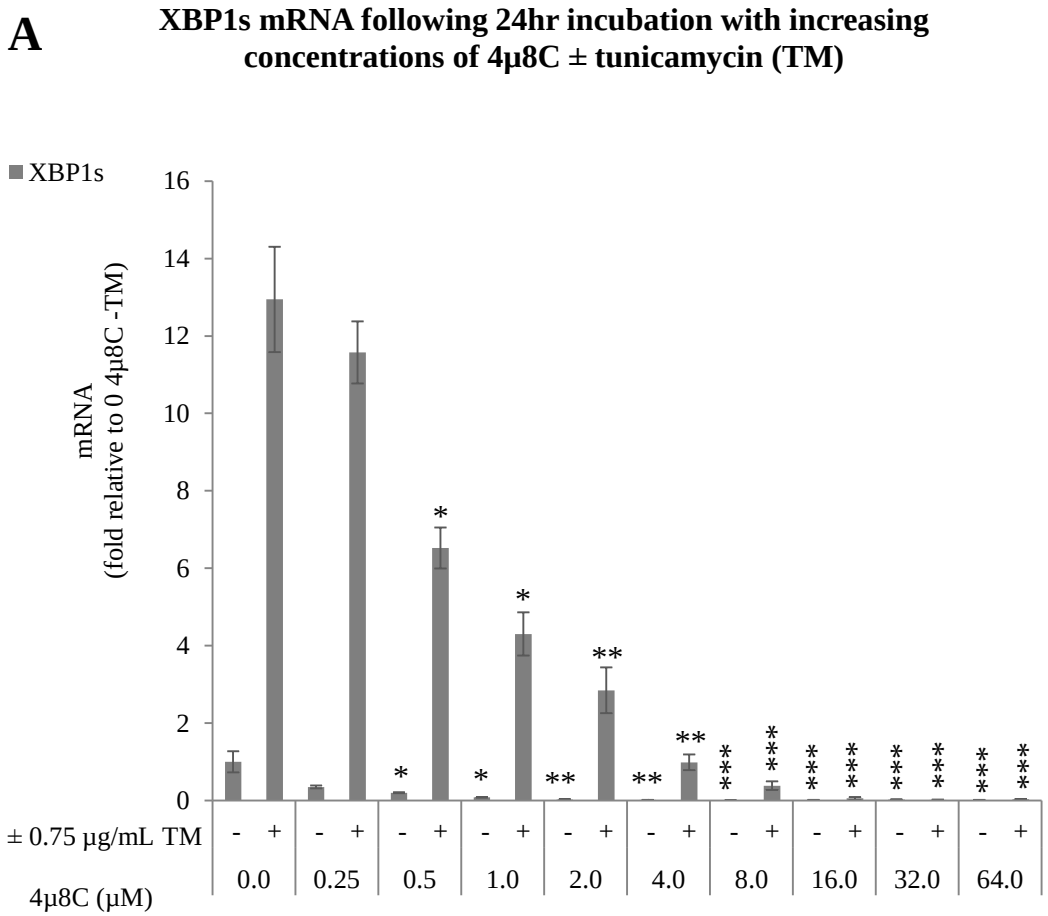
ER stress gene expressions measured (figure 5.2.1 A – E). In the absence of 4μ8C, TM increased XBP1s expression 13-fold higher than control. 4μ8C decreased the expression of XBP1s both without and with ER stress induction in a dose dependent way. 16 μM 4μ8C reduced ER stress induced XBP1s expression by 99 % compared to control. XBP1u decreased in the presence of 4μ8C without TM but increased in the presence of TM in a dose dependent way. In the absence of TM, IRE1α expression did not change with 4μ8C up to 16 μM, above this concentration IRE1α expression increased more with more inhibitor and so induced ER stress. In the presence of TM, IRE1α increased consistently to 4 – 5 fold higher than without TM up to 16 μM 4μ8C, above this concentration IRE1α expression increased to 8 fold higher than control. ATF6 expression did not change with TM or 4μ8C up to 16 μM, above this concentration it increased with and without TM. CHOP expression did not change without TM up to 16 μM 4μ8C, above this concentration it increased. CHOP consistently increased 30 – 50 fold in the presence of TM and up to 32 μM 4μ8C. At 64 μM 4μ8C and the presence of TM, CHOP expression increased 20 fold higher than control.

In order to assess the effect of 4μ8C on cell viability, cytotoxicity assays were conducted on ChubS7 and primary adipocytes with 4μ8C concentrations 0 – 32 μM and without or with TM (figure 5.2.1 F – G). Cell viability compared to control with 4 μM 4μ8C for 24 hours was 99 % and for 48 hours was 90 % for both primary and ChubS7 adipocytes. Cell viability in the absence of 4μ8C and with TM for 24 hours was 80 % and 60 % for primary and ChubS7 cells

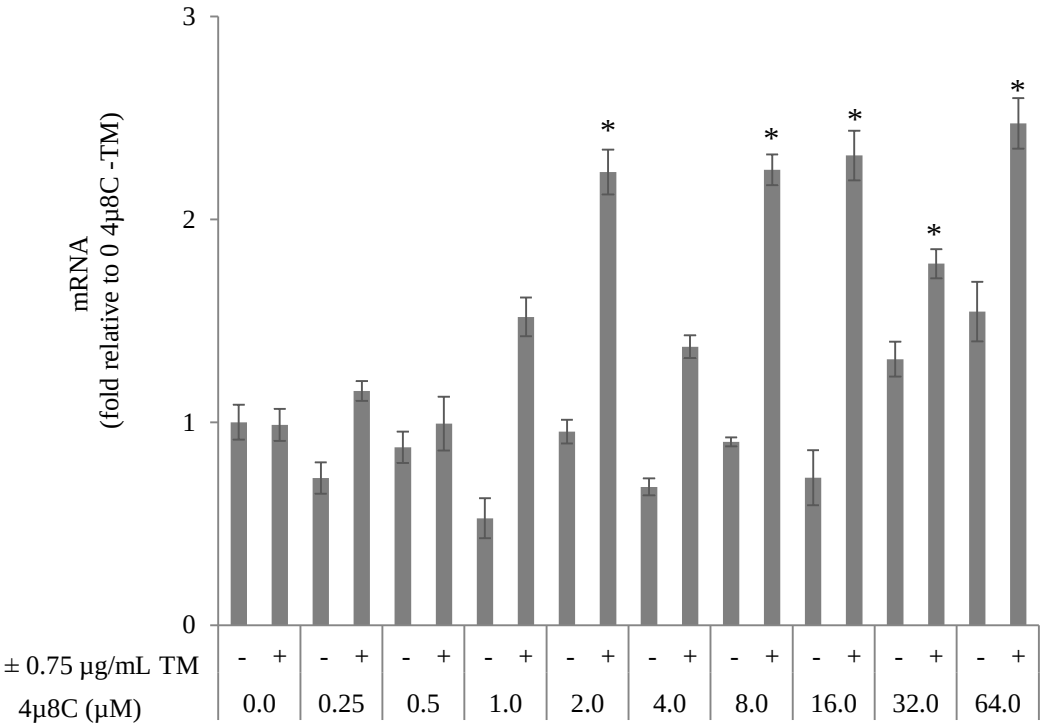
respectively. As the concentration of 4μ8C was increased cell viability decreased with dose and exposure.

The optimum concentration of 4μ8C to use in human adipogenesis experiments was decided as 4 μM because XBP1s mRNA expression was reduced by over 90 % whilst cell viability was over 90 % compared to control. All subsequent experiments used a concentration of 4 μM 4μ8C.

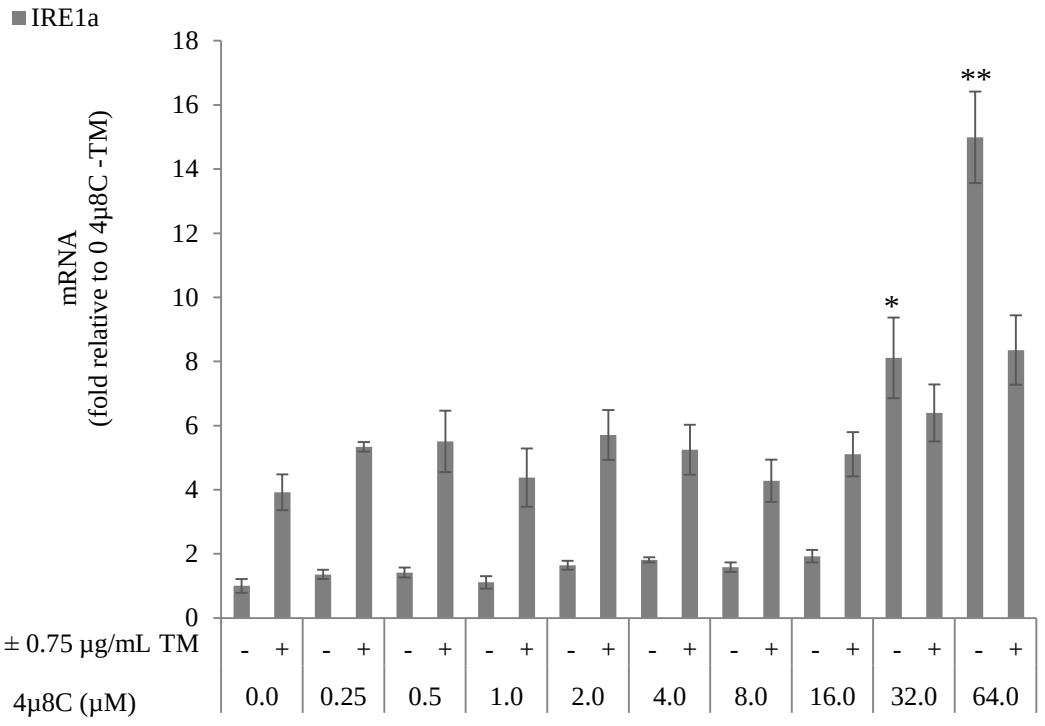
Figure 5.2.1 IRE1α endonuclease activity was specifically inhibited with the small molecule inhibitor 4μ8C in adipocytes without effecting cell viability



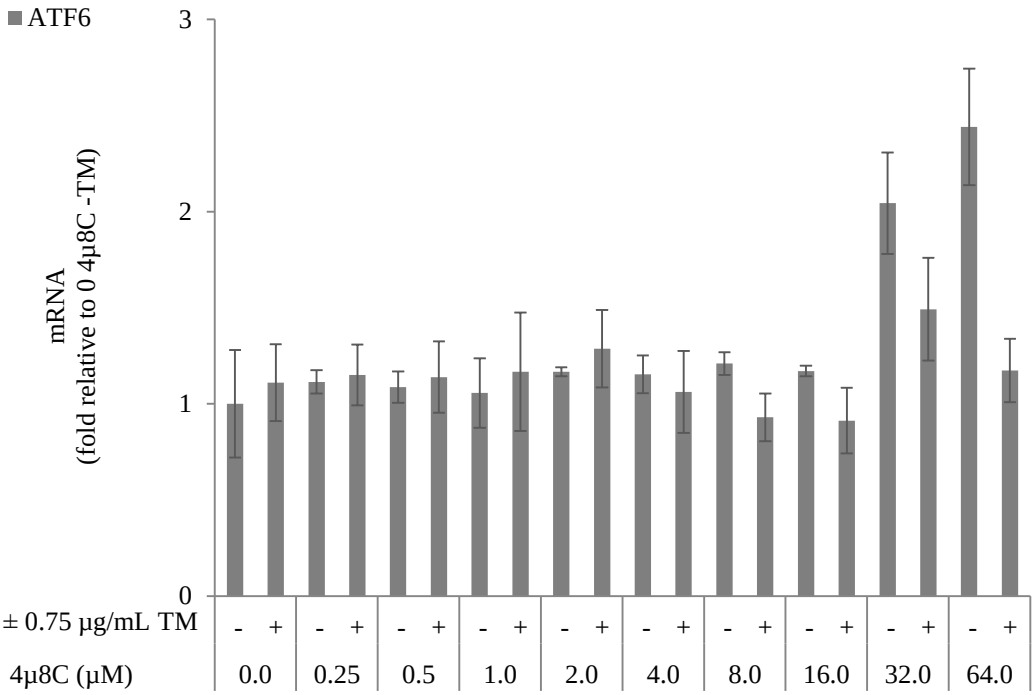
B **XBP1u mRNA following 24hr incubation with increasing concentrations of 4μ8C ± tunicamycin (TM)**



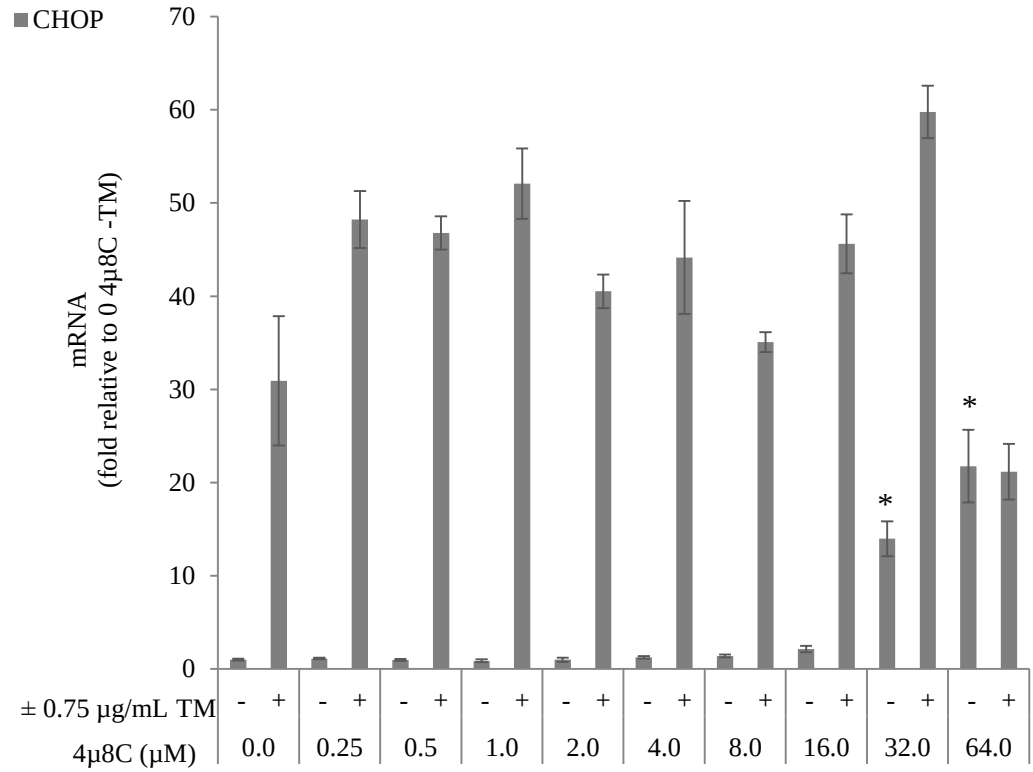
C **IRE1α mRNA following 24hr incubation with increasing concentrations of 4μ8C ± tunicamycin (TM)**



D **ATF6 mRNA following 24hr incubation with increasing concentrations of 4μ8C ± tunicamycin (TM)**

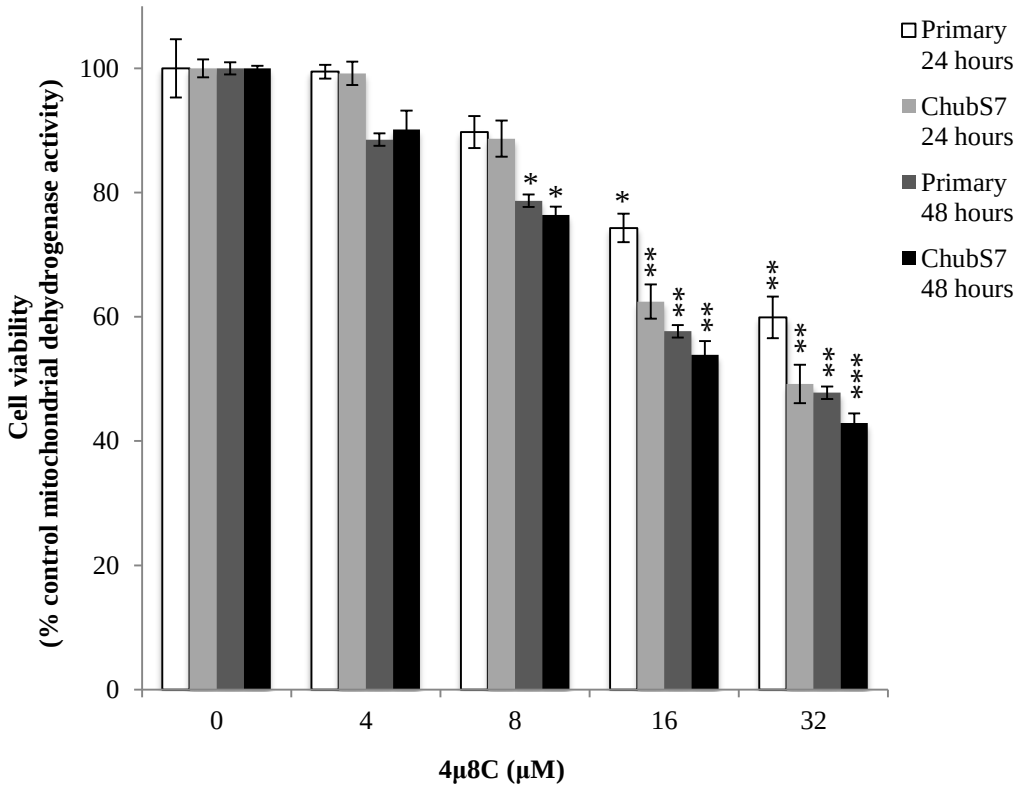


E **CHOP mRNA following 24hr incubation with increasing concentrations of 4μ8C ± tunicamycin (TM)**



F

Cell viability following IRE1α endonuclease activity inhibition



G

Cell viability following ER stress with 0.75 μg/mL tunicamycin (TM) and inhibited IRE1α endonuclease activity

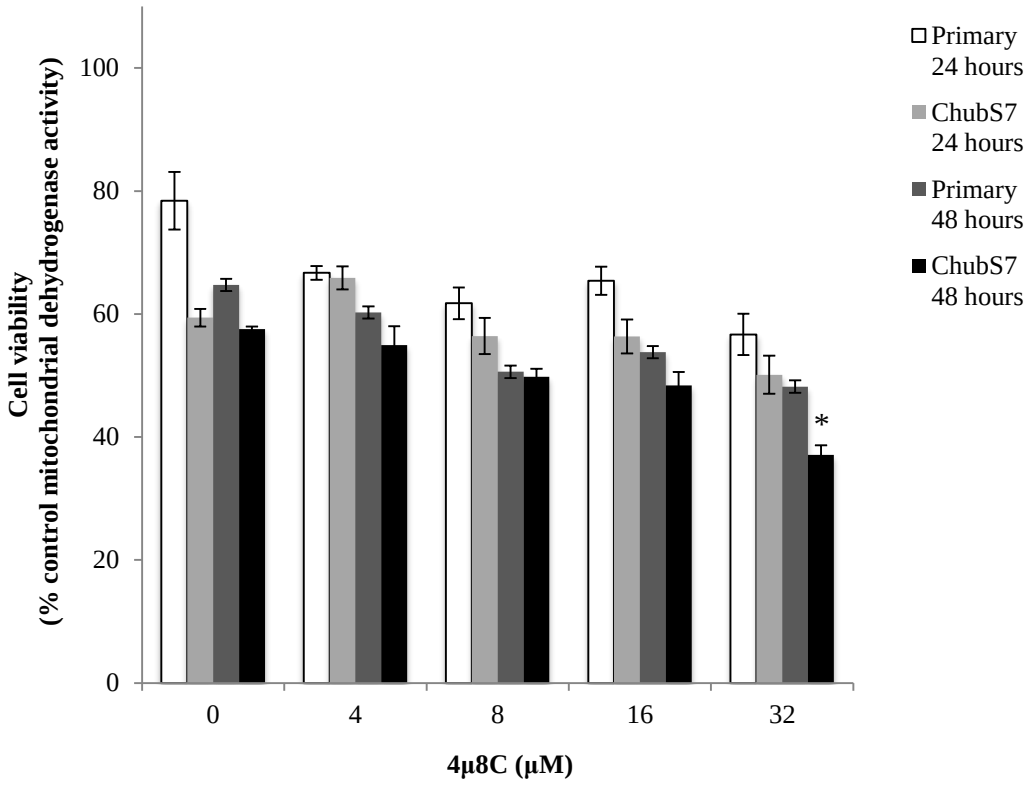


Figure 5.2.1 legend: IRE1α endonuclease activity was specifically inhibited with the small molecule inhibitor 4μ8C in adipocytes without effecting cell viability. Adipogenesis was induced in ChubS7 cells and on day 14 cells were incubated for 24 hours with 0.75 μg/mL TM or DMSO without TM (control) with 4μ8C concentrations 0 – 64 μM. mRNA expression of XBP1s (A), XBP1u (B), IRE1α (C), ATF6 (D) and CHOP (E) were measured. The expressions of mRNA without and with TM are compared to 0 μM 4μ8C without and with TM respectively. Cytotoxicity assays were conducted on ChubS7 and primary lean SAT cells with treatments starting on day 0 (preadipocytes) and day 14 after induction of adipogenesis (adipocytes). No significant differences in cell viability between day 0 and day 14 were detected. Cell viability is presented as mean of day 0 and day 14. ER stress was induced for 24 or 48 hours with 0.75 μg/mL TM or control without TM in the presence of 4μ8C, concentrations 0 – 32 μM. Cell viability was assessed by MTT assay without TM (F) and with TM (G) and significantly lower cell viability compared to without 4μ8C are indicated. Data is expressed as mean ± SE. Significantly different values are indicated as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

5.2.2 During adipogenesis 4μ8C reduced adipogenic capacity depending on time and duration of treatment

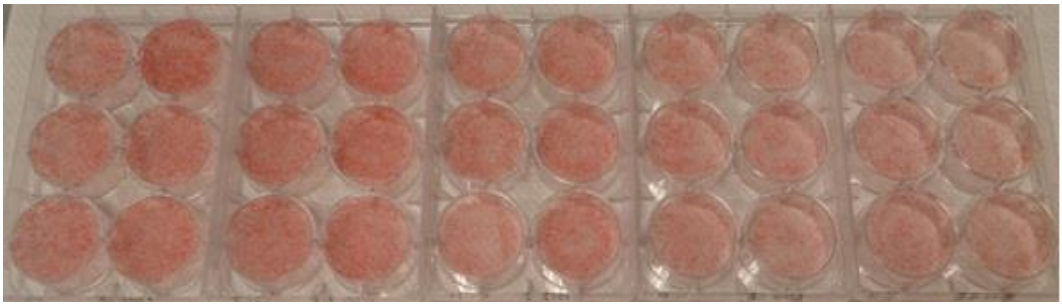
ER stress induction and inhibition of IRE1α endonuclease activity during early adipogenesis reduced adipogenic capacity (chapter 4). To determine the critical period during early adipogenesis for the IRE1α - XBP1s pathway, adipogenesis

was induced in primary SAT lean preadipocytes for 14 days in conjunction with DMSO (control) or 4 μM 4μ8C for different time periods during early adipogenesis. Adipogenesis was measured by lipid accumulation, lipolysis and insulin stimulated glucose uptake (figure 5.2.2 A – D). Treatment with 4μ8C during the initial 48 hours of adipogenesis had no significant effect on lipid accumulation, lipolysis or glucose uptake. However treatment with 4μ8C between 48 hours and 96 hours after induction of adipogenesis significantly reduced lipid accumulation, lipolysis and glucose uptake. All three parameters were further reduced when 4μ8C was administered for the initial 96 hours and furthermore during the initial 144 hours. Therefore, day 0 – 4 (96 hour) inhibition was used for all further studies.

Figure 5.2.2 During adipogenesis 4μ8C reduced adipogenic capacity depending on time and duration of treatment

A

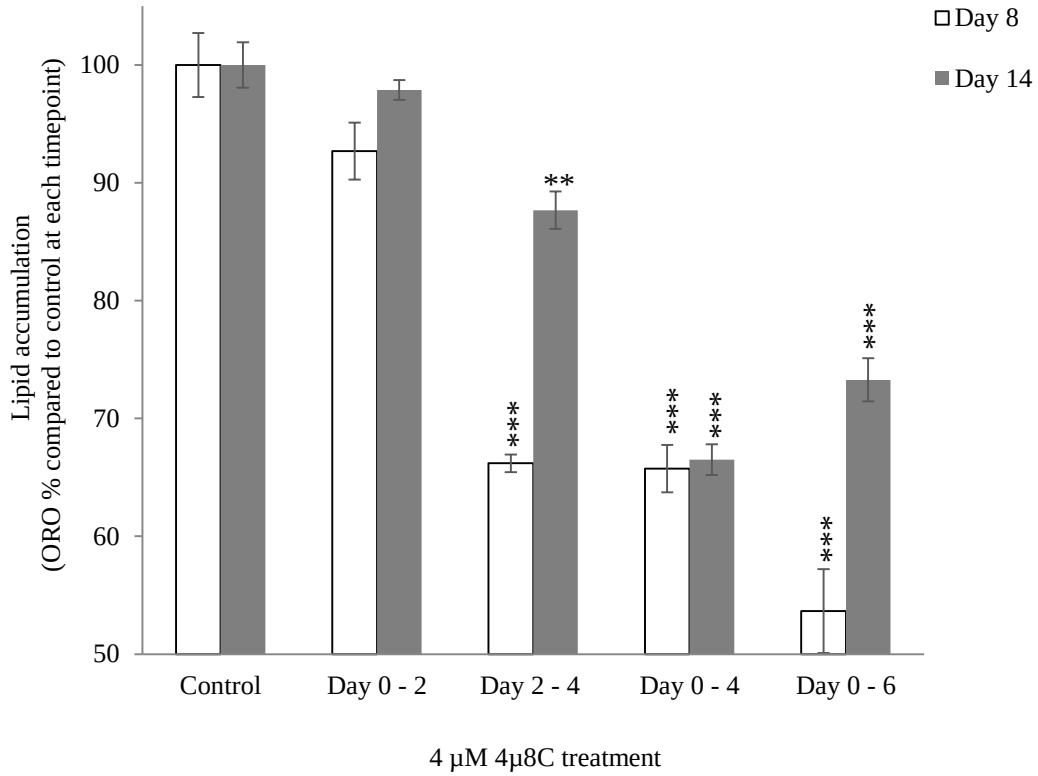
Lipid accumulation in differentiated adipocytes following 4μ8C treatment



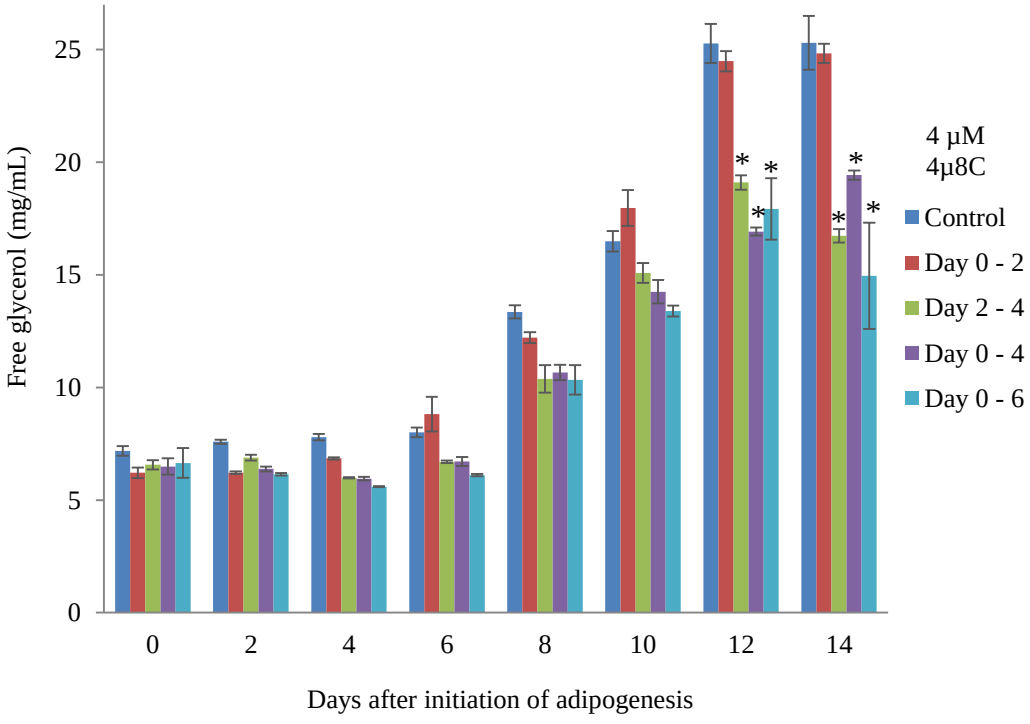
Control Day 0 – 2 Day 2 – 4 Day 0 – 4 Day 0 – 6

4 μM 4μ8C treatment

B **Lipid accumulation during adipogenesis following 4μ8C treatment**



C **Lipolysis during adipogenesis following 4μ8C treatment**



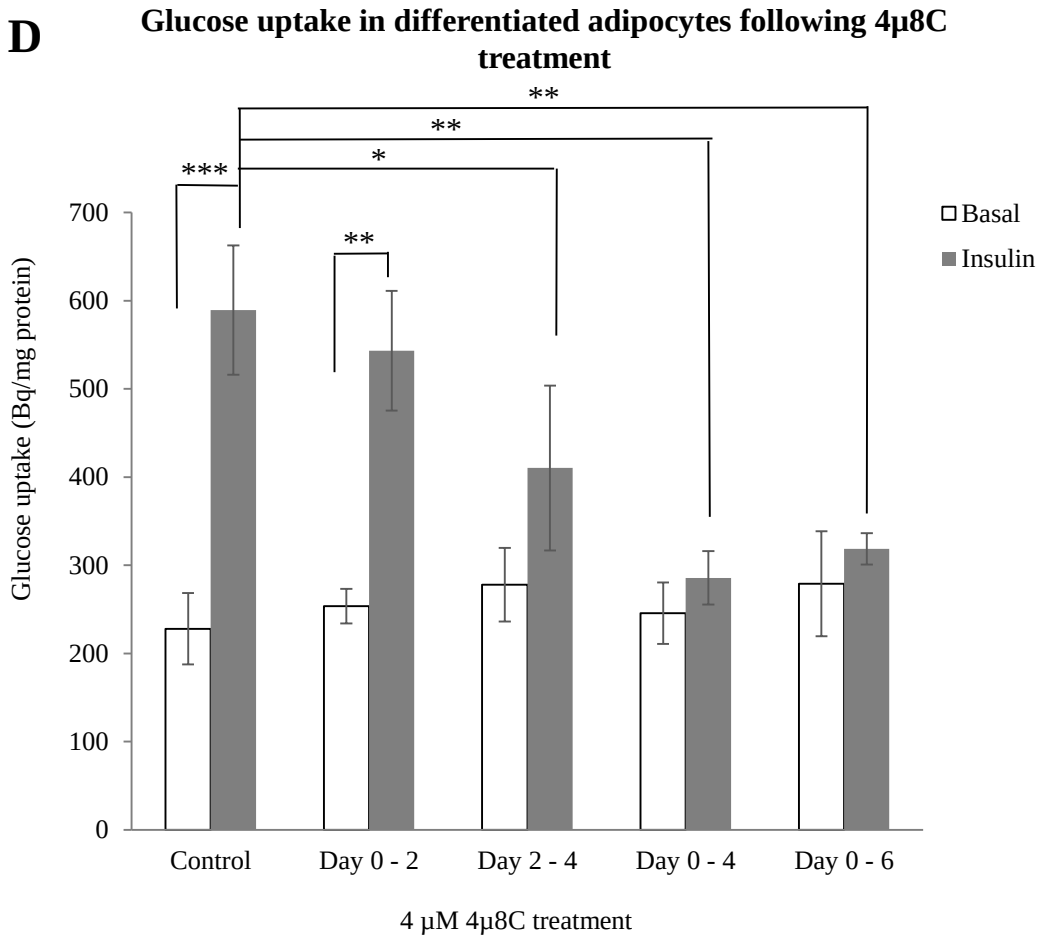


Figure 5.2.2 legend: During adipogenesis 4µ8C reduced adipogenic capacity depending on time and duration of treatment. Adipogenesis was induced in primary SAT lean preadipocytes for 14 days without (control) and with 4 µM 4µ8C for the initial 48 hours (day 0 – 2), between 48 and 96 hours (day 2 – 4), the initial 96 hours (day 0 – 4) and the initial 144 hours (day 0 – 6). Lipid accumulation was assessed on day 8 and day 14 after induction of adipogenesis (A – B), lipolysis was measured every 48 hours (C), and glucose uptake without or with insulin stimulation was measured on day 14 (D). Data is expressed as mean ± SE. Significantly different values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

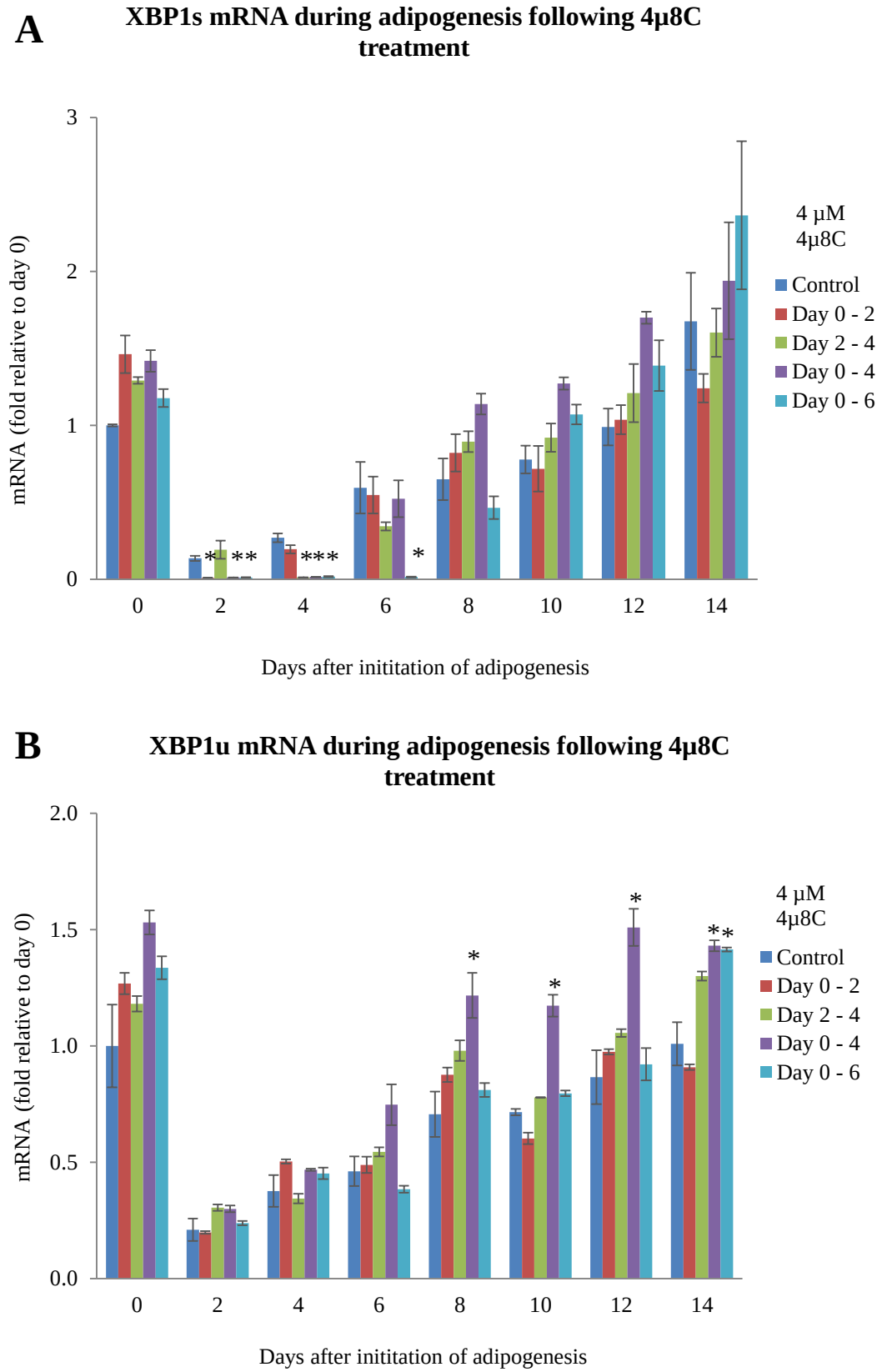
5.2.3 During adipogenesis 4μ8C inhibited XBP1s, adipogenic regulator and adiponectin mRNA expression depending on treatment duration

Inhibiting IRE1α activity during early adipogenesis reduced lipid accumulation, lipolysis and glucose uptake compared to control (figure 5.2.2). To assess what effect the inhibition had on gene expression during adipogenesis, primary adipocytes were treated with 4μ8C for different times during early adipogenesis and key ER stress and adipogenic gene expressions were measured every 48 hours for 14 days (figure 5.2.3 A – G). At every time point following 4μ8C treatment XBP1s mRNA was significantly reduced ($P<0.05$), however XBP1u mRNA did not change compared to control. After inhibitor treatments had been withdrawn XBP1s mRNA recovered to within no significant difference from control, however XBP1u mRNA increased significantly higher ($P<0.05$) than control following 96 hour inhibitor treatment. Without inhibitor treatment IRE1α and CHOP expression decreased after induction of adipogenesis and fluctuated at levels below that of day 0. Both IRE1α and CHOP expression remained the same as control following 4μ8C treatments at every time point during adipogenesis.

In control cells CEBPα expression peaked at day 8 of adipogenesis at 600 fold higher than day 0 and then gradually decreased to 400 fold higher than day 0 on day 14. CEBPα expression was significantly reduced ($P<0.05$) on day 4 and day 6 following IRE1α inhibition compared to control. CEBPα expression following IRE1α inhibition then recovered to the control expression level between days 8 and 12 but on day 14 it was significantly lower ($P<0.05$) than control for all treatment groups. PPARγ expression, in control adipocytes, increased up to day 8

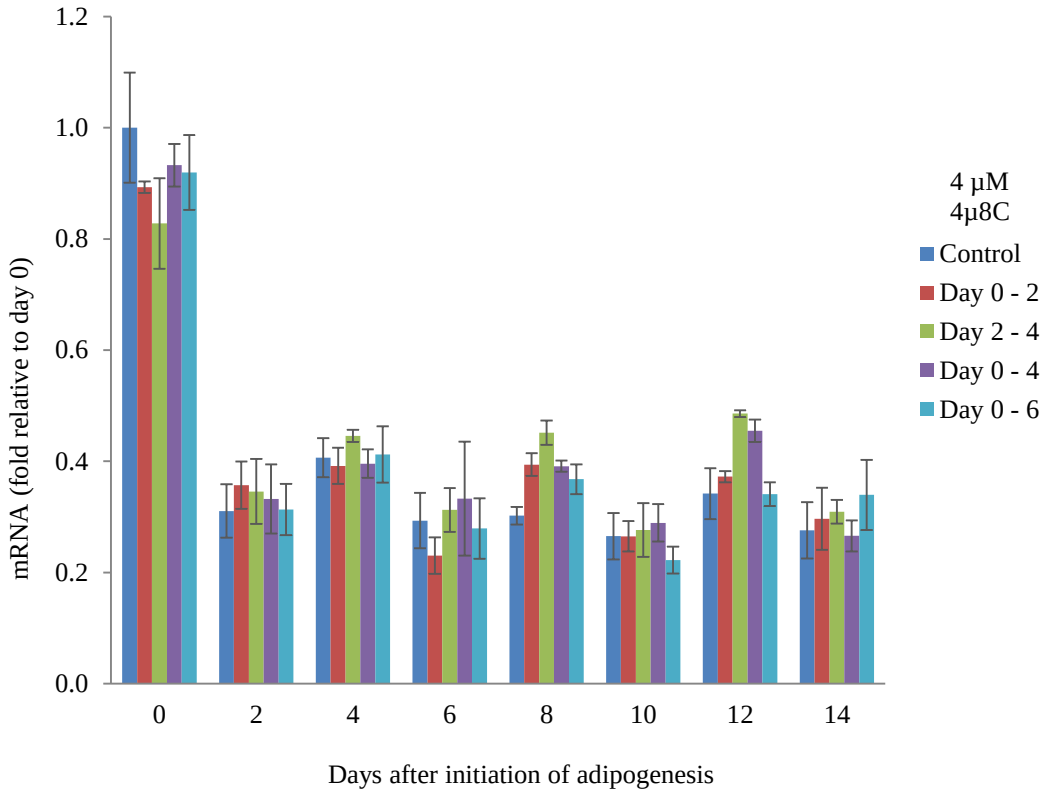
and then remained at the same level up to day 14. PPARγ was significantly lower compared to control on day 6 for adipocytes with IRE1α inhibited day 0 – 2, 0 – 4 and 0 – 6. At all other time points there was no significant difference from control to any of the treatment groups. Adiponectin mRNA was undetected on day 0 in control and all the treatment groups. The control adipocytes increased adiponectin mRNA expression on day 6 to over 3,000 fold higher than day 2. It remained around this level until day 12 and then it decreased on day 14 to 2,000 fold higher than day 2. Adiponectin expression was significantly ($P<0.05$) lower than control on day 4 and day 6 following IRE1α inhibition. Adiponectin expression remained significantly lower ($P<0.05$) than control 2 days after IRE1α inhibition had been withdrawn for day 0 – 4 and day 0 – 6 treatments. Adiponectin expression then recovered to control levels before decreasing to significantly below ($P<0.05$) control on day 12 and 14 in cell treated with 4 μM 4μ8C on days 2 – 4, 0 – 4 and 0 – 6.

Figure 5.2.3 During adipogenesis 4μ8C inhibited XBP1s and adipogenic regulator mRNA expression depending on treatment duration



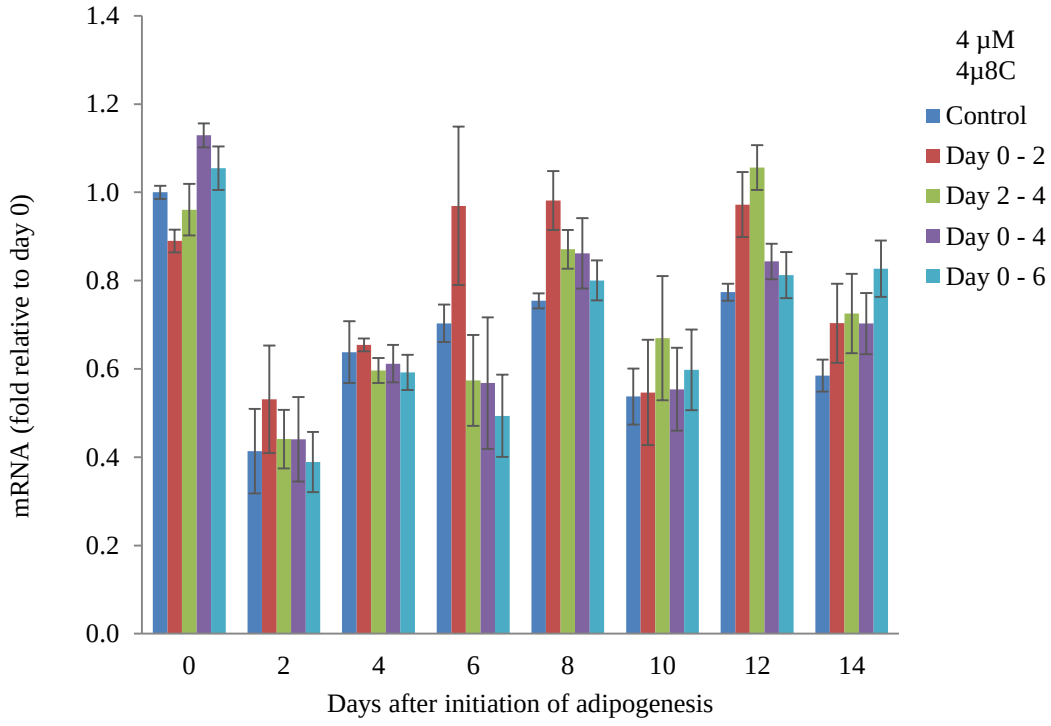
C

IRE1α mRNA during adipogenesis following 4μ8C treatment



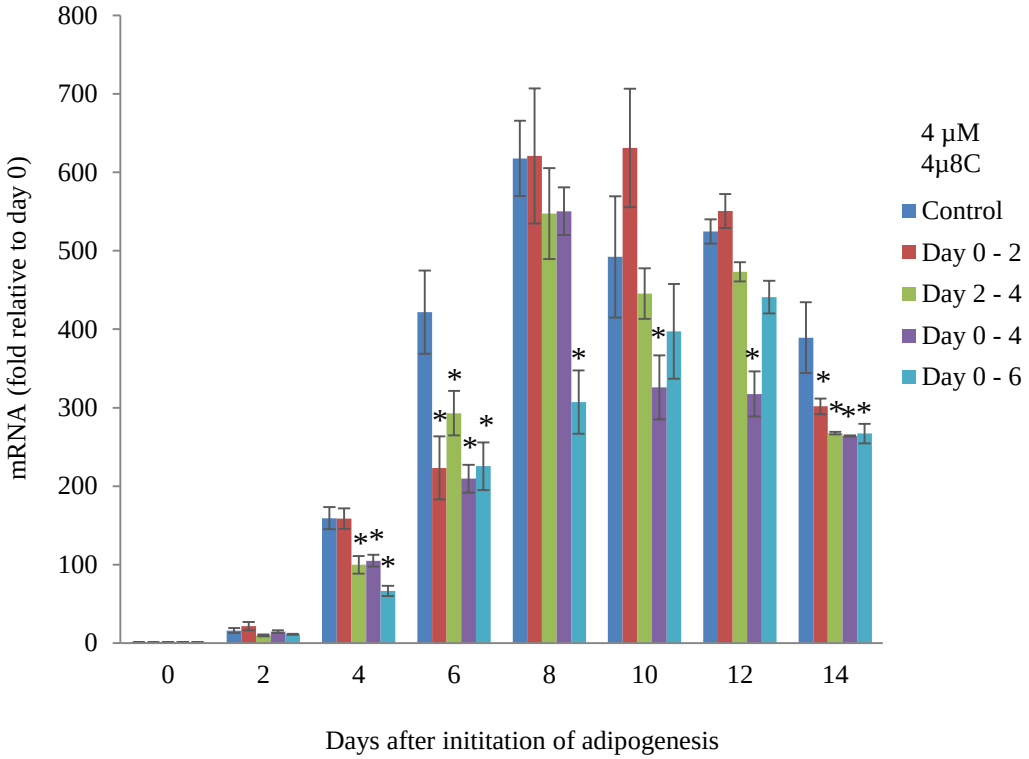
D

CHOP mRNA during adipogenesis following 4μ8C treatment



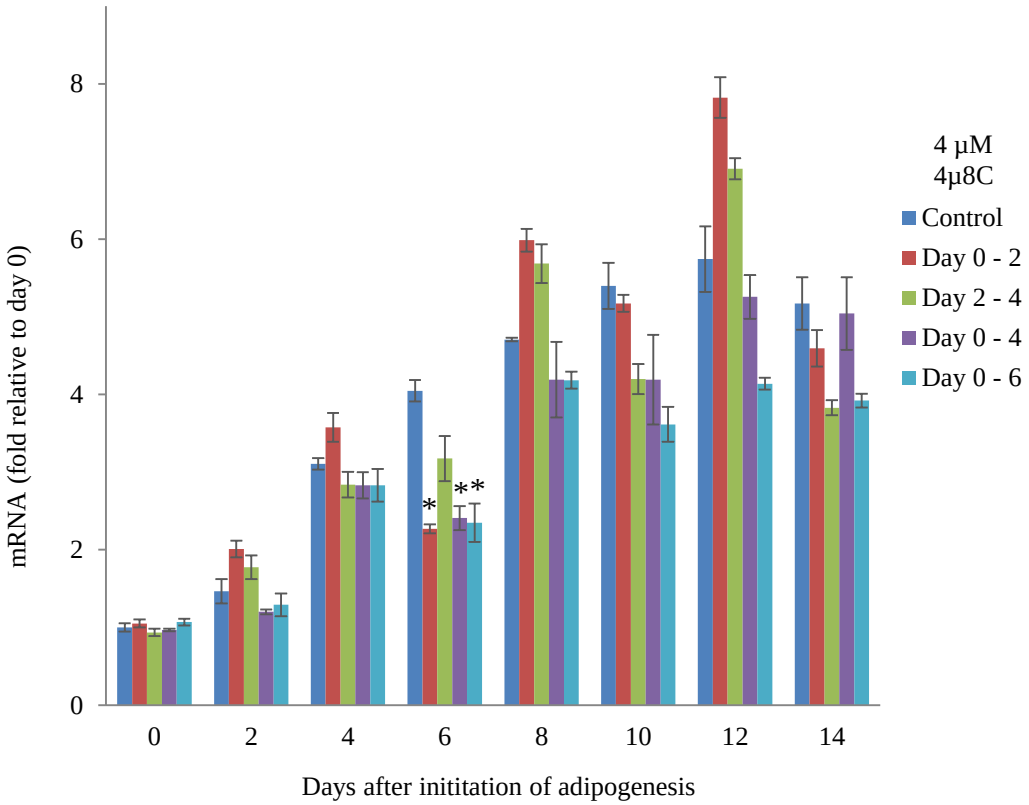
E

CEBPα mRNA during adipogenesis following 4μ8C treatment



F

PPARγ mRNA during adipogenesis following 4μ8C treatment



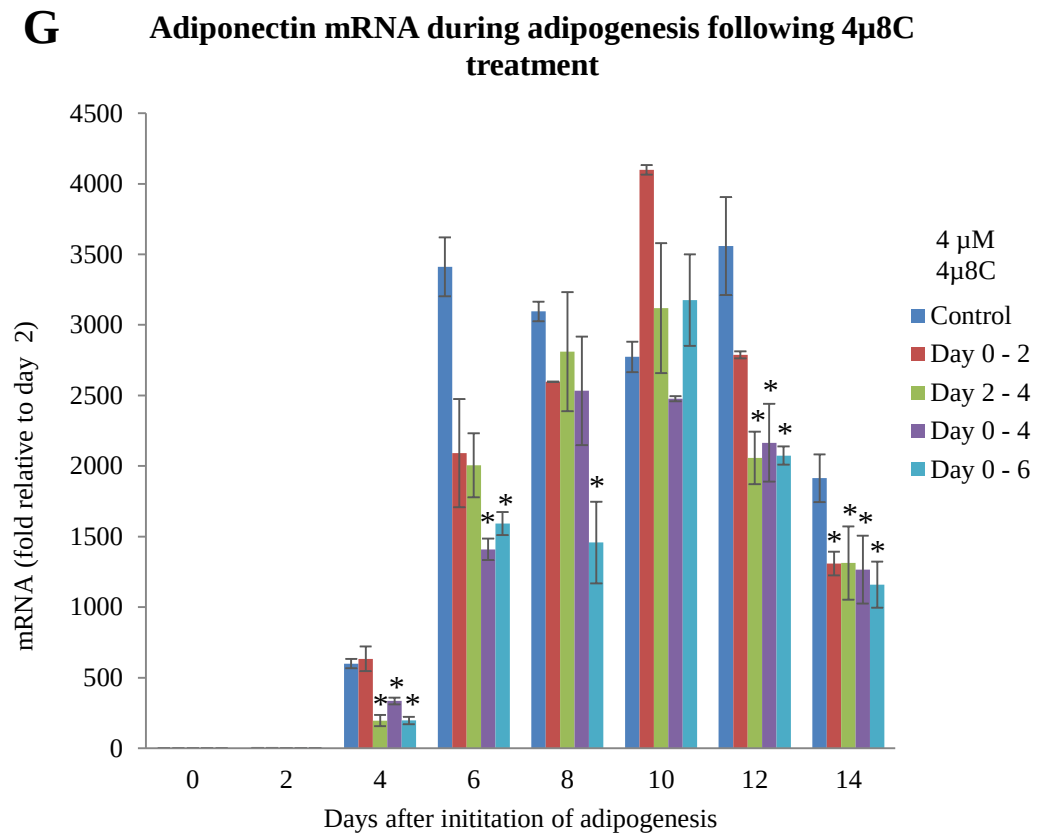
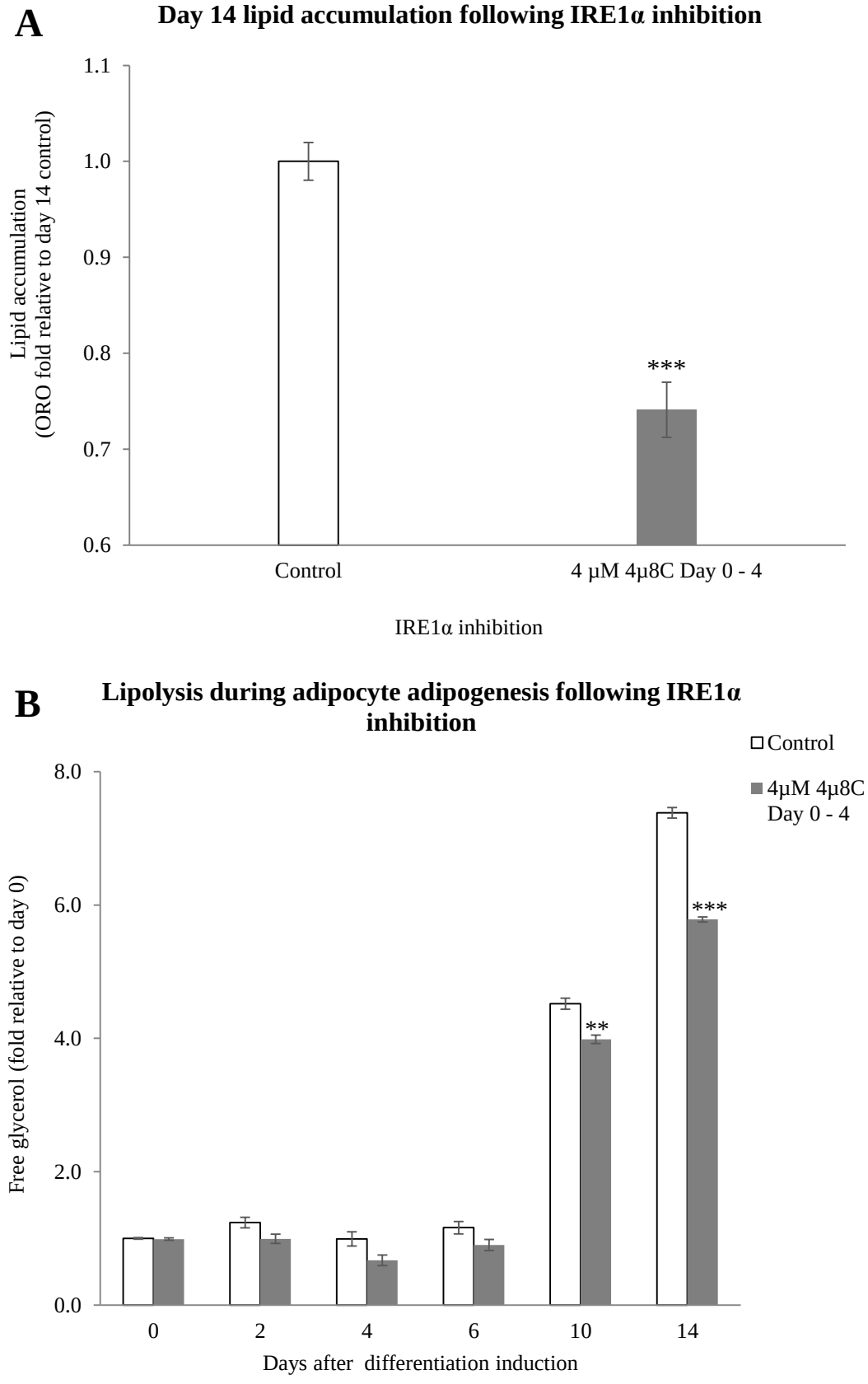


Figure 5.2.3 legend: During adipogenesis 4μ8C inhibited XBP1s and adipogenic regulator mRNA expression depending on treatment duration. Adipogenesis was induced in primary SAT lean preadipocytes for 14 days with DMSO (control) or 4 μM 4μ8C for the initial 48 hours (day 0 – 2), between 48 and 96 hours (day 2 – 4), the initial 96 hours (day 0 – 4) and the initial 144 hours (day 0 – 6). mRNA expression was measured every 48 hours for XBP1s (A), XBP1u (B), IRE1α (C), CHOP (D), CEBPα (E), PPARγ (F), Adiponectin (G). Data is expressed as mean ± SE. Significantly different values are indicated as follows: * P<0.05.

5.2.4 During adipogenesis 4μ8C consistently reduced adipogenic capacity and function in adipocytes

Inhibiting IRE1α with 4μ8C for 96 hours (day 0 – 4) after induction of adipogenesis resulted in the most profound reduction in adipogenic capacity compared to other treatment durations (figures 5.2.2 – 5.2.3). Therefore this duration of inhibition was used in subsequent studies. To confirm the preliminary outcomes of 4μ8C treatment during day 0 – 4 of adipogenesis in preadipocytes from one lean metabolically healthy subject, the treatments were repeated in SAT preadipocytes from three metabolically healthy lean subjects, each in triplicate. The preadipocytes were subjected to adipogenesis in DMSO (control) and 4 μM 4μ8C treated conditions during day 0 – 4 of adipogenesis and then lipid accumulation, lipolysis and glucose uptake were measured (figure 5.2.4 A – C). Day 14 lipid accumulation, lipolysis and insulin stimulated glucose uptake were all significantly reduced ($P<0.001$) following 4μ8C treatment compared to control.

Figure 5.2.4 During adipogenesis 4μ8C consistently reduced adipogenic capacity and function in adipocytes



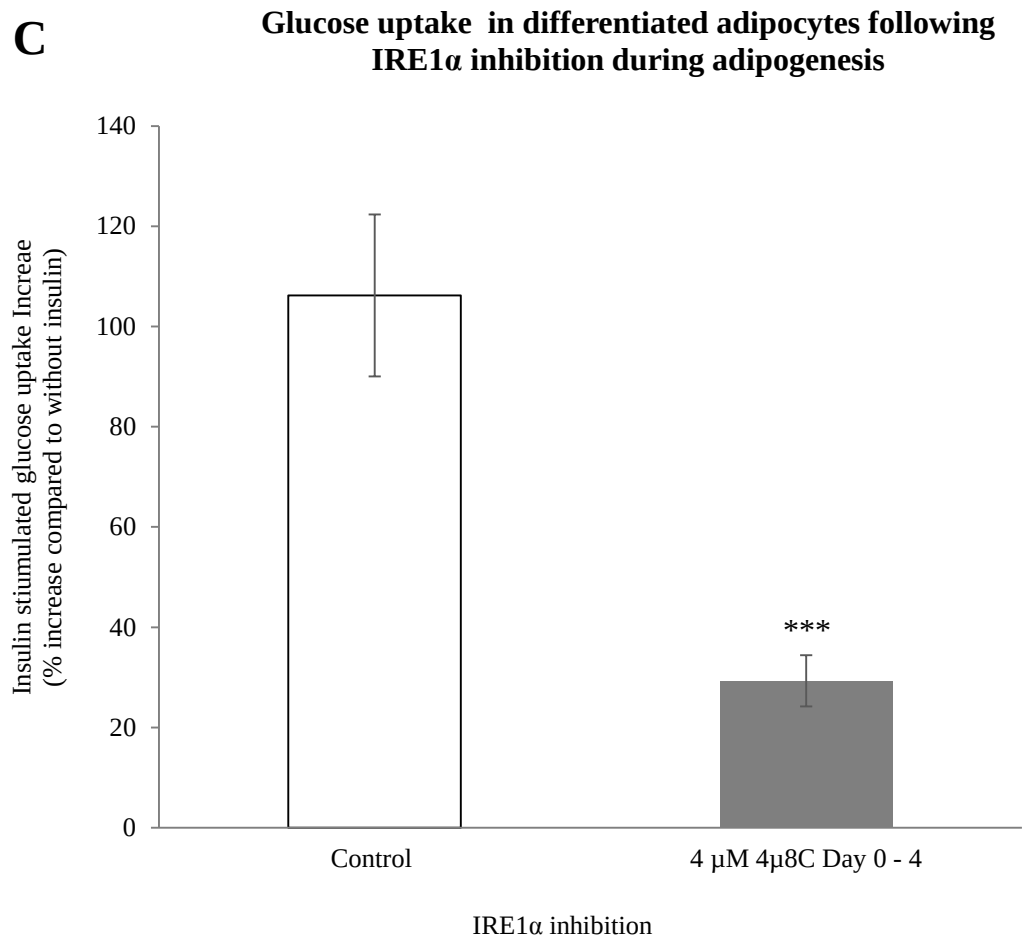


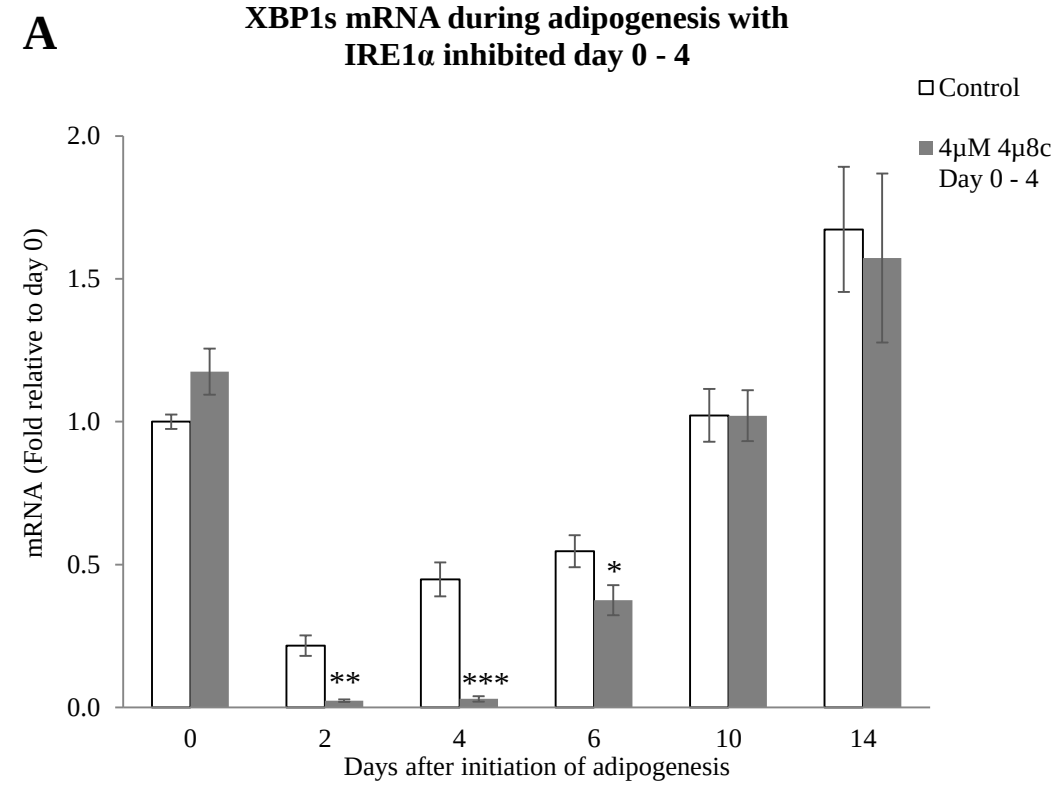
Figure 5.2.4 legend: During adipogenesis 4µ8C consistently reduced adipogenic capacity and function in adipocytes. Adipogenesis was induced in primary SAT lean preadipocytes (n=3, each in triplicate) for 14 days with DMSO (control) or 4 µM 4µ8C for the initial 96 hours (day 0 – 4). Lipid accumulation was assessed on day 14 after induction of adipogenesis (A), lipolysis was measured during adipogenesis (B), and basal and insulin stimulated glucose uptake was measured on day 14 (C). Data is expressed as mean ± SE. Significantly different values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

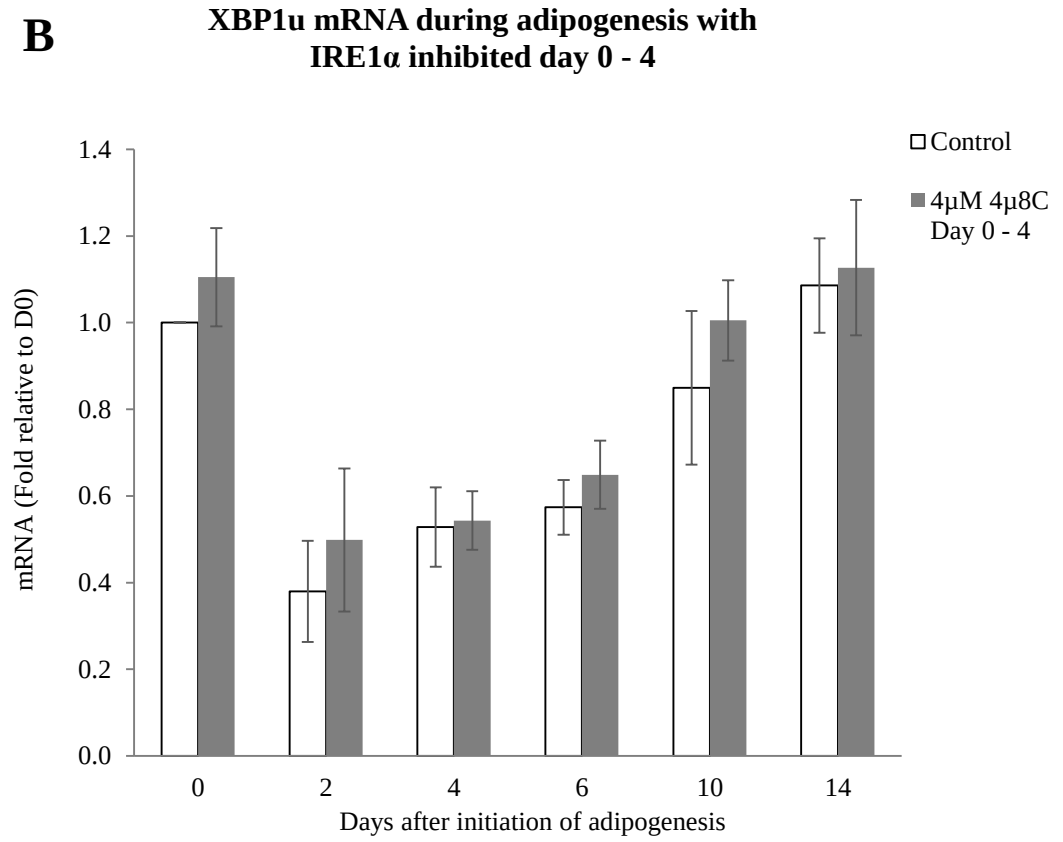
5.2.5 During adipogenesis 4μ8C inhibited XBP1s and adipogenic regulator mRNA expression

Adipogenic capacity was significantly reduced in primary human adipocytes following 4μ8C treatment (figure 5.2.4). To assess the effects of 4μ8C on ER stress and adipogenic gene expression during adipogenesis, 4 μM 4μ8C was administered for the initial 96 hours of adipogenesis and ER stress and adipogenic regulator gene expressions were measured for 14 days (figure 5.2.6 A – G). The initial six days of adipogenesis were considered vital for adipogenic capacity outcome because in control cells, adipocyte marker adiponectin gene expression peaked on day 6 (figure 5.2.3 G). Therefore gene expression was measured every 48 hours between day 0 and day 6 and every 96 hours between day 10 and day 14. XBP1s expression in control adipocytes reduced to 0.2 fold compared to day 0 on day 2 before gradually increasing to 1.5 fold higher than day 0 on day 14. Following 4μ8C, XBP1s mRNA expression was significantly lower ($P<0.01$ – $P<0.001$) than control on day 2 and day 4. The inhibitor was removed on day 4, and on day 6 the expression of XBP1s remained significantly below ($P<0.05$) control. The expression of XBP1s matched that of control on day 10 and day 14. XBP1u expression in control and IRE1α inhibited adipocytes decreased to 0.4 fold compared to day 0 on day 2 and then gradually increased to 1.1 fold compared to day 0 on day 14. IRE1α and CHOP expression in both control and IRE1α inhibited adipocytes decreased on day 2 and then fluctuated below the day 0 level for the duration of adipogenesis. There were no significant differences in XBP1u, IRE1α, or CHOP mRNA expression between control and IRE1α inhibited adipocytes for the duration of adipogenesis.

CEBPα expression in control adipocytes increased and peaked on day 6 – 10 after induction of adipogenesis, however the fold increase compared to day 0 varied between subjects and only the median control is shown. In all the subjects, IRE1α inhibition reduced CEBPα expression by approximately 50 % ($P<0.001$) from day 4 to 14 of adipogenesis compared to control. PPARγ expression gradually increased for the duration of adipogenesis in control cells, peaking on day 14 at 5 fold higher than day 0. In 4 µM 4µ8C treated adipocytes, PPARγ expression increased after initiation of adipogenesis but was significantly lower ($P<0.05$) than control from day 4 to day 14. Adiponectin was not detected on day 0 in any of the adipocytes. In control adipocytes, adiponectin increased considerably after day 2, and similarly to CEBPα, the extent of fold increase compared to day 0 varied between subjects. Following 4 µM 4µ8C treatment, adiponectin expression was reduced compared to control for the duration of adipogenesis.

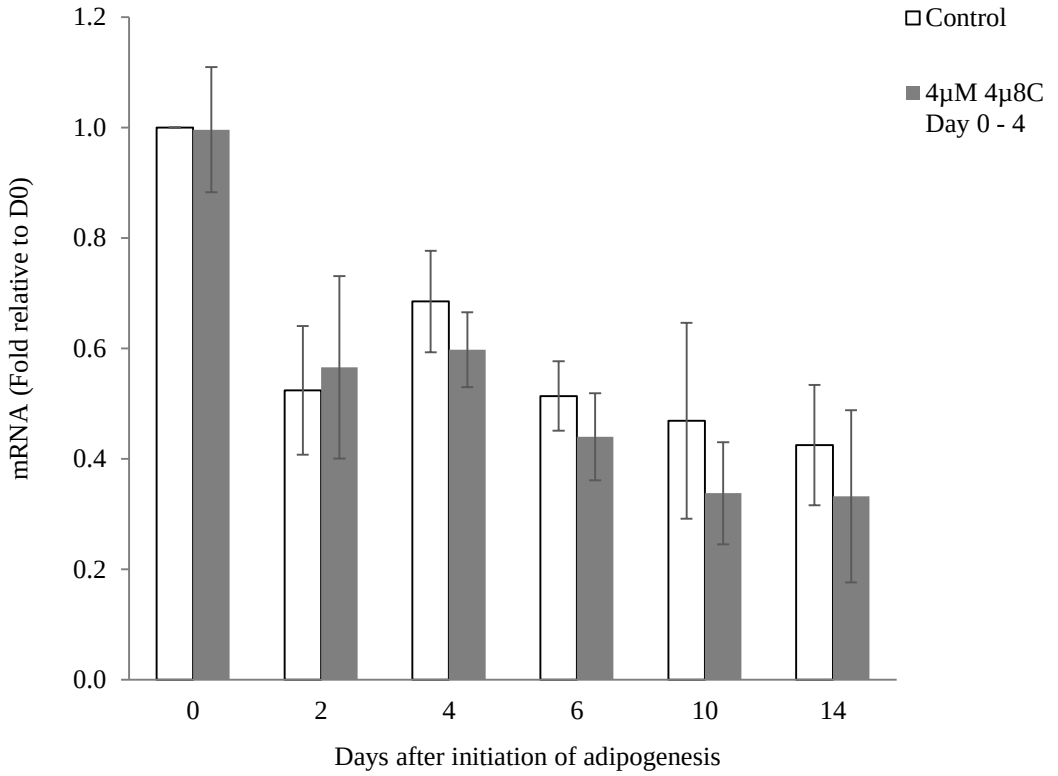
Figure 5.2.5 During adipogenesis 4μ8C inhibited XBP1s and adipogenic regulator mRNA expression





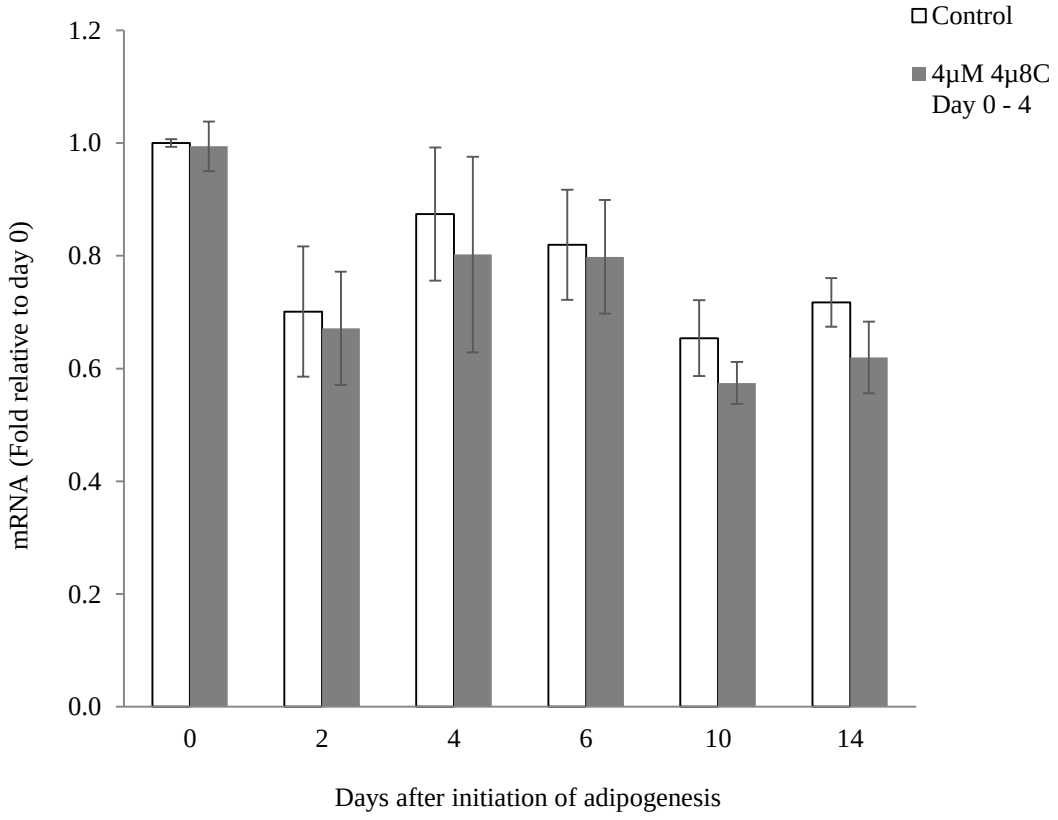
C

IRE1α mRNA during adipogenesis with IRE1α inhibited day 0 - 4



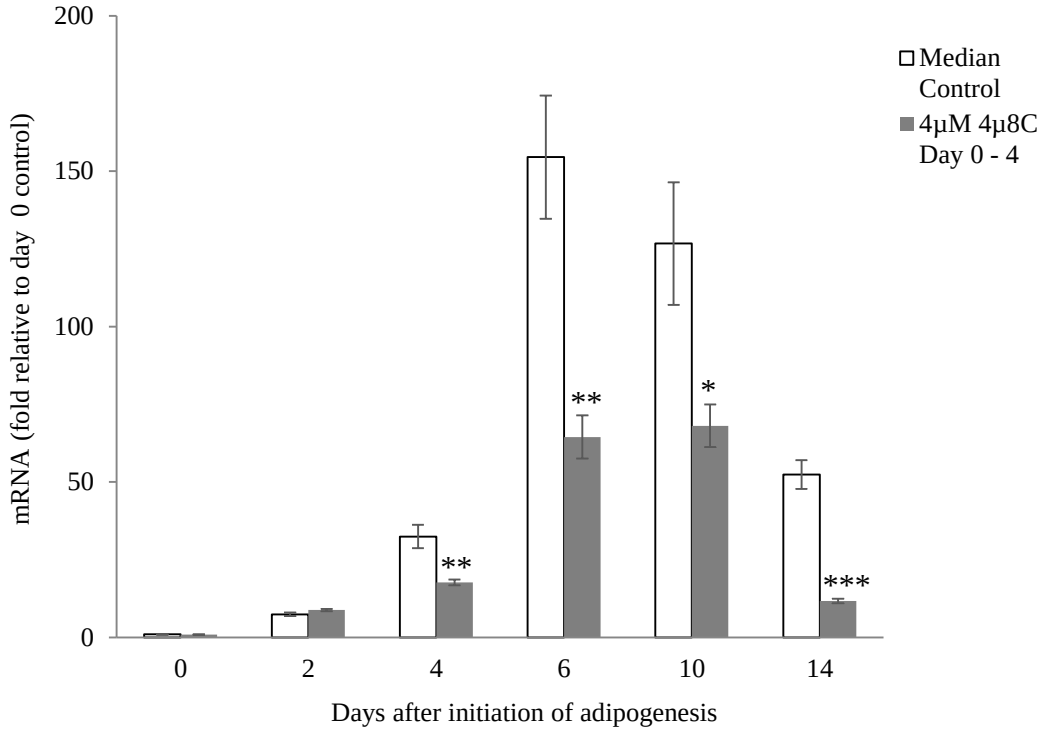
D

CHOP mRNA during adipogenesis with IRE1α inhibited day 0 - 4



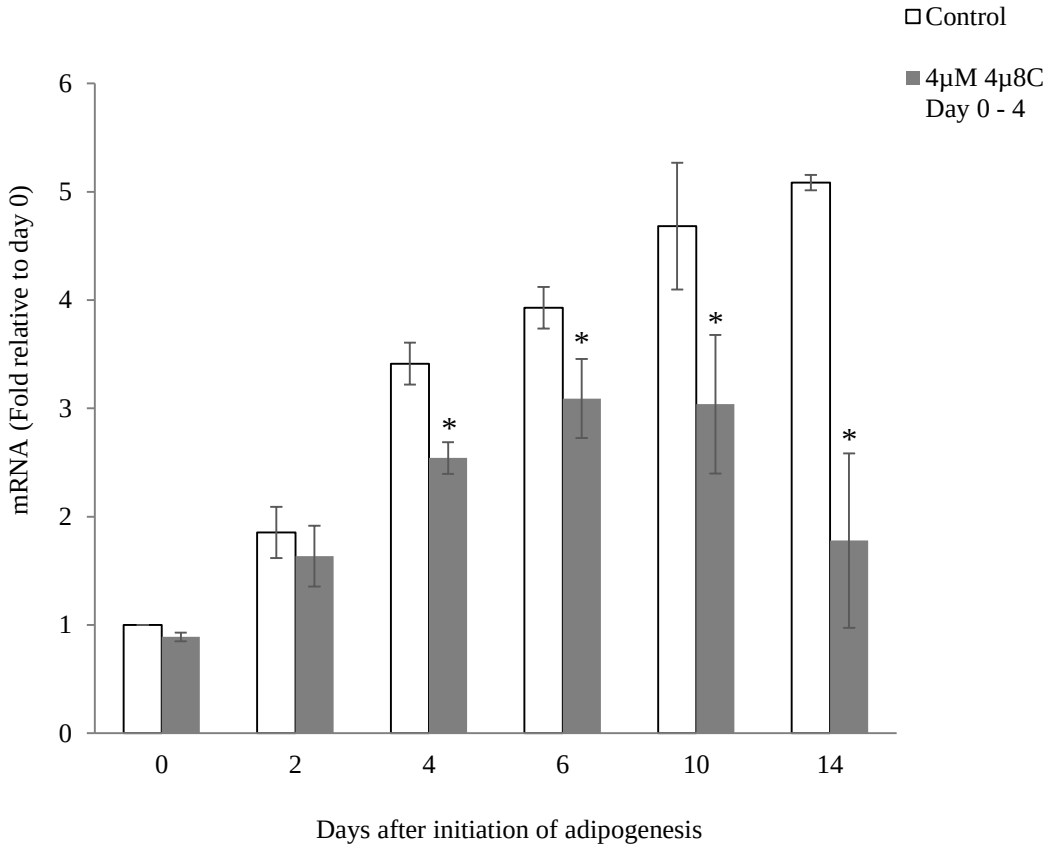
E

CEBPα mRNA during adipogenesis with IRE1α inhibited day 0 - 4



F

PPARγ mRNA during adipogenesis with IRE1α inhibited day 0 - 4



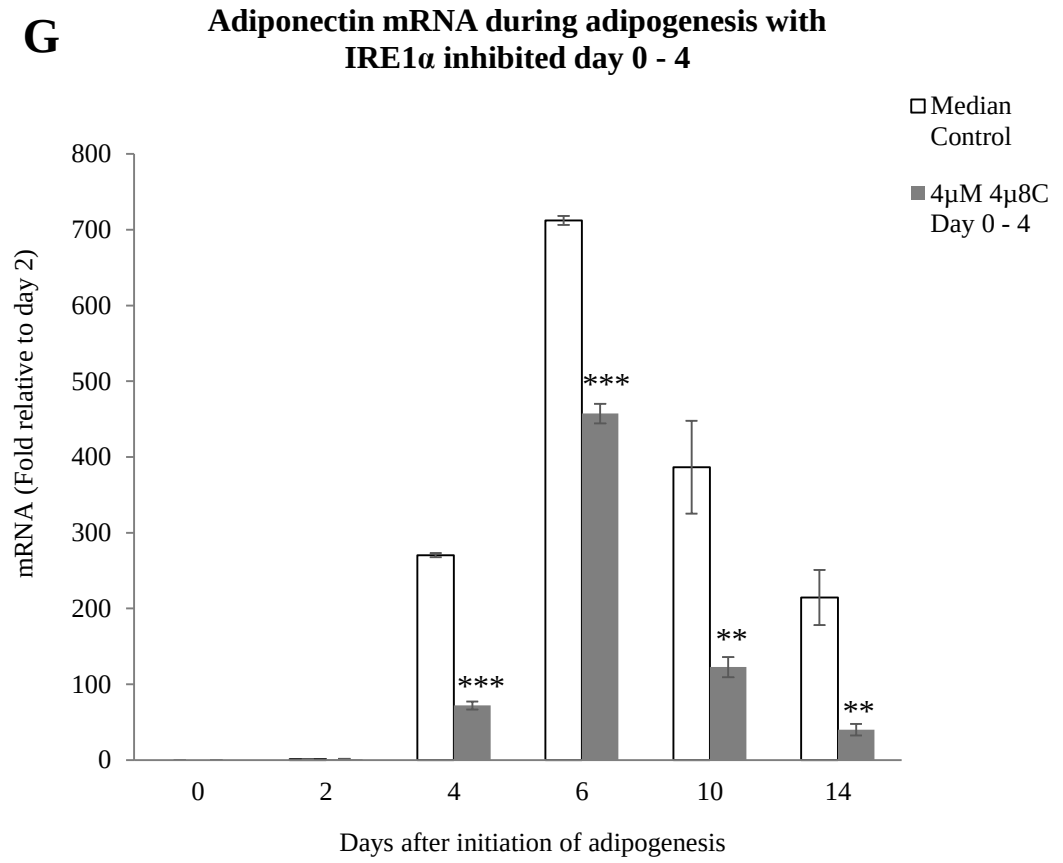
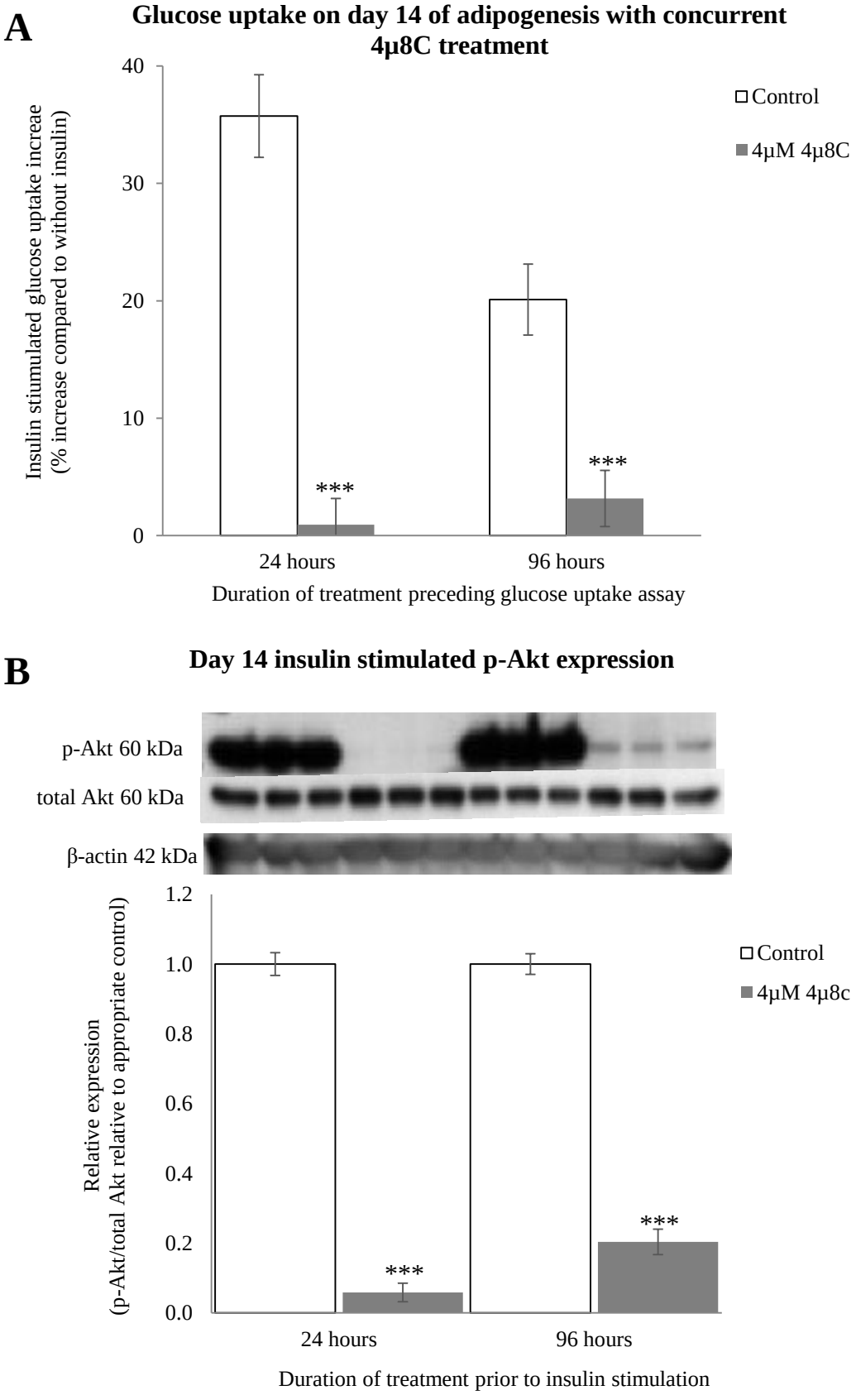


Figure 5.2.5 legend: During adipogenesis 4μ8C inhibited XBP1s and adipogenic regulator mRNA expression. Adipogenesis was induced in primary SAT lean preadipocytes (n=9) for 14 days with DMSO (control) or with 4 μM 4μ8C for the initial 96 hours (day 0 – 4). mRNA was measured during adipogenesis for XBP1s (A), XBP1u (B), IRE1α (C), and CHOP (D). Data is expressed as mean ± SE. Significantly different values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

5.2.6 4μ8C reduced insulin stimulated glucose uptake and inhibited Akt phosphorylation

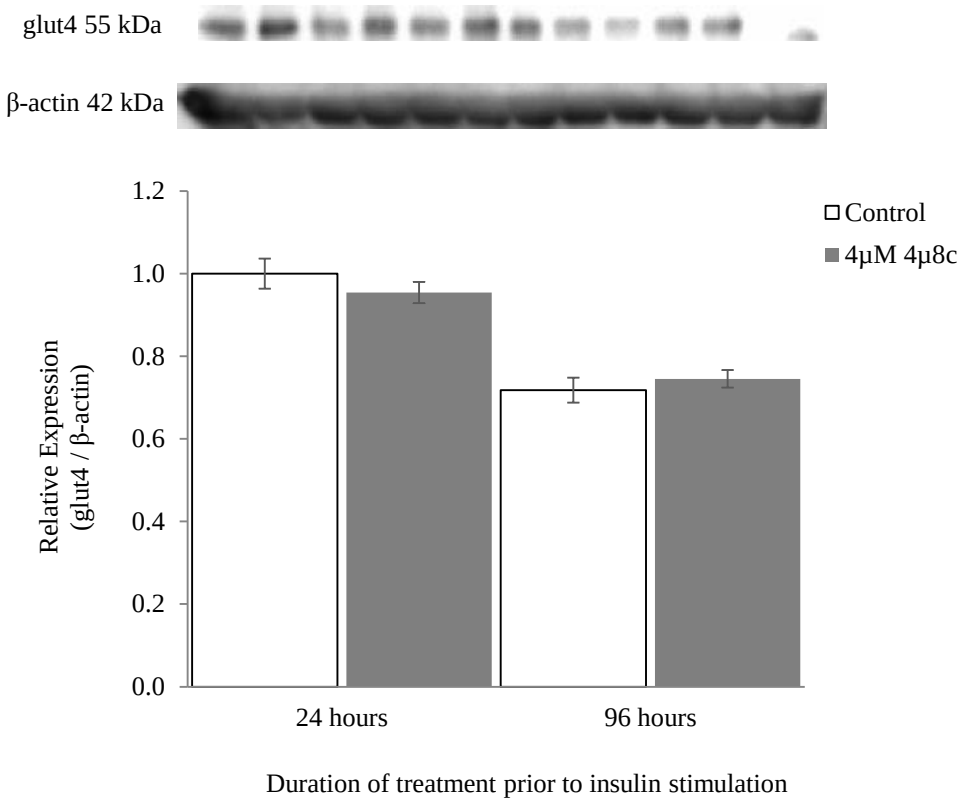
XBP1s mRNA correlated with plasma glucose in subjects after bariatric surgery (figure 3.2.3) and glucose uptake was affected after 4μ8C treatment during early adipogenesis (5.2.4 C), suggesting a possible link between the IRE1α - XBP1s pathway and glucose uptake. To investigate the role of IRE1α - XBP1s during glucose uptake, 4μM 4μ8C was added during the final 24 or 96 hours of adipogenesis up to day 14 and on day 14 insulin stimulated glucose uptake, p-Akt and glut4 proteins, and XBP1s, XBP1u and GLUT4 mRNA expression were measured (figure 5.2.6 A – D). Day 14 insulin stimulated glucose uptake was significantly reduced ($P<0.001$) following IRE1α inhibition for 24 and 96 hours. p-Akt protein normalised with total Akt was highly expressed in control adipocytes and significantly reduced ($P<0.001$) following 4μ8C treatment compared to control. There was no difference in glut4 protein expression between control and IRE1α inhibited adipocytes. XBP1s mRNA was significantly reduced ($P<0.01$) and XBP1u mRNA was significantly increased ($P<0.05$) following 4μ8C treatment compared to control. There was no difference in GLUT4 mRNA following 24 hours with 4μ8C but there was a significant reduction ($P<0.05$) following 96 hours with 4μ8C compared to control.

Figure 5.2.6 4µ8C reduced insulin stimulated glucose uptake and inhibited Akt phosphorylation



C

Day 14 insulin stimulated glut4 expression



D

Day 14 insulin-stimulated gene expression

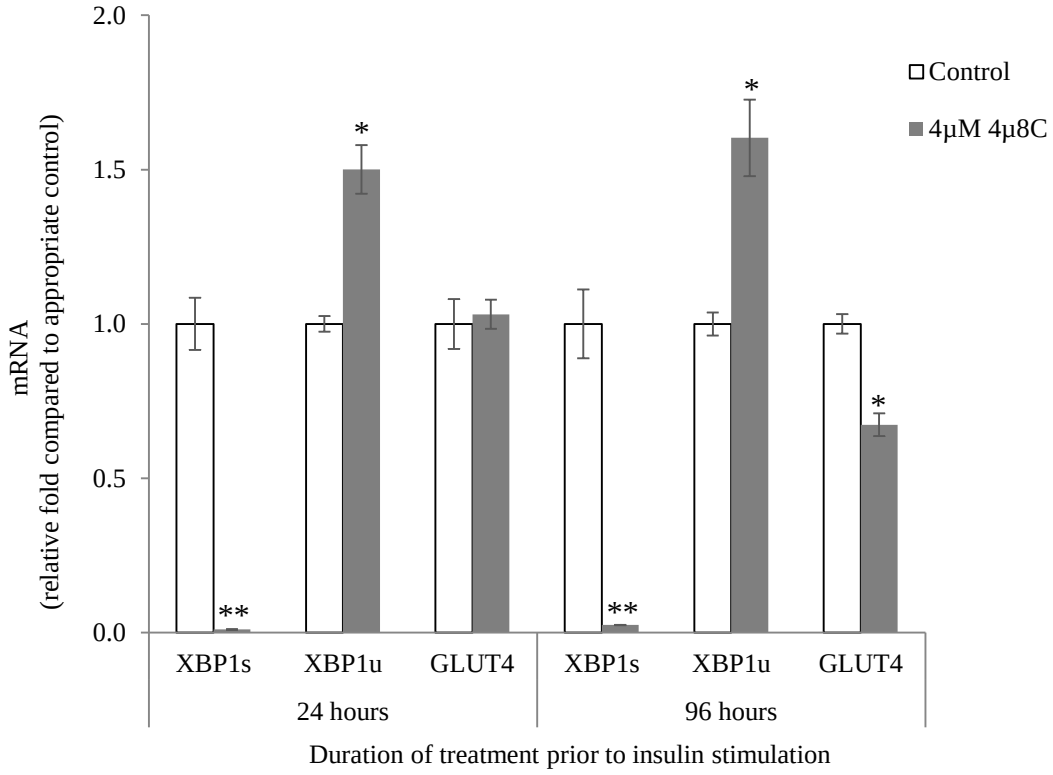


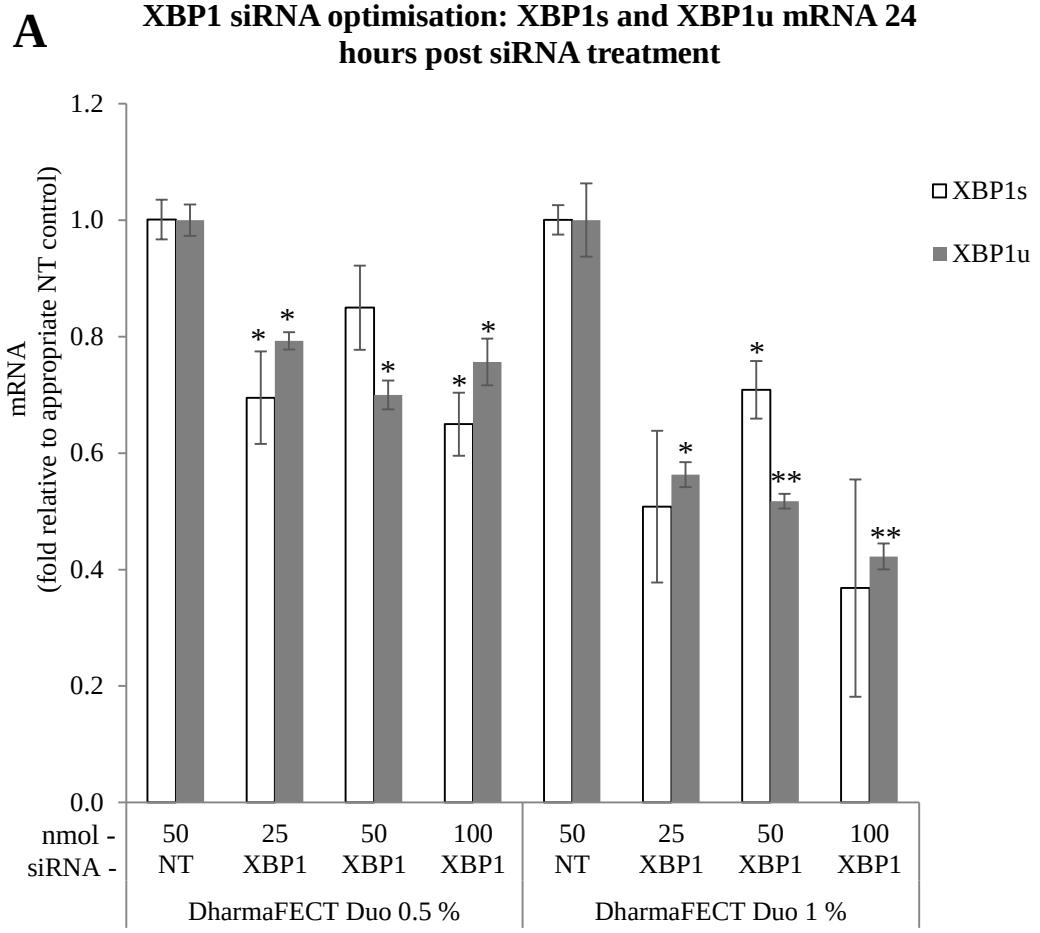
Figure 5.2.6 legend: 4μ8C reduced insulin stimulated glucose uptake and inhibited Akt phosphorylation. Adipogenesis was induced for 14 days in ChubS7 cells and treated for the final 24 hours with DMSO (control 24 hours) or 4 μM 4μ8C, or treated for the final 96 hours with DMSO (control 96 hours) or 4 μM 4μ8C. On day 14 after induction of adipogenesis, insulin stimulated glucose uptake (A), p-Akt (B) and glut4 (C) protein expression, and XBP1s, XBP1u, and GLUT4 (D) mRNA expression were measured. Data is expressed as mean ± SE. Significantly different values are indicated as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

5.2.7 XBP1 siRNA transfection during early adipogenesis reduced adipocyte function

To validate the findings using 4μ8C to downregulate XBP1s (figure 5.2.4), XBP1 siRNA treatment was performed. XBP1 siRNA treatment was optimised and administered during day 0 – 4 of adipogenesis. To ensure successful XBP1 knockdown with XBP1 siRNA, XBP1s and XBP1u mRNA were measured on day 4. Glucose uptake was measured on day 14 following induction of adipogenesis and siRNA transfection (figure 5.2.7 A – C). During optimisation both XBP1s and XBP1u mRNA were significantly reduced ($P<0.05$) following XBP1 siRNA compared to non-targeting siRNA treatment in a dose dependent way. The higher concentration of transfection reagent (1 % DharmaFECT Duo) reduced the XBP1 mRNAs more than the lower concentration (0.5 % DharmaFECT Duo). 25 nmol XBP1 siRNA with DharmaFECT Duo 0.5 % was selected to inhibit XBP1 during adipogenesis because it significantly reduced XBP1s and XBP1u mRNA whilst

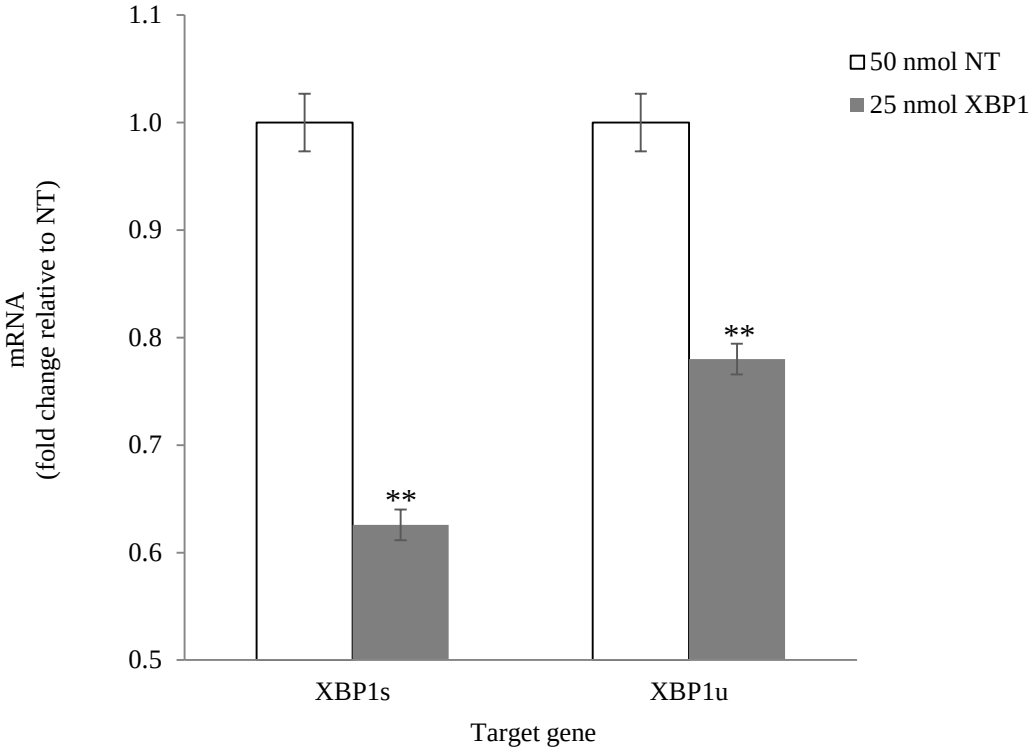
having the fewest cytotoxic effects, since it was the lowest concentration of siRNA and transfection reagent. On day 4 following XBP1 siRNA transfection XBP1s and XBP1u mRNA were significantly reduced (P<0.01) compared to non-targeting siRNA transfection. On day 14 following induction of adipogenesis, insulin stimulated glucose uptake was significantly reduced (P<0.01) following XBP1 siRNA compared to non-targeting siRNA transfection.

Figure 5.2.7 XBP1 siRNA transfection during early adipogenesis reduced adipocyte function



B

XBP1s and XBP1u mRNA expression on day 4 following XBP1 siRNA treatment on day 0 and day 2



C

Glucose uptake on day 14 of adipogenesis following transient XBP1 siRNA treatment on day 0 and 2

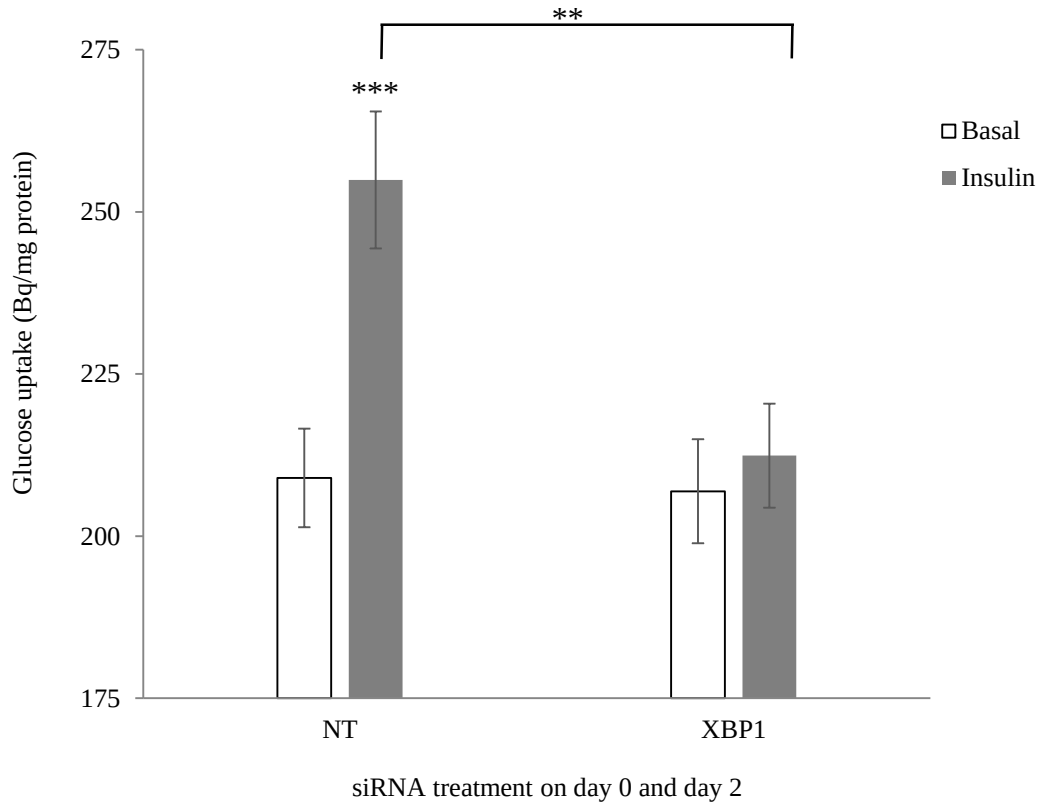


Figure 5.2.7 legend: XBP1 siRNA transfection during early adipogenesis reduced adipocyte function. Optimal XBP1 siRNA conditions were established in ChubS7 adipocytes (A) (25 nm, 50 nm, 100 nm; 25 nmol, 50 nmol, 100 nmol, NT; non-targeting siRNA, XBP1; XBP1 siRNA). Adipogenesis was induced in ChubS7 adipocytes and transfection was carried out with 50 nmol non-targeting (NT) or 25 nmol XBP1 siRNA treatment at induction (day 0) and at 48 hours after induction (day 2). XBP1s and XBP1u mRNA expression was measured at 96 hours following adipogenesis induction (day 4) (B). Adipogenic capacity was assed by functional glucose uptake under basal and insulin stimulated conditions on day 14 after induction of adipogenesis (C). Data is expressed as mean ± SE. Significantly different values are indicated as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

5.3 Discussion

This study was designed to investigate the role of XBP1s in human adipogenesis. Inhibition of XBP1s was optimised in human adipocytes with small molecule inhibitor 4μ8C and then the effects of inhibiting XBP1s during adipogenesis were assessed before investigating the molecular mechanisms and validating the findings with XBP1 siRNA. Adipogenesis is an important process to maintain WAT metabolic function. The study in chapter 4 found that the IRE1α - XBP1s pathway is indispensible for successful human adipogenesis, however the role of XBP1s in human adipogenesis is not known. Recently Cho *et al.* have shown in mouse 3T3-L1 cells that XBP1s is a key regulator of adipogenic modulator PPARγ (Cho, *et al.*, 2014). The results of this study show that XBP1s plays a key role in early human adipogenesis independently of other UPR pathways and may mediate its effects through PPARγ and CEBPα. Furthermore, XBP1s is essential for insulin stimulated Akt phosphorylation mediated glucose uptake. Therefore XBP1s is necessary for adipogenesis and transmitting insulin action and thus plays a key role in glucose uptake, metabolic health, insulin resistance and T2DM.

The small molecule inhibitor 4μ8C has been used to inhibit IRE1α endonuclease activity without effecting cell viability (Cross, *et al.*, 2012; Qiu, *et al.*, 2013; Zhang, *et al.*, 2014a). However previous reports have investigated 4μ8C in MEF cells (Cross, *et al*., 2012), macrophages from the synovial fluid of rheumatoid arthritis patients (Qiu, *et al*., 2013) and mouse pancreatic beta-cells (Zhang, *et al.*, 2014a) but not human adipocytes. Therefore appropriate pharmacological studies were conducted to ascertain a suitable dose to use in human adipocytes that inhibited XBP1s expression without inducing cytotoxic effects. This study found

that 4μ8C specifically inhibits IRE1α endonuclease activity in adipocytes; it reduced the expression of XBP1s in the presence of TM without affecting the expression of IRE1α, ATF6 or CHOP in agreement with Cross *et al.* (Cross, *et al*., 2012). In the absence of TM, 4μ8C reduced the expression of XBP1u, however in the presence of TM, 4μ8C increased the expression of XBP1u perhaps as a compensatory mechanism of the adipocytes to increase the substrate of IRE1α endonuclease activity in an attempt to restore expression of XBP1s. This study found that human adipocytes treated with 4 μM 4μ8C maintained 100 % cell viability when treated for 24 hours and over 90 % cell viability when treated for 48 hours. As the concentration of inhibitor increased, adipocyte cell viability decreased in contrast to studies in MEF cells (Cross, *et al*., 2012), however the MEF cells were only exposed to 4μ8C for 6 hours and therefore any cytotoxic effects may not have had time to take effect. The optimum concentration of 4μ8C for human adipocytes was decided as 4 μM because this concentration reduced TM induced XBP1s expression by 90 % and maintained 90 % cell viability after 48 hours of treatment. Therefore 4 μM 4μ8C was used for all subsequent studies.

This investigation found that the time and duration of XBP1s inhibition during adipogenesis affected adipogenic capacity in primary human adipocytes. Inhibiting XBP1s did not alter other UPR factors during adipogenesis and therefore its effects are likely to be mediated independently of UPR activation and via alternative mechanisms. This study shows that during adipogenesis in control cells, XBP1s significantly decreased initially until day 2 and then gradually increased to above the day 0 expression. This overall pattern of XBP1s expression during adipogenesis is the same as Cho *et al*. reported in 3T3-L1 cells (Cho, *et al*.,

2013). However, Cho *et al*. suggested that an increase in XBP1s expression during the initial onset of adipogenesis plays a role in successful adipogenesis (Cho, *et al*., 2013). This study disagrees with their hypothesis because inhibiting XBP1s during the initial 48 hours of adipogenesis had no effect on adipogenesis or adipocyte function as assessed by CEBPα, PPARγ and adiponectin mRNA expression, lipid accumulation, lipolysis, or adipocyte glucose uptake. This study found that inhibiting XBP1s during day 2 – 4 of adipogenesis significantly reduced CEBPα and adiponectin expression on day 4 and 6, reduced PPARγ on day 6 and significantly reduced lipid accumulation compared to control. Administering 4μ8C for the initial 4 days of adipogenesis resulted in significantly lower CEBPα expression on days 4 and 6, reduced PPARγ on day 6 and reduced adipogenic capacity, measured by lipid accumulation, lipolysis and glucose uptake. There was no additional impact on adipogenesis when XBP1s was inhibited for the initial 6 days of adipogenesis. Therefore collectively these results suggest that XBP1s exerts its effects during the early but not immediate early stages of adipogenesis. In context of existing literature, this suggests XBP1s acts downstream of the early activated CEBPβ (Guo, *et al.*, 2015) and may interact directly with CEBPα or PPARγ.

A link between UPR signalling and metabolism has been suggested (Hotamisligil, 2010; Lee and Ozcan, 2014). This study showed that XBP1s plays a key role during early adipogenesis in primary human adipogenesis and affects primary human adipocyte metabolism. Having found that inhibiting IRE1α endonuclease activity and subsequent depletion of XBP1s during early adipogenesis reduced primary human adipogenic capacity, it was validated further with primary human

preadipocytes from three lean metabolically healthy subjects, each with experiments repeated in triplicate. This study showed that inhibiting IRE1α with 4 μM 4μ8C during the initial 4 days of adipogenesis consistently and significantly reduced lipid uptake, lipolysis and insulin stimulated glucose uptake in primary human adipocytes. Therefore concurring with the hypothesis of a link between ER stress and insulin resistance (Flamment, *et al.*, 2012).

This investigation showed that XBP1s exerts its effects on adipogenesis independently of other UPR signalling factors and it may regulate CEBPα and PPARγ expression. These findings are the first of their kind in human adipocytes and support the mouse cell line work of Cho *et al.* that suggest XBP1s may regulate PPARγ in adipogenesis (Cho, *et al.*, 2014). Blocking XBP1s expression had no significant effect compared to control on CHOP expression, which has been shown to inhibit adipogenesis (Hou, *et al.*, 2013). All three lean metabolically healthy subjects had varying degrees of CEBPα activation during adipogenesis, however inhibition of XBP1s resulted in consistent downregulation of CEBPα compared to control. Despite the variation in CEBPα expression during adipogenesis between the preadipocytes, PPARγ expression was consistent. Nonetheless, when XBP1s was inhibited PPARγ was downregulated compared to control. Thus, XBP1s likely acts upstream of CEBPα and PPARγ in the sequential transcriptional processes involved in adipogenesis.

XBP1s has been shown to promote adiponectin multimerisation and improve glucose regulation in mice (Sha, *et al.*, 2014). Furthermore, WAT XBP1s and plasma glucose were both reduced after bariatric surgery in humans (chapter 3).

This study provides novel evidence that XBP1s is necessary for insulin stimulated glucose uptake in a controlled human adipocyte cell culture system. Insulin stimulated glucose uptake on day 14 of adipogenesis was blocked in human adipocytes when treated with 4μ8C for the final 24 and 96 hours of adipogenesis. To investigate the molecular mechanisms linking XBP1s and glucose uptake, key proteins and mRNA expressions were measured. GLUT4 mRNA expression was not affected by 24 hour XBP1s inhibition but was significantly reduced following 96 hours of inhibition compared to control, although this was not reflected at the protein level. In control adipocytes, 30 minutes following insulin addition, p-Akt was highly expressed whereas it was almost completely blocked in 4μ8C treated adipocytes irrespective of inhibitor treatment duration. This suggests that XBP1s is necessary for transmitting the acute action of insulin and therefore plays a key role in metabolism. Therefore this study provides a novel mechanism that links the UPR to metabolism and supports the hypothesis of UPR integration with metabolism (Lee and Ozcan, 2014). The findings using 4μ8C to inhibit XBP1s were supported by XBP1 siRNA transfection. XBP1s siRNA transfection during early adipogenesis in human preadipocyte cell line ChubS7 significantly reduced day 14 insulin stimulated glucose uptake compared to non-targeting siRNA transfection. A summary of findings from this study and proposed roles of UPR signalling in adipocyte metabolism are shown in figure 5.3.1.

Figure 5.3.1 Proposed roles of unfolded protein response molecular signalling in adipocyte metabolism

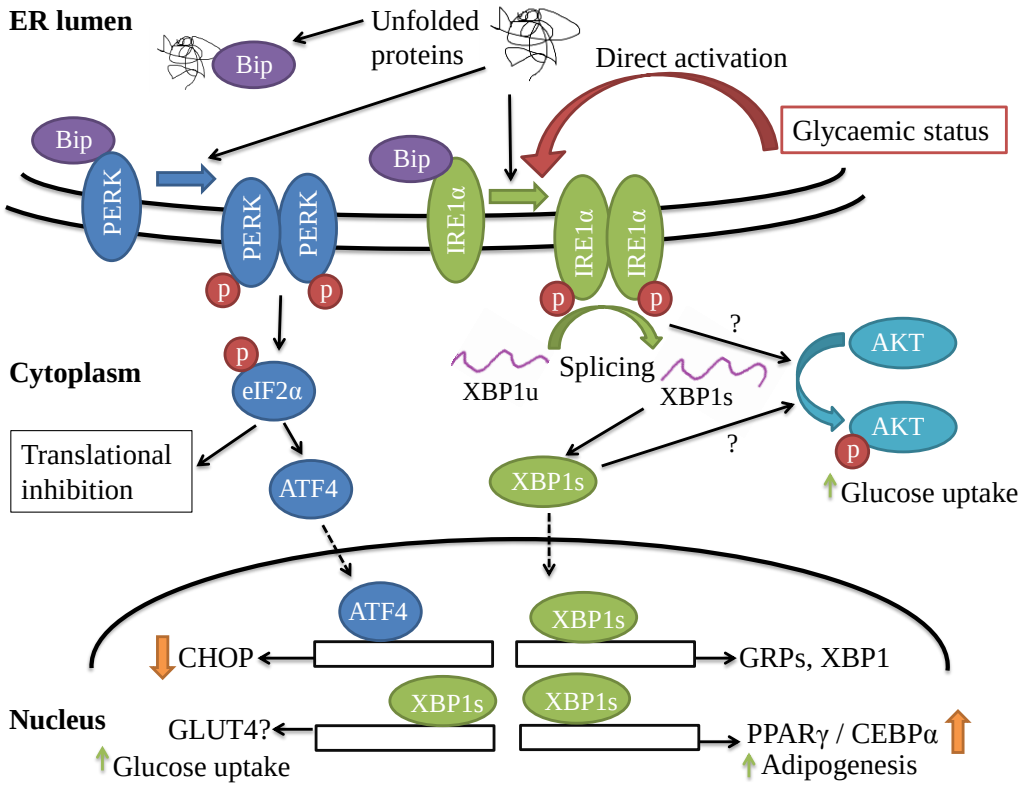


Figure 5.3.1 legend: Proposed roles of unfolded protein response molecular signalling in adipocyte metabolism. The results from this study indicate that the unfolded protein response (UPR) plays an integrated role in adipocyte metabolism. UPR may be directly activated by glycaemic status and it influences short-term glucose uptake via Akt phosphorylation and long-term adipogenesis via balancing inhibiting and activating adipogenic factors. (ER; endoplasmic reticulum, Bip; binding immunoglobulin protein – also known as GRP78; glucose regulated protein 78 kDa, PERK; protein kinase RNA-like endoplasmic reticulum kinase, p; phosphorylated, eIF2α; eukaryotic translation initiation factor 2 A, ATF4; activating transcription factor 4, CHOP; C/EBP homologous protein, IRE1α; inositol requiring enzyme 1 alpha, XBP1u; X-box binding protein 1

unspliced, XBP1s; X-box binding protein 1 spliced, GRPs; glucose regulated proteins, AKT; Akt, also known as protein kinase B (PKB), PPARγ; peroxisome proliferator-activated receptor gamma, CEBPα; CCAAT/enhancer-binding protein alpha, GLUT4; Glucose transporter type 4).

5.4 Conclusions

In summary, this study has shown that XBP1s plays a vital role upstream of CEBPα and PPARγ in human adipogenesis and that it is necessary for mediating the action of insulin. These findings are the first of their kind in human adipocytes and support the mouse cell line work of Cho *et al*. that suggest XBP1s may regulate PPARγ in adipogenesis (Cho, *et al.*, 2014). XBP1s has been shown to promote adiponectin multimerisation and improve glucose regulation in mice (Sha, *et al.*, 2014). Furthermore, WAT XBP1s and plasma glucose improved after bariatric surgery in humans (chapter 3). This study provides novel evidence that XBP1s is necessary for insulin stimulated glucose uptake in a controlled human adipocyte cell culture system. Taken together, these results support the link between UPR signalling and metabolism (Hotamisligil, 2010; Lee and Ozcan, 2014). Although further research is required, these findings have provided useful insight into the role of XBP1s in adipogenesis and metabolism and will benefit the development of clinical interventions to alleviate metabolic diseases.

Chapter 6: Wnt10b in human adipogenesis

6.1 Introduction

Adipogenesis is an important process to maintain WAT metabolic function. WAT can dynamically change in size during adult life to accommodate the energy requirements of the body (Poulos, *et al.*, 2010). Obesity complications in humans such as T2DM can result from the inability of SAT to appropriately expand and store lipids (Virtue and Vidal-Puig, 2010). Impaired WAT expandability is mainly due to defects in adipogenesis (Isakson, *et al.*, 2009). Further understanding of adipogenesis may present targets to improve metabolic regulation. The IRE1α-XBP1s UPR pathway is essential for adipogenesis in mouse cells (Sha, *et al.*, 2009) and in human cells (chapters 4 and 5). XBP1s plays an important role during early human adipogenesis and may regulate CEBPα and PPARγ (chapter 5). It has been hypothesised that reciprocal repression might exist during adipogenesis between Wnt signalling and CEBPα or PPARγ because activation of these factors can lead to a substantial reduction in Wnt downstream signalling β-catenin levels (Moldes, *et al.*, 2003). More recently it has been suggested that Wnt normally inhibits CEBPα as a negative feedback loop to reduce adipogenesis (Christodoulides, *et al.*, 2009). Cho *et al*. have shown in mouse 3T3-L1 cells that XBP1s enhances adipogenesis through the downregulation of Wnt10b (Cho, *et al*., 2013). Therefore there may be a link between XBP1s and Wnt10b in human adipogenesis, however it has not been studied.

Wnt proteins comprise a family of highly conserved secreted proteins that exert autocrine and paracrine actions via binding to cell-surface receptors (Herr, *et al.*, 2012). Wnt ligands bind to the frizzled (FZD) and low-density-lipoprotein-related protein 5/6 (LRP5/6) coreceptor complex to activate the canonical Wnt signalling

pathway. The activated receptor complex disrupts or functionally inactivates a β-catenin destruction complex. The destruction complex consists of the scaffold protein Axin, adenomatous polyposis coli (APC), glycogen synthase kinase-3 (GSK-3) and casein kinase-1 (CKI). When Wnt is not bound to its surface receptors, the destruction complex is active and regulates the phosphorylation, ubiquitination and subsequent degradation of β-catenin. When Wnt is bound to its surface receptors, the destruction complex is inhibited, β-catenin accumulates in the cytoplasm and translocates to the nucleus where is engages the TCF/LEF transcription factors to activate the Wnt transcriptional program (figure 1.5.3) (Li, *et al.*, 2012). Several TCF/LEF targets are linked to cell differentiation. For example TCF7 has been shown to regulate a self-renewal/differentiation switch that operates in the absence of Wnt signalling (Wu, *et al.*, 2012). Negative feedback regulation of the Wnt pathway is mediated through conductin, encoded by the *AXIN2* gene and also known as axin2 (Bernkopf, *et al.*, 2015). Wnt10b has been extensively studied in an adipogenesis context (Rosen and MacDougald, 2006). Wnt10b has been shown to impair WAT and BAT formation *in vivo* when overexpressed specifically in mouse adipocytes (Longo, *et al.*, 2004). Furthermore, increasing Wnt10b levels in WAT leads to decreased susceptibility to obesity in both diet-induced (Longo, *et al.*, 2004) and genetic mouse models (Wright, *et al.*, 2007). Wnt10b also seems to play a key inhibitory role in human adipogenesis. Naturally occurring mutations in the *WNT10B* gene such as C256Y are associated with early-onset obesity (Christodoulides, *et al.*, 2006). Furthermore, Wnt10b has been shown to impair human preadipocyte differentiation (Isakson, *et al.*, 2009) and abnormal expression of genes involved

in Wnt signalling have been recorded in the adipose tissue of PCOS patients (Chazenbalk, *et al.*, 2012).

Characterisation of adipogenesis regulatory processes have benefited from the discovery of key pathways and transcription factors that contribute to the adipogenic process, as reviewed by Lefterova and Lazar (Lefterova and Lazar, 2009). The transcription factors PPARγ and CEBPs are the crucial determinants of adipocyte fate. There are several inhibitors of adipogenesis including CHOP, upregulated in ER stress (Hou, *et al.*, 2013), and certain TCF/LEF proteins, activated by Wnt signalling (Farmer, 2006). Therefore a network of transcription factors and cell-cycle regulators, in concert with transcriptional coactivators and corepressors, respond to extracellular stimuli to activate or repress adipogenesis. Understanding how Wnt10b and XBP1s fit together in the complex system of adipogenesis regulation may provide pharmacological targets and therefore have clinical benefit.

Therefore the aim of this study was to investigate the role of Wnt10b in human adipogenesis and whether it is linked to XBP1s. In order to investigate the role of Wnt10b in human adipogenesis, anthropometry and a SAT biopsy were collected from a female subject with a naturally occurring WNT10B C256Y mutation rendering a non-functional Wnt10b protein, characterised by Christodoulides *et al.* (Christodoulides, *et al.*, 2006). Preadipocytes were extracted from the WNT10B C256Y SAT biopsy, cultured and adipogenesis induced and compared with preadipocytes derived from SAT of lean metabolically healthy subjects. Adipogenesis and adipocyte function were assessed by lipid accumulation,

lipolysis and glucose uptake. Expression of key adipogenic, ER stress and Wnt signalling proteins and mRNAs were measured during adipogenesis.

6.2 Results

6.2.1 Body composition and metabolic profile of a WNT10B C256Y subject

The systemic effects of non-functional Wnt10b on body composition and metabolic profile were studied. Anthropometric measurements were collected for a subject with a WNT10B C256Y mutation (table 6.2.1). The female subject was 19 years old and had a BMI of 62 kg/m^2. Her body weight (143.2 kg) and fat mass (59.8 % body weight) were greater than any of the obese T2DM subjects described in chapter 3 (table 3.2.1). However, unlike the subjects who underwent bariatric surgery, the WNT10B C256Y subject was not diabetic and had normal glucose control. Her HbA1c (40 mmol/mol) and fasting plasma glucose (4.2 mmol/L) were in the reference range for subjects with controlled blood glucose concentrations. She had a more favorable lipid profile than the obese T2DM subjects prior to their bariatric surgery. Her total cholesterol (4.5 mmol/L), LDL (2.5 mmol/L) and TAG (1.2 mmol/L) were lower, and her HDL (1.3 mmol/L) was higher than the obese T2DM subjects prior to bariatric surgery.

Table 6.2.1 Body composition and metabolic profile of a WNT10B C256Y subject

Gender	Female
Age (Years)	19
Body Weight (kg)	143.2
BMI (kg/m^2)	62
Fat Mass (kg)	57.6
Fat Free Mass (kg)	85.6
Fat Mass (% Body Weight)	59.8
Fat Free Mass (% Body Weight)	40.2
HbA1c (mmol/mol)	40
Plasma Glucose (mmol/L)	4.2
Total Cholesterol (mmol/L)	4.5
LDL Cholesterol (mmol/L)	2.5
HDL Cholesterol (mmol/L)	1.3
Triglyceride (TAG) (mmol/L)	1.2
HDL/LDL (ratio)	0.52

Table 6.2.1 legend: Body composition and metabolic profile of a WNT10B C256Y subject. Anthropometric measurements were taken for a WNT10B C256Y subject.

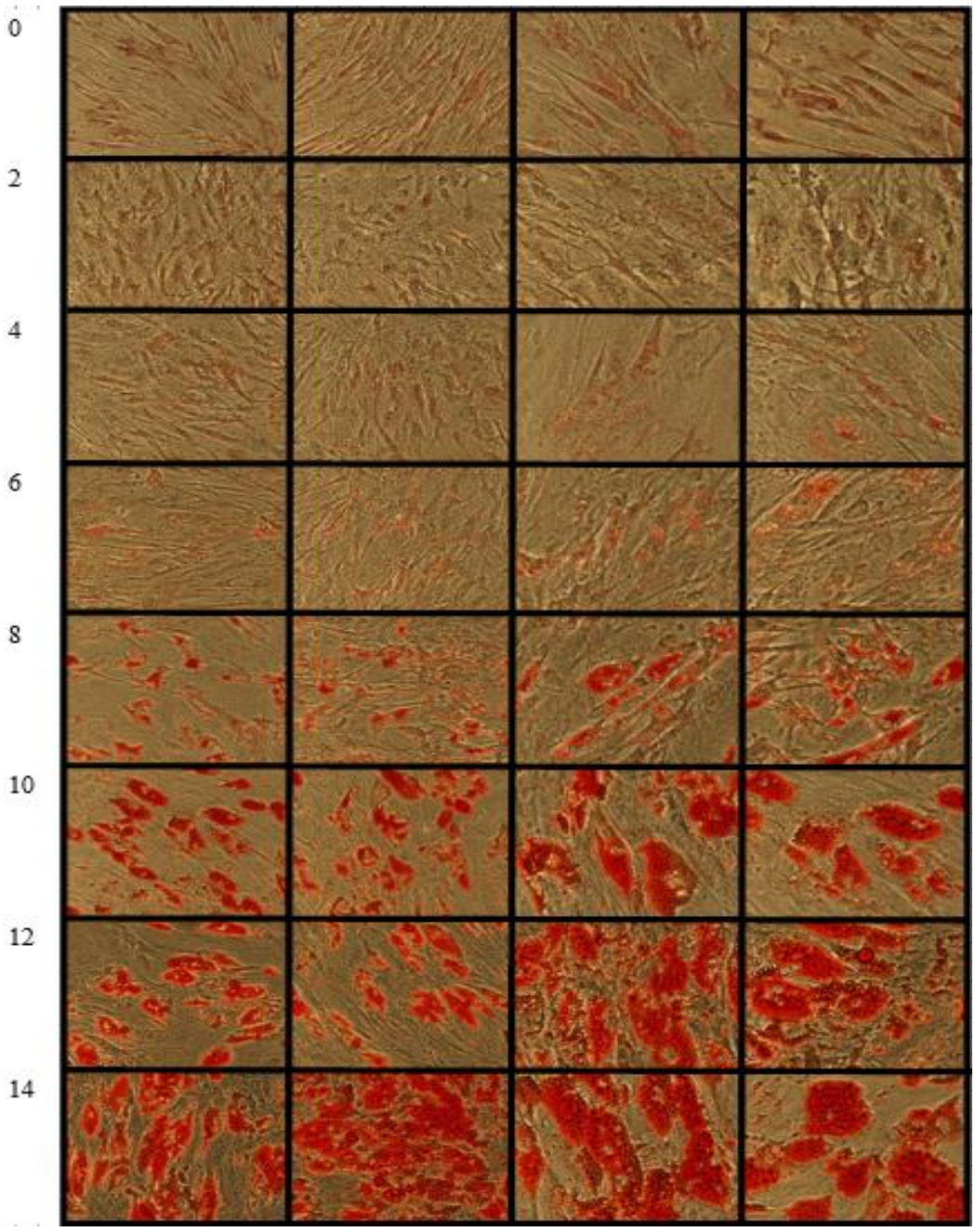
6.2.2 Functional Wnt10b was not required for adipogenesis

To assess the role of Wnt during adipogenesis, adipogenesis was induced in Abd SAT WNT10B C256Y and lean and obese preadipocytes for 14 days. Lipid accumulation and lipolysis were measured during adipogenesis and basal and insulin stimulated glucose uptake were measured on day 14 (figure 6.2.2 A – D). During adipogenesis the WNT10B C256Y adipocytes accumulated more lipid than lean or obese SAT adipocytes. They underwent the same level of lipolysis as lean while the obese control Abd SAT adipocytes underwent significantly higher

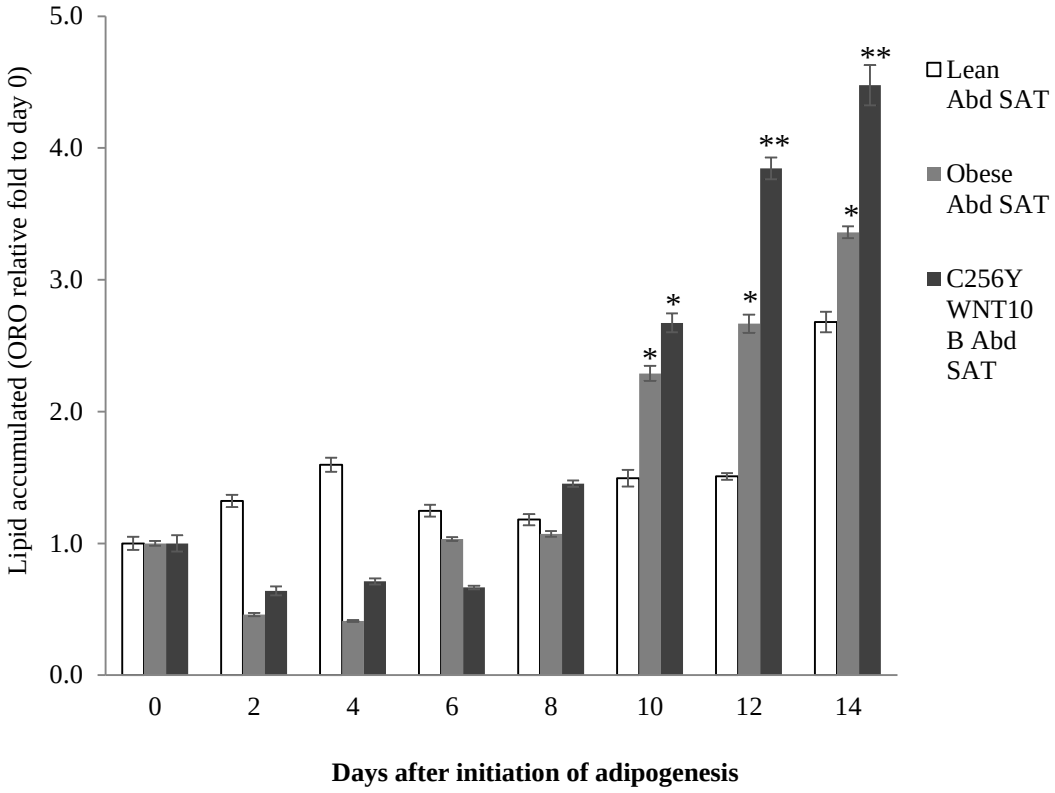
lipolysis than lean. On day 14 after induction of adipogenesis, the adipocytes had significantly higher ($P<0.01$) insulin stimulated glucose uptake compared to basal.

Figure 6.2.2 Functional Wnt10b was not required for adipogenesis

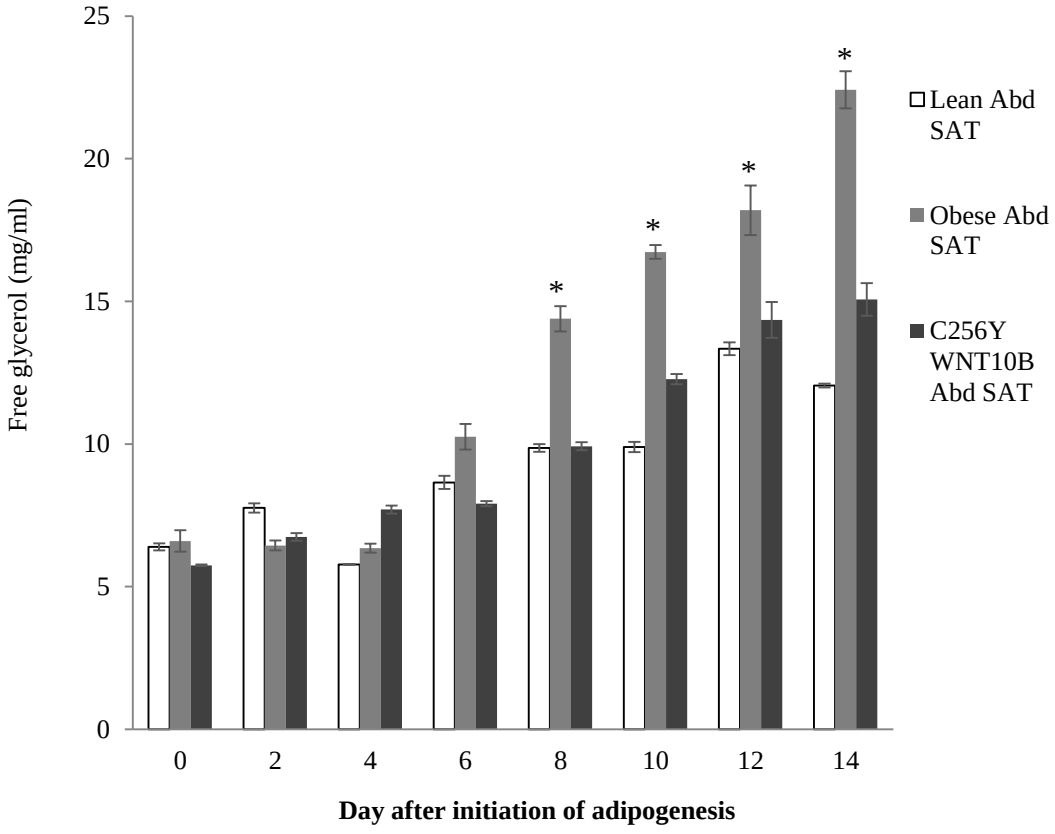
A **Lipid accumulation during WNT10B C256Y adipogenesis**



B **Adipocyte lipid accumulation during adipogenesis**



C **Adipocyte lipolysis during adipogenesis**



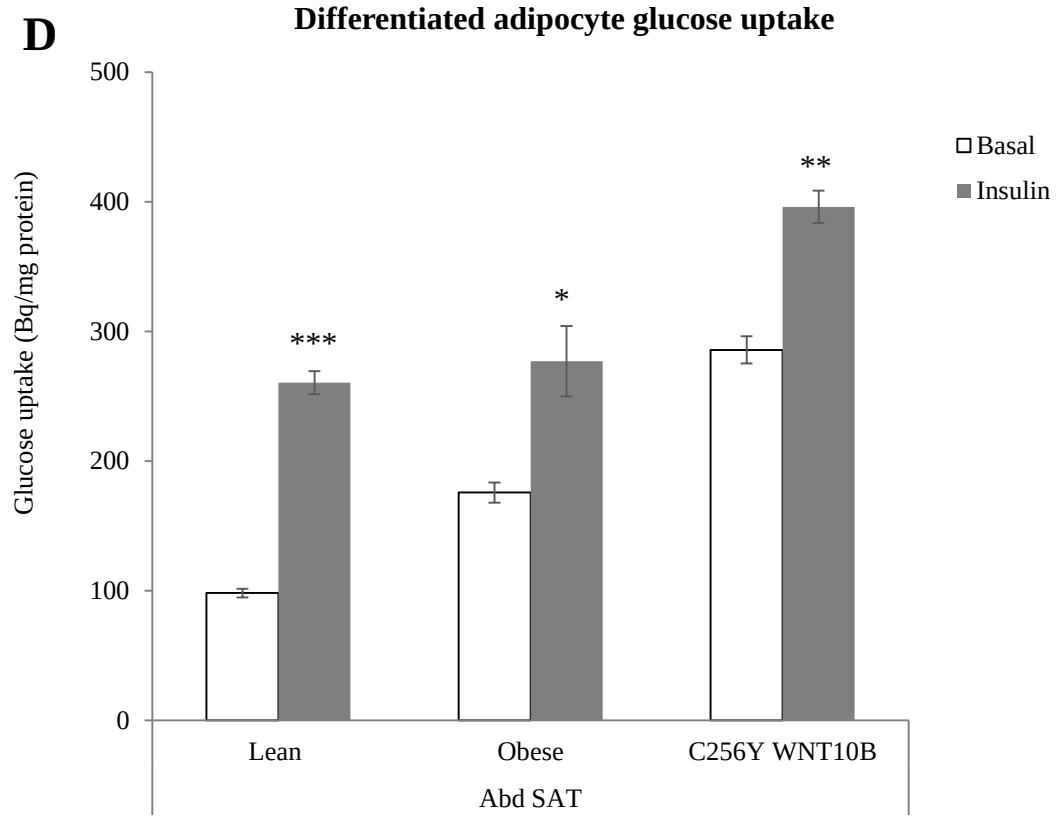
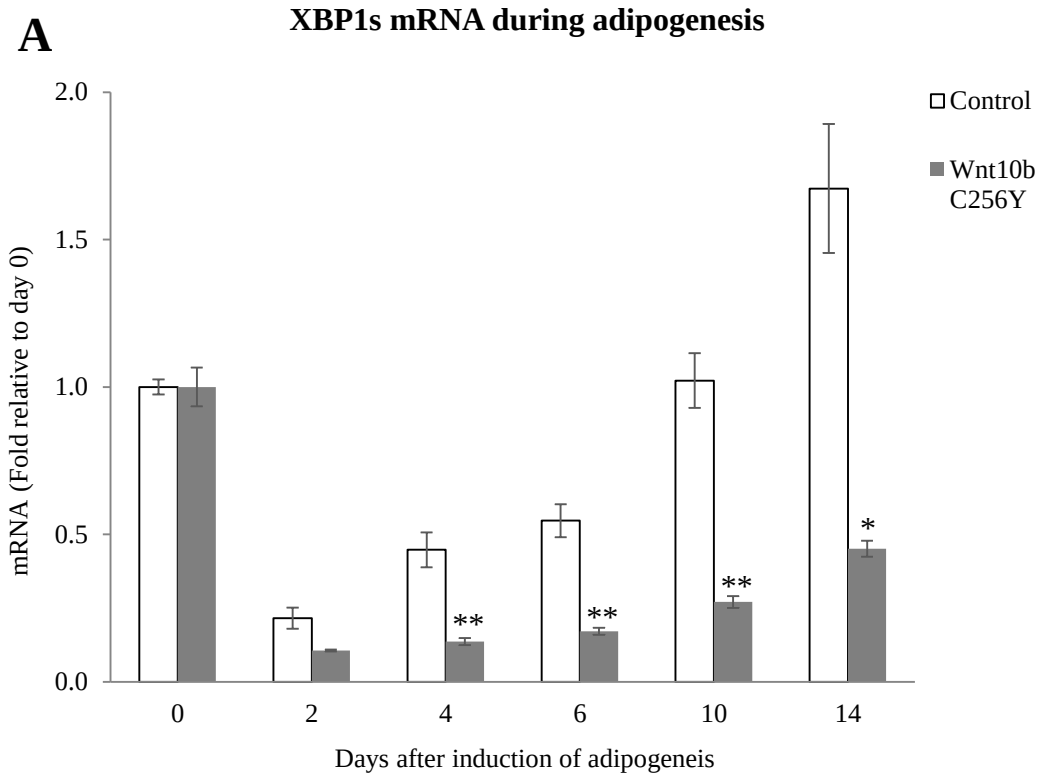


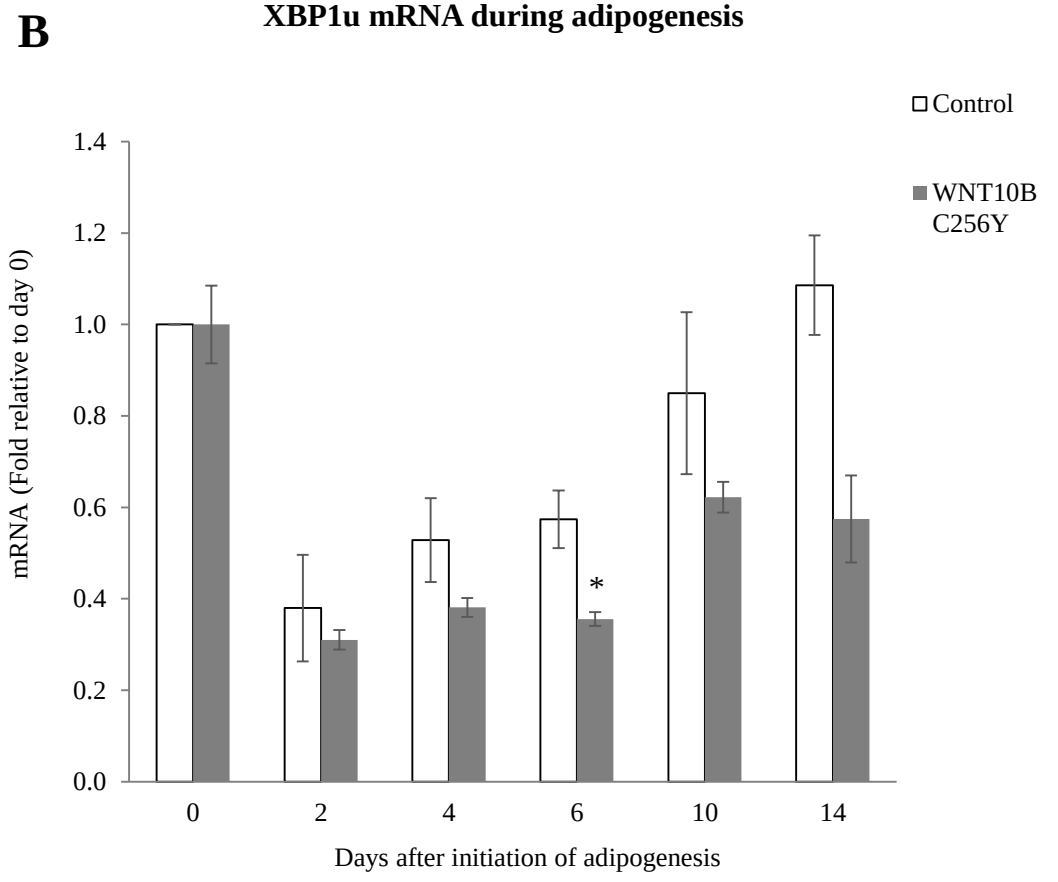
Figure 6.2.2 legend: Functional Wnt10b was not required for adipogenesis. Adipogenesis was induced in Abd SAT WNT10B C256Y and lean and obese control preadipocytes for 14 days. Lipid accumulation (A - B), and lipolysis were measured every 48 hours (C), and basal and insulin stimulated glucose uptake were measured on day 14 (D). Data is expressed as mean ± SE. Significantly different values are indicated as follows: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

6.2.3 XBP1s mRNA expression was reduced during adipogenesis in SAT WNT10B C256Y adipocytes compared to lean

To assess UPR in non-functional Wnt adipogenesis, adipogenesis was induced in SAT lean (control) and SAT WNT10B C256Y adipocytes for 14 days and key UPR gene expressions were measured (figure 6.2.3 A – D). XBP1s mRNA decreased on day 2 after induction of adipogenesis in control and WNT10B C256Y adipocytes. After day 2 XBP1s expression increased in the control cells to 1.5 fold higher than day 0 on day 14. XBP1s mRNA also increased after day 2 in the WNT10B C256Y adipocytes, however it was significantly lower ($P<0.05$ – $P<0.01$) than control cells for the remaining days of adipogenesis and on day 14 it was less than half compared to day 0. XBP1u expression was similar to XBP1s and at every time point after day 0 the WNT10B C256Y subject had lower XBP1u expression compared to control, although this only reached significance ($P<0.05$) on day 6. IRE1α and CHOP expression decreased after day 0 and remained below the day 0 level for the duration of adipogenesis in both control and WNT10B C256Y adipocytes. At almost every time point for IRE1α and CHOP, WNT10B C256Y adipocytes had lower expression levels compared to control, although they only reached significance ($P<0.05$) for IRE1α on day 6.

Figure 6.2.3 XBP1s mRNA expression was reduced during adipogenesis in WNT10B C256Y adipocytes compared to lean





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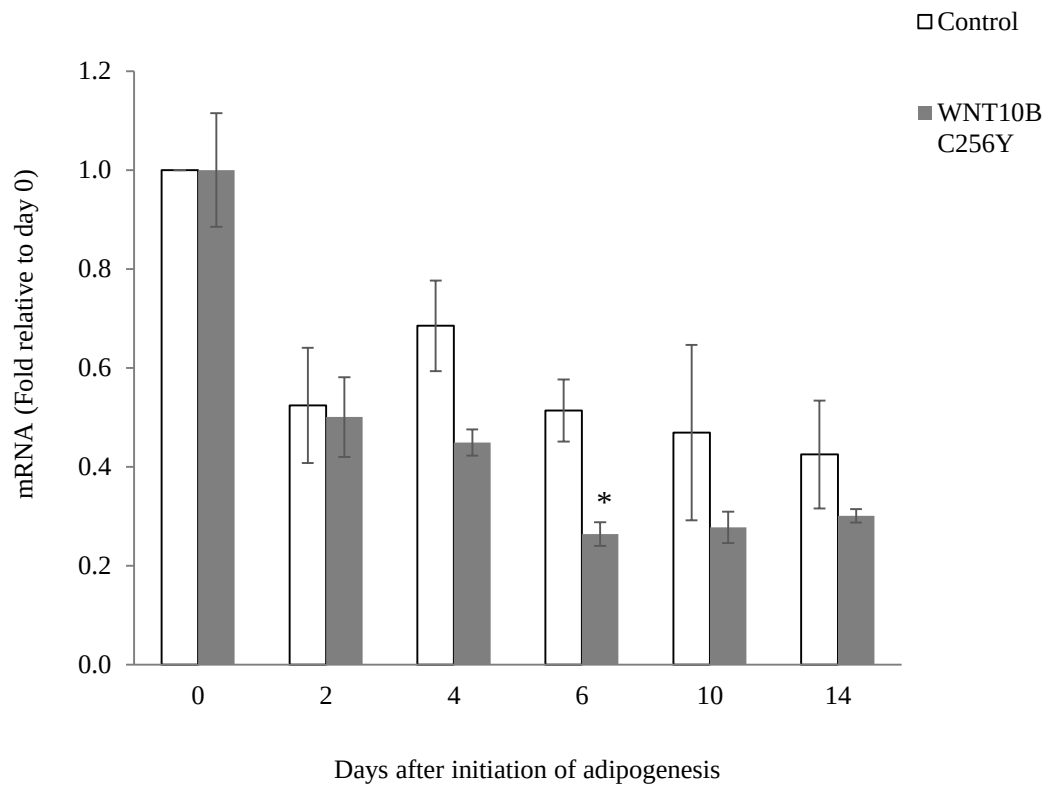

IRE1α mRNA during adipogenesis
Control
WNT10B C256Y
mRNA (Fold relative to day 0)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
0
2
4
6
10
14
Days after initiation of adipogenesis

D

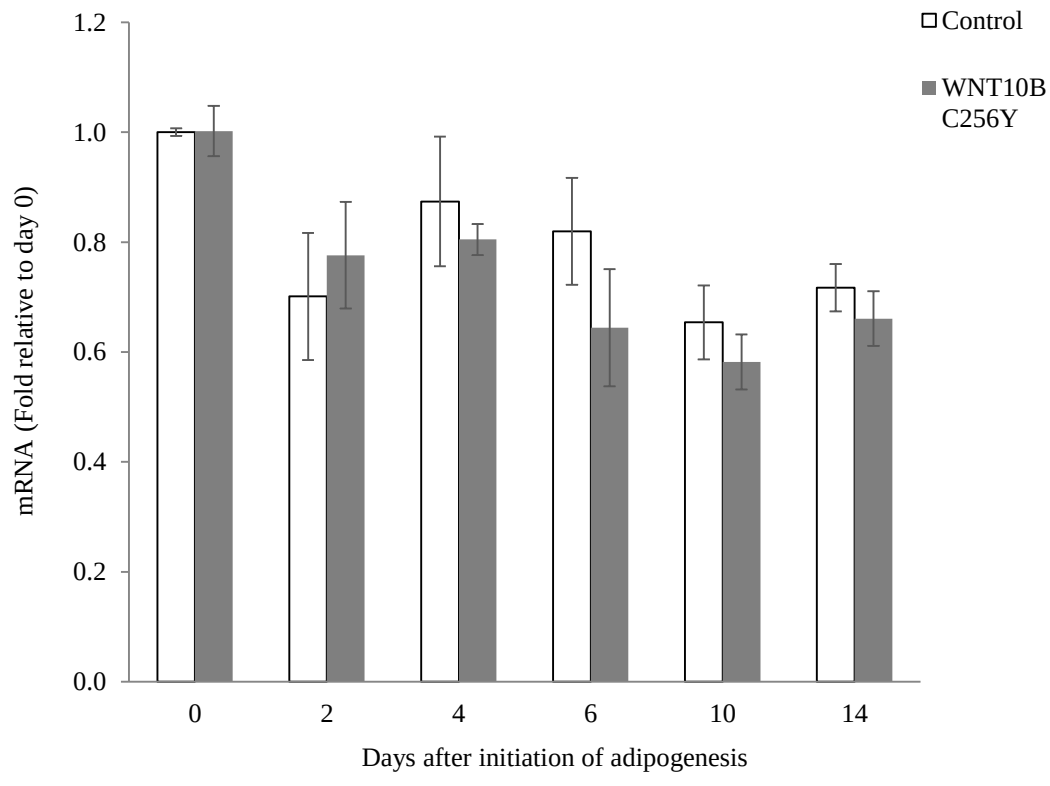
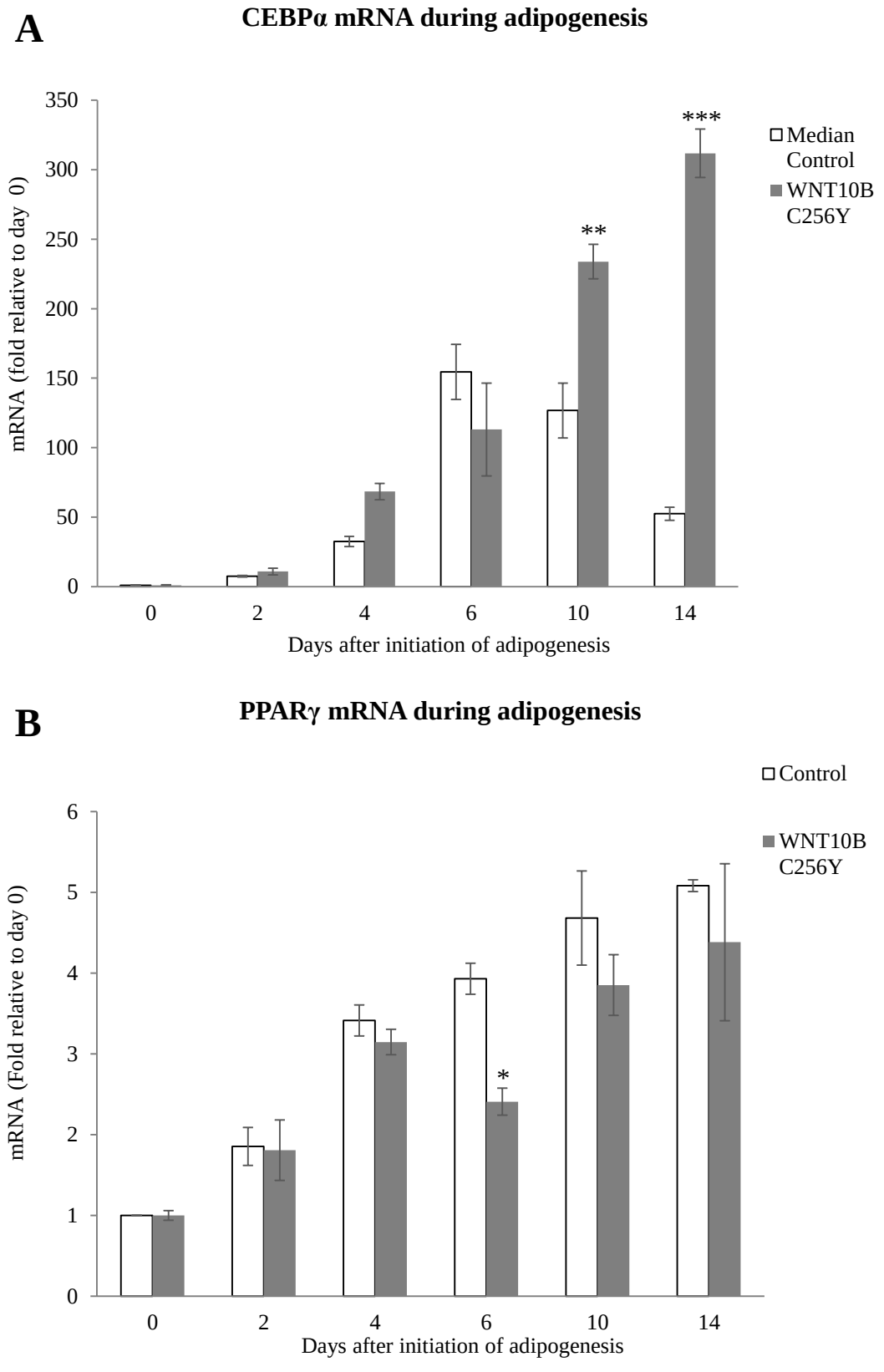

CHOP mRNA during adipogenesis
Control
WNT10B C256Y
mRNA (Fold relative to day 0)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
10
14
Days after initiation of adipogenesis

Figure 6.2.3 legend: XBP1s mRNA expression was reduced during adipogenesis in WNT10B C256Y adipocytes compared to lean. Adipogenesis was induced in lean (control) and WNT10B C256Y adipocytes for 14 days. mRNA expression was measured during adipogenesis for XBP1s (A), XBP1u (B), IRE1α (C) and CHOP (D). Data is expressed as mean ± SE. Significantly different values are indicated as follows: * $P<0.05$, ** $P<0.01$.

6.2.4 Adipogenesis regulator CEBPα gene expression was altered during adipogenesis in SAT WNT10B C256Y adipocytes compared to lean

To assess adipogenesis regulation and adipocyte gene expression in non-functional Wnt adipogenesis, adipogenesis was induced in SAT lean (control) and SAT WNT10B C256Y adipocytes for 14 days and adipogenesis regulator and adipocyte gene expressions were measured (figure 6.2.4 A – C). In WNT10B C256Y adipocytes, CEBPα expression increased gradually throughout adipogenesis unlike control adipocytes where CEBPα expression peaked on day 6 – 10 of adipogenesis (figure 5.2.7 A – C). PPARγ expression increased during adipogenesis in both control and WNT10B C256Y adipogenesis. However the WNT10B C256Y adipocytes had lower PPARγ expression at every time point during adipogenesis, although this only reached significance ($P<0.05$) on day 6. Adiponectin was not detected on day 0 in the WNT10B C256Y or control adipocytes. After day 2 the adiponectin expression profile in WNT10B C256Y adipocytes was significantly lower than the median control adipocytes.

Figure 6.2.4 Adipogenesis regulator CEBPα gene expression continued to increase during adipogenesis in WNT10B C256Y adipocytes unlike lean



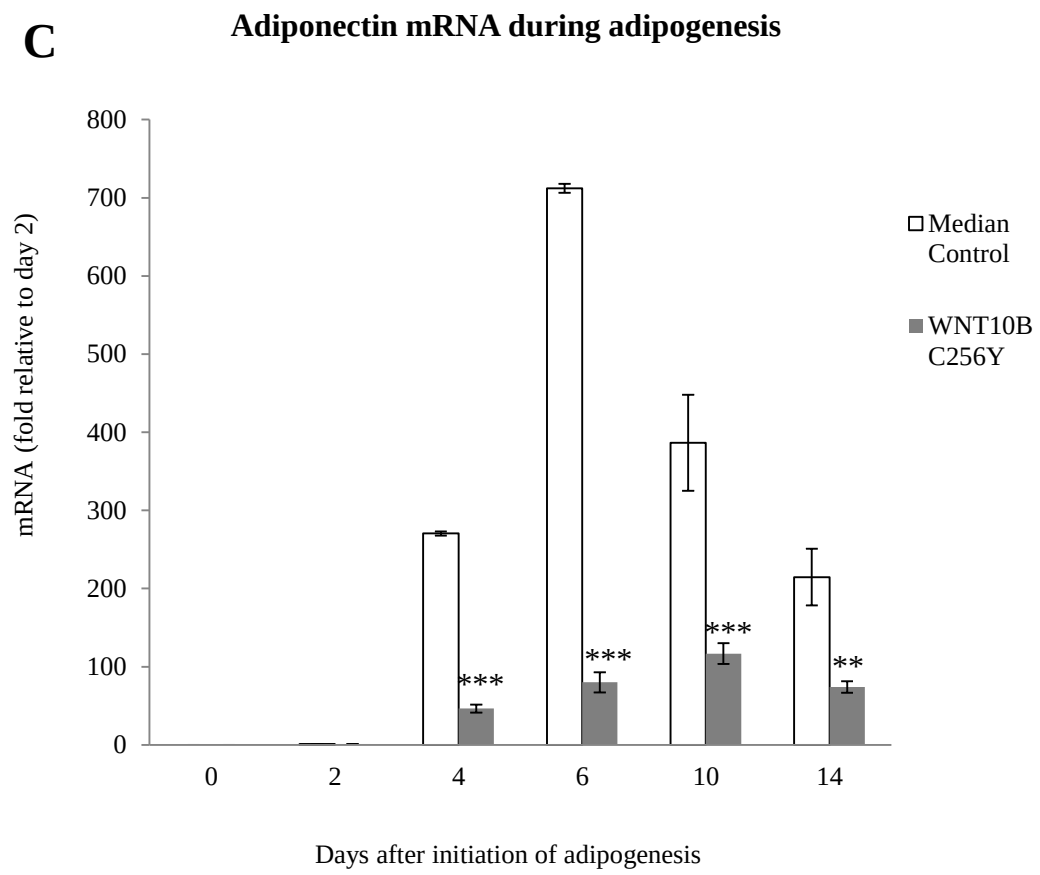
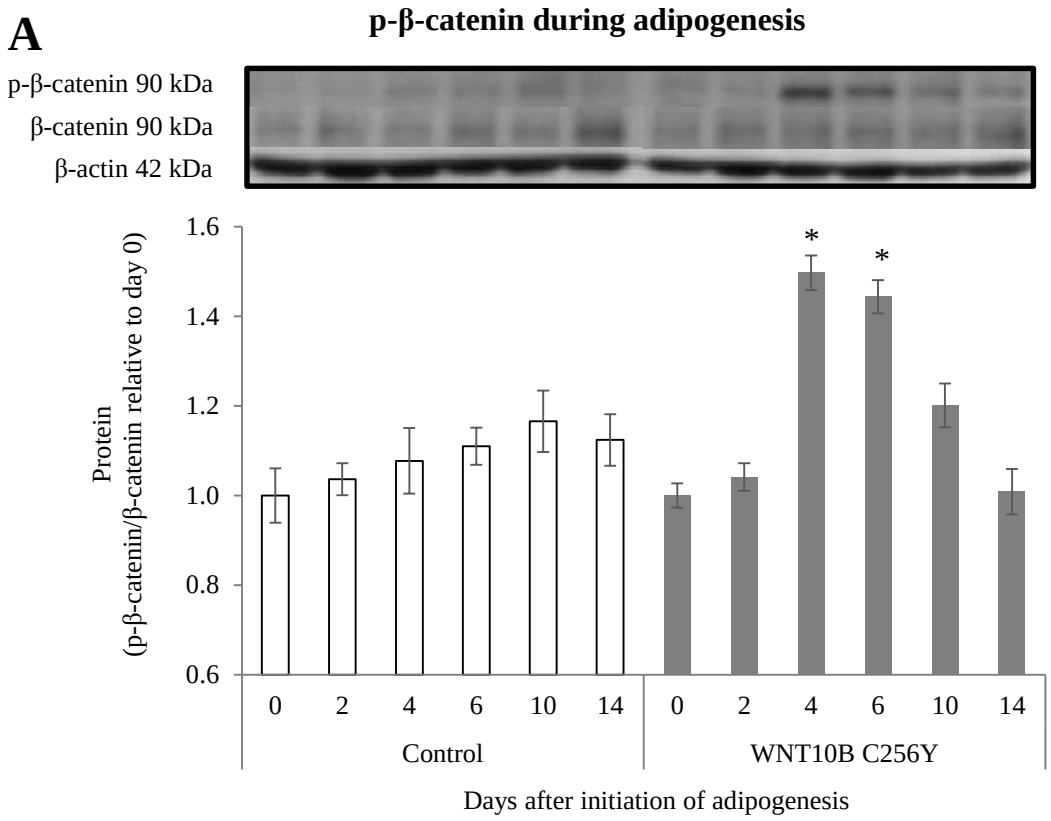


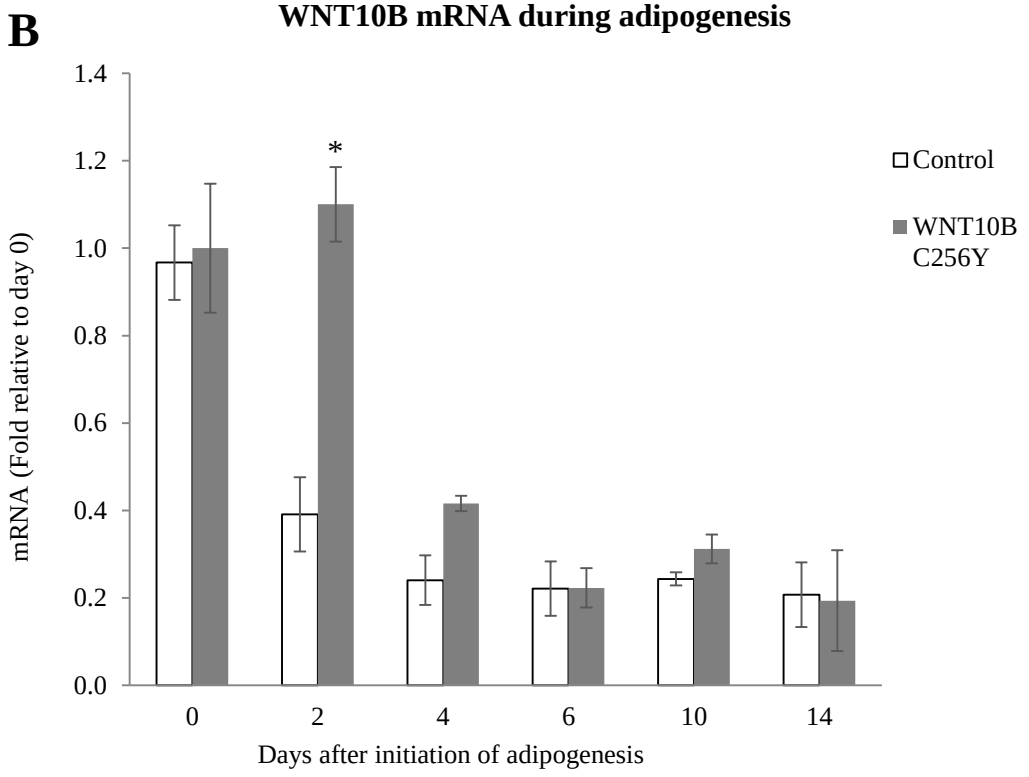
Figure 6.2.4 legend: Adipogenesis regulator CEBPα gene expression continued to increase during adipogenesis in WNT10B C256Y adipocytes unlike lean. Adipogenesis was induced in lean (control) and WNT10B C256Y adipocytes for 14 days. mRNA expression was measured during adipogenesis for CEBPα (A), PPARγ (B), adiponectin (C). Data is expressed as mean ± SE. Significantly different values are indicated as follows: * P<0.05, ** P<0.01, *** P<0.001.

6.2.5 Wnt signalling was altered during adipogenesis in SAT WNT10B C256Y adipocytes compared to lean

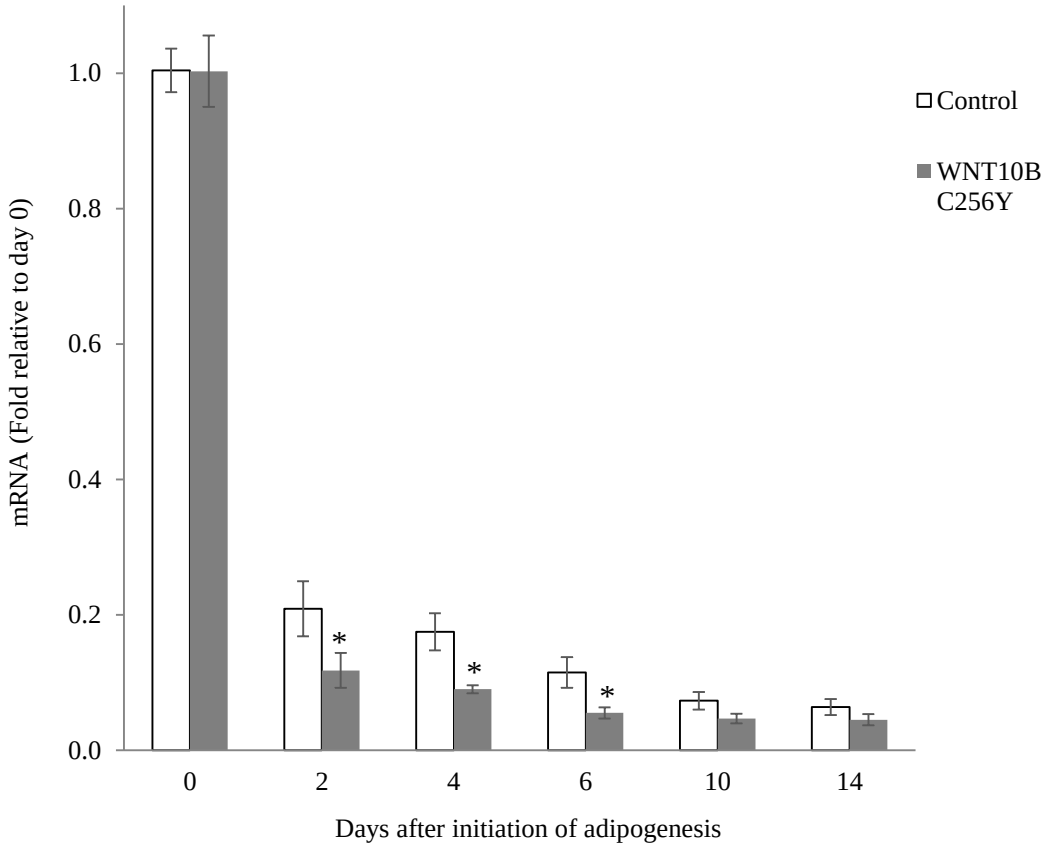
To assess Wnt signalling in adipogenesis, adipogenesis was induced in lean (control) and WNT10B C256Y adipocytes for 14 days. During adipogenesis expression of phosphorylated β-catenin and unphosphorylated β-catenin and mRNA expression of WNT10B, TCF7, and AXIN2 were measured (figure 6.2.5 A – D). In control adipocytes p-β-catenin expression relative to β-catenin increased by 10 % during adipogenesis and peaked on day 10. However in WNT10B C256Y adipocytes p-β-catenin expression relative to β-catenin increased by 50 % and peaked on day 4. The p-β-catenin expression relative to β-catenin was significantly higher ($P<0.05$) in WNT10B C256Y adipocytes on day 4 and day 6 compared to control. In control cells WNT10B and TCF7 mRNA expression decreased on day 2 after induction of adipogenesis and continued to decrease through adipogenesis. In WNT10B C256Y adipocytes WNT10B mRNA expression remained the same as day 0 on day 2, significantly higher ($P<0.05$) than control, before decreasing to within no significant difference from control adipocytes. In WNT10B C256Y adipocytes, TCF7 mRNA expression was significantly lower ($P<0.05$) than control on days 2 – 6. In control adipocytes AXIN2 mRNA expression increased after induction of adipogenesis and remained 3 fold higher than day 0 for the duration of adipogenesis. In contrast, the expression of AXIN2 in the WNT10B C256Y adipocytes remained at the same level as day 0 until increasing on day 14. At every time point during adipogenesis the WNT10B C256Y adipocytes had significantly lower ($P<0.05$ – $P<0.01$) AXIN2 expression compared to the control adipocytes.

Figure 6.2.5 Wnt signalling protein and gene expression significantly differed during adipogenesis in WNT10B C256Y adipocytes compared to lean





C


TCF7 mRNA during adipogenesis
mRNA (Fold relative to day 0)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
10
14
Days after initiation of adipogenesis
Control
WNT10B C256Y
*
*
*

D

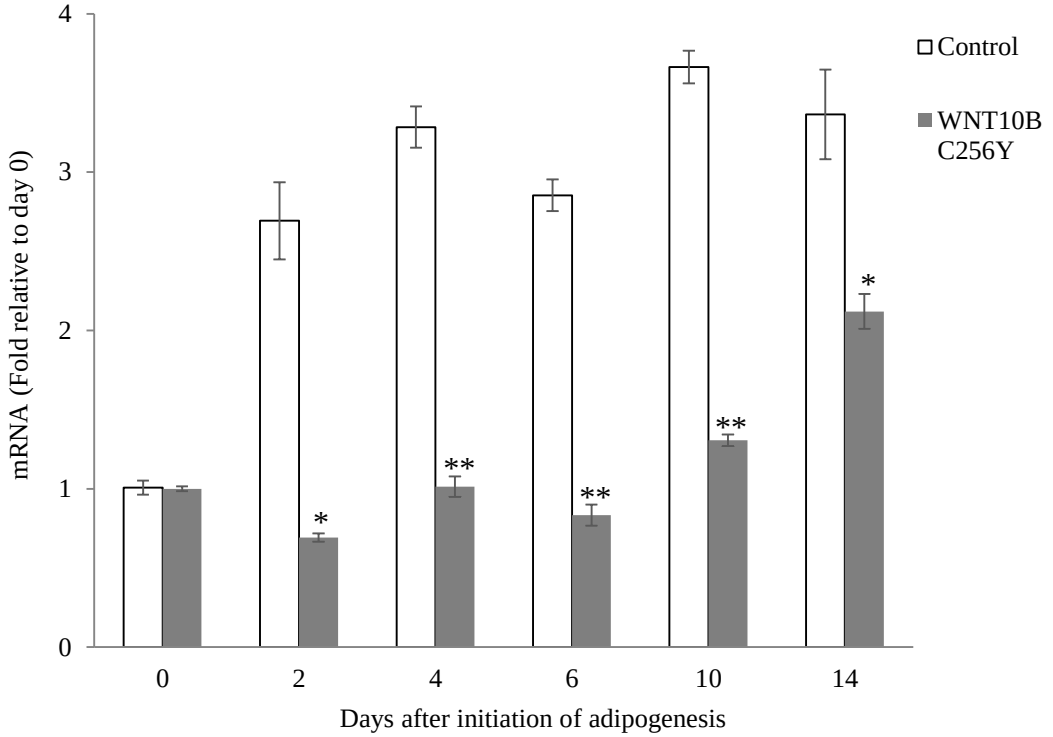

AXIN2 mRNA during adipogenesis
mRNA (Fold relative to day 0)
4
3
2
1
0
0
2
4
6
10
14
Days after initiation of adipogenesis
Control
WNT10B C256Y
*
**
**
**
*

Figure 6.2.5: Wnt signalling protein and gene expression significantly differed during adipogenesis in WNT10B C256Y adipocytes compared to lean. Adipogenesis was induced in lean (control) and WNT10B C256Y adipocytes for 14 days. During adipogenesis protein expression of phosphorylated β-catenin and unphosphorylated β-catenin (A), and mRNA expression of WNT10B (B), TCF7 (C), and AXIN2 (D) were measured. Data is expressed as mean ± SE. Significantly different values are indicated as follows: * $P<0.05$, ** $P<0.01$.

6.3 Discussion

This study was designed to investigate the role of Wnt10b in human adipogenesis and whether it is linked to XBP1s. Preadipocytes were extracted from abdominal SAT of a naturally occurring genetically defective WNT10B subject and from lean metabolically healthy subjects (control). These were then cultured and adipogenesis induced. Adipogenic capacity, adipocyte function and key adipogenic, Wnt signalling and ER stress gene and protein expressions were compared. Adipogenesis is an important process to maintain WAT metabolic function. Studies have implicated Wnt10b as a key inhibitor in human adipogenesis (Christodoulides, *et al*., 2006), (Isakson, *et al*., 2009), (Chazenbalk, *et al*., 2012) and recently Cho *et al.* have shown in mouse 3T3-L1 cells that XBP1s enhances adipogenesis through the downregulation of Wnt10b (Cho, *et al*., 2013). This study showed that functional Wnt10b was not required for adipogenesis, XBP1s expression was significantly lower and Wnt cellular signalling was altered in non-functional Wnt10b adipogenesis compared to control.

Christodoulides *et al.* reported that non-functional Wnt10b is associated with early-onset obesity (Christodoulides, *et al*., 2006). This study supports their findings. Interestingly, despite the subject's substantial obesity, they had and a healthy metabolic profile (table 6.2.1) and therefore were considered metabolically healthy but obese (MHO) (Seo and Rhee, 2014). The female subject was 19 years old and had a BMI of 62 kg/m^2. She had normal glycaemic control, measured both by HbA1c (40 mmol/mol) and fasting plasma glucose (4.2 mmol/L). She had a more favourable lipid profile than the subjects investigated in

chapter 3 (table 3.2.1). Her total cholesterol (4.5 mmol/L), LDL (2.5 mmol/L) and TAG (1.2 mmol/L) were lower, and her HDL (1.3 mmol/L) was higher than the obese T2DM subjects prior to bariatric surgery. Collectively these results suggest that the expanded WAT in the WNT10B C256Y subject is regulated and metabolically healthy. The expanded WAT may protect the subject from metabolic dysfunction in a similar way to *ob/ob* mice, when forced WAT expansion normalized glucose and insulin levels and prevented insulin resistance despite massive obesity (Kim, *et al*., 2007). The defective Wnt10b protein was thought to play a role in the vast WAT expansion because of its reported inhibitory role in human adipogenesis (Isakson, *et al*., 2009). Therefore a SAT biopsy was collected from the subject, preadipocytes were extracted and adipogenesis was studied in more detail.

During adipogenesis WNT10B C256Y preadipocytes accumulated lipid, increased lipolysis and adipocytes had significantly higher insulin stimulated glucose uptake compared to basal. Therefore the cells successfully underwent adipogenesis, functional Wnt10b was not required for adipogenesis and non-functional Wnt10b adipocytes were sensitive to insulin. During adipogenesis the SAT derived defective Wnt10b preadipocytes accumulated more lipids and underwent less lipolysis than preadipocytes derived from obese SAT and more closely resembled preadipocytes derived from lean SAT studied in chapter 4 (figure 4.2.1 D - E). Taken together, these results suggest that the Wnt10b defective adipocytes can accumulate and retain lipids better than functional Wnt10b obese adipocytes. This may in part explain why the WNT10B C256Y subject had a healthy lipid profile. The favorable lipid regulation in the adipocytes

of the defective Wnt10b MHO subject exemplifies the findings of Karpe *et al.* who convincingly discredited the hypothesis that adiposity correlates with plasma NEFA (Karpe, *et al*., 2011). Furthermore, these results suggest that plasma NEFA may correlate with adipocyte and WAT metabolic function, irrespective of adiposity. The defective Wnt10b adipocytes significantly increased insulin stimulated glucose uptake compared to basal, and therefore were sensitive to insulin. This suggests the defective Wnt10b adipocytes are able to respond to the anabolic hormone insulin and may explain why the subject had a healthy carbohydrate profile. Taken together, these findings indicate that the WNT10B C256Y subject has metabolically regulated and functional adipocytes and may explain their MHO phenotype.

This investigation showed that WNT10B C256Y adipocytes had significantly lower XBP1s expression during adipogenesis compared to control. This downregulation of XBP1s may have been due to less XBP1u substrate and also less IRE1α since both their expressions were lower in Wnt10b defective cells compared to control, although this only reached significance on day 6. This suggests that the Wnt10b defective cells may have had less ER stress during adipogenesis and subsequently less IRE1α and XBP1s expression. However, there was no difference in CHOP expression between the Wnt10b defective and control adipocytes, thus the level of ER stress and subsequent UPR activation may not have been different. Nonetheless, the diversity of ER stress induced UPR signalling likely generates suitable outcomes that are specific to the stress imposed and the homeostatic needs of the cell (Kim, *et al*., 2008). Therefore it is possible that the IRE1α - XBP1s and PERK - eIF2α - ATF4 - CHOP UPR

pathways are differentially activated during adipogenesis and the reported inhibitory role of CHOP on adipogenesis (Hou, *et al*., 2013) may act independently of the IRE1α - XBP1s UPR pathway. Cho *et al.* have shown in mouse 3T3-L1 cells that XBP1s enhances adipogenesis through the downregulation of Wnt10b, and that XBP1s and Wnt10b have a reciprocal mRNA expression profile during adipogenesis (Cho, *et al*., 2013). This study disagrees with their findings because both WNT10B and XBP1s mRNA expression decreased initially after induction of adipogenesis, suggesting an alternative upstream factor inhibits their expression. In order to investigate this further, Wnt signalling was studied.

Wnt signalling during adipogenesis in WNT10B C256Y cells was significantly altered compared to control cells and supports the findings that Wnt10b plays an inhibitory role in human adipogenesis (Christodoulides, *et al*., 2006), (Isakson, *et al*., 2009), (Chazenbalk, *et al*., 2012). WNT10B mRNA expression was measured to assess its upstream activation during adipogenesis. The expression of WNT10B mRNA in control cells decreased by day 2 of adipogenesis and stayed low for the duration of adipogenesis, however the Wnt10b defective cells remained the same as day 0 until day 2 before decreasing. This suggests that Wnt10b is suppressed upon activation of adipogenesis and the Wnt10b defective cells may have experienced a lack of inhibition during immediate early differentiation. Li *et al.* proposed that Wnt proteins mediate their action via unphosphorylated β-catenin when p-β-catenin has saturated the destruction complex (Li, *et al*., 2012). This study found no differences in unphosphorylated β-catenin expression during adipogenesis between defective Wnt10b and control cells, however

phosphorylated β-catenin (p-β-catenin) levels were significantly higher in Wnt10b defective cells on day 4 and 6 compared to control cells. In order to assess the activity of Wnt signalling, the expression of downstream target genes TCF7 and AXIN2 were measured. TCF7 expression decreased after induction of adipogenesis in both defective Wnt10b and control cells and supports the finding that TCF7 regulates a self-renewal/differentiation switch that operates in the absence of Wnt signalling (Wu, *et al*., 2012). Negative feedback regulation of the Wnt pathway is mediated through Axin2, encoded by the AXIN2 gene (Bernkopf, *et al*., 2015). Control cells increased the expression of AXIN2 from day 2 for the duration of adipogenesis, whereas the expression in defective Wnt10b cells remained the same as day 0 until day 14 and was significantly lower than control cells for the duration of adipogenesis. Therefore, collectively, these results show that Wnt signalling is significantly altered in defective Wnt10b adipocytes compared to control and may be due to dysregulated negative feedback mechanisms.

The findings in control cells in this study support the hypotheses that reciprocal repression might exist during adipogenesis between Wnt signalling and CEBPα or PPARγ (Moldes, *et al*., 2003) and that Wnt normally inhibits CEBPα as a negative feedback loop to reduce adipogenesis (Christodoulides, *et al*., 2009). Wnt10b defective cells expressed slightly lower levels of PPARγ during adipogenesis compared to control cells, although this only reached significance on day 6 and the pattern of expression was largely the same. The expression pattern of adipocyte marker adiponectin in Wnt10b defective cells largely matched that of control cells, peaking before day 14 of adipogenesis, showing that they

successfully underwent adipogenesis. Furthermore, this suggests that the WAT expansion in the WNT10B C256Y subject developed independently of upregulated adiponectin, unlike the forced expansion of WAT in *ob/ob* mouse investigation (Kim, *et al*., 2007). This study found that the expression of CEBPα in Wnt10b defective cells continued to increase throughout adipogenesis whereas in control cells it peaked on day 6 – 10 (figure 5.2.7). This suggests that CEBPα is inhibited late in regulated adipogenesis control cells and that WNT10B C256Y cells lack the ability to inhibit CEBPα. Thus, given CEBPα acts as a key adipogenic factor (Lefterova and Lazar, 2009), continued upregulation of CEBPα in the Wnt10b defective subject may have induced considerable adipogenesis and may partly explain their substantial WAT mass. Therefore collectively these data suggest that upon activation of adipogenesis in adipocytes from metabolically healthy lean subjects, Wnt signalling changes from active Wnt on to inactive Wnt off, summarised in figure 6.3.1.

Figure 6.3.1 Proposed changes in Wnt signalling during adipogenesis

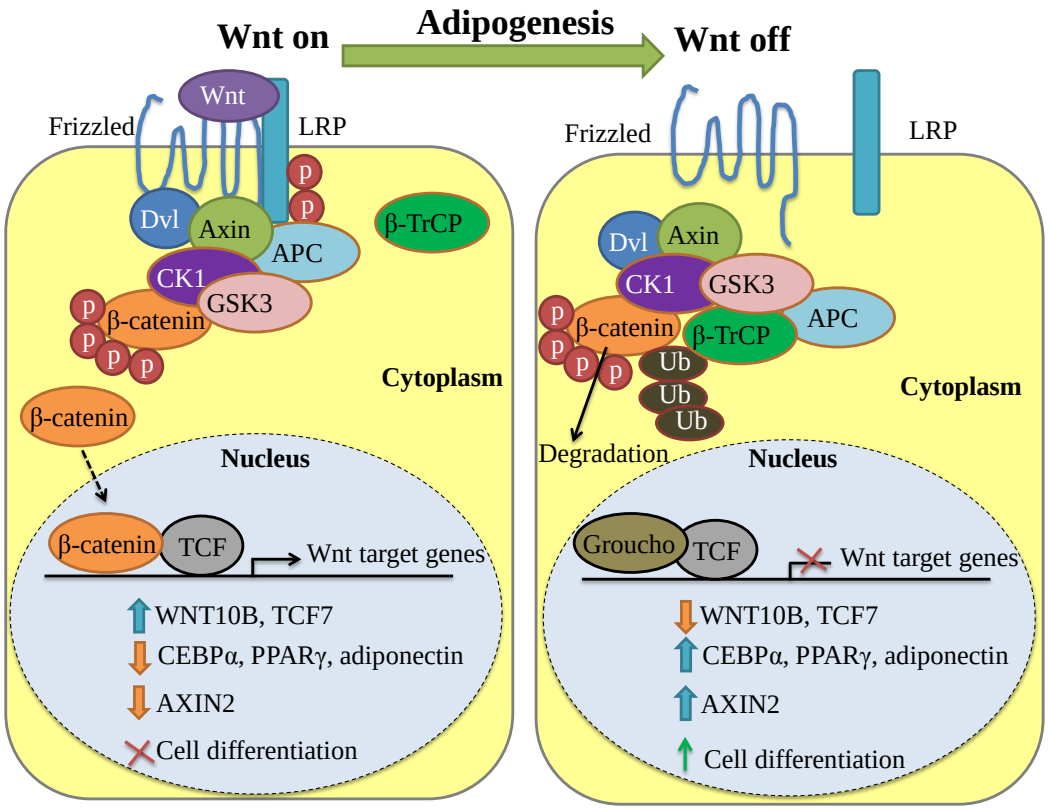


Figure 6.3.1 legend: Proposed changes in Wnt signalling during adipogenesis. The results from this study indicate that upon activation of adipogenesis Wnt signalling changes from Wnt on to Wnt off. (Wnt; Wingless-type mouse mammary tumour virus integration site family protein, LRP; low-density-lipoprotein-related protein, Dvl; dishevelled, APC; adenomatous polyposis coli, GSK-3; glycogen synthase kinase-3, CKI; casein kinase-1, β-TrCP; beta-transducin repeat containing E3 ubiquitin protein ligase, p; phosphorylated, Ub; ubiquitin, TCF; T-cell factor, WNT10B; Wingless-type mouse mammary tumour virus integration site family protein 10b, TCF; T-cell factor 7, CEBPα; CCAAT/enhancer binding protein alpha, PPARγ; peroxisome proliferator-activated receptor gamma, AXIN2; axis inhibitory protein 2.)

6.4 Conclusions

In summary, the results of this study suggest that Wnt10b plays an inhibitory role in human adipogenesis and acts independently of XBP1s. A subject with a naturally occurring WNT10B C256Y mutation rendering a non-functional Wnt10b protein was severely obese and had a metabolically healthy phonotype. This study found a reciprocal expression profile between WNT10B and CEBPα or PPARγ in preadipocytes derived from healthy lean subjects during adipogenesis and supports the hypotheses that reciprocal repression might exist during adipogenesis between Wnt signalling and CEBPα or PPARγ (Moldes, *et al*., 2003) and that Wnt normally inhibits CEBPα as a negative feedback loop to reduce adipogenesis (Christodoulides, *et al*., 2009). This study disagrees with the murine findings of Cho *et al.* that XBP1s downregulates Wnt10b (Cho, *et al*., 2013) because both WNT10B and XBP1s mRNA expression decreased initially after induction of adipogenesis. Taken together, the results in this study suggest that non-functional Wnt10b leads to dysregulated Wnt signalling, upregulation of CEBPα and subsequent enhanced adipogenesis and expanded metabolically healthy WAT. These findings provide a molecular mechanistic hypothesis to partially explain the MHO phenotype. However, further research is required to fully elucidate the roles of Wnt10b and XBP1s in human adipogenesis. Nonetheless, these studies have identified Wnt10b as an exciting potential pharmacological target to improve metabolic health and reduce the burden of insulin resistance and T2DM.

Chapter 7: Overall discussion

7.1 Overall discussion

This thesis researched the role of ER stress and consequent UPR activation in human WAT regulation in metabolic health and T2DM. Current evidence about WAT regulation and UPR activation was reviewed to identify areas where further research had the potential to benefit T2DM clinical outcomes. Translational experimental methodologies from humans to the laboratory were adopted in order to generate valid and representative information about human WAT regulation. SAT from T2DM subjects before and after bariatric surgery, paired SAT and VAT from lean and obese non-diabetic subjects and SAT from a naturally occurring non-functional Wnt10b subject were utilised alongside a human adipocyte cell line to investigate the molecular mechanisms governing WAT regulation. Standardised cell culture and analytical laboratory techniques were employed to systematically research the role of UPR activation in human WAT regulation in metabolic health and T2DM. Several novel outcomes from this body of research provide mechanistic insight into the regulation of human WAT and have identified potential therapeutic targets to improve metabolic health.

Current evidence about the role of WAT in metabolic regulation and T2DM was studied. The main outcomes of the literature review found the following: the chronic metabolic disease T2DM presents a huge burden on individuals and global society through medical, social and financial implications. Obesity, characterised by WAT expansion, is the most significant risk factor for developing T2DM. WAT is a multifunctional organ that plays a key role in metabolic health and regulating energy homeostasis. An inability of WAT to expand to accommodate excess nutrients is predominantly due to impaired

adipogenesis and results in ectopic fat deposition, lipotoxicity and insulin resistance. Adipogenesis is influenced by several extracellular and intercellular factors. Obesity associated physiological characteristics such as hyperglycaemia, endotoxemia, elevated circulating FFAs, and local hypoxia induce adipocyte ER stress. This occurs via several mechanisms including increased demand on the ER for protein synthesis and processing, lipid droplet formation and lipid partitioning, and elevated ROS production. ER stress is a key factor in the initiation of insulin resistance through inhibiting insulin action and inducing WAT inflammation and may interfere with adipogenesis. Interventions that alleviate ER stress may be suitable first-line treatments to restore ER function and metabolic regulation in metabolic diseases such as T2DM.

The most durably effective treatment for T2DM has emerged as bariatric surgery, however the mechanisms governing the improvement in glucose homeostasis have not been fully elucidated (Nguyen and Korner, 2014). Some bariatric procedures improve glycaemic control in people with T2DM beyond that expected for weight loss alone (Dixon, *et al.*, 2012). Since WAT plays a vital role in metabolic health, it was hypothesised that the improvements in glycaemic regulation after bariatric surgery may be due to alleviated ER stress in WAT and improved regulation and function. The study in chapter 3 demonstrated that both restrictive and malabsorptive bariatric interventions are effective weight loss interventions for obese T2DM patients resulting in significantly improved glucose and insulin levels six months after surgery. While malabsorptive bariatric surgery involves more complicated procedures and requires long-term vitamin and other supplementation, the restrictive bariatric procedures are less complicated and

induce almost equal benefits. In this study WAT health was better following the two restrictive procedures (LAGB and LGCP) compared to BPD as shown by higher reduction of ER stress markers as well as improved regulation of CHOP. The data from Bradley *et al.* (Bradley, *et al*., 2012) obtained from non-diabetic obese patients did not specifically support malabsorptive (RYGB) over restrictive surgery type (LAGB) and suggested that further studies were required which could apply to obese patients with T2DM. This study offers novel data in that direction indicating that while the clinical outcomes regarding parameters such as % EBL and HbA1c were better following BPD, the adipose tissue health was significantly improved following the restrictive procedures. The clinical implications of these findings in the longer term require further investigations. Overall, all the surgery types investigated significantly improved the metabolic health of obese diabetics included in this study irrespective of surgery type.

WAT health and subsequent metabolic regulation was improved in obese T2DM subjects who lost weight almost exclusively through WAT mass reduction following bariatric surgery. Therefore mechanisms governing WAT regulation were considered vital for metabolic health. Evidence suggests obesity complications in humans such as T2DM result from the inability of SAT to appropriately expand and store lipids, and consequently ectopic fat deposition and lipotoxicity contribute to insulin resistance (Hill, *et al.*, 2009; Virtue and Vidal-Puig, 2010). Impaired WAT expandability is mainly due to defects in adipogenesis (Isakson, *et al.*, 2009). Adipogenesis is an important process within WAT to allow normal turnover of adipocytes (Spalding, *et al.*, 2008) and facilitate appropriate WAT expansion to respond to the energy needs of the body (Wang, *et*

al., 2013), therefore adipogenesis in primary human preadipocytes was studied. The studies in chapter 4 showed that adipogenesis in primary human preadipocytes is influenced by both adiposity and WAT depot of preadipocyte source and that the IRE1α - XBP1s UPR pathway plays an integral role in human adipogenesis. This investigation indicated that during adipogenesis obese derived preadipocytes accumulated more lipid than lean irrespective of WAT depot, however lipolysis and insulin sensitivity were both influenced by adiposity and WAT depot. Collectively these findings support human investigations that suggest abdominal SAT is protective for insulin resistance whereas VAT mass has the opposite effect (McLaughlin, *et al*., 2011). The expressions of IRE1α and XBP1s during adipogenesis differed between depots and may in part explain the observed functional differences in adipocytes. ER stress prevented adipogenesis irrespective of preadipocyte origin and may in part explain why WAT ER stress is associated with metabolic dysfunction (Hotamisligil, 2010). The novel finding that XBP1s is a prerequisite for human adipogenesis strengthens the link between UPR and adipogenesis, although further investigation is required to elucidate the role of XBP1s in human adipogenesis. These findings provide insight into adipocyte functional differences and their integration with the UPR and therefore this information will benefit development of translational clinical interventions to improve metabolic health.

Adipogenesis in preadipocytes derived from SAT was investigated further because SAT represents the vast majority of WAT (about 85 % of all body fat (Frayn and Karpe, 2014)) and the inability of SAT to expand has been causally related to obesity complications such as T2DM (Virtue and Vidal-Puig, 2010).

Chapter 4 indicated that XBP1s is essential for adipogenesis, therefore the role of XBP1s in adipogenesis was investigated further. The studies in chapter 5 showed that XBP1s plays a vital role upstream of CEBPα and PPARγ in human adipogenesis and that it is necessary for mediating the action of insulin. These findings are the first of their kind in human adipocytes and support the mouse cell line work of Cho *et al.* that suggest XBP1s may regulate PPARγ in adipogenesis (Cho, *et al*., 2014). XBP1s has been shown to promote adiponectin multimerisation and improve glucose regulation in mice (Sha, *et al*., 2014). Furthermore, WAT XBP1s and plasma glucose reduced after bariatric surgery in humans (chapter 3). This study provides novel evidence that XBP1s is necessary for insulin stimulated glucose uptake in controlled human adipocyte cell culture system. Taken together, these results support the link between UPR signalling and metabolism (Hotamisligil, 2010; Lee and Ozcan, 2014). Although further research is required, these findings have provided useful insight into the role of XBP1s in adipogenesis and metabolism and will benefit the development of clinical interventions to alleviate metabolic diseases.

A link between XBP1s and Wnt10b in human adipogenesis was considered because Cho *et al.* have reported in mouse 3T3-L1 cells that XBP1s enhances adipogenesis through the downregulation of Wnt10b (Cho, *et al*., 2013). Therefore the role of Wnt10b in primary human adipogenesis was investigated in chapter 6. The results from this study suggest that Wnt10b plays an inhibitory role in human adipogenesis and acts independently of XBP1s. A subject with a naturally occurring WNT10B C256Y mutation rendering a non-functional Wnt10b protein was severely obese and had a metabolically healthy phonotype.

This study found a reciprocal expression profile between WNT10B and CEBPα or PPARγ in preadipocytes derived from healthy lean subjects during adipogenesis. The study supports the hypotheses that reciprocal repression might exist during adipogenesis between Wnt signalling and CEBPα or PPARγ (Moldes, *et al*., 2003) and that Wnt normally inhibits CEBPα as a negative feedback loop to reduce adipogenesis (Christodoulides, *et al*., 2009). This study disagrees with the murine findings of Cho *et al.* that XBP1s downregulates Wnt10b (Cho, *et al*., 2013) because both WNT10B and XBP1s mRNA expression decreased initially after induction of adipogenesis. Taken together, the results in this study suggest that non-functional Wnt10b leads to dysregulated Wnt signalling, upregulation of CEBPα and subsequent enhanced adipogenesis and expanded metabolically healthy WAT. These findings provide a molecular mechanistic hypothesis to partially explain the MHO phenotype. Given the expanded WAT in non-functional WNT10B C256Y subject, Wnt10b may influence appetite. Furthermore, given the subjects favourable metabolic profile considering their BMI of 62 kg/m^2, having non-functional Wnt10b may protect against T2DM. Although the subject was only 19 years old and therefore unlikely to have developed T2DM at such a young age, irrespective of severe obesity. Follow up studies of the WNT10B C256Y subject would be beneficial to ascertain whether T2DM develops. Further research is also required to fully elucidate the roles of Wnt10b and XBP1s in human adipogenesis. Nonetheless, these studies have identified Wnt10b as an exciting potential pharmacological target to improve metabolic health and reduce the burden of insulin resistance and T2DM.

7.2 Future work

This body of work has generated novel data about the role of ER stress in human adipose tissue metabolism. The results from this thesis suggest that WAT function is crucial for metabolic regulation and adipocyte ER stress signalling may play an integrative role between metabolic function and systemic homeostasis. If these results are corroborated in future investigations then specific pharmaceutical agents to ameliorate ER stress and improve metabolic function may provide substantial tangible clinical benefits. Therefore the findings in this thesis warrant further investigations.

The subjects who underwent bariatric surgery improved their metabolic profile and their WAT ER stress levels and regulation six months after surgery (chapter 3). Although the subjects appeared to have an improved metabolic profile six months after surgery, their metabolic status is likely to fluctuate with time. During the initial six months after surgery the subjects experienced a negative energy flux where more calories were used compared to being consumed. Consequently the subjects lost weight almost exclusively from a reduction in WAT mass. Their rate of weight loss is unsustainable, they cannot continue to lose weight indefinitely. Therefore there must be a certain point when their body weight stabilises. This will be partly influenced by their surgery type since their altered physiology will govern the rate at which calories from ingested food can be absorbed and stored or utilised. In addition, there are likely to be considerable WAT reorganisation events such as altered innervation, vasculature and cell composition. Since WAT plays a key integrative role in regulating metabolism (figure 1.4.3) it is likely that the altered WAT architecture will influence the subject's long-term metabolism.

For example, the reciprocal hormonal brain – WAT axis controlling appetite may be altered, leading to changes in calorie consumption. Or the immune cell repertoire may change thus altering the local cytokine production and consequently influencing the inflammatory status of the WAT. Furthermore, the regulation of adipogenesis may be altered following the changes in WAT physiology. Therefore to investigate the long-term metabolic changes following LAGB, LGCP and BPD bariatric surgery procedures further studies will be required. Annual anthropometric measurements and Abd SAT biopsies from the subjects will allow long-term follow up and assessment of metabolic profile, ER stress levels and WAT regulation. Preadipocytes can be extracted from the biopsies and adipogenesis experiments conducted to investigate the change in adipogenesis regulation over time. Furthermore, the long-term follow up may indicate which procedure is most favourable for WAT health and therefore influence clinical decision making when considering a patient for bariatric surgery.

The IRE1α – XBP1s pathway was found to play an essential role in adipogenesis and insulin stimulated glucose uptake (chapters 4 and 5). Perhaps the reason why the UPR pathway is essential for adipogenesis is because it is essential to mediate the effects of insulin signalling. This study found that insulin stimulated glucose uptake and Akt phosphorylation were almost completely blocked in the presence of IRE1α endonuclease inhibitor 4μ8C compared to control (figure 5.2.6). It is possible that 4μ8C has off-target effects and simultaneously blocks IRE1α endonuclease and insulin signalling upstream of Akt. However XBP1 knockdown with XBP1 siRNA also prevented insulin stimulated glucose uptake compared to

non-targeting siRNA (figure 5.2.7). Therefore suggesting that XBP1s does indeed play a key role in insulin stimulated glucose uptake. Further studies to pinpoint the role of XBP1s in insulin stimulated glucose uptake are required. For example, assessing IRS1 phosphorylation upstream of Akt following insulin stimulation in control adipocytes and in the presence of 4μ8C. Elucidating a role for IRE1α and/or XBP1s in insulin stimulated glucose uptake will have profound effects on clinical management. For instance, if a T2DM patient has elevated ER stress and IRE1α and XBP1s, treatment with insulin might be ineffective.

A subject with a WNT10B C256Y mutation rendering a non-functional Wnt10b protein was severely obese with a healthy metabolic profile (chapter 6). The subject's adipocytes continued to increase the expression of adipogenesis master regulator CEBPα upon activation of adipogenesis whereas the marker peaked on day 6 – 10 in preadipoctes from control lean subjects (figure 6.2.4). Furthermore, the WNT10B C256Y subject had significantly different Wnt signalling gene and protein expression during adipogenesis compared to lean control (figure 6.2.5). AXIN2 gene expression increased in lean adipocytes during adipogenesis, however it did not increase in the WNT C256Y subject. Therefore Axin2, encoded by the AXIN2 gene may act as a negative feedback regulator to 'turn off' CEBPα during adipogenesis. Further studies are required to elucidate the role of Wnt signalling and Axin2 in adipogenesis. Elucidating the signalling networks involved in adipogenesis may allow the process to be manipulated to favour the metabolic health of the patients with T2DM. For example, a regulated expanded WAT with sufficient functioning adipocytes can act like an active pool to uptake harmful lipids and glucose from the circulation, thereby protecting the subject

from the adverse effects of hyperlipidemia and hyperglycemia. The WNT10B C256Y subject had a metabolically healthy phenotype despite her massive obesity. This may be because her expanded WAT is metabolically healthy or it could be that she has dysregulated WAT but it has not had sufficient time to influence her metabolic profile because she is only 19 years old. Therefore follow up studies are required to assess the subjects metabolic profile over time. Will she be protected from insulin resistance with her expanded WAT, or will she develop insulin resistance because of her expanded WAT? Follow up studies of this rare naturally occurring mutation have the potential to provide useful information about the regulation of adipogenesis and overall WAT health.

Further studies investigating people with MONW and MHO phenotypes, PCOS and matched controls may identify additional regulatory factors in adipose tissue health and metabolism. WAT biopsies alongside anthropometric data from the aforementioned groups can be utilised to assess differences in genome using GWAS, ER stress levels assessing protein and gene expression and adipogenesis regulation by extracting stem cells and inducing adipogenesis *in vitro.* These naturally occurring differences in WAT and metabolic regulation may assist the identification of integrated pathways of metabolism. Understanding the metabolic pathways involved will allow development of therapeutics to modulate said pathways in order to improve metabolic health and thus reduce the encumbrance of T2DM.

7.3 Overall conclusions

In conclusion, this thesis has researched the role of ER stress and consequent UPR activation in human WAT regulation in metabolic health and T2DM. The main outcomes of these studies have shown: Both restrictive and malabsorptive bariatric interventions are effective weight loss interventions for obese T2DM patients and result in significantly improved glucose and insulin levels six months after surgery. WAT health is better following restrictive procedures (LAGB and LGCP) compared to BPD as shown by higher reduction of ER stress markers as well as improved regulation of CHOP. Adipogenesis in primary human preadipocytes is influenced by both adiposity and WAT depot of preadipocyte source and the IRE1α - XBP1s UPR pathway plays an integral role in human adipogenesis. XBP1s plays a vital role upstream of CEBPα and PPARγ in human adipogenesis and it is necessary for mediating the action of insulin. Wnt10b plays an inhibitory role in human adipogenesis and acts independently of XBP1s. Collectively these findings suggest that WAT function is key for metabolic health and can be impaired by ER stress; however regulated adipogenesis may serve to improve WAT function and therefore improve metabolic health. In order to confirm these findings further studies are required with larger numbers of human subjects and specific *in vivo* investigations. If confirmed, clinical trials with therapeutic targets to improve adipogenesis and therefore WAT function and metabolic health could hold significant clinical potential.

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