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The Metabolic Regulation of Cellular Ageing

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Doctor of Philosophy

ASTON UNIVERSITY

September 2015

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Thesis Summary

The incidence of obesity and type 2 diabetes (T2D) are increasing in the UK and there is evidence that these disorders influence the ageing process. The mechanisms by which obesity/diabetes might regulate ageing are poorly understood. This study aimed to investigate the links between body composition, metabolic disease and ageing, as well as the role of cellular senescence on nutrient homeostasis. Study participants had biochemical and anthropometric measurements taken. DNA analysis was used to measure telomere length (TL) using real-time PCR and plasma was used to quantify circulating factors using ELISA. Human dermal fibroblasts were made senescent and conditioned media was collected and used to treat AML-12, C2C12 and 3T3-L1 cell lines. Following treatment glucose content of cell media was measured.

TL exhibited a significant negative association amongst individuals retaining excess visceral fat ($p < 0.001$) and plasma irisin levels positively correlate and predict TL ($p = 0.01$) in the control cohort. There was no significant association between irisin and TL in the T2D cohort ($p = 0.333$), and a 3-fold higher concentration of irisin was observed in individuals with T2D in comparison to controls ($p < 0.0001$). Irisin was a statistically significant predictor of soluble E-selectin ($p = 0.003$) in type 2 diabetics. 40% Conditioned media from senescent HDF reduced the ability of C2C12 to utilise glucose after 24 and 48 hours and AML-12 hepatocytes after 48 hours ($p < 0.0001$, $p < 0.01$) quantification of circulating glycerol in 3T3-L1 adipocytes following treatment with 20% and 40% conditioned media suggests lipotoxic tendencies ($p < 0.05$ and $p < 0.001$).

In conclusion this study highlights the importance of maintaining adequate body fat in preserving TL, provides novel data regarding the role of irisin in healthy and obese/type 2 diabetic individuals and insights into the relation between ageing and insulin resistance.

Key words: Type 2 diabetes, Obesity, Telomere length, Irisin, E-selectin, Cellular senescence.

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Contents

List of Abbreviations.

(AGEs) Advanced glycation end products

(AGRP) Agouti-related protein

(AML12) alpha mouse liver 12

(AMP) Adenosine monophosphate

(ATP) Adenosine triphosphate

(BAT) Brown adipose tissue

(BBS) Bardet-Biedl syndrome

(BDNF) Brain-derived neurotrophic factor

(BIA) Bioelectrical impedance analysis

(BMI) Body Mass Index

(BRAF) B-Raf proto-oncogene, serine/threonine kinase

(cAMP) Cyclic adenosine monophosphate

(CART) Cocaine- and amphetamine-related transcript

(CDC) Centers for disease control and prevention.

(CDK) Cyclin dependent kinases

(CRP) C-reactive protein

(CT) Computed tomography

(CVD) Cardiovascular Disease

(DEPC) Diethylpyrocarbonate

(DMF) N-N-dimethylformamide

(DMSO) Dimethylsulfoxide

(DNA) Deoxyribonucleic acid

(ELISA) Enzyme-linked immunosorbent assay

(FAK) Focal adhesion kinase

(FDG) Fluorodeoxyglucose

(FFA) Free fatty acids

(FNDC5) Fibronectin domain-containing protein 5

(FTO) Fat mass and obesity associated gene

(GLUT4) Glucose transporter 4

(GSK3) Glycogen synthase kinase-3

(GWAS) Genome wide association studies

(H_2O_2) Hydrogen peroxide

(HDF) Human dermal fibroblasts

(HDL) High-density lipoproteins

(HFCS) High fructose corn syrup

(HOMA-IR)

(HUVEC) Early passage Human Umbilical Vein Endothelial Cells

(ICAM)-1 Intracellular adhesion molecule 1

(IGF) Insulin-like growth factor

(IL-6) Interleukin-6

(IL-8) Interleukin-8

(INR) Indian Rupee

(IRS-1) Insulin receptor substrate 1

(JAK) Janus kinase

(LDL) Low density lipoprotein

(MC4R) Melanocortin 4 receptor

(MCP)-1 Monocyte chemoattractant protein

(MetS) Metabolic syndrome

(mRNA) Messenger ribonucleic acid

(MSH) Melanocyte-stimulating hormone

(mTOR) The mechanistic target of rapamycin

(NEFA) Non-esterified fatty acids

(NHS) National Health Service

(NPY) Neuropeptide Y

(PBMC) Peripheral blood mononuclear cell

(PET) Positron emission tomography

(PI3-kinase) Phosphatidylinositol 3-kinase

(PGC-1α) Peroxisome proliferator-activated receptor gamma coactivator *1*-alpha (*PGC*-1α)

(PKA) Protein Kinase A

(PKB) Protein kinase B

(POMC) Pro-opiomelanocortin

(PPAR-γ) Peroxisome proliferator-activated receptor gamma

(PTEN) Phosphatase and tensin homolog

(PVN) Paraventricular nucleus

(PWS) Prader Willi Syndrome

(Rb) Retinoblastoma

(RNA) Ribonucleic acid

(ROS) Reactive oxygen species

(SAHF) Termed senescence-associated heterochromatic foci

(SASP) The senescent associated secretory phenotype

(SAT) Subcutaneous adipose tissue

(SA-β-Gal) Senescence associated β-galactosidase

(SC) Senescent cells

(SCG) Single copy gene

(SIPS) Stress induced premature senescence

(SNP) Single nucleotide polymorphism

(STAT) Signal transducers and activators of transcription

(T1D) Type 1 diabetes

(T2D) Type 2 diabetes

(TCF7L2) Transcription factor-7– like 2

(TERC) Telomerase RNA component

(TG) Triacylglycerol

(TL) Telomere length

(TLR) Toll like receptors

(TMB) Tetramethylbenzidine

(TNF-α) Tumour necrosis factor-α

(TPP1) Tripeptidyl peptidase 1

(TRF1) Telomeric repeat binding factor 1

(TRKB) Tropomyosin receptor kinase B

(UCP-1) Uncoupling protein 1

(UK) United Kingdom

(USA) United States of America

(VCAM-1) Vascular cell adhesion molecule 1

(VAT) Visceral adipose tissue

(VEGF) Vascular endothelial growth factor

(WAGR) Wilms tumour, aniridia, genitourinary anomalies, and mental retardation

(WHO) World Health Organisation

(WHR) Waist to hip ratio

List of Figures and Tables.

Chapter 1: Introduction

1.1. The modern obesity epidemic.

The term obesity describes a metabolic disorder manifested from irregular energy homeostasis [1]. Obesity is always associated with the situation where energy consumption increases beyond energy expenditure, characterised by the accumulation of excessive adipose tissue and the phenotypic state of being grossly overweight [2]. The prevalence of obesity has substantially increased in almost all societies since the mid-20th century, and due in part to the association of obesity with disability and death, obesity is regarded the fifth leading risk for global deaths [3]. The mortality associated with obesity surpasses the number of deaths due to being underweight and this has led to the identification of obesity as the largest preventable cause of premature morbidity and mortality in modern times [3, 4]. The universally applied standard for diagnosing obesity is currently the Body Mass Index (BMI), a metric calculated by dividing an individual's bodyweight (in kilograms) by the square of their height (in metres) and is widely implemented across clinical and public health applications, due to its practicality under clinical settings and in epidemiological studies [5, 6].

The wide use of BMI has helped to identify individuals at various stages of risk of developing obesity. In the UK more than 1 in 4 adults are now classified as obese (BMI > 30kg/m^2), and it is projected that by 2050, 60% of males, 50% of females and 25% of children will be obese [7-9]. The increased number of obese individuals in society in coming years will account for £9.7 billion in NHS costs, attributable to health implications associated with obesity [10, 11]. Based upon such alarming statistics it is clearly evident that despite the overt recognition of the taxing effects of obesity on both medical and social programs, Westernised societies are still succumbing to this global epidemic. Although scientific and technical advances made over the last decade have yielded many tools to extensively explore the dysregulated biochemistry underlying the obese phenotype, it has also demonstrated that interactions between genetic makeup and environment are essential for the regulation of adipose tissue mass and function [12, 13].

1.2. Obesity: genetic predisposition

1.2.1. Monogenic obesity.

Genetic associations with obesity have been unequivocally documented through genetic studies in recent years [14]. Principal studies have successfully identified Mendelian inheritance as a vehicle for an individual developing obesity, supported by findings which demonstrate increased expression of the obese phenotype amongst monozygotic twins in comparison to dizygotic twins irrespective of environmental conditions [15]. Single mutations in genes encoding proteins modulating physiological regulation of energy homeostasis and/or appetite are responsible for the development of monogenic obesity [16]. Initial knowledge concerning monogenic diseases was derived from large-scale linkage analyses in mice that had naturally occurring mutations that led to extreme adiposity attributed to recessive mutations in genes encoding leptin and/or the leptin receptor [17]. The role of leptin was first documented following experimental studies in obese *ob/ob* mice, which possessed mutations in the *ob* gene resulting in an inability to produce leptin [17, 18].

Leptin is produced by adipocytes and interacts with the leptin receptor located on neurons of the arcuate nucleus of the hypothalamus [19]. Neurons present in the arcuate nucleus can briefly be divided into two; those which promote food ingestion and decrease energy expenditure, agouti-related protein (AGRP) and neuropeptide Y (NPY) and their antagonistic counterparts [20] Pro-opiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART) [21, 22]. Leptin functions by inhibiting the AGRP and NPY neurones whilst activating POMC and CART, elucidating itself as a means of controlling dietary intake and thus energy consumption [17, 23]. The first-order neuronal targets of leptin in the arcuate nucleus include POMC and CART, combined their synergistic interactions modulate downstream effects regulating satiety and energy homeostasis [19]. Complete POMC deficiency manifests with life threatening complications from birth including, hypoglycaemia, neonatal jaundice often sustaining till later

life, increased susceptibility to infections and in extremely rare cases neonatal death [23]. Underlining previously mentioned health implications is an apparent retention of adipose tissue and hyperphagia evident in both murine and human studies. Gut-brain interrelationships and regulation of feeding behaviours are governed by interactions between leptin and melanocortin receptors (Fig 1.1) [24]. The melancortin 4 receptor (MC4R) is highly expressed in the paraventricular nucleus (PVN) of the hypothalamus, where it has a key role in the control of appetite [25]. Mutations affecting leptin-melanocortin signalling can briefly be divided into two classes, class 1 mutations correspond to intracellular retention of mutated proteins, completely abolishing MC4R signalling [26]. Class 2 mutations result in decreased constitutive activity, or exhibit an inability to respond to agonist, or both [26]. The net result manifests in destabilising the MC4R signalling axis and decreased anorexigenic activity of the receptor, responsible for the most common monogenic contributor to non-syndromic human obesity identified so far [27]. MC4R deficiency has been observed in 1-6% of obese individuals from various ethnic groups, and correlates positively with severity and age of onset [26, 28]. Studies analysing phenotypic features of patients suffering from MC4R mutations reveal a characteristic increase in fat free mass, bone density, enhanced linear growth throughout childhood and severe hyperinsulinaemia and obesity [28].

Figure 1.1 The leptin-melanocortin pathway

The central nervous system plays a primary part in regulating food intake through the brain–gut axis, with the hypothalamus acting as the central regulator, receiving both long- and short-term food intake and energy expenditure feedback from the periphery. Leptin released from adipose tissue binds to leptin receptors (LEPR) on agouti-related protein (AGRP)-producing neurons and pro-opionomelanocortin (POMC)-producing neurons in the arcuate nucleus (ARC) of the hypothalamus. Leptin binding inhibits AGRP production and stimulates the production of POMC, which undergoes post-translational modification to generate a range of peptides, including α-, β- and γ-melanocyte-stimulating hormone (MSH). AGRP and α-MSH compete for MC4R — AGRP binding suppresses MC4R activity and α-MSH binding stimulates MC4R activity. Decreased receptor activity generates an orexigenic signal, whereas increased receptor activity generates an anorexigenic signal [29].

1.2.2. Syndromic obesity.

Syndromic obesity stems from discrete genetic defects and chromosomal aberrations that can either be sex linked or autosomal [30]. More than 25 syndromic forms of obesity have been identified, each presenting with unique clinical features including mental retardation, dysmorphic features and even organ-specific developmental abnormalities [31]. However due to increased genetic heterogeneity amongst syndromes of obesity, clinical characterisation rarely defines a specific syndrome, with multiple genes deregulated in the same molecular pathway, yet producing identical phenotypes. Presented below are a few frequently observed syndromic forms of obesity, presenting with severe hyperphagia along with symptoms of hypothalamic disarray in common.

Prader Willi Syndrome (PWS) is an autosomal dominant disorder and amongst the most common forms of syndromic obesity with an incidence of approximately one in 15,000-25,000 live births [32]. Affected individuals present with diminished neonatal activity, muscular hypotonia and are often short of stature in later years of life [32]. More severe health implications associated with PWS include mental retardation and hypogonadotropic hypogonadism, with underlining obesity [33]. PWS exhibits various etiologies but is almost always associated with reduced or complete absence of expression of paternally transmitted genes on 15q11-13 [34]. Bardet-Biedl syndrome (BBS) occurrence is considerably rarer relative to PWS, with a prevalence less than 1/100,000 live births [35]. Typical symptoms associated with BBS include rod-cone dystrophy recognised as the most commonly observed phenotype, polydactyly, developmental delay, hypogonadism in males, renal abnormalities and marked obesity [36, 37]. BBS inheritance is autosomal recessive in nature and initial investigations utilising positional cloning efforts had identified BBS2 and BBS6 as two potential candidate marker genes [38, 39]. Wilms tumour, aniridia, genitourinary anomalies, and mental retardation (WAGR), is heterozygotic in inheritance and manifests due to deletion of 11p13 [40].

Alongside previously mentioned symptoms, marked hyperphagia and obesity are observed [41]. Certain individuals suffering from WAGR exhibit normal body weight and eating habits, however molecular techniques employed to compare the extent and overlap of the 11p13 deletion revealed obese patients present with decreased expression of brain-derived neurotrophic factor (BDNF) [42]. Murine studies have also reported data in unison with data establishing BDNF to possess a vital role in eating behaviours. Rios *et al*., reported mice lacking functional BDNF developed hyperphagia and obesity [43]. Furthermore mutations in the BDNF receptor Tropomyosin receptor kinase B (TRKB) also result in obesity, an observation reported in both mouse and human models [44]. Patients suffering from TRKB deficiency suffer from learning difficulties, memory loss and loss of nociception, along with typical obesity [45]. TRKB is recognised as a separate syndromic form of obesity and evidently portrays the role of BDNF in food intake, as a fundamental one [46].

1.2.3. Polygenic obesity.

The genetics governing polygenic syndromes, arise from the presence of multiple DNA variations in numerous genes [47]. By definition, a group of alleles at specific gene loci that cumulatively regulate the inheritance of a quantitative phenotype or alter the expression of a qualitative character are termed polygenic variants [47]. Existing research has identified many polygenic variants known to potentiate body weight; therefore individuals possessing many such polygenic variants are susceptible to developing obesity [48]. Unlike disorders of syndromic obesity, which arise predominantly from genetic defects either promoting hunger and/or enhanced adipose accumulation, the development of polygenic obesity is considerably dependent upon environmental influences [49]. Therefore disorders of polygenic obesity are potentially more aggressive in Westernised societies owing to an environment consisting of processed calorific foods and automated advances reducing energy expenditure, thus promoting adipose mass accumulation [50]. Genome-wide linkage studies, although useful for identifying genetic mutations underlying monogenic forms of obesity, have now been superseded by genome-wide

association studies, offering greater power and ability to detect genes of modest or small effect masked by underlying complex disorders involved in the development of obesity. The development of single nucleotide polymorphism (SNP) chip technology has facilitated the identification of numerous confirmed genes involved in obesity [47].

1.2.4. Fat mass and obesity associated (FTO) gene.

Genome-wide association studies conducted to identify genes predisposing an individual to T2D, were amongst the first to discover the FTO gene [47]. Following statistical adjustments it was then revealed that the FTO gene was in fact an indicator of increased BMI and not specifically an indicator of increased risk of developing T2D [51]. Large scale follow up studies followed to confirm these findings, integrated studies collectively analysed 13 cohorts consisting of 38,759 participants and reported the SNP's located in intron 1 of the FTO gene translated to enhanced susceptibility to the development of obesity by 31% [52]. Adults homozygous for the risk allele weigh on average 3kg more than those heterozygous for the risk allele, and had a 1.67-fold increased odds for developing obesity when compared to individuals not inheriting the risk allele [53].

The contribution of SNPs in FTO related obesity in children is equally relevant to that observed in adults [54]. Frayling *et al.*, was the first to document the persistence of SNPs in the FTO gene, found in obese children as young as 7 [55]. Studies examining the effects of the FTO gene in children have yielded interesting results regarding eating behaviours and identified children heterozygous for the risk allele exhibit hyperphagic tendencies and display selective behaviours towards calorific foods in comparison to children homozygous for the non-risk allele [56]. Links between SNPs in the FTO gene and reduced physical activity have also been established, and describe physical inactivity to accentuate the effect of the FTO gene in promoting adipose mass accumulation, further highlighting the prominent influence of the Westernised way of life on polygenic obesity [57, 58]. Despite a wealth of literature reporting associations between the FTO

gene and obesity, the precise mechanism contributing to weight gain remains elusive. Although results from animal studies have shown FTO knockout mice exhibit increased energy expenditure in comparison to wild type mice, express elevated oxygen utilisation, carbon dioxide and heat production, furthermore FTO deficient mice readily metabolise triglycerides and fatty acids [59, 60]. Therefore the animal model studies suggest that FTO gene is functionally involved in energy homeostasis. Humans predisposed to obesity conferred by SNP's in the FTO gene display reduced insulin effect on Beta cell activity, which implicates a lower cerebrocortical response to insulin [61]. Furthermore a separate study has suggested the presence of the FTO gene is vital in regulating lipolysis; increased intake of calorific foods and an uncontrolled appetite have also been reported [62].

1.3. Obesity, genetic or environmental?

Views regarding the recent increased incidence of obesity are surrounded by controversy. There exists a dichotomy in scientific opinion with respect to the increased incidence of obesity, with some opinion suggesting that the development of obesity is primarily genetic, whilst other viewpoints consider the impact of environmental influences, more specifically the increasing consumption of energy dense, calorific foods and lack of exercise as the driving force behind the obesity epidemic [63-65]. Although there exists considerable evidence in favour of both arguments, a more realistic view might suggest that human lifestyle has experienced a greater degree of evolution than human genetics has in the past 30 years. Recent sequencing of the Neanderthal genome, regarded as the closest evolutionary relatives of present-day humans, understood to have suffered extinction 40,000 years ago puts this idea into perspective. The work of Burbano *et al*., sequenced ~14,000 protein-coding positions inferred to have changed in the human lineage since the last common ancestor shared with chimpanzees [66]. The authors conclude a disparity of 88 amino acids exists between Neanderthals and modern day humans, and suspect them to be functionally irrelevant [66].

Studies comparing the Neanderthal genome to the genomes of five present-day humans from different parts of the world identified that Neanderthals shared genetic variants with present-day humans residing in Eurasia, while DNA methylation patterns varied by 0.12% between the two species [67]. Considering the Neanderthal species were largely free of metabolic disorder, although genetic variants predisposing humans to Crohn's disease, lupus and biliary cirrhosis have been implicated to be inherited via *Homo sapiens* interbreeding with the Neanderthal species, the documentation of obese Neanderthals is unheard of [68, 69]. Thus genes responsible for obesity have not been inherited from ancestral species; an alternative concept connecting genetics to obesity would then suggest large proportions of the global population in the last 20 years will have undergone spontaneous mutations in genes regulating energy homeostasis to account for the rapid spread of obesity. Considering the frequency of different genes across a population remains fairly stable for many generations a more plausible theory recognises the drastic change in dietary habits and inadequate energy expenditure.

1.3.1. The thrifty genotype.

Obesity is essentially a multifactorial condition and develops due to a combination of both genetic influence and environmental risk factors where the latter plays a constitutively larger role. A theory which urbanely amalgamates these two concepts was proposed by geneticist James V. Neel in 1962 and explicates the contribution of gene-environmental interactions responsible for metabolic disease risk in humans [70]. Moreover, Neel's work elucidated the vital contribution of dietary habits in the development of chronic obesity. Neel argued that a genotype that would promote retention of energy for future use coincided with the evolutionary mechanism of natural selection, whereby organisms capable of storing excess calories as fat would better survive times of food scarcity [70]. The thrifty genotype would thus be advantageous for hunter-gatherer populations; however in modern societies with a constant abundance of food and introduction of processed, energy dense food types, this genotype efficiently prepares individuals for a non-

existent famine. The result of this disparity between the environment in which we evolved and the environment of today is a facilitated accumulation of excess adipose tissue. An additional theory recognises the cryoprotective effects of elevated circulating glucose and glycerol on organs, protecting ancestral species from extreme temperatures [71]. The authors suggested factors that predispose to elevated levels of sugar derivatives may have been selected for as adaptive measures in exceedingly cold climates [71]. Conclusively, it should be noted that in most cases of obesity the largest contribution to positive energy balance is excess caloric intake and suggests that a significant portion of intervention and prevention strategies should be focused on controlling food intake and increasing energy expenditure.

1.4. The influence of diet and sedentary behaviour on obesity.

1.4.1 Nutritional transition.

Analysing change in human dietary habits over the course of history clearly identifies a vital contribution of food availability, processing and choice in the role of obesity. For example, hunter-gatherer species alive during the Paleolithic era and prior to the advent of agriculture and domestication of animals evolved nutritional needs specific to the foods available at that time, which may have been key to their resistance to metabolic disorder [72]. The Paleolithic diet required hunter gatherers to attain the majority of their calories from lean proteins; hunting wild animals and fishing would thus be an obvious source of food, and a requisite source of protein and animal/omega fats [72, 73]. The remaining calories would have been obtained from the consumption of carbohydrates in the form of non-starchy fruits, berries and vegetables [74]. Food groups excluded from the Paleolithic diet include dairy, whole grains, processed oils, refined sugars and alcohol, and are now considered to be at the root of the obesity crisis [75]. The consumption of such foods became evident post the Neolithic agricultural revolution which gave rise to wide-scale food-crop cultivation and domestication of animals, as a consequence of expanding populations [76].

Interestingly, artistic figurines of women recovered from the Neolithic period displayed characteristics of the obese phenotype, and such depictions may be a reflection of the first cases of human obesity in a civilised society [76, 77]. The unambiguous appearance of obesity was even more apparent during the Greek and Roman era, a time in which food as a homeostatic energy source grew into a hedonic source of pleasure, high in fats, sugars and alcohol [78]. Social pressures in accordance with wealth and opulence catalysed over-indulgence in such foods and paved the way for modern day eating behaviours. Medical manuscripts from these periods readily described clinical features reminiscent of the obese phenotype. The work of Papavramidou and Christopoulou-Aletra summarises the views of numerous physicians documented between 25BC to 9th century AD [79]. Fascinatingly, despite the lack of advanced clinical diagnostic tools, a mutual understanding amongst physicians of the time recognised excessive food consumption was the underlining cause of the obese phenotype and treatment involved, dieting, taking hot baths, vomiting, purgation and lifestyle changes [79]. These ancient ideas contributed to the elementary understanding of obesity in current times.

1.4.2. The modern day nutritional milieu

The human diet has considerably changed over the course of time. Ancestral species ate primarily to sustain physiological wellbeing and that to without substantial culinary modifications [80]. For modern day humans, behaviours governing the consumption/purchasing of foods is considerably more complex and largely influenced by preference for taste, convenience, cost and marketing ploys. Such influences on diet have vastly spread across the globe and converged to form what is known as the “Western diet”, loosely defined by increased intake of processed foods, refined carbohydrates, added sugars, increased salt content, fats, and animal-source foods [81, 82]. Statistics derived from the NHS information centre documented food purchasing and consumption habits in the UK and support this statement. Key findings published in this report outline mean consumption of saturated fat, non-milk extrinsic sugars and alcohol significantly exceeded the recommended amounts. The consumption of lean proteins, in particular oily fish is

significantly below the recommended daily amount and appears in conjunction with decreased consumption of fruit and vegetables [83]. Only a quarter of adults consume the recommended 5 portions a day of fruit and vegetables. Furthermore a decrease of 1% in household fruit purchases was reported in 2010, and is now 11.6% lower than 2007 [83, 84]. Purchases of vegetables increased by 0.4% but are 2.9% lower than in 2007. Unsurprisingly the majority of household expenditure was attributed to increased consumption of butter/oil, sugary beverages and processed packaged meals [85, 86]. Such consumption/purchasing trends are representative of both developed and developing nations and currently considered to be at the heart of the global obesity epidemic.

1.4.3. Edible oil, butter and fat.

During the later 19th century, technological advances facilitated the cost effective production of oil from oilseeds, in line with breeding techniques the ability to increase oil content from seeds accompanied the large scale availability of cheap vegetable oils. Between 1985 and 2010 individual intake of vegetable oils increased three-fold to six-fold, depending on the subpopulation studied [87]. In China, which has moderate but not high vegetable oil intake, persons age two and older now consume on average almost 300 calories and more than 30 grams of vegetable oil daily [87].

1.4.4. The sweetening of the world's diet.

Data collected from surveys undertaken around the world have indicated that over the past 20 years, concomitant with the rising rates of obesity, consumption of carbohydrates largely in the form of added sugars has significantly increased [87]. In the USA up to 75% of foods and beverages are reported to contain added caloric sweeteners [88]. In the UK soft drink consumption has risen by 30% in the past 10 years and is considered to be vital in the progression of obesity [88]. China, India, Vietnam, Thailand, and other Southeast Asian countries are currently major growth markets for the soft drink industry [88]. Consumption of high-sugar

desserts and snacks is also increasing in urban centres in both developed and developing countries [89, 90]. Malik *et al*., conducted a systematic review which analysed publications between 1966 and 2005 in a bid to better understand the association between sugar-sweetened beverages and weight gain. Findings from large cross-sectional studies, in conjunction with those from well-powered prospective cohort studies with long periods of follow-up, show a positive association between greater intakes of sugar-sweetened beverages and obesity in both children and adults [88]. Likewise reducing soft-drink consumption in schoolchildren led to a significant reduction in the prevalence of obesity [88]. Increasing evidence suggests the utilisation of high-fructose corn syrup (HFCS) by food manufacturers is contributing to the obesity epidemic [89]. Western countries have particularly fallen victim to the negative effects of HFCS with the USA have increased HFCS consumption by 1000% between 1970 and 1990 [89]. HFCS now accounts for 40% of sweeteners added to foods and is the sole caloric sweetener in soft drinks, moreover mirrors the incidence in obesity. Murine studies have been successful in identifying the differential digestion, absorption and metabolism of fructose relative to glucose and have correlated the consumption of HFCS to increased adipogenesis [90]. The mechanism proposed by the authors recognises the inability of fructose to stimulate insulin and leptin production, sequestering glucose uptake and pathways regulating food intake, but sustaining ghrelin levels. The net effect leads to enhanced lipogenesis [91, 92].

1.4.5. Processed foods.

Numerous reports have been published in recent years outlining the considerable impact of the increased processing of foods, on the obesity pandemic [93]. Reasons behind the preference of processed foods are multi-factorial however can be summarised by the need for low-cost, easily prepared and tasteful foods, at the expense of low quality and obesogenic ingredients [93, 94]. Monteiro *et al*., describes how the classification of processed foods can be sub-divided into 3 groups. Foods listed under group 1 are either unprocessed or minimally processed foods, such as fresh meat and milk, grains, pulses, fruits and vegetables [95]. Group 2 foods include oils, fats,

flours, pastas and sugars; processed physically and chemically to produce culinary and/or food industry ingredients [95]. Finally group 3 foods are the most readily consumed globally and typically undergo industrial processes to increase shelf-life and palatability, regarded as habit-forming foods. Foods within group 3 include biscuits, ice creams, chocolates, crisps and soft drinks all of which are low nutrient density, dietary fibre and high in saturated fats, sodium, and trans-fatty acids [95]. Key to their increased consumption is the increased branding, international distribution and marketing [96]. Growth in their production and consumption has been enormous in the last decades in both higher- and lower-income countries and is a significant contributor to obesity [96].

1.4.6. Eating behaviors.

Westernised eating behaviours are considered central to the increased incidence of obesity seen across the globe [97]. Additionally, increased eating frequency, binge eating and the consumption of foods from restaurants are common practices in modern societies [97, 98]. Evidence suggests both higher- and lower-income societies residing in the USA and UK exhibit increased snacking tendencies [99]. Energy dense snacks account for 20-25% of total energy intake in these countries; snacking has been reported to transition into subconscious behaviour [99]. Binge eating has been recognised by the diagnostic and statistical manual of mental disorders as of 2013 and identifies causes which readily affect westernised populations; including depression, anxiety, stress and loneliness [100]. Binge eating disorders and night eating disorders are commonly associated to obesity according to cross-sectional studies [101-103]. Studies examining the effects of eating food prepared outside the home are plentiful and the majority have been conducted in the USA. In the last 40 years US citizens have increased the amount of money spent on eating out from 26% to 56%, increases which coincide with obesity rates [104].

Studies examining British cohorts are limited however the fast food market is as influential in the UK as in the US, and of major concern is the increased availability of fast foods to children and

young adults [105]. Findings from a recent study carried out on 3600 adolescents in UK suggested that those adolescents who ate at fast food outlets tend to consume more unhealthy foods high in total fat, saturated fat, cholesterol and sodium and were likely to have higher BMI than those adolescents who did not consume fast food frequently [98]. Comparatively increased frequency of participation in the family dinner was associated with higher consumption of fruits and vegetables, fibre, folate, calcium, iron and vitamins B-6, B-12, C and E, lower consumption of saturated and trans-fatty acids and a decreased glycaemic load [106]. Patterson *et al*., summarise the impact of "eating out" on obesity and attribute the increasing portion sizes and the availability of calorie dense foods creates an obesogenic environment predisposing children and young adults to obesity [98].

1.4.7. Sedentary behavior

Much like the drastic transformation of the human diet, factors governing physical activity levels and energy expending behaviours, have too altered in favour of promoting obesity [107]. Preceding species were considerably more active employing manual efforts to complete home-based chores, hunt for food and transport [73]. Contrarily modern day humans residing in metropolitan cities are exceedingly reliant on time saving technologies, in conjunction with the rapid increase in the availability and implementation of a wide range of screen-based devices for work or leisure purposes, make it significantly difficult to expend energy, thus creating an obesogenic environment [108, 109]. Various authors have denoted the vital contribution of sedentary behaviour in propagating the obesity epidemic. Obvious examples of such behaviours include TV viewing, playing computer games, online shopping, social media applications, car travel as well as long periods spent sitting at school, home or work (Fig. 1.2.) [110]. Such changes reflect low levels of habitual physical activity and are associated with 'hypokinetic diseases' [111].

Evidence suggests that physical activity levels are low within the UK and exhibit an age associated decline, with only 40% of men and 28% of women meeting the minimum recommendations for physical activity and only 17% of men and 13% of women aged 65-74 engaging in physical activity at all [108]. Hu *et al.,* examined 3757 non-obese women, over a period of 6 years who became obese, with hours spent watching TV exhibiting a causal association. The authors reported 2 hour daily increments in watching TV were associated with a 23% increase in obesity and 2 hour daily increments in sitting at work were associated with a 5% increase in obesity [112]. Moderate exercise at home or work positively correlated with a 9% reduction in obesity, while 1 hour of brisk walking reduced obesity risk by 24%, demonstrating the drastic benefits of engaging in moderate exercise [112]. The role of sedentary behaviour in propagating the obese phenotype has been verified by numerous studies correlating sedentary behaviours with increased metabolic risk. Hours spent utilising computers and/or watching TV have been positively associated with increased fasting insulin, BMI, Homeostatic model assessment-insulin resistance (HOMA-IR) and metabolic syndrome [112-114].

Figure 1.2. Changes in activity levels over time with changes in technology.

The development of time saving (cars and internet) and time wasting technologies (gaming consoles) has resulted in a significant reduction in daily energy expenditure. A common view regards the increased automation of previously manual processes responsible for the increased incidence of metabolic disorders [78].

1.5. The epidemiology of obesity

Considering the aetiology of obesity, researchers have previously believed health implications arising from the atypical retention of adipose tissue was a cause of concern for Westernised civilisations only. However globalisation of calorific processed foods and time saving/wasting technologies has propelled obesity to epidemic proportions. Key to the identification of the increased prevalence rate of obesity is the standardised approach in measuring obesity. The World Health Organization in 1995 accepted the BMI, a simple index of weight–height relationship as the appropriate method for crudely assessing degrees of body fat sufficient to classify obesity [115] . The National Institutes of Health have defined overweight as an individual having a BMI between 25.0 and 29.9 kg/m^2; and obesity as having a BMI greater than 30.0 kg/m^2 [116, 117]. For a given BMI, adiposity varies with sex, age and ethnicity; however, BMI correlates reasonably well with body fat mass and the risk of obesity-related diseases [116]. The use of BMI is preferred over densitometry, computed tomography (CT), magnetic resonance imaging, and dual energy X-ray absorptiometry, due to its low cost and accessibility. Recent research has emphasised the effectiveness and practicality of waist to hip ratio measurements as a predictor of adiposity and metabolic risk [118, 119].

1.5.1 Global trends in obesity

Research has revealed that almost all countries are facing increasing obesity rates [120]. Initial statistics released by the WHO considered obesity as cause of concern for the Western hemisphere only. More recent figures reveal developing countries are also at risk [120]. The WHO reports approximately 115 million people suffer from obesity and/or related problems in developing countries [121]. Generally East and South-Eastern countries such as China and India have low levels of obesity, estimated to be 1 % or less, however the prevalence of obesity in countries forming the Pacific Islands can reach up to 80 % (Samoa, Fiji). Even in low-prevalence countries stark variations exist between urban and rural areas [122]. Recently, application of lower cut off of body BMI (Asian criteria of overweight: 23–25 kg/m^2 and obesity: >25 kg/m^2)

has led to a marked increase in prevalence figures in several Asian countries [123]. Data derived from the China Health and Nutrition Survey indicates a BMI increase of 1.2 in adults between 1993 and 2009 with an increase of 67.0 % in the prevalence of overweight, from 9.4 % to 15.7 %, and an increase of 168.0 % in the prevalence of obesity, from 4.0 % to 10.7 % [124]. While the overall prevalence of overweight and obesity remains low in India, a recent national urban survey of the six metropolitan cities in India reported the prevalence of overweight and obesity for adults aged ≥ 20 years old to be approximately 30% [125]. Similar estimates from Vietnam suggest that nearly one-fifth of adults in Vietnam's biggest city are overweight [126]. This indicates that India and Vietnam, along with many other countries in the region, face the dual challenge of under- and over-nutrition.

Of a greater concern is the increasing incidence of childhood obesity. For example in the USA the National Health and Nutritional Examination Survey (NHANES) revealed in 2003–2006, 31.9% of children 2–19 years old had a BMI at or above the 85th percentile of the 2000 Centers for Disease Control and Prevention BMI-for-age growth charts, and 16.3% were at or above the 95th percentile of BMI for age [127]. Since 1986 various surveys have been conducted investigating obesity amongst preschool children, and report countries comprising Latin America, the Caribbean, the Middle East and North Africa exhibit rates of obesity comparable to USA [128, 129]. Similar trends have also been observed in India, Mexico, Nigeria, and Tunisia over the past 2 decades [130-132]. The prevalence of obesity among 5 to 12 year old children in Thailand increased from 12.2 to 15.6% over a period of 2 years [133]. Increases in the prevalence of overweight among older children and adolescents have been seen also; from 4.1 to 13.9% between 1975 and 1997 in Brazil [134]. The prevalence of obesity is generally high in developed countries, with statistics showing that in 2007–2008, approximately 68% of US citizens were overweight or obese, and approximately 34% were obese [135]. In England, the prevalence of obesity among adults rose from 14.9% to 24.9% between 1993 and 2013 [136]. The rapid increase in the prevalence of overweight and obesity has meant that the proportion of adults in England

with a healthy BMI decreased between 1993 and 2013 from 41.0% to 31.2% among men, and 49.5% to 40.8% among women [137]. In England, currently 24.9% of adults are obese [138]. Contrastingly Asia has the lowest rates of obesity and the Pacific islands have the highest rates [139]. The rates of obesity in Africa and Middle Eastern countries are considerably variable. Conclusively, obesity incidence is increasing globally. The WHO established a global BMI database as of 2004; the WHO estimates that in 2005 approximately 1.6 billion people worldwide were overweight and that at least 400 million adults were obese [140]. They further project that, by 2016, approximately 2.3 billion adults will be overweight and that at least 700 million will be obese [141].

1.5.2 Gender disparities in obesity

Epidemiological studies have identified females are at greater risk of developing obesity than males [142]. According to reports published by the WHO, in 138 of 194 countries more than 50% of women were likely to be obese. Although the precise mechanisms responsible for this phenomenon are unknown, various theories have been proposed. Interestingly gestational nutritional deprivation affects males and females differently, while males respond by exhibiting decreased ability to gain weight and deposit fat, females respond oppositely [143]. Interestingly exposure to the Dutch famine (1944-1945) during early gestation was associated with higher weight, BMI and waist circumference amongst 50-year-old women but not 50-year-old men [144]. Similar results were obtained from a study examining men and women born during the Great Famine (1959-1962) in China [145]. Case and Deaton reported that South African women suffer from depression, more so than South African males, furthermore the work of Onyike *et al*., positively correlated greater degrees of depression with obesity, either a negative association or no association was observed in men [146].

Depression has been related to eating patterns and particularly hyperphagia and may lead to differential weight gain between men and women [147]. There exists evidence to suggest that

males and females do exhibit differing eating patterns, and that one's sociocultural environment likely influences these preferences. Kanter and Caballero report that women are more interested in purchasing and consuming healthier foods, however in actuality consume a larger amount of energy dense processed foods such as cookies, ice cream and chocolate, relative to men [142]. While men obtain a larger amount of their daily calorie intake from lean proteins, likely due to their greater preference for and thus consumption of meat-based products than women. The work of E. Blaak recognises that males and females metabolise lipids differently and report the oxidation of basal lipids is considerably lower in females as compared to males, thereby contributing to a higher fat storage in women [148]. Finally, postprandial fat storage may be higher in subcutaneous adipose tissue in women than in men, whereas storage in visceral adipose tissue has been hypothesised to be augmented in men. The role of sex hormones, particularly oestrogen may be largely responsible for the sexual dimorphism in both body composition and body fat distribution amongst males and females [149]. Studies have previously reported that oestrogens stimulate preadipocyte proliferation and that this effect is depot-specific and more pronounced in preadipocytes from women compared with preadipocytes from men [150]. In developing countries sociocultural beliefs/influences largely account for gender disparities in obesity. For instance in certain parts of South and East Asia, along with North and Sub-Saharan African physical inactivity and the obese phenotype is associated with high social status, fertility good health and prosperity. Contrastingly slimmer and smaller women are perceived as infertile and ill.

1.5.3. Relationship between age and the prevalence of obesity.

Advancing age is associated with an increase in adipose tissue in humans and has been recognised as a primary causative agent behind age-related metabolic disorders [151]. The age associated increase in adipose tissue may be responsible for the increased incidence of obesity with advancing age [152]. It has been reported that obesity increases with age and reaches its peak at around 55- 64 years and decreases afterwards [152]. Increasing adiposity is also accompanied by

the development of age related decrease in muscle mass, also known as sarcopenia [153]. Basal metabolic rate is determined chiefly by fat-free mass which progressively deteriorates with age [153]. Increasing age is also accompanied with a reduction in physical activity, further contributing to a decrease in muscle mass and energy expenditure; however diet is often maintained, inducing a positive energy balance promoting storage of calories in the form of fat [152]. Alongside the increase in total adipose tissue mass ageing is also associated with an altered pattern of adiposity distribution. A review by Kuk., *et al* highlights the inability of certain individuals to develop sufficient subcutaneous adipose tissue mass specifically in the lower body, thus exhibit a reduced ability to store circulating lipids and non-esterified fatty acids (NEFA) [154]. As a compensatory mechanism the increased fat content is stored in non-subcutaneous regions, giving rise to increased lipid accumulation in the visceral region [154]. Concurrently both cross sectional and longitudinal studies have confirmed an age associated increase in waist circumference largely recognised as both a risk factor and diagnostic marker of obesity and related co-morbidities [155].

1.5.4. Obesity and ethnicity.

Strong evidence exists to affirm certain ethnic minorities are at an increased risk of developing obesity in comparison to Caucasian populations. Statistics describing the incidence of obesity amongst children in the UK validate such evidence [156]. In 2010, the prevalence of obesity among 10–11-year-olds in England was 20–29% among Bangladeshi, Pakistani and black ethnic groups compared with 16–19% in Caucasian British children; among 4–5-year-olds these figures were 11–18% and 9–11%, respectively [156]. Kumar *et al*., assessed differences in obesity amongst 3019 immigrants from developing countries, residing in Oslo, Norway. Generalised obesity was most frequent among the Turks [157]. However, central obesity was most frequent among the Pakistanis and Sri Lankans. For any given value of BMI, the waist to hip ratio (WHR) was considerably higher among Sri Lankans and Pakistanis compared to Norwegian residents [157]. Statistics derived from studies undertaken in the USA reveal a larger proportion of African

American adults and children suffer from obesity in comparison to Caucasian adults and children [158]. Exact figures from the Center for Disease Control and Prevention reveal Non-Hispanic blacks have the highest age-adjusted rates of obesity (47.8%) followed by Hispanics (42.5%), non-Hispanic whites (32.6%), and non-Hispanic Asians (10.8%) in the USA [159]. Among all Asians in the United States, South Asians have the highest rates of overweight/obesity (25% among men and 37% among women) [160]. The variance in obesity amongst different ethnicities can be explained due to notable differences in lifestyle factors, for example children from ethnic minority groups in the UK engage in lower levels of physical activity than their Caucasian peers, while South Asian children report higher consumption of dietary fat and children from black ethnic groups are more likely to skip breakfast [156]. One possible explanation for the ethnic patterning of obesity-related behaviours is the effect of cultural values and norms; it has been proposed that, in order to reduce health inequalities, culturally specific efforts are required to address the issue of healthy lifestyle among high-risk ethnic groups [161].

1.5.5. Financial burden of obesity

The obese phenotype is more than a cosmetic concern; instead increased adipose mass retention predisposes an individual to numerous health risks which significantly reduce the quality and longevity of life, including cardiovascular disease, T2D and even cancer [162]. Such health implications constrain financial budgets particularly of developing countries. For instance, mortality stemming from obesity related non-communicable diseases is predicted to account for 43.3 % of all deaths in India and are estimated to cost the Indian economy $200 billion from 2005-2015 [163, 164]. In 2000, in Latin America and the Caribbean, the direct and indirect costs of diabetes (as result from obesity) were about $65.2 billion according to WHO estimation [165]. In the Pacific Islands, about $1.95 million is a cost of obesity induced diabetes in Tonga while $13.6 million in Fiji, taking about 60 % and 39 % of health budgets, respectively [163, 164]. In 2003, mainland China expended $50 billion in medical costs associated with obesity, counting nearly 25.5 % of the total costs of medical care of chronic diseases and nearly 3.7 % of national

total medical costs and expected to rise to about $112 billion by 2025 [164]. Studies examining the financial burden of obesity in Westernised countries are plentiful and more detailed and rightfully so considering the magnitude of the problem [163]. The USA leads the world in rates of overweight and obese individuals per capita, evidently reflected by the significant expenditure on obesity related healthcare. Health care costs for obese individuals are 37% higher than for people of normal weight, adding an extra $732 to the health care bill of each and every American, such expenditures are comparable to healthcare expenses associated with smoking [166].

The work of R. Strum reports health conditions associated with obesity are analogous to health implications associated with 20 years of ageing and correspond to 36 % increase in inpatient and outpatient spending while, increase medication costs by 77 % [167]. In the workplace, employers of the overweight and obese face costs of absenteeism, reduced productivity, and other obesity related complications including heart disease, osteoarthritis, gallbladder disease, hypertension, and Type 2 diabetes, resulting in $62.7 billion in doctor's visits and $39.3 billion in lost workdays each year [168]. The UK exhibits similar trends, estimates of the direct costs to the NHS for treating overweight and obesity, and related morbidity in England, have ranged from £479.3 million in 1998 to £4.2 billion in 2007 [169]. Estimates of those costs arising from the impact of obesity on the wider economy such as loss of productivity over the same time period ranged between £2.6 billion and £15.8 billion [168, 170]. In 2006/07, obesity and obesity-related illness was estimated to have cost £148 million in inpatient stays in England [170]. Modelled projections suggest that indirect costs could be as much as £27 billion in 2015 [171].

1.6. The pathophysiology of obesity

The accretion of excess adipose tissue, characteristic of the obese phenotype is chiefly determined by the balance between lipogenesis and lipolysis/fatty acid oxidation [172]. Energy expenditure in the form of exercise or fasting diminishes lipogenic responses in adipose tissue and upregulates

lipolysis and the consequential net loss of triglycerides from fat cells, offering protection against obesity [173]. Conversely the consumption of a diet high in carbohydrates stimulates lipogenesis in both liver and adipocyte tissues and is key in the manifestation of obesity [174]. Postprandial spikes in glucose levels feed directly into lipogenesis via several mechanisms; primarily, by inducing insulin secretion which actively inhibits glucagon production from the pancreas and stimulates the activity of Pyruvate dehydrogenase, and Acetyl-CoA carboxylase, which facilitate the glycolytic conversion of glucose into malonyl-CoA [175].

Malonyl-CoA provides the two-carbon building blocks that are used to create larger fatty acids [175]. It has been hypothesised that the storage of fatty acid as triacylglycerol (TG) within adipocytes protects against fatty acid toxicity; otherwise, free fatty acids would circulate freely in the vasculature and produce oxidative stress by disseminating throughout the body [176]. However the dysregulation of normal physiological nutrient homeostasis manifesting from chronic overconsumption and the enhanced sympathetic state existing in obesity eventually saturates adipocyte storage capabilities. The “spillover” effect (Fig 1.2) describes the release of excessive free fatty acids (FFA) from adipocytes, this redirects fatty acids to the liver promoting dyslipidemia, characterized by elevated plasma FFA, TGs, and small dense low density lipoprotein (LDL), and the reduction of high-density lipoproteins (HDL) which then incite lipotoxicity, as lipids and their metabolites create reactive oxygen species (ROS) within the endoplasmic reticulum and mitochondria [176]. Circulating free fatty acids have been commonly associated with the development of insulin resistance, although the precise mechanisms that establish insulin resistance are unknown. Existing research identifies the ability of FFA, via various mechanisms, to promote tyrosine phosphorylation of the insulin receptor substrate, potentially due to increased ROS production [177]. Additionally FFA function as substrates for gluconeogenesis, further influencing hyperglycemia, in the absence of sufficient insulin secretion [177]. Key to the pathogenicity of storing atypical amounts of fat is the relatively recently described endocrine nature of adipose tissue [178]. The specific secretions of adipose tissue will

be considered in a later section however the altered expression and/or secretion patterns of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) from obese adipocytes have been reported to impede lipolysis and insulin sensitivity [179]. The secretion of pro-inflammatory cytokines from adipose sites has been attributed to the increased infiltration of M1 pro-inflammatory macrophages [180]. Subsequent studies have demonstrated that M1 macrophage infiltration into adipose tissue is increased in obesity due to hypertrophic expansion, while M2 anti-inflammatory macrophages are redundant and are found in reduced numbers compared to leaner individuals [180].

Existing research has implicated fatty acid flux as a crucial contributor to macrophage recruitment to adipose tissue, a process mediated by interactions between macrophage toll like receptors (TLR) and dietary circulating saturated fatty acids, resulting in augmented cytokine production [181]. TLR deficient mice exhibit reduced macrophage infiltration of adipose tissues and inflammation [182]. In support of the role of macrophage mediated inflammation are studies which have utilised murine models and report the increased expression of intracellular adhesion molecule (ICAM-1) in response to 3 weeks of high-fat feeding [183]. Following 6 months of high fat feeding soluble ICAM-1 levels correlated with adipose mass and provide a mechanism whereby immune cells are able to adhere to capillary endothelial cells and via extravasation invade neighbouring adipocytes [183]. A prominent hypothesis recognises increased macrophage recruitment to phagocytose dead or dying adipocytes, forming crown like structures which stain positive for pro-inflammatory markers [184]. Whereas adipocyte death is rare in lean humans, it is a common hallmark of obesity and is positively correlated with adipocyte hypertrophy [185].

The inflammation accompanying obesity is critical in the manifestation of life threatening co-morbidities associated with obesity. Co-morbidities of obesity can be divided into two categories; those determined by an excess number of fat cells and therefore inflammatory in nature and responsible for the development of health implications associated with a reduced lifespan i.e.

atherosclerosis, T2D and cancer and those presenting due to an accumulation of fat mass i.e. a state of lethargy, breathlessness and psychological stress [186].

1.6.1. Atherosclerosis.

The condition atherosclerosis can be defined as a thickening of the arterial walls due to the accumulation of fatty materials, a common complication amongst the morbidly obese [187]. Existing research has correlated BMI, arterial thickness and gross atherosclerotic plaques with obesity [188]. Symptoms of atherosclerosis manifest due to the rupture of foam cells, (low density lipoprotein containing macrophages) and surrounding extracellular matrix, liberating tissue factors and platelets leading to thrombosis [188]. Stenosis of coronary arteries can prove to be fatal and result in myocardial infarction, while obstruction of blood flow can deprive target organs of both oxygen and essential nutrients [189]. Atherosclerosis is associated with potentially fatal conditions including hypertension, coronary heart disease, stroke and peripheral arterial disease [189].

1.6.2. Hypertension.

Links between hypertension and obesity have been well documented; waist circumference measurements have been reported to significantly correlate with systolic and diastolic blood pressure [190]. Obese individuals often present with rigid, constricted arteries due to ectopic fat accumulation, which impede blood flow [191]. The detection of reduced flow of blood by baroreceptors is met with a compensatory increase in heart rate to restore blood pressure. In support of this model are studies which have successfully quantified larger amounts of angiotensin II and aldosterone in the blood of obese patients [191]. Moreover CT has been utilised to identify renal sinus fatty deposits along with conformational alterations within kidneys, coupled with increased abdominal pressure secondary to central obesity which may impair nephron function and promote hypertension [192]. Moreover, the hyperfiltration observed in

obesity sets the stage for progressive glomerular loss and loss of renal function and associated increases in arterial pressure [190]. The previously mentioned pathology is further aggravated by the consumption of processed foods high in sodium, further deregulating blood pressure homeostasis by the active reabsorption of fluids in the kidney [190].

1.6.3. Cancer.

Research has provided significant evidence of increased risk for colon, postmenopausal breast, endometrial, kidney, esophageal, liver, and pancreatic cancer as well as non- Hodgkin's lymphoma and myeloma in obese individuals [193]. Completely establishing links between cancer and obesity are studies reporting that obese individual's exhibit resistance to chemotherapeutic treatment, while other studies have concluded reduced cancer risk post bariatric surgery [194]. A large number of mechanisms are currently being investigated to explain the association between obesity and cancer and are common to obesity mediated insulin resistance; including, hypoxia, pro-inflamamtory cytokine secretion, oxidative stress, hyperinsulinemia, hyperleptinaemia and the contribution of sex hormones [195]. The complex aetiology of both conditions, in conjunction with the effects of leading a sedentary lifestyle and consuming a variety of compounds in modern diets makes it increasingly difficult to determine precise pathways relating obesity to cancer [196].

Figure 1.3. A flow chart depicting the consequences of retaining excess adipose tissue.

A flow diagram by which excess adiposity is associated with increased cardiometabolic risk. Under this model, excess adiposity results in the accumulation, excessive amounts of visceral adipose tissue and the accumulation of ectopic fat depots, in areas such as the liver, pancreas etc. which go on to facilitate the development of inflammation/insulin resistance and eventually lead to CVD [197].

1.7. The significance of body composition in obesity and related co-morbidities.

It has been well documented and recognised that total adipose tissue is crucial in the propagation of symptomatic health implications giving rise to metabolic syndrome. However emerging evidence identifies specific adipose tissue depots are more closely associated with risk factors for disease than others and therefore can regulate lifespan differentially [198]. The main adipose depots of interest are located in the abdomen and can be divided into subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT); VAT can be further sub-divided into omental and mesenteric fat depots [199].

1.7.1 Subcutaneous adipose tissue.

SAT has been described as being less metabolically active in comparison to VAT, however it is more efficient in both short and long term energy storage. The main areas for subcutaneous fat deposition are the femerogluteal regions, back and anterior abdominal wall [200]. About 80% of all body fat is in the subcutaneous area and functions to store triglycerides during times of energy surplus whilst supplying energy during starvation [201]. SAT also functions as a buffer for FFA and protects against lipotoxic effects [202]. Enlarged adipocytes are typically observed in patients with metabolic disorder and are an indication of adipogenic potential in subcutaneous tissue and can be the trigger for increased macrophage infiltration and inflammatory process activation [202]. Apovian *et al*., reports individuals bearing larger amounts of subcutaneous adipose tissue, as in obesity are associated with systemic hyperinsulinemia, impaired endothelium-dependent flow-mediated vasodilatation, and elevated plasma C-reactive protein (CRP) levels, a marker of systemic inflammation [203]. Gealekman *et al*. reported that the angiogenic capacity of subcutaneous abdominal adipose tissue decreased with increasing BMI but that this did not change in visceral adipose tissue [204]. In addition, a decrease in angiogenic capacity correlated with insulin resistance which suggests that impairment in subcutaneous adipose tissue

angiogenesis may contribute to metabolic complications of obesity [204]. However unlike VAT, SAT has also been associated with health benefits; larger subcutaneous thigh fat mass has been reported to exhibit protective effects. The Health, Aging, and Body Composition Study reported that large subcutaneous thigh fat was independently correlated with improved glucose homeostasis in males and favourable lipid profile in both males and females. A separate study conducted in Australia examined associations between waist and hip circumferences to components of metabolic syndrome. After adjustment for age, BMI and waist circumference, a larger hip circumference was associated with a lower prevalence of diabetes and dyslipidaemia. A review by Gunawardana discussed how healthy adipose tissue offers resistance against insulin resistance, as adipokine secretions may be able to compensate for the lack of functional insulin in diabetes. Studies strongly suggest that adiponectin plays a protective role against insulin resistance and cardiovascular disease. Adiponectin, an abundant adipokine with reported beneficial properties, decreases insulin resistance by decreasing triglyceride content in muscle and liver in obese mice via fatty-acid combustion and energy dissipation in muscle. Also, adiponectin increased the ability of insulin to stimulate glucose uptake, through increased glucose transporter 4 (GLUT4) gene expression.

1.7.2. Visceral adipose tissue

CT measures of both SAT and VAT have been shown to be correlated with multiple metabolic risk factors, although risk factor correlations with VAT are significantly stronger than those for SAT [205]. VAT, has also been documented to provide information above and beyond simple clinical anthropometrics, including BMI and waist circumference and accounts for differences in risk factor stratification among individuals who are obese [205]. Visceral fat depots, including omental and mesenteric adipose tissue, represent a risk factor for the development of CVD and type 2 diabetes (T2D) [206]. This was found in particular in obese diabetic individuals, where it was linked to a significant up regulation of leptin and down regulation of adiponectin gene expression in VAT compared to SAT [206]. SAT and VAT also differ in metabolic activity,

which can largely be accounted for by the increased mitochondrial density in VAT, an observation made in both human and murine studies [207]. Despite being smaller than SAT cells, VAT is bio-energetically more active and responsive to substrates of the electron transport chain and may contribute to the production of larger amounts of ROS [207]. Visceral adipose tissue mass correlates significantly with development of insulin resistance, while total or subcutaneous tissue mass does not [208]. It has been thoroughly confirmed that the adipocytes of visceral fat tissue are more lipolytically active than subcutaneous adipocytes and thus contribute more to the plasma free fatty acid levels [209]. As a result, excess visceral fat will enhance the level of free fatty acid delivered to the liver, thus increasing hepatic glucose and very low density lipoprotein particles (VLDLs) output, and impair the hepatic insulin response [210]. Various studies have documented an age-associated increase in VAT which is known to contribute to metabolic syndrome irrespective of sex and ethnicity [211]. The detrimental effects of VAT as opposed to subcutaneous fat are attributable to its increased potential to secrete pro-inflammatory cytokines also referred to as adipokines [211]. These adipokines, also include TNF- α, IL-6, and vascular endothelial growth factor (VEGF) amongst many others, all of which combined or independently aid the development of metabolic disease, via inflammation, increased cell proliferation and insulin resistance [212].

A review by Bremer *et al*., highlights IL-6, IL-1, TNF-α and CRP as key mediators in the development of metabolic syndrome. IL-6, induced by increased IL-1 and TNF-α concentrations has been shown to hinder insulin signalling by directly interacting with insulin receptor substrate 1 (IRS-1) and inhibiting glycogenesis in the liver [213]. While neutralisation of TNF-α significantly increases insulin sensitivity, IL-6 has also been linked to the production of C-reactive protein and fibrinogen, further propagating inflammation [213]. In conclusion increased visceral fat promotes the development of metabolic abnormalities i.e. diabetes, hypertension, cardiovascular disease, all of which are well known to reduce lifespan. Surgical removal of visceral fat ameliorates the negative impact of pro-inflammatory cytokines and protects against previously discussed health implications. Removal of SAT exhibits no such effect [214].

1.7.3. Brown adipose tissue.

Brown adipose tissue (BAT) is a specialised tissue believed to have developed as an essential thermoregulatory effector, a role it fulfils by dissipating stored energy via the production of heat during the challenge of low environmental temperatures [215]. This is in comparison to its antagonistic adipose counterpart, white adipose tissue (WAT) which is predominantly responsible for storing excess energy as triglycerides [215]. Differences in both structural and physiological characteristics are sufficient to distinguish between BAT and WAT. White adipocytes are considerably less complex, spherical cells and consist of a single lipid droplet, accounting for the majority of the cell volume [216]. Comparatively, BAT has increased vascular density compared with WAT and contains brown adipocytes, which are more complex polygonal cells with a characteristically increased mitochondrial content, responsible for the brown colour [216]. The thermogenic potential of mammals is limited to BAT and skeletal muscle, with skeletal muscle being shown to burn roughly 50 times as much glucose during cold exposure as BAT [216].

Skeletal muscles augment shivering thermogenesis by converting chemical energy to kinetic energy [217]. The thermogenic ability of BAT is attributable to the high mitochondrial content mediating proton transfer across mitochondrial membranes to produce adenosine triphosphate (ATP), in the absence of ATP production excess energy stored in the proton gradient is leaked via uncoupling protein 1 (UCP-1) and released as heat, inducing “non-shivering” thermogenesis [218]. Consequently BAT is found increasingly in small hibernating mammals and newborns, both of which have a large surface area to body mass ratio, thus are less capable of maintaining core body temperature due to insufficient skeletal muscle and inadequate basal metabolic rate to produce heat [217]. Until recently it was thought that BAT was only present in neonates, isolated in axillary, cervical, perirenal, and periadrenal regions, with minuscule amounts found in adults exhibiting little to no physiological relevance.

1.7.4. Brown fat in adults

Recent studies utilising both positron emission tomography (PET) and CT have been successful in identifying BAT deposits in adults, using radioactively labelled fluorodeoxyglucose (FDG) as a means of identifying metabolically active tissue [219]. Quellet *et al.,* designed a sophisticated protocol to determine the thermogenic role of BAT in 6 male adults under cold conditions specifically designed to minimise shivering [220]. Using PET-CT the authors reported increased glucose and non-esterified fatty acid utilisation, as an energy source to fuel BAT thermogenesis under cold conditions, in accordance with enhanced ^{11}C-acetate uptake, a marker of oxidative metabolism [220]. Interestingly the authors reported a 1.8 fold increase in total body energy expenditure mediated by BAT activity, undoubtedly highlighting the thermogenic role of brown fat in adults [220]. A recent study examined BAT distribution/activity in relation to body composition and energy metabolism in 24 healthy men, under thermoneutral conditions and mild cold exposure, using indirect calorimetry to measure energy expenditure and dual energy x-ray absorptiometry to evaluate body composition [221].

BAT activity declined as BMI increased and expressed a significant negative relation with percentage body fat, while resting metabolic rate and BAT were significantly positively correlated, highlighting the beneficial effects of BAT on resting energy expenditure and the lack of in overweight/obese subjects [221]. Thus the accumulation and/or retention of BAT can be assumed to have favourable metabolic consequences due to its role in the dissipation of excess energy as heat. Following the discovery of active BAT in human adults, there exists a potential avenue which could be therapeutically exploited to elevate from the symptoms of obesity and protect against metabolic syndrome (Fig 1.4). To confirm the identity of BAT studies have conducted immunohistochemical staining for UCP1 on tissue samples extracted from the cervical-supraclavicular region and were successful in identifying immunopositive, multilocular adipocytes [222].

Figure 1.4.The main locations of brown and white adipose tissues and areas most commonly associated with disease risk.

A) VAT surrounds organs and can be divided in omental (b), mesenteric (c), retroperitoneal ((d): surrounding the kidney), gonadal ((e): attached to the uterus and ovaries in females and epididymis and testis in men), and pericardial (f). The omental depot starts near the stomach and spleen and can expand into the abdomen, while the deeper mesenteric depot is attached to the intestine. The gluteofemoral adipose tissue (g) is the SAT located to the lower-body parts and is measured by hip, thigh, and leg circumference. WAT can also be found intramuscularly (h). Brown adipose tissue is found above the clavicle ((i): supraclavicular) and in the subscapular region (j). Although the mentioned subcutaneous and visceral adipose tissues are found in humans, depots (d) and (e) are mostly studied in rodents. (B) The adipose tissue depots that have been linked to risk of developing obesity-related diseases are indicated in red.

1.7.5. Skeletal muscle

The benefits of retaining muscle mass throughout life have long been recognised; as well as facilitating mobility and mechanical strength, skeletal muscle functions as a metabolic furnace increasing its rate of energy consumption in situations where explosive contractions are required [223]. Furthermore, abundant evidence points to a key role of altered muscle metabolism in the genesis, and therefore prevention, of many common pathologic conditions and chronic diseases [224]. Because the amount of energy consumed by muscle tissue can vary considerably due to exercise stimulated hypertrophy, increased muscle mass protects against the development of obesity and associated co-morbidities, by altering energy homeostasis in favour of energy expenditure [224].

The age-associated loss of muscle, termed sarcopenia is usually accompanied with an increase in adipose tissue mass, as metabolic rate drops and the ability to engage in physical exercise diminishes and perfectly demonstrates the vital role of skeletal muscle in contributing to energy expenditure and safeguarding against the development of obesity [225, 226]. In support of this notion is a study conducted by Berit Heitmann who associated thigh circumference, as an indirect measure of lower body muscularity with an increased risk of cardiovascular disease and coronary heart disease; in a study comprising of 1436 men and 1380 women [227]. A more recent study published in 2015 by Hamasaki *et al*., investigated correlations between lower extremity muscle mass to body weight ratio (L/W) and the ratio of lower extremity muscle mass to upper extremity muscle mass (L/U) with metabolic parameters related to obesity [228]. The authors conclude, both high L/W and L/U ratio were indicative of improved metabolic parameters related to obesity including systolic blood pressure, triglyceride, high-density lipoprotein cholesterol, insulin resistance, and waist circumference [228]. Muscular strength quantified by one-repetition maximal measures for leg and bench presses, inversely associated with metabolic syndrome incidence, independent of common confounding factors such as age and body size, in a follow up study recruiting 3233 men between the age of 20-80 [229].

The molecular mechanisms responsible for fat free mass mediated protection from obesity are complex and involve cell signalling pathways governing oxidative stress resistance, anti-inflammatory cytokine production and enhanced GLUT4 mediated glucose uptake and glycogen storage, all of which have been associated with the development if not the progression of obesity and associated health implications [229].

1.8. The role of Adipokines and myokines in obesity.

Adipokines are factors produced and secreted by adipocytes and mykoines are factors produced and secreted by myocytes [230]. These factors can be peptide or non-peptide in structure [230]. Several studies have shown that the secretion of adipokines and myokines can influence the development and progression of disorders stemming from obesity including T2D and CVD [178]. To date, more than 300 adipokines have been identified, a list far too extensive to consider in detail and therefore those adipokines most influential in obesity will be discussed [178]. Considerably fewer myokines have been identified and the current global hypothesis is that myokines play a role in protection against the diseasome of physical inactivity [230].

1.8.1. Adipokines

1.8.1.1. Leptin.

Leptin is the product of the *ob* gene and is predominantly produced by adipose tissue, playing a role in satiety and body mass regulation as discussed previously [231, 232]. Through interactions with its receptor in the hypothalamus (via Janus kinase (JAK)-signal transducers and activators of transcription (STAT)), leptin offers protection against the negative health implications associated with increased adiposity i.e. insulin resistance [232]. Leptin activates, 5'-activated protein kinase (AMPK) protein kinase, an intracellular signalling molecule capable of inhibiting acetyl-CoA carboxylase, thus reducing malonyl-CoA concentrations and decreasing lipogenesis

[233]. However the observed protective role of leptin is rendered defective in obese humans due to leptin desensitisation [233]. Leptin plasma concentration and mRNA expression in adipose tissue are directly related to obesity severity [234]. Reviews by Rabe *et al.*, discuss similar observations in obese rodents which demonstrated insulin resistance, as a result of attenuation of leptin sensitivity in the brain, leading to excess triglyceride accumulation in muscle, liver and the pancreas [235]. Leptin has also been associated with inhibiting insulin's interactions with insulin receptors on adipocytes. A potential mechanism behind this observation may be due to at the genetic level, the leptin receptor and insulin response loci appear to be closely co-localised on chromosome 1 [236].

With high leptin levels down-regulating the receptor gene and diminishing the acute insulin response, this may in turn have implications for insulin sensitivity [236]. Hyperleptinaemia has been associated with pro-inflammatory responses in obese adipose tissue, the proposed mechanism recognises leptin's ability to regulate TNF-α production and macrophage recruitment [237]. Leptin has also been reported to trigger monocyte chemoattractant protein (MCP)-1 expression, and endothelial cell proliferation and migration [238]. In conclusion hyperleptinemia exhibits deleterious effects in obese individuals and facilitates the pathophysiology of obesity [237].

1.8.1.2. Adiponectin

Adiponectin is the most abundant adipokine secreted by adipose tissue. Interestingly, adiponectin is found in lower concentrations in the circulation of obese individuals yet is found excessively in lean, fit individuals **[239]**. Adiponectin has been identified as having anti-diabetic properties, promoting insulin sensitivity among other beneficial effects **[240]**. Adiponectin's role in glucose metabolism is mediated by adiponectin receptors, adipoR1 expressed ubiquitously and adipoR2 predominantly found in the liver **[241]**. Interactions between adiponectin and adipoR2 in the liver, results in a cascade of molecular interactions beginning with the activation of AMPK resulting in

down-regulation of genes encoding gluconeogenic and lipogenic enzymes and molecules **[241]**. AdipoR2 expression has been noted to increase glucose uptake in the liver by up-regulating glucokinase **[241]**. Essentially adiponectin decreases insulin resistance by decreasing hepatic triglyceride content and glucose production, while in muscle contributes to fatty-acid combustion and energy dissipation **[242]**. Fu *et al*., further support adiponectin's role in insulin sensitivity, by reporting adiponectin's ability to stimulate glucose uptake, through increased glucose transporter 4 (GLUT4) gene expression and increased GLUT4 recruitment to the plasma membrane **[243]**. In addition to the insulin sensitising effects adiponectin is also vascular-protective and inhibits the development of atherogenic processes by down regulating the expression of cellular adhesion molecules and by exhibiting anti-TNF-α effects **[243]**. However adiponectin circulates in plasma as a low-molecular weight trimer, a middle-molecular weight hexamer, and high-molecular weight 12- to 18-mer, and these forms were postulated to differ in biological activity **[244]**.

1.8.1.3. Other adipokines with metabolic influences

The increased secretion of TNF-α from adipocytes is a reflection of the increased infiltration of macrophages and lymphocytes, rather than an enhanced rate of production [245]. Nevertheless TNF-α is pivotal in the manifestation of insulin resistance; by atypical phosphorylation of IRS-1 [246]. Evidence supporting such claims stems from studies which have deleted TNF-α and/or TNF-α receptors and reported vast improvements in insulin sensitivity in both high fat fed mice and leptin deficient *ob/ob* mice [247]. Furthermore studies conducted in humans conclude adipose tissue TNF-α expression correlated with BMI, percentage of body fat, and hyperinsulinemia, whereas weight loss decreased TNF-α levels [235]. Increased IL-6 concentrations have been reported to reduce insulin-dependent hepatic glycogen synthesis and glucose uptake in adipocytes [235]. More recent evidence has outlined the role of IL-6 in cancer progression, IL-6 was seen to have roles in tumour microenvironment regulation and production of breast cancer stem cell-like cells and may be the link between obesity and tumour progression [248]. Mice administered the

recombinant adipokine resistin exhibit deregulated glucose homeostasis in hepatocellular and myocellular tissues and neutralisation of resistin resulted in increased insulin sensitivity [249]. *In vitro* studies conducted in adipocytes reveal resistin reduces glucose uptake in response to insulin, anti-resistin antibodies produced an opposing effect [249]. Resistin knockout mice fed a high fat diet develop obesity and insulin resistance, however exhibit reduced fasting glucose in comparison to matched-weight controls [250, 251]. Visfatin is an adipokine secreted predominantly from visceral fat stores and correlates significantly with obesity and insulin resistance [252]. Surprisingly visfatin mimics the actions of insulin and can induce glucose uptake by both adipocytes and myocytes and can inhibit gluconeogenesis in the liver and highlights the potential benefits of visceral adipose tissue, although literature often present contrasting views [253]. Like insulin visfatin regulates glucose uptake via the insulin transduction pathway and even binds to insulin receptors with the same affinity but at a differing site [253]. Increased visfatin production from visceral fat may function as a compensatory mechanism to declined beta-cell function and that obese individuals experience visfatin resistance [254].

1.8.2. Myokines.

1.8.2.1. Irisin

Bostrom *et al* recently identified Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) induced overexpression of the Fibronectin domain-containing protein 5 (FNDC5) gene following aerobic exercise in mice [255]. The FNDC5 gene encodes a type 1 membrane protein that is proteolytically cleaved and secreted into the circulation, and can bind undetermined receptors on white adipocytes [256]. Irisin is thought to be a myokine, and is referred to as such in current literature, however there is a paucity of evidence demonstrating its release [257]. Irisin has been reported to manifest an anti-obesity effect by imposing a BAT phenotype upon WAT leading to increased energy expenditure via thermogenesis [258]. This potential manipulation of cellular energy balance therefore has the potential to induce a calorie

restriction (CR) like state via modulation of WAT function, as excess energy would be dissipated as heat upon exposure to irisin [259]. Irisin has also been reported to reduce fasting glucose concentrations and improve insulin sensitivity in both murine and human models, potentially mitigating the detrimental effects of obesity and T2D [260]. Although the precise mechanisms behind this observation are not completely understood, the ability of irisin to increase expression of UCP1 and thus cause a 'browning' shift in WAT may be likened to caloric restriction. Irisin may therefore impact the ageing process, by releasing stored energy through non-shivering thermogenesis and thus mimicking CR mechanisms, which have been proven to promote longevity [261]. Irisin administration has therefore been proposed to be a potential future therapeutic target that may function to protect against age associated metabolic disorders. By creating a calorie deficit it could be speculated that irisin may also promote longevity by the same mechanism [16]. However there remains a significant amount of confusion regarding firstly the source of irisin secretion and secondly the role of irisin in individuals suffering from metabolic complications. In addition to production by muscle, irisin release has been observed from adipose tissue, suggesting that irisin is an adipokine as well as a myokine [262, 263].

Evidence of a positive correlation between irisin and BMI has been reported [264] whilst others have found either no association [265] or a negative association [266]. As in muscle, irisin release from WAT may be stimulated by exercise training and is reduced in fasted animals (12). The role of irisin in diabetes is controversial, with initial reports suggesting that circulating irisin levels in T2D may be lower than in healthy individuals [267, 268]. However, studies performed in obese individuals, some of whom had T2D, have reported elevated levels. Most recently, published data have suggested that irisin may also have a role in atherosclerotic cardiovascular disease [269] and could predict cardiovascular disease risk [270]. This finding is of importance as individuals with T2D are at an increased risk of death by cardiovascular disease [271]. As irisin is a potential target for the treatment of obesity, further appreciation of the role of irisin in obesity and diabetes is required.

1.8.2.2. Other myokines with metabolic influences

The neurotrophin BDNF and its receptor, the Tyrosine receptor kinase B (TrkB) are most widely expressed in the brain [46]. Increasing evidence suggests that BDNF may be influential in regulating central metabolic pathways but as a regulator of skeletal muscle [272]. Interestingly low levels of circulating BDNF are also found in obese and T2D individuals [273]. Farooqi highlights BDNF is a key modulator of hypothalamic pathways governing body composition and energy homeostasis and more specifically report BDNF levels enhance glucose utilisation in skeletal muscle [274]. Such results may explain molecular mechanisms behind the benefits of exercise which are still largely unknown. Exercise stimulated IL-15 release offers great resistance against the development of obesity/T2D and is even implemented in therapeutic approaches against metabolic disorders [275]. IL-15 exhibits its effects, by modulating both skeletal muscle structure and function (Fig. 1.5) [276].

Furthermore IL-15 has been reported to regulate glucose homeostasis by upregulating GLUT4 transporters and increasing glucose uptake in C2C12 muscle cells [277]. Both resistance training and aerobic exercise have been correlated to increased IL-15 concentrations, in mice and human studies and have been associated with a reduction in visceral adipose tissue [276, 278]. While IL-6 secretion from adipocytes is deleterious in nature, IL-6 secretion from myocytes exhibits beneficial effects including anti-inflammatory effects [279]. Generally exercise stimulates the production and secretion of anti-inflammatory cytokines, an effect which can be evoked with recombinant IL-6 treatment *in vitro* [280]. IL-6 can reduce levels of TNF-α and up regulate production of anti-inflammatory cytokines IL-1ra and IL-10 [281]. Mechanistic studies reveal IL-6 facilitates glucose uptake via GLUT4 translocation and fat oxidation via increased activation of AMPK and/or phosphatidylinositol 3-kinase (PI3-kinase) [282]

Figure 1.5. The various roles of exercise induced myokines and their metabolic consequences.

IL-4, IL-6, IL-7, IL-15 and LIF promote muscle hypertrophy. Myostatin inhibits muscle hypertrophy and exercise leads to liver secretion of the myostatin inhibitor follistatin. BDNF and IL-6 are involved in AMPK-mediated fat oxidation, IL-6 stimulates lipolysis and IL-15 stimulates lipolysis of visceral fat. IL-6 also enhances insulin-stimulated glucose uptake and stimulates glucose output from the liver, but only during exercise. IL-6 also increases insulin secretion by inducing the expression of GLP-1. IL-6 has anti-inflammatory effects as it inhibits TNF production. IGF-1, FGF-2 are involved in bone formation, and follistatin-related protein 1 (FSTL-1) improves endothelial function and revascularisation of ischaemic blood vessels. Irisin has a role in 'browning' of white adipose tissue [283].

1.9. Type 2 diabetes (T2D)

The prevalence of T2D, a co-morbidity of obesity, is increasing worldwide. T2D is a preventable but incurable disease that affects approximately 2.9 million people within the UK, and has been associated with significantly reduced lifespan [284]. T2D is a chronic metabolic disorder traditionally associated with older adults, although younger individuals are being diagnosed with increasing frequency [285]. T2D can be defined at its simplest as a condition that leads to the presence of elevated blood glucose levels [285]. This increase in blood glucose is usually caused by a combination of insulin resistance, altered endogenous glucose output and failure of the endocrine portion of the pancreas to compensate for this change in insulin sensitivity [286]. Like obesity, T2D often develops due to a combination of environmental risk factors and genetic predisposition [287]. There is sufficient evidence to declare obesity and the sedentary lifestyle and calorific diet that often accompany it, as the principle risk factor for developing T2D [285, 286]. The close association between obesity and T2D has led to use of the term, “diabesity”, as more than half of T2D patients are diagnosed with obesity prior to diagnosis of T2D itself, therefore adipose tissue should play a crucial role in the onset of T2D.

1.10. Aetiology of T2D.

The aetiology of T2D involves a series of complex interactions between a number of factors, including arguably the most influential to the progression of T2D, leading a diabetogenic lifestyle. This lifestyle comprises reduced physical activity and excessive calorie consumption.

1.10.1. Genetics of T2D.

Genome wide association studies (GWAS) analysing SNP between cohorts of T2D volunteers and healthy volunteers have been successful in identifying 36 genes associated with an increased risk of developing T2D, a list far too extensive for the purpose of this study. The strongest

associations are currently found for gene variants in the loci of transcription factor-7– like 2 (TCF7L2), initially identified in an Icelandic population, and potassium channel, voltage gated KQT-like subfamily Q, member 1 (KCNQ1), first described in Asian populations with subsequent replications in many other cohorts [288, 289]. *TCF7L2* gene was identified in 2006 by Grant *et al*., and is recognised as the strongest genetic contributor to T2D across all racial groups [290]. Carriers of one risk allele of the SNPs rs7903146 have an approximately 40% higher T2D risk than homozygous carriers of the protective allele [290]. The precise genetic defect that causes the association of *TCF7L2* with T2D remains unclear, although silencing *TCF7L2* expression by siRNA correlates with reduced insulin output whereas overexpression stimulated insulin secretion in both human and mice islets [291]. It is therefore clear that the effect of the TCF7L2 risk allele is closely related to a reduction in insulin secretion. Tong *et al*., conducted a meta-analysis, studying the data from 25 publications involving 35,843 cases of T2D and 39,123 controls, compromising of Caucasians, North Europeans, East Asians, Indians, and Africans [292].

The results from their study revealed four variants of *TCF7L2* gene which are all associated with T2D, and indicates a multiplicative genetic model for all the four polymorphisms, as well as suggests the *TCF7L2* gene is involved in near 1/5 of all T2MD [292]. The *KCNQ1* gene codes for the pore-forming alpha subunit of a voltage gated potassium channel that is expressed in a number of tissues, notably, the heart, pancreas, kidneys and intestine [289]. Yasuda *et al*., conducted a multistage GWAS study in 1,612 Japanese individuals with T2D and 1,424 normally healthy controls, analysing a total 100,000 SNPs [293]. The authors report the most significant association was obtained with SNPs in KCNQ1, and dense mapping within the gene revealed that rs2237892 in intron 15 showed the most significance [293]. Interestingly the authors found significant correlations with the KCNQ1 risk allele and impaired lipid metabolism with decreased HDL and increased LDL along with higher levels of total cholesterol [293]. Ohshige *et al*., have

reported positive correlations between diabetic neuropathy and KCNQ1, however this observation requires further validation [294].

1.10.2. Obesity.

Existing research has identified obesity as a potent risk factor for the development of T2D and it has been estimated that obesity accounts for 85 percent of the overall risk of developing T2D [295]. Therefore factors facilitating the development of the obese phenotype, including a sedentary lifestyle and the excessive consumption of calorific foods are central to the manifestation of T2D [296]. Studies have reported individuals homozygous for the FTO gene, characteristic of individuals genetically predisposed to obesity often present with impaired glucose homeostasis and aggressive insulin resistance [296]. Reasons behind the increased incidence have been discussed previously in great deal. In summary, the recent adoption of the Western diet globally consisting of increased consumption of processed foods, high in trans/saturated-fats, salt and sugar in conjunction with the implementation and utilisation of time saving/wasting technologies has resulted in energy imbalance in favour of energy retention as opposed to expenditure [295, 297]. The net effect leads to increased adipose tissue, leading to β-cell damage and impairment of insulin receptor signalling [298].

1.11. The pathophysiology of T2D.

The reduced ability to produce insulin and a reduction in insulin sensitivity is routinely diagnosed in patients suffering from T2D [299]. An area of much controversy is which is more influential and which manifests first. Understandably a difficult predicament considering both defects are invariably present in individuals diagnosed with T2D. Initial studies trying to identify the root of T2D sought to investigate a group of indigenous Americans, also known as Pima Indians; recognised for being highly susceptible to developing T2D [300, 301]. The conclusions drawn from such studies identified a marked increase in insulin resistance with little or no significant

change in insulin output. At which point insulin resistance was recognised as the primary defect in T2D [301, 302]. However succeeding work implemented advanced research tools and conducted studies highlighting a falling insulin secretory response following an oral glucose test and up to 60% decreased postprandial insulin output [303, 304]. It is understandable that beta-cell dysfunction plays an imperative role in the manifestation of T2D, considering the precise mechanisms regulating normoglycemia [305]. To summarise, the current concept is both beta-cell dysfunction and insulin resistance occur very early in the course of T2D long before blood glucose values reach a level that is defined as prediabetes.

1.11.1. T2D: β-cell dysfunction

The pancreas is an organ with dual function and consists of two types of parenchymal tissue [306]. The islet of Langerhans exhibit endocrine function and produce various hormones, while the acinar cells display exocrine function and secret digestive enzymes [307]. Islets contain five different endocrine cell types, (alpha, beta, delta, pp and epsilon cells) which modulate changes in plasma nutrient levels with the release of a carefully balanced mixture of islet hormones into the portal vein [308, 309]. The pancreatic β-cells make up the majority of the islet of langerhans and are responsible for producing insulin, the major hormone responsible for carbohydrate, fat and protein metabolism [310]. Pancreatic β-cell dysfunction, thus insulin deficiency has been an area of research interest for many years, since it has been recognised as an imperative risk factor for the development of T2D [311]. The United Kingdom prospective diabetes study highlighted the significance of β-cell impairment in the development of T2D, by reporting β-cell dysfunction at least 10 years before the onset of hyperglycaemia, confirming its role in the pathophysiology of T2D [312]. Blood glucose levels rise post-prandially and glucose enters the β-cell across the plasma membrane via its respective transporter (GLUT-2) and is then a substrate for the rate limiting step in glucose induced insulin secretion, glucokinase [313]. This means that glucose metabolism is mainly aerobic allowing glycolytically produced pyruvate to enter the mitochondria to fuel ATP production via oxidative phosphorylation [314].

The ATP produced via this pathway increases the ATP/ADP ratio within the cytosol which leads to closure of ATP-sensitive K^+-channels in the plasma membrane [314]. Closure of K^+ channels depolarises the plasma membrane and activates cyclic adenosine monophosphate (cAMP) along with specific catalytic subunits of protein kinase A (PKA) facilitating the opening of the mitochondrial permeability transition pore, a multi-protein complex, which functions as a mitochondrial $Ca2^+$ release channel thus aids exocytosis of insulin containing vesicles [314-316]. This process has been recognised defective in individuals with T2D (Fig.1.6).

1.11.2. Contributors to T2D β-cell dysfunction.

The cellular mechanisms contributing to β-cell dysfunction have mostly been investigated *in vitro* due to the increased difficulty to obtain primary cells from humans. However despite experimental drawbacks several theories have been proposed to be potentially influential in the manifestation of β-cell dysfunction.

Figure 1.6. A simplified representation of the mechanism behind glucose mediated insulin secretion, from pancreatic-β cells.

A molecule of glucose is rendered into pyruvate via glycolysis and is shuttled in the mitochondria, directly into the Krebs cycle, yielding ATP molecules which facilitate the closure of ATP sensitive K^+ channels. The net effect leads to the depolarisation of the plasma membrane activating cAMP along with specific catalytic subunits of PKA facilitating the opening of the mitochondrial permeability transition pore, a multi-protein complex, which functions as a mitochondrial Ca^{2+} release channel thus aids exocytosis of insulin containing vesicles [317]

1.11.3. Glucotoxicity

Glucotoxicity in the context of T2D is recognised as the deleterious nature of chronic hyperglycaemia on β-cell function and insulin action [318]. *In vitro* studies investigating the effects of chronically elevated glucose levels often report an associated increase in ROS and a subsequent increase in cytoplasmic DNA fragmentation, increased expression of pro-apoptotic proteins (Bax) relative to anti-apoptotic proteins (Bcl-2) and significant alterations in mitochondria morphology and volume [319]. Several glucose related pathways have been identified which result in the increased production of ROS including enhanced autoxidation, oxidative phosphorylation, glycosylation, and the glucosamine pathways [318]. Separately hyperglycaemia-induced mitochondrial superoxide activates UCP2-mediated proton leak, thus lowering ATP levels and impairing the closure of K^+ channels and preventing depolarisation of plasma membranes, thus inhibiting glucose-stimulated insulin secretion [320]. The addition of antioxidant molecules abrogates the apoptotic effects of excess glucose and restore insulin secretion from β-cells [320].

Existing research has elucidated individuals suffering from T2D exhibit a metabolic shift towards dysregulated nutrient homeostasis and a compensatory increased flux through the hexose biosynthetic pathway, resulting in increased levels of intra and extracellular glucosamine [321]. Glucosamine levels have been correlated with impaired activation of the insulin receptor and the net result leads to impaired phosphorylation of IRS proteins [318]. The endoplasmic reticulum (ER) is responsible for completing fundamental processes governing the production of the mature form of insulin, following endopeptidase mediated excision of the C peptide domain on molecules of proinsulin [320]. Glucotoxicity induces ER stress, identified by the increased quantification of x-box protein 1 and heat shock protein 5 in islets from T2D patients; potentially decreasing insulin production and Golgi apparatus mediated exocytosis [318]. Data from the Weir laboratory shows profound effects of hyperglycemia to change the transcriptional pattern of a multitude of beta-

cell genes termed beta-cell dedifferentiation, the effects of which remain to be fully understood [322].

1.11.4. Lipotoxicity.

The lipotoxicity associated with excess adiposity has been discussed previously and is fundamental in the pathogenesis of obesity, likewise lipotoxicity plays an integral role in the pathogenesis of T2D; β-cell function and is just one mechanism connecting obesity to T2D. Chronic exposure of pancreatic β-cells to FFA, as a result of poor dietary regulation of fat intake and the consequent fatty acid spill over effect elicits multiple mechanisms of toxicity, including accumulation of malonyl–CoA and long-chain fatty-acyl-CoA in β-cells and increased fatty acid oxidation resulting in an increased generation of ROS [318]. Murine islets subjected to high FFA treatment exhibit decreased expression of mRNA coding for fatty acid oxidation promoters, including peroxisome proliferator–activated receptors, carnitine palmitoyl transferase 1 and acyl CoA oxidase whilst mRNA expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), acetyl CoA carboxylase and fatty acid synthase, the transcription factors for lipogenic enzymes are upregulated [320, 323]. Mouse models of T2D also exhibit increased expression of pro-apoptotic genes and caspase activity in a similar fashion to hyperglycaemia induced cell death [319].

Direct mechanisms relating elevated FFA to β-cell death involve the increased hydrolysis of sphingomyelin and de novo production of ceramide, two conditions which arise in times of triglyceride excess [324]. Ceramide can induce apoptosis by activating NFκB, which upregulates the expression of nitric oxide synthase [324]. The increased formation of nitric oxide forms peroxynitrite, capable of disrupting mitochondrial depolarisation and actively upregulating apoptotic and necrotic pathways. Furthermore studies have demonstrated both ceramide and peroxynitrite are capable of inducing cellular senescence, another potential mechanism behind β-cell dysfunction [325]. The work of S. Del Prato recognises an equal contribution of both

glucotoxicity and lipotoxicity. However a high glucose level must be present for production of malonyl-CoA, which is required in sufficiently large amounts to promote adipogenesis and inhibit fatty acid oxidation. Otherwise, the excess fatty acids would be oxidized, and thus detoxified [319].

1.11.5. β-cell exhaustion.

Chronic insults leading to β-cell dysfunction/loss are met with a decrease in insulin secretion and hyperglycaemia [326]. Following significant β-cell damage, β-cells are able to compensate by enhancing cell replication, neogenesis, hyperplasia and hypertrophy with the aid of external stimuli including hormones, growth factors and glucose levels [326]. T2D mice often display an enlarged pancreas, specifically β-cell mass, verifying the role of excessive β-cell proliferation in T2D [327]. However β-cell compensation eventually leads to β-cell exhaustion; defined as gradual decline with the progression of diabetogenic effects [319]. This decline can be categorised into specific phases, beginning with stable adaptation which is characterised a decrease in glucose stimulated insulin secretion as well as morphological alterations [319]. Furthermore stable adaption is met with a reduction in key enzymes for glucose metabolism and an increase in enzymes governing gluconeogenesis and lactate production, along with increased expression of pro-apoptotic genes and transcription factors modulating inflammatory responses [319].

The next phase of β-cell exhaustion, unstable early decompensation, is met with a further decrease in β-cell mass/function and is met with a consequential increase in glucose and in a feedback loop further facilitates β-cell deterioration and reduced insulin output [328, 329]. As insulin mRNA falls with increasing hyperglycaemia, there is evidence that insulin biosynthesis becomes rate limiting for secretion [328]. However regular exercise and calorie control can reduce the extent of β-cell damage and patients are able to return to stable adaption [329]. Weir & Bonner state that

decompensation is the final stage of β-cell exhaustion and presents with hypotrophy and hypoplasia resulting in minimal insulin secretion. Murine studies reveal considerable β-cell dedifferentiation in correlation with hyperglycaemia. Morphometric studies on post-mortem pancreases of patients with T2D provide convincing evidence that β-cell mass is reduced to less than 50% of that of control subjects due to a combination of increased apoptosis, decreased neogenesis and proliferation [329].

1.11.6. T2D: Insulin resistance.

The binding of insulin to the insulin receptor induces autophosphorylation at several tyrosine residues located inside the cell, resulting in the activation and mobilisation of PI 3-kinase to the plasma membrane bringing it in the vicinity of its physiological substrate phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P2) which it phosphorylates to generate PtdIns(3,4,5)P3 [330]. The chain of events then leads to the activation of protein kinase B (PKB) and the subsequent phosphorylation of PKB at Thr308 and Ser473, mediated by by the protein kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1) [331, 332]. PKB is a vital regulator of glycogen synthesis, and does so by phosphorylating glycogen synthase kinase-3 (GSK3), which is inactivated following its phosphorylation by PKB [333]. Inactivation of GSK3 by PKB results in dephosphorylation of glycogen synthase through the action of protein phosphatases and hence the activation of glycogen synthesis. GSK3 also phosphorylates and inhibits a guanine nucleotide exchange factor, eIF2B that controls the initiation stage of protein translation [333, 334]. Insulin induces the dephosphorylation of eIF2B at the site phosphorylated by GSK3, thereby stimulating the synthesis of protein from amino acids [335]. Thus insulin-dependent inactivation of GSK3 underlies the insulin induced synthesis of glycogen and protein [335]. Relevant to the role of glycose uptake is the PKB mediated activation of PI 3-kinase which stimulates the recruitment of GLUT4 to the cell [336]. This process is rendered defective in T2D giving rise to insulin resistance, below are the main contributors identified by current research.

The first study to suggest a primary role for elevated FFA in the development of insulin resistance was conducted in 1963 by Randle *et al* [337]. Randle's hypothesis was supported by different studies, it manifested that insulin resistance can be induced within hours through lipid infusion or weeks through a high fat feeding regimen. Increased intake of dietary fat results in increase FFA oxidation resulting in increased ratio of mitochondrial acetyl-CoA to pyruvyl-CoA [337]. The net effect is an increase in citrate concentration and the accumulation of glucose 6-phosphate that results in inhibition of hexokinase the rate limiting enzyme regulating glycogen synthesis and the glycolytic pathway [337]. FFA induce NFκB activation, highlighting the involvement of inflammatory cytokines in propagating insulin resistance [337, 338]. Inflammation and oxidative stress present as by-products of glucolipotoxicity, makers of both are increasingly found in obese individuals suffering from T2D [339]. Mitochondrial production of hydrogen peroxide (H_2O_2) is a considerable source of oxidative stress in T2D individuals as is increased NADPH-derived superoxide production [340]. The inflamed phenotype is more closely related to the retention of exceeding amounts of adipose tissue [339, 340]. Various authors have reported that inflammatory cytokines impede insulin signalling by facilitating phosphorylation of serine residues on IRS-1 [341, 342]. Likewise oxidative stress hinders insulin response in a similar manner, however at smaller doses for short periods of time H_2O_2 has been reported to augment glucose uptake *in vitro* [341, 342].

Studies implementing NMR technology have verified a significant loss of functional mitochondria amongst patients suffering from T2D and a causative factor behind ectopic fat accumulation in muscle and liver; potentially due to the inability to oxidise metabolic substrates and produce ATP [343]. The reason for this defect may be genetic as T2D individuals exhibit decreased expression of peroxisome PPAR-γ and Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [343]. Microarray studies have supported such claims and report reduced expression of PGC-1-responsive genes in obese Caucasians with impaired glucose tolerance and T2D [344]. These data support the idea that insulin resistance in humans might arise from defects in mitochondrial function, which in turn lead to increases in intracellular FA

metabolites (fatty acyl-CoA and diacylglyerol) that disrupt insulin signaling in the muscle as well as the liver as discussed previously [344].

1.12. The epidemiology of T2D.

Much like initial concerns regarding rising obesity levels, T2D was once considered a metabolic disorder rising in Western civilisations only. Understandably with increasing incidence of obesity globally, a consequential increase in T2D has also been observed. Unlike obesity, health implications associated with T2D are a larger cause of concern. Generally, the injurious effects of hyperglycemia are separated into macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) [345]. Therefore it is of great necessity that T2D is diagnosed before the onset of disorders affecting both health and lifespan. Currently an effective diagnostic framework exists which is also responsible for the increased incidence rate [346].

Follow up tests usually involve a fasting glucose test, during which a blood sample is drawn pre and post a glucose drink [347]. Non-diabetics will generally have less than 6 mmol/l before the test and less than 7.8 mmol/l two hours after the test, while diabetic will have more than 7 mmol/l before the test and more than 11 mmol/l two hours after the test [347]. Tests analysing levels of glycated haemoglobin (HbA1c) are more accurate and provide an insight into glucose homeostasis over the course of 2-3 months [348]. An HbA1c level of 6.5% (48mmol/mol) or above indicates T2D an HbA1c level of 6-6.4% (42-47 mmol/mol) would indicate that a person has a high risk of developing diabetes [347, 349].

1.12.1. Global trends in T2D.

Statistics describing the incidence of T2D, derived from the International Diabetes Federation (IDF) estimate T2D incidence for adults between the ages of 20 and 79 worldwide for 2013 was 382 million and it is expected to affect 592 million people by 2035 [350]. According to the WHO 90 percent of all diabetes cases in the world are attributed to T2D and 175 million people are thought to have undiagnosed T2D [350]. The IDF report that India, China, the United States, Indonesia, Japan, Pakistan, Russia, Brazil, Italy and Bangladesh all harbour in excess of 10 million people suffering from T2D and are at the forefront of the T2D epidemic [350]. With the increasing adoption of the Western lifestyle many emerging economies are now exhibiting increased prevalence of T2D [351, 352]. The ten countries with the highest prevalence rates of T2D, relative to population size include Tokelau, Micronesia, Marshall Islands, Kiribati, Cook Islands, Vanuatu, Saudi Arabia, Nauru, Kuwait and Qatar [351-353].

The greatest percentage increase in rates of diabetes will occur in Africa over the next 20 years. Unfortunately, at least 80% of people in Africa with diabetes are undiagnosed. In general, global trends display an increased rate of T2D in urban areas (64%) in comparison to rural areas (36%) and may be reflection of lifestyle factors in such areas [353, 354]. Since 2013 more than 3.2 million people have been diagnosed with T2D in the UK, roughly equating to 1 in 17 people, while 850,000 people remain undiagnosed [353]. A longitudinal study conducted by Holden *et al*., monitored T2D incidence over the course of 20 years in the UK and reveals in 1991 there existed 169 people with T2D per 100,000 persons per year and since then has increased to a little more than 3-fold to 515 people per 100,000 a year [355]. It has been predicted that by 2025 5 million people will be suffering from T2D in the UK [355].

1.12.2. Age-related increased susceptibility to T2D.

Advancing age presents as a considerable risk factor in the development of T2D and therefore T2D is recognised as an age-related metabolic disorder [356]. Taking the demographics from the USA as an example in 2011 63% of the adult incident cases of diabetes were diagnosed between the ages of 40 and 64 years [357, 358]. About 16% were diagnosed at age 18–39 years, and about 21% were diagnosed at age 65–79 years [357, 358]. Guidelines delivered by the NHS outline individuals over the age of 40 are at increased risk for developing T2D and attribute this risk to the decrease in physical activity, reduction in muscle mass and poorly maintained nutrient homeostasis in conjunction with rising obesity rates, often presenting with advancing age [359]. However, the incidence of T2D is increasing more rapidly in adolescents and young adults than in other age groups [360]. The disease is being recognised increasingly in younger persons, particularly in highly susceptible racial and ethnic groups and the obese. In some areas, T2D is more prevalent amongst pre-pubertal children, teenagers, and young adults, in comparison to type 1 diabetes (T1D) [360].

1.12.3. Ethnicity and T2D.

The prevalence of T2D varies substantially within ethnic groups and is not restricted by geographical isolation, instead exhibiting a strong trend worldwide. To understand the role of ethnicity in T2D, populations residing in the USA and UK provide a valid model due to enormous ethnic diversity. A recent study conducted by Cox *et al*., collected data from 335 general practices in England and Wales and report a fivefold variation in risk of T2D existed between different ethnic groups compared with the white reference group [361]. More specifically the results identified Bangladeshi, Pakistani, Indian and African males were at an increased risk of T2D in comparison to the white reference group [361]. Recent statistics from the Centre for Disease Control (CDC) describe T2D as being more prevalent amongst Native Americans, African Americans, Hispanics, and Asian Americans than among Caucasians in the USA [362]. Asian

Americans have a nine percent higher risk of diabetes [363]. Hispanics have a 12.8 percent higher risk, and non-Hispanic blacks have a 13.2 percent higher risk of diabetes than non-Hispanic white adults in the USA [364]. These data mirror the strong links between ethnicity and obesity, and in fact may be a reflection of increased incidence of T2D in the same ethnic groups, the precise reasons predisposing certain ethnic groups to T2D remain elusive. The influence of genetics, cultural differences and lifestyle factors such as diet are all potential contributors to disease risk.

1.12.4. Gender disparities in T2D.

Initial research suggested females are at a larger risk of developing T2D in comparison to males. However as research progressed it became evident that more males are diagnosed with T2D [365]. For example, whilst the prevalence of diabetes was over 19% in men over 50 years of age by 2005 in a population-based study in Ontario, Canada, the corresponding prevalence for women was just under 16% [366]. A recent Korean study reporting data from 2005 showed T2D prevalence in above 30 year olds to be around 7.9% in women but 10.2% in men with the biggest differences in the 40–59 year olds, where, remarkably, male T2D prevalence was around double that in females [367]. Statistics collected in 2009 show that 2.4 per cent (around 92,960) of men in England aged 35-44 have diabetes compared to 1.2 per cent (around 47,000) of women of the same age [368-370]. It was found for a given BMI males retain more visceral adipose tissue relative to subcutaneous adipose (insulin sensitive) tissue in comparison to women [371]. It has also been documented that males, generally are more insulin resistance and exhibit elevated fasting blood glucose levels [371]. The role of sex hormones in this phenomenon may be a potential mechanism behind the observation as, increased testosterone has been associated with increased retention of central adipose tissue; on the contrary oestrogen has been reported to aid adipose re-distribution in favour of subcutaneous adipose storage and protecting against visceral fat accumulation [149].

1.12.5. Mortality in T2D.

T2D presents with a variety of disorder which can significantly reduce quality of life but also reduce lifespan. Individuals suffering from T2D in England and Wales are 37.5 percent more likely to die early relative to individuals free of disease [372]. The biggest contributor to increased mortality in T2D is CVD accounting for 52–80 percent of deaths, followed by renal disease 10–20 percent of mortality, and cerebrovascular disease 15 percent, which is approximately twice that seen in the population without T2D [373]. Nwaneri *et al*., conducted a meta-analysis of recently published reviews assessing mortality in T2D patients residing across Europe, USA, Canada and New Zealand, as well as Japan, Fuji, Argentina and Israel [374]. The authors report a two-fold increase in the mortality risks in type 2 diabetes when compared with the general population. Specific statistics from the UK reveal T2D contributed to 23,300 additional deaths in 2010-11 in England [374]. In 2010, diabetes was mentioned as a cause of death in a total of 234,051 death certificates. Statistics from the international diabetes federation declare 1 million deaths due to T2D in India during 2011-2012 [375]. In 2012 diabetes was the direct cause of 1.5 million deaths. Total deaths from diabetes are projected to rise by more than 50% in the next 10 years [376].

1.12.6. Financial burden of T2D.

T2D is associated with acute and chronic complications; combined contribute to an enormous financial burden, not only affecting patients and their families but entire financial budgets and healthcare systems. Taking the UK as an example, the total spending associated with diabetes equates to £9.8 billion, costs associated with T2D alone account for £8.8 billion, while the rest is attributed to type 1 diabetes. Approximately 80 percent of this amount is spent on dealing with co-morbidities and associated complications, (£7.7 billion) while the remainder is spent on treatment regimens (£2.1 billion) [377, 378]. The indirect costs, defined as loss of working hours and/or productivity due to ill health, associated with T2D in the UK is estimated to be £9 billion

[379, 380]. With vastly growing rates of obesity and therefore T2D total health care costs associated with T2D are projected to increase from £8.8 billion to £16.9 billion in 2035 [379, 380].

Statistics from the USA exhibit a similar trend; for the approximately 16.5 million people with T2D, the annual national cost is $159.5 billion attributed to healthcare related expenditures and $65 billion in reduced productivity [381]. Cost estimates for individuals diagnosed with T2D equate to $9,677, in America this diabetes burden represents a hidden tax in the form of higher health insurance premiums and reduced disposable income [381]. In India, a recent study showed that total annual expenditure by patients on diabetes care was, on average, Indian Rupee (INR) 10,000 (£100) in urban areas and INR 6,260 (£62) in rural areas [382]. An increase of 113% was observed in the total expenditure between 1998 and 2005 in the urban population [382]. A review by J. Singh reports the average expenditure on T2D care in India equates to 180,000 million INR. With little to no subsidies the majority of healthcare bills are paid from household incomes [383].

The incidence rate of metabolic disorders are generally lower in East Asia, in 2013 China spent $38 billion on T2D, 6 times less than USA, however prevalence is gradually increasing. In support of this statement is data reporting in 1993 the direct medical costs associated with T2D were $0.25 billion, accounting for 0.07% of the GDP [384, 385]. In 2008 the direct costs amassed to $8.65 billion accounting for 0.21% of the GDP. Hospitalisation stay for diabetes patients is two times longer than non-diabetics and is largely responsible for the increased cost [386]. It is apparent that there exists a substantial financial burden associated with T2D, sapping funds from healthcare departments worldwide and of a larger concern is the fact that such expenses can be avoided by cost effective preventative methods such as diet and exercise [387-389].

1.13. Ageing

1.13.1. The ageing crisis.

Most living organisms experience the inevitable biological process of ageing and in humans this can be defined as a multi-factorial progression into a physiologically inferior state, in comparison to their younger counterparts [390]. As muscle mass, bone density and the capability to withstand physiological stresses decline an inability to fight off infection rises as does vulnerability to various degenerative disorders [391]. Over the last decade most research in the field of ageing has focused on prolonging lifespan in lower organisms and preventing the onset of diseases that disproportionately affect an individual's age in human subjects [392-394]. National Statistics reveal that there has been a steady incline in the number of individuals reaching age 65 and beyond in the last 25 years within the UK. The percentage of the population aged 65 and over increased from 15 % in 1984 to 16 % in 2009, an increase of 1.7 million people. Statistical projections suggest a further surge in the number of elderly by 5½ million in the coming 20 years [395-398].

There are two integral contributors to population ageing; increased social/government pressure to have smaller families paralleled by increased longevity thus falls in mortality [399, 400]. Population ageing is a global phenomenon and is not limited to developed countries as the number of ageing individuals is growing in virtually all nations, supported by statistics derived from The US Census Bureau report, which stated the older population grew by an average of 870,000 people each month in 2008 worldwide [401, 402]. With decreasing birth rates and an ageing community the UK's independent population has significantly reduced, while the dependent population is increasing, accounting for increased expenditure on pensions, care homes and the most integral contributor the health services [398, 403]. The UK can expect to spend approximately £50 billion each year to provide services for its ageing population, (Office for National statistics, elder people 2011, and statistical bulletin). A report by Anderson and Hussey

estimates that the average cost of providing hospital and community health services for a person aged 85 years or more is approximately three times greater than for a person aged 65 to 74 years [404]. While enhancements in nutrition and drug development, disease screening, along with increasing scientific evidence and positive research outcomes, have achieved the goal of prolonging lifespan to a certain extent, it remains to be understood why certain individuals experience healthy ageing whereas others endure rapid physiological deterioration. Studies have outlined various factors, responsible for the onset of premature ageing amongst individuals, including physiological stress, genetics and environmental factors with no one aspect receiving unanimous support and in actuality may comprise of a particular factor which develops due to a combination of all 3.

1.13.2. Theories of ageing.

Research into the fundamental concept of ageing has identified several potential theories as to why we age. However, to date a unified consensus remains to be agreed upon, instead scientific opinion regarding theories of ageing have been divided into two main categories, the programmed theory and the damage or error theory, although numerous other sub-theories exist such as antagonistic pleiotropy [405].

1.13.2.1. Antagonistic pleiotropy.

George Williams' antagonistic pleiotropy theory of aging proposes that cellular damage and organismal aging are caused by pleiotropic genes, or genes with multiple phenotypic effects [406]. According to the antagonistic pleiotropy theory of ageing, natural selection has favoured genes conferring increased odds of successful reproduction early in life to the organism at the cost of deterioration in later life. Examples of antagonistic pleiotropy include individuals suffering from Huntington's disease, a rare neurodegenerative disorder characterized by a high

number of CAG repeats within the Huntingtin gene [407]. The onset of Huntington's is usually observed post-reproductive age and generally involves involuntary muscle spasms, cognitive difficulties and psychiatric problems. Incidentally, the high number of CAG repeats is associated with increased activity of p53, a tumor suppressing protein that participates in apoptosis. It has been hypothesized that this explains the lower rates of cancer among Huntington's patients [406].

1.13.2.2. The programmed theory.

The programmed theory can be defined as a set course of biological deterioration following adolescent development, governed by alterations in gene expression and protein function vital in biological pathways regulating maintenance, repair and cellular defence responses [405]. Furthermore the programmed theory can be further subdivided into three other theories which will be briefly discussed.

1.13.2.3. Programmed longevity.

The programmed longevity theory recognises ageing, not as a gradual loss of body function, but instead as a scripted series of events, genetically programmed to potentiate evolution [405]. The effect of these events is ageing and death due to recombination and mutations leading to the existence of some individuals better adapted to environmental conditions and thus further evolved [405]. The programmed longevity theory is supported by little experimental evidence, and is instead supported by observations. For example, a wide range of plant species die shortly after flowering and some animal species exist, among them insects, worms and fish, in which death occurs immediately after procreation [405]. Other examples include organisms exhibiting increased lifespan, such as mutated *Drosophila* which are able to produce long-lived progeny and the opposing extreme are human patients suffering from progeria like syndromes [408, 409]. These examples represent ageing and death as a programmed event, much like cellular apoptosis; fundamental to normal development and growth. A review by Roland Prinzinger states the

dramatic increase in lifespan in recent times, is not necessarily due to an increase potential lifespan, but due to a reduction in mortality by diseases, accidents, starvation and succumbing to predators [410]. Davidovic *et al*., hypothesises that two subgroups exist in the general population: the first with a "normal" genetic make-up and aging pattern, and the other with delayed aging, "the privileged group", who exhibit genetic stability and are protected against DNA mutations, methylation, oxidant mediated damage and telomere attrition [411]. Thereby protecting genes responsible for metabolic, cell survival/signalling, and endocrine processes [411].

1.13.2.4. Endocrine theory.

The endocrine theory proposes that the biological clock governing lifespan is largely modulated by the secretion and actions of hormones [405]. Considering the endocrine nature of adipose and skeletal muscle tissues and their influence on the manifestation and development of metabolic disorders, the endocrine theory may have value. Unlike the programmed longevity theory the endocrine theory has more scientific evidence in its support. In rodents, mutations in genes involved in hormone-signalling pathways can substantially increase lifespan and also show reductions in age-related conditions such as diabetes, memory loss and cancer [402]. Mice with mutations that disrupt the development of the pituitary gland, which produces growth hormone, show increased longevity, as do mice that lack the receptor for growth hormone [412]. Studies surgically removing the pituitary gland in mice reveal increased longevity and upregulation of pathways reminiscent of calorie restriction [412]. The work of Diana van Heemst outlines the significance of the insulin and insulin-like growth factor (IGF) signaling (IIS) pathway plays a major role in the control of longevity [413]. Mutations diminishing the insulin/IGF-1 signalling pathway in *Caenorhabditis elegans* significantly enhances lifespan an effect also reported in *Drosophila melanogaster* fruit fly [413].

1.13.2.5. Immunological theory

The immunological theory of ageing suggests that age-associated health implications arise as a direct result of a deterioration of the immune system [405]. It is well documented that the effectiveness of the immune system peaks at puberty and gradually declines thereafter with advance in age, with the eventual loss of immune function termed immunosenescence [414]. The innate immune response of inflammation has been recognised as a contributing risk factor in the progression of various age associated diseases including osteoporosis, sarcopenia, T2D, Alzheimer's disease and atherosclerosis [415]. A view proposed by the immunological theory of ageing suggests inflammation exhibits hormetic functions while at lower levels and is largely responsible for offering protection against invading pathogens, whilst enhanced lifespan results in chronic activation of inflammation and the overproduction of inflammatory molecules which can also cause immune-related inflammatory diseases and eventually death later [392, 414]. In 1969 Roy Wolford discussed the importance of immunosenescence in age-associated disease susceptibility and progression, the effects of which are very much relevant today [416]. The immunological theory of ageing describes senescence of clonotypic immunity as being responsible for the inability to mount an effective immune response in advancing age [416]. Chronic antigen loads throughout the course of life have been postulated to be a possible driving force of immunosenescence, which in conjunction with age-associated atrophy of the thymus (thymic involution) would consequently result in reduced naïve T cells and the increased circulation of expanded clones of memory and effector, antigen-experienced T cells [417]. Furthermore, ageing is met with a reduction in hematopoietic stem cells, phagocytes and number and function of natural killer cells [418]. Therefore the repertoire of cells available to respond to antigenic challenge from previously un-encountered pathogens is reduced giving rise to more infections in later life and poor health leading to a reduction of lifespan [418, 419].

1.13.2.6. The damage or error theory of ageing.

The theories which comprise the damage or error theory suggest that the gradual deterioration of human physiology is due to environmental damage which accumulates over time and contributes to ageing; these theories include the wear and tear theory, cross-linking theory and the theory of free radicals [405].

1.13.2.6.1. The wear and tear theory.

The wear and tear theory was proposed by Dr. August Weismann in 1882 and suggests that ageing is a result of overuse and abuse of the body, an effect which is possibly fuelled by the consumption of energy dense foods, alcohol, pollution in the atmosphere, ultra-violet rays and numerous other psychological stresses [420]. However, individuals refraining from such damage are not exempt from ageing instead endure a reduced rate of damage in comparison to individuals leading an unhealthy lifestyle [405]. The analogy often given to explain the wear and tear theory compares the human body to an automobile which over the course of time and excessive use will suffer from corrosion and mechanical wear. Even with normal moderate use the automobile will still experience wear and tear only at a slower rate. Evidence in support of the wear and tear theory is sparse and not without its downfalls and fails to address the ability of the human body to repair itself along with the wide interspecies variation in longevity [405, 411]. Also certain organisms apparently do not age or age very slowly while others have an inbuilt programmed self-termination programme following reproduction.

1.13.2.6.2. The cross linking theory.

The cross linking theory was proposed in 1942 by Johan Bjorksten and certain aspects of it may still be of relevance in gerontology research conducted today [421]. The cross linking theory is applicable to both external and internal biology. With increasing age, tendons, skin, and even

blood vessels lose elasticity [421]. This is due to the formation of cross-links between or within the molecules of collagen that give elasticity to these tissues and may contribute to the appearance of tough, leathery and yellow skin with advancing age [421]. Cross linking may also be responsible for cardiac enlargement and the hardening of collagen, which may then lead to the increased susceptibility of a cardiac arrest [422]. The glycosylation theory which may potentially be recognised as a separate but related theory describes the cross linking of glucose molecules with proteins, significantly dampening the activity and function of proteins [422]. These interactions result in the production of advanced glycation end products (AGEs), commonly found in large amounts amongst individuals suffering from vascular complications, cardiovascular disease, obesity, T2D and Alzheimer's disease [422].

1.13.2.6.3. The free radical theory

Oxidative stress was first described as a contributor to ageing by Denham Harman who postulated the free-radical theory of ageing [423]. A theory which states, continuous production of ROS, by-products of general metabolism would eventually overwhelm antioxidant molecules [423]. This disruption in balance generates a state of oxidative stress, resulting in DNA damage to the extent of cell death and a common contributor to ageing [423]. The interaction between oxidative stress, DNA damage and telomere shortening has been exhibited by Zglinicki and Schewe, who reported increased oxidative stress accelerated telomere shortening in fibroblasts *in vitro* [424]. Excessive exposure to oxidative stress is known to hinder telomerase function, on the contrary, antioxidants significantly prolong telomerase activity whilst simultaneously reduce telomere shortening *in vitro* [424]. The free radical theory is more widely accepted, largely due to a wealth of literature documenting its involvement in various age related disorders [425]. Overactive mitochondria mediated increase in ROS has been implicated in both obesity and T2D. Miyazawa found mitochondrial superoxide anion production can lead to organ atrophy and dysfunction via mitochondrial- mediated apoptosis [426]. ROS also play a significant role in the pathogenesis of atherosclerosis and other disorders which present with an inflammatory nature [426].

1.14. Cellular ageing: the telomere theory

While multiple theories of aging have been proposed, currently there is no consensus on this issue. Many of the proposed theories interact with each other in a complex way and arguably the most effective marker of ageing is chronological age [427]. However the use of chronological age, as an approach to predict morbidity and/or mortality can be erroneous [428]. Although years lived correlate considerably with risk of developing health implications which disproportionately affect an individual's life span, chronological age fails to explain inter-individual inconsistencies in longevity [427]. Furthermore, human lifespan is determined by a multitude of factors, to assume years lived is the sole determinant of lifespan is seemingly unscientific. For instance genetic variability, psychological wellbeing and nutritional background are all vital contributing factors in determining lifespan, all of which are unaccounted for when using chronological age alone to predict longevity. Research into an alternative, more prognostic marker of longevity, that can be quantified on an interval scale and can fluctuate in association with life style factors, has led numerous studies to investigate telomere length (TL) [429].

1.14.1. The telomere complex.

Telomeres form vital components of eukaryotic chromosomes, distinctively found on the ends and consisting of non-coding hexanucleotide repeats TTAGGG that are extended 9-15kb in humans and end in a 50-300 nucleotide 3' single guanine strand overhang [430]. The overhanging segment of the telomere complex is able to fold back onto itself and lodge itself in between the telomere helix to form a 'T-loop'; and is also responsible for the end-capping structure of telomeres [430, 431]. Supporting the T-loop structure are evenly distributed shelterin complexes which consist of the telomeric repeat binding factor 1 (TRF1) and TRF2 which bind to double stranded segment of telomeric DNA [432, 433]. Other components of the shelterin complex include protein protection of telomeres (POT1) which binds directly to the single-stranded telomeric DNA and interacts with tripeptidyl peptidase (TPP1) [430]. Repressor activator protein

1 (Rap1) binds TRF2, and TRF1- interacting nuclear factor 2 (TIN-2) is a central component of the complex interacting with TRF1, TRF2 and TPP1 [430]. Combined, the shelterin complexes in conjunction with the T-loop structure form a telomere protective cap, which allows DNA repair proteins to distinguish between chromosome ends and DNA damage, therefore insuring genomic stability and protecting against recombination, exonuclease degradation and end-to end fusion [430].

Figure 1.7. Simplified diagram depicting the structure of the telomere and its location on the chromosome along with the terminal end of the telomere concealing the terminal single-stranded segments with help of the shelterin complex [430]

1.14.2. End replication problem

TL has been extensively proposed as a biomarker of cellular ageing, since telomeres shorten with each round of cellular division due to what is known as the “end replication problem” [432]. The end replication problem can be summarised as follows. Prior to DNA replication, double stranded DNA is unwound by helicase enzymes and separated into two individual strands, which are antiparallel to one another, the backbone of one strand runs from 5'-3' while the complimentary strand runs 3'-5'. Unfortunately, DNA polymerase, the enzyme responsible for replicating DNA, can only elongate from a 3’ hydroxyl group and therefore make DNA in a 5'-3' direction, also known as the leading strand [434, 435]. Furthermore DNA polymerase requires RNA primers upstream from the site to be replicated to provide a 3’ hydroxyl group and initiate replication. However this is problematic when replicating the antiparallel strand, also known as the lagging strand since the DNA polymerase cannot replicate DNA 3’-5’[430, 434].

During DNA replication this is solved by synthesizing small pieces of DNA also known as Okazaki fragments ahead of the replication fork on the lagging strand [436]. RNA primers provide 3’ hydroxyl groups at regular intervals and using a “back-stitching” mechanism the lagging strand is replicated. The leading strand is replicated continuously 5’-3’ completely to the end of the template[436]. However the extreme end of the lagging strand DNA cannot be copied because this segment is occupied by the RNA primer required to provide a 3’ hydroxyl group, which is subsequently removed and remaining 3’ hydroxyl groups of Okazaki fragments are joined to complete a copy of the template DNA [437]. Therefore at the end of the chromosomes there is no 3’ hydroxyl available to prime DNA synthesis. Because of this inability to completely replicate the end of the lagging strand, chromosomes progressively shorten with each round of cellular division accounting for the loss of approximately 50-150bp [438]. This is "the end replication problem" and it is solved by putting a DNA cap on the ends of DNA called a telomere

which does not code for any protein, thus when this information is lost it does not have severe consequences for the cell [438, 439].

1.14.3. Telomere attrition and ageing.

Over the course of time and many cellular divisions, the telomere cap is completely eroded and vital genetic information required for cell viability/function is lost [440, 441]. Cells harbouring chromosomes with shortened telomeres reach replicative senescence also known as the Hayflick limit, described as an inability to proliferate despite the presence of mitogenic substrates [441]. The induction of cellular arrest is mediated by the activation of DNA repair mechanisms i.e. tumour suppressor checkpoints. At the first checkpoint of the cell cycle, cells are rendered senescent via activated p53, which binds DNA and simultaneously up-regulates transcription of genes WAF1/CIP1 encoding p21 [442, 443]. p53 and p21 combine to inhibit cellular proliferation by holding cells in the G1 phase of the cell cycle, p21 conjugates to both G1- S phase cyclin dependent kinases (CDK) and S phase CDK's and inhibits their activation [442, 443]. G1 –S phase cyclins are vital for progression from G1 to S phase of the cell cycle, supported by a study conducted by Noda *et al.*, which reported increased levels of both p53 and p21 in senescent cells, cells successfully bypassing checkpoint 1 reach second mortality stage and are cleared via apoptosis, thus limiting the replicative capacity of somatic cells [444-446].

Telomere dynamics facilitate genomic integrity during DNA replication, in the absence of telomeres, genomic DNA is at risk of degradation due to the end replication problem [447]. Therefore telomeres function as buffering zones not only protecting genomic DNA but form the balance between cellular senescence and immortalisation, while cellular senescence is largely responsible for ageing and limiting replicative lifespan of somatic cells and is one extreme, the other extreme is uncontrolled proliferation of cells and the concept of immortalisation which is

associated with increased telomerase expression and the development of cancer [447-449]. The process of telomere restoration was introduced in 1984 by Olovnikov and described as a natural process mediated by a ribonucleoprotein enzyme known as telomerase, responsible for restoring telomere length in a variety of tissues, utilising internal RNA (TERC) as a template for reverse transcription mediated by a reverse transcriptase catalytic subunit (TERT) (Fig.1.9.) [450]. Telomerase was reported to synthesise nucleotide sequences complimentary to chromosome ends, whilst exhibiting healing properties by inserting telomere sequences into damaged DNA [450]. Therefore it seems telomerase could prove to be a vital contributor in reversing the effects of ageing, supported by research suggesting anti-telomerase therapy administered to cancer patients deemed beneficial results and prevented relapse [451]. In *vivo* telomerase activity peaks during embryogenesis conversely is significantly suppressed postnatally to the extent where it is insufficient to overcome telomere attrition however remains functional in stem cell pools facilitating the generation of specific cell sub-sets as per the physiological needs of the body. Thereby elucidating TL as an ample means of investigating cellular ageing in obese/diabetic individuals [452].

Figure 1.8. A diagrammatical representation of the telomerase complex and it's elongation [430]**.**

1.14.4. Evidence of telomere length regulating cellular ageing.

Telomere attrition has been shown to progressively lead to a form of cellular senescence, a permanent cell cycle arrest, resulting in a loss of replicative and proliferative capacity [453]. Existing literature has successfully identified shorter telomeres amongst individuals suffering from; progeria and/or progeria like disorders which abnormally accelerate ageing such as Hutchinson-Gilford syndrome, Werner syndrome and ataxia telangiectasia [454]. Malnutrition, psychological instability, low socio-economic status and even organ failure have been positively associated with increased telomere reduction [455-457]. Furthermore TL amongst cells derived from grafts donated from older donors exhibit reduced TL in comparison to younger donors [458]. TL thus has the potential to function as both, a prognostic marker of longevity and physiological deterioration. A wealth of literature is emerging which recognises factors such as diet, social economic status and psychological stress as considerable determinants of TL, and is therefore not solely regulated by age [456, 457]. Somatic cancer cells, which lack normal DNA damage response mechanisms, continue to divide despite critically short telomeres by upregulating of telomerase or utilising the alternative lengthening of telomeres mechanism [459]. Studies have found shortened telomeres in many cancers, including pancreatic, bone, prostate, bladder, lung and kidney, compensated by increased telomerase expression [460-462]. Telomere dynamics would be better suited as diagnostic tool and predictor of age-related diseases if the differing rate of telomere attrition amongst individuals was considered along with causative mediators and therefore be indicative of biological health and could help identify individuals at risk of age related disease and mortality.

1.15. Cellular senescence.

Cellular senescence was first described by Leonard Hayflick approximately 40 years ago in a pioneering study that presented two prescient ideas [446]. Firstly, cells void of limited cellular division exhibit escape from senescent like changes and can only be achieved by somatic cells

which have developed cancer properties [463]. Secondly the cessation of cell growth *in vitro* may be reminiscent of cellular senescence *in vivo*[441]. There now exists strong evidence to affirm the role of cellular senescence in chronic inflammation and both tumour progression and suppression, highlighting the relevance and complexity of cellular senescence in organismal ageing [464]. Furthermore recent evidence derived from murine studies has declared the specific removal of senescent cells (SC) can reverse the ageing phenotype and delay the onset of multiple age-related pathologies [465]. Age-related cellular senescence is chiefly governed by the attrition of TL and more specifically the end replication problem, discussed previously [466, 467]. However telomere attrition/dysfunction is not the sole determinant of cellular senescence, instead senescence can be induced by a variety of conditions. The arrest of cellular division prior to the erosion of the telomere complex is known as premature senescence and with respect to modern lifestyles and the increased incidence of age related disorders is a more tangible contributor to health pathologies of advancing age [467-469]. The three most influential types will be discussed below.

1.15.1. Stress induced premature senescence (SIPS)

SIPS is a rather broad concept encompassing numerous stresses which cause sub-lethal cell damage and prevent cellular replication [470]. Although the precise mechanisms leading to cell cycle arrest are equivocal, *in vitro* studies have proven valuable in demonstrating the various contributors to SIPS [471]. For instance explanted cells have to adapt to an artificial environment in culture, characterised by abnormal concentrations of nutrients, growth factors, oxygen concentration, fluctuations in temperature, in conjunction with the absence of surrounding cell types and extracellular matrix components [472, 473]. All of the previously mentioned factors can result in the manifestation of SIPS, independent of TL. Toussaint *et al*. recognised that certain proliferative cell lines including human melanocytes and human retinal pigment epithelial cells undergo SIPS during culture when exposed to hydrogen peroxide, ethanol and homocysteine [472-474]. *In vivo* development of SIPS has largely been attributed to oxidative damage, however

the involvement of radiation, heat, toxins and chemotherapeutic agents has been implied without mechanistic insights [471]. It is believed such stressors induce DNA damage: via the ATM/ATR pathway a known regulator of the cell cycle, increased activation of the p53 tumour suppressor, the $p16^{INK4A}$ pathway and recent research has declared the involvement of p38 MAP kinase [475]. The final effectors involved include the increased phosphorylation of cyclin-dependent kinase inhibitors, retinoblastoma (Rb) and altered gene expression in favour of cell cycle arrest. A similar state has been observed in cancer patients receiving chemotherapy, proven by the induction of $p16^{INK4A}$ [476].

1.15.2. Oncogene-induced senescence (OIS).

Oncogenes derive from mutations incurred in normal genes, which then have the potential to transform cells with additional mutations [477]. The physiological response of normal cells is to undergo senescence under the influence of oncogenes [478]. This was first observed when cells overexpressing oncogenic forms of cytoplasmic mitogenic signal transducers and pro-proliferative nuclear proteins underwent senescence [479]. Therefore OIS manifests as a protective mechanism which induces a state of cellular arrest before atypical stimulation of cell growth occurs [480]. The mechanisms regulating OIS in humans do not seem to be universal across cell types and genetic contexts, although the involvement of p53 and $p16^{Ink4A}$ DNA damage pathways is imperative [481]. However unlike replicative senescence, OIS cannot be bypassed by expression of hTERT, confirming its independence from telomere attrition [482]. The majority of evidence supporting OIS stems from *in vitro* experiments however recent *in vivo* studies have further contributed to the role of OIS. Mice exhibiting enhanced proliferative signals in association with the loss of tumour suppressor protein Phosphatase and tensin homolog (PTEN), develop benign lesions consisting of senescent cells [483]. Studies conducted in humans also support the role of OIS as a tumour suppressor as benign naevi in human skin contain cells that express oncogenic B-Raf proto-oncogene, serine/threonine kinase (BRAF) and are senescent

[484]. OIS has also been reported to counteract the induced conversion of primary cells into pluripotent stem cells, therefore limiting the generation of cancer stem cells.

1.16. Characteristics of Senescent cells.

1.16.1. Growth arrest.

The primary characteristic of senescent cells is the inability to progress through the cell cycle and is an indispensable marker for the identification of cellular senescence both *In vitro* and *vivo*[485]. Although the use of cell cycle arrest may not be used solely to determine a senescent population as terminal differentiation similarly results in cell cycle arrest. However, unique to cellular senescence is the ability to remain metabolically active despite a long term exit from the cell cycle [486]. Contrary to initial understanding which declared cellular senescence as irreversible arrest and therefore completely opposite to quiescence, a state in which cells may revert back to proliferating in the presence of mitotic stimuli, more recent studies declare potential mechanisms for reversing growth arrest [486, 487]. Typically features of senescence growth vary between species and genetic backgrounds, however in general cells exhibiting increased expression of tumour suppressor proteins (p53, p21, $p16^{Ink4A}$) are found increasingly in SC [487]. SC also repress genes that encode proteins that stimulate or facilitate cell cycle progression for example, replication dependent histones, cyclin A, cyclin b and proliferating cell nuclear antigen [473].

1.16.2. Apoptosis resistance.

Apoptosis can be defined as the process of programmed cell death and is vital to both organismal development, as well as integral to apoptotic clearance of auto-reactive immune cells and virus infected or transformed cells [488]. Much like senescence, apoptosis is a programmed response to cellular stress, however it functions as an antagonistic counterpart involved in cell clearing [488]. The increased number of SC commonly found in ageing organisms implies that SC have

acquired resistance to apoptotic clearance [489]. For instance human fibroblasts often used to investigate cellular senescence exhibit resistance to ceramide, oxidative stress, and deprivation of nutrient and growth factors however epithelial cells do not and are killed under such circumstances [489].

Hampel attributes the resistance to apoptosis exhibited by SC to the inability to internalise IGF-binding proteins, which has been quantified to greater extents in cell media obtained from SC and has been reported to enhance apoptotic cell death in tumour cells via internalisation and activation of intracellular regulators of apoptosis [490]. The works of Marcotte *et al*., and Ryu *et al*., attribute the increased apoptosis resistance to maintenance of Bcl2 protein levels and reduced expression of caspase 3 [491, 492]. The senescent associated secretory phenotype (SASP) which will be discussed later may also regulate SC survival.

1.16.3. Altered morphology.

Cells undergoing cellular senescence exhibit a vastly altered morphology, which will vary between cell-subsets but certain morphological changes are characteristic [493]. For example SC can become large, flat, and multinucleated. Cho *et al*., investigated the structural determinants of SC and reported that the expression of integrin β_1 and focal adhesion kinase (FAK) were increased and that the phosphorylations of FAK and paxillin, hallmarks of focal adhesion formation, were also increased in senescent human fibroblasts [493]. The authors go on to recognise a fundamental role of caveolin-1 in propagating the senescent phenotype. Caveolin-1 is a scaffolding protein and one of the main components of the plasma membrane. Utilising RNA silencer specifically knocking out caveolin-1 returned cells to a younger more healthy morphology [493, 494].
The authors conclude caveolin-1 regulates focal adhesion kinase activity and actin stress fibre formation in senescent cells [493, 494].

1.16.4. Senescent associated beta-galactosidase (SA-βgal) and Senescence-associated heterochromatic foci (SAHF).

The commonly thought of 'gold standard' for characterising senescent cells is the upregulation of SA-βgal a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides only in senescent cells [495]. This marker is detectable using histochemical staining and was first described by Dimri *et al.*, following the observation that at pH 6.0 a blue-dyed precipitate results in the presence of its chromogenic substrate X-Gal [496]. However existing research has identified SC to possess atypical chromatin structure. SC can inhibit the expression of genes encoding proteins which stimulate cellular replication in an E2F dependent manner. E2F is a transcription factor vital for the progression of cell cycle, SC exhibit repression of E2F target genes due to increased phosphorylation of Rb, leading to reorganisation of chromatin into discrete foci termed senescence-associated heterochromatic foci (SAHF) [497]. SAHF can be readily identified in vitro using the DNA dye 4′,6-diamidino-2-phenylindole [497].

1.16.5. Senescent associated secretory phenotype (SASP).

The most distinctive feature of SC is the profound alterations in their secretome [498]. Irrespective of causative factors all SC upregulate the production of numerous cytokines and chemokines, including TNF-α, IL-6, MMPs, MCP-1 and IGFBPs, which have been quantified to greater extents in late passage human fibroblasts or fibroblasts from patients suffering from progeria-like syndromes [498]. However the secretory nature of SC is an area of senescence biology requiring much work, considering the full range of functions ascribed to members of the SASP varies considerably, arguably between cell sub-sets but also within same cell type and therefore the physiological effects of SASP are complex [499]. A review by Tchkonia *et al*., defines the divergent implications of SASP using two concepts, the first is antagonistic pleiotropy defined previously [500]. The second concept is SASP can coordinate both positive and negative effects on organismal physiology, depending on context [500]. Existing literature has described

SASP to induce both autocrine and paracrine signalling, to exhibit pro-tumorigenic and tumour-suppressive effects as well as pro-and anti-inflammatory signalling [500]. Evidence in support of this statement reveals the proliferative rate, migration, and invasion of premalignant cells are enhanced when they are co-cultured with, or grown in medium conditioned by, senescent fibroblasts [499, 501]. On the contrary as discussed previously the initiation of cellular senescence in response to DNA damage or in a bid to suppress oncogene expression, manifests as a cancer suppression mechanism, an effect which could be reinforced by the SASP in an autocrine feedback loop [486]. The paracrine activities of SC are responsible for both senescence reinforcement in non-senescent cells via IL-1β and can also promote tumorigenesis via increased secretion of cytokines IL6 and IL8, VEGF, and the metalloprotease MMP3 [486]. The SASP can induce systemic inflammation, and disrupt tissue architecture particularly in aged and obese individuals [502].

On the contrary, in younger individuals SASP has proven to be instrumental in repairing tissue damage, promotion of dead cell clearance and inhibition of fibrosis following hepatic insults [502]. Furthermore elevated IL-6 and IL-8 promote cellular senescence and protect against the development of cancer [502]. The secretome of senescent cells includes MCP-1 and stimulates the immune system specifically macrophages to clear dead or dying cells [503]. However chronic exposure to IL-6, also a SASP secretion, inhibits macrophage function [503]. It is therefore evident that cellular senescence exhibits both positive and negative effects on organismal health in a complex network of interactions. Studies are required to delineate upstream mechanisms causing the SASP, explore differences in the SASP among cell types and more importantly investigate the role of cellular senescence in relation to glucose homeostasis and contribution to disorders of advancing age, including obesity and T2D. The effects of SASP on insulin resistance are poorly studied, with existing literature identifying the SASP as a significant source of inflammatory cytokines and deregulators of immune function, the role of the SASP in metabolic disorders is potentially of grave importance.

1.17. Links between ageing and metabolism.

1.17.1. Changes in body composition and ageing.

Disruptions in metabolic pathways can significantly contribute to and regulate the ageing process [504]. Advancing age is almost unanimously associated with an increase in adipose tissue in humans and has been recognised as a primary causative agent behind age related metabolic disorders [505]. Increasing adiposity is also accompanied by the development of age-related decrease in muscle mass, also known as sarcopenia [505]. Basal metabolic rate is determined chiefly by fat-free mass which progressively deteriorates with age [506]. Increasing age is also accompanied with a reduction in physical activity, further contributing to a decrease in muscle mass and energy expenditure; however diet is often maintained, inducing a positive energy balance promoting storage of calories in the form of fat [507, 508].

Alongside the increase in total adipose tissue mass ageing is also associated with an altered pattern of adiposity distribution [509]. A review by Kuk *et al.*, highlights the inability of certain individuals to develop sufficient subcutaneous adipose tissue mass specifically in the lower body, thus exhibit a reduced ability to store circulating lipids and NEFA [154]. As a compensatory mechanism the increased fat content is stored in non-subcutaneous regions, giving rise to increased lipid accumulation in the visceral region [154]. Concurrently both cross sectional and longitudinal studies have confirmed an age associated increase in waist circumference and existing research identifies there are clear differences in the fat storage patterns between different ethnic groups [154, 508, 510]. The empirical data provided by Hairston *et al.*, 2009 supports this theory. White, black and Hispanic women over 60 years of age exhibited an increase in waist circumference by 4cm, 5cm and 3.1cm over the course of 5 years. Waist circumference is an imperative predictor of disease susceptibility, men who have a waist circumference of 40 inches and women, of 35 inches are at increased risk of developing metabolic disease [511].

The relationship between health risk and waist circumference, to certain extents is governed by a subsequent increase in visceral adiposity [512]. Various studies have documented an age-associated increase in visceral fat mass, which is known to contribute to metabolic syndrome irrespective of sex and ethnicity [512-514]. The detrimental effects of visceral fat as opposed to subcutaneous fat are attributable to its increased potential to secret pro-inflammatory cytokines, adipokines [515]. These adipokines, two of which have been discussed previously, leptin and adiponectin, also include TNF- α, IL-6, and vascular endothelial growth factor (VEGF) amongst many others, all of which combined or independently aid the development of metabolic disease, via inflammation, increased cell proliferation and insulin resistance [515, 516]. A paper by Bremer *et al.,* highlights IL-6, IL-1, TNF-α and C-reactive protein as key mediators in the development of metabolic syndrome [517]. IL-6, induced by increased IL-1 and TNF-α concentrations has been shown to hinder insulin signalling by directly interacting with IRS-1 and inhibiting glycogenesis in the liver [517]. While neutralisation of TNF-α significantly increases insulin sensitivity, IL-6 has also been linked to the production of C-reactive protein and fibrinogen, further propagating inflammation [518].

1.17.2. Ageing and energy balance: nutrient restriction.

One prominent theory behind healthy ageing is that of calorie restriction [519]. Calorie restriction mediated longevity has recently received considerable attention. Principle findings declared, restricting calories delayed sexual maturation significantly and impacted longevity in rats [520]. Subsequent work has shown that consuming a calorie restricted diet can prolong lifespan in almost all lower organisms including yeast, worms and flies [521, 522]. More recent analysis of the mechanism by which this occurs has identified, up-regulation of the sirtuin family of genes, responsible for encoding a series of histone deacetylases [523]. The SIR2 mammalian ortholog SIRT1 has been in investigation ever since and the activation of SIRT1 NAD^+ deacetylase has been reported to increase cellular resiliency to oxidative stress, a process mediated by cellular interactions with members of the Fork head transcription factors [524, 525]. More specifically,

deacetylation of FOXO3 has been shown to provide protection from cell cycle arrest by upregulating DNA repair factors and enhancing resistance to oxidative stress [523, 526]. Cohen *et al*., reported a substantial elevation in SIRT1 expression in calorie restricted rats, and recognised SIRT1 as an inhibitor of stress-induced apoptosis, thereby protecting against the loss of irreplaceable somatic cells and promoting organismal longevity [527]. To further advocate the role of negative energy balance in healthy ageing are studies investigating the role of BAT and ageing. BAT functions to expel excess energy as heat rather than store it, as does WAT [528].

Animal studies have associated the age related deterioration of thermoregulatory control to BAT atrophy as well as reduced UCP1 activity [216]. Furthermore it has been demonstrated a lack of BAT and UCP1 increased susceptibility to the development of obesity and insulin resistance in laboratory mice [216]. Human studies provide similar findings, BAT exhibits an inverse relationship with both age and BMI, suggesting a protective role against the development of obesity and thus T2D, two metabolic disorders commonly associated with advancing age [219]. Feige *et al*., chemically enhanced SIRT1 expression in mice and documented a marked up-regulation in BAT lipid oxidation, deeming advantageous against obesity and insulin resistance [529]. A more specific sirtuin to BAT, SIRT3 may play a larger role in energy balance and ageing [530]. SIRT3 expression is enhanced under both cold exposure and calorie restriction, whilst simultaneously elevating UCP1 activity [530]. SIRT3 regulates thermogenesis in BAT by reducing ROS production and increasing cellular respiration. SIRT3 negative adipocytes exhibit no such effect [530]. Supporting the beneficial effects of enhanced UCP1 activity is a study conducted by Molina *et al.*, who in 2012 reported, mice carrying additional copies of PTEN a tumour suppressor gene, have hyperactive BAT and high levels of UCP1, orchestrated by the PI3K/AKt/Foxo pathway and activation of UCP1 transcriptional promoter PGC1α [531]. This increase in energy expenditure protected the mice from onset of metabolic pathologies like obesity and T2D, and subsequently prolonged life span. Energy homeostasis is therefore an integral component of ageing, whereby positive energy balance can disrupt normal metabolism

and facilitate metabolic disease, thus ageing. Contrastingly negative energy balance has been recently documented to influence healthy ageing, exempt from metabolic syndrome but also to increase life-span.

1.17.3. Ageing and energy balance: nutrient excess.

Nutrient excess predisposes an individual to a variety of metabolic disorders which disproportionately reduce longevity, for example in nutrient excess, insulin resistance is often seen, forming the basis for the age-related increase in risk for metabolic disease and T2D. The mechanistic target of rapamycin (mTOR) pathway plays a pivotal role in nutrient sensing, energy metabolism and glucose homeostasis [532]. Aberrant activation of mTOR signalling has been linked to several age related diseases including T2D, cancer, Alzheimer's disease, Parkinson's disease and CVD, leading to studies on the role of this pathway in metabolism, aging, and life span [533, 534]. Increasing evidence suggests that the mTOR signalling pathway is activated by nutrient excess and drives biogenesis mediated by the mTORC1 effector, S6K1 [535]. Interestingly, S6K1 -/- mice exhibit similar gene expression profiles to those of calorie restricted mice, with females showing extended longevity and reduced age-related diseases [536, 537]. Inhibition of the mTOR signalling pathway by genetic or pharmacological intervention extends life span in invertebrates, including yeast, nematodes, and fruit flies. Reduction of mTORC1 activity using genetic mouse models or with rapamycin treatment is associated with a reduction in age-related cancers and improved glucose homeostasis and protection against the development of obesity [537]. Further studies are required to achieve such effects in humans, nevertheless these data provide further links between absence of an enzyme which regulates response to nutrient excess, and successful ageing cross sectional studies have been successful in identifying significant correlations between markers of advancing age and metabolic disorders independent of chronological age. Increasingly, scientific investigations have examined TL a biomarker of cellular ageing and associated telomere attrition with vascular ageing, arterial stiffening,

atherosclerosis, and cardiovascular mortality [538, 539]. Obesity has been implicated as a considerable risk factor for developing the previously mentioned health implications and therefore is recognised as a determining factor of accelerated ageing [540]. Peters *et al*., reports forty year old females lost 7.1 years and 40 year old males lost 5.8 years due to obesity [541].

There is a growing body of evidence that obesity/T2D can influence the ageing process at the cellular level itself [542]. Lee *et al*., published convincing data, documenting rises in fasting blood glucose; fasting serum lipids and lipoprotein were inversely correlated with TL [542]. Fitzpatrick *et al*., report decreased mean terminal restriction fragment length amongst patients suffering from cardiovascular disease in comparison to healthy controls, furthermore an increasing number of senescent cells have been quantified amongst individuals suffering from cardiovascular complications in comparison to age matched controls [538]. There is evidence that replicative senescence pathways involving p53, p16 and p21 play a central role in disease progression and graft outcome, independent of chronological age in patients suffering from renal disease and kidney dysfunction [543]. Kuhlow *et al* report young adult mice which are deficient for the TERC subunit of telomerase exhibit impaired glucose tolerance and impaired glucose-stimulated insulin secretion due to increased cellular apoptosis [544]. Elmadhun and collegues report metabolic syndrome is associated with increased apoptosis signalling, decreased survival signalling, and increased cell death, molecular mechanisms most commonly associated with advancing age [545].

It is therefore clearly evident metabolic disorders can influence organismal ageing by giving rise to health implications which can severely shorten lifespan and reduce the quality of life. It will be of interest to examine the effects of metabolic disorders on organismal ageing to help identify early predictors of ageing and also clarify, whether the metabolic disorders develop as a result of ageing or do the metabolic disorders influence ageing themselves.

1.18. Aims of the study

The broad aim of this study was to investigate the metabolic regulation of cellular ageing in healthy and obese/type 2 diabetic volunteers and alternatively to investigate how cellular senescence can affect nutrient homeostasis in metabolic cell lines. In the former the primary aim was to identify anthropometric and biochemical measures which could be used to predict TL. In an attempt to identify a biomarker of ageing which could provide more valuable information in comparison to chronological age in a cohort consisting of both obese/type 2 diabetic and non-diabetic participants. More specifically to elucidate the role of the recently discovered myokine irisin and its potential effects on TL in healthy and obese/type 2 diabetic participants. In the latter of the study the primary aim was to investigate whether conditioned media collected from SC could influence glucose utilisation in the AML-12, C2C12 cell lines, as well as effect lipolysis and glucose utilisation in the 3T3-L1 cell line.

The outcomes from this study will help clarify the role of body composition and associated adipo/myokines and markers of endothelial dysfunction in cellular ageing. The study will go on further to improve the understanding of the role of irisin in cellular ageing, while elucidating the relationship between cellular senescence in regulating glucose homeostasis with the expectation of providing knowledge which may assist in future research aiming to perturb the affects of cellular senescence and develop novel treatments.

Chapter 2: Methods

2.1. Study participants

Eighty one healthy participants (44 males and 37 females, age 18-83 years) with a mean body mass index (BMI) of between 20 and 30 kg/m^2 were recruited from the local community in Birmingham, England. None of the participants in the present study were obese (BMI>30), pregnant, T2D, previously diagnosed with cancer, suffered from immune disorder, were recently hospitalised or treated with oral corticosteroids. Eighty four type 2 diabetic participants (45 males and 39 females, age 25-76 years) with a mean BMI of 31.5 kg/m^2 were recruited from the Heart of England NHS trust, Birmingham, England. To control for physical activity all participants refrained from exercise for at least 12 hours prior to recruitment this ensured that physical activity was not a confounding factor. The study was approved by both Aston University and Staffordshire NHS Research Ethics Committees and written informed consent was given by all participants according to the principles of the Declaration of Helsinki. Subjects were asked to fast for a minimum of 8 hours prior to recruitment to the study. Each participant is provided a unique subject identifier, and volunteer specific data is recorded on a subject information sheet.

2.2. Anthropometric measures

Bioelectrical impedance analysis (BIA), using a segmental multiple frequency analyser (BC-601 Bioimpedenace Analyser Tanita®), was performed on all subjects to measure segmental fat mass (FM), fat free mass (FFM) and visceral fat score (calculated by the manufacturer's software as 1-59; a score of 1-12 is considered healthy, 13- 59 considered as being an indication of excess visceral fat). Abdominal fat and muscle readings are subtracted from other segmental readings and therefore represent the body trunk. Height and weight was measured in order to determine (Body Mass Index) BMI.

2.3 Blood collection

Venous blood was collected from all volunteers by venepuncture into K^+-EDTA coated blood collection tubes (sterile 9mL, Vacutainer, Becton Dickinson, UK). Plasma was separated by

centrifugation within 30 minutes of blood withdrawal (1300 x RCF for 10 minutes), aliquoted into 0.5mL aliquots and stored at -80°C until required.

2.4 Blood Glucose analysis

Fasting whole blood glucose was measured using an Accucheck Advantage blood glucose meter. Healthy participants with a fasting blood glucose of >6.1mmol/l glucose were excluded from the study, ensuring no recruitment of diabetic individuals.

2.5 DNA isolation

Aliquots of whole blood were used for peripheral blood mononuclear cell (PBMC) genomic DNA extraction using the QIAamp® DNA blood mini kit (Qiagen®, # 51104,) according to manufacturer's instructions. Briefly, blood samples were treated with proteinase K and buffer AL to remove protein and polysaccharides, briefly vortexed and incubated at 56°C for 10 minutes. DNA was precipitated by ethanol, applied to a column provided in the kit followed by washes with buffers AW1 and AW2, and then dissolved in 200µL of elution buffer (10 mM Tris•Cl; 0.5 mM EDTA; pH 9.0). Isolated DNA was quantified using the NanoDrop-1000 (NanoDrop Technologies, USA) and diluted in pure water to a concentration of 5ng/µl and stored at -80°C.

2.6 Telomere length (TL) Assay

Relative TL was measured using real-time polymerase chain reaction (RT-PCR). PCR reactions were set up using previously published primer pair telg and telc (Appendix 7.2; final concentrations 900nM each), combined with the single copy gene (SCG) albumin primer pair albu and albd (final concentrations 900nM each), in a 25µl PCR reaction, consisting of 1X Precision qPCR Mastermix (0.025 U/µl Taq Polymerase, 5 mM $MgCl_2$, dNTP Mix 200µM each dNTP) and 5ng of template DNA on the Stratagene MX 3000P sequence Real-Time PCR Fluorescent Detection System. SYBR® Green (492nm-516nm). Samples for both the telomere and single-copy gene amplifications were performed in duplicate, with duplicates of a non-

template control included in each run. Dissociation curve analysis was performed on each sample at the end of each run to verify specificity of the PCR. The ratio of telomere to the normalising genomic control sequence (T/S ratio) was calculated as described by Cawthon [546] to provide an indication of relative telomere length.

The thermal cycling profile was Stage 1: 4 minutes at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; and Stage 3: 32 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C with signal acquisition. The 74°C reads provided the cycle threshold value (Ct) values for the amplification of the telomere template. The thermal profile is then modified for signal acquisition of the SCG. 10 s at 84°C, 15 s at 88°C with signal acquisition. The 88°C reads provided the Ct values for the amplification of the SCG template, this method is a modification of duplex PCR. After thermal cycling, raw data was exposted from the thermal cycler to a Microsoft Excel spreadsheet. The T/S ratio for an experimental DNA sample is T, the average of the cycle threshold value for the telomere product, divided by S, the average of the cycle threshold value for the single copy gene product. The Ct value is defined as the number of cycles required for the fluorescent signal to cross the threshold, i.e. exceeds background level. As each experimental sample was assayed in duplicate, two T/S results were obtained for each sample. Average T/S is expected to be proportional to the average telomere length per cell.

2.7 Enzyme-linked immunosorbent assay (ELISA)

Plasma levels of adipokines and markers of endothelial dysfunction were measured using commercially available ELISA kits for the quantification of leptin, adiponectin, soluble thrombomodulin, C-Reactive protein (CRP) and soluble E-Selectin (ES) (Duoset; R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's protocol. In short, ninety-six–well microtiter plates were coated overnight at room temperature with the working concentration of the appropriate capture antibody and then blocked with reagent diluent (1% BSA in sterile PBS) for one hour. A standard curve was prepared and plasma samples were diluted

with reagent diluent according to plasma reference ranges and standards provided by distributors. 100µl of both standards and diluted samples were added to the wells. After a 2 hour incubation period the microtiter plates were washed 3 times using wash buffer (0.05% Tween® 20 in PBS) and re-incubated with 100µl biotinylated detection antibody for 2 hours. Plates were washed again and 100µl streptavidin conjugated to horseradish-peroxidase was added for 20 minutes. Colour formation was achieved by the addition of equal parts of Reagent A (H_2O_2) and Reagent B (Tetramethylbenzidine (TMB)) and was stopped by adding 1M sulphuric acid stop solution (Sigma®, # 339741). Optical density values were measured at 450 nanometer on an optical plate reader. Plasma irisin (Phoenix Peptides, # EK-067-29, Germany) and insulin (Mercodia, # 10-1247-01, Sweden) concentrations were also assessed by ELISA following protocols provided by the manufacturers.

2.8 Homeostatic model assessment (HOMA)

HOMA is computer based model, developed from Turner and Holaman's physiological data which was used to devise a mathematical feedback model capable of estimating the degree of insulin resistance (HOMA-IR) insulin sensitivity (HOMA-S) and beta cell function (HOMA-β) from plasma glucose and insulin levels observed in an individual. The HOMA computer model allows users to compute the previously mentioned parameters by inserting the plasma glucose concentration in mmol/l and plasma insulin concentration in pmol/l into the following equation.

$$\text{HOMA} - \text{IR} = \frac{\text{Glucose x insulin}}{22.5}$$

$$\text{HOMA} - \beta = \frac{20 \text{ x Insulin}}{\text{Glucose} - 3.5} \%$$

2.9. Cell Culture

2.9.1 Early passage Human Umbilical Vein Endothelial Cells (HUVEC) – pooled

One cryo-vial containing 5 x 10^5 HUVEC was obtained from Cellworks (Cellworks # ZHC-2301) and stored in liquid nitrogen upon arrival. HUVEC were grown in human large vessel endothelial cell basal medium (Cellworks # KC1015), supplemented with human large vessel endothelial cell growth supplement (Cellworks # KC1016) and antibiotic supplement consisting of 1X Amphotericin B/Gentamycin (Cellworks # KC1019). Prior to thawing the cryo-vial, a T-25 flask was prepared with 6ml of growth media and left to pre-equilibrate at 37°C in a 5% CO_2 humidified incubator. After 15-20 minutes one cryo-vial was thawed and the contents were transferred into the T-25 flask and fed with growth media every 48 hours.

2.9.2 Human Dermal Fibroblasts

Human dermal fibroblasts (HDF) isolated on the 3/4/2011 from the foreskin of a healthy male newborn were purchased from Zenbio at passage 2 (Cat # DF-N-F, Lot #, DFMF0217118) and stored under liquid nitrogen. The contents of the vial (500,000 cells) were cultured at 37ºC, 5% CO_2 in a T-25 flask using Dulbecco's Modified Eagle Medium (DMEM), high glucose (4.5g/l) (PAA Laboratories GmbH, # E00912-2761) supplemented with 10% fetal bovine serum (Biosera #, S08460S1810) 1x L-Glutamine 200mM (PAA Laboratories GmbH, # M00410-2744, 100x) and 1x Penicillin-Streptomycin (PAA Laboratories GmbH, #P01010-3161, 100x) growth media. Upon becoming confluent cells were transferred into a T-75 flask and the media was refreshed every three days.

2.9.3 AML12 hepatocytes.

One cryo-vial containing 2.5 x 10^6 AML12 (alpha mouse liver 12) hepatocyte cells was obtained from American Type Culture Collection (ATCC # CRL-2254) The contents of the vial were cultured at 37ºC, 5% CO_2 in a T-25 flask. AML12 cells are cultured in DMEM/Ham's Nutrient Mixture F-12, 1:1 with 2.5mM L-glutamine (PAA Laboratories GmbH, # M00410-2744, 100x), supplemented with 10% fetal bovine serum (Biosera #, S08460S1810), 40ng/ml dexamethasone (Sigma® # D8893) and 1X Insulin-Transferrin-Selenium (ITS) cocktail (Gibco, life technologies # 41400-045) at final concentrations of 5µg/ml insulin, 5µg/ml transferrin, 5ng/ml selenium. The cells were started as per manufacturer's instructions under aseptic conditions and growth media was refreshed every two days.

2.9.4. C2C12 Myoblasts

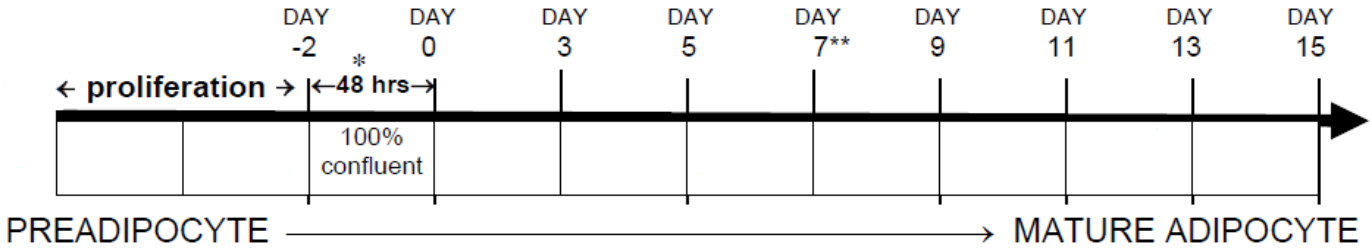
C2C12 myoblasts, derived from mouse muscle tissue were obtained from American Type Culture Collection (ATCC® # CRL-1772™) The contents of the vial were cultured at 37ºC, 5% CO_2 in a T-25 flask and started up in a T-25 flask, using DMEM, high glucose (4.5g/l) (PAA Laboratories GmbH, # E00912-2761) supplemented with 10% fetal bovine serum (Biosera #, S08460S1810) 1x L-Glutamine 200mM (PAA Laboratories GmbH, # M00410-2744, 100x) and 1x Penicillin-Streptomycin (PAA Laboratories GmbH, #P01010-3161, 100x) growth media. Cultures were grown to approximately 70-80% confluence and then split into a T-75 flask. The C2C12 cell line differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins. In order to induce differentiation, the cells are grown in regular growth medium containing 2% horse serum as opposed to 10% FBS.

2.9.5. 3T3-L1 pre-adipocytes

3T3-L1 pre-adipocytes (Zen bio # SP-L1-F) at passage 8 were obtained from Zen bio and stored immediately under liquid nitrogen. One cryo-vial containing 5 x 10^5 cells was cultured at 37ºC, 5% CO_2 in a T-25 flask using preadipocyte medium consisting of the following components. DMEM, high glucose (4.5g/l) (PAA Laboratories GmbH, # E00912-2761) supplemented with 1% bovine calf serum (1x l-glutamine 200mM (PAA Laboratories GmbH, # M00410-2744, 100x) and 1x penicillin-streptomycin (PAA Laboratories GmbH, #P01010-3161, 100x) growth media. Once the cells became approximately 60-70% confluent the cells were transferred into a T-75 flask and the media was refreshed every other day as per manufacturer's instructions.

2.9.6 Differentiation protocol

For differentiation experiments, 3T3-L1 cells at passage 11 were seeded into 24 well plates. Cell density was undetermined; as cells were required to become 100% confluent in order to induce growth arrest, through contact inhibition prior to clonal expansion and terminal differentiation. 2 days post confluence, designated day 0, cells were induced to differentiate with DMEM/Hams' F-12 (1:1, v/v), supplemented with 10% fetal bovine serum (Biosera #, S08460S1810), 1x penicillin-streptomycin (PAA Laboratories GmbH, #P01010-3161, 100x), biotin (3.3µM) (Sigma® # B4639), pantothenic acid (0.17 µM) (Sigma® # P5155), human insulin (1µg/ml) (Sigma® # I0516) dexamethasone (0.25µM) (Sigma® # D8893), isobutylmethylxanthine (IBMX) (0.5mM) (Sigma® # I7018), and rosiglitazone (2µM) (Santa Cruz # sc-202795). Differentiation media was replaced after 3 days with adipocyte media, DMEM/Hams' F-12 (1:1, v/v) supplemented with previously mentioned components at the same concentrations, except IBMX and rosiglitazone which were not included in the media. Adipocyte media was refreshed every 2 days, while the 3T3-L1 cells were photographed at 3 day intervals using a light microscope at high magnification (400x), up until day 15 when cells were fully differentiated into mature adipocytes. The growth and differentiation feeding schedule is illustrated below.



Pre-adipocyte media	**Differentiation media**	**Adipocyte media**
DMEM, high glucose	DMEM/Ham's F-12	DMEM/ Ham's F-12
10% BCS	10% FBS	10% FBS
1x Penicillin/Streptomycin	Biotin	Biotin
	Pantothenate	Pantothenate
	Human Insulin	Human Insulin
	Dexamehasone	Dexamethasone
	1x Penicillin/Streptomycin	1x Penicillin/Streptomycin
	IBMX	
	Rosiglitazone	

Figure 2.1. Differentiation protocol for 3T3-L1 pre-adipocytes.

3T3-L1 cells were grown in pre-adipocyte media till they were 100% confluent; cells were left for 48 hours post 100% confluence and then treated with differentiation media for a period of three days. Cells were then grown in adipocyte media which replaced every 2 days.

2.10 Cell culture treatment

2.10.1 HUVEC Irisin treatment

Human recombinant irisin was purchased from Cayman chemical (Cayman chemical # 11451), supplied in 50mM Tris (pH 8), containing 150mM sodium chloride and 20% glycerol at 0.2mg/ml. To avoid protein degradation irisin was aliquoted and stored at -80°C. Upon reaching 70-80% confluence HUVEC were seeded into 6-well plates and treated with low (20ng/ml) and high (200ng/ml) concentrations of recombinant irisin for 4 and 24 hours. Following the respective time period, media was taken off the cells and stored, whilst the cells were trypsinised and incubated for 5 minutes at 37°C in a 5% CO_2 humidified incubator. Upon cell detachment, the trypsin was neutralized with cell growth media and this solution was transferred to 1.5ml Eppendorf and spun at 500xg for 5 minutes. The cell supernatant was removed and stored as were the cell pellets at -80°C.

2.10.2 Fibroblast hydrogen peroxide treatment (H_2O_2)

Existing literature has referenced H_2O_2 as a proficient chemical inducer of cellular senescence in various cell lines including HDF. There exists, however, a fine line between inducing cellular senescence and cytotoxic cell death, therefore in a preliminary experiment, a range of concentrations were tested to determine the optimal concentration capable of inducing senescence. In summary, HDF at passage 5 were seeded into a 24 well plate in growth media, cell number was not determined, instead HDF were grown to confluence to avoid variability of H_2O_2 toxicity (H_2O_2 toxicity is inversely related to cell density until cells reach confluence). Cell media was replaced with media treated with H_2O_2 (Sigma® # H1009) at final concentrations of 0µM, 50µM, 60µM, 70µM, 80µM, 90µM, 100µM and 110µM in triplicate. Treatment media was removed following one hour incubation, each well washed with PBS three times and replaced

with DMEM. HDF were incubated for four days and then split in a 1:2 ratio and further incubated for 24 hours before measurement of senescence associated beta galactosidase activity.

2.10.3 Fibroblast doxorubicin hydrochloride treatment.

Doxorubicin hydrocholoride (Tocris # 2252) was dissolved in water to form a stock concentration of 1mM. As previously mentioned a 24 well plate was set up with HDF and left to grow till confluence. Then growth media was replaced with treatment media containing doxorubicin at the following concentrations 0µM, 0.25µM, 0.5µM, 0.75µM, 1µM, 1.25µM, 1.5µM and 1.75µM in triplicate. Treatment media was removed following 45 minute incubation and replaced with DMEM. HDF were incubated further for 24 hours to measure senescence associated beta galactosidase activity.

2.10.4 Fibroblast etoposide treatment.

Etoposide (Tocris # 1226) was dissolved in Dimethyl Sulfoxide (DMSO) to form a stock concentration of 1mM. As previously mentioned a 24 well plate was set up with HDF and left to grow till confluence. Then growth media was replaced with treatment media containing Etoposide at the following concentrations 0µM, 1µM, 5µM, 10µM, 15µM, 20µM, 25µM and 30µM in triplicate. Treatment media was removed following 45 minute incubation and replaced with DMEM. HDF were incubated further for 24 hours to measure senescence associated beta galactosidase activity.

2.10.5 AML-12, C2C12 and 3T3-L1 SASP treatment.

AML12 cells were seeded in 24 well plates and allowed to grow till 100% confluence. Cells were treated with normal DMEM high glucose media as a control, 20% (80% regular growth media)

of the media collected from non-senescent fibroblasts after 24 hours, 20% of the media collected from non-senescent fibroblasts after 48 hours, 20% of senescence media obtained from senescent fibroblasts after 24 hours and 20% of senescence media obtained from senescent fibroblasts after a period of 48 hours. This plate setup was repeated to produce two identical plates one of which was incubated for 24 hours and the other for 48 hours. Two other plates were set up in the same fashion, except for the cells were treated with 40% conditioned media (60% regular growth media) and incubated for 24 and 48 hours. Following incubation the cell media was collected and stored at -80°C and the cells were trypsinised, pooled into a 1.5ml Eppendorf and centrifuged at 500 x g for 5 minutes. The resulting supernatant was removed and the remaining cell pellet was stored at -80°C.

2.10.6 Collection of senescence associated secretory phenotype containing media.

24-well plates were seeded with HDF in DMEM high glucose media and allowed to reach confluence. At this point the cells were either treated with 1.5µM doxorubicin or regular growth media as a control and incubated in a 37ºC, 5% CO_2 humidified incubator for 45 minutes. Following incubation, both the treatment and control media were removed and the cells were washed three times with sterile PBS, the cells were then incubated in regular DMEM high glucose media for a period of 24 and 48 hours. Following the respective time periods the cell media was collected into 50ml tube and centrifuged at 500 x g for 5 minutes, in order to pellet any cell and/or cell debris. The media was then filter sterilised using a 20ml syringe and 0.2µm filter, to ensure that there was no crossover of cellular material. The media was then aliquoted into 1.5ml Eppendorfs and stored at -80°C.

2.11 Aged and young mice tissues

C57BL/6N mice tissues were bought from ShARM (source, Taconic), specifically skeletal muscle tissue (ShARM # 020600036), liver tissue (ShARM # 021300036) and white adipose

tissue (ShARM # 021500036) were purchased for both young (4 months) and old (19 months) male mice. Mouse tissues were received with a material transfer agreement in place and were stored at -20°C upon arrival.

2.12 RNA isolation from cell pellets.

RNA extraction and purification from cell pellets was conducted using the E.Z.N.A. ™ Total RNA kit (Omega # R6834-01) according to manufacturer's instructions. In summary, pelleted cells were lysed with the addition of 350µl of TRK lysis buffer and vortexed for complete dissociation of the cell pellet. An equal volume (350µl) of 70% ethanol was added to the lysate and triturated repeatedly to ensure complete mixture of ethanol. The sample was then added to a HiBind RNA column inserted into a 2ml collection tube and centrifuged at 10,000 x g for 60 seconds at room temperature and the flow through was discarded. Following centrifugation 500µl of RNA wash buffer 1 was added directly to the HiBind RNA column and centrifuged at 10,000 x g for 60 seconds. After disposing the flow through, 500µl of RNA wash buffer 2 was added onto the HiBind RNA column and spun at 10,000 x g for 60 seconds. This step was then repeated before the HiBind RNA column was centrifuged at maximum speed for 2 minutes, to completely dry the HiBind matix. The column was then transferred into a clean RNase free 1.5ml Eppendorf and the RNA was eluted by the addition of 50µl of diethylpyrocarbonate (DEPC) treated water, followed by centrifugation for 1 minute at 14,000 x g. Isolated RNA was quantified using the NanoDrop-1000 (NanoDrop Technologies, USA).

2.13 RNA isolation from tissues.

RNA isolation using TRIzol reagent was completed according to a well-established method published by LifeTechnologies. The tissues were cut using sterile scissors, into small sections weighing 50mg. The samples were transferred into sterile 50ml tubes and 1ml of TRIzol was added prior to homogenisation using a power homogeniser. For RNA extraction of muscle tissue

an additional isolation step was conducted before phase separation. Following tissue homogenisation the samples were centrifuged at 12,000 x g for 10 minutes at 4°C, the upper fatty layer was discarded and the supernatant was transferred to a new tube. This additional step removes insoluble materials from the sample. Next the homogenised samples were left to incubate at room temperature for 5 minutes before the addition of 200µl of chloroform; each sample was then shaken by hand for 15 seconds and incubated at room temperature for 3 minutes. The samples were then centrifuged at 12,000 x g for 10 minutes at 4°C, separating the sample into three phases; the aqueous phase containing RNA was removed. Subsequently 500µl of 100% isopropanol was added to the aqueous phase and the sample was incubated at room temperature for 10 minutes, followed by another centrifugation step at 12,000 x g for 10 minutes at 4°C. The supernatant formed in the previous step was removed leaving a RNA pellet which was washed with 1ml of 75% ethanol, the sample was then briefly vortexed and centrifuged at 7500 x g for 5 minutes at 4°C. The supernatant was removed and the RNA pellet was air dried for 10 minutes. The remaining RNA pellet was re-suspended in 40µl of RNase-free water and incubated at 60°C for 15 minutes. Isolated RNA was quantified using the NanoDrop-1000 (NanoDrop Technologies, USA).

2.14 RNA reverse transcription

To reverse transcribe RNA into cDNA, the precision nanoscript reverse transcription kit was used (PrimerDesign # RT-nanoScript). For each RNA sample the following reagents were added to a 0.2ml thin walled PCR tube, see table 2.1 below.

Component	1 Reaction
RNA template (500ng/µl)	X µl
Reverse transcription primer	1.0 µl
RNAse/DNAse free water	X µl
Final volume	**10 µl**

Table 2.1. Reverse transcription reaction mixture.

The samples were then heated at 65°C for 5 minutes using a thermostatically controlled heat block, and promptly returned to dry ice. In order to initiate the extension step the following mastermix was prepared (Table 2.2).

Component	1 reaction
nanoScript 10X Buffer	2.0µl
dNTP mix 10mM	1.0µl
DTT 100mM	2.0µl
RNAse/DNAse free water	4.0µl
nanoScript enzyme	1.0µl
Final volume	**10**µl

Table 2.2 Extensions step reaction mixture.

10µl of this mixture was added to each sample on ice, briefly vortexed, followed by a pulse spin to ensure the reaction mixture was at the bottom of the tube and incubated at 55°C for 20 minutes, followed by a heat inactivation step at 75°C for 15 minutes in the PCR thermocycler.

2.15 Reverse transcription PCR.

cDNA was produced following reverse transcription, as described in section 2.14 and was diluted 1 in 10 with RNase free water and stored at -80ºC until needed for amplification. PCR was completed using the components outlined in section 2.6. A full list of primers can be found in Appendix 7.2. Samples were analysed on a Stratagene MX3000P thermal cycler. The thermal profile was set as follows. 10 minutes at 95ºC, 15 seconds at 95ºC and 1 minute at 60ºC for 40 cycles, 30 seconds at 95ºC, 30 seconds at 55ºC and 30 seconds at 95ºC. Data analysis was conducted using REST 2009 software available from QIAGEN.

2.16 Cell culture assays.

2.16.1 Senescence associated β-galactosidase (SA-β-Gal) assay.

SA-β-Gal staining was performed using β-Gal staining kit (invitrogen # K1465-01) according to manufacturer's instructions. Briefly, growth media was removed from the cells and washed with PBS, then the cells were fixed in 1X fixative solution (2% formaldehyde and 0.2% glutaraldehyde, diluted in PBS) and incubated for 10 minutes at room temperature. The cells were rinsed twice with PBS to ensure the removal of fixative solution and incubated for 24 hours at 37ºC, 5% CO_2 with staining solution containing, 400mM potassium ferricyanide, 200mM magnesium chloride and 20mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) dissolved in N,N'-dimethylformamide (DMF) final concentration of 1mg/ml. Cells were then examined for the development of senescent morphology and blue colour. For long-term storage, staining solution was removed and the wells were overlaid with 70% glycerol and stored at 4ºC. The cells were photographed at low magnification (200x) with the use of a light microscope.

2.16.2 Glucose assay

Glucose utilisation post treatment with senescent and non-senescent media was investigated using the glucose (GO) assay kit (Sigma® # GAGO-20), in AML-12, C2C12 and 3T3-L1. The assay was completed as per manufacturer's instructions with slight modifications. Briefly, the glucose oxidase/peroxidase reagent (Sigma® # G 3660) was reconstituted in 39.2ml of deionised water. The o-dianisidine reagent (Sigma® # D 2679) was reconstituted with 1ml of deionised water and 0.8ml of o-dianisidine was added to the oxidase/peroxidase reagent and inverted to mix. Using the glucose standard solution (Sigma® # D 3285) a 4 point standard curve was set up at the following concentrations 40µg/ml, 30µg/ml, 20µg/ml, 10µg/ml and a blank. Media from cells was diluted 1/200 in deionised water and 50µl of standards and diluted samples were added to separate wells in duplicate, followed by the addition of 100µl of glucose oxidase peroxidase solution. The plate was incubated at 37ºC, 5% CO_2 in a humidified incubator for 30 minutes.

Next the reaction was stopped using 100µl of 12N sulfuric acid, eliciting a color change from brown to pink which was read at 540 nm using an optical plate reader.

2.16.3 Glycogen assay

The ability of each cell line used in this study, to store glucose as glycogen post treatment with or without senescent media was analysed using the glycogen assay kit (Enzychrom # E2GN-100). The assay was completed as per manufacturer's instructions. In summary cells treated with or without senescent media were pelleted and stored on ice. Cell pellets were homogenised using 2.5g/L sodium fluoride, 25mM citrate, pH 4.2 on ice, the cell suspension was then centrifuged at 14,000g for 5 minutes to remove cell debris and 10µl of the supernatant was used for the assay. A glycogen standard curve was prepared at the following concentrations 200µg/ml, 150µg/ml, 100µg/ml, 50µg/ml and 0µg/ml diluted in dH_2O. Samples were diluted in dH_2O 1/10. 10µl of both diluted samples and standards were pipetted into a 96well plate and mixed with 90µl of working reagent, consisting of 90µl Assay Buffer, 1µl Enzyme A, 1µl Enzyme B and 1µl Dye Reagent. The plate was then incubated at 30 minutes at room temperature and read on an optical plate reader at 570nm.

2.16.4 PrestoBlue cell viability assay

PrestoBlue cell viability reagent (Life Technologies # V4) was used to determine if the media derived from NF (non-senescent fibroblasts) and SF (senescent fibroblasts) could reduce cell viability in the AML-12, C2C12 and 3T3-L1 cell lines. Cells were seeded into two 96 well plates and induced to differentiate, as outlined previously. Cells were treated with either normal media as a control, media derived from non-senescent fibroblasts after 24 and 48 hours, and media derived from senescent fibroblasts after 24 and 48 hours. 200µM etoposide was used as positive control to induce cell death. The cell viability assay was completed as per manufacturers

instructions. Cell treatments were removed and then 10x PrestoBlue dye was diluted down to 1x and added directly to cells. Cells were left to incubate for 2 hours and then the optical density was read at two frequencies 570nm and 600nm on a microplate reader.

2.16.5 MitoSOX™ Red mitochondrial superoxide indicator.

MitoSOX red mitochondrial superoxide indicator (Life Technologies # 1575871) was used to determine if senescent media could manipulate cellular respiration, more specifically if senescent media was able to influence reactive oxygen species production. C2C12 cells were grown to confluence and differentiated. Six treatments were established, differentiated cells were treated with either normal media as a control, media derived from non-senescent fibroblasts after 24 and 48 hours, and media derived from senescent fibroblasts after 24 and 48 hours. 5 µM doxorubicin was used as positive inducer of reactive oxygen species. To begin with a 5mM stock solution of mitochondrial superoxide indicator was prepared in dimethylsulfoxide (DMSO). This stock solution was further diluted to 5µM in PBS. Upon completion of the respective treatment incubation periods, cells were overlaid with the working concentration of mitochondrial superoxide indicator and left to incubate at 37ºC, 5% CO_2 in a humidified incubator for 20 minutes. Following staining cells were then washed three times using warm PBS and then photographed using a fluorescent microscope. ImageJ software was used to quantify fluorescence.

2.16.6 Mitotracker mitochondrial probe.

MitoTracker red CMXRos (life technologies # 1453322) was used to stain mitochondria in live differentiated C2C12 cells. 5 cell treatments were administered, cells were treated with either normal media as a control, media derived from non-senescent fibroblasts after 24 and 48 hours, and media derived from senescent fibroblasts after 24 and 48 hours. The experiment was conducted as per manufacturer's instructions, briefly a stock solution of Mitotracker Red

CMXRos was prepared using DMSO at a concentration of 1mM. A working concentration was prepared at a concentration of 100nM in serum free media and heated to 37ºC. Cells were then incubated at 37ºC, 5% CO_2 in a humidified incubator for 45 minutes, following the 45 minute incubation treatment media was removed and the cells were washed with warm PBS and replaced with pre-warmed media. Cells were then photographed using a fluorescent microscope at 100x magnification, exciation wavelength 579 (nm) emission wavelength 599 (nm). ImageJ software was used to quantify fluorescence.

2.16.7 Glycerol assay

To test whether SASP can induce lipolysis in 3T3-L1 adipocytes, media was removed from mature adipocytes cultured under 20% and 40% SASP for 24 and 48 hours. Circulating glycerol a prominent marker of adipocytes undergoing lipolysis was measured using free glycerol reagent (Sigma # F6428). The protocol was adjusted to accommodate the experiment in a 96 well plate. In summary, a 7 point standard curve was prepared ranging from 0.26mg/ml to 0mg/ml. Standards, samples and blank were pipetted into a 96 well plate, followed by 200µl of free glycerol reagent. The plate was then incubated at 37ºC, 5% CO_2 for 10 minutes, and the absorbance read at 540nm.

2.16.8 Nile Red staining

Nile red (Sigma® # 72485) was used to stain 3T3-L1 cells to qualitatively assess differences in lipid droplet size following treatment with senescent media. Briefly 3T3-L1 cells were seeded into 24 well plates and differentiated according to the differentiation protocol outlined previously. Post day 15 of differentiation the cells were treated with 40% senescent media for a period of 24 and 48 hours as discussed previously. Following the treatment cell media was removed and the cells were washed with PBS, prior to the addition of 4% paraformaldehyde in PBS on ice. The 3T3-L1 cells were fixed for 15 minutes and the cells were washed three times with PBS. A Nile

red stock solution was prepared at 1mg/ml in methanol, a working solution was prepared using PBS at a final concentration of 1µg/ml. 400µl of Nile red was added to each well and incubated for 30 minutes at 37ºC, 5% CO_2 and photographed on a confocal microscope. ImageJ software was used to quantify fluorescence.

2.16.9 Statistical tests.

All data generated during the clinical study was primarily analysed using Pearson's bivariate correlations to determine significant associations and put into a stepwise backward linear regression to determine the most significant predictor of the independent variable. Data was analysed using IBM SPSS version 6 and diagrammatical representations were generated using GraphPad prism. PCR data was analysed using Qiagen software REST 2009. Statistical analysis was performed using a two tailed, unpaired student's t-test when comparing the difference between two group means. When comparing column and row means from more than two samples the two way analysis of variance followed by Tukeys Multiple Comparisons test was used. A $P<0.05$ was considered statistically significant. A 95% confidence interval was sued for the difference between all selected pairs of means in the t-test and the ANOVA/Tukey's post test.

Chapter 3: Results

Investigation into metabolic and ageing markers in a healthy, non-obese population

3.1 Introduction

Energy homeostasis has long been recognised as a fundamental modulator of longevity and morbidity [547]. Whilst calorie restriction extends lifespan in a variety of lower organisms including some mammals, caloric excess is thought to be able to have an opposing effect [548-550]. The consequences of aberrant calorie consumption in humans translate directly into changes in body composition [551]. For example, in environments of increased energy intake a consequential increase in adipose mass is observed, whilst a calorific deficit created through reduced calorie intake and/or increases in physical activity can shift the balance towards a reduction in fat tissue and increased muscle mass [552-554]. The significance of body composition, in propagating the development of metabolic disorders which disproportionately affect an individual's lifespan is becoming increasingly apparent.

Adipose tissue, is now recognised to be at the nexus of mechanisms regulating oxidative stress, inflammation, metabolic disease and therefore health span [503, 555, 556]. Accumulating excess adipose tissue can accelerate the onset of multiple age related disorders, including obesity, T2D, cardiovascular disease and cancer [557-559]. The endocrine nature of adipose tissue may also contribute towards either improved health and longevity or facilitating physiological deterioration and metabolic disease. For example leptin and adiponectin, two protein hormones thought to function as opposing forces and substantially influence the propagation of metabolic dysfunction [243, 560]. Hyperleptinemia has been strongly associated with glucose intolerance, insulin resistance and cardiovascular complications [561]. Hyperadiponectinemia on the contrary has been implicated to have cardioprotective effects, induce insulin sensitivity, improve glucose homeostasis and generally protect against the development of metabolic syndrome [243, 562]. A common observation amongst individuals with elevated adipose mass is a marked increase in visceral adipose tissue, previously described as a considerable source of pro-inflammatory cytokines and a significant causative agent in the manifestation of metabolic dysfunction [563-

565]. Research has provided evidence for reduced TL, an established genetic marker of cellular ageing, amongst individuals suffering from metabolic syndrome [566]. Increased telomere attrition has previously been correlated with vascular ageing, arterial stiffening, atherosclerosis, and cardiovascular risk [453, 566-568]. More specifically elevated fasting blood glucose, increased serum lipids and hyperinsulinemia, all parameters of metabolic disease have been inversely correlated with TL [453, 569]. Increased adipose mass has been implicated as a considerable risk factor for developing the previously mentioned health implications and therefore is recognised as a determining factor of accelerated ageing [570]. These data highlight the strong influence dysregulated metabolism can have on healthy ageing. Interventions which attenuate the accumulation of excess adipose tissue and visceral fat, such as engaging in exercise or consuming a calorie restricted diet protect against the development of metabolic disease, thus may promote longevity or increased healthspan [571-573]. Murine models have been used to demonstrate that surgical removal of visceral fat alleviates the symptoms of T2D, upregulates insulin sensitisation and improves glucose homeostasis, an outcome which has been reproduced in calorie restricted mice [574, 575].

The biological benefits of following a calorie restricted diet are apparent, however, due to the lack of adherence to such a strictly regimented diet, recent research is increasingly aimed towards determining the feasibility and efficacy of natural and/or pharmacological calorie restriction mimetic molecules [523]. Resveratrol, 2-deoxyglucose and metformin have all been reported to mimic the effects of calorie restriction, however due to increased inter-individual and intra-individual variances and a lack of longitudinal studies, the beneficial effects of calorie restriction mimetics on longevity warrant further investigation [576]. One potential mechanism, yet to be investigated is the facilitation of energy dissipation through the thermogenic activity of BAT. BAT has developed as an essential thermoregulatory effector, by dissipating stored energy through the production of heat during the challenge of low environmental temperatures, in comparison to its antagonistic adipose counterpart, WAT which is predominantly responsible for

storing excess energy as triglycerides [577]. The thermogenic ability of BAT is attributable to increased mitochondrial density facilitating proton transfer across mitochondrial membranes to produce ATP [578]. Once ATP production reaches a point of saturation excess energy stored in the protons is leaked via UCP-1 and released as heat, inducing "non-shivering" thermogenesis [220]. Human BAT activity was recognised to peak at two years of age, at which point its metabolic function progressively declines with advancing age, until being limited in adolescents and being completely non-functional in adults [216]. Recent studies utilising PET and CT have been successful in discovering BAT in adults, using radioactively labelled fluorodeoxyglucose (FDG) as a means of identifying metabolically active brown fat [222]. Using PET-CT the authors reported increased glucose and nonesterfied fatty acid utilisation, as an energy source to fuel BAT thermogenesis under cold conditions, in accordance with enhanced ^{11}C-acetate uptake, a marker of oxidative metabolism [579]

Bostrom *et al* recently identified, PGC1-α induced overexpression of the FNDC5 gene following aerobic exercise in mice [580]. The FNDC5 gene encodes a type 1 membrane protein that is proteolytically cleaved and secreted into the circulation, termed irisin [580, 581]. Similarly to adipose tissue, skeletal muscle has been identified as an endocrine organ, with the ability to release myokines [581]. Irisin is thought to be a myokine, and is referred to as such in current literature, however there is a paucity of evidence demonstrating its release. Irisin has been reported to manifest an anti-obesity effect by imposing a BAT phenotype, by binding WAT via undetermined receptors and up regulating energy expenditure via increased thermogenesis [582]. It has been demonstrated a lack of BAT and UCP1 increased susceptibility to the development of obesity and insulin resistance in laboratory mice [583]. Human studies provide similar findings, BAT exhibits an inverse relationship with both age and BMI, suggesting a protective role against the development of obesity and thus T2D, two metabolic disorders commonly associated with advancing age [579] .

Irisin may be able to rejuvenate the age associated decrease of metabolically active BAT, resulting in an increment in net energy expenditure, accompanied by a decrease in WAT accumulation, a considerable risk factor for the development of obesity and associated co-morbidities.

3.2 Aims and Hypothesis

Previous research has revealed that obesity significantly reduces lifespan, and that excess fat stored within the abdomen, particularly visceral fat increase the risk of serious and chronic condition such as T2D, cardiovascular disease and cancer. However the exact relationship between adipocyte mass distribution and its association with ageing is poorly understood. Furthermore, it is not clear whether subcutaneous or visceral fat has a larger influence on lifespan dynamics. The primary aim of this chapter was to investigate the potential role of body composition in the ageing process in a healthy, non-obese cohort. By measuring the distribution of fat and muscle using segmental bio-impedance, and TL in a cohort of healthy volunteers, in a bid to clarify the relationship between body composition and ageing at the cellular level. More specifically the role of circulating leptin, adiponectin, and irisin, along with parameters of glucose homeostasis were investigated to understand their role in ageing at the cellular level.

Additionally the relationship between markers associated with endothelial dysfunction and inflammation with telomere length were examined, in a bid to probe associations between body composition and markers of disease risk. Understanding the association between these markers of cell dysfunction and ageing is important for early identification of individuals at risk of enduring premature ageing or an advanced rate of cellular ageing. This chapter will investigate the relationship between body composition and ageing and provide new insights into the mechanisms by which metabolic factors affect the ageing process.

The hypotheses for this study are therefore:

1) Markers of adiposity will negatively correlate with TL

2) Visceral fat score will negatively correlate with TL

3) Circulating irisin will positively correlate with global muscle mass and TL.

3.3. Methods

For complete details of experimental methodology please refer to chapter 2 section 2.1 to 2.8.

3.4. Results

3.4.1 Comparative analysis of anthropometric measurements and biochemical analysis, between healthy male and female volunteers.

Gender differences in anthropometric and biochemical characteristics are exhibited in Table 1. Both males and females were of similar age (44 ± 15.9 and 43 ± 15.4). The males recruited in this study were significantly taller (177 ± 6.9cm, p = <0.01), weighed more (80.9 ± 10.4kg, p = <0.01) and therefore had a higher BMI (25.4 ± 2.5, p = <0.01) in comparison to female volunteers recruited (166 ± 7.8cm, 64 ± 9.2kg and 23 ± 2.8) respectively. As expected females exhibited higher global fat percentages (30 ± 5.9%, p = <0.01) and abdominal fat percentages (26.7 ± 7.4%, P = <0.01), in conjunction with elevated concentrations of the adipokine leptin (11.6 ± 7.33ng/ml, p = <0.01) relative to males (20.6 ± 9.1%, 20.8 ± 7.0% and 5.89 ± 5.81ng/ml). Plasma adiponectin concentrations reveal a sexual dimorphism, with female volunteers having higher levels than males (4.3 ± 2, 2.8 ± 1.4), differences in leptin to adiponectin ratio remained non-significant. Male subjects displayed an expected higher global muscle mass (61.6 ± 7.4kg, p = <0.01) abdominal muscle (33.7 ± 4.0kg, p = <0.01) and a higher visceral fat score (7.6 ± 4.2, p = <0.01) relative to females, in line with existing literature. Interestingly, despite significant differences in both muscle mass and body fat percentage, circulating irisin concentrations were similar between males and females, (46.7 ± 28ng/ml and 46.7 ± 36.3, p = 1).

There were no significant differences in fasting blood glucose, fasting insulin concentration and percentage pancreatic beta cell function between males and females, however males were significantly more insulin resistant (3.5 ± 1.7, p = <0.01) and less insulin sensitive (124.7 ± 68.7, p = <0.01) than recruited female subjects (0.7 ± 0.4 and 161.4 ± 73). With respect to markers of endothelial dysfunction and inflammation measured both males and females had similar concentrations of soluble thrombomodulin, E-selectin and C-reactive protein, all of which were within the normal reference range for healthy individuals.

Healthy Volunteers	Males	Females	Significance
Cohort Size (n)	43	38	
Age (years)	44 ± 15.9	43 ± 15.4	P 0.7
Height (cm)***	177 ± 6.9	166 ± 7.8	**P = <0.001**
Weight (Kg)***	80.9 ± 10.4	64 ± 9.2	**P <0.001**
Body Mass Index (kg/m^2)***	25.4 ± 2.5	23 ± 2.8	**P <0.001**
Total Fat (%)***	20.6 ± 9.1	30 ± 5.9	**P <0.001**
Total Muscle (kg)***	61.6 ± 7.4	41.9 ± 4.5	**P <0.001**
Abdominal (Trunk) Fat (%)***	20.8 ± 7.0	26.7 ± 7.4	**P <0.001**
Abdominal (Trunk) Muscle (kg)***	33.7 ± 4.0	23.4 ± 3.7	**P <0.001**
Visceral Fat Score (0-60)***	7.6 ± 4.2	4.7 ± 2.4	**P <0.001**
Fasting Blood Glucose (mmol/l)	4.6 ± 0.6	4.4 ± 0.8	P = 0.2
Fasting Insulin (mU/L)	8.6 ± 3.9	6.9 ± 3.8	P = 0.051
HOMA β (%)	115 ± 55.4	103.9 ± 49.2	P = 0.3
HOMA IR***	3.5 ± 1.7	0.7 ± 0.4	**P <0.001**
HOMA S*	124.7 ± 68.7	161.4 ± 73	P = 0.02
Telomere Length (T/S ratio)*	2.2 ± 0.5	2 ± 0.3	P = 0.03
Irisin (ng/ml)	46.7 ± 28	46.7 ± 36.3	P = 1
Leptin (ng/ml)***	5.89 ± 5.81	11.6 ± 7.33	**P <0.001**
Adiponectin (µg/ml)	2.8 ± 1.4	4.3 ± 2	**P <0.001**
Leptin/Adiponectin ratio	0.0052 ± 0.0068	0.0054 ± 0.0048	P=0.7
Thrombomodulin (ng/ml)	6.2 ± 2.8	5.6 ± 3.3	P = 0.3
E-selectin (ng/ml)	36.5 ± 31.5	37.9 ± 35.9	P = 0.8
C-reactive protein (µg/ml)	1.15 ± 0.9	0.94 ± 0.75	P = 0.2

Table 3.1 Clinical characteristics and metabolic profiles of male and female subjects studied.

Data is presented as mean ± S.D. for normal continuous variables. A t-test was conducted to test for significant differences between individual variables. Significant differences are highlighted by * and, correspond to a P value of < 0.05. ** Corresponds to a p value < than 0.01 and *** corresponds to a P value < 0.0001.

3.4.2 Anthropometric and biochemical cohort characteristics

Cohort characteristics are displayed in Table 2. The 81 healthy volunteers recruited for this study, mean age of 43 years and mean BMI of 24.3 kg/m^2, exhibited bioimpedance-derived values typical of normal body composition, notably normal proportions of total muscle mass and adipose distribution. Visceral fat scores were within the acceptable range for healthy individuals (1-12), as per determined by the BC-601 Bioimpedanace Analyser Tanita®. Equally fasting blood glucose levels were below 7 mmol/l the widely accepted threshold for diagnosing diabetes. Mean T/S ratio values (2.14 ± 0.47) showed a tight distribution while plasma concentrations of irisin (46.7 ± 32.4 ng/ml), leptin (8.5 ± 7.2 ng/ml), soluble thrombomodulin (5.9 ± 3ng/ml) and E-selectin (36.9 ± 33.2 ng/ml) showed a wider interindividual variation.

	Healthy Volunteers
Cohort Size (n)	81
Men (n)	43
Women (n)	38
Age (years)	43 ± 15.8
Height (cm)	172 ± 9.5
Weight (Kg)	73 ± 13.1
Body Mass Index (kg/m^2)	24.3 ± 2.9
Total Fat (%)	25.1 ± 9.2
Total Muscle (kg)	52.3 ± 11.7
Abdominal (Trunk) Fat (%)	23.6 ± 7.9
Abdominal (Trunk) Muscle (kg)	28.9 ± 6.5
Visceral Fat Score (0-60)	6.2 ± 3.8
Fasting Blood Glucose (m/mmol)	4.5 ± 0.7
Fasting Insulin (mU/L)	7.9 ± 4
HOMA β (%)	110.6 ± 52.8
HOMA IR	2.2 ± 12.3
HOMA S	141.9 ± 73.7
Telomere Length (T/S ratio)	2.14 ± 0.47
Irisin (ng/ml)	46.7 ± 32.4
Leptin (ng/ml)	8.6 ± 7.2
Adiponectin (µg/ml)	3.44 ± 1.87
Leptin/Adiponectin ratio	0.005 ± 0.005
Thrombomodulin (ng/ml)	5.9 ± 3
E-selectin (ng/ml)	36.9 ± 33.2
C-reactive protein (µg/ml)	1.03 ± 0.8

Table 3.2. Clinical characteristics and metabolic profiles of healthy subjects studied.

Anthropometric and biochemical analytes measured in this study. Data is presented as mean ± S.D. for normal continuous variables.

3.4.3 Associations with T/S ratio

Pearson's bivariate correlations between T/S ratio, age, anthropometric measures and biochemical analytes are displayed in Table 3.3. Significant associations are expressed as X,Y scatterplots (Figures 3.1-3.21) and significant association are given in table 3.4 Age ($p < 0.001$), height ($p = 0.036$), total body fat ($p = 0.023$), total muscle ($p = 0.043$), abdominal fat ($p = 0.036$) visceral fat ($p = <0.0001$), leptin ($p = 0.024$) irisin ($p = 0.01$), thrombomodulin ($p = 0.012$) and E-selectin ($p = 0.021$) displayed significant correlation with T/S ratio. Significant associations between thrombomodulin, E-selectin and T/S ratio were most likely due to type-1-error, as a few data points are largely influencing the linear correlations. Significance with 95% confidence was designated at $p \leq 0.05$.

		TS	Age	height	weight	BMI	G_fat	G_muscle	A_fat	A_muscle	V_fat	Lep	Adp	LAR	F_bg	F_in	HOMA_B	HOMA_IR	HOMA_S	Irisin	Throm	E_S	CRP
TS	Pearson Correlation	1	-.560	.233	.110	-.064	-.253	.225	-.234	.193	-.393	-.250	.009	-.176	-.157	.153	.193	-.117	.090	.285	.279	.256	.060
	Sig. (2-tailed)		.000	.036	.329	.572	.023	.043	.036	.084	.000	.024	.939	.115	.160	.172	.084	.298	.423	.010	.012	.021	.592
Age	Pearson Correlation	-.560	1	-.132	-.001	.126	.252	-.158	.328	-.134	.729	.132	.141	.070	.334	-.108	-.255	.113	.016	-.163	-.209	-.337	-.078
	Sig. (2-tailed)	.000		.238	.991	.264	.023	.158	.003	.235	.000	.242	.225	.534	.002	.338	.022	.316	.889	.146	.061	.002	.490
height	Pearson Correlation	.233	-.132	1	.752	.246	-.309	.793	-.160	.758	.170	-.273	-.246	-.110	.044	.208	.163	-.031	-.013	.044	.171	-.007	.022
	Sig. (2-tailed)	.036	.238		.000	.027	.005	.000	.153	.000	.130	.014	.032	.328	.697	.062	.146	.781	.905	.697	.126	.948	.844
weight	Pearson Correlation	.110	-.001	.752	1	.809	-.002	.868	.111	.767	.510	-.007	-.396	.154	.198	.190	.126	-.007	-.156	.044	.141	-.087	.157
	Sig. (2-tailed)	.329	.991	.000		.000	.983	.000	.324	.000	.000	.953	.000	.169	.077	.090	.262	.951	.164	.699	.209	.442	.161
BMI	Pearson Correlation	-.064	.126	.246	.809	1	.326	.552	.383	.438	.620	.261	-.404	.358	.294	.073	-.012	.029	-.219	.027	.066	-.154	.190
	Sig. (2-tailed)	.572	.264	.027	.000		.003	.000	.000	.000	.000	.019	.000	.001	.008	.519	.916	.797	.050	.812	.561	.170	.090
G_fat	Pearson Correlation	-.253	.252	-.309	-.002	.326	1	-.450	.854	-.471	.326	.562	.058	.332	.192	-.099	-.122	-.035	-.069	-.022	-.024	-.143	-.071
	Sig. (2-tailed)	.023	.023	.005	.983	.003		.000	.000	.000	.003	.000	.619	.002	.085	.378	.280	.757	.539	.845	.830	.203	.530
G_muscle	Pearson Correlation	.225	-.158	.793	.868	.552	-.450	1	-.355	.925	.269	-.299	-.406	-.001	.062	.213	.201	.009	-.096	.043	.132	.007	.146
	Sig. (2-tailed)	.043	.158	.000	.000	.000	.000		.001	.000	.015	.007	.000	.996	.580	.056	.073	.937	.395	.702	.241	.951	.195
A_fat	Pearson Correlation	-.234	.328	-.160	.111	.383	.854	-.355	1	-.394	.471	.500	.010	.258	.256	-.137	-.240	.015	-.057	.044	-.019	-.194	-.010
	Sig. (2-tailed)	.036	.003	.153	.324	.000	.000	.001		.000	.000	.000	.934	.020	.021	.221	.031	.891	.615	.697	.869	.083	.927
A_muscle	Pearson Correlation	.193	-.134	.758	.767	.438	-.471	.925	-.394	1	.209	-.329	-.324	-.061	.083	.242	.200	.015	-.091	.051	.097	-.006	.129
	Sig. (2-tailed)	.084	.235	.000	.000	.000	.000	.000	.000		.062	.003	.004	.588	.461	.029	.074	.892	.420	.650	.391	.956	.250
V_fat	Pearson Correlation	-.393	.729	.170	.510	.620	.326	.269	.471	.209	1	.206	-.159	.294	.374	.062	-.086	.140	-.155	-.036	-.103	-.331	.080
	Sig. (2-tailed)	.000	.000	.130	.000	.000	.003	.015	.000	.062		.065	.170	.008	.001	.580	.448	.211	.168	.747	.359	.003	.479
Lep	Pearson Correlation	-.250	.132	-.273	-.007	.261	.562	-.299	.500	-.329	.206	1	.052	.636	.178	.018	-.061	-.044	-.195	-.069	.026	-.113	.270
	Sig. (2-tailed)	.024	.242	.014	.953	.019	.000	.007	.000	.003	.065		.654	.000	.111	.874	.586	.697	.081	.540	.819	.316	.015
Adp	Pearson Correlation	.009	.141	-.246	-.396	-.404	.058	-.406	.010	-.324	-.159	.052	1	-.350	-.115	.030	.090	.042	-.028	.086	.074	.114	.087
	Sig. (2-tailed)	.939	.225	.032	.000	.000	.619	.000	.934	.004	.170	.654		.002	.322	.795	.437	.722	.814	.462	.525	.328	.453
LAR	Pearson Correlation	-.176	.070	-.110	.154	.358	.332	-.001	.258	-.061	.294	.636	-.350	1	.089	.032	.030	-.051	-.214	-.084	.020	-.039	.198
	Sig. (2-tailed)	.115	.534	.328	.169	.001	.002	.996	.020	.588	.008	.000	.002		.432	.779	.792	.650	.055	.456	.861	.729	.077
F_bg	Pearson Correlation	-.157	.334	.044	.198	.294	.192	.062	.256	.083	.374	.178	-.115	.089	1	.191	-.498	-.043	-.278	-.022	.090	-.084	.043
	Sig. (2-tailed)	.160	.002	.697	.077	.008	.085	.580	.021	.461	.001	.111	.322	.432		.087	.000	.703	.012	.843	.425	.457	.702
F_in	Pearson Correlation	.153	-.108	.208	.190	.073	-.099	.213	-.137	.242	.062	.018	.030	.032	.191	1	.631	.037	-.655	-.096	.137	-.005	-.044
	Sig. (2-tailed)	.172	.338	.062	.090	.519	.378	.056	.221	.029	.580	.874	.795	.779	.087		.000	.741	.000	.393	.222	.961	.695
HOMA_B	Pearson Correlation	.193	-.255	.163	.126	-.012	-.122	.201	-.240	.200	-.086	-.061	.090	.030	-.498	.631	1	.009	-.289	-.015	.057	.086	-.044
	Sig. (2-tailed)	.084	.022	.146	.262	.916	.280	.073	.031	.074	.448	.586	.437	.792	.000	.000		.935	.009	.897	.610	.446	.697
HOMA_IR	Pearson Correlation	-.117	.113	-.031	-.007	.029	-.035	.009	.015	.015	.140	-.044	.042	-.051	-.043	.037	.009	1	-.252	.075	.014	-.065	-.010
	Sig. (2-tailed)	.298	.316	.781	.951	.797	.757	.937	.891	.892	.211	.697	.722	.650	.703	.741	.935		.023	.503	.901	.564	.928
HOMA_S	Pearson Correlation	.090	.016	-.013	-.156	-.219	-.069	-.096	-.057	-.091	-.155	-.195	-.028	-.214	-.278	-.655	-.289	-.252	1	.059	-.046	.151	-.088
	Sig. (2-tailed)	.423	.889	.905	.164	.050	.539	.395	.615	.420	.168	.081	.814	.055	.012	.000	.009	.023		.600	.684	.179	.432
Irisin	Pearson Correlation	.285	-.163	.044	.044	.027	-.022	.043	.044	.051	-.036	-.069	.086	-.084	-.022	-.096	-.015	.075	.059	1	.135	.055	.020
	Sig. (2-tailed)	.010	.146	.697	.699	.812	.845	.702	.697	.650	.747	.540	.462	.456	.843	.393	.897	.503	.600		.228	.627	.856
Throm	Pearson Correlation	.279	-.209	.171	.141	.066	-.024	.132	-.019	.097	-.103	.026	.074	.020	.090	.137	.057	.014	-.046	.135	1	.668	.095
	Sig. (2-tailed)	.012	.061	.126	.209	.561	.830	.241	.869	.391	.359	.819	.525	.861	.425	.222	.610	.901	.684	.228		.000	.397
E_S	Pearson Correlation	.256	-.337	-.007	-.087	-.154	-.143	.007	-.194	-.006	-.331	-.113	.114	-.039	-.084	-.005	.086	-.065	.151	.055	.668	1	.120
	Sig. (2-tailed)	.021	.002	.948	.442	.170	.203	.951	.083	.956	.003	.316	.328	.729	.457	.961	.446	.564	.179	.627	.000		.285
CRP	Pearson Correlation	.060	-.078	.022	.157	.190	-.071	.146	-.010	.129	.080	.270	.087	.198	.043	-.044	-.044	-.010	-.088	.020	.095	.120	1
	Sig. (2-tailed)	.592	.490	.844	.161	.090	.530	.195	.927	.250	.479	.015	.453	.077	.702	.695	.697	.928	.432	.856	.397	.285	

Table 3.3. Pearson's Bivariate correlation matrix

Two-tailed pearsons bivariate correlations between T/S ratio, age, anthropometric measures and biochemical analytes. Negative associations are denoted by -. Significance was set at p = ≤0.05, N = 81.

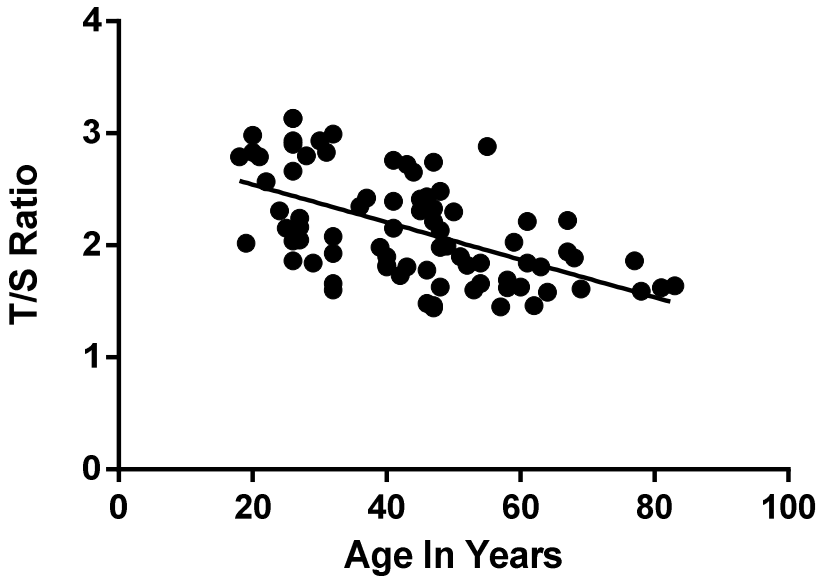


Figure 3.1. correlation of age with telomere length.

Pearson's bivariate correlation represented as a linear regression X, Y scatterplot. In the healthy cohort it was observed a significant negative correlation between chronological age and telomere length ($p = 0.0001$, $R^2 = 0.3$, $n = 81$).

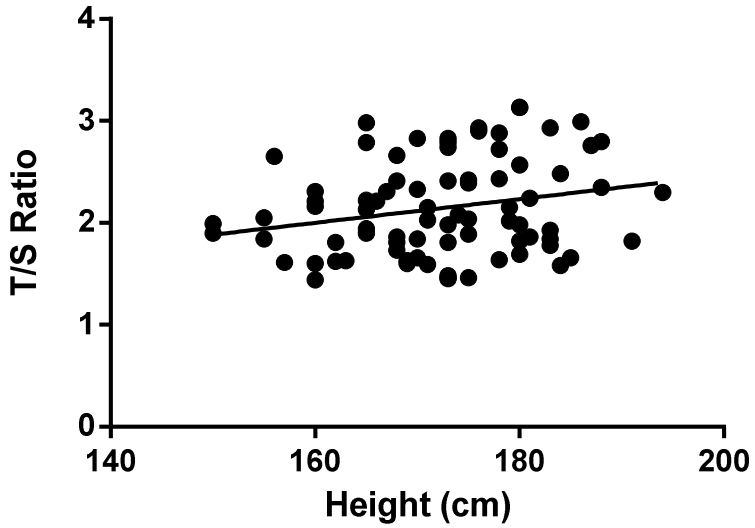


Figure 3.2. Correlation of height in cm with telomere length.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. Height in cm exhibited a significant positive correlation with T/S ratio in the healthy volunteer cohort ($p = 0.03$, $R^2 = 0.05$, $n = 81$).

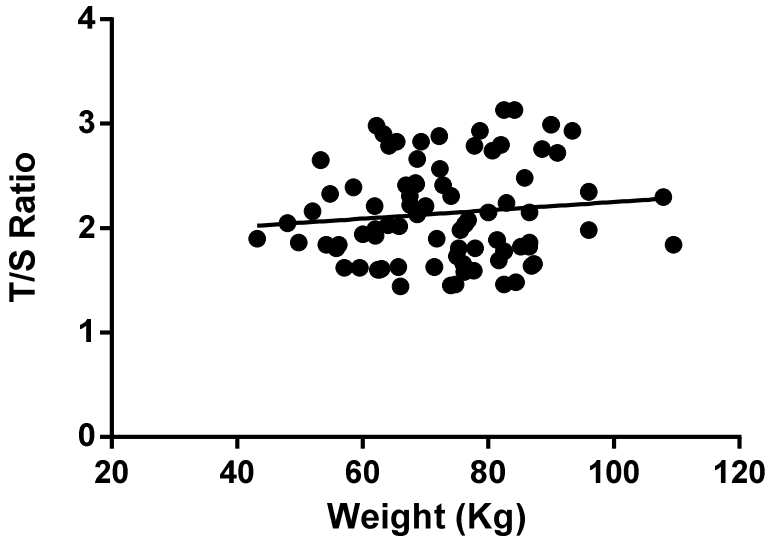


Figure 3.3. Correlation of weight in kg with telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Weight in kg did not exhibit any association with T/S ratio in the healthy volunteer cohort ($p = 0.3$, $R^2 = 0.01$, $n = 81$).

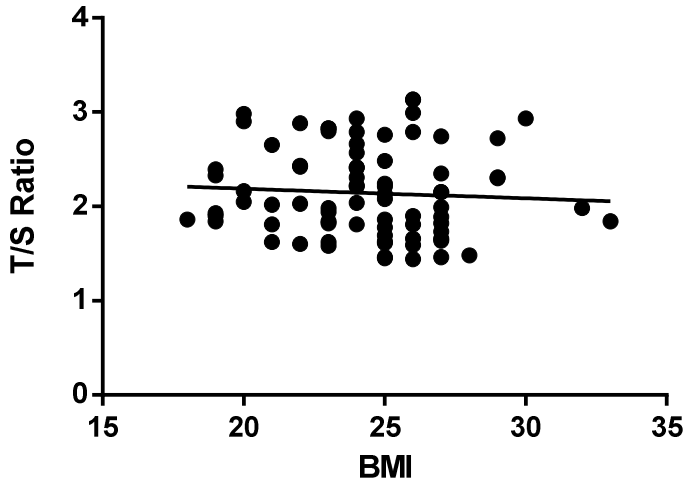


Figure 3.4. Correlation of BMI with telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. BMI failed to exhibit any association with T/S ratio in the healthy volunteer cohort ($p = 0.5$, $R^2 = 0.004$, $n = 81$).

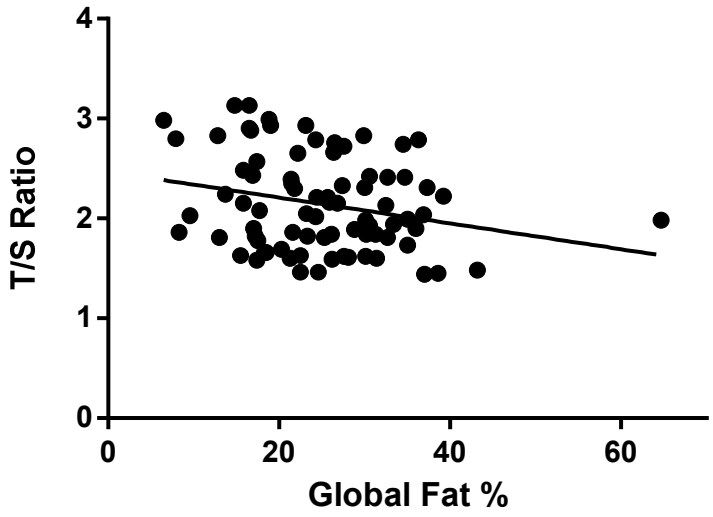


Figure 3.5. Correlation of global Fat % with telomere length.

Pearson bivariate correlations represented as linear regression X, Y scatterplot. Global fat % exhibited a significant negative correlation with T/S ratio in the healthy volunteer cohort ($p = 0.02$, $R^2 = 0.06$, n =81).

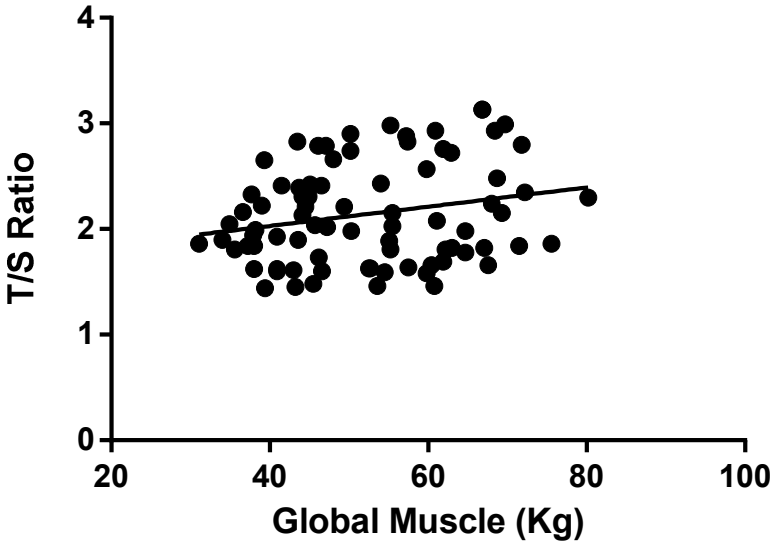


Figure 3.6. Correlation of global muscle (kg) with telomere length.

Pearson bivariate correlations represented as linear regression X, Y scatterplot. Global muscle mass exhibited a significant positive correlation with T/S ratio in the healthy volunteer cohort ($p = 0.04$, $R^2 = 0.05$, n =81).

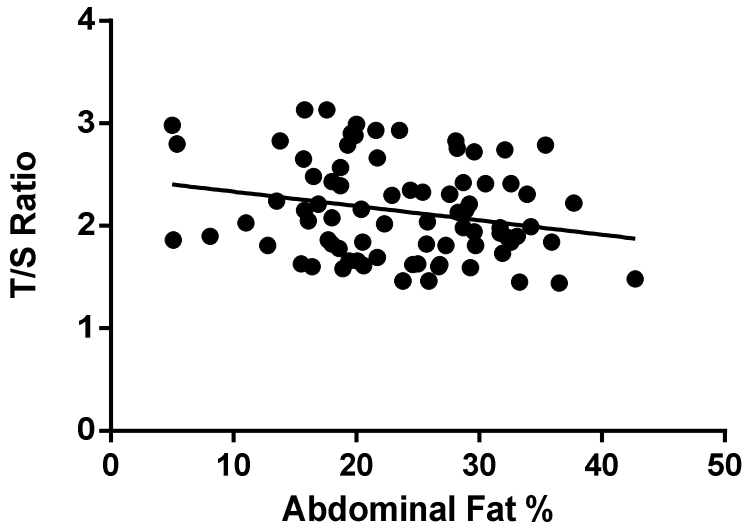


Figure 3.7. Correlation of abdominal fat percentage with telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Abdominal fat % exhibited a significant negative correlation with T/S ratio in the healthy volunteer cohort ($p = 0.03$, $R^2 = 0.05$, $n = 81$).

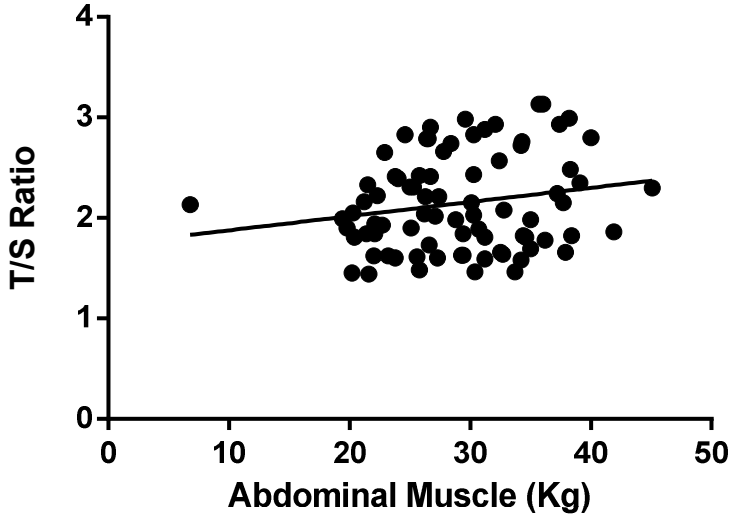


Figure 3.8. Correlation of abdominal muscle (kg) with telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Abdominal muscle in kg did not exhibit a significant association with T/S ratio in the healthy volunteer cohort, ($p = 0.08$, $R^2 = 0.03$, $n = 81$).

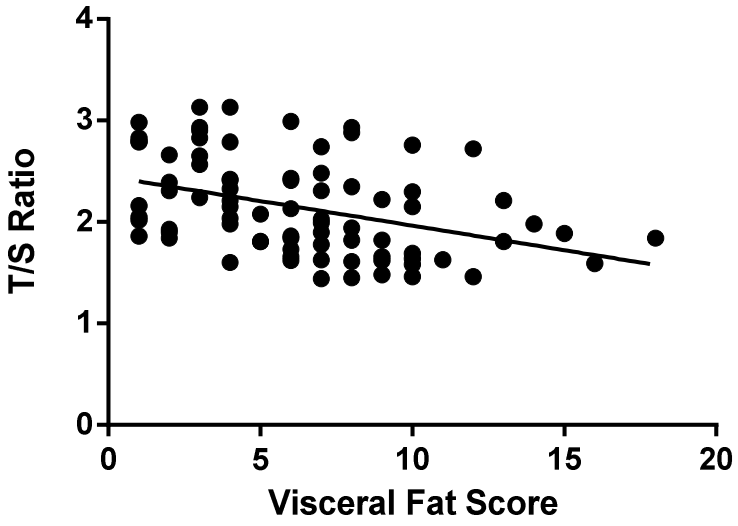


Figure 3.9. Correlation of visceral fat score with telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Visceral fat score exhibited a significant negative correlation with T/S ratio in the healthy volunteer cohort ($p = 0.0003$, $R^2 = 0.1$, $n = 81$).

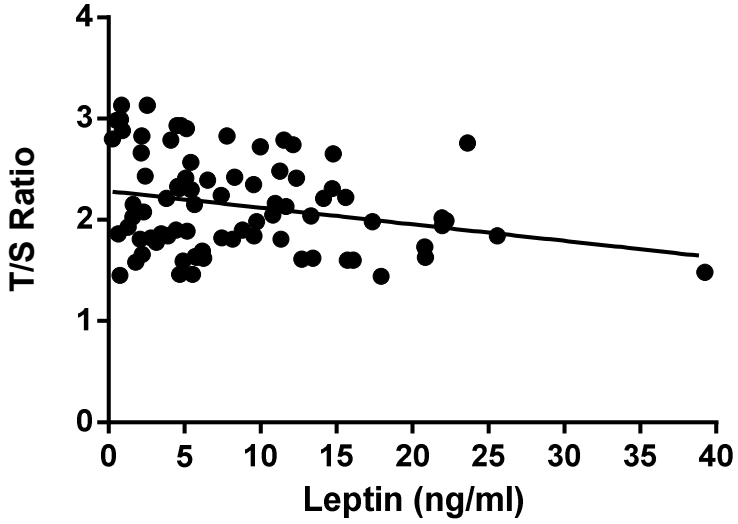


Figure 3.10. Correlation of circulating leptin with telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Circulating leptin exhibited a significant negative association with T/S ratio in the healthy volunteer cohort ($p = 0.02$, $R^2 = 0.06$, $n = 81$).

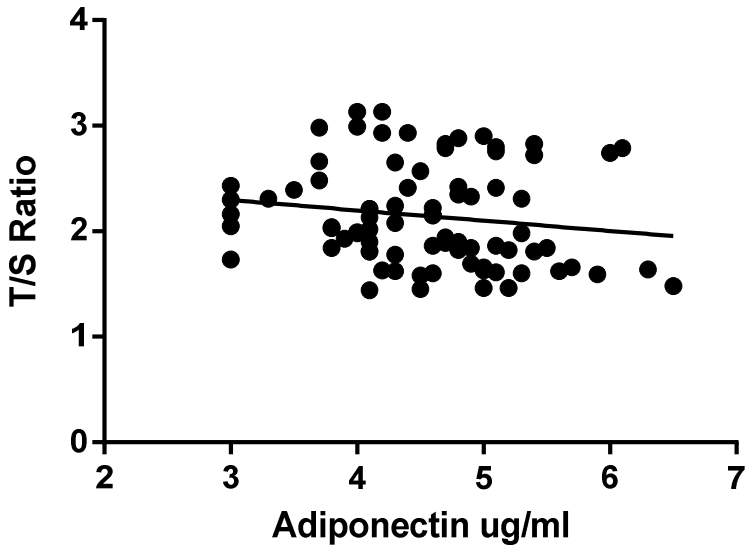


Figure 3.11. Correlation between circulating adiponectin and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Circulating adiponectin levels did not exhibit any association with T/S ratio in the healthy volunteer cohort ($p = 0.1$, $R^2 = 0.02$, n= 81).

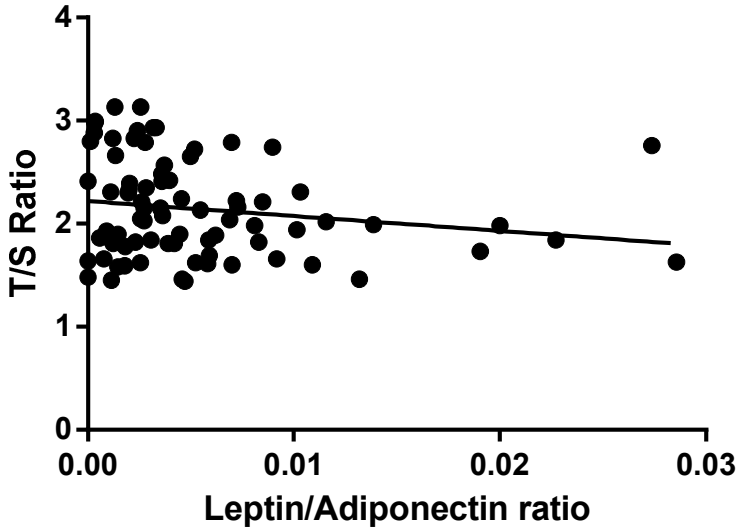


Figure 3.12. Correlation between leptin:adiponectin molar ratio and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. The leptin:adiponectin ratio did not exhibit any association with T/S ratio in the healthy volunteer cohort ($p = 0.11$, $R^2 = 0.03$, n= 81).

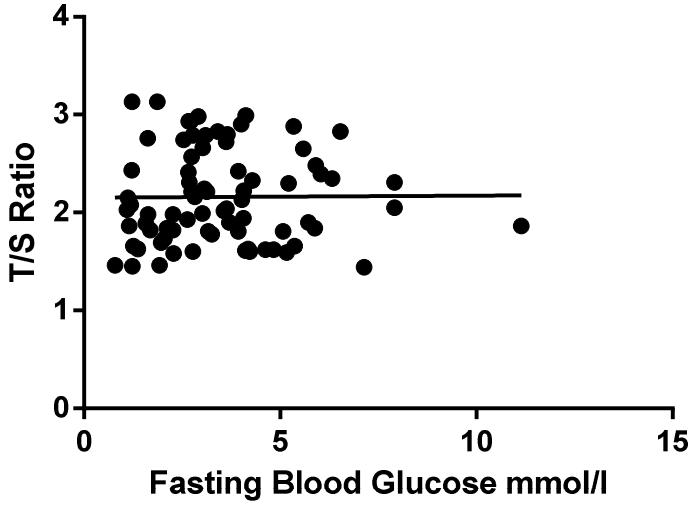


Figure 3.13. Correlation between fasting blood glucose and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Fasting blood glucose did not exhibit a significant association with T/S ratio in the healthy volunteer cohort ($p = 0.1$, $R^2 = 0.02$, $n = 81$).

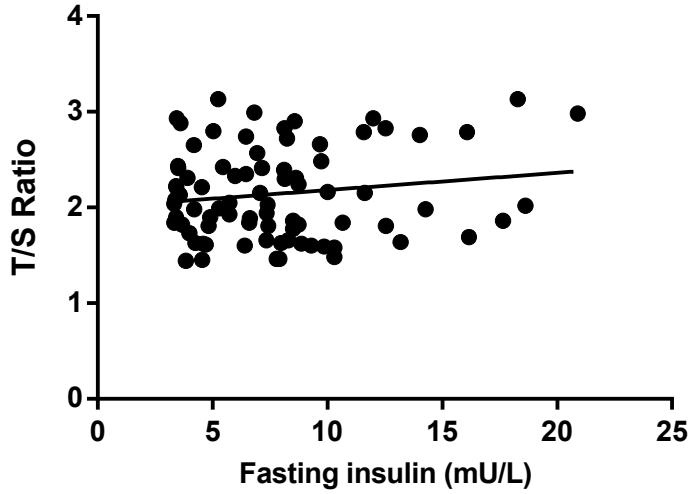


Figure 3.14. Correlation between fasting insulin and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Fasting insulin (mU/L) did not exhibit a significant association with T/S ratio in the healthy volunteer cohort ($p = 0.1$, $R^2 = 0.02$, $n = 81$).

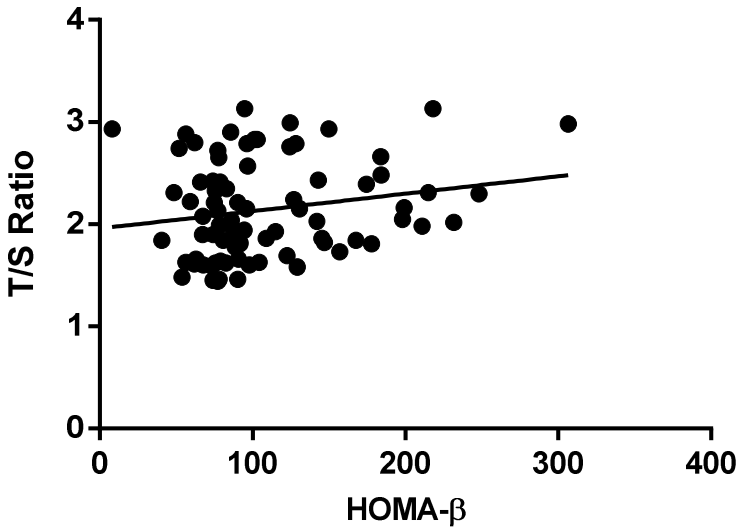


Figure 3.15. Correlation between HOMA-β and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. HOMA-β did not exhibit a significant association with T/S ratio in the healthy volunteer cohort ($p = 0.08$, $R^2 = 0.03$, n= 81).

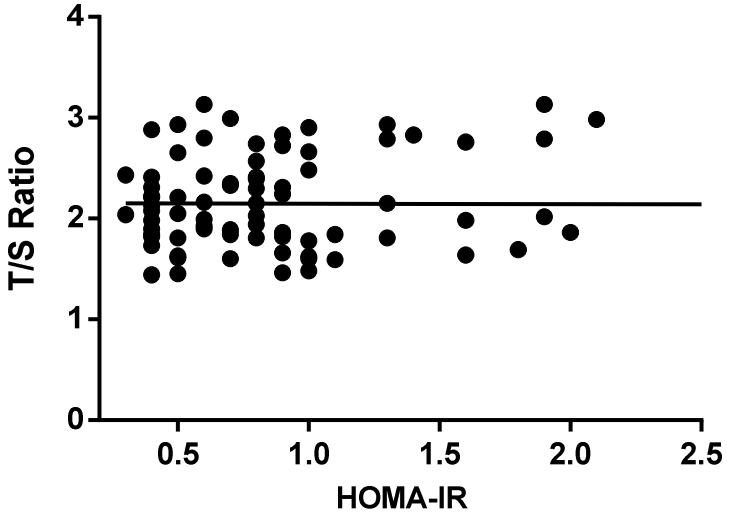


Figure 3.16. Correlation between HOMA-IR and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. HOMA-IR did not exhibit a significant association with T/S ratio in the healthy volunteer cohort ($p = 0.3$, $R^2 = 0.01$, n= 81).

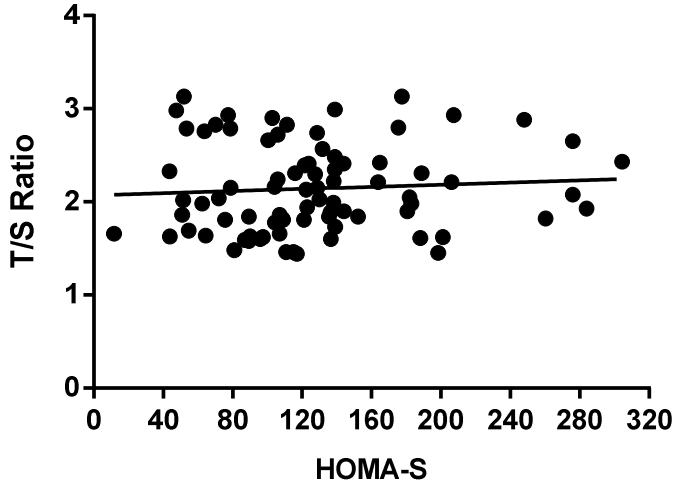


Figure 3.17. Correlation between HOMA-S and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. HOMA-S failed to exhibit a significant association with T/S ratio in the healthy volunteer cohort ($p = 0.5$, $R^2 = 0.005$, n= 81).

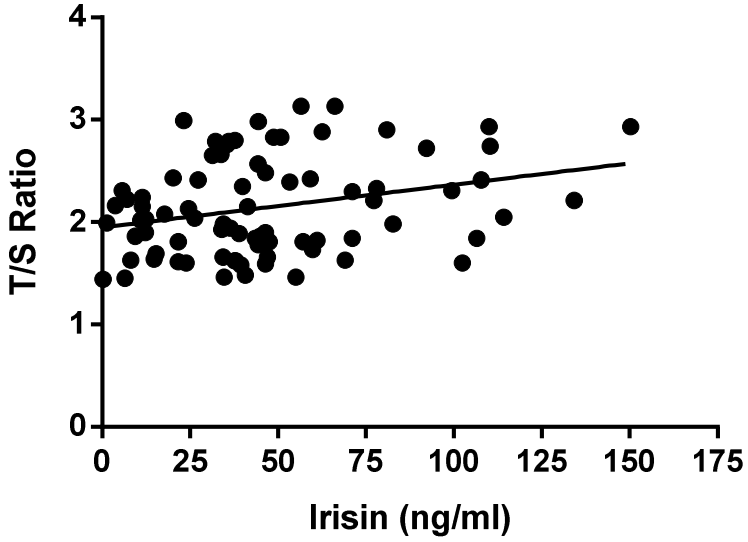


Figure 3.18. Correlation between circulating Irisin and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Circulating irisin exhibited a significant positive correlation with T/S ratio in the healthy volunteer cohort, this association is novel and has not been reported previously ($p = 0.01$, $R^2 = 0.08$, n=81).

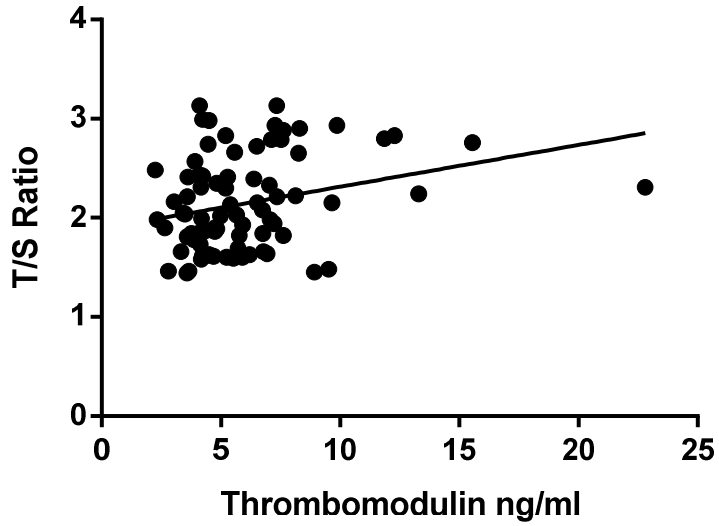


Figure 3.19. Correlation between circulating thrombomodulin (ng/ml) and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Circulating thrombomodulin (ng/ml) exhibited a significant positive correlation with T/S ratio in the healthy volunteer cohort ($p = 0.01$, $R^2 = 0.07$, n= 81).

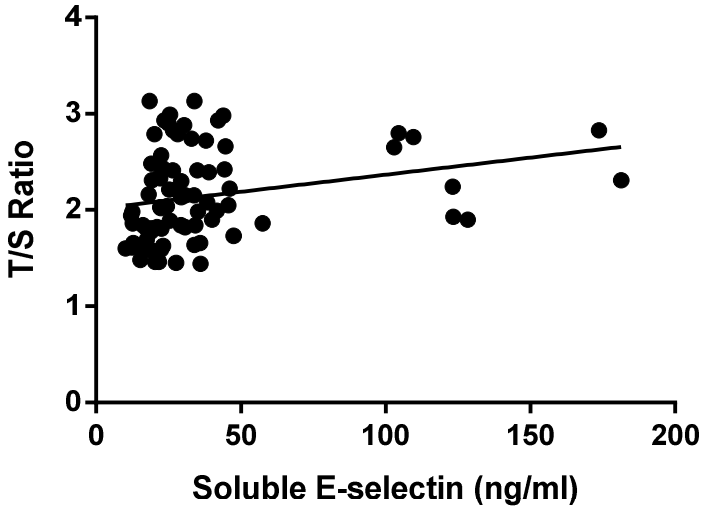


Figure 3.20. Correlation between soluble E-selectin (ng/ml) and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Soluble E-selectin (ng/ml) exhibited a significant positive correlation with T/S ratio in the healthy volunteer cohort ($p = 0.02$, $R^2 = 0.06$, n= 81).

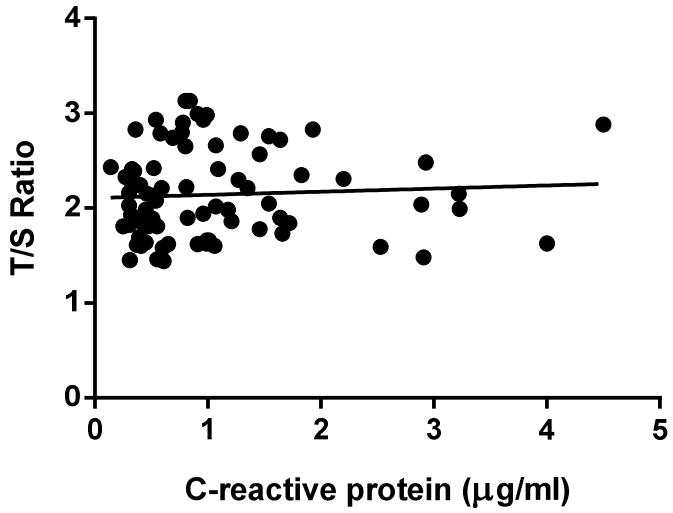


Figure 3.21. Correlation between circulating C-reactive protein and telomere length.

Pearson bivariate correlation represented as linear regression X, Y scatterplot. Circulating C-reactive protein (µg/ml) did not exhibit a significant correlation with T/S ratio in the healthy volunteer cohort ($p = 0.5$, $R^2 = 0.003$, $n = 81$).

Measurement	Association with T/S ratio	Significance
Age (years)	-0.560	<0.001
Height (cm)	0.233	0.036
Total Fat (%)	-0.253	0.02
Total Muscle (kg)	0.225	0.04
Abdominal (Trunk) Fat (%)	-0.234	0.03
Visceral Fat Score (0-60)	-0.393	<0.001
Leptin (ng/ml)	-0.250	0.02
Irisin (ng/ml)	0.285	0.01
Thrombomodulin (ng/ml)	0.279	0.012
E-selectin (ng/ml)	0.256	0.021

Table 3.4. Summary of all significant Pearson's bivariate correlations observed in this study.

All statistically significant associations between anthropoimetric/biochemical analyst measured in this investigation and TL are summarised herein. Positive correlation coefficients imply an increase with increase TL and negative correlation coefficients suggests a decrease in the respective variable with a decrease in TL. Significance was set at $p \leq 0.05$.

3.4.4. Correlation of multiple factors with telomere length - stepwise backward multiple linear regression

Using statistical package IBM SPSS version 20 stepwise backward multiple linear regression analysis was conducted. Using this method the dependent variable was identified as T/S ratio and statistically significant correlations determined using Pearson's bivariate correlations were entered as the independent variables (Table 4) into the regression model. The stepwise backward function enters all variables into the model and then subsequently eliminates each variable based upon the effect of the variable on the significance of the model. This method eliminates all variables which are poor at predicting T/S ratio. The model summary highlights that 38% of the variability in T/S ratio can be predicted by the final variables selected by the model. The analysis of variance indicates the final combination of variables selected that are most efficient in predicting T/S ratio, have been incorporated into a statistically significant model with a P value of <0.0001.

Model Summary				
Model	**R**	**R Square**	**Adjusted R Square**	**Std Error of the Estimate**
6	0.617	0.380	0.356	0.37929

Table 3.5. Model summary for healthy, non-obese volunteers.

The final combination of variables selected that are best able to predict T/S ratio, account for 38% of the variability in the T/S ratio's.

ANOVA				
Model	**Sum of Squares**	**df**	**Mean Square**	**Significance**
Regression	6.799	3	2.266	< 0.000
Residual	11.077	77	0.144	
Total	17.876	80		

Table 3.6. The analysis of variance table for both male and female models.

ANOVA analysis shows that for non-diabetic volunteers, the variables (see Table 5) selected are significant predictors of T/S ratio. This confirms the significance of the model.

Significant correlations determined using the Pearson's bivariate correlation test, age, height, total fat, total muscle, abdominal fat, visceral fat, leptin and irisin (Table 3.4) were analysed using the backward multivariable linear regression, to test which of these significant association could predict T/S ratio. The backward multivariable linear regression identified age and circulating irisin as the only significant predictors of T/S ratio in the model, p <0.0001 and p = 0.04 respectively. Interestingly age exhibited a negative beta coefficient of -0.506, whilst irisin exhibited a positive beta coefficient of 0.191 with regards to T/S ratio. The positive beta value associated with irisin denotes the dependent variable (T/S ratio) has increased in response to an increase in the independent variable (irisin), this is novel association and suggests irisin has a role in modulating telomere length and therefore influencing healthy ageing. A negative beta value, as associated with the independent variable chronological age suggests the dependent variable has decreased in response to an increase in the independent variable, which is an association which would be expected and has been previously reported. Circulating leptin was included in the final model however was a not a significant predictor of T/S ratio, with a p value of 0.064; however with a negative beta coefficient the data suggests that leptin too would express a similar trend to the association between age and T/S ratio. Biologically a higher leptin concentration would coincide with increased adipose mass, a well-recognised contributor to telomere attrition and decreased lifespan. All other variables were excluded from the analysis.

Coefficients				
Variable	**Beta**	**Standard Error**	**Significance**	**95% confidence interval**
Age	-0.506	0.003	<0.0001	-0.021 - -0.01
Leptin	-0.170	0.006	0.064	-0.023 – 0.001
Irisin	0.191	0.001	0.04	0.000136 – 0.005

Table 3.7. Stepwise backward linear regression analysis using T/S ratio as a dependent variable and significant correlations as independent variables.

The backward stepwise multivariable regression eradicated all variables which were unable to predict T/S ratio and selected Age (p =<0.0001) and irisin (p = <0.04) as the two most significant variables that were able to predict T/S ratio.

3.5 Discussion

This study was undertaken in an attempt to better understand any associations between ageing (as measured by telomere length), anthropometric measurements, and circulating molecules associated with metabolism, in healthy volunteers in order to examine how body composition and associated circulating factors may be able to regulate ageing at the cellular level and whether circulating irisin, a newly discovered myokine can influence cellular ageing. The primary findings of this section of the study can be summarised as the following; the reduction in TL with chronological age is well recognised and, as expected, was confirmed by the inverse relationship between age and TL in this cohort (Fig.3.1, $p < 0.0001$), confirming that the technique used provides expected data. TL also exhibited a significant negative association amongst individuals retaining excess body fat (Fig.3.5. $p = 0.02$), to a lesser extent abdominal fat (Fig.3.7 $p = 0.03$) and the strongest association was observed with visceral fat (Fig.3.9 $p < 0.001$).

Lean body mass, in the form of muscle was conversely positively correlated with TL (Fig.3.6 $p = 0.04$) and to the authors knowledge this is a novel observation. In spite of these associations, none of the anthropometric measurements obtained were significant predictors of TL and it is therefore difficult to assign great significance to them in a cohort of this size. They suggest a pattern, adipose tissue and muscle having opposing relationships with TL, but no directionality of that relationship. A significant inverse correlation was also observed between TL and circulating leptin (Fig.3.10 $p = 0.02$), although again leptin could not mathematically predict TL. This study is the first to investigate potential associations between plasma irisin and TL. A novel finding of this study is that plasma irisin levels positively correlate with TL (Fig. 3.18 $p = 0.01$) and furthermore can predict TL in a mathematical model adjusted for chronological age.

The precise mechanisms responsible for the reduced TL amongst individuals retaining large amounts of subcutaneous and visceral adipose tissue observed herein remains unclear; however oxidative stress and inflammation have been implicated by previous research [430, 542]. Adipose tissue of late has been recognised as a metabolically active endocrine organ. Amongst the many bio-peptides and hormones it is able to release, adipose tissue also serves as a reservoir of inflammatory cyto/chemokines, particularly visceral adipose tissue [555, 584]. Events leading to adipose tissue inflammation have been investigated extensively in murine models. Rodents consuming a calorific diet upregulate the production of inflammatory cyto/chemokine and extracellular matrix modifying protein within days. Subsequently initiating the augmentation of $CD8^+$ effector T lymphocytes, along with a concomitant increase in pro-inflammatory cytokines [585]. Fontana *et al* reported visceral fat is a considerable source of IL-6 and TNF-α secretion, moreover IL-6 concentrations correlated with circulating CRP. Combined or individually these inflammatory cytokines have been associated with dysregulated glucose homeostasis, insulin resistance and cardiovascular complications, facilitating the development of age related disorders as well as directly contributing to premature ageing [586].

An integral source of inflammatory cytokine generation from adipose tissues stems from the increased infiltration of classically activated M1 macrophages, into adipose tissue [587, 588]. A review by Zeyda & Stulnig documents the role of various chemotactic proteins and receptors in facilitating macrophage migration to adipose tissue, of which the chemotactic chemokines, Mcp-1,2,3, RANTES and MIP-1α have been quantified to greater extents in individuals with increased central adiposity in comparison to leaner participants [570, 587]. Upon activation, macrophages initiate a positive feed-back loop, secreting more inflammatory cytokines that lead to further production of MCP-1 and other chemokines, inducing further macrophage, recruitment, infiltration and inflammation, in a cascade effect [589].

The adipokine leptin, reported here to be negatively correlated with TL, further facilitates this situation and has been reported to encourage inflammation. Circulating leptin can be best described as an adipokine with hormetic functions, while at lower doses leptin regulates dietary intake and has been documented to have beneficial effects on both the innate and adaptive immune system [590, 591]. At higher concentrations leptin has been associated with increased production of proinflammatory cytokines TNF-α, IL-1 and IL-6, and in a positive feedback loop these cytokines increase the expression of leptin mRNA which subsequently sustains leptin production [590, 592, 593]. Leptin is evidently a positive regulator of acute inflammation however has also been associated with chronic inflammation. Increasing concentrations of leptin are found in patients suffering from inflammatory disorders including multiple sclerosis, Crohn's disease and osteoarthritis[591].

It may be speculated participants recruited in this study that were recorded as carrying larger adipose stores secrete larger amounts of leptin, and are therefore potentially subjected to a chronic low grade inflammation. Excess visceral fat may further exacerbate this effect and in reality may be a vital contributor to systemic inflammation. Excessive cell turnover is a hallmark of inflammatory episodes, it is possible the increased metabolic needs of a larger individual elicit angiogenesis, in combination with the plethora of pro inflammatory cytokines secreted marginally by subcutaneous and predominantly by visceral adipose tissue promote WBC proliferation. Firstly to maintain the larger circulatory blood pool of an individual with a greater surface area, and secondly as a direct result of the increased circulation of pro-inflammatory cytokines. The combined effect leads to the increase in total number of circulating WBCs and the resulting cell turnover are responsible for the increase in telomere erosion. Furthermore accumulated exposure to inflammation is marked by heightened oxidative stress, a phenomenon that individually presents as a parameter of advancing age [594].

Oxidative stress, and more specifically reactive oxygen species (ROS) display a strong biochemical interplay with inflammation. As visceral fat mass expands, the larger number of adipocytes secreting increasing levels of ROS have been positively correlated with inflammatory adipokines [595]. However ROS are far more prominent modulators of telomere dynamics, because telomeric sequences consist largely of guanine residues thus are susceptible to oxidative attack due to the low electron potential of guanine, contributing substantially to cellular senescence [596, 597]. Senescent cells harbour up to 30% more oxidative modified guanine in their DNA and four times as many free 8-oxo-deoxyguanosine bases [598]. Furthermore, cells harbouring telomeric DNA subjected to chronic ROS exposure have been reported to become deficient in DNA repair capabilities [599]. Excessive exposure to oxidative stress is also known to hinder telomerase function, on the contrary, antioxidants significantly prolong telomerase activity [600, 601]. *in vitro* studies have identified the beneficial effects of antioxidant vitamins on TL maintenance, and report ascorbic acid derivatives promote replicative life span in vascular endothelial cells [602].

Fig 3.22. Hypothetical model of the events leading to adipose tissue inflammation and their effects on telomere biology.

The cellular stress encountered during increased adiposity, including replicative stress, glucose toxicity and the production of metabolites influences the secretion of chemokines which trigger an immune response. The augmentation of $CD8^+$ lymphocytes propels an increase in pro-inflammatory cytokines eliciting an immune reaction lead by the classically activated predominantly pro-inflammatory M1 macrophages which engulf the adipose tissue and upregulate the expression of macrophage chemotactic proteins MCP-1 etc. The net result is an overall increase in inflammation and a consequential increase in oxidative stress, resulting in telomere dysfunction the impairment of stem cell regeneration and a loss of cell proliferation and repair. The combined effect leads to systemic inflammation along with enhanced oxidative stress facilitating the development of metabolic disorder along with accelerated cellular ageing.

Enhanced muscle mass can be obtained as a direct result of regular physical exercise, activity that normally results in a concomitant decrease in adipose tissue and therefore an overall reduced load of inflammatory cytokines and ROS [603]. It would not be unrealistic to assume individuals engaging in regular exercise avoid the accelerated rate of telomere erosion triggered by oxidative stress and inflammatory episodes. At the time of writing this study is the first to report a positive association between skeletal muscle mass and TL in healthy volunteers and provides evidence that the benefits of fat free mass translate onto TL. Rosa *et al* recently conducted a mouse study to evaluate the effect of exercise on mRNA and protein expression of IL-10, TNF-α and IL-6 in different types of skeletal muscle. The authors reported, moderately intense bouts of exercise significantly down regulate the expression of inflammatory cytokine mRNA and protein expression in exercised mice, particularly in type 2 muscle fibres. A separate investigation reproduced these results in humans and reported reduced concentration of serum TNF-α, IL-1β, IL-6 and iNOS in the skeletal muscles of exercised participants [604]. The authors concluded the anti-inflammatory effect of exercise attenuates the catabolic wasting associated with chronic heart failure. IGF-1 mediated suppression of toll-like receptor mediated inflammation cascades may in part be responsible for the anti-inflammatory effects of exercise [413]. IGF-1 itself has been reported to be positively associated with TL [605].

Energy consumed during bouts of exhaustive exercise is replenished by increased oxygen consumption by mitochondria to facilitate cellular respiration, resulting in a consequential increase in free radical formation [606]. However there now exists considerable evidence to suggest low grade ROS generated during exercise function to increase the tolerance of ROS themselves and help to induce adaptation (Fig 21). This is largely due to the activation of MAPKs (p38 and ERK1/ERK2), resulting in the activation of NF-κB and increase in endogenous antioxidant enzymes including, superoxide dismutase, glutathione and peroxidase [607, 608]. Valle *et al* suggests PGC-1α, could be involved in the transcriptional regulation of the mitochondrial antioxidant defence system, following exercise [609].

-α

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Figure 3.23. The benefits of moderate ROS exposure, induced by exercise.

Low dose exposure to ROS activates signaling mechanisms which confer adaption to ROS by upregulating antioxidant molecules i.e. glutathione peroxidase and superoxide dismutase. Low levels of ROS induce upregulation of IGF-1, which induces muscle growth, differentiation, contributes to the oxidant-resistant phenotype as well as down regulating inflammatory cytokines. ROS can also enhance expression of transcriptional coactivator PGC1-α which regulates genes involved in energy metabolism preventing oxidative damage and chronic diseases. Mild ROS exposure can activate AMPK and GLUT4 translocation to the surface of the plasma membrane, facilitating increased glucose uptake by skeletal muscle. ROS also facilitates calcium release and contributes to increased muscle contraction and strength.

Bostrom *et al* identified the integral role of PGC-1α in the series of events which lead to the eventual secretion of irisin, from what currently is thought to be from skeletal muscle. At the time of writing this study was the first to examine the association between plasma irisin and TL. Plasma irisin levels in our cohort were shown to be only correlated with TL, and no association was observed with any other factor measured. Since plasma irisin correlates with TL ($p = 0.01$) and can also predict TL ($p = 0.04$), irisin may serve as a hormone which can prevent stress induced cellular senescence. Although the precise mechanisms through which irisin can modulate TL in PBMCs is as yet unknown. Existing data has shown that irisin activates signalling pathways associated with the regulation of cellular proliferation including p38 MAPK which has previously been shown to regulate expression of human telomerase reverse transcriptase [610, 611]. It is also possible that the association reported here is due to indirect effects involving WAT. Because irisin secretion is believed to be modulated by PGC-1α following exercise and is secreted from skeletal muscle, although no such association was observed in this study, the benefits of increased serum irisin concentration on TL may in part be due to the antioxidant and anti-inflammatory effects instigated by exercise discussed previously. Whether irisin can elicit an anti-inflammatory or antioxidant effect warrants further investigation.

Other studies have reported increased circulating irisin reduces fasting glucose concentrations and improves insulin sensitivity in both mice and humans; potentially mitigating the detrimental effects of obesity and T2D, two metabolic disorders associated with decreased TL [580, 612, 613]. Although the precise mechanisms behind this observation are not completely understood, the ability of irisin to increase expression of UCP1 and thus cause a 'browning' shift in WAT may be likened to caloric restriction. This manipulation of cellular energy balance has the potential to induce a CR-like state via modulation of WAT function, as excess energy would be dissipated as heat upon exposure to irisin. Irisin may therefore impact the ageing process, by releasing stored energy through non-shivering thermogenesis and thus mimicking CR mechanisms, which have been proven to promote longevity [523, 614]. Supporting this concept is a study which reported an inverse association between calorie intake and leukocyte TL; this

recognised the abrogating effect of oxidative stress and inflammation [615].Furthermore, CR has been shown to delay telomere shortening in rodents, whilst simultaneously upregulating the TERT responsible for elongating the telomere sequence [615, 616]. If the benefits of irisin are to be mediated via mechanisms similar to or of calorie restriction, the effect of increased irisin concentration on SIRT1 expression requires investigation. The SIR2 mammalian ortholog SIRT1 functions as a NAD+ deacetylase increasing resiliency to oxidative stress, a process mediated by cellular interactions with members of the Fork head transcription factors [617, 618]. More specifically deacetylation of FOXO3 is known to provide protection from cell cycle arrest by upregulating DNA repair efficiency and enhancing resistance to oxidative stress [619]. SIRT1 has been reported to improve TL maintenance in vivo and aid genome preservation by augmenting homologous recombination at telomeres, centromeres and chromosome arms [620].

Within this study several anthropometric and biochemical parameters failed to exhibit correlations with TL, that otherwise might have been expected to. The data from this study did not find a significant correlation between BMI and TL. BMI has long been utilised as a clinical diagnostic tool and its credibility warrants re-evaluation. The results herein clearly demonstrate that body composition, is not only a more detailed account of an individual's biological make up but also serves as much more accurate measure of physiological state. The conventional method of measuring height and weight to deduce BMI does not take into account, how much muscle; fat and more important visceral fat are contributing to the weight measurement. Previous studies have also failed to find correlations between BMI and TL.

Given the anti-inflammatory, anti-oxidant and cardioprotective effects of adiponectin a positive association with TL might have been expected but was not observed. There seems to be controversy surrounding the beneficial effects of adiponectin. Adiponectin circulates in the system in multimeric isoforms, at high, middle and low molecular weights, with different studies reporting different isoforms as clinically significant [621-623]. The low molecular weight isoform was analysed in this study and although has been reported to be biologically relevant, the

high molecular weight isoform is generally regarded to have insulin sensitising and cardio protective effects [624]. It may be possible no correlation between TL and adiponectin was observed due to the incorrect isoform being investigated. On the contrary various studies have reported increasing concentrations of the high molecular weight isoform in the elderly, implying ageing is associated with increased adiponectin. Thus adiponectin secretion may be a compensatory mechanism in individuals suffering inflammatory and oxidative attack and therefore its role in longevity is of more prominence in patients suffering metabolic dysfunction. Broer *et al*, collated data from seven independent studies examining 11,448 healthy participants, examining associations between adiponectin and TL. Adiponectin initially displayed a borderline correlation with TL, a relationship which was lost upon the removal of one outlier study [625].

Five parameters of glucose homeostasis were assessed in this study, none of which correlated with TL. Because the participants recruited in this study were healthy and free of metabolic disorder, factors secondary to retaining excess adipose and visceral mass, and associated with accelerated ageing such as hyperglycaemia, hyperinsulinemia, increased insulin resistance and decreased beta cell function were not observed in this study. This suggests that adipose mass is the primary modulator of TL and only at higher levels of body fat do the previously mentioned indicators of glucose homeostasis influence TL. However it is expected that individuals suffering from complications like obesity and T2D are at increased risk of accelerated ageing and insulin sensitivity, beta cell function, glucose/insulin concentrations are likely to be vital contributors to TL. CRP, soluble thrombomodulin and E-selectin were analysed in an attempt to identify if markers of inflammation and/or endothelial dysfunction, two phenomena strongly associated with advancing age could predict TL. All three, according to current literature should exhibit negative associations with TL, however none actually did correlate with TL. The role of circulating E-selectin in adipose mass associated inflammation may not be significant or E-selectin shedding and endothelial activation may occur only at higher levels of body fat that would not be acceptable in the healthy control population. In which case E-selectin may be a pivotal determinant of TL in an obese/diabetic cohort.

Likewise circulating levels of CRP detected in the healthy cohort may have been considerably below the threshold required to induce changes in TL. CRP release from hepatocytes is a non-specific acute response to inflammation, CRP rises within two hours of the onset of inflammation, up to a 100 to 1000-fold, and peaks at 48 hours [626]. Its half-life of 48 hours is constant, and therefore its level is determined by the rate of production and hence the severity of the precipitating cause. The CRP concentrations observed in this investigation were narrowly distributed with the majority of volunteers displaying CRP concentrations within the healthy reference range. It may be likely chronic exposure to CRP at higher concentrations is required to manifest enhanced telomere erosion, such as in individuals suffering from metabolic dysfunction.

3.6 Conclusion

The data presented here demonstrates stark anthropometric variability amongst individuals of good health, and successfully identifies adipose tissue and skeletal muscle mass as two opposing facrors, each associated with TL. Whilst increased adipose tissue was associated with decreased TL and enhanced cellular ageing, the latter has been reported to safeguard against accelerated ageing here and promote longevity. Furthermore the significance of adipose tissue distribution came to light as, central adiposity, in particularly visceral fat displayed the most significant negative correlation with TL. At this current stage it can be merely assumed that adipocyte mediated oxidative stress and inflammation are the predominant contributors to telomere erosion. Therefore it is of both interest and necessity to analyse more markers of both oxidative stress and inflammation in order to confirm the association between adipose related oxidative stress and inflammation and TL. The benefits of exercise on general physiological wellbeing have long been understood. The positive association and ability of irisin to predict TL provides a novel mechanism by which exercise may inhibit age-related decline.

Exogenous irisin administration has potential as a future therapeutic treatment that may function to protect against age associated metabolic disorders, in particular obesity and T2D by creating a calorie deficit and promote longevity.

Chapter 4: Results

The Metabolic Regulation of Cellular Ageing in Type 2 Diabetic Patients

4.1 Introduction

There has been a substantial rise in the incidence of metabolic disorders, particularly obesity and T2D in recent years, with current reports forecasting further increases in the future [627]. Obesity often develops as a result of a dysregulation in energy balance, whereby energy consumption is in excess of energy expenditure, leading to increased adipose tissue mass [628]. This excess adipose tissue provides a considerable source of reactive oxygen species and inflammatory peptides, two vital contributors in the development of T2D [629]. Obesity and T2D are often closely related and, coupled with their association with increased morbidity and mortality, are a cause for great concern. Existing research has already demonstrated that individuals diagnosed with obesity and/or T2D experience a reduction in lifespan, primarily due to health implications associated with these disorders, such as cardiovascular disease, endocrine/reproductive complications and psychiatric and neurological disorders [630, 631]. There also now exists growing evidence that obesity/T2D may be able to accelerate the ageing process itself [632, 633]. Results from chapter 3 highlight body fat percentage and visceral fat score, inversely correlated with TL, a genetic marker of cellular ageing. On the contrary fat free mass conversely correlated with TL, in a cohort of 81 healthy volunteers.

Various studies have reported reduced TL amongst patients suffering from obesity/T2D, however there are also numerous studies which report opposite findings and therefore, published literature is currently divided regarding TL and obesity/T2D [634, 635]. Both obesity and T2D are associated with numerous health implications which are known to reduce lifespan such as Alzheimer's disease, cancer and cardiovascular complications [636-638]. Almen *et al*., conducted GWAS to investigate whether DNA methylation patterns vary with both age and obesity. The authors conclude obesity influences age driven epigenetic changes, thus providing molecular links between ageing and obesity. Interestingly the author's document diverse methylation patterns in the telomerase catalytic sub-unit amongst obese and lean individuals [639].

If individuals suffering from such metabolic disorders are experiencing an accelerated rate of ageing due to the pathophysiology of obesity/T2D, interventions reducing the severity of these disorders would prove beneficial in restoring an adequate rate of ageing and prevent rapid deterioration. Dietary and exercise interventions reduce the risk of developing obesity and T2D, the recent discovery of the peptide hormone irisin, stimulated by PGC1-α expression in myocytes following exercise, may be able to provide a mechanistic insight on this effect [640]. Irisin has been reported to bind to undetermined receptors on WAT and induce "browning", by enhancing UCP1 expression, consequently increasing uncoupled respiration. These changes promote energy expenditure via thermogenesis [640]. Results from chapter 3 discovered plasma irisin levels can predict TL in healthy non-obese individuals, suggesting that irisin can both positively influence ageing and regulate energy balance [641].

On the contrary many studies are surfacing which completely oppose this theory. Recently published research has identified WAT as an additional source of irisin secretion [263, 642]. The authors report both subcutaneous and visceral adipose tissues were able to secrete irisin post exercise, and the ability to do so was significantly reduced in fasting animals [263]. The general perception regarding irisin in research is one of confusion; however media coverage has hailed irisin as a wonder treatment capable of eradicating metabolic disease, particularly useful in treatment of obesity and T2D owing to its ability to induce browning of WAT. With contrasting views regularly published regarding irisin's therapeutic capabilities and recent data highlighting the detrimental effects of irisin on cardiovascular health, the pharmacological implementation of irisin as a treatment for obesity and T2D may prove to be more hazardous to health than useful, or may be of no use at all [263, 640, 643]. Therefore it is of great necessity that the role of irisin in individuals suffering from obesity and T2D be clarified.

4.2 Aims and Hypothesis

The aim of this part of the study was to investigate the relationship between metabolic disease and ageing, and to compare findings in a cohort of obese/T2D to the healthy cohort described in Chapter 3. More importantly the main aim of this chapter is to elucidate the role of TL in obese/T2D volunteers, by examining associations between body composition, leptin, adiponectin, irisin and markers of endothelial dysfunction including soluble thrombomodulin, C-reactive protein and E-selectin, along with homeostatic model assessment with TL. With the purpose of clarifying whether circulating TL correlate with measures of adipose mass or skeletal muscle. Probing associations between adipokines, markers of inflammation and endothelial dysfunction, will help clarify whether obese/T2D patients experience enhanced cellular ageing relative to healthy controls and whether obese/T2D volunteers can reap the anti-ageing effects of irisin. Finally, using HUVEC the effect of increased irisin concentration on the gene expression of four endothelial adhesion proteins, ICAM-1, PECAM-1, P-selectin and E-selectin was assessed. The purpose of this investigation was to bridge the gap in knowledge currently correlating increased circulating irisin concentration with the development of cardiovascular complications. The results from this study may reveal elevated irisin concentration as an intermediary step leading to systemic inflammation.

The hypotheses for this study were;

1) T2D volunteers will exhibit shorter relative TL in comparison to non-diabetic volunteers.

2) Circulating irisin concentrations will be lower in T2D volunteers and correlate with muscle mass.

3) HUVEC treated with a high concentration of irisin will exhibit increased expression of endothelial adhesion proteins.

4.3 Methods

For complete details of experimental methodology please refer to chapter 2, section 2.1-2.8, 2.9, 2.10.1, 2.12, 2.14-2.15.

4.4 Results

4.4.1 Comparison of biochemical and anthropometric measurements in healthy individuals and individuals with type 2 diabetes.

Clinical cohort characteristics can be found in Table 4.1. Seventy nine T2D volunteers (42 males and 37 females) were recruited in this study and compared to the eighty one previously described healthy volunteers. The type 2 diabetic cohort was significantly older with a mean age of 56± 12, as opposed to 43± 15.8 years for the healthy volunteer cohort. The Type 2 diabetic cohort were shorter in height and weighed more (165 ± 10cm, 87 ± 20.5kg), in comparison to non-diabetic volunteers (172 ± 9.5cm, 73 ± 13.1kg). The type 2 diabetic volunteers recruited in this study had an average BMI of 31.5 ± 5.4, whereas the non-diabetic volunteers had a significantly lower average BMI of 24.3 ± 2.9. A BMI score above 30 is a well-established indicator of the obese phenotype and therefore the majority of type 2 diabetics involved in this study were also obese.

Global body fat percentages were significantly higher in the type 2 diabetic cohort (34.9 ± 9.3) in comparison to the non-diabetic cohort (25.1 ± 9.2). Individuals from both cohorts were shown to have similar amounts of both total muscle and abdominal muscle. The healthy volunteers had an average total muscle mass of 52.3 ± 11.7kg and the type 2 diabetic volunteers had an average total muscle mass of 53 ± 11.8kg, with no significant difference between the two ($p = 0.7$). Healthy volunteers had on average 28.9 ± 6.5kg of abdominal muscle in comparison to 29 ± 5.7kg in the diabetic cohort, again exhibiting no statistically significant difference ($p = 1$). The comparable amounts of total and abdominal muscle between the two study cohorts suggests, differences in BMI are irrespective of muscle tissue and the greater adipose mass found in the type 2 diabetic participants is predominantly accountable for the higher BMI score. Non-diabetic participants had visceral fat scores within the healthy range 6.2 ± 3.8, whereas the type 2 diabetic volunteers, on average were above this range 13.2 ±5.5 respectively.

Individuals within the T2D volunteers cohort demonstrated typical biochemical characteristics of type 2 diabetic patients, including higher fasting blood glucose and fasting insulin concentrations, decreased percentage β cell function, increased insulin resistance and decreased insulin sensitivity in comparison to non-diabetic volunteers (Table 4.1). Additionally T2D volunteers exhibited higher concentrations of circulating leptin (1490 ± 1327ng/ml) in comparison to non-diabetic volunteers (8.5 ± 7.2ng/ml) and significantly lower concentrations of circulating adiponectin (0.788± 0.399µg/ml) as opposed to non-diabetic volunteers (3.44 ± 1.87µg/ml) and therefore T2D volunteers also had a higher mean leptin to adiponectin ratio (5.1± 6.5). Markers of endothelial dysfunction and inflammation were also found in significantly higher concentrations in the type 2 diabetic volunteers, with the exception of soluble thrombomodulin (5.9 ± 3ng/ml) in comparison to (6.6 ± 6.3ng/ml) in T2D volunteers.

Anthropometric and Biochemical Analysis			
	Healthy Volunteers	**Type 2 Diabetic Volunteers**	**Significance**
Cohort Size	81	79	
Men	43	42	
Women	38	37	
Age (years) ***	43 ± 15.8	56 ± 12	**P <0.0001**
Height (cm) ***	172 ± 9.5	165 ± 10	**P <0.0001**
Weight (Kg) ***	73 ± 13.1	87 ± 20.5	**P <0.0001**
Body Mass Index (kg/m^2) ***	24.3 ± 2.9	31.5 ± 5.4	**P <0.0001**
Total Fat (%)***	25.1 ± 9.2	34.9 ± 9.3	**P <0.0001**
Total Muscle (kg)	52.3 ± 11.7	53 ± 11.8	P = 0.7
Abdominal (Trunk) Fat (%)***	23.6 ± 7.9	34.1 ± 9.4	**P <0.0001**
Abdominal (Trunk) Muscle (kg) ***	28.9 ± 6.5	29 ± 5.7	P = 1
Visceral Fat Score (0-60) ***	6.2 ± 3.8	13.2 ±5.5	**P <0.0001**
Fasting Blood Glucose (m/mmol)	4.5 ± 0.7	9.9 ± 3.8	**P <0.0001**
HbA1c (mmol/mol)	N/A	70.5 ± 17.6	N/A
Fasting Insulin (mU/L) ***	7.9 ± 4	29.1 ± 42.6	**P <0.0001**
HOMA β (%)***	110.6 ± 52.8	67.2 ± 67.9	**P <0.0001**
HOMA IR	2.2 ± 12.3	5.7 ± 21.9	P = 0.4
HOMA S (%)***	141.9 ± 73.7	74 ± 71	**P <0.0001**
Telomere Length (T/S ratio) ***	2.14 ± 0.47	1.6 ± 0.2	**P <0.0001**
Irisin (ng/ml) ***	46.7 ± 32.4	175.4 ± 131	**P <0.0001**
Leptin (ng/ml) ***	8.5 ± 7.2	1490 ± 1327	**P <0.0001**
Adiponectin (µg/ml) ***	3.44 ± 1.87	0.788 ± 0.399	**P <0.0001**
Leptin/Adiponectin ratio***	0.005 ±0.005	5.1 ± 6.5	**P <0.0001**
Thrombomodulin (ng/ml)	5.9 ± 3	6.6 ± 6.3	P = 0.2
E-selectin (ng/ml) ***	36.9 ± 33.2	50.9 ± 21.4	**P <0.0001**
C-reactive protein (µg/ml) ***	1.03 ± 0.8	4.5 ± 3.99	**P <0.0001**

Table 4.1. Clinical characteristics and metabolic profiles of Non-diabetic control and Type 2 diabetic subjects studied.

Data is presented as mean ± S.D. for normal continuous variables. Significant differences are denoted as * p value of < 0.05 . ** p value < than 0.01 and *** p value < 0.0001.

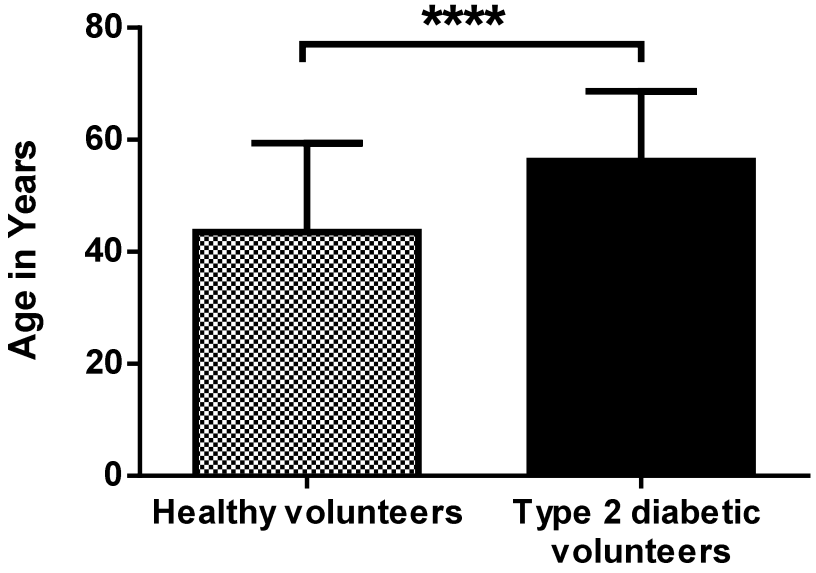

Figure 4.1. Comparison between average age in years in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$

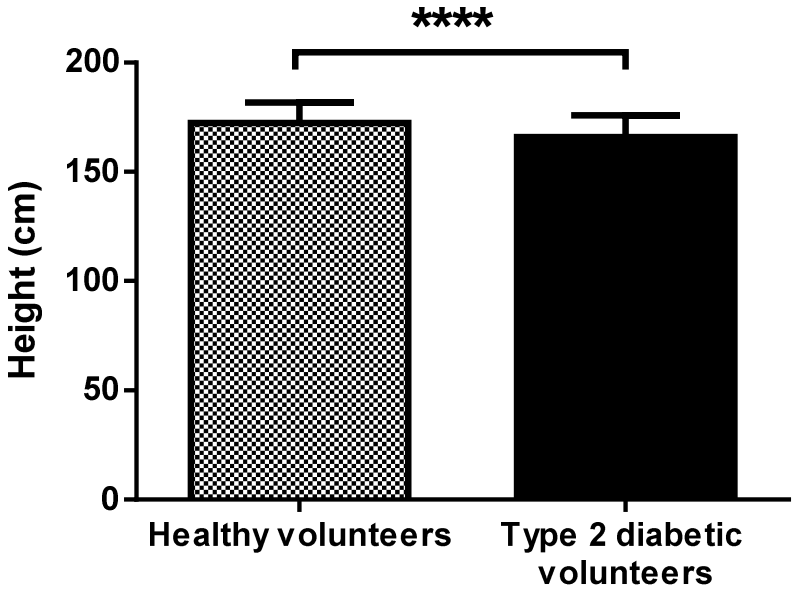

Figure 4.2. Comparison between average height in cm in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$

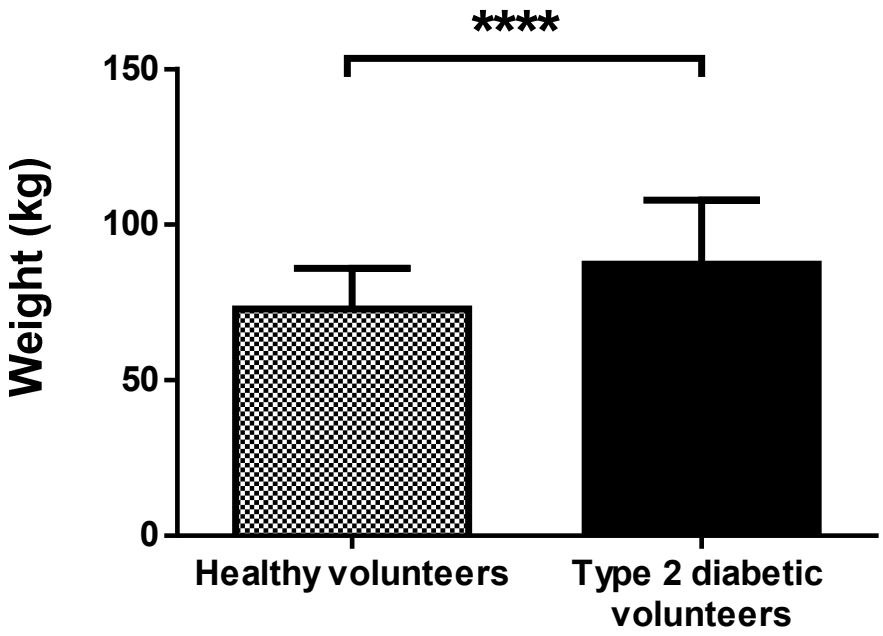

Figure 4.3. Comparison between average weight in kg in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

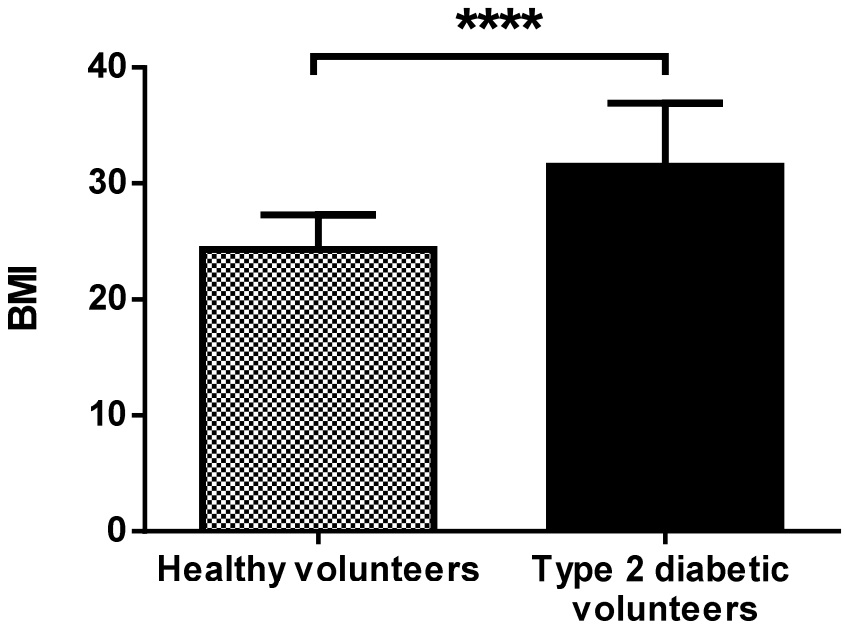

Figure 4.4. Comparison between average BMI in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

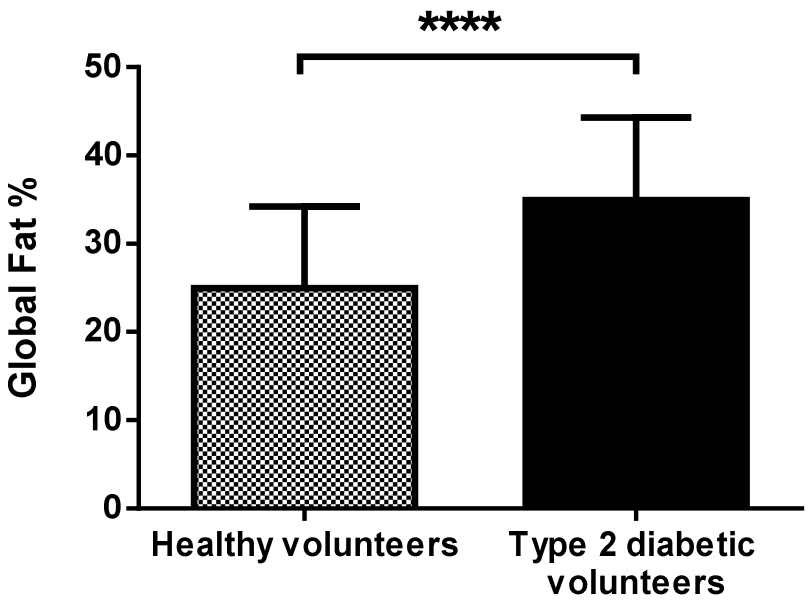

Figure 4.5. Comparison between average global fat % in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$

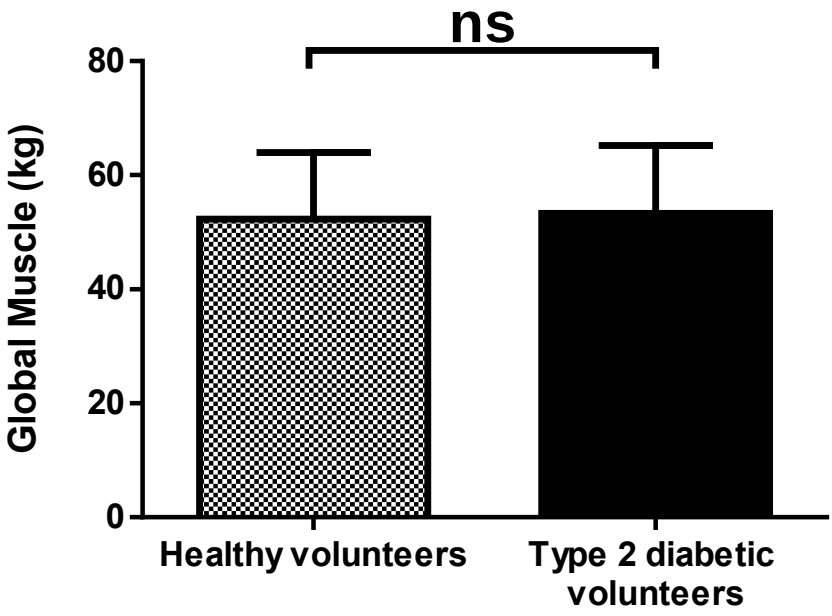

Figure 4.6. Comparison between average global muscle (Kg) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p = 0.6027$). Significance was set at $p < 0.05$.

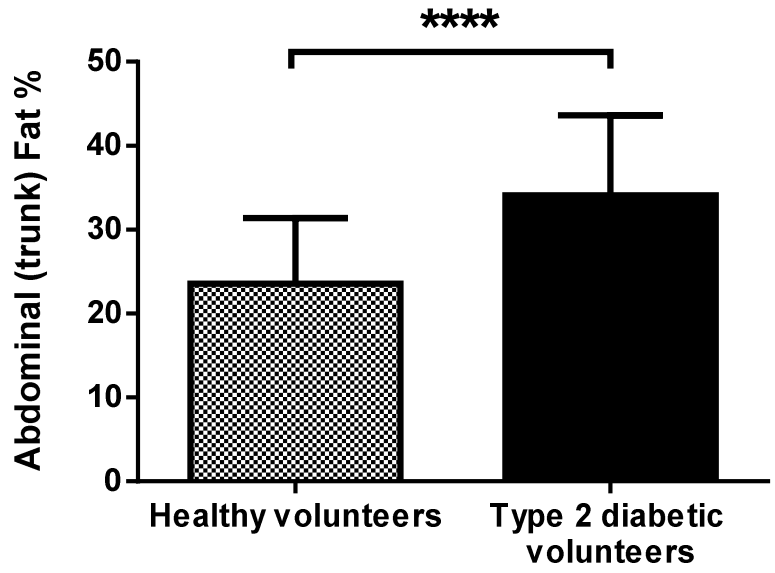

Figure 4.7. Comparison between average abdominal fat % in healthy and type 2 diabetic volunteers.

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$

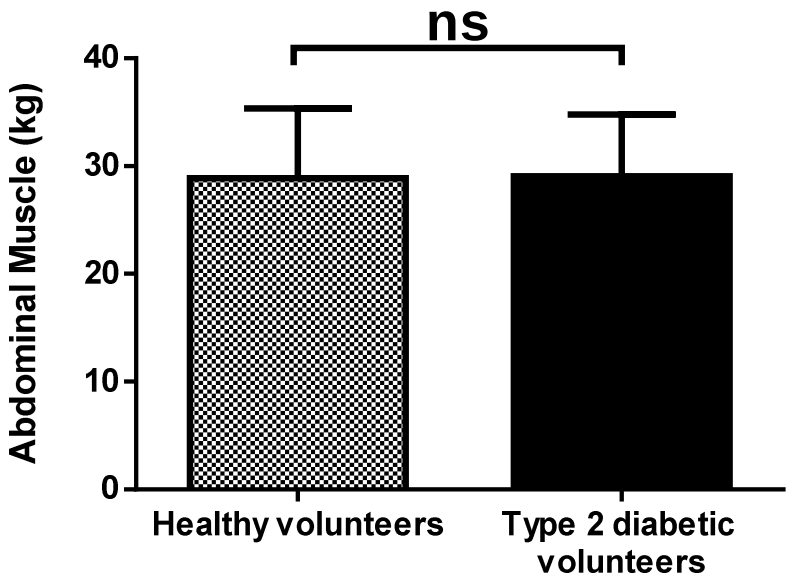

Figure 4.8. Comparison between average abdominal muscle (Kg) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p = 0.8716$). Significance was set at $p < 0.05$.

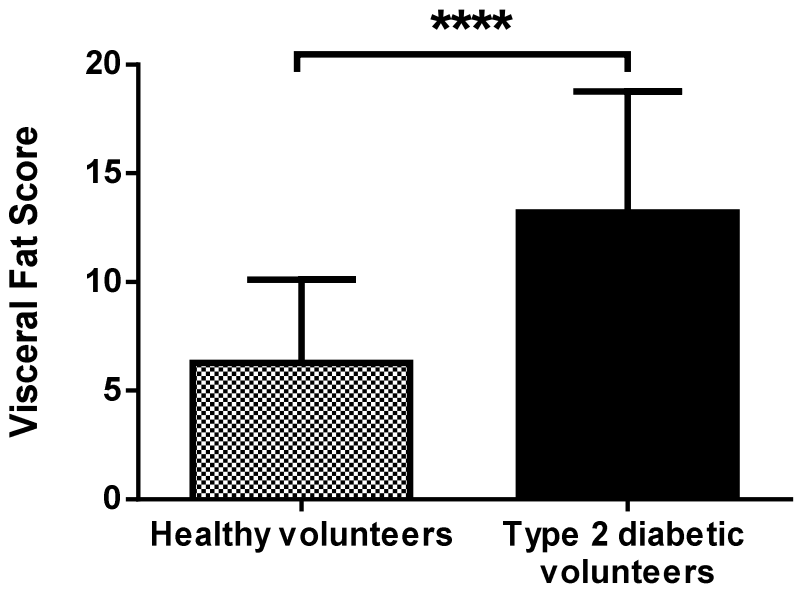

Figure 4.9.Comparison between average visceral fat score in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

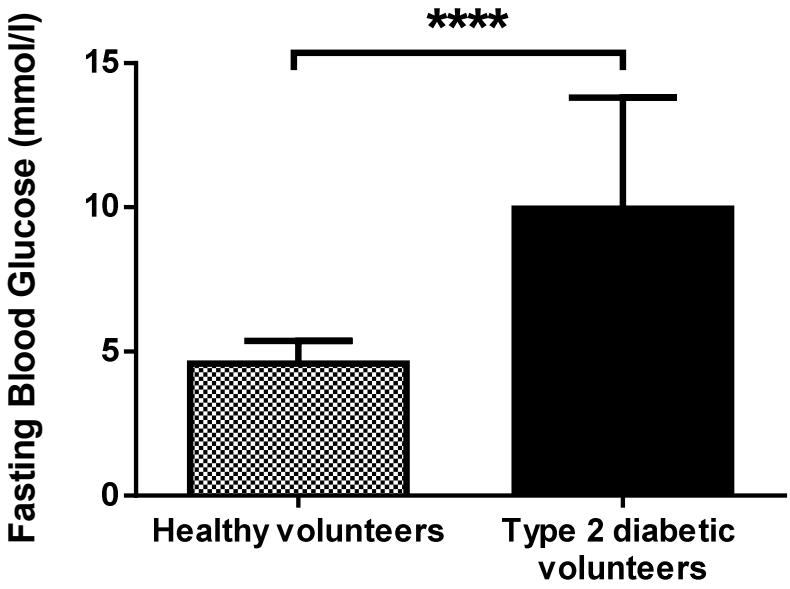

Figure 4.10. Comparison between average fasting blood glucose (mmol/l) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

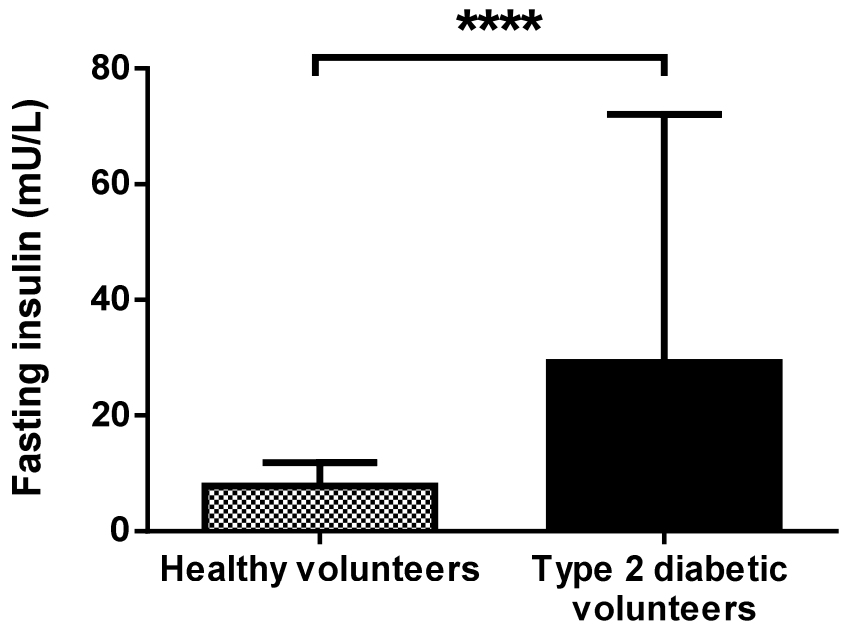

Figure 4.11.Comparison between average fasting blood insulin (mU/L) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

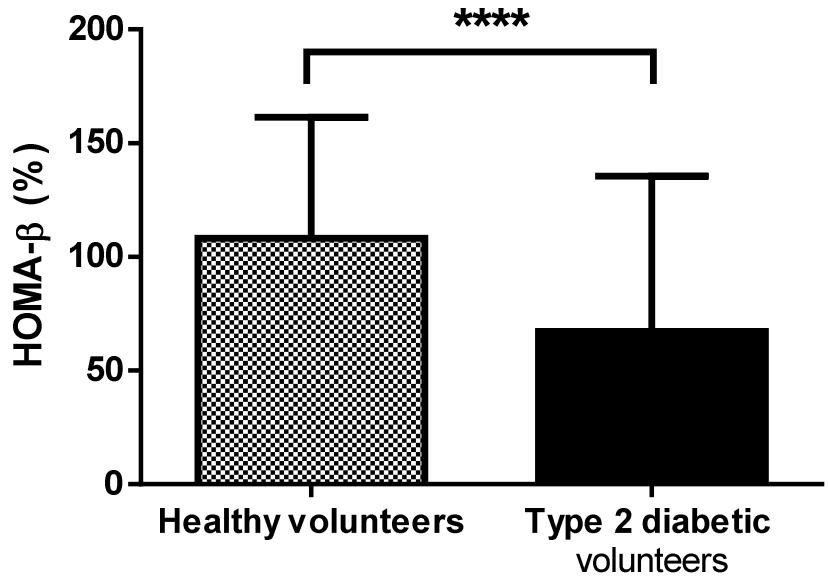

Figure 4.12. Comparison between average percentage β-cell function in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

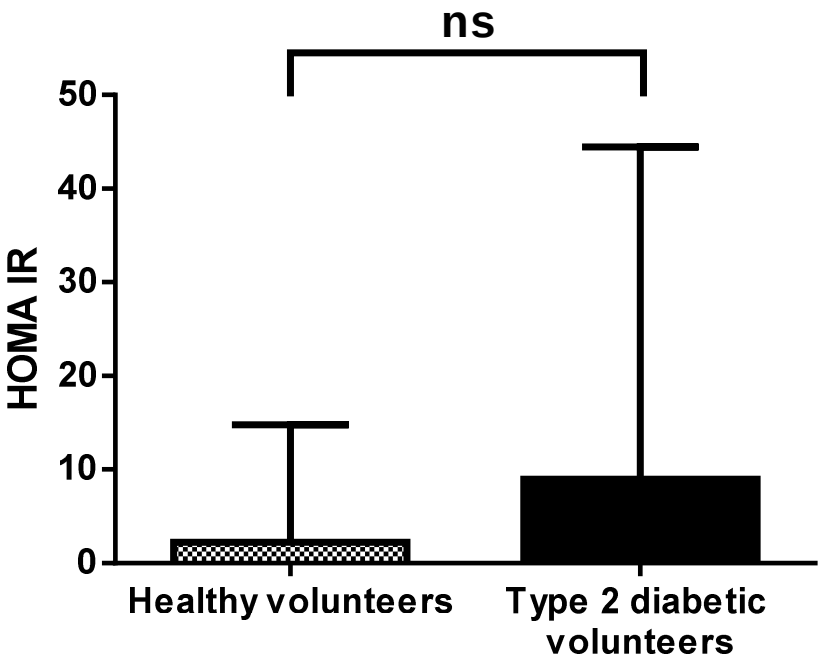

Figure 4.13. Comparison between average insulin resistance score in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD (p = 0.1082). Significance was set at p <0.05.

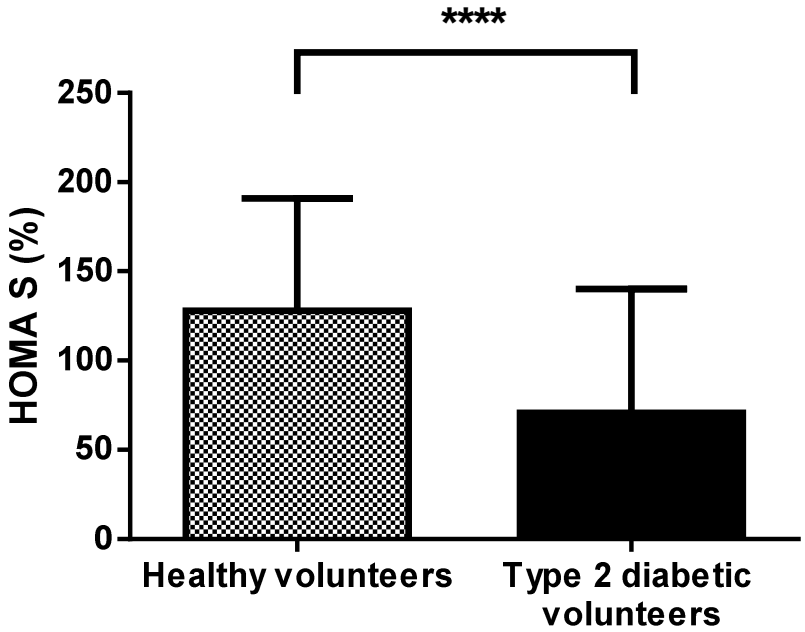

Figure 4.14. Comparison between average percentage insulin sensitivity in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD (p < 0.0001). Significance was set at p <0.05.

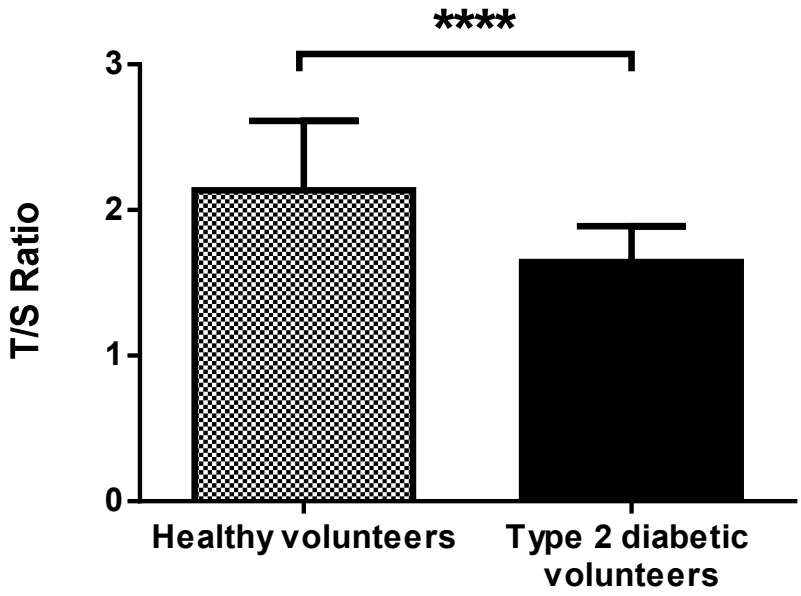

Figure 4.15. Comparison between average T/S ratio in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

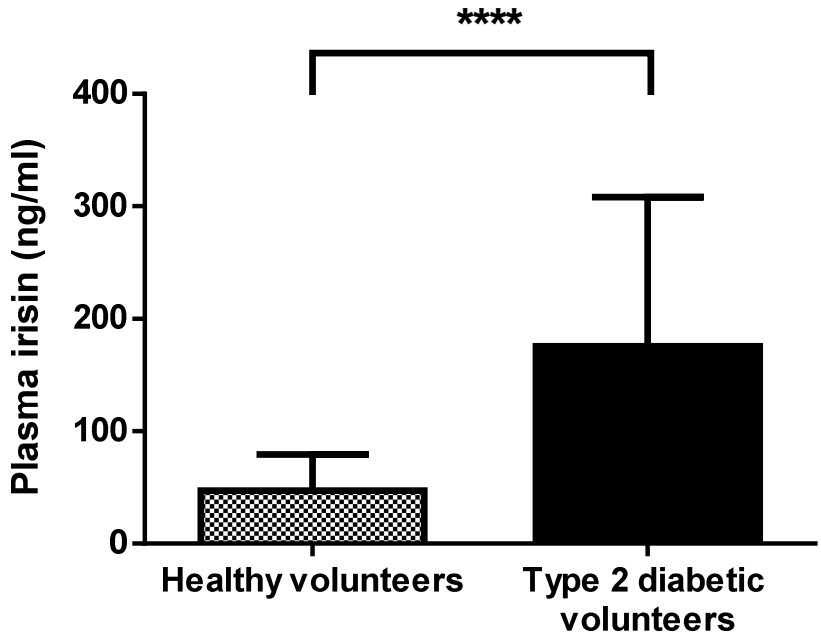

Figure 4.16. Comparison between average irisin concentration (ng/ml) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

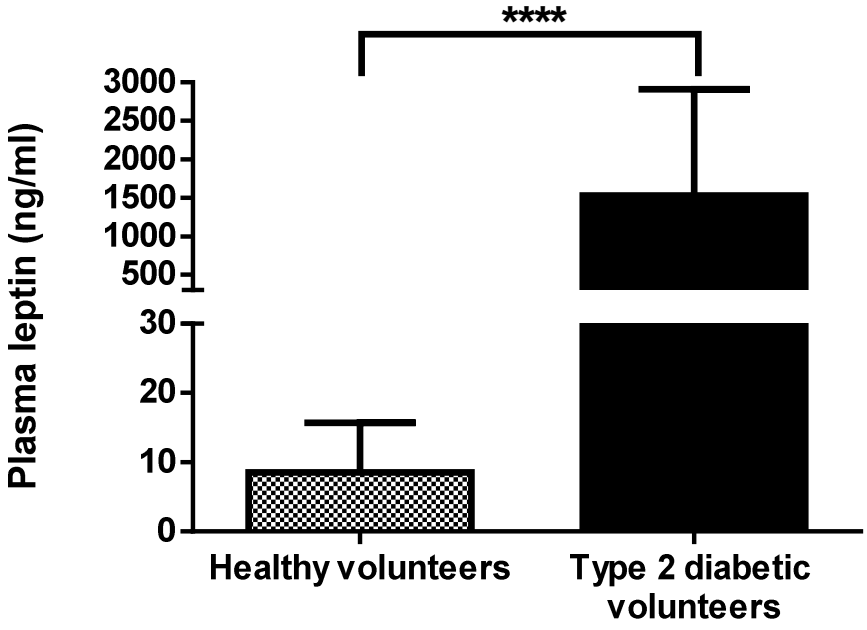

Figure 4.17. Comparison between average leptin concentration (ng/ml) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

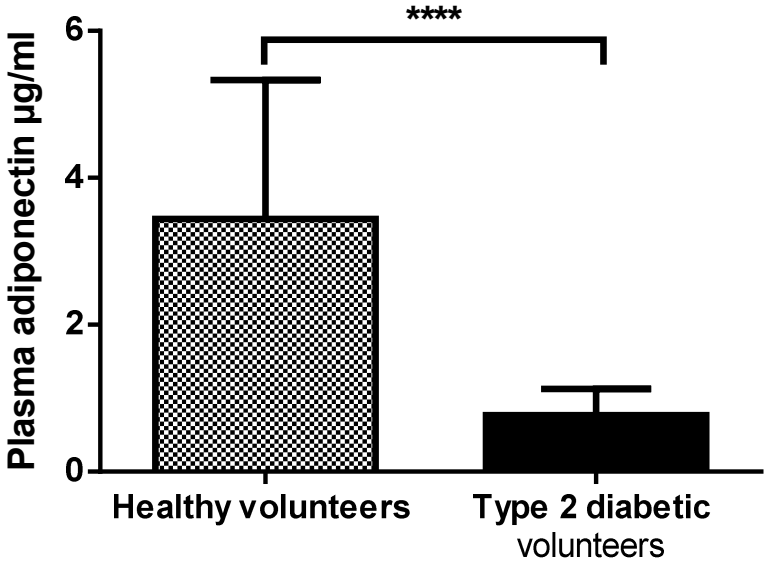

Figure 4.18. Comparison between average plasma adiponectin concentration (µg/ml) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p = 0.0001$). Significance was set at $p < 0.05$.

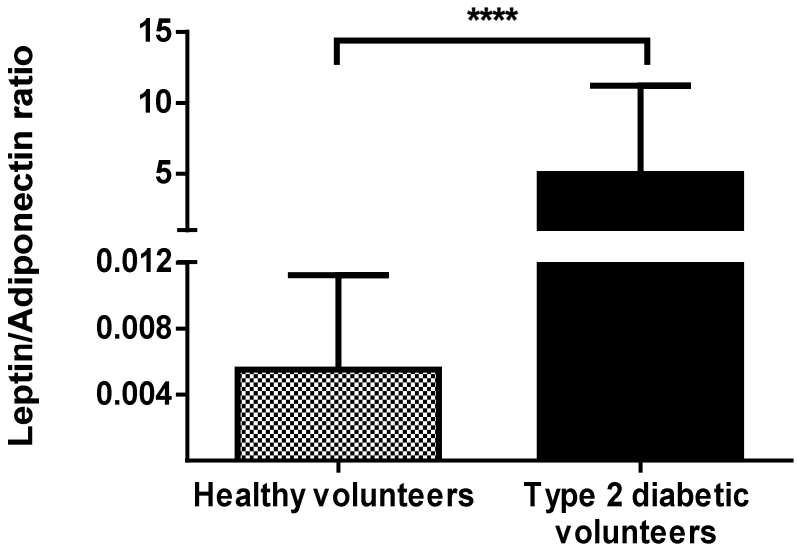

Figure 4.19. Comparison between average leptin/adiponectin ratio in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p < 0.0001$). Significance was set at $p < 0.05$.

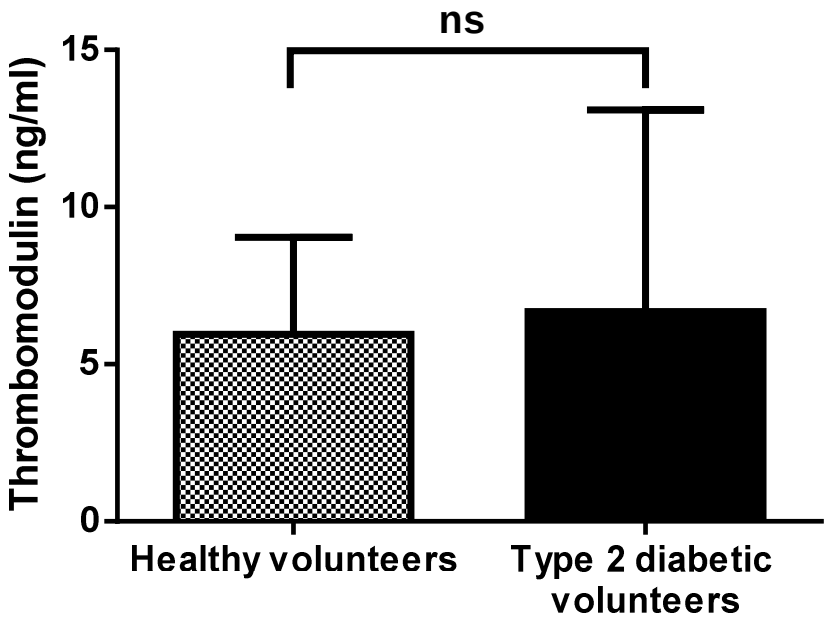

Figure 4.20. Comparison between average thrombomodulin concentration (ng/ml) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD ($p = 0.3590$). Significance was set at $p < 0.05$.

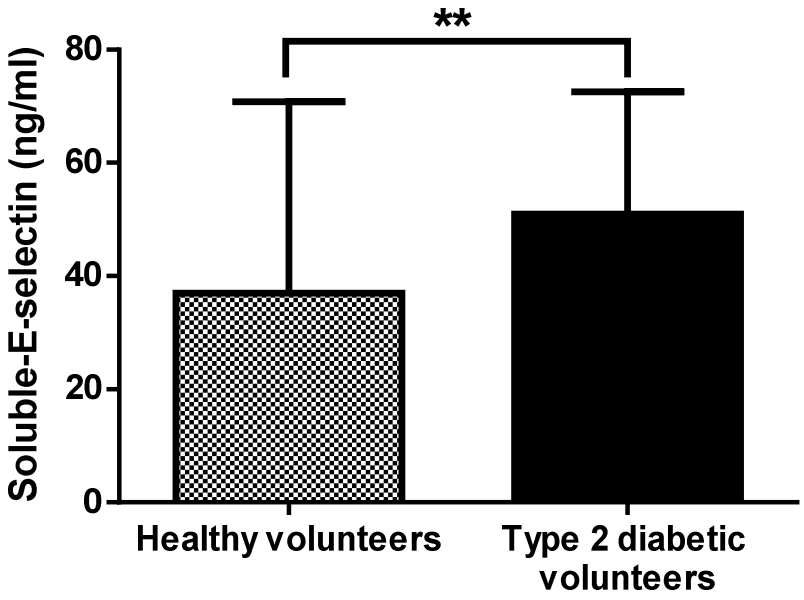

Figure 4.21. Comparison between average soluble E-selectin concentration (ng/ml) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD (p = 0.0022). Significance was set at p <0.05.

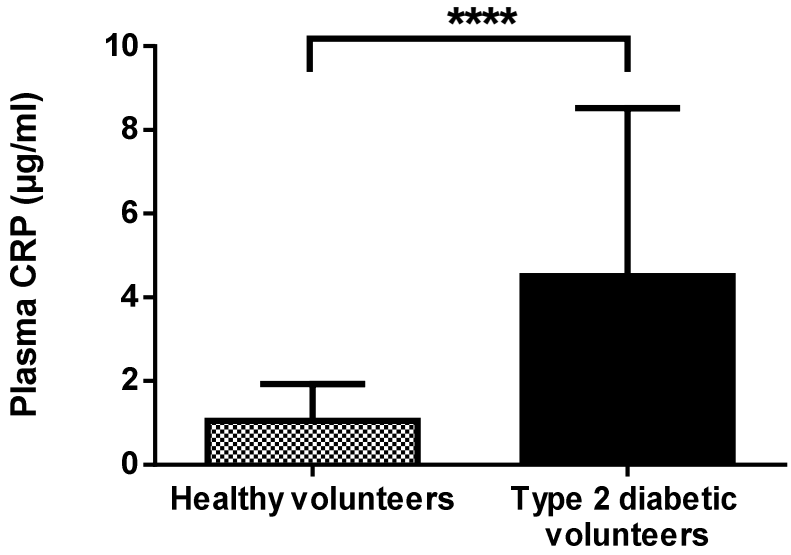

Figure 4.22. Comparison between plasma CRP (µg/ml) in healthy and type 2 diabetic volunteers

Healthy volunteers n = 81, T2D volunteers n = 79, bar represent mean ± SD (p < 0.001). Significance was set at p <0.05.

4.4.2. Associations of plasma irisin with biochemical and anthropometric factors in type 2 diabetic volunteers.

To ascertain whether the same mathematical relationship between irisin and TL that was shown in Chapter 3 existed in this cohort of individuals with diabetes, a Pearson's bivariate correlations test was conducted to assess significant associations between plasma irisin and anthropometric, biochemical measures and T/S ratio in this cohort of T2D volunteers (Table 4.3). Circulating irisin concentration was $\log_{10}$ transformed in order to decrease heteroscedasticity, thereby following a more even distribution. Unlike in the healthy cohort recruited for this study, there was no significant co-linearity between T/S ratio and chronological age. Additionally log irisin and T/S ratio did not express any significant correlation within the T2D cohort (Fig. 4.24).

Anthropometric and biochemical measures which exhibited significant positive associations with natural log transformed circulating irisin concentration include; BMI ($p = 0.04$), total fat percentage ($p = 0.033$), HbA1c ($p = 0.032$) and E-selectin ($p < 0.0001$). Visceral fat score ($p = 0.006$) displayed a significant negative association with plasma irisin levels, as did age ($p = 0.001$) and leptin ($p = 0.02$). No other factors measured in this study displayed any significant association with plasma irisin.

		logirisin	TS	Age	height	weight	BMI	G_fat	G_muscle	A_fat	A_muscle	V_fat	Lep	Adp	LAR	F_bg	HBA1C2	F_in	HOMA_B	HOMA_IR	HOMA_S	Throm	E_S	CRP
logirisin	Pearson Correlation	1	-.110	-.364	-.171	.089	.223	.241	-.134	.062	-.173	-.307	-.251	-.174	-.041	-.030	.284	.049	-.007	-.196	.021	-.161	.434	.056
	Sig. (2-tailed)		.333	.001	.133	.437	.048	.033	.238	.590	.132	.006	.026	.145	.721	.791	.032	.670	.953	.086	.858	.156	.000	.626
TS	Pearson Correlation	-.110	1	-.137	.009	.050	.041	-.094	.142	-.070	.182	-.022	-.279	-.177	-.127	-.080	.036	.127	.003	-.129	.012	.116	-.012	.192
	Sig. (2-tailed)	.333		.229	.937	.664	.719	.411	.213	.542	.112	.852	.013	.138	.267	.489	.791	.265	.981	.261	.920	.308	.918	.090
Age	Pearson Correlation	-.364	-.137	1	.164	-.072	-.175	-.126	.032	.029	.090	.454	.145	.366	-.044	-.161	-.439	-.138	-.031	.028	.092	-.140	-.486	.126
	Sig. (2-tailed)	.001	.229		.148	.527	.123	.267	.779	.804	.436	.000	.201	.002	.702	.159	.001	.226	.789	.808	.425	.220	.000	.270
height	Pearson Correlation	-.171	.009	.164	1	.646	.190	-.226	.834	-.013	.772	.539	.143	-.056	.006	-.072	-.099	-.115	.112	.099	-.061	.147	-.025	-.104
	Sig. (2-tailed)	.133	.937	.148		.000	.094	.045	.000	.911	.000	.000	.208	.641	.961	.530	.463	.311	.329	.387	.595	.197	.829	.361
weight	Pearson Correlation	.089	.050	-.072	.646	1	.862	.392	.775	.503	.663	.736	-.006	-.209	-.026	-.056	.157	.039	.092	-.115	-.059	.088	.210	.072
	Sig. (2-tailed)	.437	.664	.527	.000		.000	.000	.000	.000	.000	.000	.956	.079	.822	.625	.244	.733	.422	.317	.606	.441	.063	.527
BMI	Pearson Correlation	.223	.041	-.175	.190	.862	1	.672	.443	.688	.285	.560	-.072	-.210	-.044	-.038	.230	.148	.041	-.170	-.077	.024	.291	.162
	Sig. (2-tailed)	.048	.719	.123	.094	.000		.000	.000	.000	.012	.000	.529	.077	.700	.744	.086	.192	.721	.136	.502	.837	.009	.154
G_fat	Pearson Correlation	.241	-.094	-.126	-.226	.392	.672	1	-.241	.916	-.379	.263	.009	-.049	-.008	-.011	.177	.106	-.084	-.181	-.100	-.099	.235	.169
	Sig. (2-tailed)	.033	.411	.267	.045	.000	.000		.032	.000	.001	.020	.939	.682	.946	.926	.189	.351	.464	.114	.383	.387	.037	.137
G_muscle	Pearson Correlation	-.134	.142	.032	.834	.775	.443	-.241	1	-.077	.939	.563	.025	-.175	-.018	-.050	.031	-.015	.200	-.016	-.042	.204	.050	-.034
	Sig. (2-tailed)	.238	.213	.779	.000	.000	.000	.032		.501	.000	.000	.825	.142	.873	.664	.816	.892	.080	.889	.717	.071	.665	.765
A_fat	Pearson Correlation	.062	-.070	.029	-.013	.503	.688	.916	-.077	1	-.165	.537	.113	-.029	.008	.077	.051	.091	-.135	-.104	-.112	-.052	.173	.176
	Sig. (2-tailed)	.590	.542	.804	.911	.000	.000	.000	.501		.153	.000	.325	.809	.942	.508	.708	.430	.243	.367	.334	.652	.130	.123
A_muscle	Pearson Correlation	-.173	.182	.090	.772	.663	.285	-.379	.939	-.165	1	.511	.029	-.116	-.025	-.084	.007	.023	.279	-.009	-.034	.225	-.008	-.130
	Sig. (2-tailed)	.132	.112	.436	.000	.000	.012	.001	.000	.153		.000	.805	.339	.831	.473	.959	.840	.015	.941	.769	.049	.948	.259
V_fat	Pearson Correlation	-.307	-.022	.454	.539	.736	.560	.263	.563	.537	.511	1	.169	.002	.014	.021	-.155	-.010	-.008	-.029	.003	.063	-.115	.076
	Sig. (2-tailed)	.006	.852	.000	.000	.000	.000	.020	.000	.000	.000		.139	.985	.906	.859	.255	.932	.946	.803	.980	.584	.318	.511
Lep	Pearson Correlation	-.251	-.279	.145	.143	-.006	-.072	.009	.025	.113	.029	.169	1	.013	.633	.294	-.155	.000	-.115	.044	-.285	.272	-.136	-.390
	Sig. (2-tailed)	.026	.013	.201	.208	.956	.529	.939	.825	.325	.805	.139		.915	.000	.009	.251	.998	.315	.701	.011	.015	.233	.000
Adp	Pearson Correlation	-.174	-.177	.366	-.056	-.209	-.210	-.049	-.175	-.029	-.116	.002	.013	1	-.491	-.233	-.197	.035	.045	.184	.144	.076	-.258	-.011
	Sig. (2-tailed)	.145	.138	.002	.641	.079	.077	.682	.142	.809	.339	.985	.915		.000	.051	.158	.773	.710	.125	.230	.525	.029	.929
LAR	Pearson Correlation	-.041	-.127	-.044	.006	-.026	-.044	-.008	-.018	.008	-.025	.014	.633	-.491	1	.346	.067	-.034	-.197	-.032	-.129	-.033	-.045	-.231
	Sig. (2-tailed)	.721	.267	.702	.961	.822	.700	.946	.873	.942	.831	.906	.000	.000		.002	.619	.769	.083	.783	.261	.770	.696	.041
F_bg	Pearson Correlation	-.030	-.080	-.161	-.072	-.056	-.038	-.011	-.050	.077	-.084	.021	.294	-.233	.346	1	.133	.147	-.449	-.028	-.277	.076	.181	-.104
	Sig. (2-tailed)	.791	.489	.159	.530	.625	.744	.926	.664	.508	.473	.859	.009	.051	.002		.328	.198	.000	.808	.014	.511	.112	.367
HBA1C2	Pearson Correlation	.284	.036	-.439	-.099	.157	.230	.177	.031	.051	.007	-.155	-.155	-.197	.067	.133	1	.140	.195	-.088	-.063	-.057	.270	.076
	Sig. (2-tailed)	.032	.791	.001	.463	.244	.086	.189	.816	.708	.959	.255	.251	.158	.619	.328		.300	.150	.519	.646	.672	.042	.572
F_in	Pearson Correlation	.049	.127	-.138	-.115	.039	.148	.106	-.015	.091	.023	-.010	.000	.035	-.034	.147	.140	1	.288	-.043	-.404	.044	.246	-.040
	Sig. (2-tailed)	.670	.266	.226	.311	.733	.192	.351	.892	.430	.840	.932	.998	.773	.769	.198	.300		.010	.709	.000	.698	.029	.729
HOMA_B	Pearson Correlation	-.007	.003	-.031	.112	.092	.041	-.084	.200	-.135	.279	-.008	-.115	.045	-.197	-.449	.195	.288	1	-.099	-.238	.212	.001	-.019
	Sig. (2-tailed)	.953	.981	.789	.329	.422	.721	.464	.080	.243	.015	.946	.315	.710	.083	.000	.150	.010		.387	.036	.063	.992	.871
HOMA_IR	Pearson Correlation	-.196	-.129	.028	.099	-.115	-.170	-.181	-.016	-.104	-.009	-.029	.044	.184	-.032	-.028	-.088	-.043	-.099	1	-.221	.050	-.037	-.134
	Sig. (2-tailed)	.086	.261	.808	.387	.317	.136	.114	.889	.367	.941	.803	.701	.125	.783	.808	.519	.709	.387		.052	.664	.750	.243
HOMA_S	Pearson Correlation	.021	.012	.092	-.061	-.059	-.077	-.100	-.042	-.112	-.034	.003	-.285	.144	-.129	-.277	-.063	-.404	-.238	-.221	1	-.247	-.219	.181
	Sig. (2-tailed)	.858	.920	.425	.595	.606	.502	.383	.717	.334	.769	.980	.011	.230	.261	.014	.646	.000	.036	.052		.029	.054	.113
Throm	Pearson Correlation	-.161	.116	-.140	.147	.088	.024	-.099	.204	-.052	.225	.063	.272	.076	-.033	.076	-.057	.044	.212	.050	-.247	1	.239	-.273
	Sig. (2-tailed)	.156	.308	.220	.197	.441	.837	.387	.071	.652	.049	.584	.015	.525	.770	.511	.672	.698	.063	.664	.029		.034	.015
E_S	Pearson Correlation	.434	-.012	-.486	-.025	.210	.291	.235	.050	.173	-.008	-.115	-.136	-.258	-.045	.181	.270	.246	.001	-.037	-.219	.239	1	-.162
	Sig. (2-tailed)	.000	.918	.000	.829	.063	.009	.037	.665	.130	.948	.318	.233	.029	.696	.112	.042	.029	.992	.750	.054	.034		.153
CRP	Pearson Correlation	.056	.192	.126	-.104	.072	.162	.169	-.034	.176	-.130	.076	-.390	-.011	-.231	-.104	.076	-.040	-.019	-.134	.181	-.273	-.162	1
	Sig. (2-tailed)	.626	.090	.270	.361	.527	.154	.137	.765	.123	.259	.511	.000	.929	.041	.367	.572	.729	.871	.243	.113	.015	.153	

Table 4.2. Pearson's Bivariate correlation matrix

Two-tailed pearsons bivariate correlations between T/S ratio, age, anthropometric measures and biochemical analytes. Negative associations are denoted by -. ** Correlation is significant at the 0.01 level.* Correlation is significant at the 0.05 level

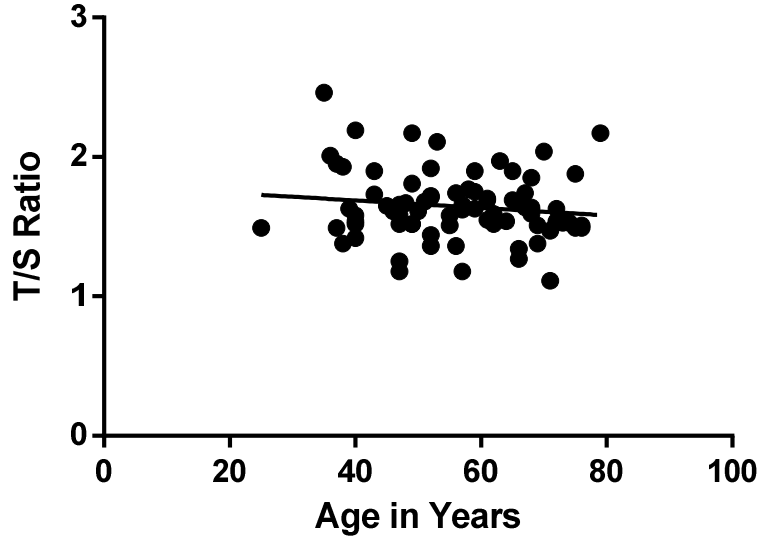

Figure 4.23. Correlation of age in years with telomere length.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.229$, $R^2 = 0.01878$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

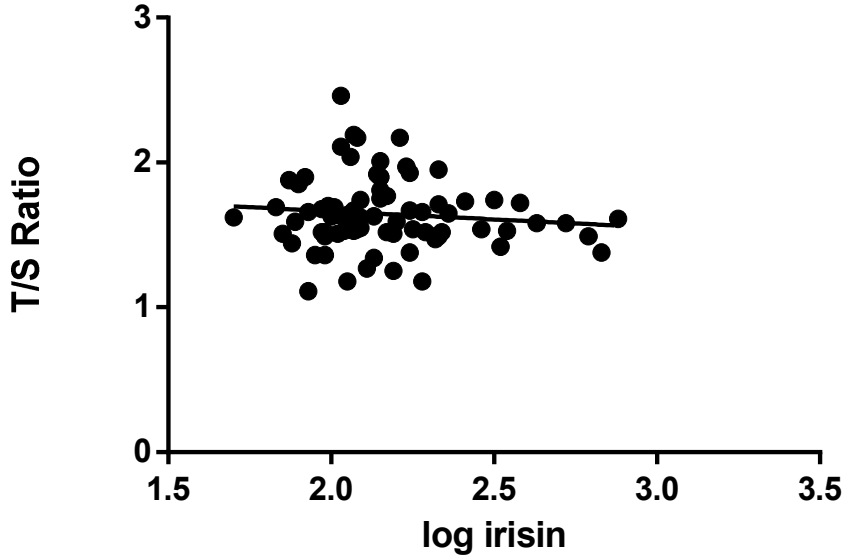

Figure 4.24. Correlation between log irisin with telomere length.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.333$, $R^2 = 0.01147$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

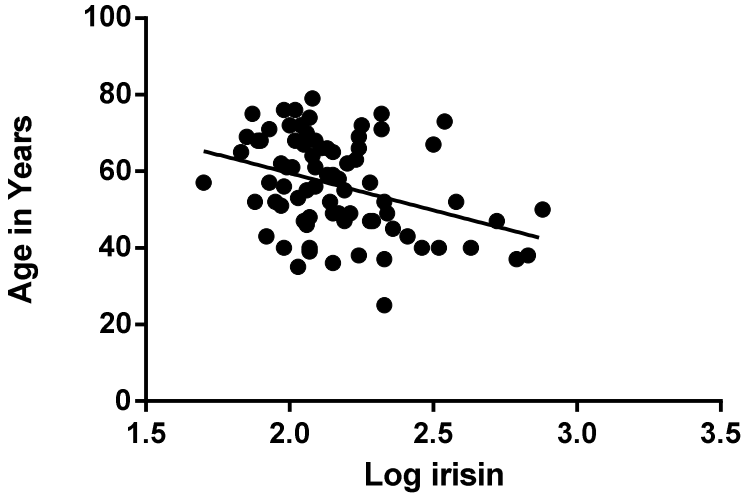

Figure 4.25. Correlation between log irisin and age.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.001$, $R^2 = 0.1326$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

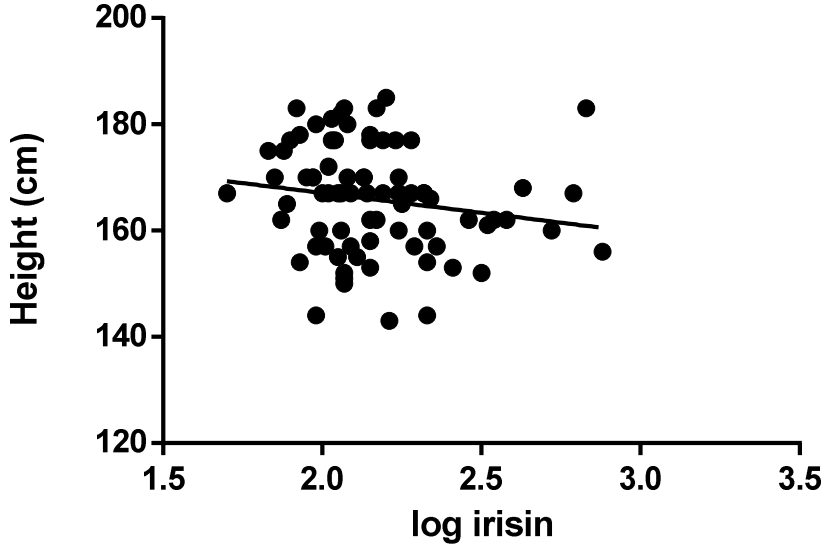

Figure 4.26. Correlation between log irisin and height in cm.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.133$, $R^2 = 0.02947$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

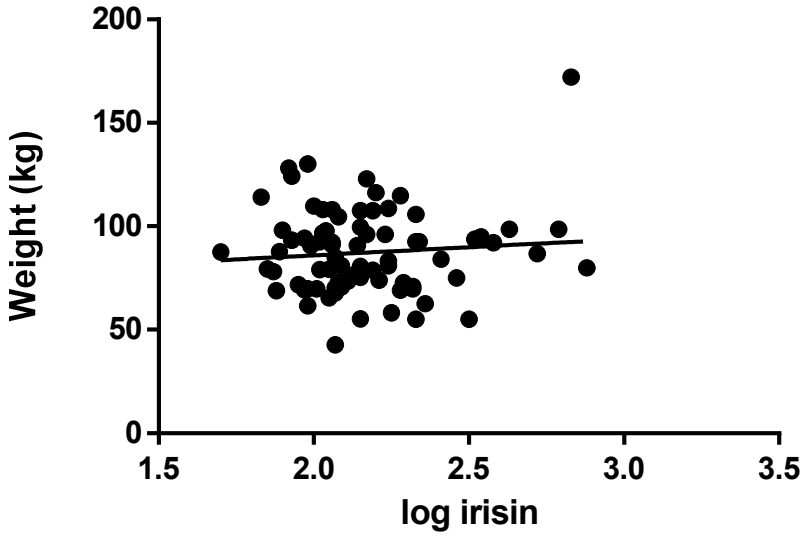

Figure 4.27. Correlation between log irisin and weight in kg.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.437$, $R^2 = 0.007842$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

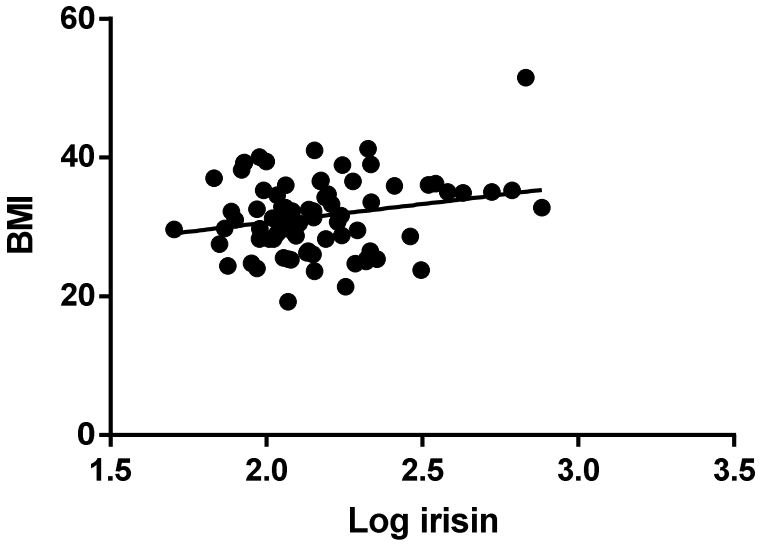

Figure 4.28. Correlation between log irisin and BMI.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. In our type ($p = 0.048$, $R^2 = 0.05228$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

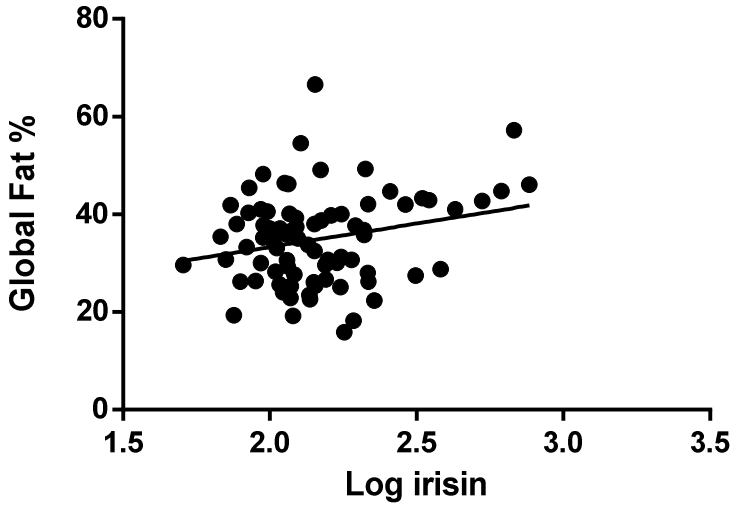

Figure 4.29. Correlation between log irisin and global fat percentage.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.033$, $R^2 = 0.05794$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

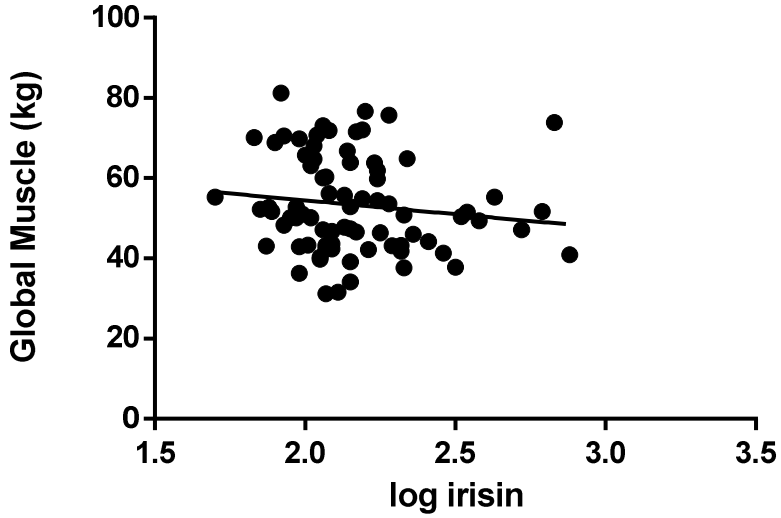

Figure 4.30. Correlation between log irisin and global muscle in kg.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.238$, $R^2 = 0.01803$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

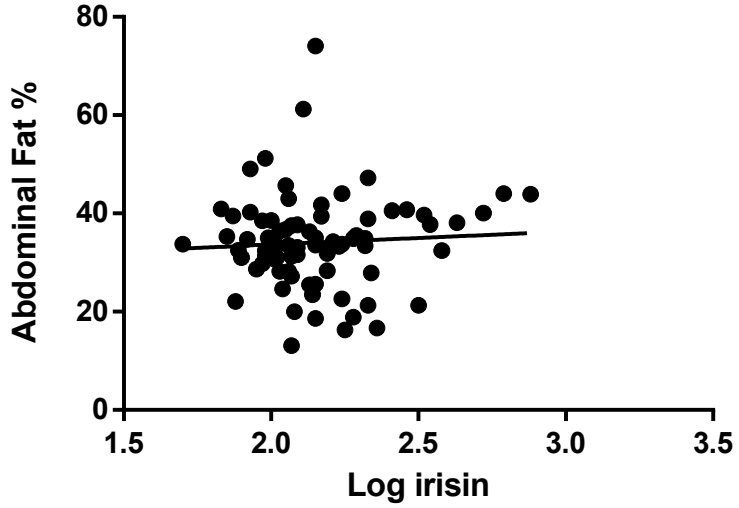

Figure 4.31. Correlation between log irisin and abdominal fat percentage.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.59$, $R^2 = 0.003893$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

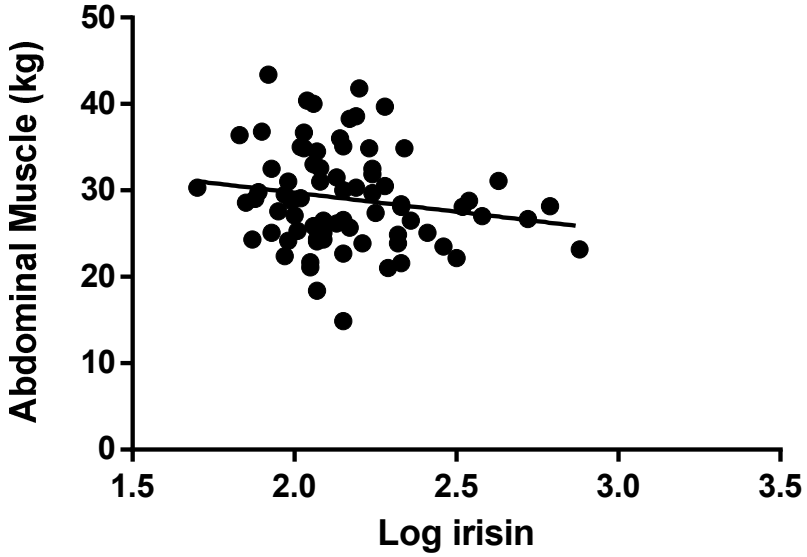

Figure 4.32. Correlation between log irisin and abdominal muscle.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.132$, $R^2 = 0.02976$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

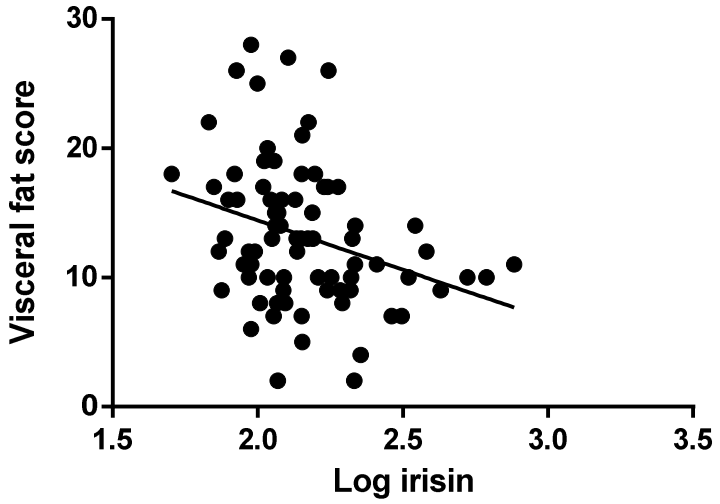

Figure 4.33. Correlation between log irisin and visceral fat score

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.006$, $R^2 = 0.09408$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

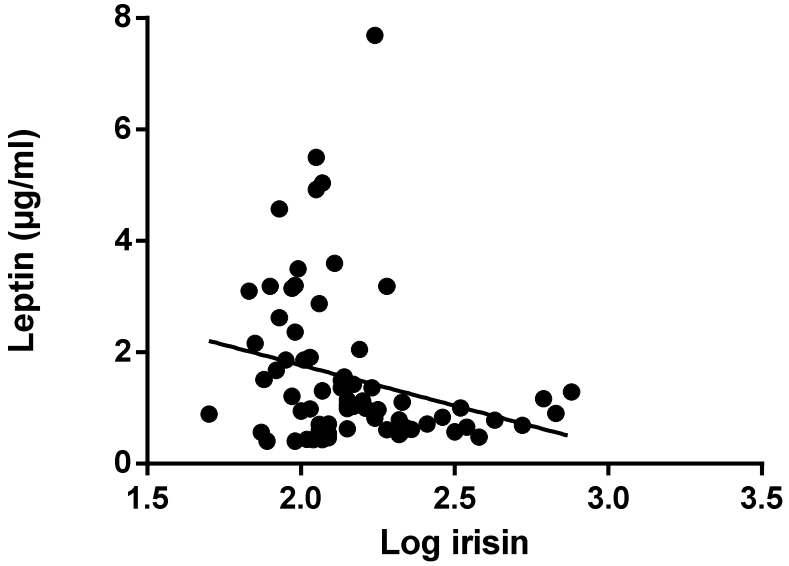

Figure 4.34. Correlation between log irisin and leptin

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.026$, $R^2 = 0.06$) T2D volunteers n = 79. Significance was set at $p < 0.05$.

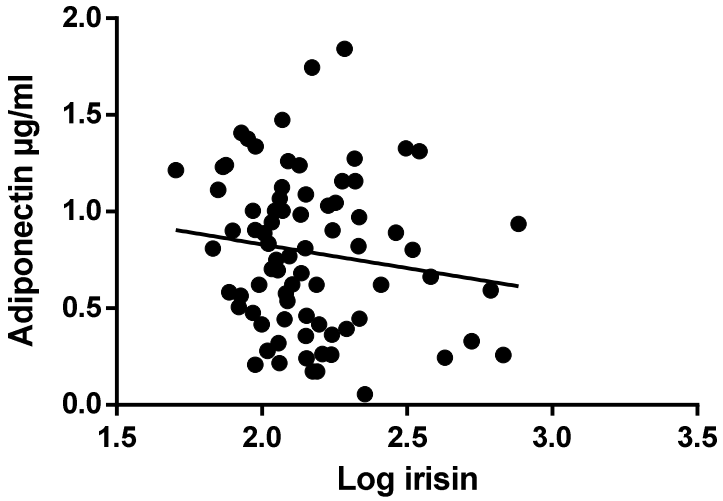

Figure 4.35. Correlation between log irisin and adiponectin

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.2$, $R^2 = 0.02$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

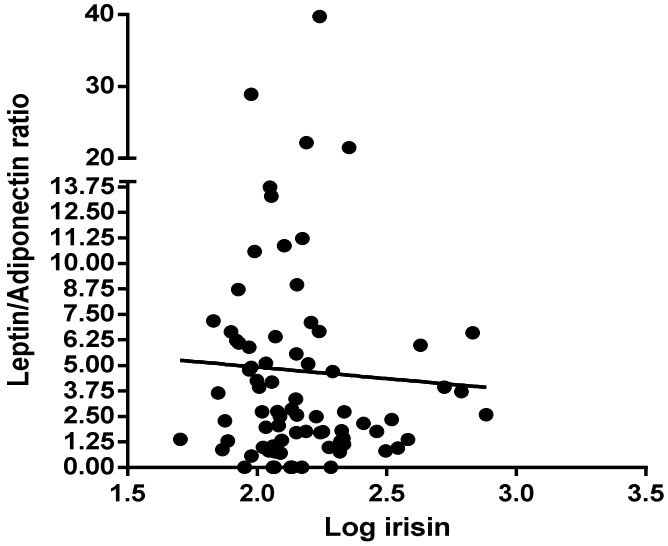

Figure 4.36. Correlation between log irisin and the leptin/adiponectin ratio

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.7$, $R^2 = 0.001$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

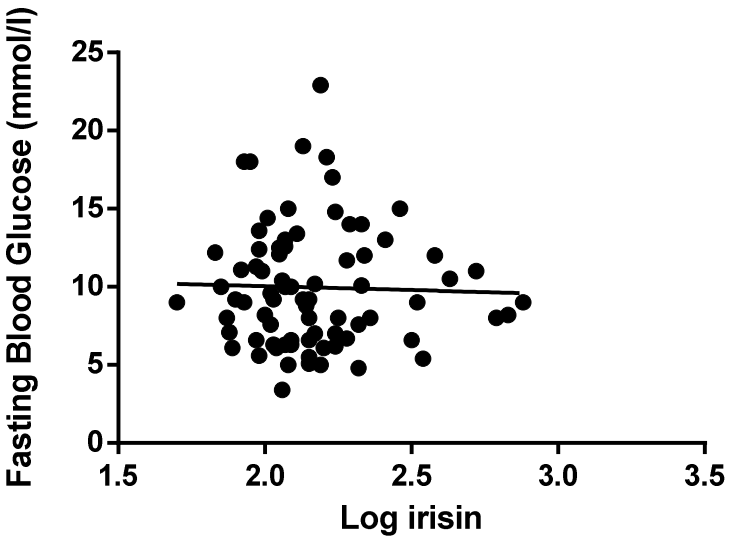

Figure 4.37. Correlation between log irisin and fasting blood glucose.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.791$, $R^2 = 0.0009$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

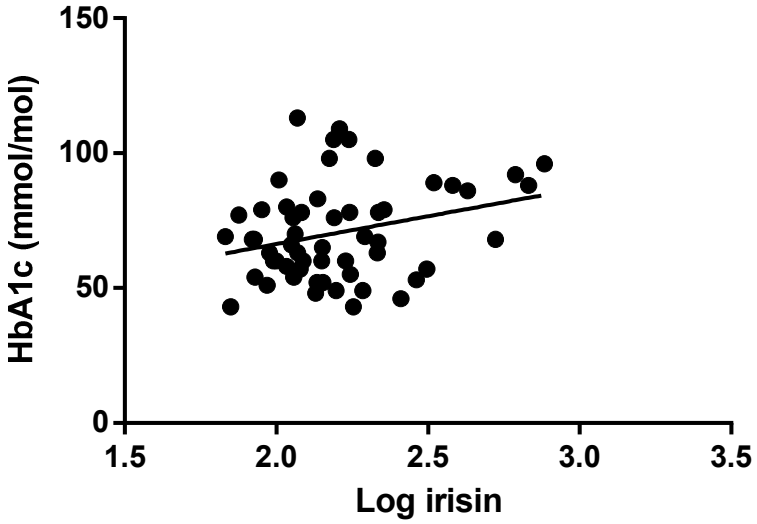

Figure 4.38. Correlation between log irisin and HbA1c.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.032$, $R^2 = 0.08068$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

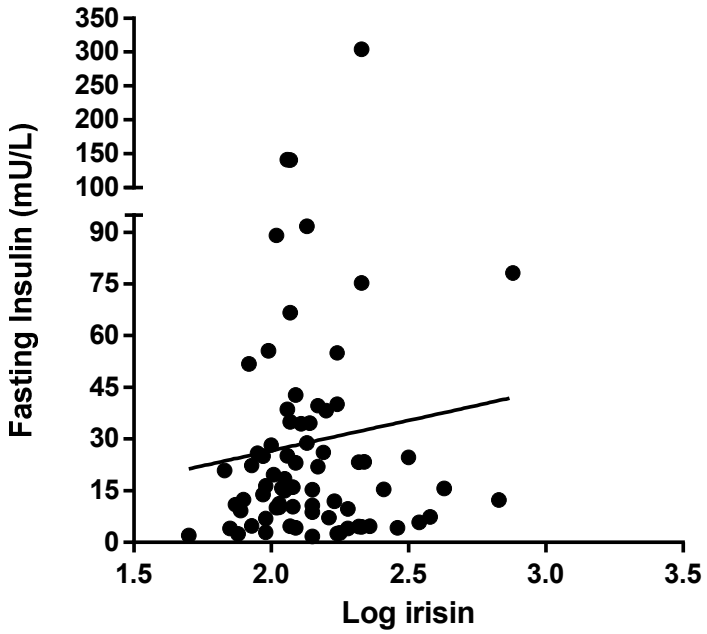

Figure 4.39. Correlation between log irisin and fasting insulin.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.67$, $R^2 = 0.007821$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

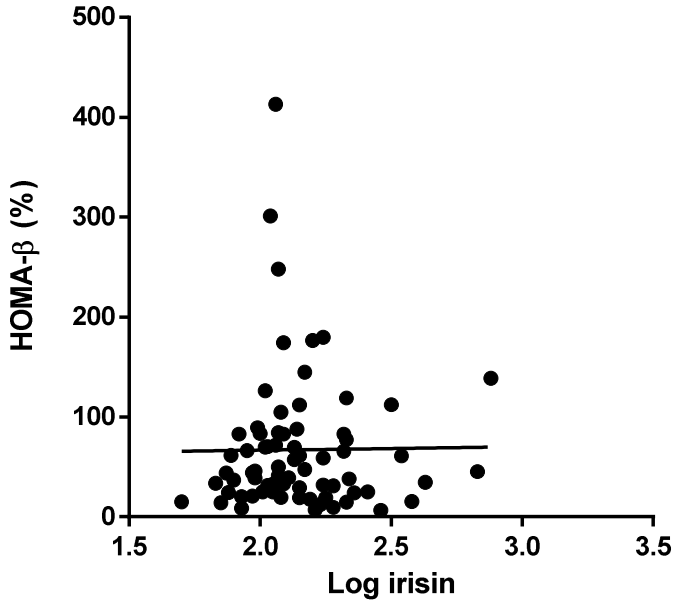

Figure 4.40. Correlation between log irisin and HOMA-β.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.953$, $R^2 = 0.0001171$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

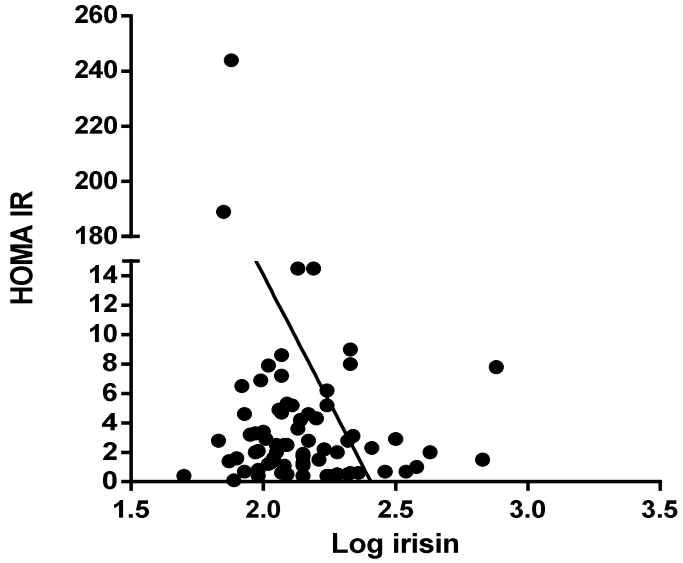

Figure 4.41. Correlation between log irisin and HOMA IR.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.086$, $R^2 = 0.04485$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

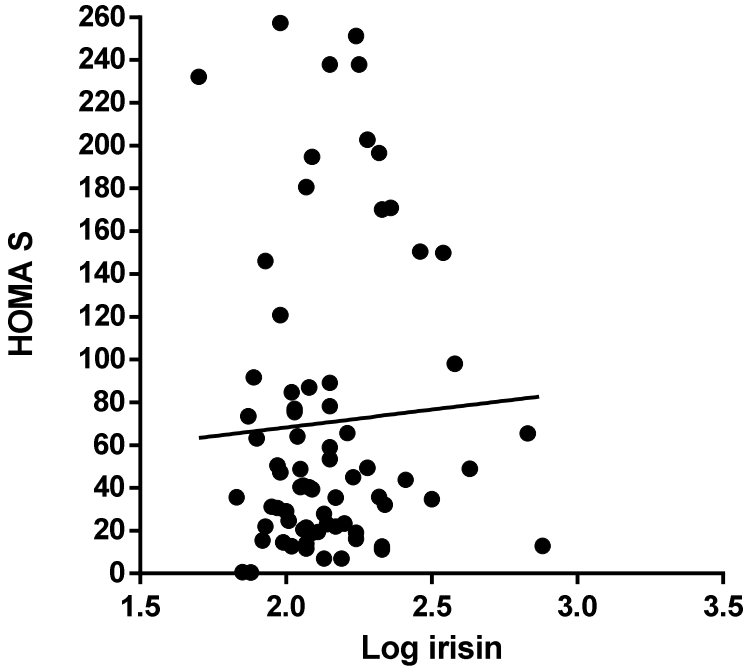

Figure 4.42. Correlation between log irisin and HOMA S.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot ($p = 0.858$, $R^2 = 0.002720$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

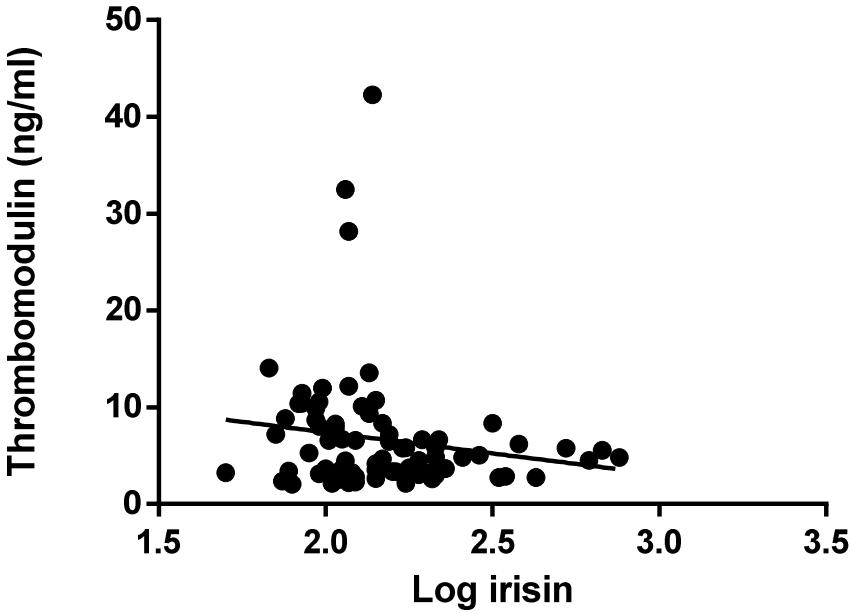

Figure 4.43. Correlation between log irisin and thrombomodulin.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = 0.156$, $R^2 = 0.02513$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

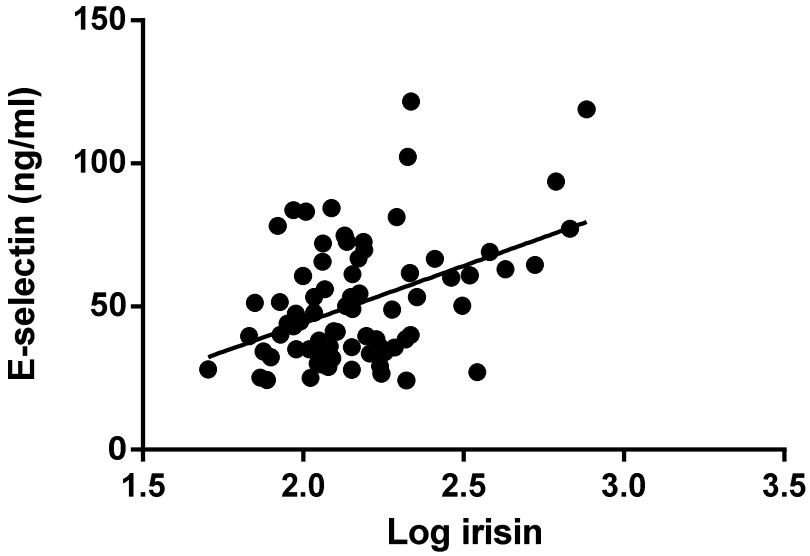

Figure 4.44. Correlation between log irisin and soluble E-selectin.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = < 0.0001$, $R^2 = 0.1887$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

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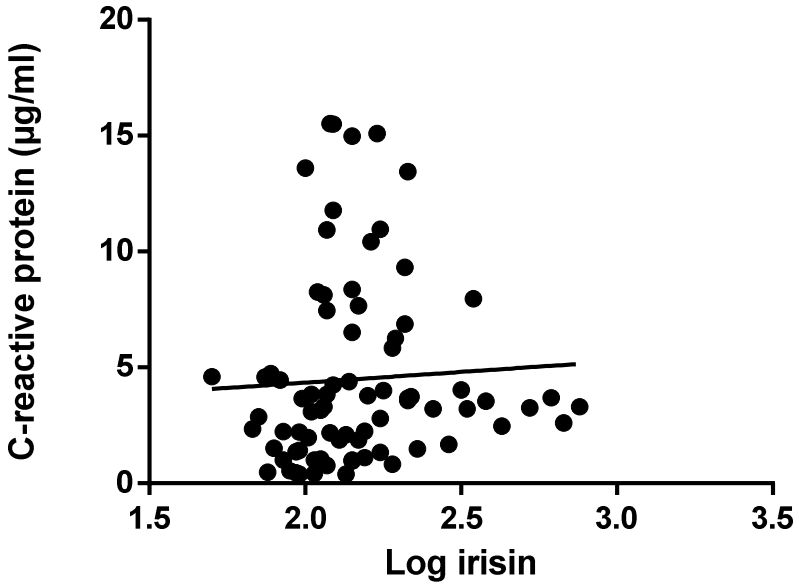


Figure 4.45. Correlation between log irisin and C-reactive protein.

Pearson bivariate correlation represented as a linear regression X, Y scatterplot. ($p = < 0.626$, $R^2 = 0.002881$). T2D volunteers n = 79. Significance was set at $p < 0.05$.

Measurement	Association with Log irisin	Significance
Age	-0.364	0.001
BMI	0.223	0.04
Global fat %	0.241	0.03
Visceral fat score	-0.3	0.006
HbA1c (mmol/mol)	0.28	0.03
Leptin (µg/ml)	-0.24	0.02
E-selectin (ng/ml)	0.43	<0.001

Table 4.3 Results of Pearson's bivariate correlations analysis

Pearson's bivariate correlations between anthropometric/biochemical parameters and log plasma irisin concentration. Data are represented as positive or negative correlation coefficients with p values displaying statistical significance.

4.4.3 Correlation of multiple factors with soluble E-selectin - stepwise backward multiple linear regression.

Results from the Pearson's bivariate correlations (Table 4.4) reveal that soluble E-selectin exhibited a negative association with log transformed plasma irisin. The statistical methodology was adapted from chapter 3. The dependent variable selected was soluble E-selectin concentration, in an attempt to investigate whether the novel association between soluble E-selectin and log irisin and the independent variables were significant associations determined using the Pearson's bivariate correlations test, the backward function was chosen which would remove the least significant variable until only the most significant predictors of soluble E-selectin remained.

Model Summary				
Model	**R**	**R Square**	**Adjusted R Square**	**Std Error of the Estimate**
6	0.493	0.243	0.214	19.74

Table 4.4. Model summary for non-diabetic volunteers.

The final combination of variables selected that are best able to predict T/S ratio, account for 38% of the variability in the T/S ratios.

ANOVA				
2Model	**Sum of Squares**	**df**	**Mean Square**	**Significance**
Regression	6.799	3	2.266	< 0.000
Residual	11.077	77	0.144	
Total	17.876	80		

Table 4.5. The analysis of variance table for both male and female models.

ANOVA analysis shows that for non-diabetic volunteers, the variables selected are significant predictors of T/S ratio. This confirms the significance of the model.

4.4.4 Significant predictors of soluble E-selectin.

The stepwise backward linear regression analysis revealed that the sole significant predictor of soluble E-selectin was circulating irisin (p = 0.003), despite correcting for age, BMI, visceral fat, HbA1c and leptin. Log irisin concentration had a positive beta value of 0.382, a positive beta value suggest an increase in circulating irisin concentration would also lead to an increase in soluble E-selectin, an increase which could be statistically predicted using circulating irisin concentration. The only other variable included in the most significant model was global fat %, which also had positive beta coefficient and that retaining increased adipose tissue could be used as a predictor of soluble E-selectin. However this association was non-significant with p = 0.071.

Coefficients				
Variable	**Beta**	**Standard Error**	**Significance**	**95% confidence interval**
Global Fat %	0.228	0.328	0.071	-0.053 – 1.262
Log Irisin	0.382	11.904	0.003	12.837 – 60.590

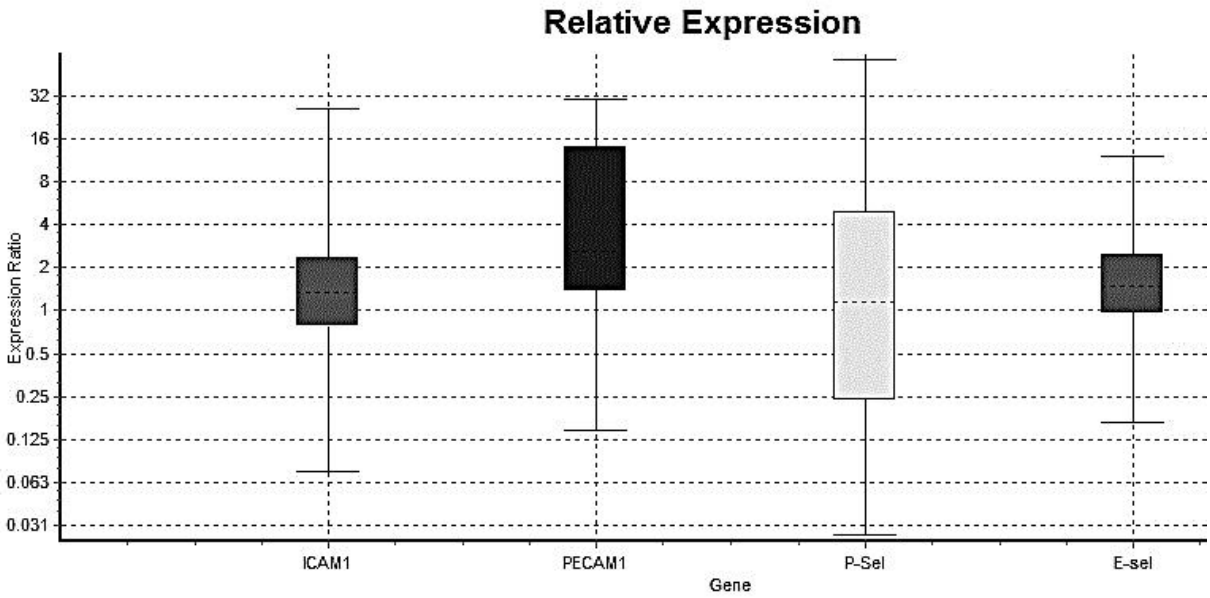
Table 4.6. Stepwise backward linear regression analysis using E-selectin concentration as the dependent variable and significant correlations as independent variables.

The backward stepwise multivariable regression eradicated all variables which were unable to predict E-selectin and only selected log irisin (p =<0.003) as the most significant variable which was able to predict E-selectin ratio.

4.4.5 High but not low irisin concentraton induces E-selectin mRNA expression in primary endothelial cells.

HUVECs treated with 20ng/ml irisin, representative of circulating irisin in a healthy individual, for a period of 4 hours failed to alter gene expression of any of the cell adhesion markers. Comparatively, HUVECs treated with 200ng/ml irisin, a concentration within the range of circulating concentrations observed in the T2D cohort, significantly upregulated gene expression of E-selectin after 4 hours ($p = 0.034$) but not other cell adhesion markers. At 24 hours incubation E-selectin levels had returned to baseline.

A)



B)

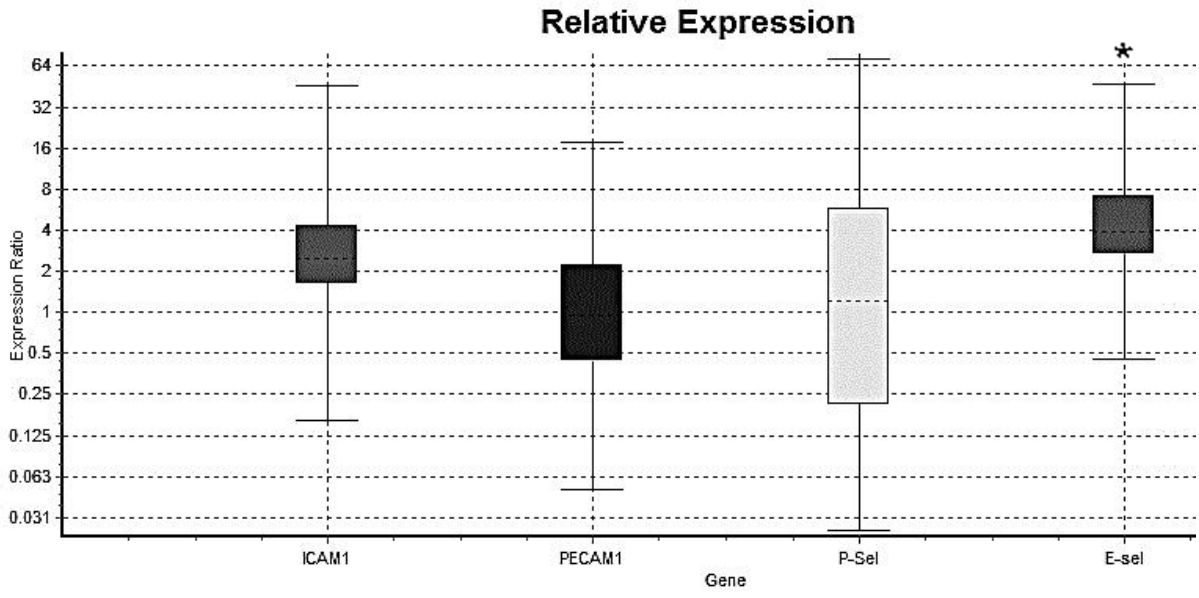
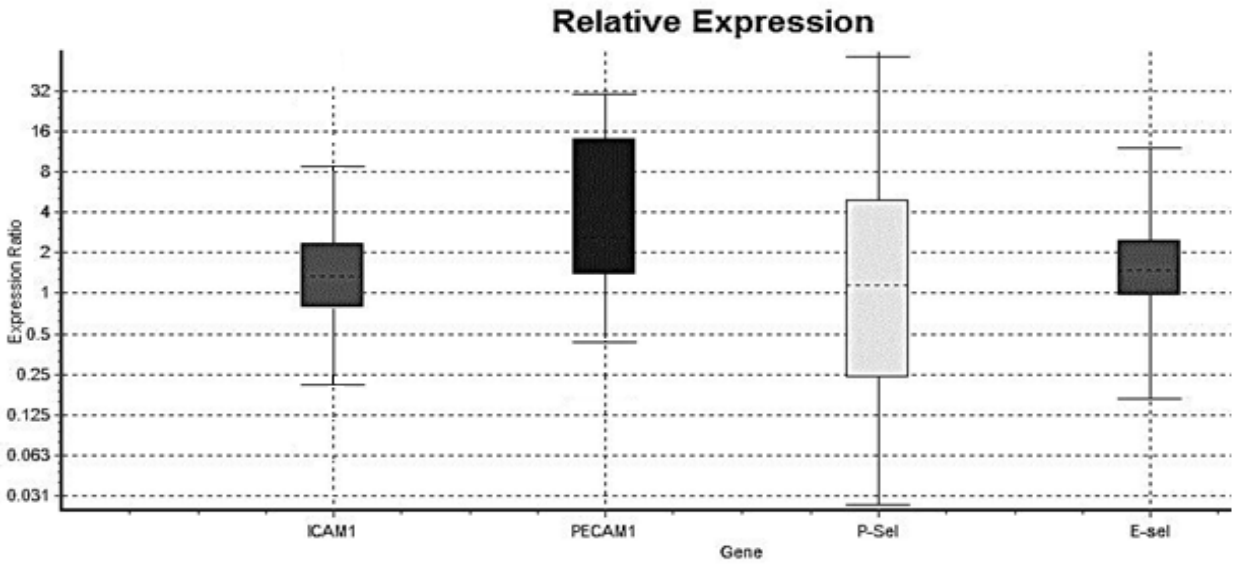


Figure 4.46. Exposure to 200ng/ml irisin for 4 hours induces E-selectin gene expression.

Real-time PCR analysis of irisin treated HUVEC ICAM1, PECAM1, P-selectin and E-selectin mRNA expression levels. (A) Exposure to 20ng/ml irisin for 4 hours, (B) Exposure to 200ng/ml irisin for 4 expression compared to control ($p=0.034$). Values normalised to actin and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta (YWHAZ). Boxplots represent mean ± SD. N=4. Significance was set at $p < 0.05$.

A)



B)

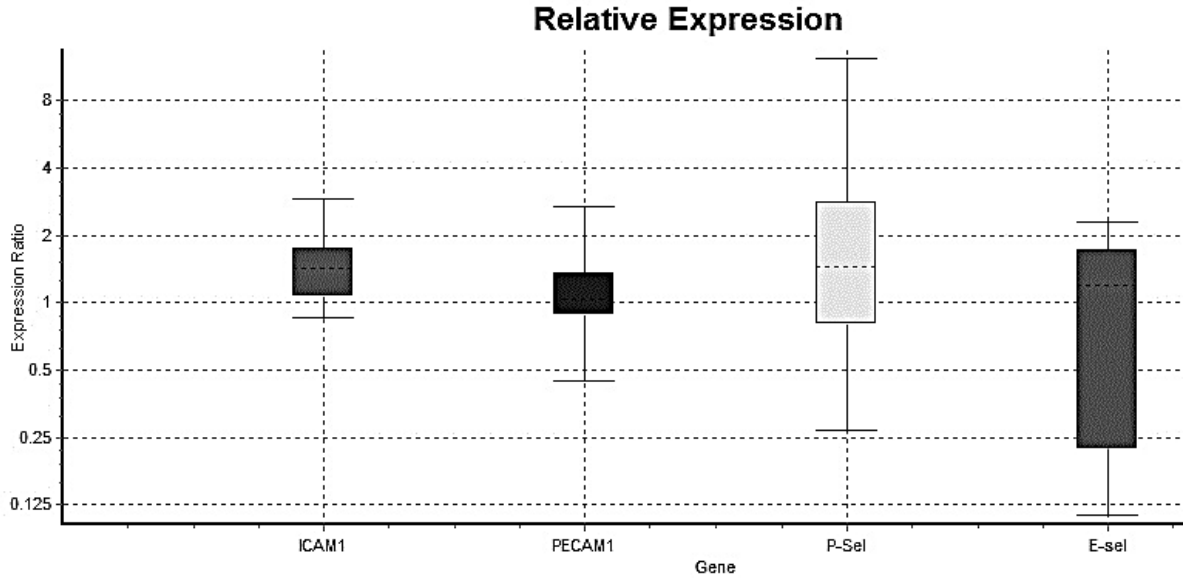


Figure 4.47. Exposure to 200ng/ml irisin for 24 hours did not alter cell adhesion gene expression.

Real-time PCR analysis of irisin treated HUVEC ICAM1, PECAM1, P-selectin and E-selectin mRNA expression levels. (A) Exposure to 20ng/ml irisin for 24 hours, (B) Exposure to 200ng/ml irisin for 24 expression compared to control (p=0.034). Values normalised to actin and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, Zeta (YWHAZ). Boxplots represent mean ± SD. N=4. Significance was set at $p < 0.05$.

4.5 Discussion

The results from this study provide new insight into the role of irisin in obesity and T2D. Data presented here shows a greater than 3-fold higher concentration of circulating irisin was observed in individuals with T2D in comparison to healthy controls (Fig. 4.16) ($p < 0.0001$). Interestingly, there was no significant association between irisin and TL in the T2D cohort (Fig. 4.24) ($p = 0.333$), although a significant association was observed in non-diabetic controls. Pearson's bivariate correlations revealed irisin to exhibit statistically significant positive associations with HbA1c (Fig. 4.38) ($p = 0.03$), BMI (Fig. 4.28) ($p = 0.04$) and global fat % (Fig. 4.29) ($p = 0.03$) and negative associations with visceral fat score (Fig. 4.33) ($p = 0.006$). The most significant association was observed with marker of endothelial dysfunction/inflammation, soluble E-selectin (Fig. 4.44) ($p <0.001$). Furthermore irisin was a statistically significant predictor of soluble E-selectin ($p = 0.003$). This study is the first study to report this novel association and sheds light on a potential mechanism between excess adipose tissue, elevated circulatingn irisin concentration and cardiovascular risk.

This association was further validated using real-time PCR analysis to assess the effect of irisin on endothelial cells. Irisin treated HUVECs exposed to 200ng/ml irisin for 4 hours displayed a 4-fold increase in E-selectin mRNA expression in comparison to control ($p = 0.034$). E-selectin expression levels had returned to baseline after 24 hours (Fig.4.46). Low irisin (20ng/ml) did not induce any change in E-selectin expression levels. Previous research has shown that E-selectin expression after exposure to pro-inflammatory stimuli follows a similar pattern of a peak within 4-6 hours and a return to baseline after 24 hours, suggesting that irisin is acting like a pro-inflammatory cytokine in these cells. The primary findings of this study identified T2D volunteers exhibited reduced TL relative to healthy volunteers. Although the mean age of the T2D cohort was higher; significant negative correlations between TL and age reported previously in healthy volunteers were lost in T2D volunteers [641]. The implications of this result suggest mechanisms

beyond age related cellular divisions are responsible for the reduced TL found in T2D. Obesity and T2D are disorders which carry a significant inflammatory burden and oxidative stress, two factors which have been well established to excel telomere attrition [644, 645]. Although TL did not correlate with markers of endothelial dysfunction/ inflammation in this study, other markers of inflammation have previously been correlated to reduced TL. Salpea *et al*., measured TL in 242 T2D patients and conclude shorter TL is associated with the presence of T2D and this could be partially attributed to the high oxidative stress in these patients. They further report an association with the expression of the *UCP2* functional promoter variant with the TL implying a link between mitochondrial production of reactive oxygen species and shorter TL in T2D [646].

Testa *et al* report patients with T2D had significantly shorter TL than both patients without diabetes complications and healthy control subjects. Moreover, among patients with diabetes complications, TL became significantly and gradually shorter with the increasing number of diabetes complications [647]. Studies offering mechanistic insights into reasons behind reduced TL amongst obese/T2D patients are rare and require further investigation. Comparing the two cohorts it is clearly evident there are stark differences in both anthropometric measurements and biochemical analytes between healthy participants and obese, glucose intolerant participants. Measures of visceral and subcutaneous adipose mass, BMI and consequently the leptin/adiponectin ratio were clearly elevated amongst T2D participants, along with insulin resistance, markers of endothelial dysfunction and inflammation. These observations were expected and validate the fact that an accurate representation of a T2D cohort was in fact recruited for this study. Interestingly, despite similar muscle mass readings plasma irisin levels were more than 3-fold higher amongst subjects with T2D relative to non-diabetic volunteers.

The relationship between irisin and markers of energy metabolism have been explored earlier by other investigators [582, 648, 649]. The results reported herein oppose findings reported by Liu

et al and Choi *et al*., who suggested T2D patients have reduced concentrations of irisin [650, 651]. The lack of anthropometric measurements taken in both of these studies prohibited the authors to comment on the influence of skeletal muscle or adipose tissue on irisin secretion. It is noteworthy that both of the studies recruited non-obese T2D participants, the reported BMI in both studies combined averages to be 26, generally recognised as borderline overweight [652]. The mean BMI reported in this study is 31.5. The differences between observations reported here may be in part relate to these phenotypic differences. Significant positive associations reported within this study between plasma irisin levels and measures of obesity, including BMI and total body fat percentage support this claim.

These observations suggest circulating irisin levels are primarily determined by degree of adiposity in individuals suffering from obesity/T2D, the significant negative association with visceral adipose tissue implies that irisin secretion is predominantly subcutaneous in nature. These findings are consistent with previous reports. Roca-Rivada *et al* were amongst the first authors to present irisin as an adipokine and reported increased irisin secretion in adipocytes derived from diet induced or genetically obese mice, moreover this group found subcutaneous adipose tissue to secrete 40% more irisin in comparison to visceral fat [263, 642, 653] . Nevertheless the precise mechanisms regarding irisin secretion from adipose tissue remain to be elucidated and are difficult to conceive considering the strong evidence previously associating irisin secretion with skeletal muscle tissue. The increased irisin concentrations reported herein may be a result of a compensatory mechanism to account for innate defects in metabolism or decreased energy expenditure in obese/T2D patients. More specifically the larger amounts of adipose mass associated with obese/T2D individuals may be directly influencing circulating irisin concentrations, considering the strong associations with parameters of adiposity reported here. Based upon these results and the work of Bostrom *et al*., it seems the muscle/adipose irisin secretion ratio is largely dependent upon the physiological situation.

Thus, with exercise training, muscle tissue would strongly affect irisin levels as in non-obese healthy individuals, whereas in atypical BMI cases such as obesity/T2D, adipose tissue may actively secrete irisin. In conjunction with this concept are the findings of Stengel *et al*., who conducted a rather simplistic study but with great effect. The authors report plasma irisin levels fluctuate considerably with varying BMI, with anorexic individuals secreting the least irisin and obese individuals producing significantly higher amounts [264]. Furthermore irisin over secretion was reported amongst obese animals [263]. In support of this study is a separate investigation analysing irisin levels amongst 107 participants without metabolic syndrome (MetS) and 44 participants with MetS. The authors report significantly higher baseline irisin levels amongst subjects with MetS than in healthy control volunteers. Irisin was associated negatively with adiponectin and positively with parameters of ill health for instance; BMI, blood pressure, fasting glucose, triglycerides and homeostasis model assessment for insulin resistance [654]. These data convolute the current understanding of irisin.

Crujeiras *et al*., designed a sophisticated protocol whereby anthropometric measurements along with plasma irisin levels were measured before and after a hypocaloric dietary intervention in a cohort of obese men and women. Primary findings showed positive associations between plasma irisin, weight, BMI, waist circumference, and fat mass. More importantly irisin levels coincided with body weight reduction after the dietary treatment and again returned to baseline levels in those patients regaining the lost weight as fat mass [655]. Iglesia *et al*., support these findings and report plasma irisin depletion under energy restriction is associated with improvements in lipid profile in MetS patients, an effect which can also be achieved by bariatric surgery [656]. However the seeming lack of correlations between irisin and other well characterised adipokines, e.g., leptin and adiponectin in this study, highlights the potential influence of unknown obesity/T2D related factors also involved in irisin secretion. In either case it appears abnormal irisin secretion is only contributing to underlining metabolic defects seeing as increased irisin concentrations would be expected to dissipate stored energy via non-shivering thermogenesis and protect against metabolic disorder. In line with this theory is the fact that circulating irisin levels have previously

been demonstrated to be an independent and strong positive predictor of the metabolic syndrome (MetS), with individuals in the highest irisin tertile having up to 9.5 times higher chances to develop MetS [657]. Given the beneficial effects of irisin on glucose/fatty acid metabolism, insulin secretion/sensitivity in animal models, it has been proposed that the elevated irisin levels in obesity and the MetS might represent a state of "irisin resistance", similar to the well-established insulin resistance in T2DM and leptin resistance in obesity [263, 642]. The lack of association between irisin and TL in this T2D cohort aids this concept and implies that the beneficial effects of irisin in delaying cellular senescence are lost amongst individuals with an atypical metabolism, potentially due to irisin resistance. In the present study, a strong positive correlation was noted between HbA1c and plasma irisin levels. Plasma irisin levels have previously been correlated with increased fasting insulin and glucose concentrations, furthermore a separate study was able to predict insulin resistance, as assessed by HOMA-IR using plasma irisin concentrations [658]. Although both glucose and insulin were measured in this study no such association was observed here. While it is highly unlikely that circulating irisin is directly facilitating haemoglobin glycosylation and it is possible increased irisin secretion is a compensatory mechanism to counteract elevated glucose concentrations and associated metabolic disturbances in obese/T2D individuals but is rendered ineffective due to irisin resistance. Sesti *et al*, has associated increased irisin concentrations with carotid intima-media thickness, an indicator of vascular atherosclerosis in a cohort of 192 adults. Individuals diagnosed with T2D are at increased risk of developing cardiovascular disease (CVD), according to the work of Sesti, increased irisin concentrations facilitate the development of CVD [659]. However there is much need for the validation of these results.

Although other studies have reported increased levels of circulating irisin amongst individuals suffering from cardiovascular complications, more specifically myocardial infarction, the precise molecular mechanisms yet remain unknown. Other authors have likewise claimed that the increase in irisin under obesity conditions may indicate a physiological adaptation to improve

glucose tolerance, which is often impaired in obese subjects, whether irisin can modulate, appetite, insulin sensitivity or glucose uptake remains to be seen [660, 661]. Although Polonsky reports converse associations between irisin and insulin and inverse correlations between irisin and ghrelin, described as the “hunger hormone” and antagonistic counterpart of leptin. Advance glycation end products are routinely found in the blood of diabetic patients, these glycated proteins are rendered ineffective and have been linked to the development of T2D, Alzheimer’s disease and accelerated cellular ageing [662, 663]. It is worth assessing whether circulating irisin may also become glycosylated in the blood stream and its biological function impaired, as a result excessive secretion ensues to compensate for an increasing amount of biologically non-functional irisin. It is well established that insulin resistance presents as a pathophysiological feature in the obese phenotype and manifests as the primary contributing factor in the development of T2D [664-666].

Insulin insensitivity has causally been linked to CVD predisposition; however the mechanisms unifying the diverse effects of insulin resistance are not well defined [667-669]. Existing research has elucidated the strong influence of subclinical inflammation on the development of both T2D and CVD [670, 671]. A specific mechanism whereby inflammation may be able to instigate the progression of these metabolic disorders is via endothelial dysfunction [672, 673]. The data reported here highlights positive associations between circulating irisin and soluble E-selectin, a prominent marker of endothelial dysfunction. Moreover, stepwise backward multi-variable linear regression analysis revealed soluble E-selectin as the only significant predictor of circulating irisin. These findings have crucial implications on the current understanding of irisin and reveal a prospective link between elevated irisin concentrations and cardiovascular complications, via E-selectin mediated endothelial dysfunction. Increased adipose mass, typically found in abundance amongst T2D patients is a significant contributor to the level of blood inflammatory cytokines, consequently upregulating the expression of cellular adhesion molecules along the vascular endothelium [674]. Furthermore plasma levels of E-selectin, ICAM-1 and vascular cell

adhesion molecule 1 (VCAM-1) have been quantified to greater extents in cross-sectional studies recruiting patients suffering from T2D [675-677]. Separately Dosi *et al* support these findings, by using ultrasound assessment of endothelial dependent flow mediated dilation of the brachial artery; the authors conclude endothelial dysfunction was considerably higher amongst T2D volunteers in comparison to non-diabetic controls. Positive correlations were also observed with BMI and waist to hip ratios. E-selectin cell surface expression has previously been denoted to correlate strongly with soluble levels in times of endothelial distress and inflammation [678, 679]. In support of this statement is data reported in this study. Using HUVEC cells as a model of the endothelium, irisin concentrations comparable to concentrations observed in the T2D cohort were able to upregulate E-selectin gene expression, 4-fold following 4 hours of exposure relative to control ($p = 0.034$).

Furthermore flow cytometric analysis conducted in this study revealed a 15.57% increase in E-selectin cell surface expression following 4 hour incubation with 200ng/ml irisin, in comparison to control. The increased irisin concentrations observed amongst the T2D cohort within this study are a reflection of enhanced adipose mass and may directly be influencing circulating E-selectin levels by upregulating inflammatory pathways. The role of E-selectin in influencing cardiovascular complications is currently limited to the already well established role of E-selectin in mediating an inflammatory response [680, 681]. Unlike other cell adhesion molecules, E-selectin is generally absent and exclusively found in the activated endothelium during times of inflammation [682]. The author postulates high irisin concentrations evoke E-selectin expression as expressed by the HUVEC model, and facilitate leukocyte rolling, adhesion, and transmigration into the sub-endothelial space, progressively leading to the formation of atherosclerotic plaques (Fig 4.49). A study supporting the author's hypothesis examined ICAM-1, VCAM-1 and E-selectin concentrations in patients who had been diagnosed with unstable angina reports increased concentrations of E-selectin on the day of admission and further increases at a follow up test ten days later in comparison to healthy controls [683].

The detrimental effects of increased irisin are not however limited to enhanced susceptibility to cardiovascular disease; recent experimental evidence has been successful in correlating endothelial dysfunction with increased blood pressure and insulin resistance [684, 685]. Supporting this claim are studies documenting reduced risk of endothelial dysfunction and more specifically a reduction in circulating soluble E-selectin levels following treatment with statins, angiotensin-converting enzyme inhibitors and metformin, commonly prescribed drugs to control glucose homeostasis. It is possible therefore that elevated concentrations of irisin not only pose a considerable risk in developing CVD but also stem the progression of T2D in a positive feedback loop.

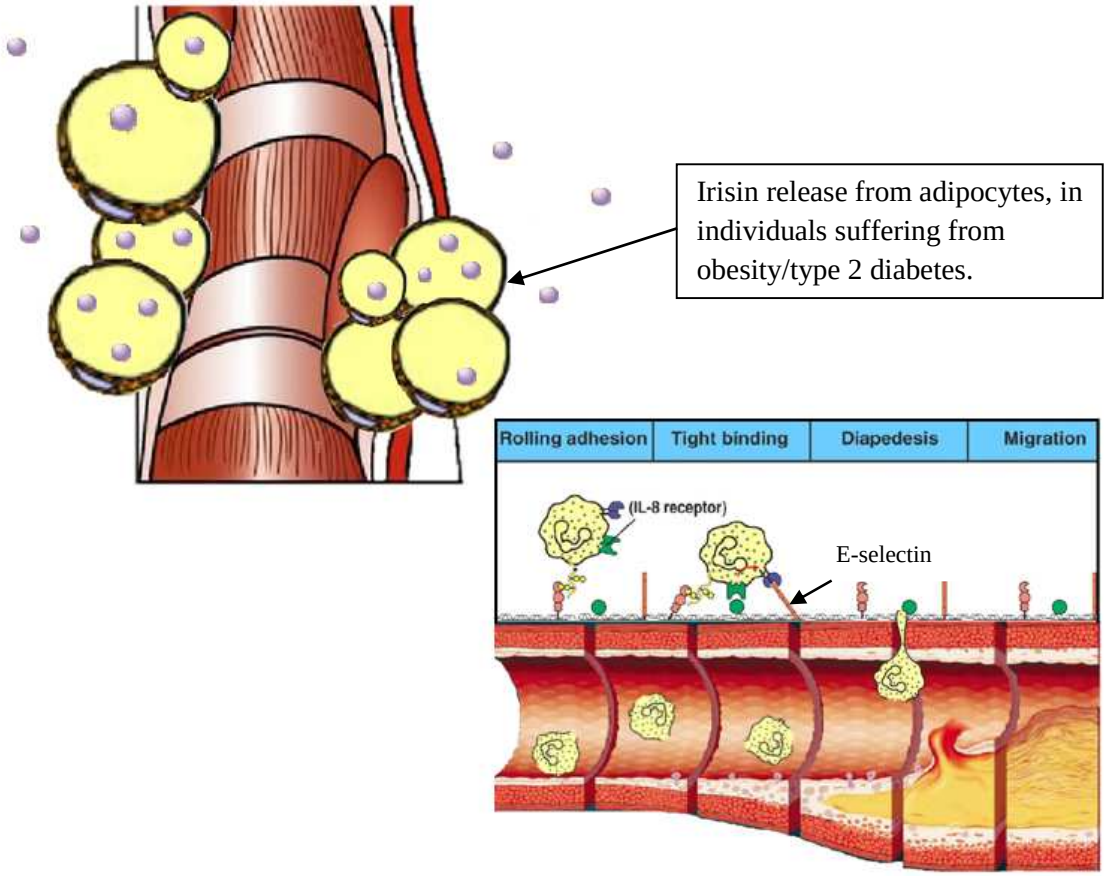


Fig 4.49. Hypothetical model of the events leading to increased inflammation and potential cardiovascular complications, as a result of enhanced circulating irisin concentration amongst T2D patients.

Increased irisin secretion from irisin resistant adipocytes, upregulates the expression of cell adhesion molecule E-selectin across the endothelium surface. Although the precise mechanisms responsible for this are unknown, enhanced irisin secretion from adipocytes may be to compensate for innate metabolic defects or enhanced caloric intake, as commonly observed in obese/T2D individuals. Irisin may be exhibiting hormetic functions and exhibit beneficial effects on glucose homeostasis and insulin sensitivity at lower concentrations, while at higher concentrations propagate an inflammatory immune response. Obese/T2D individuals are often at an increased risk of atherosclerosis, the rise in irisin mediated E-selectin gene expression and soluble E-selectin may be aiding the development of atherosclerotic plaques, via facilitating leukocyte adhesion, binding, and migration into the endothelium.

4.6. Conclusion

The work of other researchers discussed previously provides sufficient evidence to suggest T2D participants are at an increased risk of developing cardiovascular complications. A potential mechanism or a facilitator of this risk, may be increased irisin secretions which promote E-selectin mediated endothelial dysfunction and inflammation. The novel association reported in this study is the first to highlight a potential role for irisin to instigate an inflammatory response; these findings undoubtedly raise concerns regarding the implementation of recombinant irisin as a therapeutic intervention to help obese T2D patients lose weight. Instead future research should look into measures of reducing circulating irisin concentrations and eradicating irisin resistance experienced by T2D patients. Furthermore the role of irisin mediated oxidative stress and inflammation should be considered.

Chapter 5: Results

The Effect of the Senescence-Associated Secretory Phenotype (SASP) on Glucose Homeostasis in Metabolic Cells.

5.1 Introduction

Cellular senescence is defined as irreversible proliferative arrest, despite the presence of mitogenic stimulants [686]. Cellular senescence serves as *in vivo* tumor suppression mechanism by restricting aberrant proliferation and consequently is a significant modulator of organismal ageing [686, 687]. Senescent cells (SC) differ considerably from quiescent somatic cells. A distinct marker of SC is a radically altered, flattened and enlarged morphology along with increased expression of β-galactosidase, referred to as senescence associated β-galactosidase (SA- β gal) [688, 689]. Existing research has identified numerous molecular phenotypes associated with SC including altered gene expression, chromatin reorganisation and deregulated protein processing [690]. Although we develop senescent cells throughout the lifespan, advancing age is associated with an accumulation of senescent cells potentially due to a failing of ageing immune cells to clear senescent cells from the body [487]. The presence of senescence-associated markers at sites of age related pathologies has been reported previously, confirming the importance of this potentially mechanism of ageing. Evidence for the presence of SC overlying atherosclerotic plaques, engulfing chrondrocyte clusters and replacing mitotic satellite cells provides further links between cellular senescence and age related disorders, atherosclerosis, arthritis and sarcopenia [473, 691-693]. Furthermore, senescent cells can influence the growth and angiogenic ability of premalignant cells, inducing carcinogenesis in ageing tissues and exhibit a decreased ability to migrate, which enhances susceptibility to impaired wound healing a common complication of advancing age [694, 695].

Currently it is unclear precisely how accumulating SC may influence the development of age related disorders. However, their ability to persist in circulation despite losing biological function may be a vital contributing factor. There are at present no published data regarding the survival time of senescent cells *in vivo*, although various studies have attributed their enhanced survival in circulation to increased resistance to apoptotic clearance [696, 697].

Although existing research has identified characteristics common to all SC, it is noteworthy that the genetic variability, influence of growth and transcription factors gives rise to a multitude of somatic cells and may also govern cell-type specific effects of entering into cellular senescence. For example senescent vascular endothelial cells exhibit diminished nitric oxide synthase activity, a vital regulator of vascular function, a decline in its production predisposes an individual to cardiovascular complications [698]. Likewise the increased accumulation of senescent stem cells significantly hampers the proliferative capacity of a specific cell subset, depletion of cardiomyocyte cell pools would be expected to have debilitating effects on cardiac function and induce age associated cardiovascular complications [699]. The biological impact of the senescent phenotype is likely to be largely dependent upon the non-autonomous effects of SC [700]. The ability of senescent cells to release into the circulation a plethora of growth factors and extracellular matrix (ECM)-degrading proteins, has been reported to significantly impact neighboring cells, more specifically the release of matrix metalloproteinases degrade collagen and elastin, vital components of the ECM [701, 702].

Some of the most significant detrimental effects of SC are attributed to the ability of SC to secrete a complex mix of pro-inflammatory cytokines, commonly referred to in the scientific literature as the senescent associated secretory phenotype (SASP) [703]. It is likely the secreted cytokines not only impact tissues in close proximity but extend the range of targets SC can impact by enforcing widespread tissue dysfunction and by inducing senescence in non-senescent cells [501]. The work by Gallin recognises the self-regulatory role of the SASP, mediating gene transcription in favor of increased pro-inflammatory cytokine production, initiating a positive feedback loop encouraging SC to secret more pro-inflammatory cytokines [704]. Therefore SC sustain a chronic low grade inflammatory phenotype often observed and vital in the propagation of disorders affecting aged individuals. Demonstrating this point are cause and effect studies correlating drastic increases in inflammatory cytokine production from SC with atherosclerosis, osteoporosis and hepatic fibrosis [705, 706].

It seems that the link between cellular senescence and disease development/progression is an SASP mediated inflammatory response. Numerous age associated disorders present with an inflammatory background. Commonly associated with advancing age is a marked increase in insulin resistance, this reduced ability to regulate glucose homeostasis progresses to manifest as T2D, reported to be inflammatory in nature correlating inflammation with disorders of ageing [670, 707]. Considerable data, reported in the late 1950's and 60's highlighted the role of inflammation in T2D development. Experiments showing that adipose tissue–derived pro-inflammatory cytokines such as TNF-α could cause insulin resistance in experimental models provided the necessary impetus to initiate research linking inflammation to diabetic pathogenesis [344, 708]. This initial study paved the way for future research, which has consolidated the role of pro-inflammatory cytokines in interfering with insulin receptor signalling.

Furthermore existing research has identified T2D patients are at an increased risk of accumulating senescent cells relative to non-diabetic individuals [709]. Yuan *et al* report T2D patients experience an accelerated onset of senescence of endothelial progenitor cells, a vital contributor to cardiovascular complications commonly experienced by patients suffering with T2D [710]. Further evidence associating cellular senescence with T2D recognises a reduced proliferative ability of the pancreatic beta cell with advancing age, thus reduced net output of insulin in response to elevated glucose concentrations [711]. However SASP mediated proliferative arrest amongst non-senescent insulin target tissues and their effect on T2D progression has not been investigated. Is it possible that increased number of SC induce widespread cellular senescence and by doing so increase the proportion of insulin non-sensitive cells to sensitive cells, reducing their ability to utilise glucose, particularly primary targets of insulin i.e. hepatocytes, adipocytes and skeletal muscle cell. The concluding effect, not only facilitates increased insulin resistance but predisposes the affected individual to glucotoxcity related health implications, a common occurrence in ageing.

5.2 Aims and Hypothesis

Increasing numbers of senescent cells have been linked to age related health implications, impaired tissue function and predisposition to tissue disease development. Integral to the manifestation of age related pathologies is the ability of senescent cells to avoid immune detection and, of more interest is the senescence-associated secretory phenotype (SASP) involving the production of pro-inflammatory cytokines which may induce senescence arrest in neighbouring non-senescent tissue environments. Dysregulated glucose homeostasis is a hallmark of T2D and is commonly associated with advancing age. The aim of this section of the present study is to elucidate the role of the SASP in metabolic dysfunction and its role in propagating senescence in non-senescent cells, and whether the SASP can alter glucose metabolism. The authors sought to determine the effect of SASP on glucose homeostasis in mouse hepatic, adipose and skeletal muscle cell lines, furthermore the effect of chronological age on metabolic and senescent related gene expression will be assessed in mouse, hepatic, adipose and skeletal muscle tissue.

The hypotheses for this study were:

1. The SASP is able to alter glucose homeostasis in C2C12, AML-12 and 3T3-L1 cell lines.

2. The expression of metabolic genes required for normal physiological function in tissues are down regulated in aged mice, while genes translating to the senescent phenotype would be increased and the converse is expected in younger mice tissue.

5.3 Methods

For complete details of experimental methodology please refer to chapter 2, section 2.9.2-2.9.6, 2.10.2-2.11, 2.13-2.15, 2.16-2.16.8.

5.4 Results

5.4.1 Induction of senescence in human dermal fibroblasts (HDF) using hydrogen peroxide, etoposide and doxorubicin hydrochloride.

Existing literature has identified hydrogen peroxide (H_2O_2) as a proficient chemical inducer of cellular senescence in various cell lines including HDF. As H_2O_2 is a potent DNA damaging agent a concentration curve was tested in HDF to determine a suitable dose of H_2O_2 that would be induce senescence but not cell death. Following 45 minutes of treatment, cells were stained with β-galactosidase, the gold standard for identifying senescent cells, a non-treatment control was setup which contained no hydrogen peroxide (Fig 1a). 50, 60 and 70µM treatments were ineffective in inducing cellular senescence, as a large proportion of cells did not express β-galactosidase activity (Fig 1b, Fig 2a and b). 80µM treatment was better at inducing SA- β gal staining while 90µM treatment exhibited an even spread of senescent cells. 100 and 110µM were more cytotoxic and caused widespread cell death (Fig 4a and b). In general the use of H_2O_2 was not wholly successful, and as cells must be passaged following H_2O_2 treatment in order to induce senescence, this prolongs the time taken to induce senescence and therefore other DNA damaging agents were investigated.

Etoposide is a cytotoxic anti-cancer drug that binds to topoisomerase II, preventing DNA re-ligation and causing DNA strands to break. Again, a concentration curve was established to determine the optimum concentration capable of inducing cellular senescence without inducing widespread cell damage. 1, 5 and 10µM treatments induced very little cellular senescence and large empty areas suggested increased cell death (Fig.5b, and 6c and d). 15 and 20µM significantly altered cell morphology (Fig.7e and f), whilst higher concentrations induced large scale cell death (Fig 8g and h). The use of etoposide was therefore found to be problematic and an alternative chemical was required.

Doxorubicin hydrochloride is also a chemotherapeutic agent which inhibits DNA replication and was clearly the better of the three treatments used in this study to induce senescence. While 1.5 and 1.75µM induced cellular senescence a significant amount of cell death was also observed. Alternatively, other treatment concentrations used including 0.25, 0.5, 0.75, 1, 1.25 µM all upregulated the number of senescent cells as determined by SA β-gal activity. 1.25µM proved to be the optimum concentration to induce cellular senescence (Fig 11f.) with even distribution of β-galactosidase expression and minimal cell death. Another advantage of using doxorubicin over previously tested DNA damaging agents is the enhanced rate of senescence induction. A 45 minute incubation with doxorubicin is sufficient for upregulating features characteristic of SC, without the need to further passage the cells following treatment.

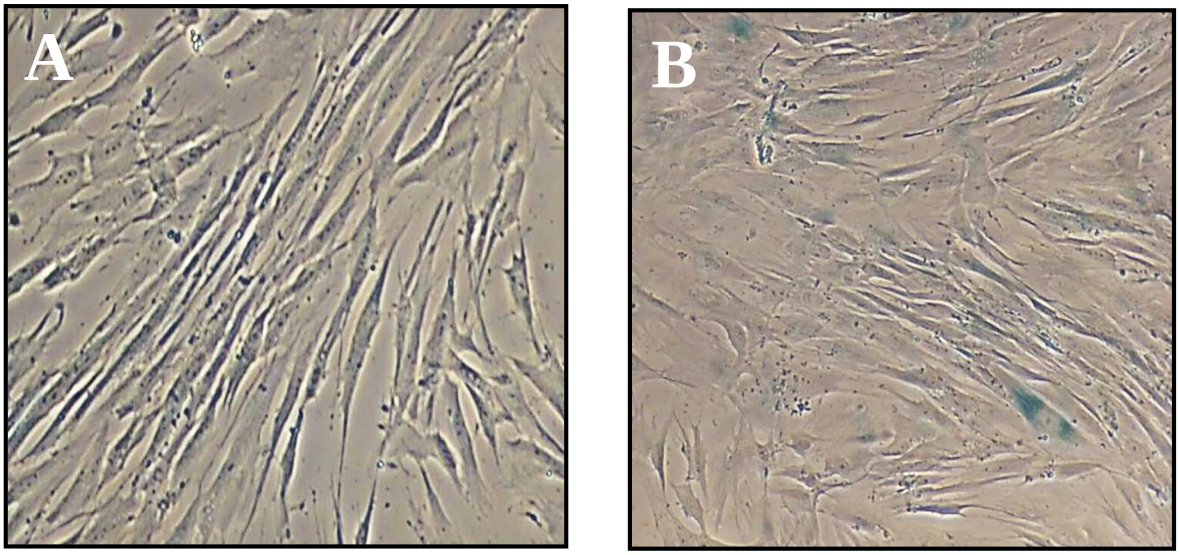


Figure 5.1. SA-β-Gal expression in HDF treated with hydrogen peroxide.

HDF were treated with hydrogen peroxide prior to β-Gal staining and then photographed at 100x magnification. A) negative control. B) 50µM hydrogen peroxide treatment.

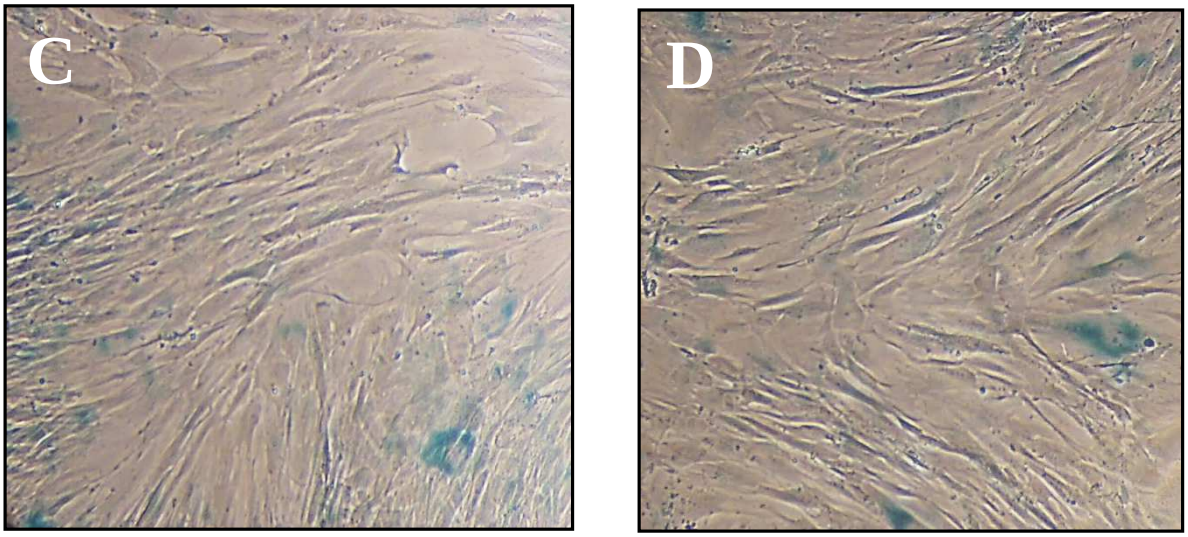


Figure 5.2. SA-β-Gal expression in HDF treated with hydrogen peroxide.

HDF were treated with hydrogen peroxide prior to β-Gal staining and then photographed at 100x magnification. C) 60µM hydrogen peroxide treatment. D) 70µM hydrogen peroxide treatment.

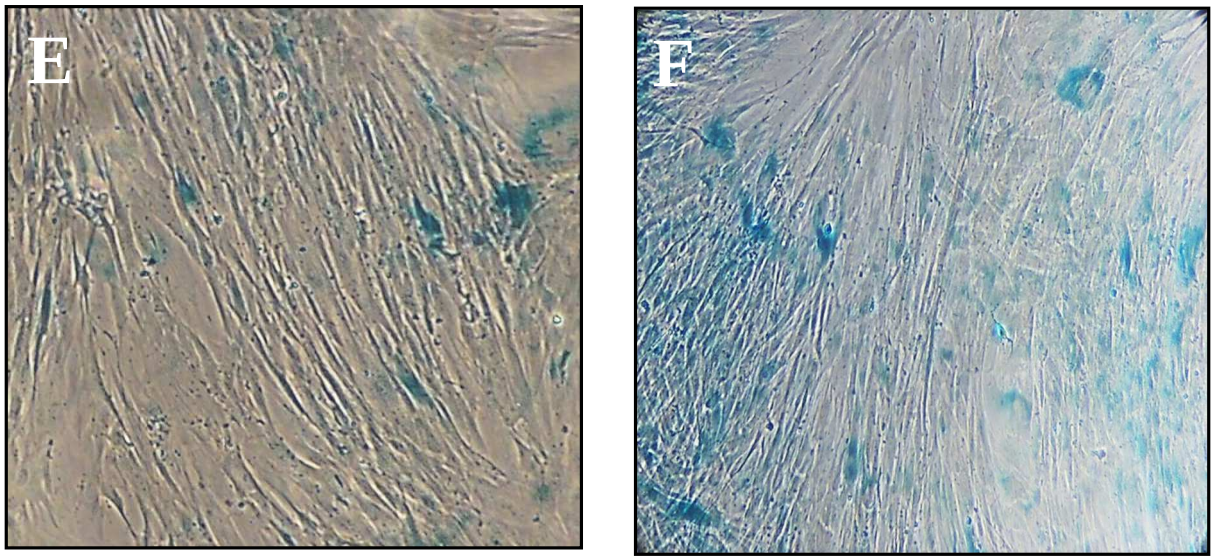


Figure 5.3. SA-β-Gal expression in HDF treated with hydrogen peroxide.

HDF were treated with hydrogen peroxide prior to β-Gal staining and then photographed at 100x magnification. E) 80µM hydrogen peroxide treatment. F) 90µM hydrogen peroxide treatment.

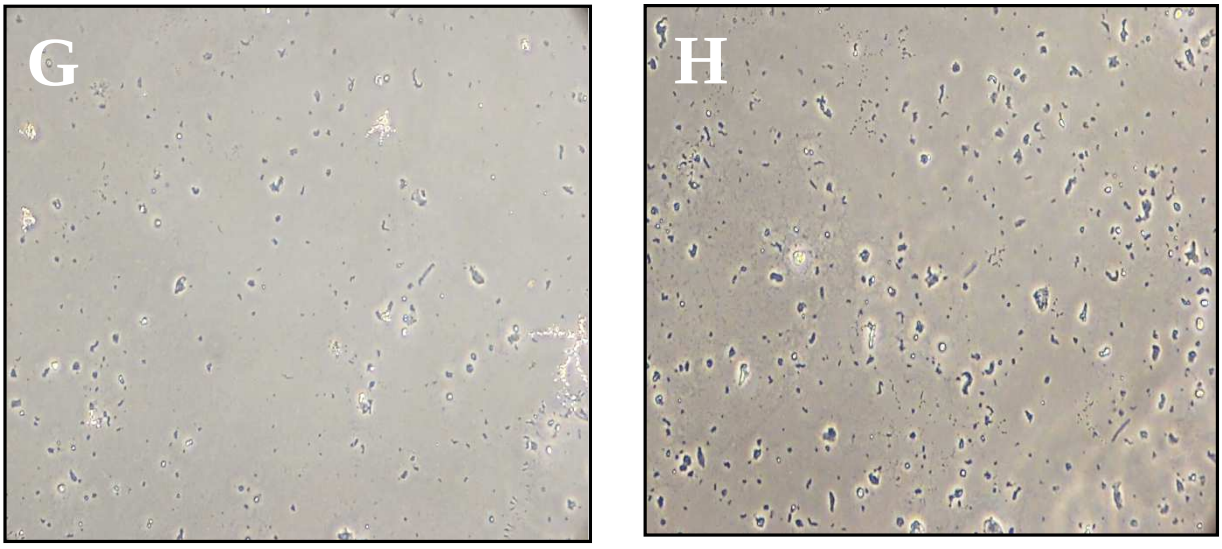


Figure 5.4. SA-β-Gal expression in HDF treated with hydrogen peroxide.

HDF were treated with hydrogen peroxide prior to β-Gal staining and then photographed at 100x magnification. G) 100µM hydrogen peroxide treatment H) 110 µM hydrogen peroxide treatment.

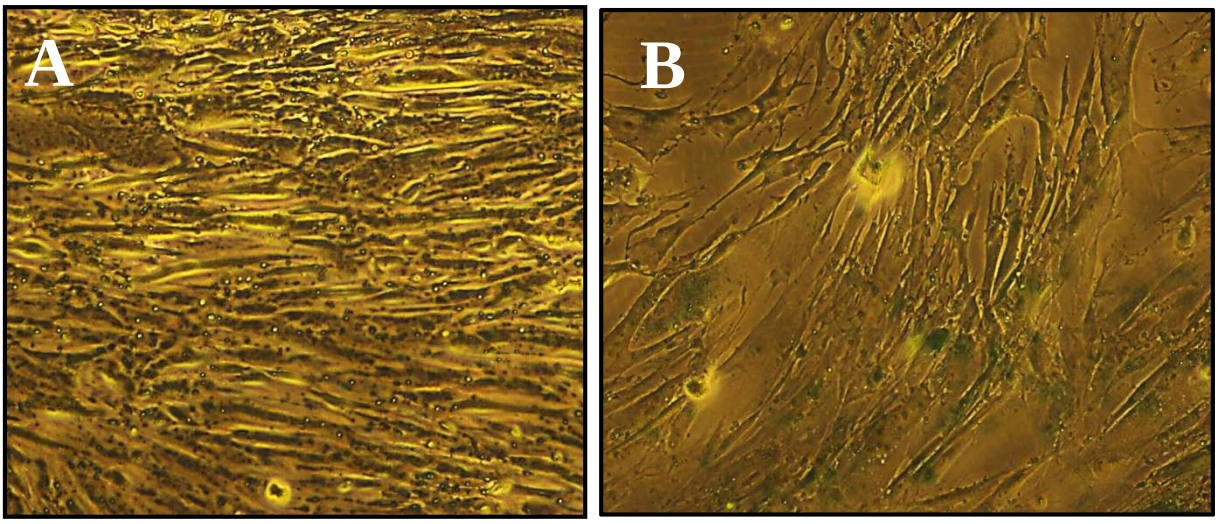


Figure 5.5. SA-β-Gal expression in HDF treated with etoposide.

HDF were treated for 45 minutes with etoposide prior to β-Gal staining and then photographed at 100x magnification. A) Etoposide negative control. B) 1µM etoposide treatment.

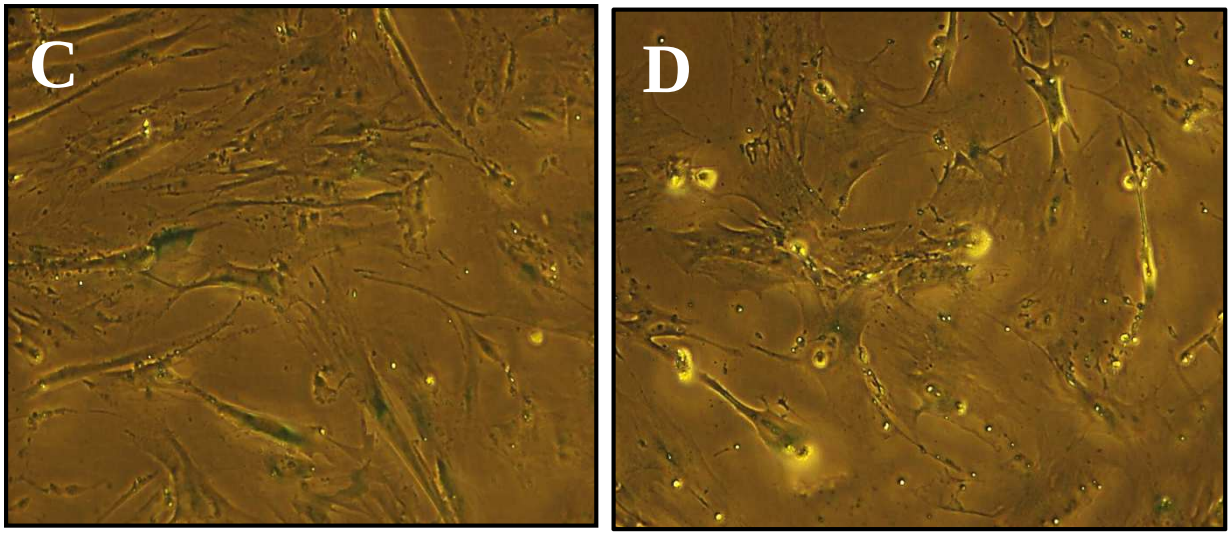


Figure 5.6. SA-β-Gal expression in HDF treated with etoposide.

HDF were treated for 45 minutes with etoposide prior to β-Gal staining and then photographed at 100x magnification. C) 5µM Etoposide, D) 10µM etoposide treatment.

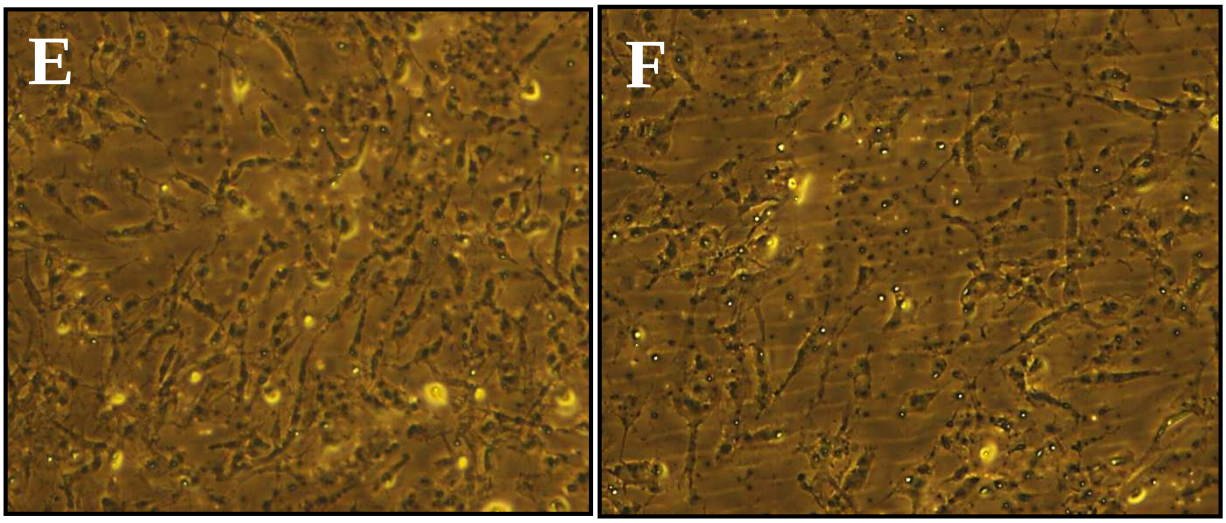


Figure 5.7. SA-β-Gal expression in HDF treated with etoposide.

HDF were treated with etoposide prior to β-Gal staining and then photographed at 100x magnification. E) 15μM etoposide treatment, F) 20μM etoposide treatment.

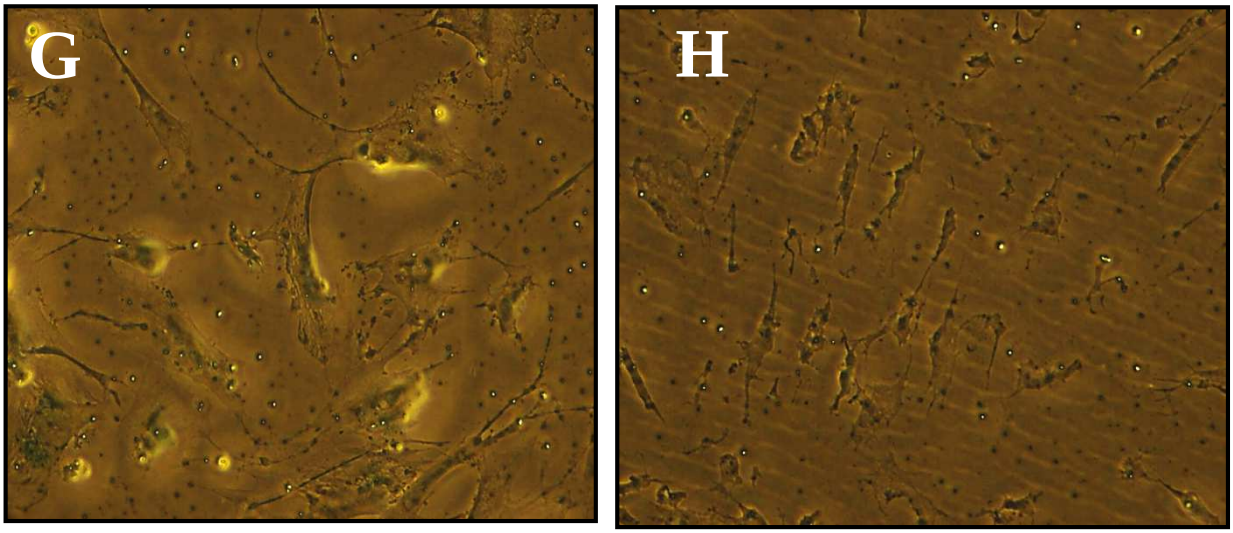


Figure 5.8. SA-β-Gal expression in HDF treated with etoposide.

HDF were treated with etoposide prior to β-Gal staining and then photographed at 100x magnification. G) 25μM etoposide treatment, H) 30μM etoposide treatment.

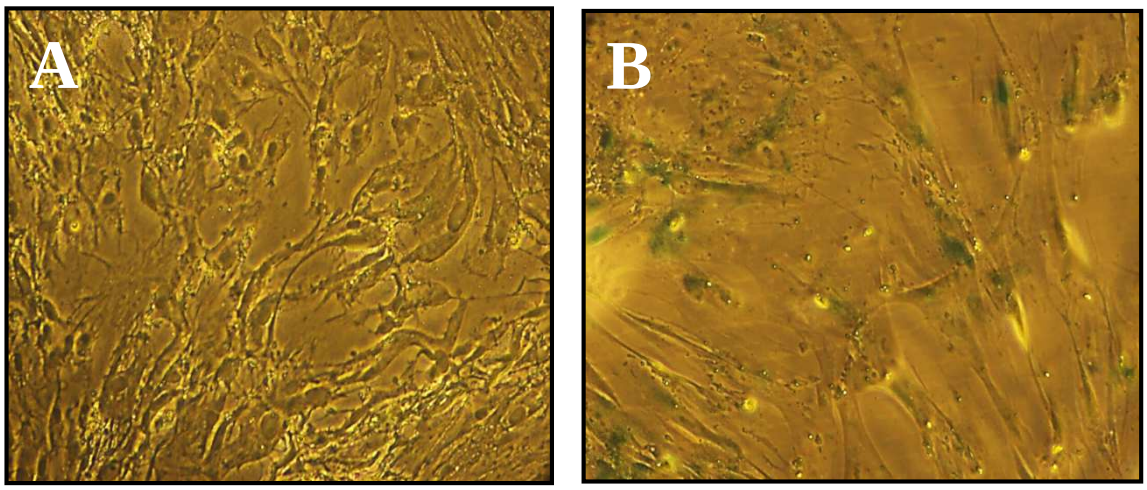


Figure 5.9. SA-β-Gal expression in HDF treated with doxorubicin hydrochloride.

Cells were treated with Doxorubicin hydrochloride prior to β-Gal staining and then photographed at 100x magnification. A) Doxorubicin hydrochloride negative control. B) 0.25µM doxorubicin hydrochloride treatment.

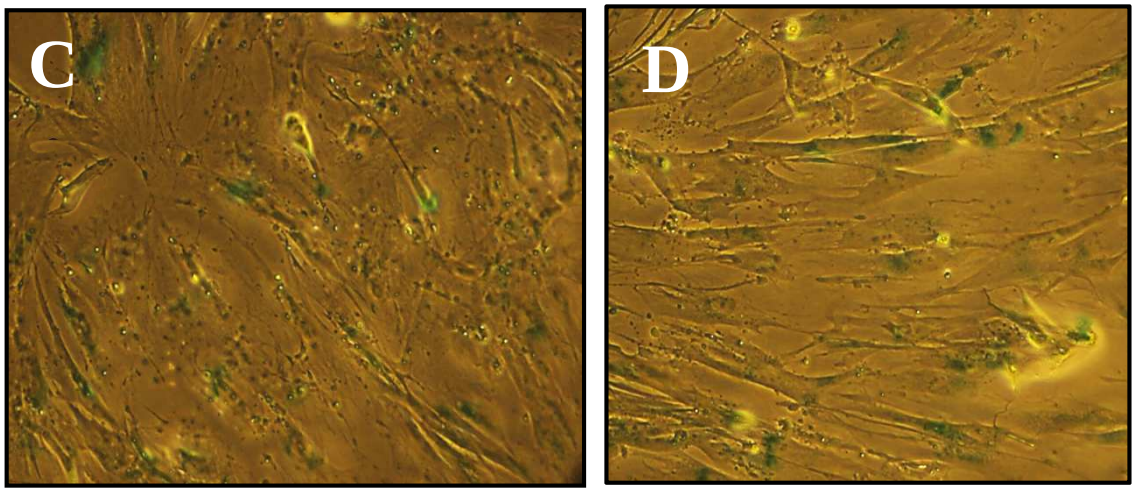


Figure 5.10. SA-β-Gal expression in HDF treated with doxorubicin hydrochloride.

Cells were treated with hydrogen peroxide prior to β-Gal staining and then photographed at 100x magnification. C) 0.5µM doxorubicin hydrochloride. D) 0.75µM doxorubicin hydrochloride treatment.

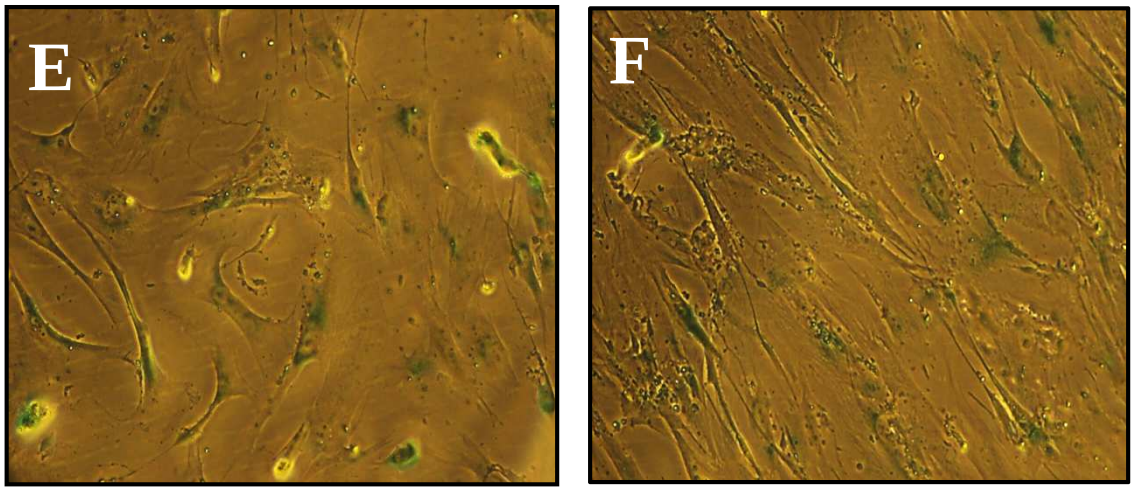


Figure 5.11. SA-β-Gal expression in HDF treated with doxorubicin hydrochloride.

Cells were treated with hydrogen peroxide prior to β-Gal staining and then photographed at 100x magnification. E) 1µM doxorubicin hydrochloride. F) 1.25µM doxorubicin hydrochloride treatment.

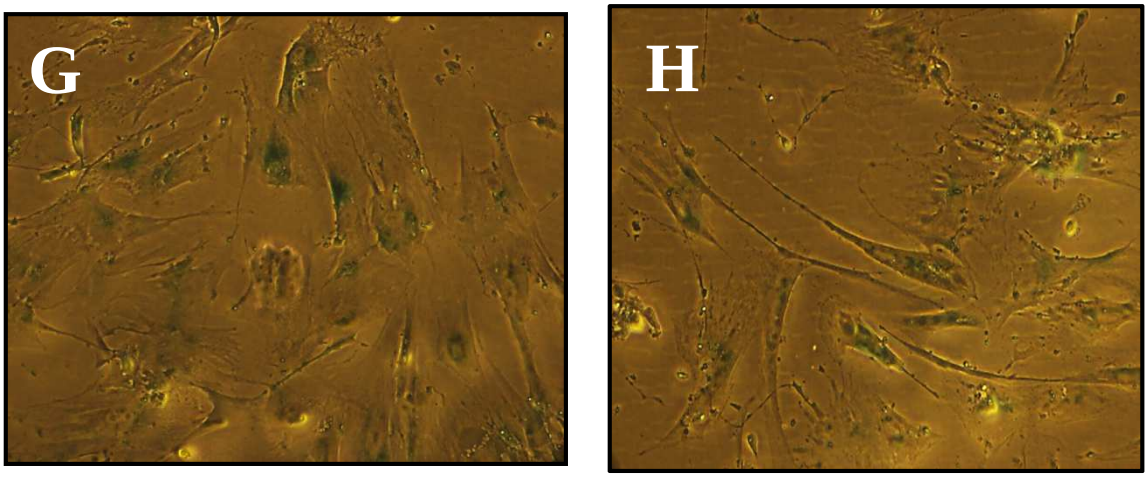


Figure 5.12. SA-β-Gal expression in HDF treated with doxorubicin hydrochloride.

Cells were treated with hydrogen peroxide prior to β-Gal staining and then photographed at 100x magnification. E) 1.5µM doxorubicin hydrochloride. F) 1.75µM doxorubicin hydrochloride treatment.

5.4.2. AML-12 hepatocytes treated with 20% and 40% SASP from senescent fibroblasts for 24 and 48 hours.

AML-12 hepatocytes treated with 20% conditioned media taken from SC collected over 24 or 48 hours, did not demonstrate any alteration in glucose content of media after 24 or 48 hour treatment in comparison to cells treated with media taken from confluent non-senescent fibroblasts (Fig 5.13). Treatment with 40% conditioned media taken from senescent fibroblasts after 24 hours did not affect glucose uptake following 24 and 48 hour incubations either, however AML-12 hepatocytes incubated with 40% conditioned media collected from senescent cells over 48 hours exhibited a significantly increased glucose content in the media after 48 hours ($p < 0.05$), suggesting a reduction in glucose uptake in comparison to hepatocytes treated with control, non-senescent media (Fig 5.14).

5.4.2.1.40% SASP treatment did not affect cell viability in AML-12 hepatocytes following 24 and 48 hour incubations.

AML-12 hepatocyte cell viability remained unchanged following treatment with 40% conditioned media from senescent cells for 24 and 48 hours (Fig. 5.15 and 5.16). AML-12 cells treated with conditioned media from non-senescent fibroblasts were equally as viable as AML-12 cells treated with conditioned media from senescent fibroblasts. Significant cell death was observed in cells treated with 200µM etoposide used as a positive control to induce cell death ($p = <0.001$). As there was no change in cell viability the reduced ability of AML-12 hepatocytes to uptake glucose is not a reflection upon a reduced number of cells, instead another mechanism is responsible for the reduced ability to utilise glucose.

5.4.2.2 Glucose utilisation was restored in AML-12 hepatocytes following co-incubation of 40% SASP and p38 inhibitor.

AML-12 hepatocytes incubated with 40% conditioned media from SC for 48 hours in the presence of 100nM p38 inhibitor restored the glucose content of media to levels observed in cells exposed to conditioned media taken from non-senescent fibroblasts ($p = < 0.01$).

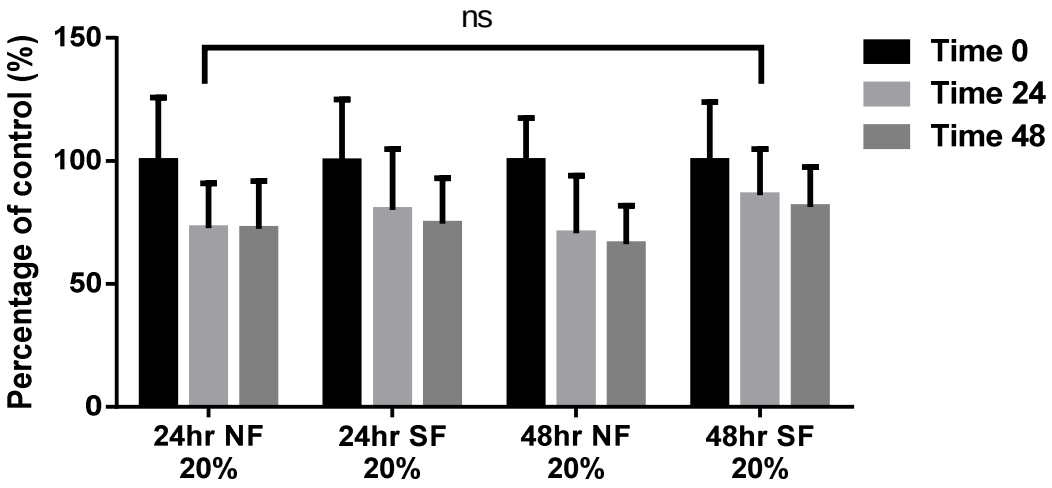


Figure 5.13. Glucose utilisation by mouse AML-12 hepatocytes following treatment with 20% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

AML-12 hepatocytes treated with 20% SF retrieved after 24 and 48 hours and incubated for 24 and 48 hours. Following respective incubations glucose content of cell media was quantified and compared to control cells treated with NF control. Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SD. Significance was set at p = <0.05, N = 4.

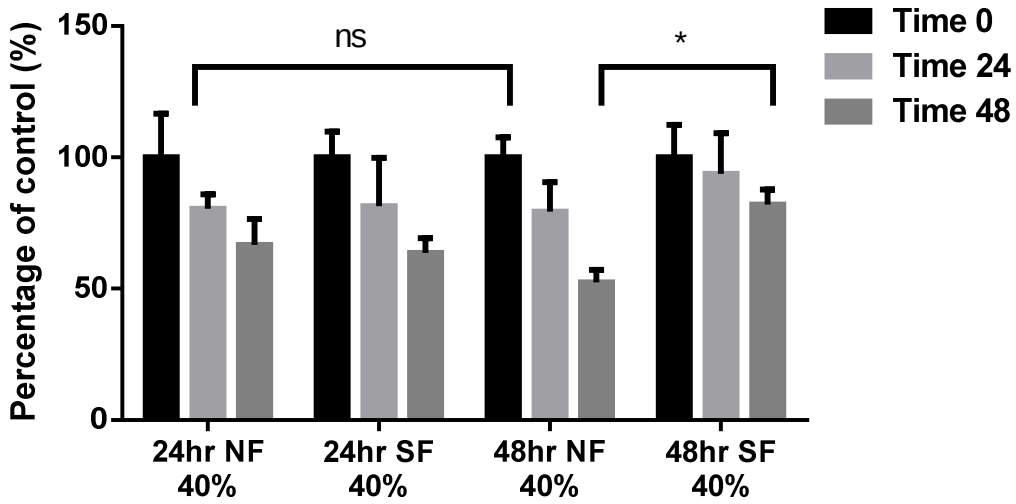


Figure 5.14. Glucose utilisation by mouse AML-12 hepatocytes following treatment with 40% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

AML-12 hepatocytes treated with 40% SF retrieved after 24 and 48 hours and incubated for 24 and 48 hours. Following respective incubations glucose content of cell media was quantified and compared to control cells treated with NF control p = <0.05. Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SD. Significance was set at p = <0.05, N = 4.

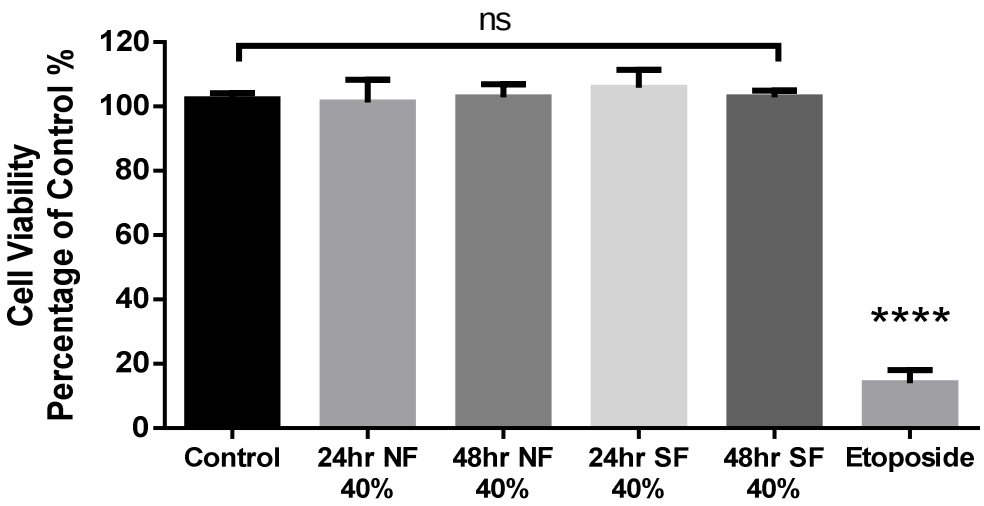


Figure 5.15. AML-12 cell viability following treatment with 40% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 24 hours.

AML-12 cell viability was determined following treatment with 40% NF and SF for 24 hours. Cells treated with 200µM Etoposide exhibited a positive control (p = 0.0001).

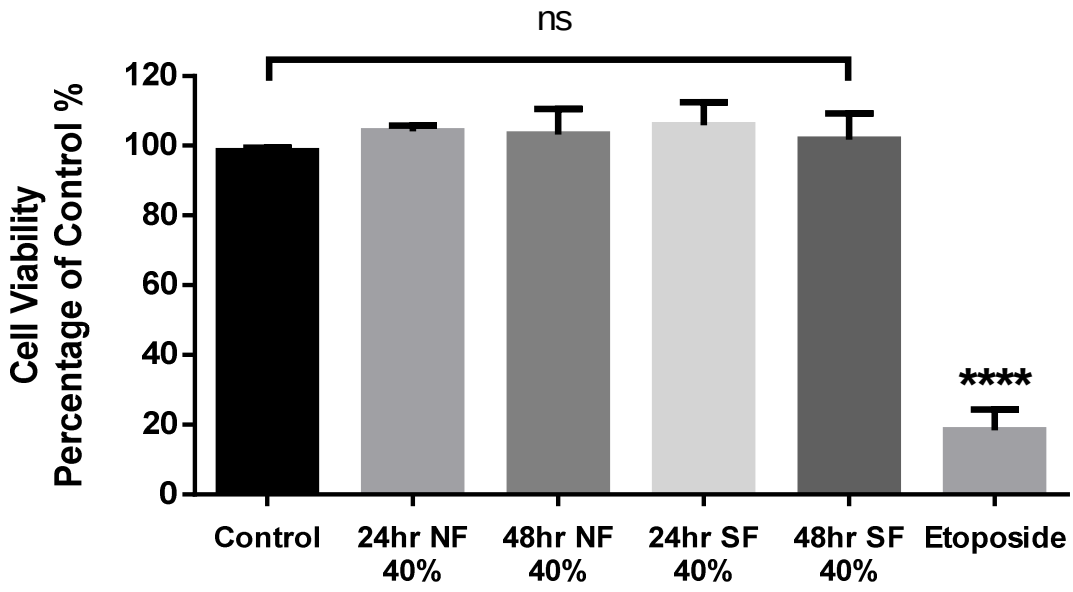


Figure 5.16. AML-12 cell viability following treatment with 40% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

AML-12 cell viability was determined following treatment with 40% NF and SF for 48 hours. Cells treated with 200µM Etoposide was used as a positive control (p = 0.0001).

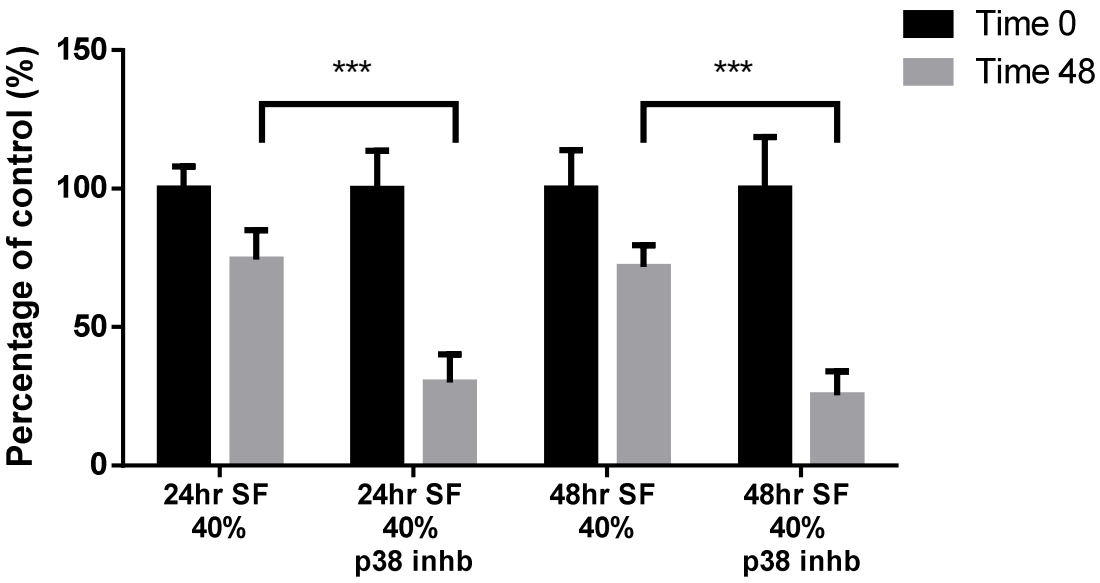


Figure 5.17. Glucose utilisation by mouse AML-12 hepatocytes following treatment with 40% conditioned media from senescent fibroblasts (SF) with p38 inhibitor and non-senescent normal fibroblast (NF) control media over 48 hours.

Differences in glucose utilisation were compared between AML-12 hepatocytes treated with 40% SF retrieved after 24 and 48 hours and incubated for 48 hours in the presence of 100nM p38 inhibitor and AML-12 cells treated with 40% SF alone. Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Significance was set at $p = <0.05$, $N = 4$.

5.4.3. 40% SASP, retrieved from senescent fibroblasts after 24 and 48 hours reduced glucose uptake in C2C12 mouse skeletal muscle cells following 24 and 48 hour incubations.

C2C12 mouse myoblasts were induced to differentiate over a period of 4 days (Fig. 5.18). Upon extensive myotube formation, C2C12 cells were treated with 20% conditioned media taken from SC after 24 or 48 hours for a period of 24 and 48 hours and glucose content of cell media measured. 20% conditioned media failed to alter glucose uptake in C2C12 cells following 24 and 48 hour incubations in comparison to conditioned media taken from non-senescent fibroblasts (Fig. 5.19). 40% conditioned media retrieved from SC after 24 and 48 hours failed to alter glucose utilisation following the 24 hour incubation, instead glucose concentration displayed a time dependent decrease as would be expected. SASP collection after 48 hours and used at 40% however significantly reduced the uptake of glucose by these mature skeletal muscle cells following 24 and 48 hour incubations ($p = <0.001$) as larger amounts of glucose were found within cell media following respective incubation periods, in comparison to skeletal muscle cells treated with media from non-senescent cells (Fig. 5.20).

5.4.3.1. 40% SASP treatment did not affect cell viability in C2C12 skeletal muscle cells following 24 and 48 hour incubations.

Having observed a reduced ability of C2C12 muscle cells to utilise glucose following treatment with 40% conditioned media from SC, to test whether the decrease in glucose uptake may be influenced by a reduced number of cells, cell viability following treatment with 40% conditioned media from SC was assessed. There was no statistically significant difference in cell viability between mature C2C12 muscle cells treated with control, no treatment media and muscle cells treated with 40% conditioned media from SC for 24 and 48 hour incubations (fig 5.21 and 5.22). 200µM etoposide was used as a positive control and was the only treatment to significantly reduce cell viability ($p = < 0.001$). The potential cytotoxic effects of the conditioned media can be discarded with regards to its influence on reducing the ability of C2C12 muscle cells to uptake

glucose and these results highlight a mechanism independent of cell death, responsible for altering glucose homeostasis.

5.4.3.2. Glucose utilisation was restored in C2C12 myocytes following co-incubation of 40% SASP and p38 inhibitor.

C2C12 myocytes incubated with 40% SASP for 48 hours in the presence of 100nM p38 inhibitor exhibited glucose utilisation comparable to C2C12 myocytes treated with non-senescent control media. There was a significant reduction in glucose concentration in media retrieved from C2C12 myotubes treated with SF media/p38 inhibitor in comparison to 40% SF alone (Fig. 5.23).

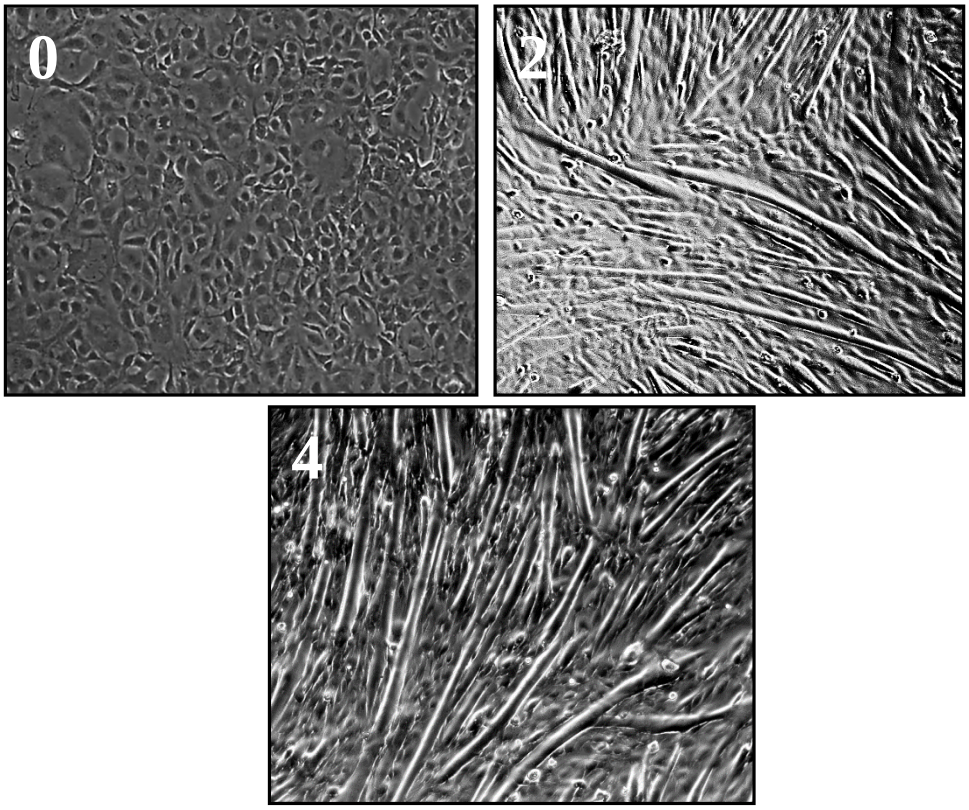


Figure 5.18. C2C12 mouse myoblasts differentiation.

C2C12 myoblasts were grown using DMEM high glucose (4.5g/l) supplemented with 10% fetal bovine serum, L-Glutamine 200mM and 1x Penicillium-Steptomycin until they reached 100% confluence. C2C12 differentiation was initiated by replacing 10% fetal bovine serum with 2% horse serum, growth media was replaced every 24 hours and the cells were incubated for a period of 4 days. A) Day 0, cells are 90-100% confluence, B) Day 2, cell differentiation is evident and the formation of contractile myotubes is apparent and C) Day 4, widespread mature myotube formation can be seen.

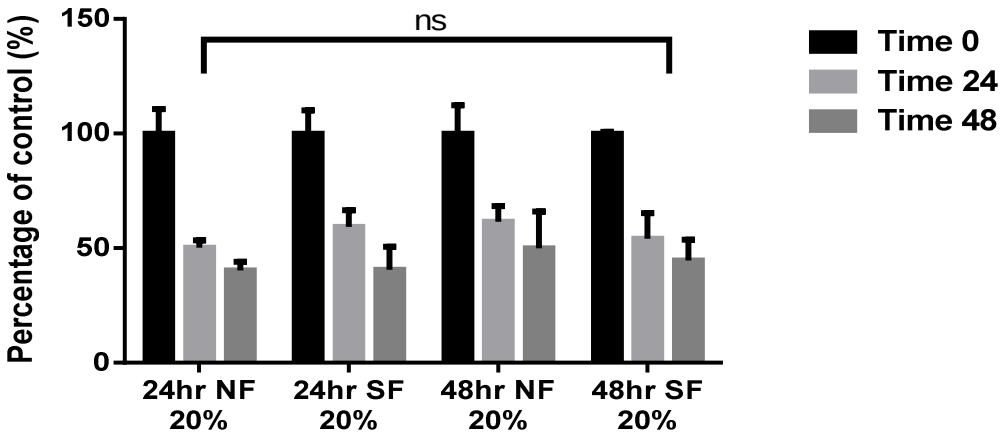


Figure 5.19. Glucose utilisation by C2C12 mouse myoblasts following treatment with 20% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

C2C12 myotubes treated with 20% SF retrieved after 24 and 48 hours and incubated for 24 and 48 hours. Following respective incubations glucose content of cell media was quantified and compared to control cells treated with NF control. Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Significance was set at p = <0.05, N = 4.

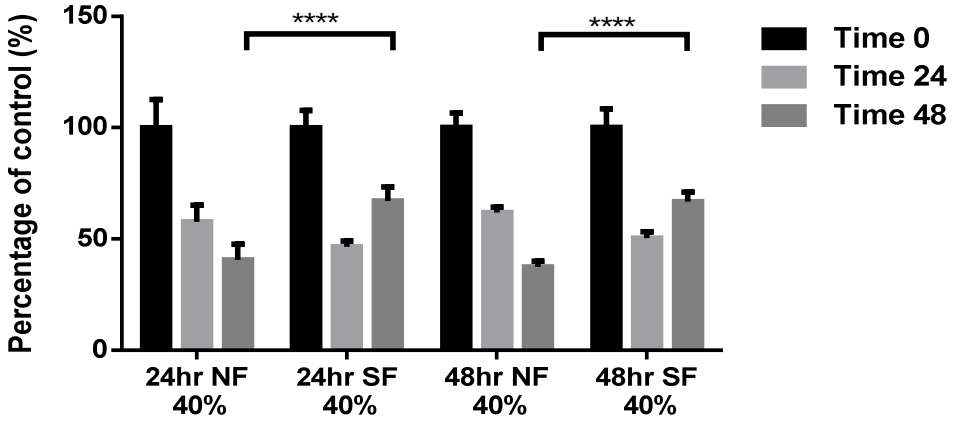


Figure 5.20. Glucose utilisation by C2C12 mouse myocytes following treatment with 40% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

C2C12 myotubes treated with 40% SF retrieved after 24 and 48 hours and incubated for 24 and 48 hours. Following respective incubations glucose content of cell media was quantified and compared to control cells treated with NF control (p = <0.001). Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Significance was set at p = <0.05, N = 4.

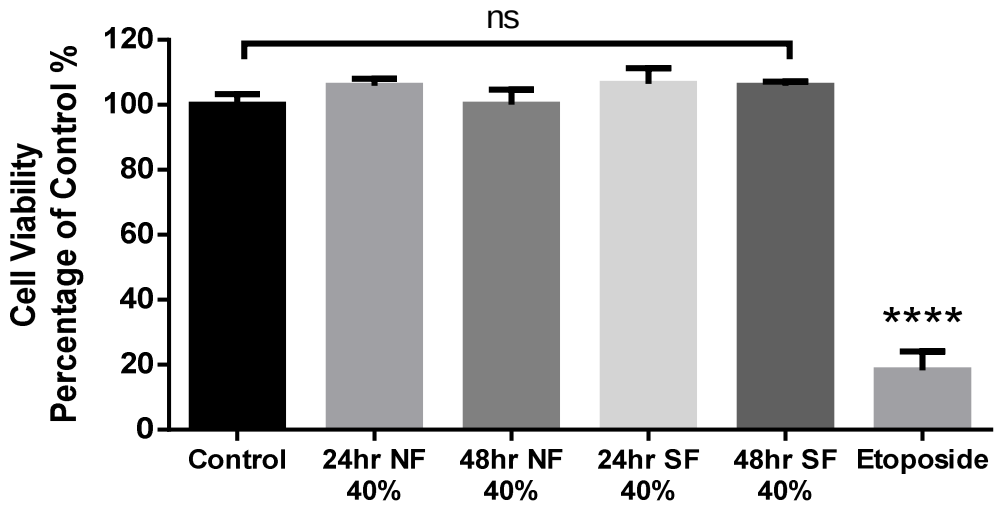


Figure 5.21. C2C12 cell viability following treatment with 40% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 24 hours.

C2C12 cell viability was determined following treatment with 40% NF and SF for 24 hours. Cells treated with 200µM Etoposide was used as a positive control ($p < 0.0001$).

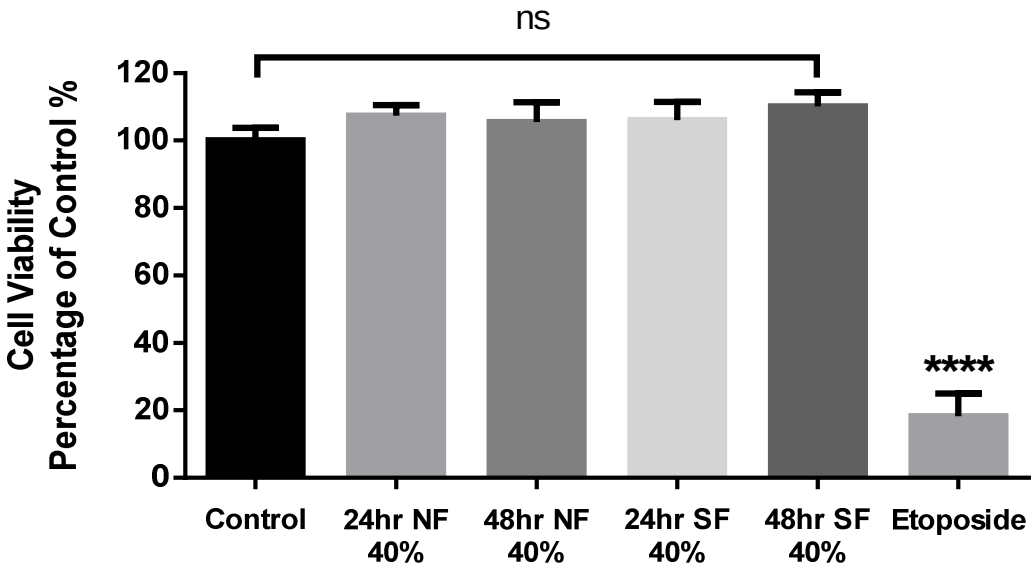


Figure 5.22. C2C12 cell viability following treat with 40% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

C2C12 cell viability was determined following treatment with 40% NF and SF for 48 hours. Cells treated with 200µM Etoposide was used as a positive control ($p < 0.0001$).

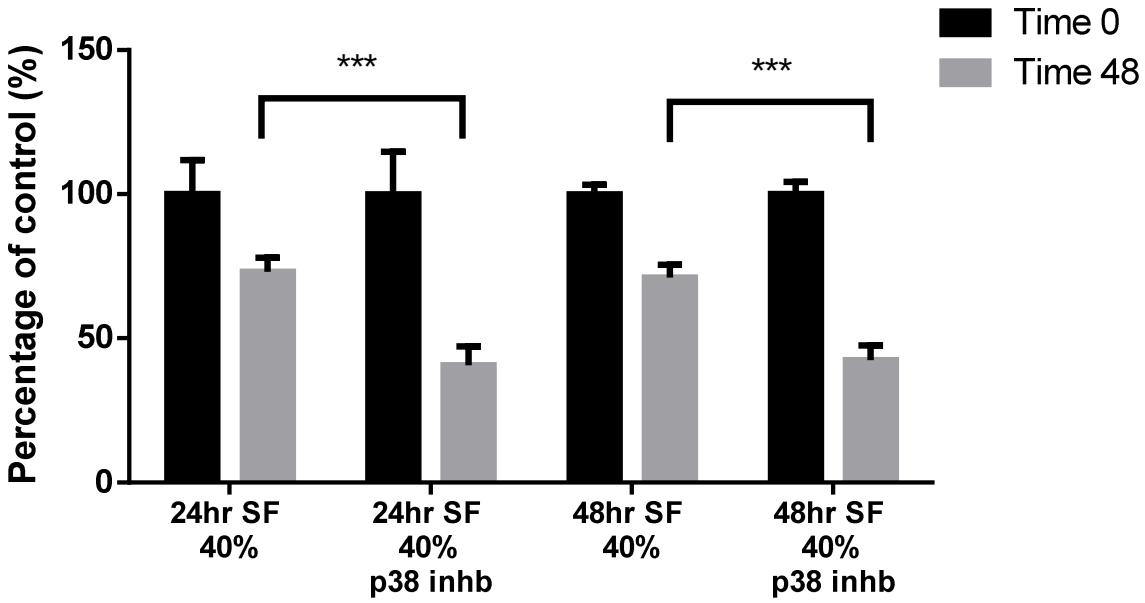


Figure 5.23. Glucose utilisation by mouse C2C12 myocytes following treatment with 40% conditioned media from senescent fibroblasts (SF) with p38 inhibitor and non-senescent normal fibroblast (NF) control media over 48 hours.

Differences in glucose utilisation between C2C12 myocytes treated with 40% SF retrieved after 24 and 48 hours and incubated for 48 hours in the presence of 100nM p38 inhibitor and C2C12 myocytes treated with 40% SF alone. Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Significance was set at $p < 0.05$, N = 4

5.4.3.3. Exposure to SASP induces reactive oxygen species (ROS) in C2C12 cells.

Mature C2C12 skeletal muscle cells were treated with conditioned media from non-senescent fibroblasts and 40% conditioned media from SC for 24 and 48 hours and then stained for superoxide formation using MitoSOX red indicator. A non-treatment control was established by treating C2C12 cells with normal media, and superoxide staining was absent (Fig. 5.24a and Fig. 5.25a). C2C12 cells treated with control media, derived from non-senescent fibroblasts after 24 and 48 hours exhibited very little superoxide staining, possibly a by-product of general metabolism (Fig. 5.24/5.25 b and c). C2C12 cells treated with conditioned media from SC recovered after 24 and 48 hours and used at 40%, substantially upregulated superoxide formation evident from the widespread red staining (Fig. 5.24/5.25 d and e). 5µM doxorubicin was used as a positive control, a known inducer of reactive oxygen species (Fig. 5.24 and 5.25f) and produced large scale MitoSOX absorption comparable to the absorption observed with 40% conditioned media from SC treatment. Control media, along with media taken from non-senescent cells failed to evoke ROS production as was expected (Fig. 5.24/5.25 a, b and c). 40% conditioned media from SC treatment resulted in increased superoxide production, evident from the increased uptake in the red dye (Fig. 5.25 d and e). 5µM doxorubicin was used for a positive control.

5.4.3.4. 40% SASP treatment failed to increase mitochondrial biogenesis.

To assess whether conditioned media from SC could alter mitochondrial biogenesis/function C2C12 cells treated with 40% conditioned media from SC were also stained with mitotracker red, a fluorescent dye that specifically stains active mitochondria, thus an indicator of membrane potential. 40% conditioned media from SC treatment for 24 and 48 hours did not significantly upregulate membrane activity/potential relative to control (Fig. 5.26 and 5.27). Quantification of total cell fluorescence using ImageJ revealed no significant difference in mitochondrial membrane potential between cells treated with 40% conditioned media from normal cells and cells treated with 40% conditioned media from senescent cells, following both 24 and 48 hour

incubations (Fig 5.28 and 5.29). C2C12 cells were also differentiated under 40% conditioned media from SC and treated with conditioned media 48 hours following differentiation to assess whether the conditioned media could alter mitochondrial number pre/during or post differentiation. In either case conditioned media treatment was unable to significantly upregulate mitochondrial biogenesis (Fig. 5.30 and 5.31) relative to C2C12 cells treated with conditioned media from non-senescent cells. Quantification of total cell fluorescence using ImageJ image analysis software revealed no significant difference in mitochondrial potential between cells treated with conditioned media from senescent cells in comparison to cells treated with conditioned media from non-senescent cells (Fig. 5.32 and 5.33).

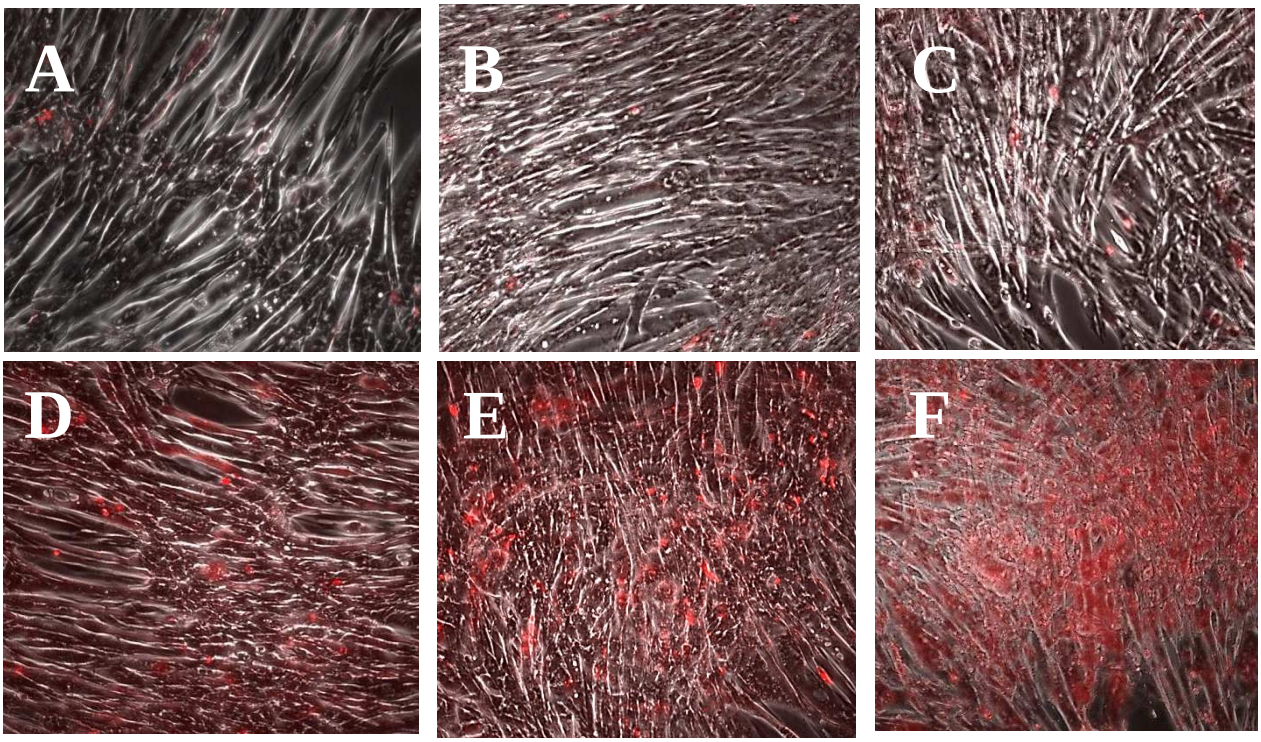


Figure 5.24. Differentiated C2C12 mouse muscle cells stained with MitoSOX red mitochondrial superoxide indicator dye following treatment with 40% SF for 24 hours.

C2C12 mouse myoblasts were grown to 100% confluence, designated as day 0 and then induced to differentiate by the addition of differentiation media. A) control media, B) Non-senescent media collected after 24 hours from fibroblasts, C) Non-senescent media collected after 48 hours from fibroblasts, D) media collected after 24 hours from senescent fibroblasts (40%), E) media collected after 48 hours from senescent fibroblasts (40%) and F) 5µM doxorubicin as a positive control. Cells were incubated for 24 hours prior to the addition of MitoSOX red and left to incubate for 20 minutes. Pictures were taken on a fluorescence microscope at 100x magnification.

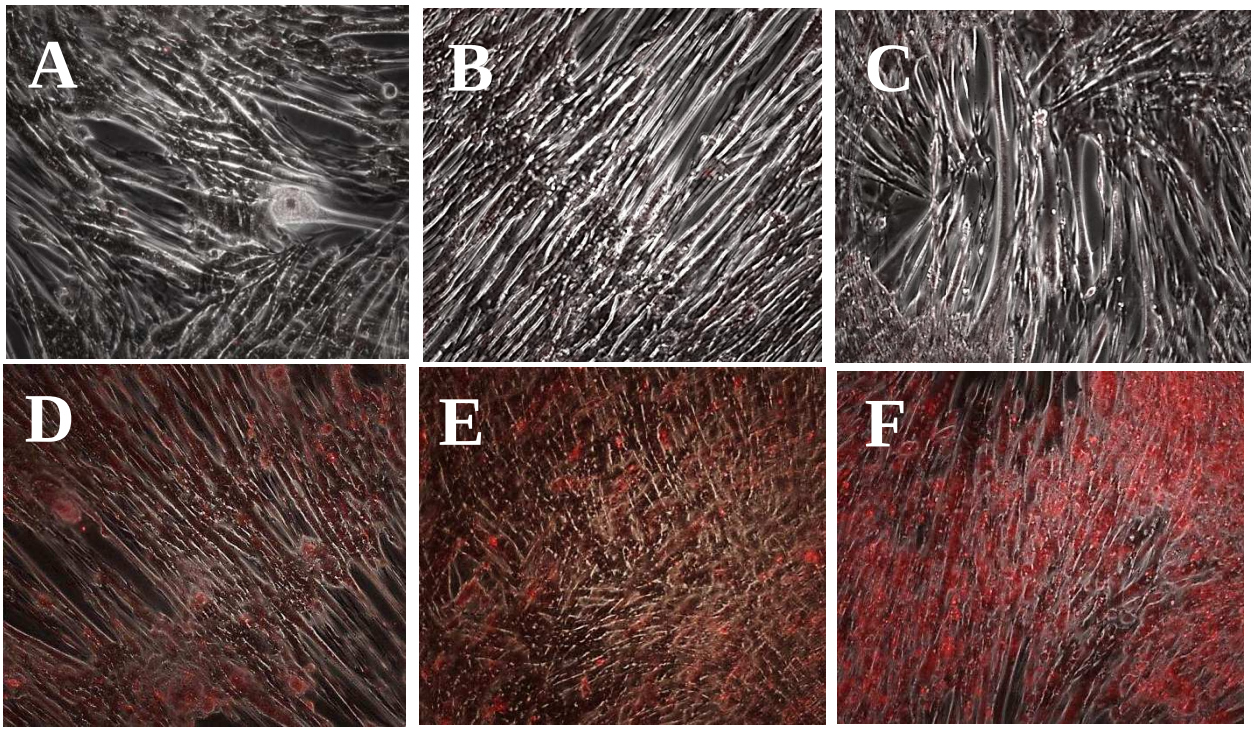


Figure 5.25. Differentiated C2C12 mouse muscle cells stained with MitoSOX red mitochondrial superoxide indicator dye following treatment with 40% SF for 48 hours.

C2C12 mouse myoblasts were grown to 100% confluence, designated as day 0 and then induced to differentiate by the addition of differentiation media. A) control media, B) Non-senescent media collected after 24 hours from fibroblasts, C) Non-senescent media collected after 48 hours from fibroblasts, D) media collected after 24 hours from senescent fibroblasts (40%), E) media collected after 48 hours from senescent fibroblasts (40%) and F) 5µM doxorubicin as a positive control. Cells were incubated for 24 hours prior to the addition of MitoSOX red and left to incubate for 20 minutes. Pictures were taken on a fluorescence microscope at 100x magnification

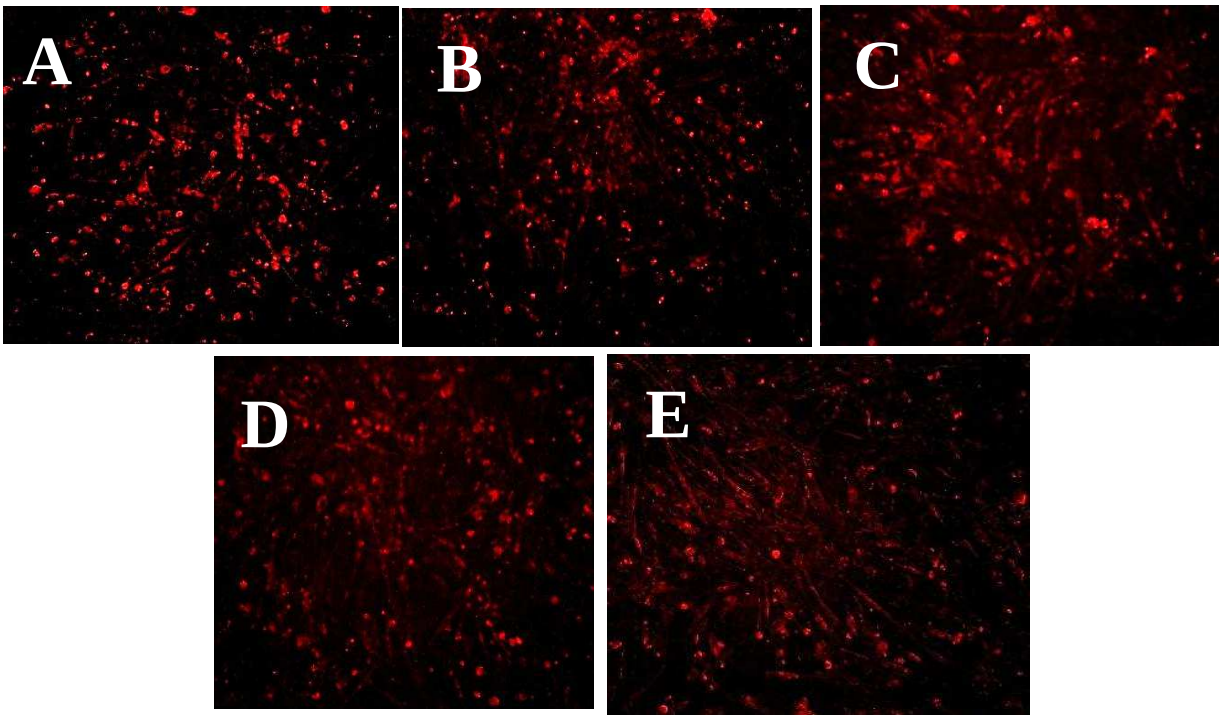


Figure 5.26. Differentiated C2C12 mouse muscle cells stained with Mitotracker red mitochondrial indicator following treatment with 40% SF for 24 hours.

C2C12 mouse myoblasts were grown to 100% confluence, designated as day 0 and then induced to differentiate by the addition of differentiation media. A) control media, B) Non-senescent media collected after 24 hours from fibroblasts, C) media collected after 24 hours from senescent fibroblasts (40%), D) Non-senescent media collected after 48 hours from fibroblasts and E) media collected after 48 hours from senescent fibroblasts (40%). Cells were treated for 24 hours prior to the addition of Mitotracker red and left to incubate for 45 minutes. Pictures were taken on a fluorescence microscope at 100x magnification.

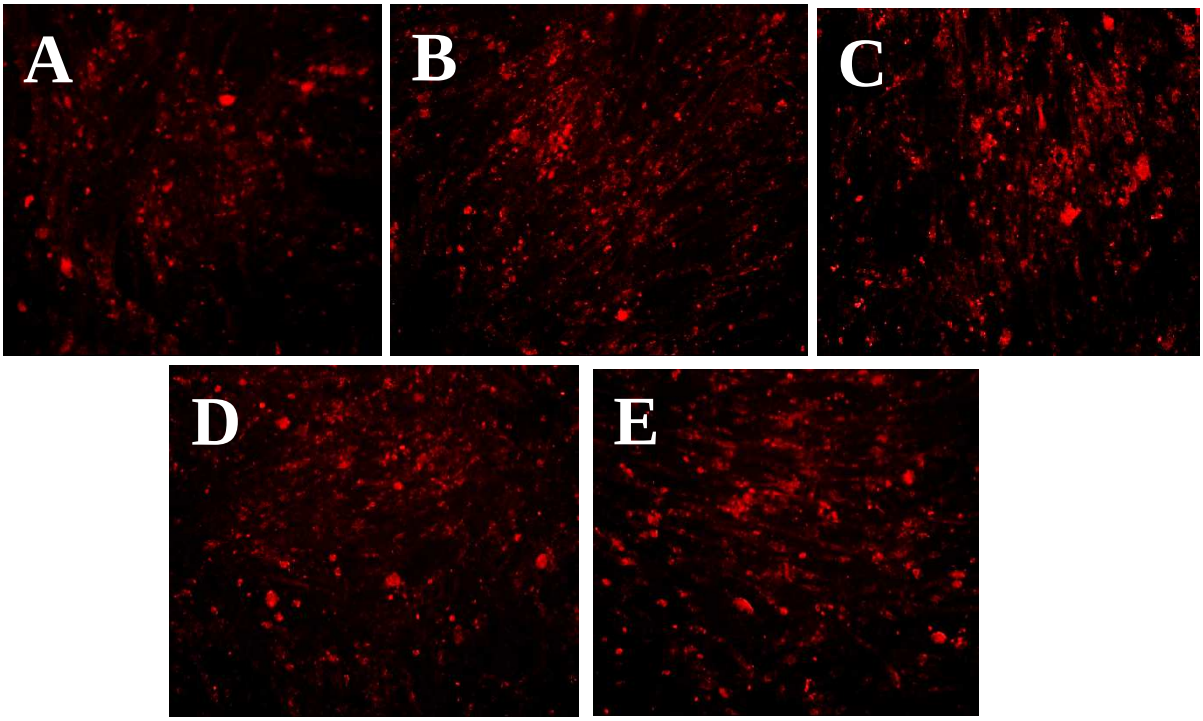


Figure 5.27. Differentiated C2C12 mouse muscle cells stained with Mitotracker red mitochondrial indicator following treatment with 40% SF for 48 hours.

C2C12 mouse myoblasts were grown to 100% confluence, designated as day 0 and then induced to differentiate by the addition of differentiation media. A total of 5 treatments were administered A) control media, B) Non-senescent media collected after 24 hours from fibroblasts, C) media collected after 24 hours from senescent fibroblasts (40%) D) Non-senescent media collected after 48 hours from fibroblasts and E) media collected after 48 hours from senescent fibroblasts (40%). Cells were treated for 48 hours prior to the addition of Mitotracker red and left to incubate for 45 minutes. Pictures were taken on a fluorescence microscope at 100x magnification.

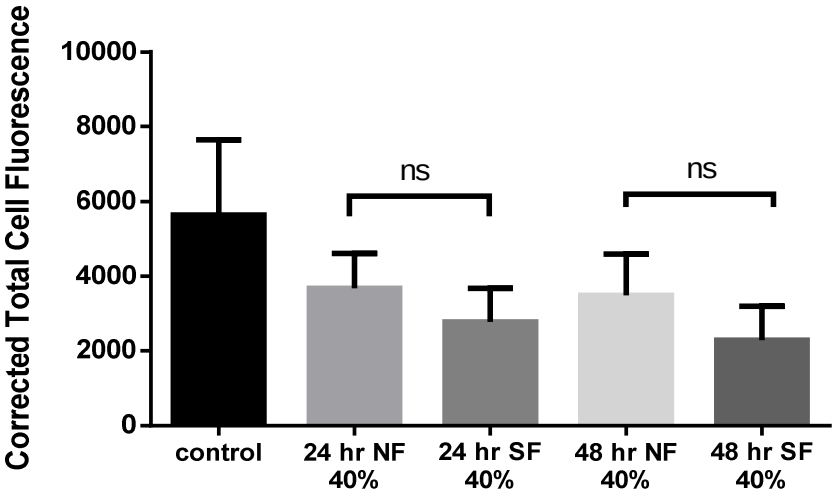


Figure 5.28. Corrected total cell fluorescence of differentiated C2C12 mouse muscle cells stained with Mitotracker red mitochondrial indicator following treatment with 40% SF for 24 hours.

Mitochondrial potential was assessed in differentiated C2C12 myotubes treated with 40% SF retrieved after 24 and 48 hours and incubated for 24 hours. One-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Fluorescence quantified using ImageJ software. Significance was set at $p < 0.05$, N = 4

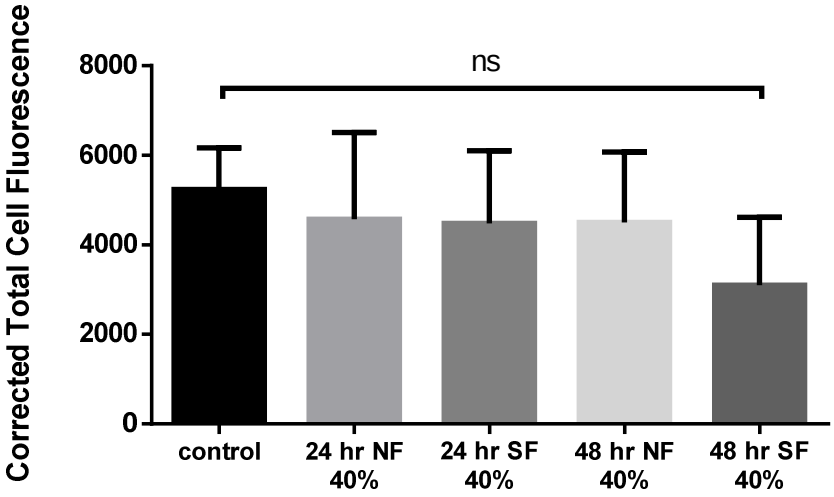


Figure 5.29. Corrected total cell fluorescence of differentiated C2C12 mouse muscle cells stained with Mitotracker red mitochondrial indicator following treatment with 40% SF for 48 hours.

Mitochondrial potential was assessed in differentiated C2C12 myotubes treated with 40% SF retrieved after 24 and 48 hours and incubated for 24 hours. One-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Fluorescence quantified using ImageJ software. Significance was set at $p < 0.05$, N = 4

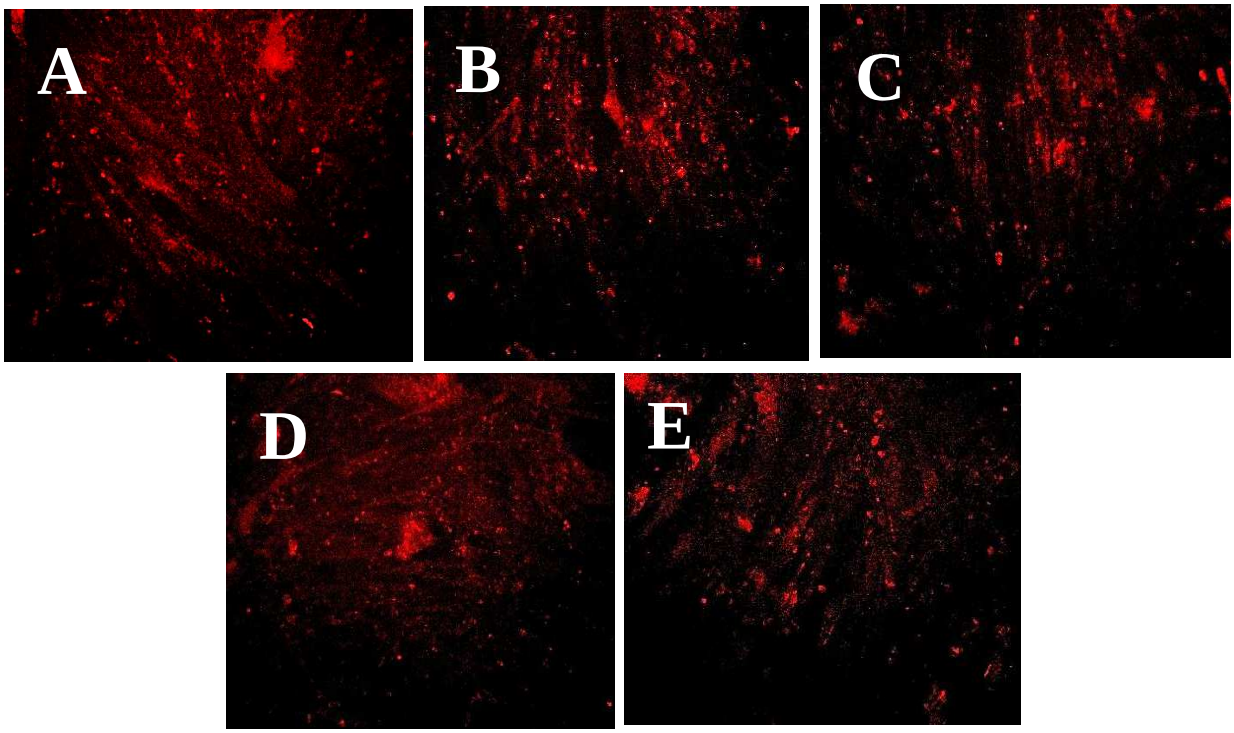


Figure 5.30. Differentiated C2C12 mouse muscle cells stained with Mitotracker red mitochondrial indicator following treatment with 40% SF for 48 hours post differentiation.

C2C12 mouse myoblasts were grown to 100% confluence, designated as day 0 and then induced to differentiate by the addition of differentiation media. A) control media, B) Non-senescent media collected after 24 hours from fibroblasts, C) media collected after 24 hours from senescent fibroblasts (40%), D) Non-senescent media collected after 48 hours from fibroblasts and E) media collected after 48 hours from senescent fibroblasts (40%). Cells were treated for 48 hours post differentiation, followed by the addition of Mitotracker red and left to incubate for 45 minutes. Pictures were taken on a fluorescence microscope at 100x magnification.

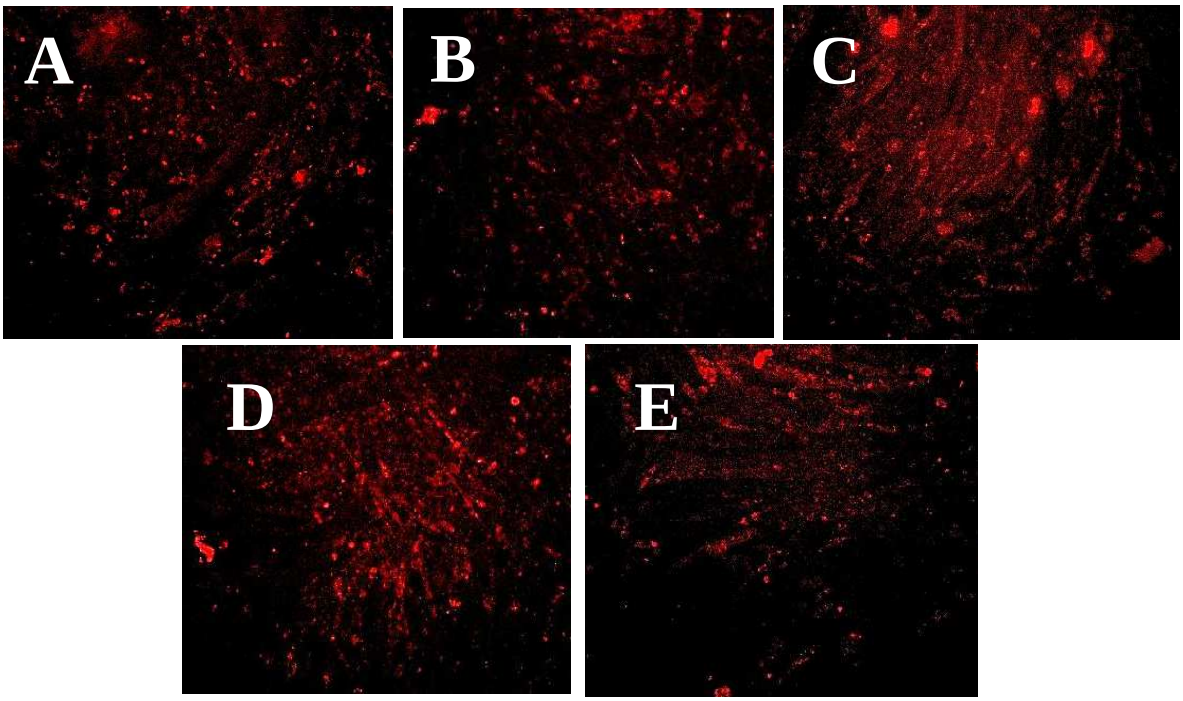


Figure 5.31. C2C12 mouse muscle cells differentiated under 40% SF and then stained with Mitotracker red mitochondrial indicator.

C2C12 mouse myoblasts were grown to 100% confluence, designated as day 0 and then induced to differentiate by the addition of differentiation media. A) control media, B) Non-senescent media collected after 24 hours from fibroblasts, C) media collected after 24 hours from senescent fibroblasts (40%) D) Non-senescent media collected after 48 hours from fibroblasts and E) media collected after 48 hours from senescent fibroblasts (40%). Cells were treated for 48 hours post differentiation, followed by the addition of Mitotracker red and left to incubate for 45 minutes. Pictures were taken on a fluorescence microscope at 100x magnification.

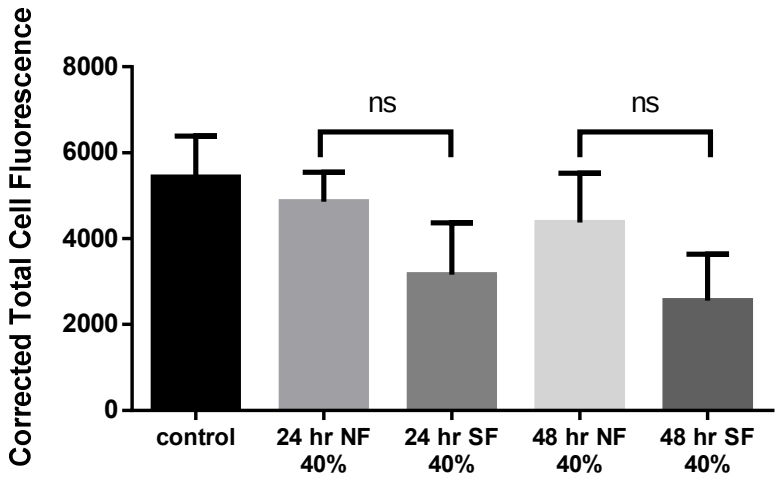


Figure 5.32. Corrected total cell fluorescence of differentiated C2C12 mouse muscle cells stained with Mitotracker red mitochondrial indicator following treatment with 40% SF for 48 hours post differentiation.

Mitochondrial potential was assessed in differentiated C2C12 myotubes treated with 40% SF retrieved after 24 and 48 hours and incubated 48 hours post differentiation. One-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Fluorescence quantified using ImageJ software. Significance was set at $p < 0.05$, N = 4

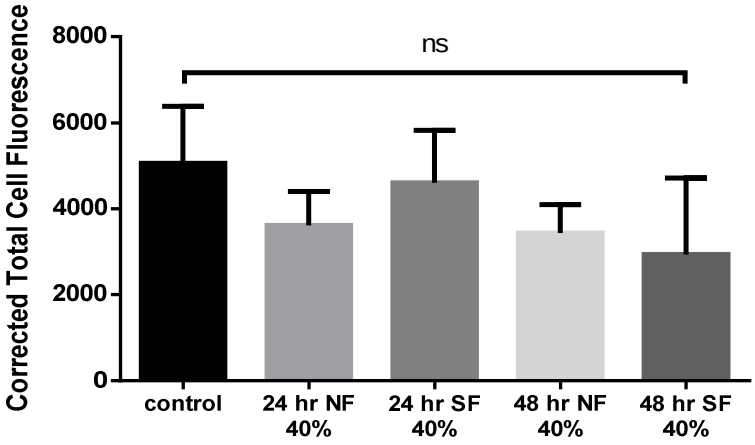


Figure 5.33. Corrected total cell fluorescence of C2C12 myoblasts differentiated under 40% SF stained with Mitotracker red mitochondrial indicator.

Mitochondrial potential was assessed in C2C12 myoblasts during differentiation treated with 40% SF retrieved after 24 and 48 hours. One-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Fluorescence quantified using ImageJ software. Significance was set at $p < 0.05$, N = 4

5.4.4. 3T3-L1 adipocytes treated with 20% and 40% conditioned media from senescent fibroblasts for 24 and 48 hours, did not exhibit any change in ability to utilise glucose.

3T3-L1 mouse pre-adipocytes were differentiated over the course of 15 days (Fig. 5.34), mature adipocytes were identified by microscopy. Glucose uptake following treatment with 20 and 40% conditioned media from SC for 24 and 48 hours remained unchanged relative to glucose uptake in 3T3-L1 cells treated with non-senescent control media (Fig 5.35 and 5.36).

5.4.4.1 3T3-L1 adipocytes stained with Nile red following 20% and 40% conditioned media treatment from senescent fibroblasts, expressed significant difference in lipid content after 48 hours of incubation only.

Mature 3T3-L1 adipocytes were treated with 40% conditioned media from SC and stained with Nile red to investigate whether the SASP could influence lipolysis. Nile red is a fluorescent stain which specifically stains intracellular lipids for quantitative analysis. 3T3-L1 cells treated with normal cell culture media, and media collected from non-senescent fibroblasts stained profusely with Nile red and a large distribution of lipid laden adipocytes were observed (Fig. 5.37a, b and c). Conditioned media retrieved after 24 hours and used at 40% did not alter adipocyte lipid content or morphology (Fig. 5.37d), on the contrary conditioned media collected at 48 hours of inducing senescence and used at 40% significantly reduced the number of adipocytes staining with Nile red, evident from the large areas of unstaining observed ($p = < 0.05$) (Fig. 5.37e). 3T3-L1 cells treated with 40% conditioned media for 48 hours exhibited a similar trend, as control and non-senescent media did not affect adipocyte lipid content or morphology (Fig. 38a, b and c). A significant reduction in fluorescent staining was observed following treatment with conditioned media collected from senescent cells after 48 hours in comparison to cells treated with media from non-senescent cells (Fig. 38d and e). ImageJ software analysis reveals a significant reduction in lipid droplets amongst 3T3-L1 cells treated with 40% conditioned media for 24 and 48 hours (Fig. 5.39 and 5.40).

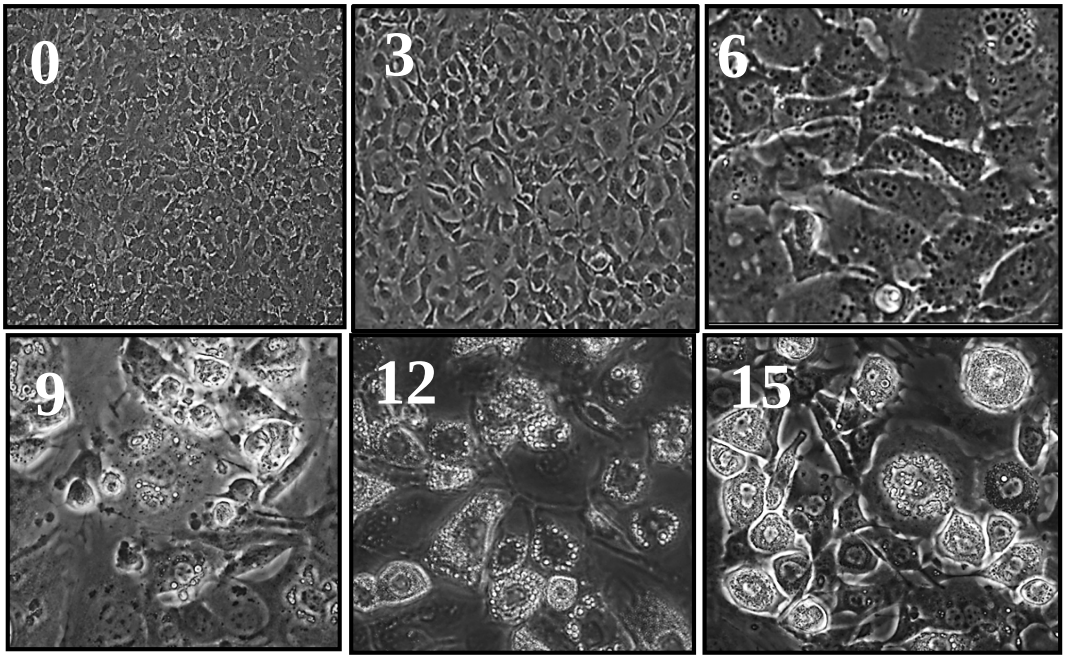


Figure 5.34. 3T3-L1 mouse pre-adipocyte differentiation.

3T3-L1 mouse pre-adipocytes were grown to 100% confluence, designated as day 0 and then induced to differentiate by the addition of differentiation media. The 3T3-L1 cells were differentiated for a total of 15 days and pictures were taken on a confocal microscope, at 100x magnification for day 0 and 3, then 400x at 3 day intervals till day 15. At day 9, increases in cell size are apparent, day 12 lipid formation and day 15 considerable lipid storage and change in cell size and morphology. The complete 3T3-L1 feeding schedule has been detailed in chapter 2.

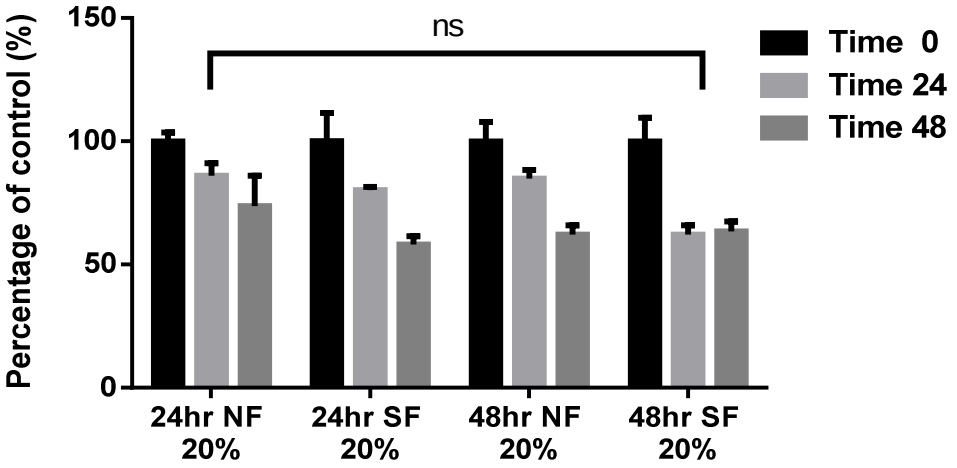


Figure 5.35. Glucose utilisation by 3T3-L1 adipocytes following treatment with 20% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

3T3-L1 treated with 20% SF retrieved after 24 and 48 hours and incubated for 24 and 48 hours. Following respective incubations glucose content of cell media was quantified and compared to control cells treated with NF control. Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Significance was set at $p < 0.05$, N = 4.

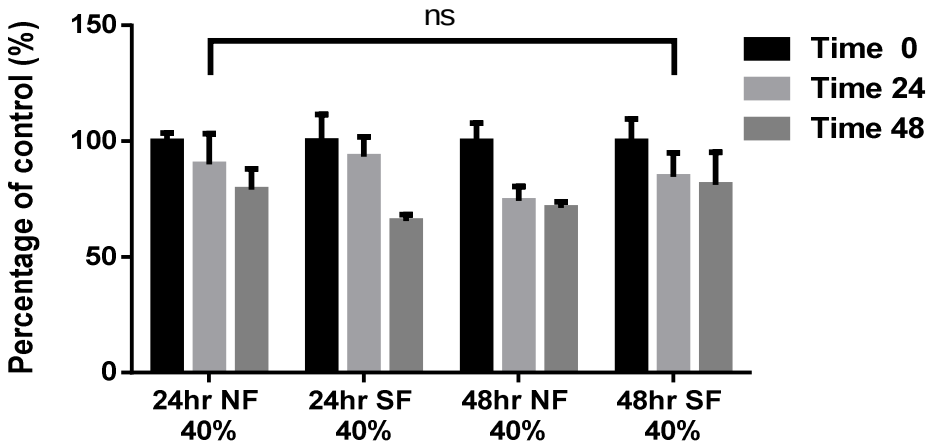


Figure 5.36. Glucose utilisation by 3T3-L1 adipocytes following treatment with 40% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

3T3-L1 treated with 40% SF retrieved after 24 and 48 hours and incubated for 24 and 48 hours. Following respective incubations glucose content of cell media was quantified and compared to control cells treated with NF control. Two-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Significance was set at $p < 0.05$, N = 4.

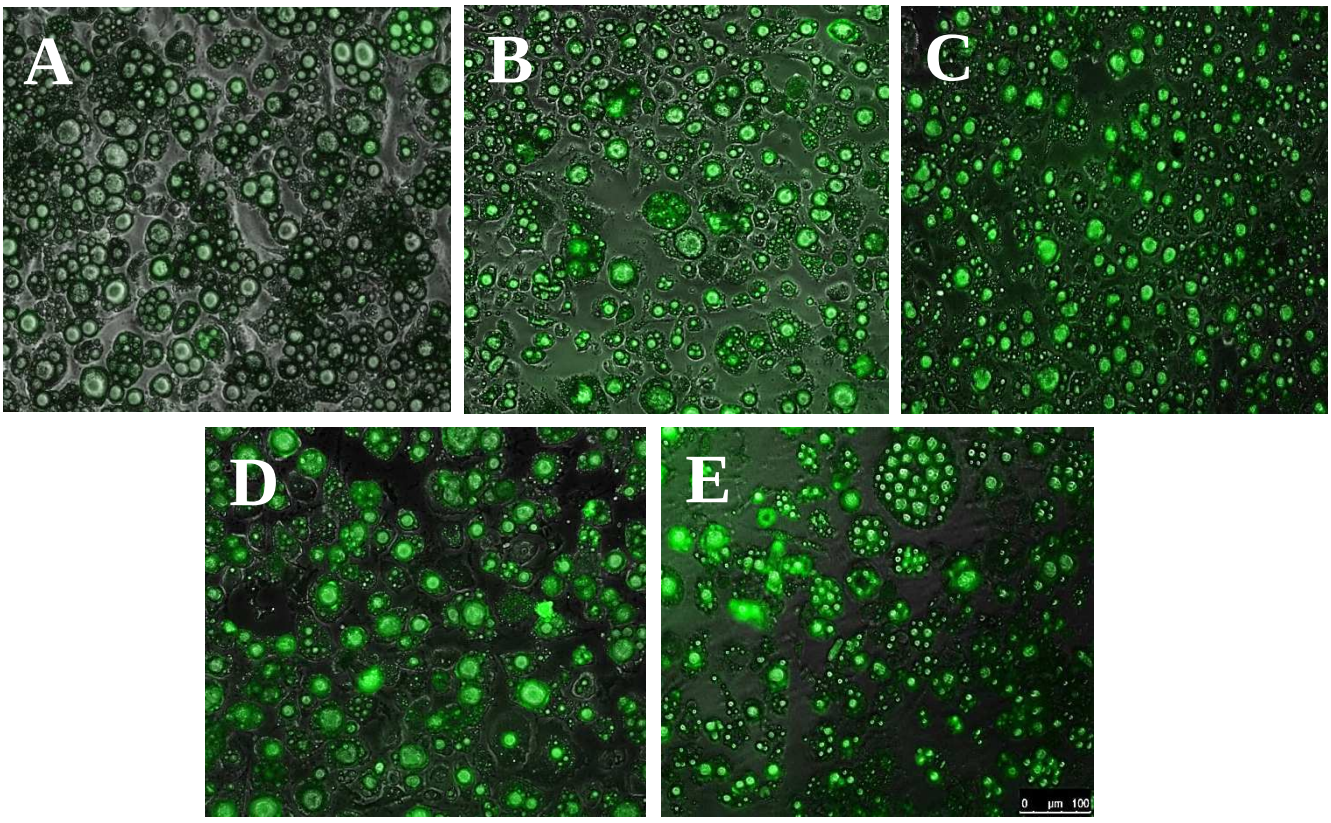


Figure 5.37. Mature 3T3-L1 mouse adipocytes stained with Nile Red following treatment with 40% SF for 24 hours.

3T3-L1 mouse pre-adipocytes were grown to 100% confluence, designated as day 0 and then induced to differentiate by the addition of differentiation media. 3T3-L1 cells were differentiated for 15 days and subsequently treated with 40% senescent media for 24 hours, followed by Nile red staining. Pictures were taken on a confocal microscope at 100x magnification. A) control media, B) Non-senescent media collected after 24 hours from fibroblasts, C) senescent media collected after 24 hours from fibroblasts, D) media collected after 48 hours from non-senescent fibroblasts and E) media collected after 48 hours from senescent fibroblasts.

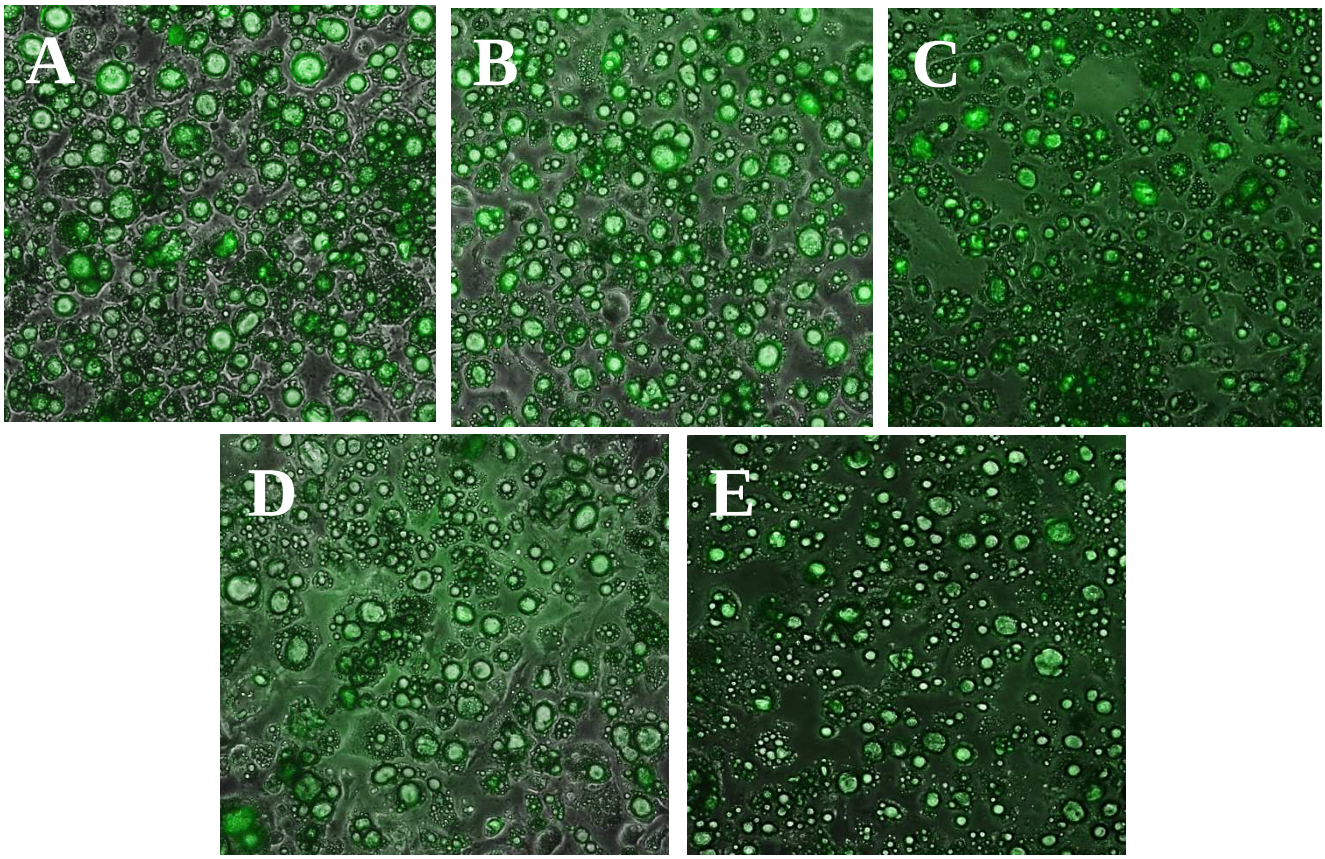


Figure 5.38. Mature 3T3-L1 mouse adipocytes stained with Nile Red following treatment with 40% SF for 48 hours.

3T3-L1 mouse pre-adipocytes were grown to 100% confluence, designated as day 0 and then induced to differentiate by the addition of differentiation media. The 3T3-L1 cells were differentiated for a total of 15 days and then treated with 40% senescent media for 48 hours, followed by Nile red staining. Pictures were taken on a confocal microscope at 100x magnification. A) control media, B) Non-senescent media collected after 24 hours from fibroblasts, C) senescent media collected after 24 hours from fibroblasts, D) media collected after 48 hours from non-senescent fibroblasts and E) media collected after 48 hours from senescent fibroblasts.

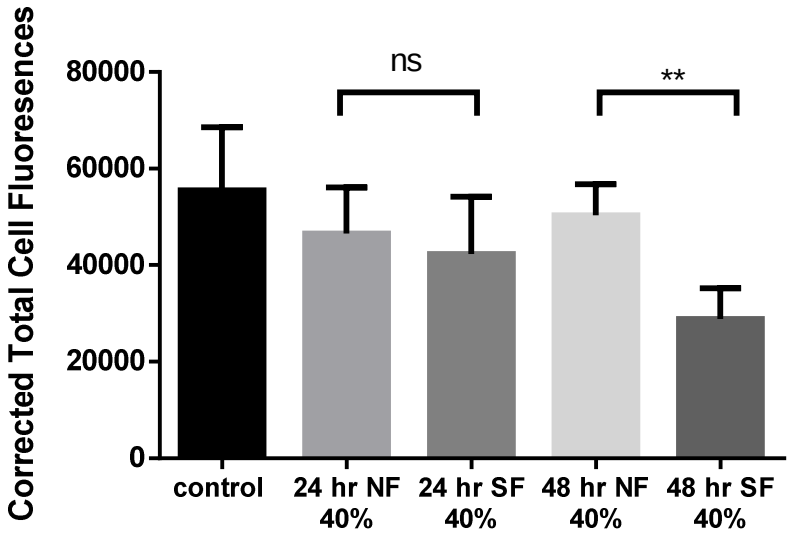


Figure 5.39. Corrected total cell fluorescence of differentiated 3T3-L1 cells stained with Nile red following treatment with 40% SF for 24 hours post differentiation.

Adipocyte lipid content was measured in differentiated 3T3-L1 cells treated with 40% SF retrieved after 24 and 48 hours and incubated 24 hours. One-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Fluorescence quantified using ImageJ software. Significance was set at $p < 0.05$, N = 4

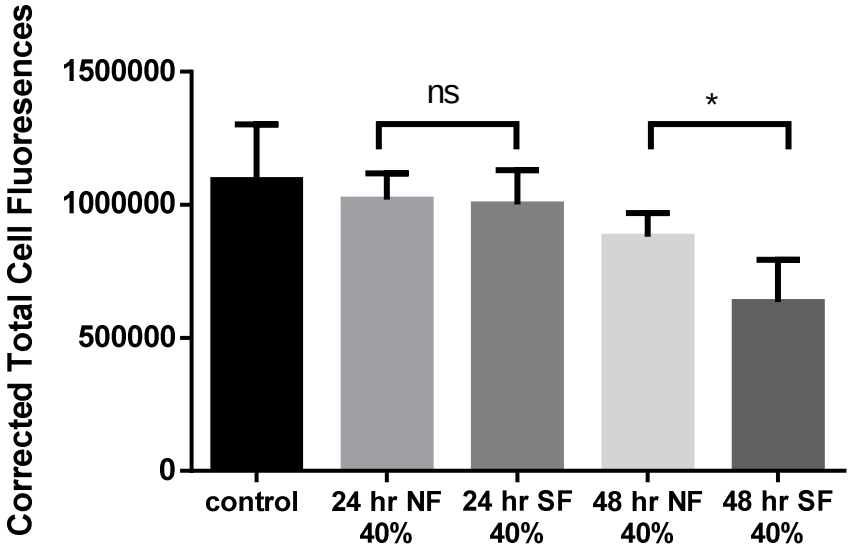


Figure 5.40. Corrected total cell fluorescence of differentiated 3T3-L1 cells stained with Nile red following treatment with 40% SF for 48 hours post differentiation

Adipocyte lipid content was measured in differentiated 3T3-L1 cells treated with 40% SF retrieved after 24 and 48 hours and incubated 48 hours. One-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Fluorescence quantified using ImageJ software. Significance was set at $p < 0.05$, N = 4

5.4.4.2. 20% and 40% SASP induces lipolysis in 3T3-L1 adipocytes following 48 hour incubation.

Free glycerol was measured in the media of 3T3-L1 adipocytes following treatment with 20 and 40% conditioned media collected from senescent fibroblasts after 24 and 48 hours. 20% and 40% conditioned media collected from senescent fibroblasts after 24 hours did not induce lipolysis in 3T3-L1 adipocytes (Fig. 5.41 and 5.42). However treatment with 20% and 40% conditioned media collected after 48 hours and incubated with cells for 48 hours significantly induced lipolysis, reflected by increased glycerol content in the media (Fig. 5.41 and 5.42). Adipocytes treated with 20% conditioned media for 48 hours underwent lipolysis to a lesser extent in comparison to adipocytes treated with 40% conditioned media. Media from 3T3-L1 cells treated with 20% conditioned media from SC for 48 hours exhibited 310% more glycerol in comparison to media from 3T3-L1 cells treated with 20% conditioned media ($p < 0.01$), Media from 3T3-L1 treated with 40% conditioned media from SC for 48 hours exhibited 705% more glycerol in comparison to media from 3T3-L1 cells treated with 40% conditioned media from non-senescent cells ($p < 0.001$). The moderate variances observed in lipid staining between 3T3-L1 adipocytes treated with 40% conditioned media from SC and control non-senescent media indicated lipolysis but due to the qualitative nature of the test remained inconclusive. Increased glycerol quantification following conditioned media treatment validates the role of SASP in inducing lipolysis, that too in a dose and time dependent manner.

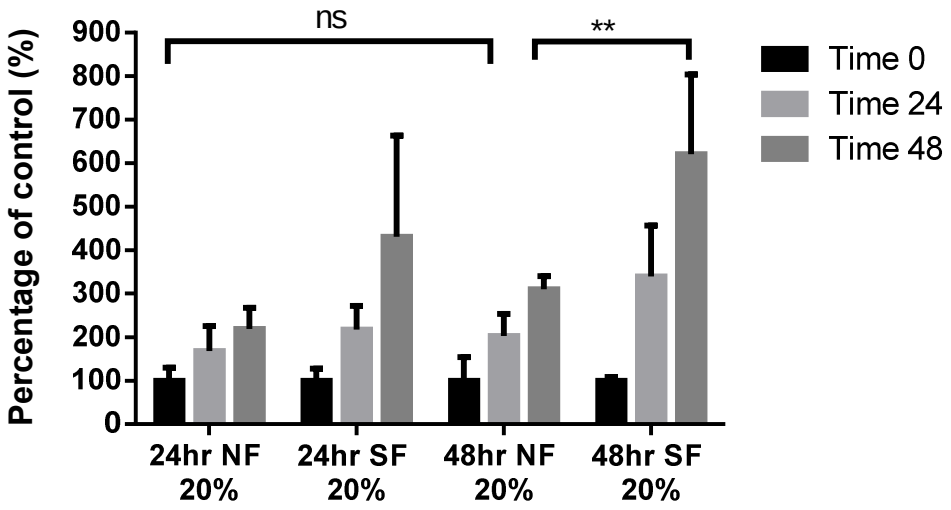


Figure 5.41. Glycerol release by 3T3-L1 cells following treatment with 20% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

Media glycerol content was measured in differentiated 3T3-L1 cells treated with 20% SF retrieved after 24 and 48 hours and incubated 48 hours. One-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Fluorescence quantified using ImageJ software. Significance was set at $p < 0.05$, N = 4

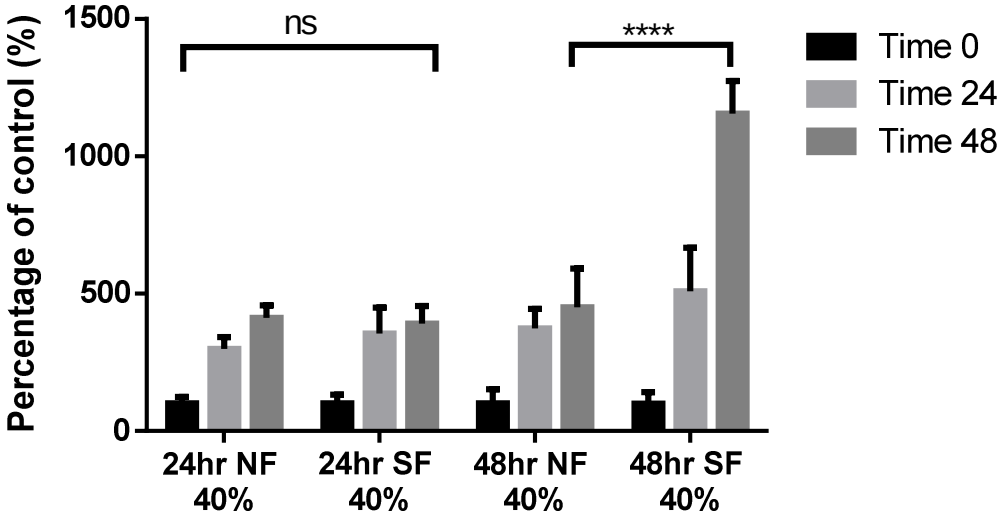


Figure 5.42. Glycerol release by 3T3-L1 cells following treatment with 40% conditioned media from senescent fibroblasts (SF) and non-senescent normal fibroblast (NF) control media over 48 hours.

Media glycerol content was measured in differentiated 3T3-L1 cells treated with 40% SF retrieved after 24 and 48 hours and incubated 48 hours. One-way ANOVA and Tukey's multiple comparisons test was conducted to test for significance, data are presented as SEM. Fluorescence quantified using ImageJ software. Significance was set at $p < 0.05$, N = 4

5.4.5. Aged mouse hepatocellular tissue exhibits decreased expression of the insulin receptor gene and Phosphoenolpyruvate carboxykinase 1 gene.

The expression of 5 metabolic genes was assessed in young and old mice hepatocyte tissue, in a bid to discover age related changes in metabolic function. While no significant difference in gene expression was observed in genes coding for; the long-chain-fatty-acid-CoA ligase 1 enzyme (ASCL1), fatty acid synthase (FASN) and carnitine palmitoyltransferase 1A (CPT1a) with respect to chronological age (Fig.5.43 a). The gene encoding the insulin receptor was significantly downregulated ($p < 0.05$) in the liver tissues of older mice (19 months old) relative to younger mice (4 months old), while phosphoenolpyruvate carboxykinase 1 (PCK1) gene expression was down-regulated in aged mice tissue ($p < 0.01$). These data highlight advancing age is met with a reduction in insulin receptor expression in the liver, as per consequence a reduced ability to store glucose as glycogen would also be expected. Likewise reduced carboxykinase 1 expression would only further deregulate glucose homeostasis.

5.4.5.1. Aged mice skeletal muscle tissue muscle displays a reduced gene expression of insulin receptor gene and GLUT4 gene. Young mice exhibits increased expression of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha gene (PGC1-α), while aged.

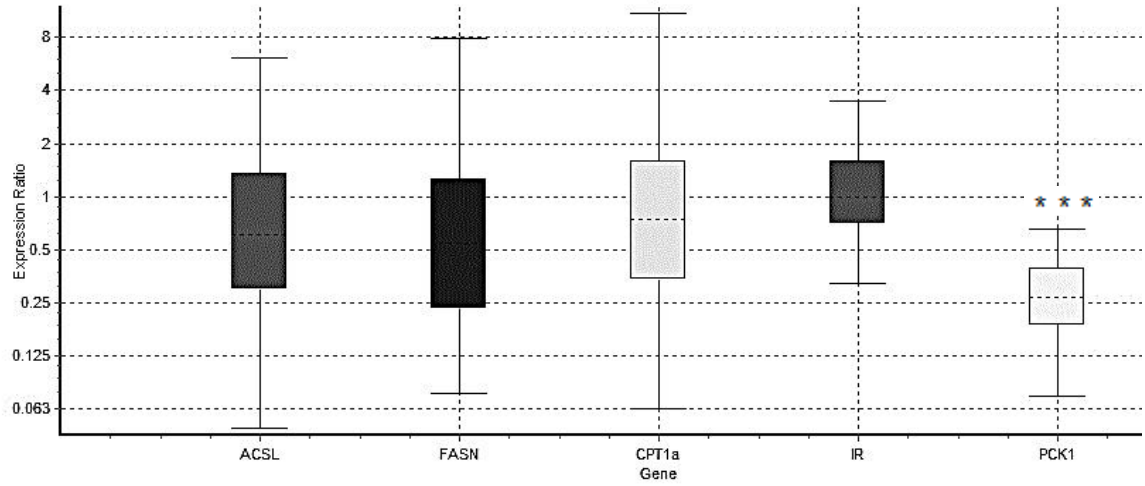
Having assessed metabolic gene expression in young and aged hepatocellular tissue, the experiment was repeated in another insulin target tissue (Fig.5.44 a). The results reveal an increased gene expression of muscle PGC-1α amongst younger mice ($p = 0.05$) which potentially reduces with advancing age and then stabilises. Significant reductions in glut4 and insulin receptor gene expression were observed in aged skeletal muscle mice tissue, ($p = 0.05$, $p < 0.001$). These data imply advancing age is met with a reduced ability to take up glucose in skeletal muscle due to reduced glucose transportation, in concert with reduced insulin receptor expression. It can therefore be deduced that age associated insulin resistance and dysregulated glucose homeostasis is largely influenced by reduced gene expression for proteins vital in insulin sensitivity and

glucose uptake. Reductions in hexokinase gene expression with age were almost significant at p = 0.06 and highlight a potential inability to utilise glucose.

5.4.5.2. Aged adipose tissue exhibits reduced peroxisome proliferator-activated receptor gamma gene expression (PPARg) only.

Aged adipose tissue expressed reduced gene expression of the PPARg gene only ($p = 0.05$), while the expression of the sterol regulatory element binding transcription factor 1, fatty acid binding protein, fatty acid synthase, lipoprotein lipase and CCAAT/enhancer-binding protein alpha genes remains unchanged with between young and old mice. PPARg is a gene vital for regulating fatty acid synthesis and storage by adipocytes and a decreased expression over time may manifest with lipotoxic effects.

A)



B)

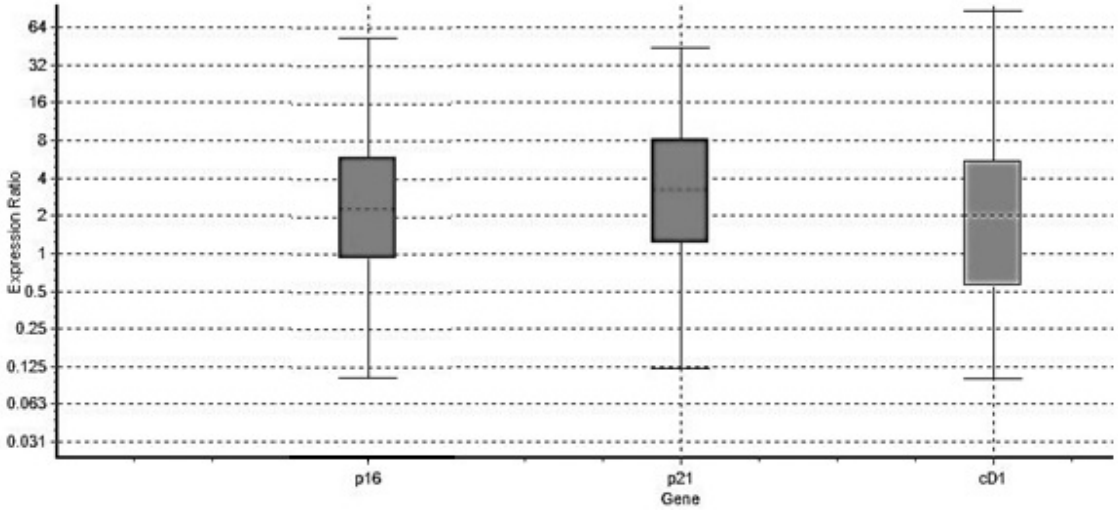
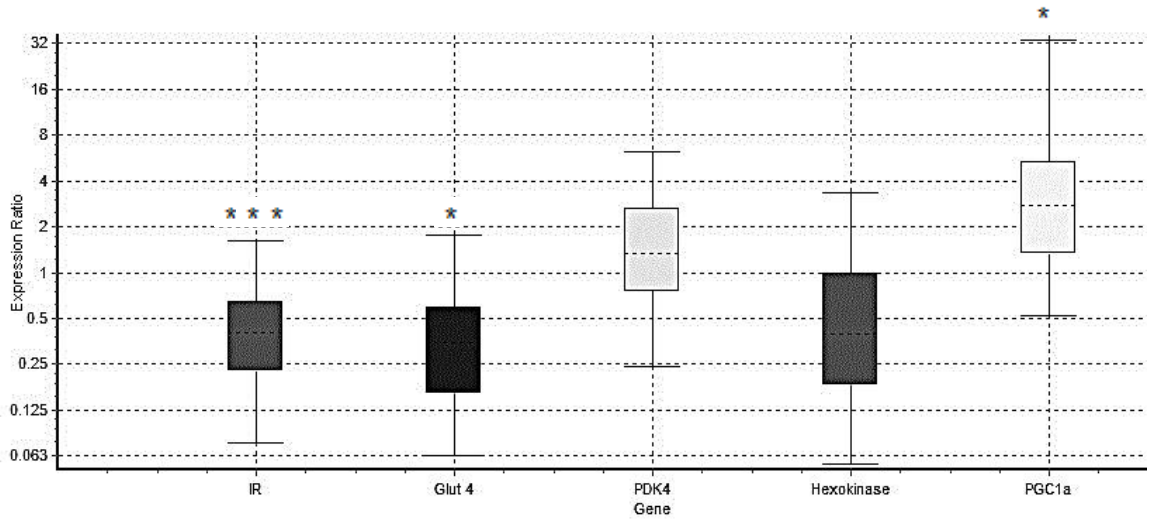


Fig 5.43. Relative gene expression of metabolic and senescent genes analysed in young and old, mice hepatocellular tissue.

A graphic representation of gene expression data obtained by relative expression software tool (REST) analysis. The figure shows boxplots of gene expression where the top and bottom walls of each box indicate the 75th and 25th percentiles, whereas the dotted line represents the median. Whiskers above and below the box extend to the 90th and 10th percentiles. Metabolic genes studied include, long-chain-fatty-acid-CoA ligase 1 enzyme (ASCL1), fatty acid synthase (FASN), carnitine palmitoyltransferase 1A (CPT1a) phosphoenolpyruvate carboxykinase 1 (PCK1) and Insulin receptor (IR).

A)



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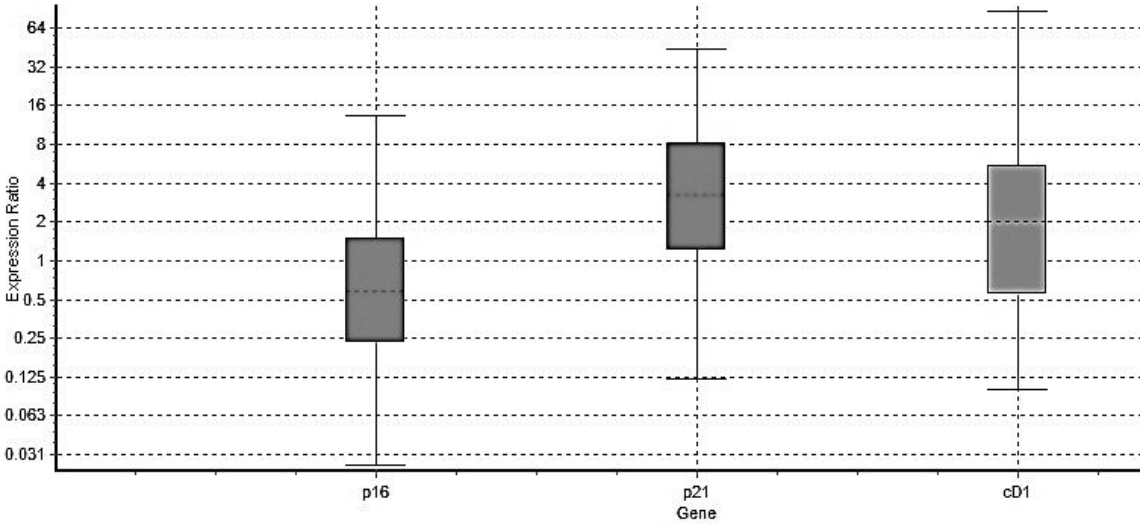
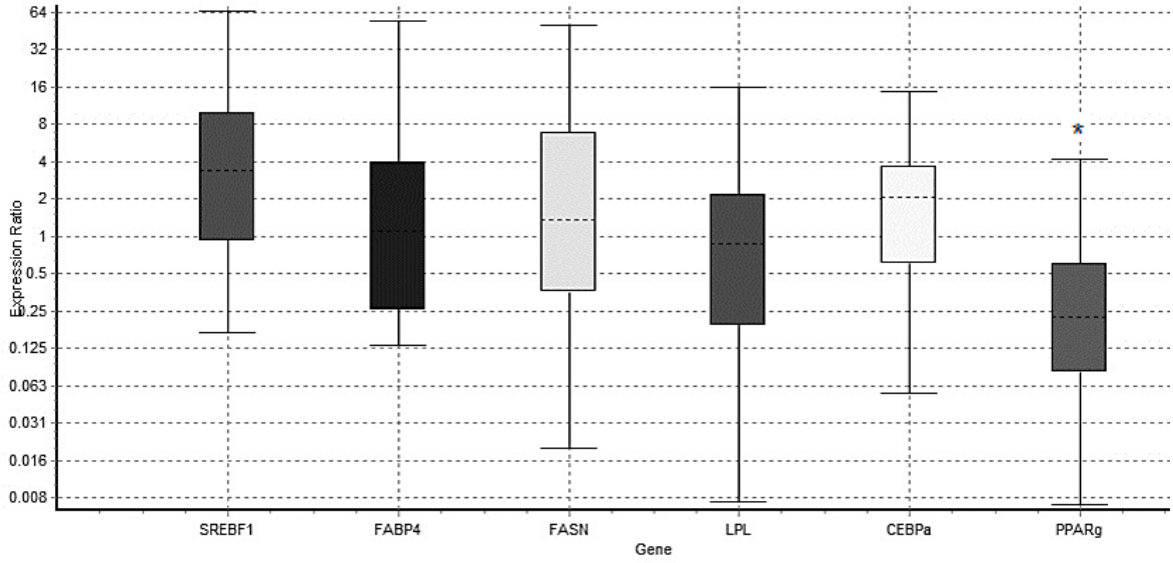


Fig 5.44. Relative gene expression of metabolic and senescent related genes analysed in young and old, mice skeletal muscle tissue.

A graphic representation of gene expression data obtained by relative expression software tool (REST) analysis. The figure shows boxplots of gene expression where the top and bottom walls of each box indicate the 75th and 25th percentiles, whereas the dotted line represents the median. Whiskers above and below the box extend to the 90th and 10th percentiles. Metabolic genes studied include Insulin receptor (IR), Glucose transporter (GLUT4) Pyruvate Dehydrogenase Kinase (PDK4), Hexokinase, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α).

A)



B)

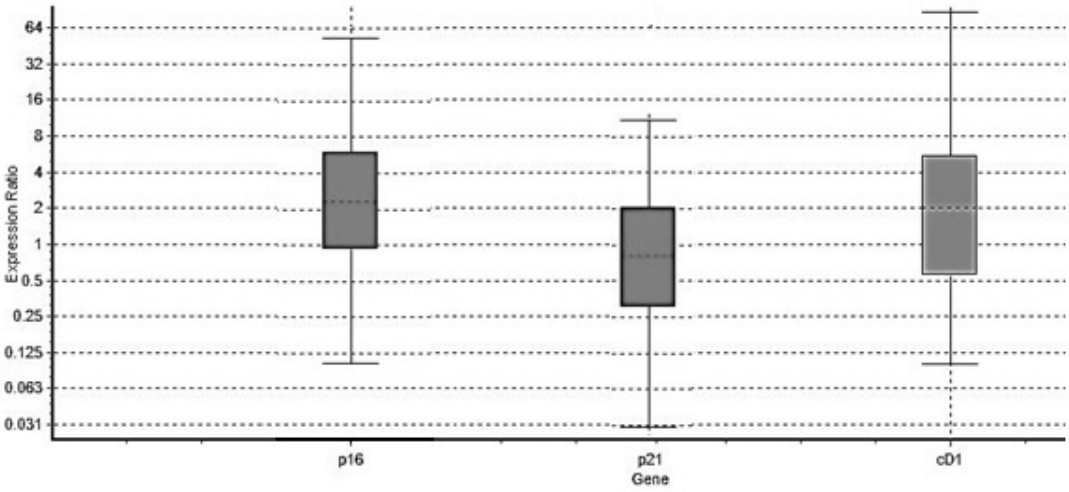


Fig 5.45. Relative gene expression of metabolic and senescent genes analysed in young and old, mice adipose tissue.

A graphic representation of gene expression data obtained by relative expression software tool (REST) analysis. The figure shows boxplots of gene expression where the top and bottom walls of each box indicate the 75th and 25th percentiles, whereas the dotted line represents the median. Whiskers above and below the box extend to the 90th and 10th percentiles. Sterol regulatory element-binding transcription factor 1 (SREBF1), fatty acid binding protein (FABP4), Fatty acid synthase (FASN), Lipoprotein Lipase (LPL), CCAAT/enhancer-binding protein alpha and Peroxisome proliferator-activated receptor gamma (PPARg).

5.5. Discussion

Existing research has established the significant impact of cellular senescence throughout various stages of organismal ageing. Evidence based research highlights the integral contribution of the cell autonomous effects of senescence in various age related health implications including; oocyte depletion mediated female reproductive senescence and senescent microglial mediated Alzheimer's disease. However the non-autonomous effects of SC have been less extensively investigated and are potentially more detrimental in propagating the senescent phenotype across non-senescent tissues, and therefore of more interest. The data reported in this study identifies an important role for the SASP in altering glucose homeostasis in insulin sensitive tissues. Cell media obtained from C2C12 myocytes exposed to 40% conditioned media from SC for 24 and 48 hours (Fig. 5.20) and AML-12 hepatocytes after 48 hours (Fig. 5.14) exhibited significantly higher concentrations of glucose in comparison to control media ($p < 0.0001$, $p < 0.01$) independent of a change in cell viability (Fig 5.16-17 and 5.20-21), suggesting a reduced ability to utilise glucose, p38 inhibitors restored glucose utilisation similar to control treatment (Fig 5.17, 5.23).

Further investigations using C2C12 myocytes revealed SASP to be a considerable source of reactive superoxides (Fig. 5.24-25) independent of enhanced mitochondrial activity (Fig. 5.26-33). Glucose utilisation remained unchanged in 3T3-L1 adipocytes cells treated with 20% or 40% conditioned media (Fig. 5.35-36), however fluorescent lipid staining (Fig. 5.37-40) and more importantly quantification of circulating glycerol following 20% and 40% conditioned media (Fig. 5.41-42) suggests lipotoxic tendencies ($p < 0.05$ and $p < 0.001$).

Separately metabolic genes vital for tissue function were assessed in young (4 months) and old (19 months) liver, muscle and fat tissues from mice. Phosphoenolpyruvate carboxykinase 1 (PCK1) was the only gene down-regulated in aged mice liver tissue (Fig. 5.43) ($p < 0.01$). PGC1α expression was significantly upregulated in young skeletal muscle tissue ($p = 0.05$), while both

Glut4 ($p = 0.05$) and insulin receptor gene expression ($p = <0.01$) decreased in aged skeletal muscle tissue (Fig.5.44). Finally aged adipose tissue expressed reduced gene expression of the PPARg gene only (Fig.5.45) ($p = 0.05$). Changes in genes associated with cellular senescence remained non-significant between mice of young and old age. The data here recognises advancing age is met with reduced expression of genes required for the normal physiological function of liver, muscle and fat tissue.

The primary outcome from this investigation presents the SASP as a potential mediator between ageing and the increase in age related insulin resistance, predominantly via the reduced ability of skeletal muscle cells and to a lesser extent hepatocytes, to utilise free glucose. Although β-cell failure is critical in the manifestation of T2D, it remains secondary to skeletal muscle insulin resistance which is believed to be the primary defect in the development of T2D and clinically evident before β-cell failure and overt hyperglycemia ensue [711, 712]. Evidence supporting these claims report, glucose tolerant offspring of parents with T2D exhibit moderate to severe skeletal muscle insulin resistance along with reduced glycogen synthesis capabilities, while hepatic and adipocyte insulin sensitivity remain unchanged [712, 713]. Current understanding of the SASP recognises it as a considerable source of inflammatory and growth remodelling factors which are able to alter tissue architecture [714]. Circulating proteins commonly associated with SASP include, TNF-α, IL-6, IL-8, MMP, MCP-1 and IGF binding proteins to name a few [703, 715]. Recent work has identified several signal transduction pathways activated by pro-inflammatory cytokines associated with the obese phenotype, but are common to the SASP and can impede on insulin receptor signalling in skeletal muscle, liver, and adipose cells [701, 716]. A majority of these complex and interrelated pathways appear to converge at the level of insulin receptor substrate-1. For example TNF- α, commonly found in SASP is known to activate serine kinases such as JNK, p70 26 kinase and p38 mitogen-activated protein kinase, which go on to phosphorylate serine residues of IRS-1, negatively regulating normal signalling through the insulin receptor/IRS-1 axis in skeletal muscle tissues [717-719].

The net result impairs phosphorylation of protein kinase B (Akt), an integral step required for the translocation of GLUT4 transporter and concomitant glucose uptake [720]. Similarly, inflammatory stimuli mediated enhanced activity of JNK1 leads to serine phosphorylation of IRS-1 impairing insulin sensitivity in hepatocytes [721]. Furthermore pro-inflammatory stimuli can initiate insulin resistance via enhanced NFκB and IKKβ signalling in hepatocellular tissues; on the contrary inhibition of IKKβ by high dose aspirin treatment restores insulin sensitivity [722]. The author assumes SASP inhibits glucose utilisation in otherwise insulin sensitive tissues via pro-inflammatory cytokines and the signalling pathways they activate. Interestingly co-incubation of SASP with p38 inhibitor restored glucose utilisation in both C2C12 myocytes and AML-12 hepatocytes. Glucose uptake was comparable to C2C12 and AML-12 treated with non-senescent media and indicates inhibiting p38 signalling pathways either reverse cellular senescence, upregulate mechanisms facilitating glucose uptake and/or inhibit the production of pro-inflammatory cytokines.

While data affirming the reversal of cellular senescence due to p38 is unheard of, a more plausible theory, backed by scientific evidence recognises inhibiting p38 signalling pathways are able to diminish the inflammatory response and facilitate glucose uptake [723]. The work of C.J. Carison and C.M. Rondinone report pharmacological inhibition of p38 in insulin resistant 3T3-L1increased basal glucose uptake as well as glucose uptake in response to a subsequent insulin stimulation, an effect which manifests due to increased GLUT 1 and 4 expression [723]. A review by Yong *et al.*, documents the involvement and contribution of p38 MAPK in asthma, rheumatoid arthritis, systemic inflammation, inflammatory bowel disease, brain inflammation and stroke [724]. The p38 MAPK pathway plays a central role in the expression and activity of pro-inflammatory cytokines such as TNF-a, IL-1, IL-2, IL-6, IL-7, and IL-8, inhibiting p38 signalling will undoubtedly reduce the inflammatory burden and therefore supress IR signalling [725, 726]. The data reported in this study identifies the SASP as a considerable source of ROS more specifically superoxide formation, and a prospective mechanism behind senescence induced insulin resistance. Interestingly ROS present as positive inducers of the senescent phenotype via

p53-depedent pathways and considerable evidence exists verifying the role of ROS in propagating insulin resistance [727]. Current evidence of ROS promoting insulin resistance in *vivo* stems from studies examining the effects of obesity on insulin resistance.

The work of Houstis *et al*., treated *ob/ob* mice with manganese (III) tetrakis porphyrin an antioxidant, and noticed improved insulin sensitivity independent of alterations in adipose mass [728]. Hoehn *et al*., have reported transgenic mice overexpressing superoxide dismutase in liver and skeletal muscle tissue were protected against the insulin desensitising effects of ROS produced from consuming a high fat diet [729]. Mitotracker data collected during this study failed to demonstrate any SASP related changes in mitochondrial activity or number, implying ROS production is independent of hyperactive mitochondria. A plausible theory suggests a chemoattractant effect of pro-inflammatory cytokines on circulating neutrophils, facilitating migration to senescent sites and upregulating ROS production. Physiologically, neutrophils are short lived however, IL-6 and IL-8 constitutively produced by SC and commonly found in SASP, have been associated with prolonging neutrophil survival significantly [730]. The influence of oxidative stress on insulin signalling is complex and involves numerous transcription factors and stress-sensitive signalling pathways including, NF-κB, JNK/IKKβ, and p38 MAPK, all of which intersect the insulin pathway at the level of IRS-1 [344]. The proposed mechanism whereby SASP influences insulin signalling, like the effect of pro-inflammatory cytokines involves phosphorylation of specific serine/threonine residues on IRS-1 which promote IRS-1 degradation [731]. Downstream effects involve the suppression of phosphatidylinositol 3-kinase, which has been reported to be a significant inducer of insulin resistance when not activated, in addition to suppressing Akt activation [732]. The net result is reduced GLUT4 expression and decreased glucose uptake following a superoxide insult (Fig.38). In concert with gene expression data acquired during this study, it is evident advancing age is met with reduced gene expression of the insulin receptor gene and GLUT4 in mice skeletal muscle tissue, further exasperating SASP mediated insulin resistance.

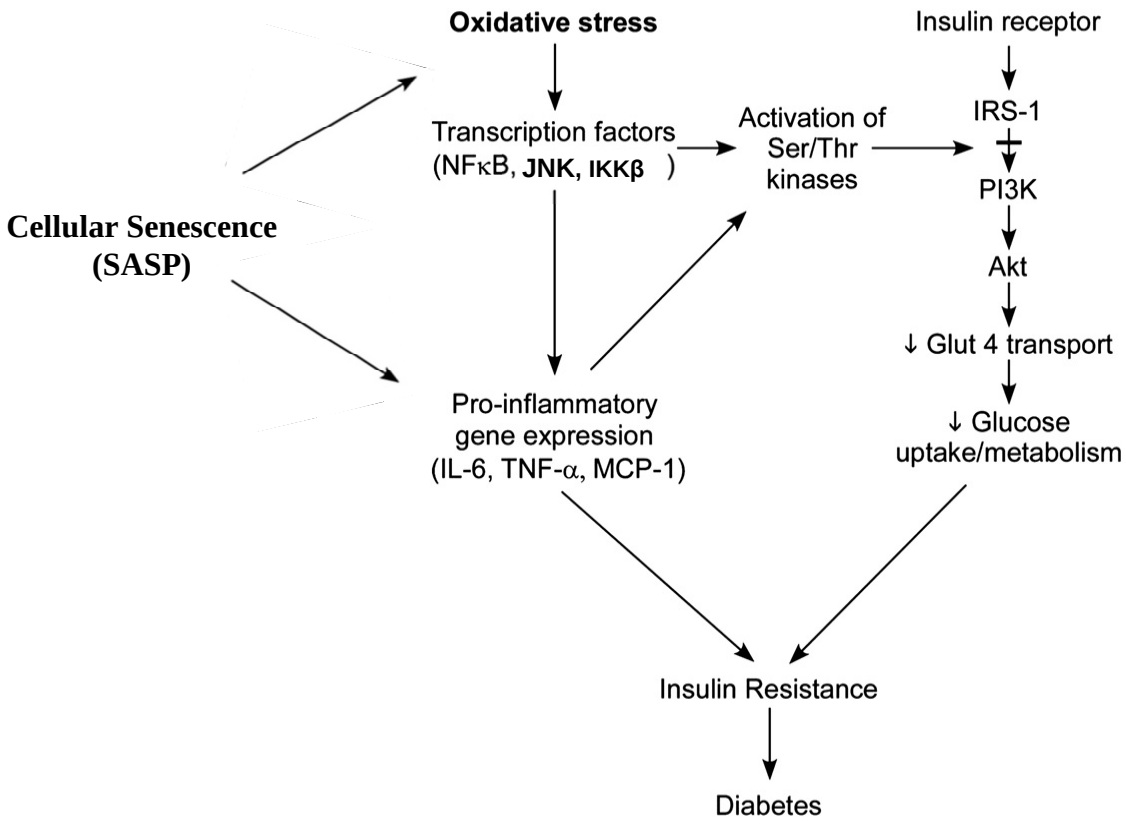


Fig 5.46. Simplified schematic outlining the associations between cellular senescence and pathways leading to insulin resistance and diabetes.

SASP mediated insulin resistance is not limited to pro-inflammatory cytokine secretion, instead ROS also contribute significantly to insulin resistance by promoting serine phosphorylation of the insulin receptor, thus mediating heterologous inhibition of insulin receptor substrate-1 signaling, which in turn counter regulates the insulin response. Excessive oxidative stress, commonly associated with advancing age also promotes p53 dependent cellular senescence which provides an alternative mechanism to SASP mediated insulin resistance.

An additional mechanism recognised in this study, contributing to age related insulin resistance and lipotoxicity, includes the lipolytic effects of the SASP on mature adipocytes. The pro-inflammatory and oxidative nature of the SASP has already been elucidated and serves as a prominent candidate behind SASP mediated lipoytic effects [733]. Grisouard *et al*., support this theory, the team treated mature adipocytes with lipopolysaccharides to induce an inflammatory response and reported a dose dependent increase in circulating glycerol [734]. Interestingly inhibitors of IKKβ or NF-κB inhibited LPS-induced glycerol release as did inhibitors of protein kinase A (PKA) and the hormone-sensitive lipase (HSL) [734]. Furthermore lipolytic agents were reported to enhance LPS-induced mRNA expression of pro-inflammatory cytokines [734]. In summary the molecular pathways orchestrating SASP facilitated inflammatory responses are common to lipolysis and in a positive feedback loop reinforce the expression of pro-inflammatory cytokines, further upregulating lipolysis.

The role of PKA and HSL also tie in with ROS mediated lipolysis. Krawczyk *et al*., identify the vital role of ROS in regulating lipolysis, more specifically antioxidants employed to neutralise ROS, decreased lipolysis in adipocytes [735]. The team also established PKA mediated phosphorylation of HSL facilitated it's translocation from the cytosol to lipid droplets where lipolysis is to occur, an effect which was abrogated by scavenging ROS [735]. A review by Poglio *et al*., supports these observations and acknowledges adipocytes are particularly sensitive to ROS, which may account for the cytotoxic effects observed, relative to the non-significant change in cell viability observed in hepatocytes and myocytes [736]. A decreased ability to supress lipolysis leads to increased local fatty acid concentrations, enhancing β-oxidation and the amount of ROS [737]. The net result establishes a vicious cycle where ROS positively regulate lipolysis which leads to a further increase in ROS. It is also noteworthy advancing age is met with reduced antioxidant capabilities and therefore only amplifies the lipolytic effects of SASP [738].

Excessive lipolysis is a considerable risk factor for developing age related metabolic disorder [739]. Enhanced lipolysis leads to considerable release of free fatty acids that are transported to the liver and responsible for hepatocellular insulin resistance, alongside fatty liver disease [740]. Free fatty acids not only sequester the inhibitory effects of insulin on gluconeogenesis, but can enter the circulation and enforce insulin resistance in skeletal muscle tissue, as well as propagating hypertension and ultimately atherosclerosis [741, 742]. Gene expression data reported in this study recognises expression of the PPARG gene was significantly down regulated with advancing age. The PPARG gene is vital for fatty acid storage and glucose metabolism, more specifically is vital for lipid uptake and lipogenesis [743]. Evidently PPARG knockout mice fail to generate adipose tissue when fed a high-fat diet and are at an increased risk of developing previously mentioned health implications [744].

The detrimental effects of reduced PPARG expression can somewhat be explained by a controversial view considering low grade obesity to offer protection against the development of T2D, and represents this relationship with a J-shaped curve [745]. This suggests individuals with extremely low or high levels of adipose tissue are at an increased risk of developing metabolic disorders, while slightly overweight individuals are at no such risk. The proposed mechanism behind the theory recognises individuals with slightly larger amounts of adipose tissue are better able to neutralise elevated circulating glucose levels and protect against the ectopic accumulation of free fatty acids in liver and skeletal muscle [745]. Conclusively SASP mediated degradation of adipocytes in combination with reduced PPARG gene expression with advancing age, lead to lipotoxic disorders and significantly enhance the risk of developing insulin resistance.

5.6. Conclusion

The data reported in this study evidences a potentially important role for senescent cells and more specifically their secretome in altering nutrient homeostasis in insulin sensitive cells. The proposed mechanism behind the dysregulated glucose utilisation in hepatocytes and myocytes includes the insulin desensitising effects of pro-inflammatory cytokines, documented in existing literature and increased ROS production reported in this study itself, in a dose and time dependent manner. The effect of SASP on adipocytes exhibited cytotoxic tendencies and demonstrate links between advancing age and lipotoxicity related health implications. Gene expression data collected in this study analysing variations amongst young and aged mouse, hepatocyte, myocyte and adipocyte tissues, reveals advancing age is met with reduced expression of several metabolic genes relevant for regular glucose homeostasis. In combination, the results from this study identify the SASP as a mediator between ageing and the increase in age related insulin resistance, amplifying the deleterious effect of ageing on vital genes required for effective glucose homeostasis.

Chapter 6:

Conclusions and Future work.

Advancing age is unanimously associated with the increased incidence of a wide range of morbidities [746]. The development of medical interventions, eradication of diseases previously thought to be fatal and the increased manufacture and implementation of automated technologies, in conjunction with a better understanding of nutrition has resulted in a significant increase in human lifespan [747]. It is clear however that to some extent such increases in longevity are accompanied by an increasing prevalence of metabolic disorders and their associated comorbidities, giving rise to an increasingly dependent population suffering from ill health and contributing significantly to healthcare expenditures and expenditures related to the rehabilitation of the elderly [109, 110].

As a result of these parallel trends a rational postulation suggests causative relations between advancing age and the increased incidence of metabolic disorders and possibly vice versa. It is therefore imperative to identify individuals experiencing an accelerated rate of ageing and/or at an increased risk of developing metabolic disorder, with the expectation that medical intervention may be able to delay the onset of disease and deterioration. However due to the seeming lack of conclusive results stemming from studies investigating the associations between markers of ageing amongst individuals suffering from metabolic disorders, in conjunction with the documentation of conflicting views, relations between metabolic disorders and their effect on ageing remain poorly understood. This study adds novel and interesting data to the emerging field of ageing research. Firstly this study agrees with existing data demonstrating TL as a candidate biomarker of cellular ageing in 'healthy' individuals, and thus advocates future implementation in similar areas of research. Furthermore the data presented here, also highlights the significance of body composition in comparison to the more widely utilised BMI, not only as a more informative measure of physiological health but as a determinant of TL and therefore ageing. Studies like this are of paramount importance, currently international and domestic healthcare practices utilise BMI as an imperative diagnostic tool to identify individuals at the risk of developing obesity.

As stated previously obesity has been recognised as a fundamental risk factor in the manifestation of numerous life threating health implications, including; T2D, CVD and cancer. Therefore accurate identification of individuals likely to develop obesity is vital, allowing for early medical intervention and can significantly reduce the financial burden of obesity related co-morbidities. However it is becoming increasing apparent that the use of BMI can be erroneous. The BMI reading takes into account weight (kg) and height (m^2) only and therefore completely disregards the contribution of muscle mass to weight. Individuals of a more athletic built are often presented with a falsely high BMI, while slender individuals who carry excess body fat around their waists will be presented with a falsely low BMI. Furthermore BMI readings cannot distinguish between SAT and VAT, the latter documented to be considerably more detrimental to health. BMI also fails to take into account body fat distribution as existing research has elucidated retaining excess central adipose tissue is unfavourable, although waist to hip readings may provide an indication of excess central adiposity, waist to hip readings are seldom taken in clinical practices.

Body composition readings taken using bio-impedance, as done in this study can overcome all previously stated shortcomings and that too at an affordable price. More studies utilising bio-impedance to take body composition measurements are required in order to induce the widespread utilisation of such body composition analysers. The body composition analyser used during this study is small and cost effective, and requires very little training to use, thereby is perfect for healthcare practices, particularly general practitioners. The use of body composition analysis will be able to give patients a more detailed account of their health, the efficiency of a training or diet regime, thus help identify patients at an increased risk of developing metabolic complications. The data presented here showing TL in the obese/T2D cohort clearly highlights increased telomere attrition in comparison to healthy participants, implying an accelerated rate of ageing amongst the former. Significant negative associations between adipose tissue and TL, and positive associations between skeletal muscle mass and TL amongst non-diabetic individuals indicates the potential benefits to longevity and increased healthspan of maintaining a healthy

body composition, potentially through diet and exercise. The lack of significant correlations between TL and anthropometric measurements, markers of endothelial dysfunction and circulating adipo/myokines within the obese/T2D cohort suggests factors governing TL in healthy individuals differ to those regulating TL in obese/T2D individuals. Although the current study is unable to comment on the specific contributors to reduced TL, existing literature advocates the contribution of a multitude of factors are responsible for the accelerated rate of cellular ageing experienced by individuals with metabolic disorders, most likely due to factors stemming from the pathology of the disorder [671, 748]. Obesity and T2D are two disorders inflammatory in nature with a high oxidative burden and may be influential in determining TL amongst patients [749]. These findings highlight issues which are of grave importance in today's society. Statistics derived from government sources and research institutes recognise the increasing prevalence of metabolic disorders particularly T2D, in conjunction with an ageing population the number of people living with T2D will only increase [750].

Future predictions point towards large proportions of the population in both developing and developed nations suffering from T2D and associated co-morbidities and therefore significantly consuming healthcare financial budget as well as resources aimed towards pensions, care homes etc. Data reported in this study will be useful in identifying individuals at risk of accelerated ageing and may be able to predict mortality in a longitudinal study. Future studies should aim to use TL as a biomarker of ageing and disease and design longitudinal studies, whereby TL measurements could be taken at pre-set intervals over the course of time to identify differing rates of telomere attrition between individuals suffering from obesity/T2D. Maybe then, on the back of sufficient evidence diagnostic approaches within clinical settings may consider TL analysis, with the potential for point of care testing. Other key findings produced by this study provide novel insights into the role of irisin in both normal and abnormal metabolic states. The strong positive correlation and the ability of irisin to predict TL in the control cohort suggests irisin potentially exhibits anti-ageing properties. Currently the source of irisin secretion is an area of

debate with existing literature providing views in favour of both adipose tissue and skeletal muscle. Because irisin did not correlate with any anthropometric measurements, the source of secretion remains inconclusive. Future studies will need to identify and reach a general consensus regarding whether irisin is in fact produced from exercised skeletal muscles or not and if so is a particular exercise regime preferable to optimise irisin secretion. Also irisin sensitivity is worth investigation if in fact irisin secretion originates from adipose tissues, then in the context of this study healthy volunteers are clearly sensitive to irisin however individuals suffering from metabolic disorders are less sensitive. Why that may be the case requires investigation. Till then, the correlation between irisin and TL is considered an indirect measure of exercise mediated longevity. Due to the observational nature of the work the mechanism behind this association remains unknown; however existing literature denotes oxidative stress and inflammation as considerable contributors to telomere attrition, whether irisin can mitigate the ill effects of both is an area of research yet to be ventured.

Measurements of BAT and biomarkers of BAT in conjunction with questionnaires conducted aimed at collating qualitative data regarding, self-assessed health, quality of diet and exercise may have aided to further clarify the role of irisin in ageing. Fibroblast growth factor 21 (FGF21) has been reported to regulate PGC-1α and browning of WAT [648]. Moreover FGF21 has been noted to stimulate glucose uptake and an increase in circulation has been reported post exercise in humans[648]. Wisse and Schwartz highlight BDNF is a key modulator of hypothalamic pathways governing body composition and energy homeostasis and more specially report BDNF levels enhance glucose utilisation in skeletal muscles [751]. Plasma quantification of FGF21 and BDNF may have proven useful to correlate with circulating irisin levels, as well as with TL and will have allowed the author to provide strong evidence in support of the benefits of BAT and calorie restriction like mechanisms involved in longevity. Qualitative data compiled from questionnaires will have allowed the author to obtain a better understanding of research volunteers and help establish links between diet, exercise and clinical history with TL with the

potential of identifying certain foods or exercises which result in elongated or shorter telomeres. Furthermore future studies investigating the role of irisin in SIRT1 activation and/or the restriction of insulin/IGF-1 signaling pathways, recognised to promote longevity may help elucidate the precise mechanisms responsible for elevated irisin mediated increases in TL. The work of Qiang *et al* recognises brown remodelling of white adipose tissue by sirt1-dependent deacetylation of PPARγ and according to the authors knowledge is the only paper to have conducted research in this area and provides potential links between irisin and SIRT1 [752]. It is also of paramount importance to probe associations between irisin concentrations and telomerase expression/activity. The source of irisin secretion in non-diabetic volunteers cannot be confirmed from the data in this study. However the data generated from the obese/T2D individuals highlights irisin circulates at 3-fold higher concentrations relative to healthy subjects and its levels correlate well with subcutaneous adipose depots and correlates positively with HbA1c, two prognostic measures consistent with the obese/T2D phenotype. Although no such conclusion can be drawn from the data generated in this study, a plausible explanation behind the increased irisin concentrations reported amongst obese/T2D volunteers is volunteers suffering from metabolic disorders are irisin resistant.

Other authors who have reported data in concert with data reported in this study have also suggested irisin resistance amongst individuals suffering from metabolic disorders, and claim the increased concentration of irisn is due to a compensatory response, to expel excess energy in the form of enhanced thermogenic activity from BAT, and much like leptin, irisin has lost physiological sensitivity amongst obese/T2D patients [269, 753]. However no concrete evidence yet exists affirming the existence of irisin resistance, future studies should aim to provide empirical data supporting irisin resistance. The culture of adipose explants from diabetic patients, or adipocyte cell lines grown under hyperglycaemic conditions could be treated with recombinant irisin to assess the expression of genes actively involved in browning of WAT, such as PGC-1α, UCP-1 etc. Also following exogenous irisin administration to obese/T2D patients, weight loss

and CT mediated scanning of BAT could be conducted to confirm irisin resistance. The postulated irisin resistance with respect to this particular study may explain the non-significant association between irisin and TL. It therefore seems obese/T2D individuals, not only experience an accelerated rate of ageing but are resistant to the protective effects of circulating irisin. Another novel observation reported in this study recognises the increase in circulating irisin levels amongst obese/T2D may negatively impact health and upregulate inflammatory processes. Such conclusions are based upon the strong correlation between irisin levels and soluble E-selectin, an inflammatory marker and a considerable risk factor for developing atherosclerosis. HUVEC cells treated with high concentrations of irisin exhibit 4-fold increased expression of E-selectin. Further studies are required to completely elucidate the role of irisin in obese/T2D patients.

Experimental methodology which may prove useful in investigating the role of irisin in cardiovascular complications, includes using apolipoprotein or lipoprotein deficient mice, which naturally develop CVD, such mice could be injected with irisin to compare the extent of cardiovascular disease to mice not treated with irisin. Such experiments could also be done in irisin knockout mice, likewise following irisin over expression biomarkers of CVD and inflammation could be quantified. Koenig and Khuseyinova outline various biomarkers to look out for at specific stages of atherosclerosis, the data reported in this study reports a stark upregulation in E-selectin following high Irisn treatment and would be associated with inflammatory cyto/chemokines upregulating the recruitment of monocytes [754]. The quantification of IL-1, MCP-1, TNF-α in conjunction with cellular adhesion molecules sICAM and selectins may help identify the effects of irisin on CVD. During this study flow cytometry was conducted to assess the cell surface expression of E-selectin (CD62E) on HUVEC cells following incubation with high concentration of irisin. These experiments need to be repeated to produce an n of three and therefore provide a statistical answer, as to whether increased irisin concentrations can upregulate cell surface expression of E-selectin and will further compliment the gene expression data reported in this study.

Using western blotting to assess protein expression of E-selectin following irisin treatment of HUVEC would also help identify relations between irisin and inflammation. The ability of irisin to upregulate soluble E-selectin could be further analysed to investigate the role of elevated irisin concentrations on monocyte/neutrophil recruitment in a trans-well microenvironment, to assess whether irisin expresses chemotactic properties to facilitate extravasation *in vitro*.

The effects of such studies could then be modulated by transgenic over-expression and silencing of irisin, to confirm the role, if any of irisin on propelling an inflammatory response. The data presented here suggests that general energy/nutrient homeostasis can dictate cellular ageing. The later aspects of this study set out to investigate how cellular senescence can influence nutrient metabolism. According to the authors knowledge the data reported in this study is novel and identifies that the SASP differentially affects different cell types. Glucose uptake was significantly down regulated in hepatocellular and skeletal muscular tissues when treated with SASP, an effect which was abrogated in the presence of p38 inhibitors. Adipocytes are considerably more sensitive to SASP and underwent lipolysis, cell viability remained unchanged in hepatocellular and skeletal muscle tissues. SASP treatment did not affect cell viability but was identified as a considerable source of superoxide species, although the source of ROS was not identified, in line with the data reported in this study and pre-existing knowledge regarding the SASP, may be responsible for dysregulating glucose homeostasis and inducing lipolytic effects.

Statistics from governing bodies are increasingly reporting the increase in the number of individuals reaching 65 and beyond, contrastingly met with a reduction in birth rate. The net result is an increase in the proportion of elderly individuals relative to the younger generation. The number of senescent cells increases with age and therefore in an ageing population, individuals are amassing a large number of senescent cells. The data reported here highlights senescent cells may potentially be driving dysregulated nutrient homeostasis, by inducing insulin resistance in

insulin sensitive tissues. Numerous research papers have documented an age associated insulin resistance, without elucidating the specific mechanisms. Future research should continue to investigate the role of cellular senescence in dysregulating nutrient homeostasis as the precautions of an elderly insulin resistant population has the potential to be catastrophic. Insulin resistance has been associated not only with T2D but Alzheimer's disease, liver disorders and even cancer. By identifying a relation between cellular senescence and insulin resistance, along with identifying a potential mechanism which could be targeted by pharmacological interventions, the results from this study are of grave importance. The results from this study warrant further investigations which can further explain the role of cellular senescence in metabolic disorders. Experiments which could not be completed due to time/financial constraints form the basis of future studies which could help further establish the role of cellular senescence in metabolic dysfunction. Firstly it is essential to characterise the SASP and identify the specific secretions of senescent fibroblasts. There is currently a general appreciation of the constituents of SASP but studies investigating cell type specific secretions along with differences in secretion between, replicative senescence and DNA damage induced senescence are required.

In the context of this study, experiments investigating whether p38 could inhibit SASP mediated lipolysis could not be completed but are essential to fortify the significance of the p38 pathway in cellular senescence. More sophisticated techniques need to be employed to measure ROS production from SC and studies investigating mitochondrial function in SC would provide additional information with regards to the source of ROS production. Additionally the role of the SASP in monocyte recruitment may also shed light on this matter. To further investigate the role of SASP in insulin resistance and glucose homeostasis, PCR analysis of genes encoding proteins fundamental for glucose uptake (IR, GLUT4, Glucokinase) should be conducted, using cell pellets from cells treated with SASP.

Glycogen content also quantifiable from cell pellets would complement experiments investigating glucose uptake conducted in this study, because a potential limitation of this work is the inability to account for glucose produced by the cells, particularly the AML-12 cell line. In general, studies delaying the accumulation of SC and thus reducing the release of SASP offer potential strategies for mitigating the deleterious effects of advancing age. The role of calorie restriction in cellular senescence needs further investigation, considering recent research has proposed calorie restriction could exert part of its beneficial effect on health span by delaying cellular senescence. Studies aiming to completely eradicate the development of SC may prevent the onset of numerous age related pathologies however compromise protection against the development of cancer. Therefore future studies should aim to neutralise existing SC and therefore indirectly reduce the SASP burden. Genetic inactivation of *p16*Ink4a in BubR1 progeroid mice has proven to delay the onset of aging, due to a decreased number of p16Ink4a-positive senescent cells [755]. The authors provide further evidence of the involvement of cellular senescence in age associated pathologies by producing a transgene called *INK-ATTAC* designed to increase susceptibility to drug induced apoptosis in senescent cells expressing the *p16*Ink4a gene. Lifelong removal of p16Ink4a-positive senescent cells delayed the onset of fat loss, skeletal muscle deterioration, and cataract formation in BubR1 progeroid mice.

In conclusion the data reported in this study establishes the significance of body composition in cellular ageing, a measure which should be considered over the utilisation of BMI. Furthermore irisin exhibits hormetic function, exhibiting anti-ageing properties in healthy individuals but at considerably higher concentrations as in obese/T2D propagating the basis of an inflammatory response. Further work is required to confirm whether recombinant irisin could be used as a therapeutic intervention to prevent age related pathologies and whether irisin functions as an potential target for drug delivery in obese/T2D to reduce inflammation. The SASP positively contributes to the development of irregular glucose homeostasis and lipotoxicity and therefore may be integral in the age associated increase in insulin resistance.

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7.0 Appendices

7.1. Flow cytometry

Flow cytometric analysis was conducted to assess if 200ng/ml irisin could induce cell surface expression of E-selectin. Three cell treatments were administered to HUVEC, including a non-treatment control, TNF-α positive control well documented to upregulate E-selectin expression and finally 200ng/ml irisin. Cells were treated for 4 hours. Staining with a isotype control exhibited very little staining on all three cell treatments as expected, while anti-CD31 a cell surface marker known to be present on HUVEC exhibited considerably higher percentage of cell staining on all three cell treatments (0 = 99.72%, TNF-α = 99.87% and irisin = 99.89%). Cells exhibiting cell surface E-selectin was assessed using an anti-CD62E antibody, 9.71% of non-treated cells exhibited E-selectin expression. The positive control TNF-α induced cell surface expression of E-selectin on 96.47% of cells. HUVEC treated with 200ng/ml irisin induced E-selectin expression on 17.29% of cells following a 4 hour incubation. Irisin increased cell surface expression of E-selectin by 15.57% following 4 hours, these results suggest either the beginning of E-selectin surface expression or declining E-selectin expression following a transient rise.

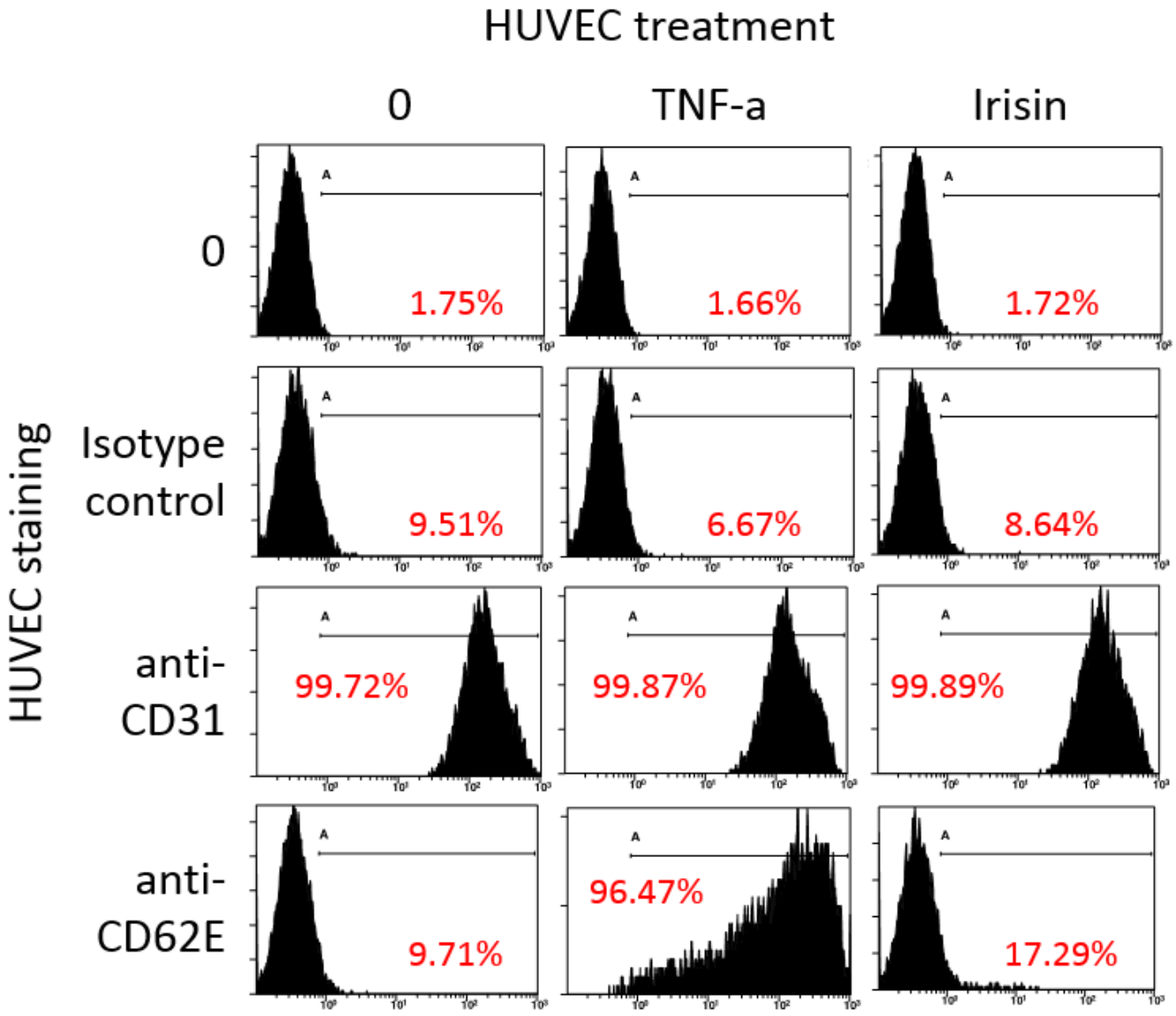

Fig 7.1 The effect of 200ng irisin treatment on CD62E cell surface expression.

HUVEC cells treated with TNF-α (10ng/ml) and 200ng/ml irisin to assess cell surface expression of CD62E. No treatment and an isotype control were used as negative controls and cells were stained with anti-CD31 as a positive control.

7.2 List of primers used

Purpose	Primer
Telomere forward and reverse primers	F: ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT R:, TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
Albumin forward and reverse primers	F: CGGCGGCGGGCGGCGCGGGCTGGGCG R: GCCCGGCCCGCCGCGCCCGTCCCGCCG
P16 forward and reverse primers	F: CAACGCACCGAATAGTTACG R: CAGCTCCTCAGCCAGGTC
CYcD1E forward and reverse primers	F: CACACGGACTACAGGGGAGT R: CACAGGAGCTGGTGTTCCAT
p21 forward and reverse primers	F: CGAGAACGGTGGAACTTTGAC R:CAGGGCTCAGGTAGACCTTGC
Hexokinase forward and reverse primers	F: GACCCGAGGCATCTTCGA R: AGCAGCGCTAATCGGTCACT
Pdk4 forward and reverse primers	F: GATTGACATCCTGCCTGACC R: CATGGAACTCCACCAAATCC
Glut 4 forward and reverse primers	F: ACATACCTGACAGGGCAAGG R: CGCCCTTAGTTGGTCAGAAG
IR forward and reverse primers	F: AATGGCAACATCACACACTACC R: CAGCCCTTTGAGACAATAATCC
PGC-1α forward and reverse primers	F: GAGTCTGAAAGGGCCAAGC R: GTAAATCACACGGCGCTCTT
IR forward and reverse primers	F: AATGGCAACATCACACACTACC R: CAGCCCTTTGAGACAATAATCC
ACSL forward and reverse primers	F: CAGAACATGTGGGTGTCCAG R: GTTACCAACATGGGCTGCTT
CPT1a forward and reverse primers	F: ACGGAGTCCTGCAACTTTGT R: GTACAGGTGCTGGTGCTTTTC
PCK1 forward and reverse primers	F: ATCATCTTTGGTGGCCGTAG R: TGATGATCTTGCCCTTGTGT

E-selectin forward and reverse primers	F: AGAGGTTCCTTCCTGCCAAG R: CAGAGCCATTGAGGGTCCAT
P-selectin forward and reverse primers	F: CGCCTGCCTCCAGACCATCTTC R: CTATTCACATTCCAGAAACTCACCACAGC
ICAM-1 forward and reverse primers	F: GACTCCAATGTGCCAGGCTT R: TAGGTGCCCTCAAGATCTCG
PECAM1 forward and reverse primers	F: ATTGCAGTGGTTATCATCGGAGTG R: CTCGTTGTTGGAGTTCAGAAGTGG