Understanding and evaluating cardio-metabolic benefits imposed by surgery, medication and adipose tissue metabolism

by

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Abbreviations

ACE	Angiotensin converting enzyme
ACEI	Angiotensin-converting-enzyme inhibitor
ADRP	Differentiation-related protein
AGT	Angiotensinogen
AGT II	Angiotensin II
AMPK	AMP_activated protein kinase
ANG I	Angiotensin I
ARB	AngiotensinII receptor blockers
AT1	Angiotensin II type I
AT2	Angiotensin II type 2
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body mass index
BPD	Biliopancreatic diversion
bpm	Beats per minute
BPST	BPS-tween
BSA	Bovine serum
CNS	Central nervous system
CRP	C-reactive protein
DHT	Dihydrotestosterone
EDTA	Ethylenediaminetetraacetic
EFSD	European foundation for the study of diabetes
ELISA	Enzyme linked immune sorbent assay

ER Endoplasmic reticulum

ERK Extracellular-regulated kinases

EU/mL Endotoxin unites per mililitre

FA Fatty acid

FABP4 Fatty acid binding protein 4

FAIMS Field Asymmetric ion mobility spectrometry

FFAs Free fatty acids

FGF Fibroblast growth factors

FGF-basic Fibroblast growth factor-basic

FGFR FGF receptor

FPG Fasting plasma glucose

FXR Farnesoid X receptor

GH Growth hormone

GLP-1 Glucogan-like peptide-1 receptor agonist

GLUT1 Glucose transport protein 1

HbA1c Glycosylated haemoglobin

HFD High fat diet

HOMA-IR Homeostatic model assessment

IBMX Isobutylmethlxantine

IDF International diabetes federation criteria

IFNs Interferons

IKK Inhibitory kappa B kinase

IL Interleukin

IL-6 Interleukin-6

IRAK-4 Interleukin-1 receptor-associated kinase 4

IRF Interferon-regulatory factor

IκB	Inhibitory kappa B
JNK	C-Jun N-terminal kinase
KHB	Krebs-Henseleit
KO	Knockout
LAGB	Laparoscopic adjustable gastric banding
LAL	Amebocyte lysate
LBP	LPS-binding protein
LDL	Low-density lipoprotein
Lir	Liraglutide
LPS	Lipopolysaccharides
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MD2	Myeloid differentiation factor 2
mmHg	Millimeter of mercury
MyD88	Myeloid differentiation factor 88
n	Number
Na3VO4	Sodium vanadate
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
ng	Nanogram
nM	Nanomolar
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
pg/mL	Picogram per mililetre

PGC1α Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPARα Peroxisome proliferator-activated receptor alpha

RAS Renin angiotensin system

RIP1 Receptor-interacting protein 1

RIPA Radioimmunoprecipitation

ROS Reactive oxygen species

rpm Revolutions per minute

SAT Subcutaneous adipose tissue

SE Standard error

SG Sleeve gastrectomy

SIRT1 Sirtuin-1

SNS Sympathetic nervous system

SVM Support vector machine

T2DM Type 2 diabetes mellitus

TAK1 Transforming growth factor-beta-activated kinase 1

TBK1 TRAF-family member-associated NF-κB activator-binding kinase 1

TG Triglycerides

TIR Toll/interleukin-1 receptor

TLR Toll-like receptor

TNF Tumour nicrosis factor

TNF-α Tumour necrosis factor alpha

TRAF6 Receptor-associated factor 6

TRAM TRIF-related adaptor molecule

TRIF TIR domain-containing adaptor-including interferon

TZDs Thiazolidinedione

UCP1 Uncoupling protein 1

VOCs	Volatile organic compounds
WAT	White adipose tissue
WHO	World health organisation
WHR	Waist to hip ratio
β-klotho	BetaKlotho
µg	Microgram
µl	Microlitre
µM	Micromolar
µU/L	Microunits per litre

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Declaration

This thesis is submitted to the University of Warwick is support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

List of Data Provided and/or Analysis Carried out by Collaborators:

- Field asymmetric mobility spectrometry and data analysis for the urinary volatile organic compounds, were carried out by Dr. James Covington's group.

List of Publications:

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2. Sahar Azharian, Alice Murphy, Philip G. McTernan, Milan K. Piya & Gyanendra Tripathi. *Effects of bariatric surgery on FGF-19 and FGF-21 levels in obese diabetic women*. Poster: American Diabetes Association, Orlando, 2018, San Diego, 2017.

Abstract

Obesity is the single most important risk factor for the development of cardio-metabolic disease. As white adipose tissue dysfunction plays an essential role in inflammation and metabolic health, it would be beneficial to therefore investigate the influence of surgery, endocrine hormones, and medication that mitigate adipose tissue inflammation and as a result improve cardio-metabolic disease. As such subcutaneous adipose tissue (SAT) biopsies and anthropometric and biochemical data were collected from obese-diabetic women before and after bariatric surgery. Inflammatory markers, endotoxin, cardio-metabolic markers, fibroblast growth factor (FGF)-19 and FGF-21, AGT protein expression and insulin sensitivity, were assessed by utilizing ELISA, western blot, anthropometric and biochemical data analysis. Collectively the findings of this study identified that combination (restrictive and malabsorptive) type of bariatric surgery compared to restrictive procedures, lead to the persistence of an inflammatory status, with less improvements in cardio-metabolic components, lower disease resolution and lack of significant improvement in FGF-19 and FGF-21 endocrine hormones, at 6 months post surgery. Therefore despite the significant weight loss with the combination type of surgery, it may still place patients at risk of cardiovascular disease at 6 months post surgery. In regards to medications that may affect adipose tissue inflammation, effects of liraglutide on adipose tissue specific angiotensinogen (AGT) was analysed to assess the possible antihypertensive qualities of this medication. The findings of this study indicated that liraglutide can down-regulate AGT in a time and dose depended manner in obese mature adipocytes. Furthermore liraglutide appeared to reduce cell proliferation, and improve insulin sensitivity. Finally, recognising the likelihood of improvement in Type 2 diabetes mellitus (T2DM) status post bariatric surgery is not only beneficial in supporting the preoperative weight loss criteria, but it will also identify individuals that may need more support post operatively to maintain improved cardio-metabolic outcomes. Therefore having a convenient, non-invasive, and cost effective detection and predictive test for T2DM, in such a circumstance can be useful. Urinary volatile organic compounds (VOC) have been used for the detection of many diseases such as different types of cancers, therefore the potential of VOCs for detecting T2DM was analysed. Urine from the same bariatric cohort was collected and tested by field asymmetric ion mobility spectrometry. The findings indicated that VOCs have the capability of distinguishing pre from post operation urine, in addition to post-operative T2DM status was predicted with the use of pre-operative VOCs. Therefore VOCs have the potential of being used as a screening and detection test for T2DM, and as a predictive test for the outcome of T2DM status post-bariatric surgery.

CHAPTER 1: Background

1. Introduction

The global rise in obesity is contributing to the increase in cardio-metabolic disease such as insulin resistance, hypertension, dyslipidaemia and cardiovascular disease. Notably obesity, T2DM and hypertension, also known as the “deadly trio’’ orchestrate together to exacerbate metabolic dysfunction and disease. The pathogenesis of disease through the “deadly trio’’ occurs often utilising many common intracellular pathways between them. Yet often screening tools for early detection of obese diabetic individuals is problematic. Noting that provision of a non-surgical and cost effective monotherapy for these individuals is currently absent.

The key element for increased metabolic risk lies in how the body stores and regulates fat (adipose tissue) function, which will be explored further. Adipose tissue is an active endocrine organ, in which in humans predominately stores white adipose tissue (WAT) and interacts with the local and systemic environment through regulation of cytokines (known as adipocytokines) and endocrine hormones. This chapter will explore the role of adipose tissue and how it leads to obesity-related cardio-metabolic disease, and how the renin angiotensin system, a key regulator of blood pressure, is important in lipid metabolism and adipogenesis. Further discussion will review the impact of factors that alter the regulation of adipose tissue-renin angiotensin system, when influenced by gut derived bacterial fragments, endotoxin/lipopolysaccharides (LPS), in the circulation; which are raised in cases of obesity and cardio-metabolic disease.

This chapter will also explore the effectiveness of the current medicines available to treat obesity-related hypertension, ranging from variety of antihypertensive, weight-loss, and anti-diabetic medications such as Liraglutide, in addition to more invasive treatments such as different types of bariatric surgery for the treatment of obesity related cardio-metabolic disease will be further discussed.

There will also be a focus on the importance and yet difficulties of early detection of hyperinsulinemia and pre-diabetes state in diabetic individuals, and how current screening strategies are inefficient, invasive and/or expensive. This review will set the scene to consider alternative strategies to provide detection and prediction of disease

using volatile organic compounds (VOCs) produced in urine. Finally, this chapter will detail the research aims for the studies conducted in this thesis.

1.1 Obesity and cardio-metabolic syndrome

The 21st century is currently faced with serious public health challenges; this is in part due to the rising population numbers and life expectancy coupled with increased obesity levels. The World Health Organisation (WHO) has estimated that globally more than 1.9 billion adults are overweight, of these over 600 million are obese (WHO,2015). The impact of obesity has also affected children to such an extent, that the prevalence of childhood overweight and obesity has risen substantially worldwide in less than one generation (Lobstein, T. *et al.*,2015, WHO,2015) making obesity a global paediatric health risk factor.

Obesity is a state of chronic inflammation, consisting of excessive body fat (adipose tissue) accumulation, which causes damaging effects to health while shortening life expectancy. Currently people categorised as either overweight (BMI≥25Kg/m^2) and/or obese (BMI≥30 Kg/m^2) are linked to higher rate of mortality worldwide than those considered as lean healthy individuals (WHO,2015). Increasing body mass index (BMI), which is a measure of weight (kilograms) relative to height (meters), seems to have an almost linear relationship with components of cardio-metabolic disorders such as elevated blood pressure (≥ 130/85 mmHg) and impaired glucose concentrations (fasting plasma glucose ≥ 5.6 mmol/L), dyslipidaemia (Triglyceride ≥ 1.7 mmol/L and high density lipoprotein (HDL) < 1.29 mmol/L (in female)), and increase in central obesity (waist ≥ 80 cm in European female)(Alberti, K. G. *et al.*,2006, Bays, H. E. *et al.*,2007, Chung, J. O. *et al.*,2012, Nordestgaard, B. G. *et al.*,2012). Longitudinal studies have shown that the presence of these cardio-metabolic risk factors during paediatric years is a precursor to chronic diseases such as T2DM and cardiovascular disease in adults (Morrison, J. A. *et al.*,2007, Sun, S. S. *et al.*,2008).

At present, the National Health Service is spending approximately £6 billion on obesity and cardio-metabolic disease and by 2030 the cost of obesity is expected to increase to £12 billion (McKinsey Consultancy,01/2015). Given the health and economical burdens

of obesity it is imperative to further understand the role of adipose tissue regarding development of cardio-metabolic disorders, such as, obesity related-hypertension for possible identification of novel molecular target for the treatment of this disease.

1.2 Adipose tissue as an endocrine organ

Adipose tissue is a complex endocrine organ, and its metabolic function is now recognised beyond the classical actions of fat storage (Hassan, M. *et al.*,2012) and thermoregulation. Adipose tissue is predominantly composed of fat cells (adipocytes) and stromal pre-adipocytes. However, there are other important components such as macrophages, connective and nerve tissues, endothelial, stromovascular, and immune cells, which act as the building blocks of this tissue (Frayn, K. N. *et al.*,2003).

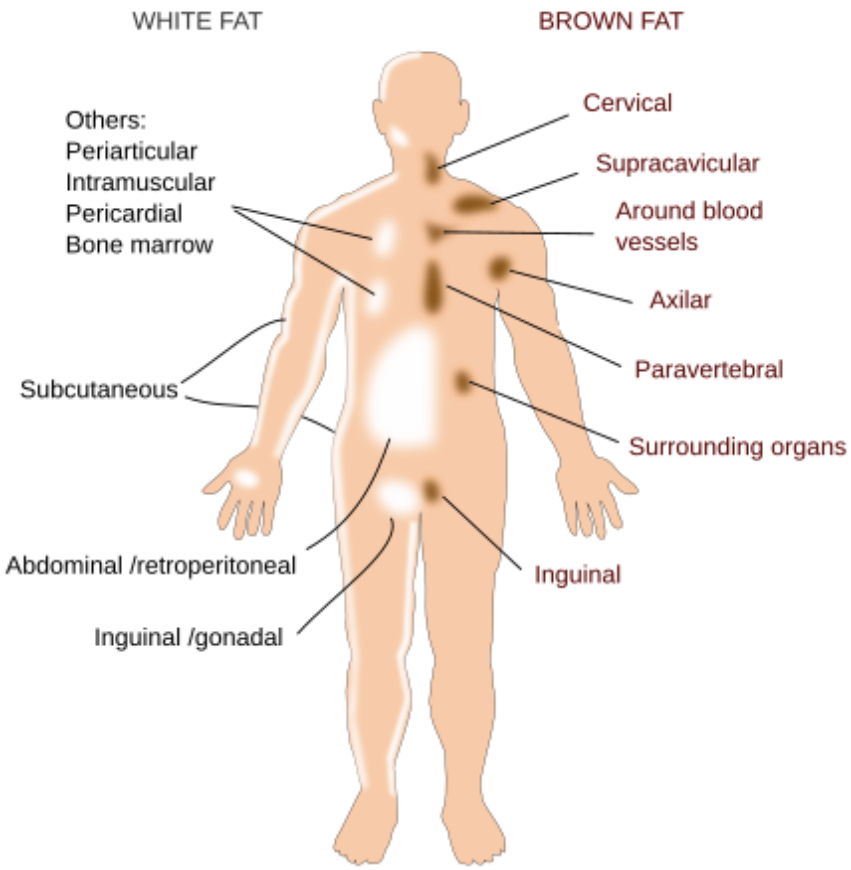
Adipocytes are known to secrete a range of bioactive peptide, known as adipokines, which can act locally (autocrine/paracrine) and systemically (endocrine). In addition to these signals, the various receptors expressed by adipose tissue provide the opportunity of responding to signals from different hormone systems as well as the central nervous system (Kershaw, E. E. *et al.*,2004). Therefore, this tissue contains the metabolic machinery to enable interaction with other organs. These interactive network connections permits adipose tissue to be involved in an array of biological processes such as differentiation of pre-adipocytes into adipocytes (adipogenesis), development of new blood vessels (angiogenesis), extracellular matrix dissolution and reformation, steroid metabolism, immune response, in addition to its original thought function of energy homeostasis (Cao, Yihai,2007, Zhong, J. *et al.*,2010).

In this section, metabolism of this multifunctional endocrine tissue and the hormones that can govern this metabolism will be discussed.

1.2.1 Adipose tissue structure and metabolism

Depending on morphology, function, development, and location, adipose tissue is divided into two categories. Firstly, located in the interscapular, axillar, perineal, paravertebral, cervical and distributed in arterials and surrounding organs is brown adipose tissue (BAT) (Pacheco, M.M *et al.*,2018). The abundance of this tissue with mitochondria provides the substantial energy production, which is utilised in the form of heat production often known as non-shivering thermogenesis (Saely, C. H. *et al.*,2012).

In contrast to BAT, secondly is WAT, which contains adipocytes with large single lipid droplets and much less mitochondria in comparison with BAT. WAT is located in several depots throughout the body such as abdominal, inguinal, perirenal, retrosplenial and gonadal(Pacheco, M.M *et al.*,2018). This tissue is principally considered as either subcutaneous adipose tissue (SAT), which lies below the basal skin membrane, or visceral adipose tissue situated inside the abdominal cavity and between the organs.



1.2. 1 White and brown adipose tissue distribution in human body.

White adipose tissue is dispersed all over the body, with the SAT and visceral intra-abdominal being the main depots for fat storage. Brown adipose tissue is abundant in infancy (interscapular) and although less present in adulthood, it is located in the cervical, supraclavicular and paravertebral. Figure from (Pacheco, M.M *et al.*,2018).

WAT has the ability to fluctuate energy supply in the form of uptake and storing energy during fed state and inversely break down and releasing energy in a fasted state. WAT plays an important role in systemic energy regulation, which it does through regulating anabolic and catabolic pathways to reserve energy in the body in the form of triglycerides (TG). WAT adipocytes take up energy in the form of lipids (nonesterified fatty acids-NEFA) and carbohydrates (glucose) from the blood. This energy is mainly stored as TG, which is subsequently broken down through lipolysis and released as NEFA upon conditions of high metabolic demand or negative energy balance.

Adipocytokines with endocrine functions such as adiponectin, leptin, tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and proteins of the renin angiotensin system (RAS), have the capability which include altering WAT metabolism (Saltiel, A.

R.,2001, Yamauchi, T. *et al.*,2001, Banerjee, R. R. *et al.*,2004). As an example, TNF-α represses genes involved in uptake and storage of NEFAs and glucose and supress genes for transcriptional factors involved in adipogenesis and lipogenesis (combination of fatty acid (FA) and TG synthesis) in adipose tissue (Ruan, H. *et al.*,2002, Kershaw, Erin E. *et al.*,2004). Proteins of RAS secreted from adipose tissue not only contribute to blood pressure regulation; they are also positively correlated with adiposity in humans (Engeli, S. *et al.*,2003, Goossens, G. H. *et al.*,2003). The effects of these proteins on adipose tissue inflammation and in other processes will be discussed in further detail later on in this chapter.

Mediating WAT adipocyte metabolism can also occur through hormonal and neuronal stimuli that initiate a cross talk between systemic metabolic organs, in which WAT predominantly supplies the body's energy demands. For example during the fasting state glycogen stores in the liver becomes depleted causing a liver-brain-adipose neural axis, that can shift energy source from carbohydrates to TG in adipocytes to maintain energy balance (Izumida, Y. *et al.*,2013). Inversely, in the fed state glycogen is accumulated in the liver, and lipogenesis is stimulated in adipocytes (Liu, M. *et al.*,2014). Hormonal stimuli such as those observed in the fibroblast growth factor, (FGF) family regulate glucose, and lipid metabolism via WAT during periods of nutritional stress.

1.2.2 Endocrine regulation of white adipose tissue metabolism

Recognition of WAT as an energy storage depot, and as an endocrine organ exposes adipocytes to various degrees of endocrine, metabolic, and neuronal regulations. While insulin (Kim, W. *et al.*,2008, Guo, S.,2014) is known as a classic endocrine regulator, due to exerting its dramatic effects on lipid metabolism in WAT, there are other vital key endocrine regulators that can also control WAT physiology and metabolism.

FGF-19 and FGF-21 are secreted signalling proteins that have the capability of targeting multiple tissues by predominantly acting as endocrine hormones. The ability of binding to both FGF receptors (FGFR) and transmembrane co-receptor betaKlotho (β-klotho)

specifically permits FGF-19 and FGF-21 to exert their regulatory effects on WAT metabolism. Although both hormones are stimulated under different conditions, they present significant functional overlap regarding insulin sensitivity and glucose tolerance improvements, weight reduction, lipid, and energy metabolism (Zhang, F. *et al.*,2015). Under conditions of metabolic stress such as T2DM, obesity prolonged fasting and cardio-metabolic disease; FGF-19 levels are reduced, while simultaneously FGF-21 is elevated in human subjects (Gallego-Escuredo, J. M. *et al.*,2015).

Human FGF-19 (mouse ortholog FGF-15) is a regulator of bile acid synthesis. As a response to feeding, bile acid activates farnesoid X receptor (FXR) expression in the terminal ileum of the small intestine, resulting in increased transcription of FGF-19 (Inagaki, T. *et al.*,2005). Upon secretion into the blood circulation FGF-19 binds to FGFR4/ β-klotho and activates various signalling cascades such as extracellular-regulated kinases (ERK) and c-Jun N-terminal kinase (JNK) within liver cells (Song, Kwang-Hoon *et al.*,2009). By doing so FGF-19 supresses bile acid, glucose, and triglyceride synthesis while stimulating glycogen synthesis and fatty acid oxidation(Degirolamo, C. *et al.*,2016).

Even though the major site of endogenous functions of FGF-19 is the liver, this endocrine hormone can also exert its biological effects in WAT. Several mouse studies have indicated that pharmacological effects of FGF-19 depends on WAT binding and activation of the FGFR1-β-klotho complex to improve blood glucose homeostasis (Wu, Xinle *et al.*,2009, Wu, Ai-Luen *et al.*,2011, Adams, Andrew C. *et al.*,2013). Chronic treatment of obesity with FGF-19 has resulted in increased energy expenditure and weight reduction followed by improvements in lipid profile (Nies, Vera J. M. *et al.*,2015), however, it remains unclear whether these effects are solely mediated via WAT, or further signalling through other metabolic tissues such as liver is required.

Secreted by the liver, FGF-21 applies its regulatory effects via signalling in various tissues. However, during episodes of metabolic stress the main focus of FGF-21 action appears to be on WAT. Similarly to FGF-19, circulating FGF-21 primarily binds with a high affinity to the FGFR1-β-klotho complex of mature adipocytes (Yang, C. *et*

al.,2012). Binding to this ternary complex initiates multiple FGF-21 regulatory actions on WAT metabolism:

1. FGF-21 stimulates insulin-independent glucose uptake within both mouse and human adipocytes (Kharitonenkov, Alexei *et al.*,2005, BonDurant, Lucas D. *et al.*,2017). It does this by phosphorylation of the ERK 1/2 pathway and activating Glucose Transport Protein 1 (GLUT1), resulting in increased glucose uptake. Studies have indicated that obesity impairs FGF21-mediated GLUT1 expression in WAT, therefore, creating a FGF-21 resistant state (Fisher, ffolliott M. *et al.*,2010, Ge, X. *et al.*,2011).

2. As a response to fasting or fibrate drugs, FGF-21 has an inhibitory effect on lipolysis in WAT (Potthoff, Matthew J. *et al.*,2012). In this state the pituitary gland secretes growth hormone (GH) in order to stimulate lipolysis in adipocytes to yield free fatty acids (FFAs). As a response to this action, hepatic FGF-21 production is induced by peroxisome proliferator-activated receptor alpha (PPARα) activation. Increase in hepatic FGF-21 levels therefore sends a negative feedback signal to terminate GH-induced lipolysis in adipocytes (Chen, W. *et al.*,2011). This feedback control maintains the balance of lipid distribution among adipose tissue and liver, in addition to preventing increased hepatic steatosis and lipotoxicity caused by sustained FFAs accumulation. Studies also indicate that the "fine-tuning" of lipolysis in this manner may provide a possible explanation for FGF-21 promoting its insulin-sensitivity properties in human (Arner, P. *et al.*,2008).

3. FGF-21 acts as the key mediator of PPARγ actions. Contrary to the fasting state, circulating FGF-21 is mainly expressed and secreted by adipocytes in response to the fed state or thiazolidinedione drugs (TZDs) (Dutchak, Paul A *et al.*,2012). Both animal and human studies have indicated that in the latter conditions FGF-21 production is governed by PPARγ activation (Wang, H. *et al.*,2008, Zhang, X. *et al.*,2008), resulting in elevated adipocyte FGF-21 expression during fed state and reduced in fasting state, a reverse pattern to that in hepatocytes (Dutchak, Paul A *et al.*,2012).

FGF-21 knockout (KO) mice study has indicated a reciprocal regulation amongst FGF-21 and PPARγ. Based on this relationship, studies have suggested that FGF-21 presents two independent physiological functions: firstly, secreted by the liver FGF-21 behaves

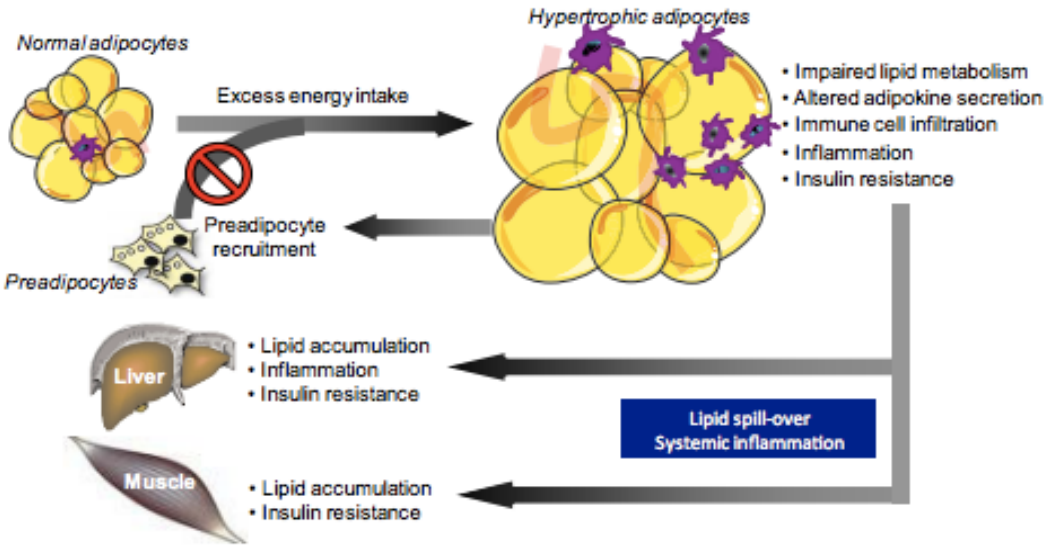
in an endocrine mode in order to synchronise the adaptive response to fasting/starvation state. Secondly, during the fed state, WAT secreted FGF-21 responses in an autocrine or paracrine mode to regulate adipocyte function (Dutchak, Paul A *et al.*,2012).

4. FGF-21 is also involved in the transformation of WAT adipocytes into a ‘’brown-like’’ state (Fisher, F. M. *et al.*,2012). FGF-21 KO mice exhibition a lack of ability to adjust to chronic cold exposure as browning of WAT reduces (Fisher, F. M. *et al.*,2012). FGF-21 achieves this effect by activation of mitochondrial brown fat uncoupling protein 1 (UCP1) via the sympathetic nerve(Lin, Xiaolong *et al.*,2017). By regulating UCP1 through the 5’-AMP_activated protein kinase (AMPK)/sirtuin-1 (SIRT1)/ peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) pathway (Bartelt, A. *et al.*,2014), this hormone can enhance mitochondrial oxidation and increase heat dissipation (Chau, M. D. *et al.*,2010).

These multifunctional governing capabilities of FGF-19 and FGF-21 on WAT metabolism and on other tissues, collectively arise during obesity and its related cardio-metabolic disease state. Understanding the physiological roles and molecular pathways essential to the actions of these two hormones will contribute to the development of better therapeutic strategies for these diseases.

1.3 White Adipose tissue dysfunction and inflammation

In obesity WAT dysfunction is considered the main cause of cardio-metabolic disease (Lewandowska, E. *et al.*,2016). Adipose tissue dysfunctional is characterised by adipose tissue lipid metabolism impairments, reduction in adipose tissue blood flow, and elevated production of pro-inflammatory cytokines by hypertrophic adipocytes (adipocytes increased in size) and adaptive and innate immune infiltration (Figure 1.3 A) (Goossens, Gijs H. *et al.*,2015). These impairments induce insulin resistance locally within the adipose tissue, as well as, on a larger scale in the kidney, liver, and skeletal muscle through lipotoxicity, leading to systemic low-grade inflammation.



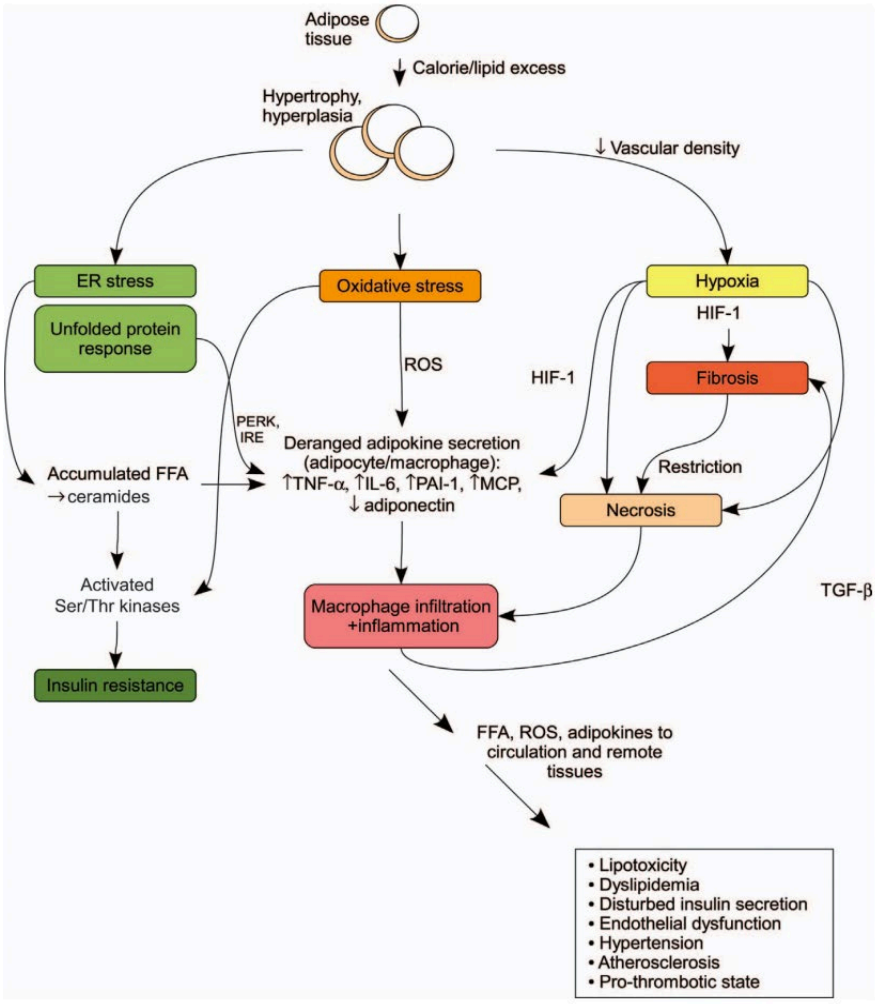
1.3.A. Adipose tissue dysfunction in obesity.

Excess energy intake leads to weight gain and increase in adipocyte size (adipocyte hypertrophy), which is accompanied by modifications in adipokine secretion and lipid metabolism disturbances resulting in a pro-inflammatory phenotype. The pro-inflammatory factors, which disrupt adipocyte differentiation, are further boosted with immune cell infiltration leading to inflammation and insulin resistance locally. These effects can be harmful to the entire body as well, since lipid spillover in the circulation and subsequent lipid accumulation in the non-obese tissues can result in insulin resistance and obesity related cardio-metabolic disease. Figure from (Goossens, Gijs H. *et al.*,2015).

During this low-grade inflammation hypertrophic adipocytes secrete chemotactic adipokines, which recruit macrophages from the blood into adipose tissue. It is considered that these macrophages contribute significantly more to the inflammatory response than residential macrophages in adipose tissue (Lumeng, C. N. *et al.*,2007). These macrophages and adipocytes secrete monocyte chemoattractant protein-1 (MCP-1), which has a key role in the development of adipose tissue inflammation (Kalupahana, N. S. *et al.*,2011). An increase in MCP-1 expression is associated with increase in macrophage adipose tissue infiltration (Surmi, Bonnie K. *et al.*,2008). Additionally, MCP-1 production is stimulated by increased amount of circulating FFA, insulin and pro-inflammatory TNF-α, IL-6, all of which have a positive correlation with adipocyte size. Simultaneously while this takes place, the production of adipocytokine adiponectin, a key contributor to insulin sensitivity and cell size, is reduced (Xu, H. *et al.*,2003).

Therefore, MCP-1 secreted by adipocytes stimulates a substantial macrophage infiltration of adipose tissue, causing an increase in typical macrophage cytokine release leading to an increase in adipose tissue inflammation (Xu, H. *et al.*,2003). Additionally, TNF-α acts as a key pro-inflammatory cytokine further adding to this condition by reducing adiponectin gene expression, leading to reduced adiponectin anti-inflammatory activity (Mlinar, B. *et al.*,2007).

Besides MCP-1 secretion from adipocytes, hypoxia, which develops due to failure of vasculature expansion alongside adipocyte hypertrophy (Pasarica, M. *et al.*,2009), and necrosis, can be the additional mechanisms for the macrophage recruitment (Figure 1.3 B). Research suggests that inducing fibrosis by hypoxia could be an early upstream event of macrophage infiltration (Halberg, N. *et al.*,2009) with excessive fibrosis leading to a decrease of capillary density causing hypoxia, which subsequently leads to inflammation (Pasarica, M. *et al.*,2009). As fibrosis usually occurs in a constant state of inflammation stimulus, it has been considered as a secondary event (Wynn, Thomas A.,2007).



1.3.B. Overview of excess caloric intake leading to cardio-metabolic disease.

Obesity leads to adipocyte increasing in size and numbers activating hypoxia, oxidative stress and Endoplasmic reticulum (ER) stress. These three pathways lead to macrophage infiltration and inflammation, which would ultimately lead to different obesity related cardio-metabolic disease. Figure from (Mlinar, B. et al.,2011).

MCP-1 as the key player in mediating recruitment of macrophages during WAT inflammation also has a critical role in accelerating the development and progression of obesity-related insulin resistance, oxidative stress, and endoplasmic reticulum stress, all of which play a vital role in pathogenesis of chronic cardio-metabolic disease.

1.3.1 Adipose tissue dysfunction, Endoplasmic reticulum stress and insulin resistance

Excess caloric intake through weight gain can lead to the development of endoplasmic reticulum (ER) stress due the sustained pressure placed on the cellular environment to produce more functional proteins to manage the excess calorie intake within adipose tissue. Ultimately continual ER stress can propagate inflammation and insulin resistance (Kawasaki, N. *et al.*,2012). The role of ER in adipocytes in addition to protein synthesis and folding is controlling FFA uptake, TG synthesis, lipid droplet formation, and regulation of *de novo* cholesterol and FFA synthesis (de Ferranti, S. *et al.*,2008). Adipocyte hypertrophy enhances protein synthesis, however, hypertrophy can cause insufficient chaperon protein production, which support correct protein folding. As such accumulation of misfolded proteins can occur in the cytosol, leading to cellular dysfunction. Ultimately, an uncontrolled unfolded protein response in adipocyte will lead to apoptosis to take place (Gregor, M. F. *et al.*,2007), followed by activation of inflammation via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Deng, J. *et al.*,2004, Hu, Ping *et al.*,2006). Prior to an apoptosis event, ER stress can also lead to inhibition of lipid formation leading to excess FFA release from adipocytes into the circulation. A substantial increased in circulating FFA, coupled with an increase in TNF-α leads to conversion of FFA into ceramides and other substrates (Hotamisligil, G. S.,2006, Watt, M. J. *et al.*,2006), causing activation of serine/threonine kinases followed by phosphorylation of insulin receptor and insulin receptor substrates; therefore causing interruptions in the insulin signalling (Mlinar, B. *et al.*,2007, Capurso, C. *et al.*,2012). Whilst studies highlight that a reduction in FFA plasma concentrations results in an improvement in insulin sensitivity in obese and T2DM subjects. Additionally, reduction of FFA by medication also improves insulin signalling in muscle within the insulin resistant subjects (Liang, H. *et al.*,2013).

1.3.2 Adipose tissue dysfunction and oxidative stress

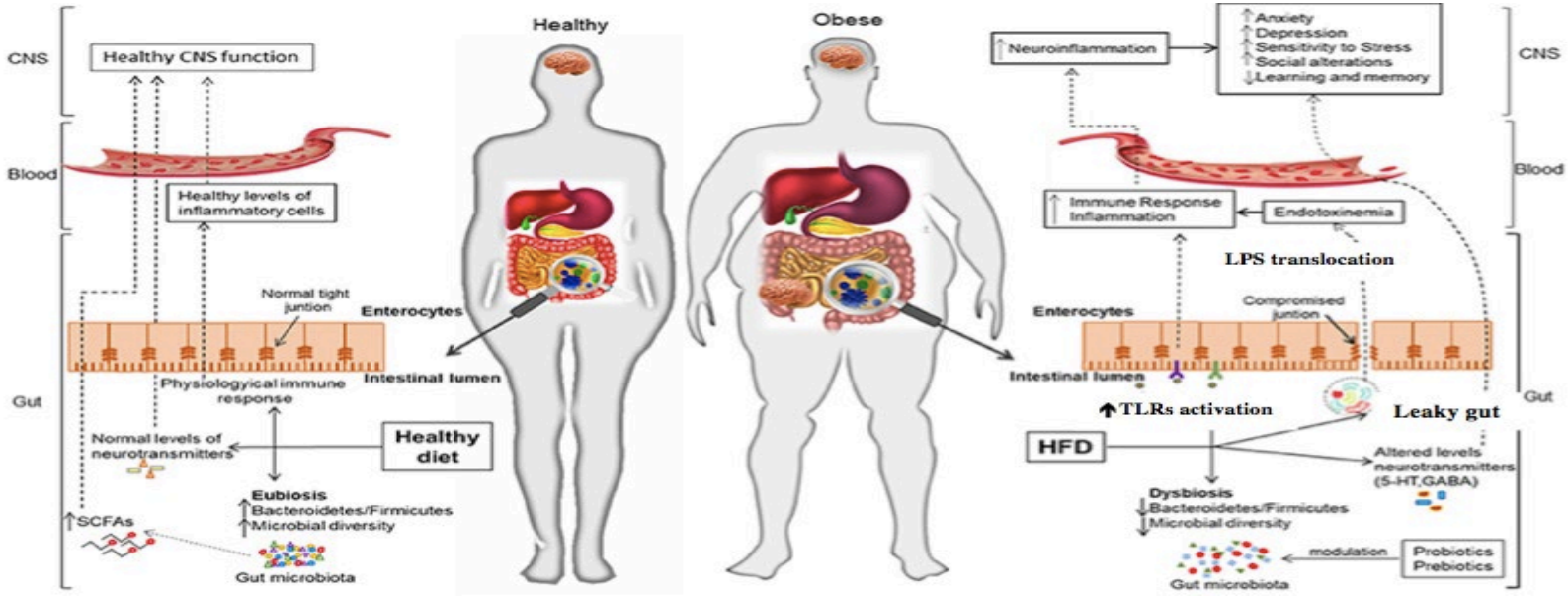
The disequilibrium between the production of reactive oxygen species (ROS), and the body's ability to detoxify these reactive intermediates cellular damage via antioxidant neutralisation is called oxidative stress (Dias, Vera et al.,2013). Studies have indicated that obesity can induce oxidative stress resulting in irregular adipokine production, which contributes to insulin resistance and the development of cardio-metabolic disease (Esposito, K. et al.,2006). Biomarkers of oxidative stress such as C-reactive protein (CRP) have been shown to be higher in obese individuals and have a direct correlation with body fat percentage, BMI, TG levels, and low-density lipoprotein (LDL oxidation) (Pihl, E. et al.,2006); additionally antioxidant defence markers are inversely correlated with central obesity (Chrysohoou, C. et al.,2007, Hartwich, J. et al.,2007). Furthermore, it has been reported that diets high in carbohydrates and fats consumed by obese subjects induces a significant increase in oxidative stress and inflammation (Patel, C. et al.,2007).

There are few mechanisms by which obesity produces oxidative stress; the first of these is the secretion of angiotensin II by adipose tissue, which stimulates the oxidase activity of nicotinamide adenine dinucleotide phosphate (NADPH), resulting in ROS production in adipocytes (Morrow, J. D.,2003). Another mechanism is via the mitochondrial and peroxisomal oxidation of fatty acids that create ROS in oxidation reactions (Duvnjak, M. *et al.*,2007). Whilst again, high fat diets and build-up of cellular damage during weight gain are alternative mechanisms generating ROS and altering oxygen metabolism (Khan, N. I. *et al.*,2006). In adipose tissue through these mechanisms an environment is created, in which there is a high ROS production and low antioxidant capacity that can lead to multiple abnormalities such as impaired insulin signalling, decrease mitochondrial biogenesis, inflammation and atherosclerosis (Fernández-Sánchez, Alba *et al.*,2011), thus similarly to ER stress, associating excess caloric intake to metabolic dysfunction.

1.3.3 white adipose tissue dysfunction and endotoxin

Endotoxin (also referred to as LPS) is a gram-negative bacterial fragment derived from the outer cell membrane (Raetz, Christian R. H. *et al.*,2002) that can enter the circulation by crossing the gut mucosa. LPS can act as a primary inflammatory mediator that can contribute to metabolic dysfunction in adipose tissue as well as other tissues. In a healthy individual, LPS combined with chylomicrons (lipoproteins that transport dietary lipids through the intestine barrier (Ghoshal, S. *et al.*,2009)) and dietary lipids, pass through the intestinal barrier then move into the systemic circulation, whereby they are eliminated by the liver cells.

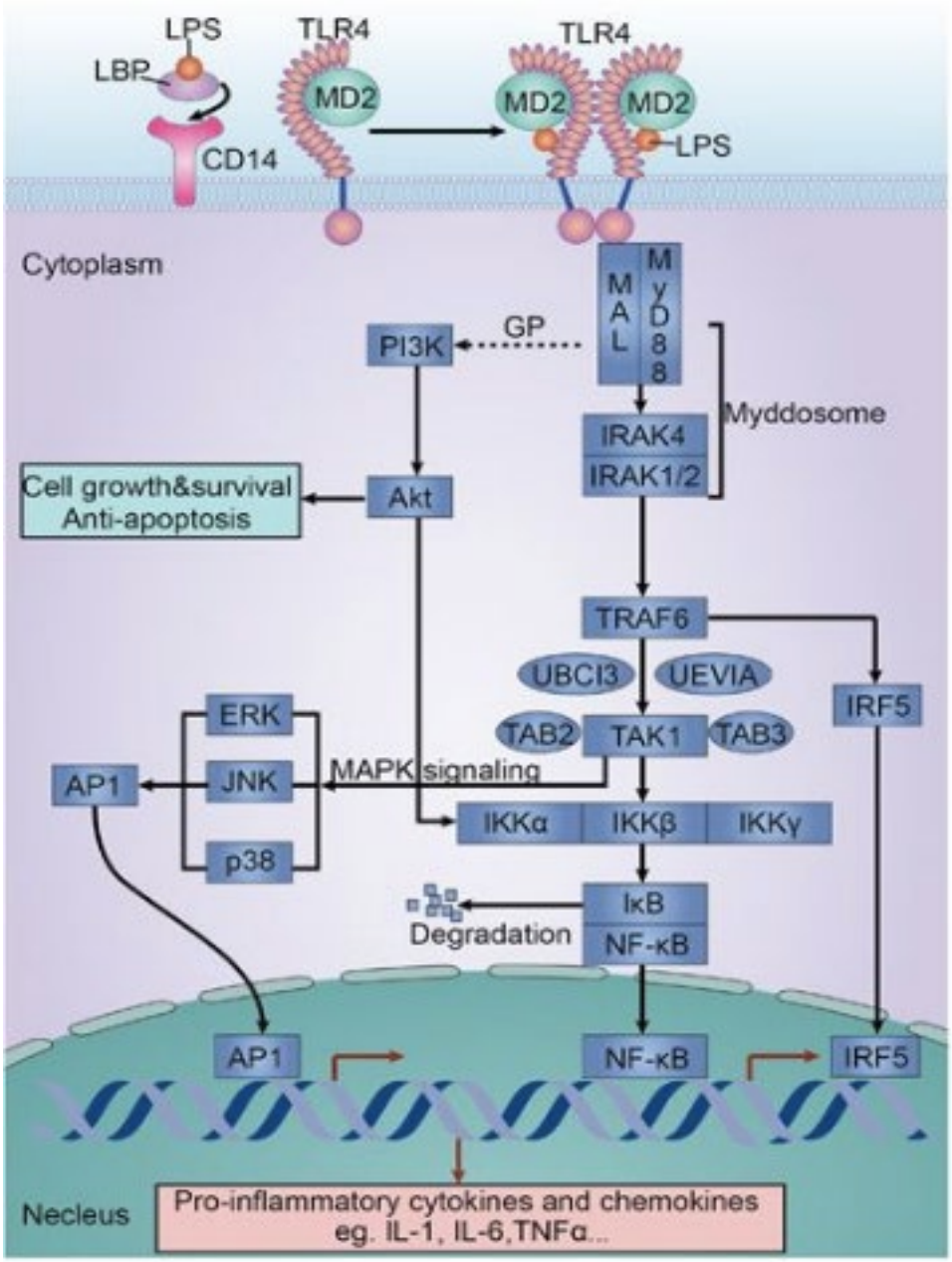
However, in obese individuals liver lipotoxicity reduces the capability of removing LPS, leading to elevated levels of circulating this endotoxin (Piya, M. K. *et al.*,2013). Therefore, increase in dietary saturated fatty acids in combination with elevated levels of circulating LPS facilitates chronic low-grade systemic inflammation. This inflammation leads to activation of the toll-like receptor 4 (TLR4) pathways, secretion of pro-inflammatory cytokines, and increase in intestinal permeability - also known as a leaky gut (Creely, S. J. *et al.*,2007, Baker, A. R. *et al.*,2009, Youssef-Elabd, E. M. *et al.*,2012). As leaky gut cannot protect the internal environment of the intestine, bacteria and toxins followed by other molecules can enter the bloodstream (Agustí, Ana *et al.*,2018). This change in gut composition, due to leaky gut, has been identified as the main contributor to obesity and its cardio-metabolic disease (Figure 1..3.3.1)



1.3.3. 1 The effect of endotoxin on obese versus healthy individuals.

In obesity high fat diets (HFD) and stress can lead to reduction in the intestinal microbiota diversity (Dysbiosis) resulting in change in the neurotransmitters released which can have a negative effect on psychological mood. Additionally dysbiosis can result in toll-like receptor (TLR) activation, leaky gut, and increase in LPS absorption. These effects contribute to endotoxaemia, which increase inflammation and immune response followed by neuroinflammation and changes in the central nervous system (CNS). A healthy diet leads to increase in gut microbiota diversity (Eubiosis) and therefore prevents leaky gut, supports immune homeostasis, and normal CNS function. Figure from (Agustí, Ana *et al.*,2018)

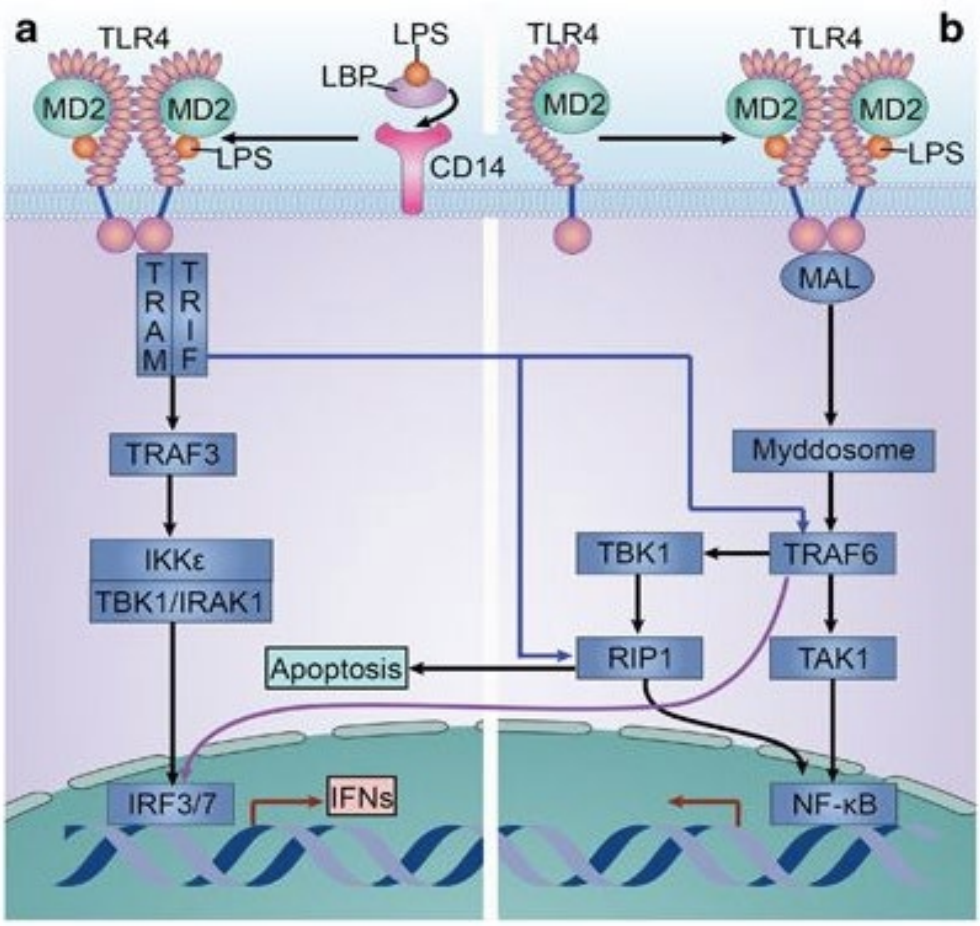
As mentioned above, TLR4 has a vital role in increasing leaky gut in obesity. The activation of TLR4 by LPS is via two signalling pathways: the myeloid differentiation factor 88 (MyD88)-dependent and MYD88 independent pathways. In the MyD88-dependent pathway, as a response to LPS, TL4 uses co receptor CD14, which only binds to LPS in the presence of LPS-binding protein (LBP). With the assistance of myeloid differentiation factor 2 (MD2), TLR4 binds to the LPS presented by the LPS-LBP-CD14 complex. At this stage, the MD2-LPS-TL4 bind to Toll/interleukin-1 receptor (TIR) domain containing adaptor protein (TIRP also known as MAL) and Myd88 in the cytoplasm. Subsequently, MyD88 binds to and activates the interleukin-1 receptor-associated kinase-4 (IRAK-4) complex, which is crucial for inflammation and host immune response (Flannery, S. *et al.*,2010, Yamamoto, T. *et al.*,2014). Then, this complex dissociates from MyD88 and interacts with tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6). TRAF6 forms a complex with transforming growth factor-β-activated kinase 1 (TAK1) and two of its binding proteins, in order to bind to two ubiquitin ligases. This binding leads to the activation and phosphorylation of inhibitory κB (IκB) kinase (IKK). The phosphorylated IκB activates the transcription of NF-κB. The released NF-κB transits into the nucleus, where it governs the expression of TNFα, IL-6 and other pro-inflammatory genes (Takeda, K. *et al.*,2004, O'Neill, L. A. *et al.*,2007).



1.3.3. 2 MyD88 dependent pathway.

LPS is presented to TLR4 via the LPS-LBP-CD14 complex. TLR4 binds to LPS with the assistance of MD2 and connects to the 'Myddosome' complex, which is combined of MyD88-MAL and IRAK4-IRAK1/2, in the Cytoplasm. IRAK4 dissociates from MyD88 and activates TRAF6. By forming a complex with TAK1, and two of its binding proteins TAB2 and TAB3, TRAF6 binds to ubiquitin ligases (UBC14 and UE1A). Activation of TAK1 results in the activation of IKKα/IKKβ/ IKKγ and, therefore IκB phosphorylation. Phosphorylated IκB is separated and directly transits into the nucleus where it mediates the expression of pro-inflammatory chemokines such as TNFα, IL-6. ERK, extracellular signal-regulated kinase; GP, glucan phosphate; IKK, inhibitory κB (IκB) kinase; JNK, c-Jun N terminal kinase; MAL, MyD88-adaptor like; UBC13, ubiquitin-conjugating enzyme 13; UEV1A, ubiquitin-conjugating enzyme variant 1A. Figure from (Yang, Y. *et al.*,2016).

The MyD88-independent pathway responds to LPS via TLR4 the same as in the dependent pathway, however, MD2-LPS-TL4 binds to the TIR domain-containing adaptor-inducing interferon /TRIF-related adaptor molecule (TRIF/TRAM) in the cytoplasm. By interacting with TRAF-family member-associated NF-κB activator-binding kinase 1 (TBK1) and IKK, TRIF phosphorylates and activates two members of the interferon-regulatory factor (IRF) (Tatematsu, M. *et al.*,2010). Activated IRFs then transit into the nucleus and produce antiviral molecules such as inflammatory cytokines and interferons (IFNs) (Takeda, K. *et al.*,2004, O'Neill, L. A.,2006). Additionally, TRIF can promote NF-κB activation through TLR4 signalling pathways. Similarly to the MyD88-dependent pathway, TRIF utilises TRAF6 to activate TAK1, which as a result leads to the NF-κB and mitogen-activated protein kinase (MAPK) pathway activation (Sato, S. *et al.*,2003). Also TRIF can activate MYD88-independent NF-κB through the adapter receptor-interacting protein 1 (RIP1) (Meylan, E. *et al.*,2004). RIP1 has the capability to bind to TRIF, causing both apoptosis and NF-κB activation (Gay, N. J. *et al.*,2014). Therefore, by activating IRFs TRIF interacts with TBK1 and IRAK1 in order to activate NF-κB through interactions with RIP1.



1.3.3. 3 the MyD88 independent pathway.

(a). LPS is presented to TLR4 via the LPS-LBP-CD14 complex. TRIF-TRAM binds to this complex in the cytoplasm. In order to phosphorylate IRF3, TRIF activates TBK and IKK. TRIF can additionally activate NF-κB through RIP1, which can also lead to cell apoptosis. **(b)** Cross talk between MyD88- dependent and independent pathways. IKK-ε, inhibitory κB (IκB) kinase ε; IRAK1, interleukin (IL)-1 receptor-associated kinase1; IRF, IFN-regulatory factor; TANK, TRAF family member-associated NF-κB activator; TRIF, TIR-domain-containing adaptor-inducing interferon-β; TLR4, Toll-like receptor 4. Figure from (Yang, Y. *et al.*,2016)

1.3.4 White adipose tissue dysfunction and hypertension

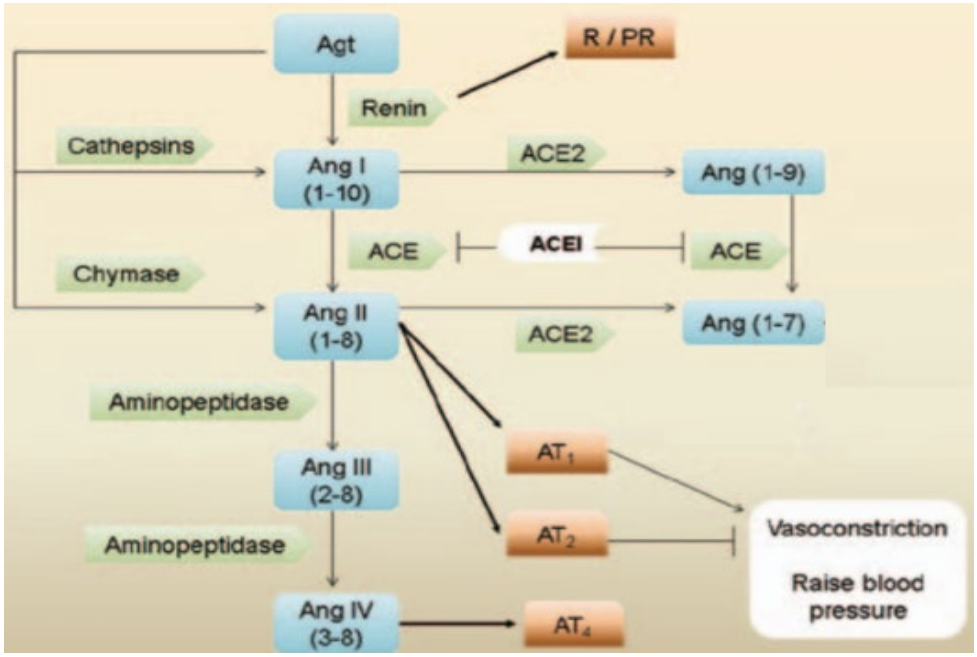
Healthy adipose tissue sustains a specifically balanced adipokine profile that governs vascular homeostasis through endocrine and paracrine signalling pathways (Linscheid, P. *et al.*,2005, Eringa, E. C. *et al.*,2007). Adipose tissue dysfunction and inflammation can alter adipocytokine expression and their physiological effects on the vessels. Obesity leads to increase in blood pressure in the majority of patients with essential hypertension (Garrison, R. J. *et al.*,1987). Hypertension occurs as the result of irregular flow/resistance relationship, whereby conductance vessels generate the systolic blood pressure, and diastolic blood pressure consists of peripheral resistance (de Simone, G. *et al.*,2001). Excess weight is positively correlated with cardiac output and blood volume, which can be due to the sympathetic nervous system (SNS) and RAS activation, in combination with renal sinus being compressed by ectopic adipose tissue (Foster, Meredith C. *et al.*,2011). SNS activation followed by the compression of the renal sinus both result in RAS activation, and pharmacological blocking of RAS or SNS has shown to significantly reduce obesity related hypertension (Davy, K. P. *et al.*,2004).

Several key players such as hyperinsulinemia, angiotensin II, FFAs and leptin have been suggested to have governing effects on obesity related hypertension. Hyperinsulinemia can cause elevated blood pressure by enhancing sodium retention (Creager, M. A. *et al.*,1985, Hall, J. E.,1993). Angiotensin II (AGT II) is a product of angiotensinogen, which is secreted by adipocytes (Cassis, Lisa A. *et al.*,2008). This potent vasoconstrictor promotes water and sodium absorption (Campbell, D. J.,1987). FFAs release and accumulation can lead to hypertension through adrenergic stimulation, increase in oxidative stress, endothelial dysfunction, or stimulation of vascular cell growth (Zhou, Junlan *et al.*,2012). Regulatory effect of leptin in blood pressure through activating SNS has been demonstrated using animal models (Carlyle, M. *et al.*,2002, Hall, J. E. *et al.*,2010).

As a result of these adipocytokine dysfunctions, changes occur in the structure and function of vessels through endothelial dysfunction, vascular smooth muscle cell proliferation and migration, and vascular inflammation. Collectively these changes cause increase in peripheral resistance, which is the fundamental contributor to elevated arterial pressure.

1.4 Adipose tissue renin-angiotensin system

RAS is the key mechanism in the control of blood pressure regulation, fluid, and electrolyte balance (Schmieder, R. E. *et al.*,2007). In lean individuals the key precursor peptide, angiotensinogen (AGT) is secreted by the liver and then cleaved by renin, which is mainly produced by the kidneys, to form angiotensin I (ANG I). ANG I is further cleaved by angiotensin converting enzyme (ACE) to produce the main effector ANG II within RAS. ANG II utilises ANG II type 1 (AT1) and type 2 (AT2) receptors to apply its physiological effects. Upon stimulation, AT1 induces vasoconstriction and secretion of aldosterone from the adrenal cortex leading to blood pressure increase along with water and sodium retention (Schmieder, R. E. *et al.*,2007). Inversely AT2 stimulation results in decrease in blood pressure (Figure 1.4).



1.4. 1A. Compound of the renin-angiotensin system.
Angiotensinogen (AGT) is cleaved into angiotensin II (ANG II) by renin and angiotensin converting enzyme (ACE). ANG II additionally can be produce by cathepsins, through a non-ACE route. ANG II can further be metabolized to small biological peptides such as ANG (1-7), ANG III (2-8) and ANG IV (3-8). ANG II activates ANG II type I (AT1) to induce vasoconstriction leading to elevated blood pressure. Inversely by stimulating AT2, ANGII reduces blood pressure. Figure from (Kalupahana, Nishan S. *et al.*,2012)

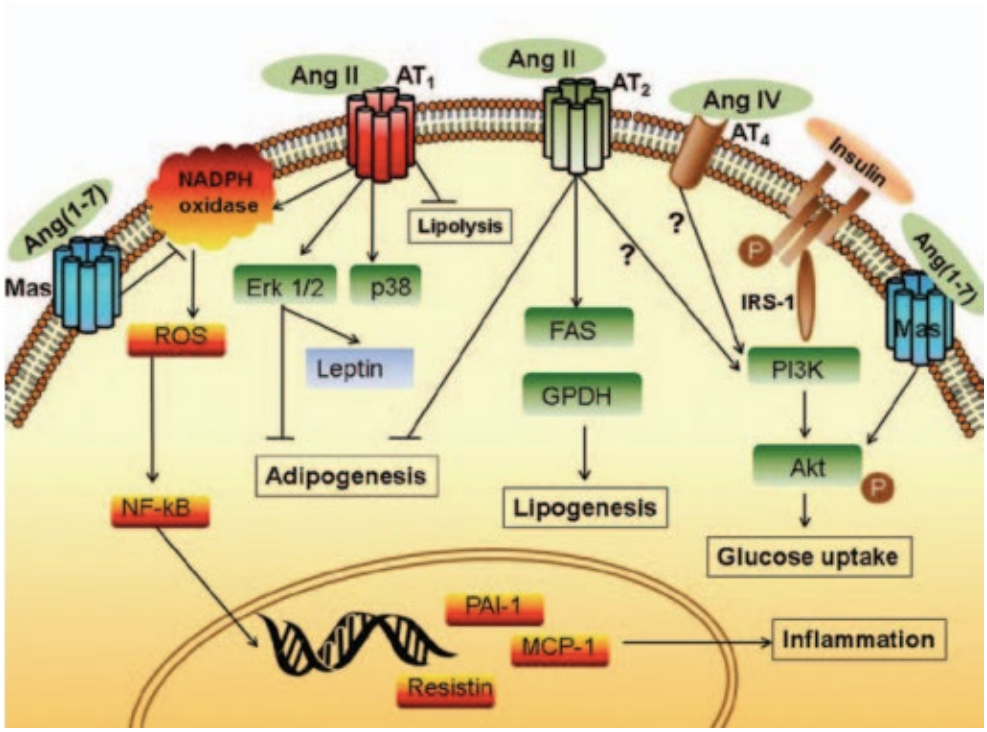
Besides liver, all or some of RAS components exist in various other tissues (Massiera, F. *et al.*,2001), therefore giving these tissues the capacity of altering blood pressure.

However, amongst these tissues WAT not only contains all the RAS components, it is also known to be the second main extra hepatic synthesis site of AGT (Massiera, F. *et al.*,2001), especially in obese individuals. Additionally, AGT secreted in adipose tissue can be cleaved by specific proteases to produce ANG II by avoiding the renin-ACE axis (Karlsson, C. *et al.*,1998), therefore, making the production of AGT the main regulatory step of adipose tissue-RAS. This step is controlled by TNF-α, insulin and other hormones (Jones, B. H. *et al.*,1997, Serazin-Leroy, V. *et al.*,2000), as well as nutrients such as fatty acids and glucose (Safonova, I. *et al.*,1997).

This structure and function of adipose tissue-RAS permits for local autocrine/paracrine regulation, whereby, adipose tissue-RAS adipocytes can influence local macrophage activation and recruitment to promote inflammation, adipogenesis, and lipid metabolism amongst other effects. In this next section, these regulatory effects of adipose tissue-RAS on inflammation, lipid metabolism and adipogenesis, in addition to the effects of endotoxins on adipose tissue-RAS will be addressed.

1.4.1 Adipose tissue-RAS and adipose tissue inflammation

High caloric intake up-regulates adipose tissue-RAS (Rahmouni, K. *et al.*,2004), giving ANG II a role in the mediation of adipose tissue inflammation. Human *in vitro* studies have shown that ANG II increases the secretion of MCP-1 by pre-adipocytes (Asamizu, S. *et al.*,2009), as well as IL-6 and IL-8 by adipocytes (Skurk, T. *et al.*,2004) through the NF-κB dependent pathway. Additionally *in vitro* murine studies indicate that ANG II increases the secretion of MCP-1 and adipocytes resistance through an NADPH oxidase and NF-κB dependent pathway (Kalupahana, N. S. *et al.*,2012). Inversely, RAS inhibition decreases macrophage infiltration and expression of MCP-1 in high fat-induced obese mice (Lee, M. H. *et al.*,2008). Taken together these findings illustrate that ANG II promotes adipose tissue inflammation in a NF-κB dependent pathway.



1.4.1. 1 Adipose tissue RAS regulation of inflammation.

ANGII activates NADPH oxidase resulting in the stimulation of ROS, leading to the activation of NF-κB pathway. This activation causes the transcription of MCP-1 and other pro-inflammatory cytokines in the nucleus, promoting an inflammatory state in the adipose tissue. Figure from (Kalupahana, Nishan S. *et al.*,2012)

1.4.2 Adipose tissue-RAS and lipid metabolism

Within RAS, energy sensing (Frederich, R. C., Jr. *et al.*,1992) and modulation of adipogenesis, lipogenesis, and lipolysis are dependent on ANG II. In an acute state of energy influx, adipose tissue stimulated AGT secretes high levels of local ANG II, which induces local vasoconstriction, and thus results in reduced rates of lipolysis (Goossens, G. H. *et al.*,2004). Inversely, in a fasting state, vasodilation occurs due to lower levels of local ANG II, which results in increase in lipolysis rates. ANG II exerts these lipolysis effects via AT1 (Yvan-Charvet, L. *et al.*,2006) and increases lipogenesis via AT2 (Jones, B. H. *et al.*,1997). In line with these effects, ANG II additionally plays a role in the uptake of insulin-stimulated glucose by adipocytes (Juan, C. C. *et al.*,2005). From these findings the paracrine effect of ANG II can be described as inhibiting lipolysis, while promoting lipogenesis, and finally, increasing lipid accumulation and inflammation in adipose tissue (Yvan-Charvet, L. *et al.*,2011).

Reports on the effects of chronic energy influx on adipose tissue-RAS have been inconsistent due to multiple factors such as regulation of AGT expression by nutritional and hormonal signals, genetic factors (Prat-Larquemin, L. *et al.*,2004), the lack of AGT overexpression in metabolically healthy obese individuals (Karelis, A. D. *et al.*,2004), and gene-environment interactions (Kalupahana, N. S. *et al.*,2011).

1.4.3 adipose tissue-RAS and adipogenesis

In a state of excess caloric intake, adipogenesis leads to adipocyte hyperplasia (increased adipocyte number), and adipogenesis inhibition in combination with increased lipogenesis cause adipose tissue dysfunction.

The effects of RAS and its components on adipogenesis have been extensively researched. Various rodent models of obesity have been used to indicate that AT1 blockers (ARB) and ACE inhibitors (ACEI), which block RAS, reduce adipocyte size (Furuhashi, M. *et al.*,2004, Zorad, S. *et al.*,2006, Munoz, M. C. *et al.*,2009). Additionally, in diabetic rats ARB has shown to increase the number of small-differentiated adipocytes (Lee, M. H. *et al.*,2008). Together these *in vivo* studies show that blocking RAS inhibits lipogenesis and promotes adipogenesis. It has been reported that transgenic mice over expressing adipose tissue AGT become relatively obese with adipocytes increasing in size (Massiera, F. *et al.*,2001) and reducing in number in comparison to wild-type mice (Yvan-Charvet, L. *et al.*,2009). Additionally, in AGT-deficient mice overexpression of adipose tissue AGT leads to reduction in adipocyte number (Massiera, F. *et al.*,2001). Taking these findings into consideration, it can be suggested that a rise in local levels of ANG II inhibits adipogenesis *in vivo*. Other research has demonstrated that the inhibitory activity of ANG II is mediated by AT2 (Yvan-Charvet, L. *et al.*,2009). Therefore, from these studies and various others it can be suggested that ANG II has inhibitory effects on adipogenesis, while promoting lipogenesis, therefore facilitating adipose tissue dysfunction.

1.4.4 Effects of endotoxin on adipose tissue-RAS

Prior studies have also shown that offspring from prenatal rats exposed to endotoxin have elevated blood pressure, coupled with an impact on weight gain, and abnormal adipocyte development (Gao, Meng *et al.*,2014). In the juvenile phase of these offspring, prenatal stimulation was noted to activate the local adipose tissue-RAS leading to adipocyte differentiation, which resulted in smaller adipocytes while stimulating hyperplasia. Inversely in the adult phase of these offspring, excessive prenatal stimulation inhibited cell differentiation causing hypertrophy. Additionally, RAS components were also activated during endotoxin stimulation. This study also highlighted that NF-κB inhibitor could reverse the influence of endotoxin stimulation during pregnancy (Gao, Meng *et al.*,2014). Further *in vivo* studies on the role of TLR4 signalling pathway in activating RAS in adipocyte cells has indicated that stimulation with endotoxin results in increase in mRNA and protein expressions of TLR4, AGT, and ATR1, therefore activation of TLR4 signalling pathway promotes the activation of local RAS in adipose cells (Luo, J. *et al.*,2014).

1.5 Obesity-related cardio-metabolic management

Most management guidelines identify weight loss as one of the most effective strategies in management of obesity related cardio-metabolic disease. The WHO has recommended that at least 5 kg reduction in initial body weight is linked to improvement in obesity risk factors (WHO,2013). For individuals with BMI ≥25Kg/m^2 diet (*e.g*. balance-diets, very low-calorie diets, low-fat/carbohydrate diets and high protein diets) (Hill, J. O.,2006), physical activity, and behavioural changes such as slower rate of eating, nutrition education, and meal planning, has appeared to be an effective way of weight loss followed by improvements in hypertension, insulin sensitivity and dyslipidaemia (Sharma, A. M.,1992). However, this regime alone is not a realistic solution for individuals with BMI ≥ 27 Kg/m^2 with comorbidity or BMI ≥ 30 Kg/m^2 (Stamler, R. *et al.*,1987), therefore, in parallel to diet and exercise, achievable weight loss for these individuals that can be maintained long term by pharmacological approaches. Bariatric surgery is mainly advised for individuals with BMI ≥ 35 Kg/m^2 with comorbidity or BMI ≥ 40 Kg/m^2 alongside behavioural changes to reduced food intake, and increase in physical activity once this is possible.

1.5.1 Obesity-related hypertension management through pharmacology

Hypertensive individuals with BMI ≥ 27 Kg/m^2 with comorbidity or BMI ≥ 30 Kg/m^2, require one or more antihypertensive medication in addition to weight loss and other lifestyle adjustments. Diuretics have shown to reduce blood pressure by reducing intervascular volume and cardiac output (Duarte, Julio D. *et al.*,2010) in obese-hypertensive individuals however, the adverse effect of this medication, which is insulin resistance and dyslipidaemia (Duarte, Julio D. *et al.*,2010) appears to be dose dependent. Inhibitors of the RAS are usually the first-line anti-hypertensive for most patients. ACE inhibitors and possibly angiotensin II receptor blockers are safe and effective treatments for reducing blood pressure, increasing insulin sensitivity and reducing diabetes risk (Lindholm, L. H. *et al.*,2002, Scheen, A. J.,2004). However, ACE inhibitors are considered to be the more suitable as they exert a variety of hypotensive

effects (Thomopoulos, C. *et al.*,2015). They inhibit RAS and the sympathetic nervous system, which leads to decrease in arterial pressure (Tsuda, K.,2012), in addition, they can positively influence conditions such as left-ventricular hypertrophy, congestive heart failure (Messerli, F. H. *et al.*,1983), renal hyperfiltration, and microalbumuria (Mokdad, A. H. *et al.*,2003).

It has been reported that β –blockers effectively reduce blood pressure in obese rather than lean hypertensive individuals, this may be that this agent reduces cardiac output and plasma renin activity, both of which are increased in obese individuals (Williams, M. *et al.*,1997) (Williams, M. *et al.*,1997). However, this medication can have adverse side effects such as weight gain, disruption in glucose metabolism and increase in triglycerides, and decrease in high-density lipoprotein cholesterol concentrations (LaGuardia, Heather A. *et al.*,2012). Therefore use of β-blockers as first line agents for obese- hypertensive individuals with T2DM is still questionable, especially since there still has not been overwhelmingly evidence regarding prevention of stroke recurrence by this medication (De Lima, L. G. *et al.*,2013). Calcium channel blockers are a preferred substituted in this category of individuals (Schumann, Sarah-Anne *et al.*,2008). Calcium channel blockers do not have metabolic adverse effects (de Courten, M. *et al.*,1993), and their use is not associated with diabetes development (Blackburn, David F. *et al.*,2006), and α-blockers have shown reduced blood pressure, improved insulin sensitivity and lipid metabolism (Blackburn, David F. *et al.*,2006), however, there has not been strong evidence for the use of both these medications as first line agents in obesity-related hypertension.

Although RAS inhibitors are the first choice of treatment for most obese-hypertensive cases, antihypertensive monotherapy is rarely sufficient to control blood pressure (Landsberg, L. *et al.*,2013, Mancia, G. *et al.*,2013). Over half of these cases are treated with two or more antihypertensive medications.

One option that may have monotherapeutic potential for obesity-related hypertension is a medication that indirectly lowers blood pressure through significant weight loss. Clinical data on anti-obesity medications, Orlistat, has shown 5-10% reduction in weight loss, which is maintained for up to two years (Jain, Suyog S. *et al.*,2011).

Orlistat is a gastrointestinal lipase inhibitor that restricts the absorption of dietary fat by about 35% (Guerciolini, R.,1997). Research has indicated that this medication in addition to weight loss, mirrors the results expected from many antihypertensive medications used as monotherapy treatment (Filippatos, Theodosios D. *et al.*,2010). Orlistat has been shown to substantially improve cardiovascular risk factors in long-term studies (Davidson, M. H. *et al.*,1999, Rossner, S. *et al.*,2000); in addition, it significantly enhances metabolic parameters both in non-diabetic (Sjostrom, L. *et al.*,1998) and T2DM patients (Hollander, P. A. *et al.*,1998). Due to lack of cardiovascular adverse effects, this medication can be well suited for the obese-hypertensive patients, however, the gastrointestinal side effects may result in discontinuation of Orlistat in some patients.

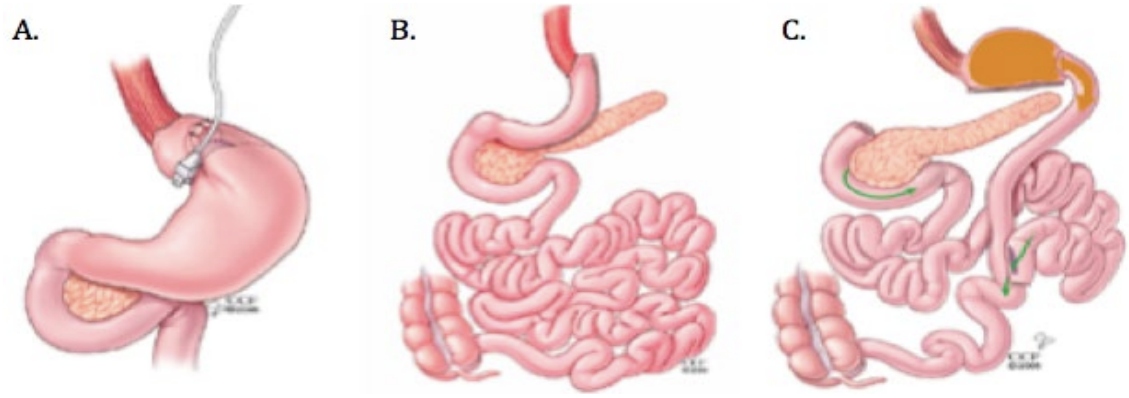
Liraglutide (Victoza), a long acting glucagon-like peptide-1 receptor agonist (GLP-1) that is used for treatment of T2DM, which has clinically been shown to sustain glycosylated haemoglobin (HbA1c) reduction in patients and lower the rates of hypoglycaemia events. Importantly it may also lead to moderate weight loss at a specific dose (1.8 mg) (Shyangdan, D. S. *et al.*,2011) and has beneficial effects on the cardiovascular system (Moretto, T. J. *et al.*,2008, Koska, J. *et al.*,2010). Recent research on rats implanted with dihydrotestosterone (DHT) to mimic a polycystic ovary syndrome model, noted that treatment with Liraglutide, resulted in significant improvement of the mean arterial pressure in these hypertensive rats, in addition to significant decrease in body weight and improved metabolic outcomes (Hoang, V. *et al.*,2015). Human studies have shown that at the dose nearly twice that used in T2DM patients, led to a weight loss of 7.8 Kg, maintained over a period of 2-years. This weight loss was associated with reduction of 12.5 mm Hg in systolic blood pressure and increase heart rate of 3 beats per minute (bpm) (Astrup, A. *et al.*,2012). Although Liragutide (Victoza) appears to be multifunctional and may be suitable as a first line treatment for obese-hypertensive individuals with T2DM, it is not recommended for chronic weight management (Victozapro,2015).

FDA approved Liraglutide injection with the dose of 3 mg (Saxenda) (Nordisk, novo,27/04/2017) is also on the market as a chronic weight management in individuals with BMI $\geq$ 27 Kg/m^2 with at least one weight-related comorbidity or BMI $\geq$ 30 Kg/m^2.

The results of the 1-year study have shown an average weight loss of 5% with this medication and significant improvements in blood pressure and other cardio-metabolic risk factors (Saxendapro,2017).

1.5.2 Obesity-related cardio-metabolic disease management through bariatric Surgery

Bariatric surgery is a suitable weight loss treatment for individuals with BMI ≥ 35 Kg/m^2 with comorbidity or BMI ≥ 40 Kg/m^2, for whom all conservative measures, including pharmacotherapy has failed. Long-term observational studies with 10 year (Hess, D. S. *et al.*,2005, O'Brien, P. E. *et al.*,2006) and 20 year (Sjöström, L.,2013) follow-up, have reported that morbidly obese individuals that undergo bariatric surgery experience up to 30% reduction in weight loss, in addition to improvements in their cardio-metabolic parameters (Henry, Robert R. *et al.*,2013). However, success of the surgery is dependent on duration of obesity and its comorbidities, gender, age, genetics, choosing the suitable surgery and postoperative behavioural and lifestyle changes (Pories, W. J. *et al.*,1992, Schauer, Philip R. *et al.*,2003).



1.5.2. 1 Different types of bariatric surgery.
Laparoscopic adjustable gastric banding (LAGB), a procedure where the size of the stomach is reduced by placing a gastric band around the stomach. **B.** Sleeve gastrectomy (SG), the size of the stomach is reduced from its original size and the small stomach pouch is reconnected to the intestine. **C.** Biliopancreatic diversion (BPD) involves the reducing of the stomach size followed by the attachment of the small stomach to the lower section of the intestine, while bypassing the upper section of the small intestine. Figure from (Eldar, S. *et al.*,2011)

Bariatric surgical procedures were traditionally divided into restrictive and malabsorptive, or a combination of both procedures. Restrictive procedures such as

sleeve gastrectomy (SG) and laparoscopic adjustable gastric banding (LAGB) involve limiting the size of the stomach and, therefore reducing the caloric intake. SG is currently the most common restrictive surgical procedure, where more than 80% of the stomach is resected (WHO,2004). The remnant small stomach patch that has the initial filling volume of less than 100 ml, is then tubularized (WHO,2004). Comparatively, less invasive than other surgeries, LAGB procedure uses an adjustable gastric band for dividing the stomach into a small pouch and a larger residual compartment (Kang, Jenny H. *et al.*,2017). Purely Malabsorptive procedures that involved diverting the biliopancreatic secretions and thus reducing nutritional absorption in the intestine, was abandoned due to the high morbidity and mortality rates (J. D. Halverson *et al.*,1978, Buchwald, H. *et al.*,2002). Combination of restrictive and malabsorptive surgery such as the highly invasive and less performed biliopancreatic diversion (BPD), firstly involve reducing the size of the stomach similar to that in the SG surgery. This is then followed by leaving the valve that releases the food to the small intestine along with the first part of the small intestine (duodenum). The ileum is then connected to the small stomach pouch, completely bypassing the upper segment of the small intestine (Anderson, Blaire *et al.*,2013). The malabsorption in this surgery occurs as most of the small intestine contains either digestive fluids without food or vice versa, with the absorption limited to the terminal ileum where these two are briefly combined in a common channel prior to entering the colon (Overduin, Joost *et al.*,2004).

Besides gastric restriction, which can lead to early satiety and reduced caloric intake, there are other mechanisms underlying the effects of these bariatric surgeries that contribute to the weight loss and improvements in cardio-metabolic diseases. The T2DM regulation post PBD surgery for instance has been suggested to be due to the hindgut hypothesis, which suggests that expedited delivery of nutrients to the distal colon produces a physiological signal that improves glucose homeostasis (Thomas, Susan *et al.*,2010). The potential mediators of this effect are the secretion of specific incretin (GLP-1 and gastric inhibitory polypeptide (GIP)) and non-incretin (peptide tyrosine tyrosin (PYY)) hormones that may reduce food intake and increase glucose tolerance (Park, Chan W. *et al.*,2011).

Impairment in the secretion of ghrelin can be another contributing factor to the improvement of cardio-metabolic disease post bariatric surgeries. Ghrelin, which is a hormone that stimulates appetite, is mainly produced from the fundus of the stomach and to a lesser extent in the duodenum (Overduin, Joost *et al.*,2004). The concentration of this hormone increases before meals and it is suppressed postprandially in proportion to the amount of calories consumed, thus highlighting a possible role in meal initiation and long-term body weight regulation (Cummings, D. E. *et al.*,2001, Callahan, H. S. *et al.*,2004). Some studies have indicated that the exclusion of fundus of the stomach that take place in SG and BPD surgeries leads to a decrease in fasting ghrelin levels post-operatively (Adami, G. F. *et al.*,2003, Langer, F. B. *et al.*,2005, Wang, Y. *et al.*,2009). Post-prandial ghrelin suppression has been speculated to play a major role in appetite reduction, excess weight loss and most importantly T2DM remission as a result of both SG and BPD surgeries. In regards to change in ghrelin levels post LAGB, some studies have reported an increase in fasting ghrelin levels(Fruhbeck, G. *et al.*,2004, Stoeckli, R. *et al.*,2004), while others have demonstrated a sharp postprandial decrease in this hormone(Leonetti, F. *et al.*,2003, Korner, J. *et al.*,2009). Additionally many studies were not able to show any changes in ghrelin levels post LAGB (le Roux, C. W. *et al.*,2006, Shak, J. R. *et al.*,2008).

In addition to ghrelin, other hormones such as FGF-19 and FGF-21 contribute to the effectiveness of bariatric surgeries in weight loss and improvements in cardio-metabolic disease (Haluzikova, D. *et al.*,2013). Studies have shown that both these hormones play a role in energy expenditure, however FGF-19 is mainly involved in glucose regulation and lipid metabolism, while FGF-21 promotes insulin sensitivity and fatty acid oxidation, post bariatric surgery (Dimitriadis, G. K. *et al.*,2017). However what remains unclear is whether the behaviour of these two hormones is surgery specific, which will be further discussed in chapter 4 of this thesis.

At different extent, these three surgeries are successful at achieving weight loss, T2DM remission, blood pressure and lipid profile improvements eventually. BPD has been reported to result in a greater percentage of excess weight loss in comparison to the other two restricted procedures. However, this is at the expense of a complicated and more time consuming surgical procedure, placing the patient at a higher risk of

complication during the operation, and a higher malnutrition/metabolic related complications post-operation (Anderson, Blaire *et al.*,2013).

1.6 Screening of obese-diabetics

The most dangerous characteristic of T2DM is that it can act as a silent killer. Many individuals who meet the current criteria for T2DM remain asymptomatic and unaware of developing this disease for many years (Engelgau, M *et al.*,2003), resulting in the late diagnosis of this disease. Late detection and diagnosis of T2DM is problematic, firstly it is highly likely to be accompanied by one or more cardio-metabolic diseases, and secondly, 80% of the cost of treating T2DM by the NHS is spent on treatments for associated complication and cardio-metabolic diseases (NHS,2016). It is therefore possible that early detection of T2DM, through screening for hyperinsulinemia and pre-diabetes state (impaired glucose tolerance and impaired fasting glucose), can significantly reduce medical expenditure, but most importantly help with early management of this disease and potentially prevent or reduce the serious complications that can effect the quality of life.

The problem is that the current tests for diagnosis of pre-diabetes and T2DM are far from perfect. For instance routinely used HbA1c test cut-off values (pre-diabetes 5.7%-6.4% (39-47 mmol/mol), Diabetes ≥ 6.5% (≥ 48 mmol/mol), and for asymptomatic adults ≥ 5.7% (39 mmol/mol) (ADA,2017) for determining disease status have proven to be controversial. Some data suggest that these cut-off values vary based on ethnicity, age, gender, and population prevalence of diabetes (Bennett, C. M. *et al.*,2007, Herman, W. H. *et al.*,2012, Waugh, N. R. *et al.*,2013). This test also appears to be very unreliable in certain cases such as hemoglobinopathies and thalassemia syndrome; as these diseases impact red blood cell survival (Herman, W. H. *et al.*,2012). Although HbA1c test is expensive and invasive due to the use of needles, it does not require individuals to fast before being tested.

However, due to the inconsistency in results, HbA1c test is usually confirmed by a second HbA1c test, fasting plasma glucose (FPG) test, or an oral glucose tolerance test (OGTT). Besides repeating tests being costly and resource intensive, the main issue is that the agreement between these three tests is poor. Some studies have observed that HbA1c would diagnose significantly fewer individuals with diabetes than OGTT and vice versa (Mostafa, S. A. *et al.*,2010, Cosson, E. *et al.*,2011, Farhan, S. *et al.*,2012), the same trend being true between FPG and HbA1c tests. The agreement between FPG and OGTT also remains poor [160, (Bernal-Lopez, M. R. *et al.*,2011).

Previously known as the ‘’gold standard” of T2DM testing, OGTT is more sensitive, however, it is time-consuming to undertake, inconvenient, and difficult to reproduce, with a low uptake in contrast to HbA1c test (Waugh, N. R. *et al.*,2013). Similarly, FPG is a preferred diagnostic tool used for many years, which has also proven to have its own challenges, such as, requiring patient compliance with fasting, invasiveness, and having a high intraindividual variability (Selvin, E. *et al.*,2007, Malkani, S. *et al.*,2012). Additionally, FPG has been noted as a weak predictive biomarker of cardio-metabolic disease than HbA1c (Bonora, Enzo *et al.*,2011). Therefore, there is a need for a convenient, non-invasive and cost effective test that would act as an early detection of T2DM and classify patients, allowing only those at need to undergo further investigations.

1.6.1 Volatile Organic Compounds as a potential screening tool

Significant development in analytical procedures for identifying VOCs in urine has created the opportunity for patients’ urine to be used as a screening and detection tool in different types of cancer (Arasaradnam, R. P. *et al.*,2014, Khalid, Tanzeela *et al.*,2015, Mazzone, Peter J.,2015, Navaneethan, U. *et al.*,2015) and infectious diseases (Guernion, N. *et al.*,2001, Banday, K. M. *et al.*,2011). Therefore, there is also an opportunity to use urinary VOCs as a non-invasive early detection-screening test for T2DM. This method of detection may encourage those at risk to seek suitable diagnosis for any disease they are likely to have, something they may not have otherwise done. This insight could therefore result in an earlier diagnosis and more importantly better

prognosis for patients. Beyond the use of VOCs to detect T2DM status, it could also be further considered as a factor to assess whether T2DM remission has occurred following lifestyle, medical or surgical weight loss (Rubino, F. *et al.*,2016, Schauer, P. R. *et al.*,2016).

Currently the most successful way to achieve T2DM remission appears to be through surgery. As surgery is expensive, many healthcare systems have eligibility criteria in place to ensure that the majority of patients achieve a successful outcome. Predicting who will achieve the best outcome is challenging, however, most bariatric clinics worldwide have a preoperative weight loss requirement, which patients must meet in order to be eligible for surgery. This requirement is put in place as it increases the probability of weight loss maintenance post-surgery (Ali, M. R. *et al.*,2007, Alger-Mayer, S. *et al.*,2008, Solomon, H. *et al.*,2009). It may also improve the status of diabetes and other co-morbidities post-surgery, however, the evidence on this is inconsistent (Brethauer, S.,2011, Ochner, C. N. *et al.*,2012). Therefore, a predictive test that would identify a patient's likelihood of improving their T2DM status post bariatric surgery would be beneficial in supporting the preoperative weight loss criteria, and identifying patients that may need more support post-operatively to sustain good health outcomes. Urinary VOCs may suggest a means for such a medical test due to their potential alignment to metabolic health risk markers.

1.7 Research Hypothesis, Amis and Objectives

1.7.1 Research Hypothesis

Adipose tissue acts as an important site for gut derived LPS to enhance inflammation and increased cardio-metabolic risk, these studies will explore the influences of surgery, endocrine hormones, and medication to mitigate this risk as well as stratify such risk factors.

1.7.2 Research Amis

1. To study the effects of different types of bariatric surgery on cardio-metabolic markers and endocrine hormones (FGF-19 and FGF-21) in obese T2DM women.

2. To examine the effectiveness of liraglutide on adipose tissue angiotensinogen and LPS in lean and obese mature adipocytes.

3. To investigate whether urinary organic compounds can be used as a biomarker for detection of T2DM status and any change within the same individual over time.

1.7.3 Objectives

1. Bariatric surgeries effect obesity related cardio-metabolic disease at different extent. Glucose homeostasis improvement after bariatric surgery typically occurs before significant weight loss (Batterham, Rachel L. *et al.*,2016), however improvement in other cardio-metabolic risk factors such as dyslipidaemia and hypertension, is very much weight dependent. Although with some bariatric surgeries significant weight loss is achieved, the resolution and the rate of improvement in such cardio-metabolic components are lower in these surgeries. In order to assess the effects of different types of bariatric surgeries on cardio-metabolic disease and whether weight reduction independently has a role on cardio-metabolic disease improvement, pre-and post-

operative SAT biopsies and anthropometric data will be collected from obese-T2DM women that had undergone one of the three different types of bariatric surgeries. Inflammatory markers and endotoxin, in addition to cardio-metabolic markers will be studied through ELISA, western blotting, anthropometric and biochemical data analysis.

2. Cardio-metabolic improvements as a result of bariatric surgery, is partially mediated by endocrine hormones, FGF-19 and FGF-21. Bariatric surgery types however impact these hormones in different ways, therefore resulting in some surgeries to be more successful at reducing the cardio-metabolic risks factors. To explore the different impacts of bariatric surgery on these hormone, pre-and post anthropometric and biochemical data, followed by serum from obese-T2DM women that had undergone one of the three different bariatric surgeries, will be collected and analysed by ELISA.

3. All components of RAS in specific AGT are produced in adipose tissue and are secreted from mature adipocytes. In obesity, adipose tissue specific AGT influences and contributes to the systemic RAS, which plays a role in pathogenesis of obesity-related hypertension and insulin resistance. Endotoxin LPS as an inflammatory mediator can induce adipose AGT expression adding to the impact of hypertension. Liraglutide used as an antihyperglycaemic and antiobesity therapy has shown to have beneficial effects on obesity related hypertension as well. Additionally this treatment appears to have an effect on RAS and its components in different tissues such as kidney and lung. It is therefore possible that firstly, liraglutide may exert its antihypertensive influence by having a direct effect on AGT and inflammatory insult of LPS in obese mature adipocytes. Secondly, liraglutide may improve insulin resistance in obese adipose tissue, further contributing to the antihypertensive quality of this treatment. To assess this, lean and obese human adipocytes will be cultured and treated with liraglutide, LPS or a combination of the two, at different time points. AGT expression will be examined by western blot analysis. Insulin sensitivity and cellular proliferation will also be examined.

4. Early detection and diagnosis of T2DM can be beneficial in the treatment of hyperglycaemia and the cardio-metabolic risks that accompany this disease, therefore reducing mortality rates. At present, a convenient, non-invasive, and cost effective early

detection test for T2DM is not available. Urinary-based volatile organic compounds have been successfully utilized for the screening and detection of variety of diseases including some cancers. Therefore there maybe a potential for volatile organic compounds to be used as detection and prediction test in T2DM status. To investigate this, pre-and post biochemical, anthropometric and urine samples will be collected from obese-T2DM women that had undergone bariatric surgery. The urine analysis will be by field asymmetric ion mobility spectrometry.

CHAPTER 2:

Subjects, materials and methodology

2.1 Ethical approval

2.1.1 Bariatric study

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

2.2 Subjects

2.2.1 Subjects who underwent bariatric surgery

At the obesity clinic Prague, Czech Republic, a group of forty-two Caucasian adult women with morbid obesity (BMI ≥35 kg/m^2) and cardio-metabolic comorbidities were recruited for the bariatric study. They underwent bariatric surgery: SG (number (n)=14), LAGB (n=14) or BPD (n=14).

Participant cardio-metabolic disease inclusion criteria were; central obesity (BMI ≥ 30 kg/m^2 does not require waist circumference measurement), triglycerides ≥ 1.7 mmol/L or taking fibrates or nicotinic acid, serum HDL-cholesterol < 1.29 mmol/L or if on specific treatment for lipid abnormality, hypertension if their BP ≥ 130/85 mmHg, or on antihypertensive medication, fasting blood glucose ≥ 5.6 mmol/L or with a previous diagnosis of T2DM. All the participants were imminently investigated prior to the surgery (baseline) along with 6 months following the procedure, during both visits anthropometry and biochemical analysis was undertaken. Serum and abdominal SAT was collected on the day of the surgery and six months post-surgery, additionally, using the bioimpedance method (Tanita TBF-300) the fat mass was measured.

2.2.2 Subjects for the liraglutide study

For the liraglutide study, abdominal SAT from lean (n=3) and obese (n=3) participants who underwent elective surgery was collected at the University Hospital Coventry and Warwickshire, in accordance with the Declaration of Helsinki. All the participants provided written and informed consent. The ethical approval for the collection of human adipose tissue was provided by The University Hospital Coventry and Warwickshire NHS trust Research and Development Department, issuing approval number SK06/9309.

2.3 Tissue culture

2.3.1 Extracting primary human preadipocytes

Under sterile conditions, a category 2 tissue culture room was used in order to minimize lysis of the fat and within 1 hour, the procedure was carried out – and as mentioned previously, adipose tissue-derived stromal cell isolation was collected(Schaffler, A. *et al.*,2007). A 50mL falcon containing 10 mL 2 mg/mL collagenase (Worthington, UK) was used to place 3 cm^3 - 10 cm^3 of WAT, which was pre-warmed to 37 C. To ensure fat pieces were no larger than 2mm in diameter, the WAT was cut using an autoclaved scissors cleaned with 70% industrial methylated spirit, and in order to reach a smooth lipid and collagenase mixture, the falcon was positioned on to a rack in a shaking water bath with a temperature of 37 °C for 30 minutes, which was briskly shaken by hand at 10 minute intervals. By filtering the mixture through sterile cotton mesh into a sterile 50 mL falcon and centrifuging it at 2000 rpm for 5 minutes allowed for the supernatant to be discarded, and the preadipocyte pellet suspended again in 5 mL lysis buffer which was incubated for 5 minutes at room temperature then centrifuged at 2000 rpm for 5 minutes. The remaining pellet was suspended in a 5mL primary adipocyte growth media, whilst the supernatant was discarded. It was then moved to a 75 cm^2 tissue culture flask (T75) (corning, UK) which contained 15 mL primary adipocyte growth

media, which had been pre-incubated in a 37 C, 5 % CO^2 humidified incubator for 15 minutes. The labeled flask was then stored in a 37 C, 5% CO^2 humidified incubator with the media being changed every 48 hours.

Collagenase:

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

From the above solution 20 ml was pipetted into a bottle of collagenase (1g), mixed and placed back into the solution. This was repeated three times until all collagenase was dissolved. The mixture was Aliquoted 10 mL into 50 mL falcons, labelled with collagenase and stored -20 °C until further use.

Lysis Buffer:

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

2.3.2 Cell culture media composition

As stated earlier, cell culture media was used (Alhusaini, S. *et al.*,2010).

Primary adipocyte growth media:

- DMEM/Ham's F-12 phenol-free medium 500 mL (Invitrogen #11039047)
- Penicillin/streptomycin/L-glutamine 100x, 5 mL (1 %) (Invitrogen #10378-016)
- Fetal bovine serum, 50 mL (10 %) (Biosera #S1810)
- Fibroblast growth factor-basic (FGF-basic), recombinant human 5 ng/mL (Fisher Scientific #VXPHG0026)
- Transferrin, human 5 μg/mL (Fisher Scientific #VX0030124SA)

Differentiation media (PromoCell Supplements):

- DMEM/Ham's F-12 phenol-free medium 500 mL (Invitrogen #11039047)
- Fetal bovine serum, 15 mL (3 %) (Biosera #S1810)
- Preadipocyte differentiation supplement pack x1 (Promocell #C39436) The media contained the following supplements at their final concentration:
- Insulin, recombinant human 0.5 μg/mL
- Dexamethasone 400 ng/mL
- D-biotin 8 μg/mL
- Isobutylmethylxantine (IBMX) 44 μg/mL
- L-thyroxine 9 ng/mL
- Ciglitazone 3 μg/mL

Nutrition media (PromoCell Supplements):

- DMEM/Ham's F-12 phenol-free medium 500 mL (Invitrogen #11039047)
- Adipocyte nutrition supplement pack x1 (Promocell #C39439). The media contained the following supplements at their final concentration:
- Insulin, recombinant human 0.5 µg/mL
- Dexamethasone 400 ng/mL
- D-biotin 8 µg/mL
- Fetal calf serum 0.03 mL/mL (3%)

2.3.3 Propagation and differentiation of preadipocytes

Given that media height above the cell layers affects adipocyte differentiation, the same volume of media was used in experiments as was in control and treatment flasks (Sheng, Xia *et al.*,2014). T75 flasks were used to isolate and culture primary human pradipocytes as mentioned earlier, and at 80% confluent the cells were passed into further 3 T75 flasks.

Trypsinization and passages of cells: using sterile phosphate buffered saline (PBS), the media was aspirated and washed thrice and pre-warmed to 37 C, then for 5 minutes it was incubated with 5 mL 0.05 % trypsin – EDTA (Life Technologies #25300-062). To form pellets of cells, the flask was gently tapped to removed the cells from the bottom and trypsin was neutralized with 15mL growth media then centrifuged at 1000 rpm for a period of 5 minutes. The cells were resuspended in 5mL growth media and moved to a new T75 with the supernatant removed. The preadipocytes were tallied using a hemocytometer, seeded at a density of 4000 cells/cm^2 onto treated polystyrene sic well culture plates (Corning) via a 2 mL primary adipocyte growth media in each well which was changed every 48 hours, and incubated in growth media for another 2 days once

confluent. Then at day 0, differentiation was initiated via the change of media to differentiation media. Throughout day 0-6 of adipogenesis media was consistently changed every 48 hours with differentiation media, then from day 6-14 it was changed to nutrition media every 48 hours.

2.3.4 Cell count

After seeded cells were trypsinized, 100 µl of sample was diluted with a 100 µl of Trypan blue 0.4% (Fisher 15250061), and the cells were the counted in the hemocytometer. Mean cell number was calculated by counting 4 replicates for each point of every experiment, and blue stained dead cells were excluded.

2.3.5 Treatments

In order to eliminate the effects of growth factors and any other components in nutrition media, they were switched to detoxification media for a period of 24 hours before the optimization treatments (DMEM/Ham's F-12 phenol-free medium containing only 3 % serum). Both treatments and control were placed in fresh detoxification media, which was made on the day of the treatment.

Mature adipocytes were treated with LPS (Sigma L2630) concentrations of (10 ng/ml and 100 ng/ml) or Liraglutide (Lir) (gifted from Novo Nordisk) or a combination of LPS and Liraglutide; (LPS 10 ng/ml + Liraglutide 10 nM), (LPS 10 ng/ml + Liraglutide 100 nM), (LPS 100 ng/ml + Liraglutide 10 nM), and (LPS 100 ng/ml + Liraglutide 100 nM) for the following lengths of time: 24hr, 48hr and 72hr, with fresh treatment exposure every 24 hours. Adipocytes in untreated media were used as controls. Filter (0.22 µM) was used to filter through all treatment samples.

2.3.6 Collection of protein

A lysis buffer was prepared via 5 mL 1x radioimmunoprecipitation (RIPA) (Millipore UK) with 100 µL of dissolved protease and phosphatase inhibitors (2 Roche Complete Mini protease inhibitor cocktail tablets and 8 mg sodium fluoride (NaF, Fisher Scientific) and 20 mg sodium vanadate (Na3VO4, Acros Organics) in 2 mL 1x RIPA) for the collection of protein. The to each well 250 µl of the protein lysis buffer was added at 4 C, then using sterile scrapers the cells were scraped for 60 seconds in each well, and the contents were collected and stored at -80 C.

2.4 Analysis of samples

2.4.1 Analysis of blood samples

Intravenous blood was taken from patients who had been on a 10 hour overnight fast, the samples were placed into chilled tubes containing EDTA with aprotinin as well as without for the measurements of glucose, insulin, and C-peptide levels, they were aliquoted and kept at -80 C till examined. Via Cobas 6000 analyzer insulin, circulating glucose, C-peptide, HbA1c and lipids were measured. As previously stated (Matthews, D. R. *et al.*,1985), for the assessment of insulin resistance (HOMA-IR), the homeostatic model was utilized as per the following equation: HOMA-IR = [fasting glucose (mmol/L) x fasting insulin (µU/L)] / 22.5. LDL cholesterol was calculated according to the Friedewald formula (Friedewald, W. T. *et al.*,1972)

2.4.2 Immunoprecipitation

For immunoprecipitation SureBeads Protein A Magnetic Beads (Bio-Rad #161-4013) were used. 100 µl of the SureBeads were resuspended and washed with PBS-Tween, then magnetized. 5 µg of angiotensinogen antibody in final volume of 200 µl was added to the beads and resuspended and rotated for 10 minutes at room temperature. The magnetized and washed beads were then added to 500 µl of treatment containing

samples, and rotated for 1 hour at room temperature. The beads were magnetized and washed three times, and the resuspended beads were transferred into new tubes. The tubes were centrifuged for few seconds, magnetized and the residual buffer was aspirated from the tubes. 40 µl 1x laemmli buffer was added to the tubes and incubated for 10 minutes at 70°C, the beads were then magnetized and the eluent was moved into new vials.

2.4.3 Protein analysis

In 250 µL protein lysis buffer, made with 5 mL 1x RIPA (Millipore UK) with 100 µL of dissolved protease and phosphatase inhibitors (2 Roche Complete Mini protease inhibitor cocktail tablets and 8 mg sodium fluoride (NaF, Fisher Scientific) and 20 mg sodium vanadate (Na3VO4, Acros Organics) in 2 mL 1x RIPA) the human WAT was homogenized and resuspended. Bio-Rad detergent 5'-compatible protein assay kit (Bio-Rad Laboratories, CA) with standard concentrations of albumin from bovine serum (BSA) (Sigma A9647) was utilized for the determination of protein concentrations and quantified using a nanospectrophotometer (GeneFlow, UK). An earlier mentioned method was used for the analysis of western blot (Alhusaini*, et al.*, 2010). In summary, 20 µg of protein samples was used to analyze the inflammatory markers TLR4, MYD88, TRAF6, IκB, NFκB, and 8 µg of protein sample was used for the AGT study. These proteins were added onto a 10% denaturing polyacrylamide gel (GeneFlow, UK). The Proteins were separated by electrophoresis and transferred at 4 ° C and 100 V for 1 hour onto a 0.4 µm pore size Immobilon-P PVDF membrane (IPVH00010 Sigma). Membranes were then cut depending on the specific protein molecular weight by using ponceau stating (P7170 Sigma), and washed by PBS thoroughly and blocked in 0.2% BPS-tween (BPST) at 4 °C for 24 hours. Membranes were then incubated in a mixture of 0.2% I-block PBS-tween and primary antibodies AGT 1:5000 for 4 hours, and TLR4 1:500, MYD88 1:1000, TRAF6 1:240, IκB 1:1000, NFκB 1:1000 overnight, at 4 ° C cold room. To confirm equal protein loading β-actin was used. PBST was used to wash the membranes six times for five minutes, and incubated in anti-rabbit IgG (whole molecule), horseradish peroxidase antibody produced in goat, IgG fraction of antiserum, buffered aqueous solution (Sigma A9169). The visualization of the bands was enabled

by a chemiluminescent detection system, ECL/ECL+ (GE Healthcare, UK), with the intensity being confirmed via the use of densitometry and the Image J software.

2.4.4 Glucose uptake assay

Glucose uptake was performed as previously described (Liu, Fang *et al.*,2001). Cells were grown to confluence in 6 well plates. These cells were then washed with PBS and incubated with Krebs-Henseleit buffer (KHB) with BSA 0.01% and glucose 5 mmol/L at 37 °C for 3 hours, for insulin and serum starvation. Mature adipocytes were then treated with DMSO (for control) and insulin 100nM (Sigma 19278). 2-deoxy-[^{3} H]D-glucose [1 μCi/μl) (PerkinElmer) was added to incubate for 10 minutes. Ice-cold PBS was used to wash the cells three times, which was then followed by transferring the cells into 4 mL of scintillation fluid. Scintillation counter was then used to measure radioactivity. Protein quantification and glucose uptake was performed with the remaining 100μL cell lysate.

The following reagents were used to make 1 L 5xKHB buffer,

Chemical	5X(nM)	5X(g/L)
NaCl	555	32.53
KCL	23.5	1.75
MgSO4	10	1.20
Na2HPO4	6	0.85

These reagents were added to 900mL of distilled and autoclaved dH_2O in a 1 L beaker, which contains a stir bar. Once dissolved the volume was adjusted to 1000mL with dH_2O and stored at 4 °C. A day prior to glucose uptake, 500 mL of the listed two reagents were made and filter sterilized;

1. 1 x KHB, low glucose – 5mM (450mg), 0.01% BSA (50mg), 10 nM Hepes

(1.19g), pH 7.4

2. 1x KHB, no glucose, no BSA, 10 mM Hepes (1.19g), pH 7.4

2.4.5 Enzyme linked immune sorbent assay (ELISA)

Blood samples were centrifuged and serum was immediately removed and aliquoted and stored at – 80 °C for future use. ELISA kit was used for the measurement of the following; CRP (DCRP00, R&D Systems), IL-6 (D6050, R&D Systems), FGF-19 (DF1900, R&D Systems) and FGF-21 (DF2100, R&D Systems). In summary, the reagents and standards were prepared. 100µl of assay diluent was added to all the 96 wells of microplate, which already have been coated with a monoclonal antibody specific for the study. Additional 100µl of standard, control or sample is the added per well, which is then incubated for 2 hours at room temperature. Each well is washed four times with wash buffer and entire fluid is aspirated. 200µl of the conjugate is added to each well and incubated in dark for 2 hours at room temperature. To each well, 50 µl of stop solution is then added. A microplate reader determined the optical density of each well. Concentrations were calculated using linear regression. All measurements were done in duplicates. The assay detection range for CRP was 0.8-50 ng/mL, coefficient of variance of 3.8% intra-assay, and 7% inter assay precision. IL-6 had a assay detection range of 3.1-300 pg/mL, coefficient of variance of 3.1% intra-assay and 2.5% inter-assay precision. FGF-19 had a detection range of 15.6-1000 pg/mL, and a coefficient of variation of 4.5 % for intra-assay and 5.5 % inter-assay precision. FGF-21 had a detection range of 31.3-2000 pg/mL, and the coefficient of variation of the intra-assay is 2.9% with 6.4% inter-assay precision.

2.4.6 Quantification of endotoxin LPS

Chromogenic quantification of endotoxin LPS (Sigma L2630) was performed by using Limulus Amebocyte Lysate (LAL) detection kit (#QCL-1000TM Lonza). In brief, samples were set to the pH range of 6.0-8.0 by using endotoxin-free sodium or

hydrochloride acid. Once the reagents are prepared, the microplates were pre-equilibrated at 37 °C in heating block adapter. 50µl of sample or standard was added to some wells, while 50µl of LAL reagent water was added to the blank wells. After 10 minutes, 100µl of pre-warmed substrate solution was added to the wells and mixed. After 16 minutes, 100µl of stop reagent is added to all wells and mixed. A microplate reader determined the absorbance of each microplate well, and the concentrations (EU/mL) were calculated by using linear regression model. All measurements were done in duplicates.

2.5 Statistical Analysis

IBM SPSS Statistics 24.0 software was used to preform all statistical analyses. To test data normality Shapiro-Wilk test was preformed. The two-tailed paired sample t-test or one-way ANOVA was used for the parametrically distributed data, and Kruskal-Wallis ranks test was used for the non-parametrically distributes data. Multivariate linear regression (forward model) was preformed for ΔFGF-19 and ΔFGF-21 as dependent variables with the variables (Δ) that had shown significant correlation with ΔFGF-19 and ΔFGF-21 as independent variables. Pearson correlation analysis was done for correlation between variables, this was followed by calculations to test the difference between two independent correlations coefficients. Changes in variables (Δ), were calculated as percentage absolute change [((post surgery-pre surgery)/pre surgery) x100]. All data is presented as mean value ± standard error of the mean (SE). $P \leq 0.05$ was considered statistically significant and significance levels are indicated as follows; p-value: *≤0.05, **≤0.01, ***≤0.001 and where more than one comparison was made: #≤0.05, ##≤0.01, ###≤0.001.

2.6 Chemical Analysis and Instruments

2.6.1 Field Asymmetric Ion Mobility Spectrometry (FAIMS)

A Lonestar (Owlstone Ltd, UK) FAIMS unit was used to analyse the headspace of pre and post-operative urinary VOC samples from all participants. The FAIMS device used a 63Ni ionization mechanism. Upon ionisation, the sample is passed through an oscillating electric field with an asymmetric high voltage waveform. Ions "zigzag" through the field and only ion species with no overall transverse motion pass through without deionising, based on properties of the waveform and the ion species. Certain properties of the waveform are then manipulated to identify the different ion species present in the analyte.

2.6.2 FAIMS Analysis

Urine samples were defrosted to 5◦C overnight. For each sample a 5ml aliquot was pipetted into a 20ml glass sample bottle. This was then placed into an Owlstone ATLAS At-Line Sampling module attached to the Lonestar FAIMS unit. The ATLAS module heats the sample to 40◦C, and introduces the headspace of the sample into the FAIMS for analysis. Four full sweeps of the dispersion field strength were carried out in succession for each sample. Before the first sample and between each sample, an air blank was run for three sweeps to allow any residue from the previous sample or other experiments to clear from the machine. No data was collected for these sweeps.

2.6.3 Data Analysis

Each sample yielded eight 512 × 51 matrices, four positive and four negative ion counts across the tested compensation voltages and dispersion field strengths, for a total of 208,896 variables per sample. As an initial step in the analysis of this data, the matrices were transformed using a discrete wavelet transformation. The levels of the transform

corresponding to the two smallest-bandwidth passes were discarded to eliminate noise. Wavelet transformation is a common step in signal processing, and can help in separating structure from noise in an image, compressing the informative variables into fewer dimensions and consequently reducing the dimensionality of the data. After transforming the data, there were 52,224 variables per sample. Similarly to Fourier transforms, wavelet transforms create a representation of an image in an alternative basis space. However, in comparison to Fourier transforms, wavelet transforms capture information about both the size (or wavelength) of structures in the image, and information about where those structures occur. This helps to concentrate the useful information in the image into fewer predictors, which helps when selecting relevant features for classification tasks. To assess the ability of FAIMS analysis of urine samples in predicting diabetes status post-surgery, HOMA-IR was considered as a classification task. For HOMA-IR, the cohort was divided into two classes. Binning the data was necessary due to the small sample size (both FAIMS and indicator data were available for 25 participants) and the high dimensionality of the FAIMS data (52,224 features), which hampered regression analysis. To perform the binning for this indicator, participants were divided into those with an indicator value which was less than or equal to the median value of the cohort (the "Low" group for that indicator), and those with a value above the median value of the cohort (the "High" group for that indicator). Participants who lacked data for this indicator were excluded from further analysis. FAIMS data was available for 25 participants, and indicator data was available from at least 24 participants for each indicator.

2.6.4 Statistical Analysis

Mann-Whitney-Wilcoxon test was used to rank features according to how well they separate the cohort into High and Low classes. The top number features were then taken forward and used to train a classification model, with n and the classification model selected using a separate cross-validation analysis. Four learning algorithms were considered: radial support vector machine (SVM), sparse logistic regression, logistic regression, and random forests. To assess the performance of HOMA-IR, 10-fold cross validation was used to produce predicted classes for each participants, with both feature

selection and model creation being carried out in the cross-validation. Classification performance was assessed by using the Receiver Operating Characteristic (ROC) curve, which is defined by the area under the curve (AUC).

CHAPTER 3:

The short-term effect of bariatric surgery on cardio-metabolic markers in obese T2DM women

3.1 Introduction

Studies have shown that measures of abdominal or central obesity such as waist to hip ratio (WHR) and most importantly measures of fat distribution (i.e. excess fat mass%) (Alberti, K. G. *et al.*,2006, Ramírez-Vélez, Robinson *et al.*,2017) are positively associated with metabolic components such as dyslipidaemia, hypertension and dysglycemia (Bener, A. *et al.*,2013, Gonzalez-Muniesa, P. *et al.*,2017, Ramirez-Velez, R. *et al.*,2017). The 'grouping' of metabolic abnormalities determined in participants with excess abdominal obesity, referred to as metabolic disease, appears to carry a significantly higher cardiovascular risk as well in comparison to the risk associated with each individual abnormality (Golden, S. H. *et al.*,2002, Sattar, N. *et al.*,2003). Within the pre-diabetic state, hyperglycaemia and dyslipidaemia have also been shown to increase the risk of cardiovascular disease (Sattar, N. *et al.*,2003). Whilst it is also evident that in the state of obesity, increase in components of the metabolic disease, results in a higher mortality rate of cardiovascular disease.

The underlining association between obesity and cardio-metabolic disease is reported to be due to a low-grade systemic inflammatory environment. This systemic inflammation has been considered in part to arise through endotoxaemia, in which gut dysbiosis triggered by high fat diets, disrupts the intestinal barrier leading to increased gut permeability, and therefore, the translocation of endotoxin also known as LPS into the blood circulation (Kallio, K. A. Elisa *et al.*,2015). LPS then promotes inflammation by activating specific pathways in adipose tissue and up-regulates pro-inflammatory cytokine secretion, resulting in obesity related cardio-metabolic disease (Eckel, Robert H. *et al.*,2011, Farb, Melissa G. *et al.*,2011, Park, Hyeong Kyu *et al.*,2017).

Studies have demonstrated that reduction in adipose tissue inflammation can arise as a result of 'weight loss'. Weight loss can have a two-fold influence on health, firstly, reduction in fatty diets results in a decrease endotoxin induced adipose tissue inflammation, and secondly reduced adipose tissue also reverses the exacerbated inflammatory response caused by the cardio-metabolic risk profile (Farb, Melissa G. *et al.*,2011). Therefore, health care professionals have placed great emphasis on preventing

or delaying cardio-metabolic diseases by advocating lifestyle changes to aid weight loss. However, in cases where weight loss through diet, exercise, and pharmacological treatments alone is unmanageable, bariatric surgery can act as an alternative therapeutic intervention; which again indirectly can impact endotoxin induced inflammation as the gut microbiota and anatomy is altered.

Although invasive, bariatric surgery results in significant long-term weight loss, which in return improves survival by 10-15 years for obese participants (Sjostrom, L. *et al.*,2007, Carlsson, L. M. *et al.*,2012, Sjostrom, L. *et al.*,2012). A long-term study assessing weight loss with a 20-year follow-up, has reported that participants who have undergone different types of surgery such as gastric bypass, LAGB and vertical-banded gastroplasty, on average, had an 18% weight loss in comparison to a weight loss of 1% in the control group that had nonsurgical obesity interventions, at 20 years (Neovius, M. *et al.*,2012). Various other studies have consistently reported that weight loss in addition to substantial improvements in T2DM, as well as other components of cardio-metabolic disease, can also lower the risk of some cancer developments (Buchwald, H. *et al.*,2004, Sjostrom, L. *et al.*,2009, Schauer, D. P. *et al.*,2017). Besides the health benefits of bariatric surgery, reduction in medication use in specific anti-diabetic and antihypertensive medication post-surgery has also lead to reduction in medication costs for obese participants (Neovius, M. *et al.*,2012).

While bariatric surgery is beneficial in improving cardio-metabolic disease, different surgeries types impact cardio-metabolic components independent of one another, in various degrees, leading to prolonged recovery in terms of cardiovascular risk markers in some cases. Therefore, participants need to be selected for each specific bariatric surgery depending on their cardio-metabolic risk profile, and additionally they need to be kept under observation for the first 6 months post-surgery as some participants may still be at cardiovascular risk.

As such, the aims of this study were to:

1. Assess the rate of improvement in cardio-metabolic components and disease resolution in obese-diabetic women post-surgery.

2. Evaluate the effect of bariatric surgery on inflammatory/cardiovascular markers.

3. Investigate the relevance of circulating endotoxin LPS on health outcomes, by assessing three different types of bariatric surgeries; SG, LAGB and BPD.

3.2 Subjects and Methods

For this study, a cohort of 42 Caucasian adult women participants with morbid obesity (BMI $\geq$ 35 kg/m^2) and cardio-metabolic comorbidities, who underwent one of the three types of bariatric surgery, SG (n=14), LAGB (n=14) and BPD (n=14).

3.2.1 Anthropometric and biochemical measurements

All measurements were performed at baseline and at 6 months post-surgery. Blood was taken from all participants after a 10 hour fast. HbA1c, serum glucose and lipids were measured using the Cobas 6000 analyzer. HOMA-IR was used to quantify insulin resistance by the following equation: HOMA-IR= fasting glucose (mmol/L) x fasting insulin (mIU/L) / 22.5 (Matthews, D. R. *et al.*,1985). LDL cholesterol was calculated by using the Friedwald formula (Friedewald, W. T. *et al.*,1972). Height and weight were measured via standardised protocol. BMI was calculated as weight (in kilograms) / height^2 (meters), and percentage excess weight loss was calculated by using the following equation: (preoperative weight - postoperative weight) / (preoperative weight - ideal body weight) x 100. Body fat mass was measured using the bioimpedance method (Tanita TBF-300; Tanita corporation). Blood pressure data, which is presented as systolic over diastolic values, with the average of three blood pressure readings with 5-minute resting period in between; these readings were taken by using an automated

device. Mean Arterial Pressure (MAP) was calculated by using: [(2diastolic blood pressure + systolic blood pressure)] / 3 (Brzezinski., Walter A.,1990).

3.2.2 Quantitation of bacterial LPS, and inflammatory markers

Chromogenic quantification of bacterial LPS (Sigma L2630) was performed by using a LAL detection kit (#QCL-1000TM Lonza). LAL LPS detection assay was carried out in duplicate with a five-part standard curve per plate, and all samples processed in the same experiment. Concentrations (EU/mL) were calculated using linear regression model. The concentrations of CRP and IL-6 were assessed through ELISA. The concentration of CRP were assessed through ELISA (DCRP00, R&D Systems), with the assay detection range of 0.8-50 ng/mL, coefficient of variance of 3.8% intra-assay, and 7% inter assay precision. The IL-6 concentration was determined by using human IL-6 ELISA (D6050, R&D Systems), with an assay detection range of 3.1-300 pg/mL, coefficient of variance of 3.1% intra-assay and 2.5% inter-assay precision. Protein analysis was preformed by using western blot method as previously described (Alhusaini, Saif *et al.*,2010). Primary antibodies utilised were: TLR4 1:500 (ab13556, UK), MYD88 1:1000 (ab2064, UK), TRAF6 1:240 (ab33915, UK), IκB 1:10000 (ab32518, UK), and NFκB 1:1000 (ab16502, UK).

3.2.3 Cardio-metabolic disease criteria

Cardio-metabolic disease was characterised by using the International Diabetes Federation criteria (IDF) (Alberti, George *et al.*,2006). Participants with central obesity (BMI $\geq$ 30 kg/m^2) does not require waist circumference measurement) fulfilled the first requirement of this criterion. Participants with triglycerides $\geq$ 1.7 mmol/L or taking fibrates or nicotinic acid fulfilled the criteria for hypertriglyceridemia. Serum HDL-cholesterol was considered low if it was < 1.29 mmol/L in women or if specific treatment for lipid abnormality was taken. Participants fulfilled the criteria for hypertension if their blood pressure $\geq$ 130/85 mmHg, or if they have been placed on antihypertensive medication from a previous diagnoses. A fasting blood glucose $\geq$ 5.6 mmol/L or with a previous diagnosis of T2DM fulfilled this criterion. IDF states that

additional measures such as CRP and IL-6 can be further used for the classification of this disease. Based on the cutoff points set by the European guidelines on cardiovascular disease prevention and the American Heart Association, participants with CRP > 3 mg/L were considered at high cardiovascular risk (Pearson, T. A. *et al.*,2003, Perk, J. *et al.*,2012). Cardio-metabolic disease was classified as ≥ 2 of these components with obesity as a diagnostic criterion.

3.2.4 Statistical Analysis

Data were reported as mean and SE. Shapiro-Wilks test was applied to normalise the data. Parametrically distributed data, and within surgery group statistical significance (pre to post), was determined by paired two-tailed t-test, while Kruskal-Wallis ranks test was used for non-parametrically distributed data. Pearson correlation analysis was done between changes in variables (Δ), which was calculated as percentage absolute change [((post surgery-pre surgery)/pre surgery) x100]. IBM SPSS Statistics 24.0 software was used to preform all statistical analyses.

3.3 Results

3.3.1 Body composition and metabolic variables

Clinical and anthropometric data obtained from 42 obese-T2DM women participants before undergoing SG (n=14), LAGB (n=14) and BPD (n=14) surgeries, and 6 months post-surgery were collected and analysed (Table 3.3.1). Although post-surgery all participants were still within the obese range (BMI $\geq$ 30 kg/m^2), significant improvements in weight markers (body weight, BMI, % Fat mass loss) were observed in all three surgeries. All participants were observed to be in T2DM remission (HbA1c $p \leq 0.01$) at 6 months with significant reduction in other diabetic markers (HOMA-IR, plasma glucose and insulin). Blood pressure improved in all surgeries, however systolic blood pressure ($p \leq 0.05$) had significantly improved in the SG group alone. In regards to the lipid profile, total cholesterol ($p \leq 0.001$), LDL ($p \leq 0.001$) and HDL ($p \leq 0.001$) significantly reduced in the BPD category, whilst significant reduction in triglycerides ($p \leq 0.01$) was only observed within LAGB. Both SG and LAGB categories have shown a significant improvement in triglyceride/HDL ratio ($p \leq 0.05$).

	SG N=14			LAGB N=14			BPD N=14		
	Pre Mean (SE)	Post Mean (SE)	p-value	Pre Mean (SE)	Post Mean (SE)	p-value	Pre Mean (SE)	Post Mean (SE)	p-value
Age (years)		53.24±7.48			53.57±11.26			50.57±5.88	
Excess weight loss (%)		17.15(1.61)			20.16(2.50)			30.67(2.24)	
Body weight (kg)	109.21(4.13)	97.61(3.51)***	<.001	119.59(5.10)	105.34(4.89)***	<.001	128.94(5.93)	108.02(4.88)***	<.001
BMI (Kg/m²)	40.41(1.38)	36.05(1.15)***	<.001	43.95(1.76)	38.31(1.88)***	<.001	47.15(2.11)	39.63(1.75)***	<.001
Fat mass (%)	48.49(.97)	44.90(.95)***	<.001	49.54(.92)	46.39(1.19)**	.001	49.59(1.03)	44.68(1.28)***	<.001
WHR (cm)	0.88(.02)	0.87(.02)	.805	0.90(.01)	0.88(.01)	.310	0.93(.02)	0.90(.01)	.361
Systolic bp (mmHg)	127.92(3.33)	118.85(4.36)*	.040	133.27(3.09)	126.00(4.59)	.106	125.91(4.84)	124.45(3.44)	.796
Diastolic bp (mmHg)	77.00(3.36)	73.42(2.89)	.411	79.67(2.10)	80.83(2.12)	.684	80.73(4.67)	73.27(2.85)	.129
MAP	93.97(3.09)	88.57(2.93)	.136	97.44(1.70)	95.72(2.36)	.422	95.79(4.41)	90.33(2.36)	.257
HbA1c (mmol/mol)	56.33(2.71)	47.93(2.66)***	<.001	53.46(2.85)	47.08(1.74)*	.015	53.85(2.69)	38.38(2.18)***	<.001
Plasma glucose (mmol/L)	8.93(.52)	7.34(.41)**	.008	9.30(.71)	7.07(.42)***	<.001	8.40(.69)	7.02(.45)	.075
Plasma insulin (mmol/L)	26.43(4.85)	16.76(2.73)**	.002	26.20(1.85)	15.21(1.58)***	<.001	31.37(5.64)	17.28(3.21)*	.035
HOMA-IR	10.95(2.28)	5.32(.83)**	.005	10.82(1.10)	5.08(.77)***	<.001	11.78(2.59)	5.42(1.26)*	.036
Total Cholesterol (mmol/L)	4.84(.19)	4.75(.20)	.309	4.83(.20)	4.54(.24)	.142	4.96(.27)	3.76(.23)***	<.001
LDL Cholesterol (mmol/L)	2.50(0.29)	2.94(.19)	.073	2.96(.17)	2.89(.22)	.764	3.24(.27)	2.27(.18)***	<.001
HDL Cholesterol (mmol/L)	1.12(.08)	1.15(.07)	.577	1.04(.06)	1.08(.07)	.378	1.06(.06)	.76(.06)**	.001
Triglyceride (mmol/L)	1.95 (.34)	1.38(.17)	.062	1.79(.20)	1.23(.13)**	.004	1.46(.17)	1.60(.18)	.364
Triglyceride / HDL ratio	2.21(.50)	1.29(.23)*	.046	1.86(.26)	1.27(.20)*	.013	1.49(.23)	3.61(1.67)	.236
C-Reactive Protein (mg/L)	4.94(.71)	4.45(.91)	.294	5.63(1.06)	5.80(1.05)	.808	11.65(1.87)	7.83(1.30)*	.028

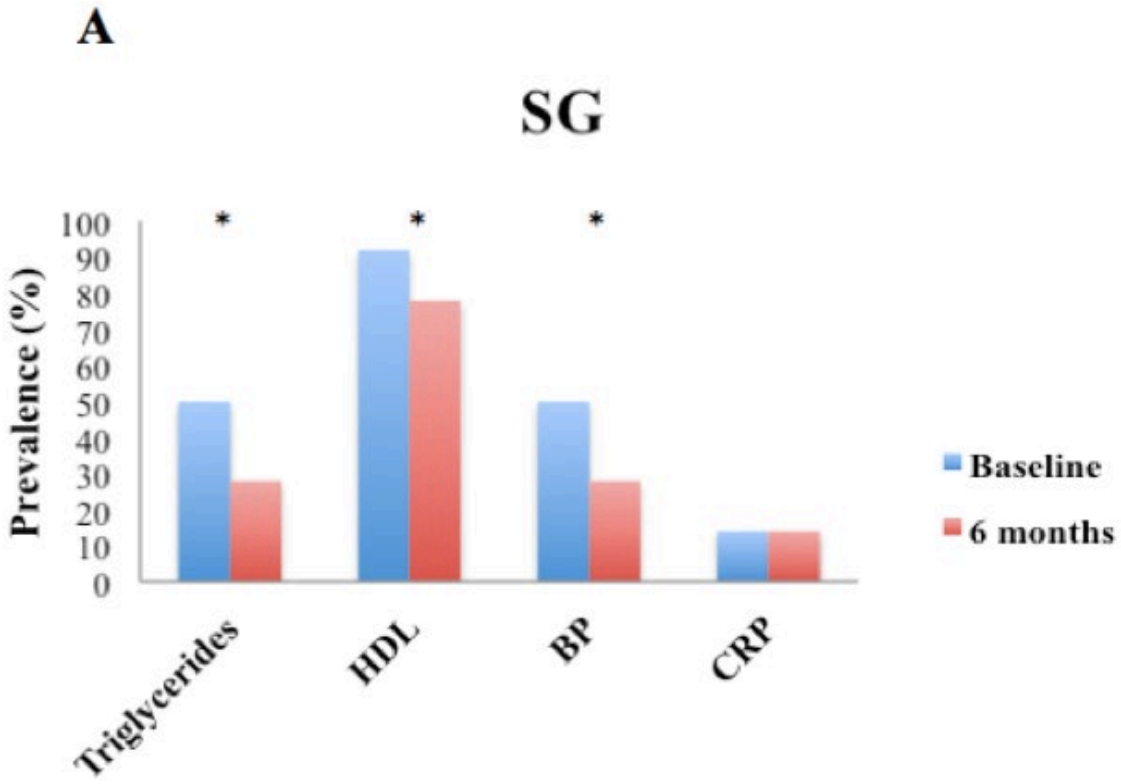
3.3. 1 Anthropometric and metabolic variables at pre (Baseline) and post (6 months) surgeries.

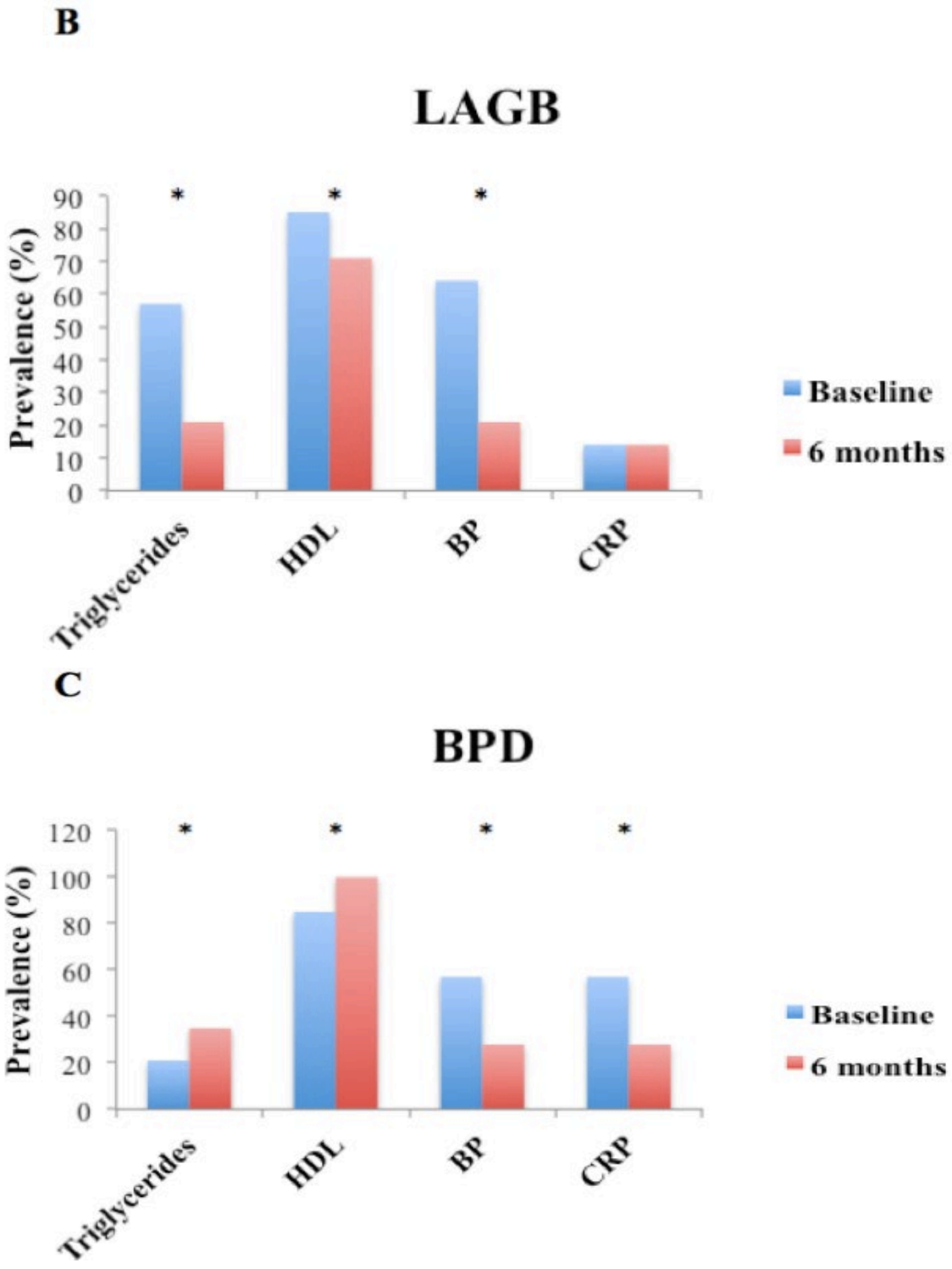
Continues variables are represented as mean and standard error. SG-sleeve gastrectomy; LAGB- laparoscopic adjustable gastric banding; BPD- biliopancreatic diversion; BMI-body mass index; WHR-waist to hip ratio; MAP-mean arterial pressure; HbA1c-glycated haemoglobin; HOMA-IR- Homeostatic assessment model of insulin. LDL-low density lipoprotein; HDL-high density lipoprotein. (* Represents p-values: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

3.3.2 Number of improvements in cardio-metabolic components, and the resolution in this disease was higher in SG and LAGB at 6 months post-surgery.

The prevalence of cardio-metabolic risk factors at 6 months post SG shows a decrease in proportion of individuals with; triglycerides ≥ 1.7 mmol/L (50% to 28%; p≤0.05), HDL < 1.29 mmol/L (92% to 78%; p≤0.05), and BP ≥ 130/85 mmHg (50% to 28%; p≤0.05) (Figure 3.3.2. A). In SG surgery 42.8% of participants achieved resolution in 3 or more components, 28.5% of participants in 2 components, and the other 28.5% attained resolution in 1 component of their cardio-metabolic disease post-surgery. In LAGB category there was a decrease in proportion of individuals with; triglycerides ≥ 1.7 mmol/L (57% to 21%; p≤0.05), HDL < 1.29 mmol/L (85% to 71%; p≤0.05), and BP ≥ 130/85 mmHg 64% to 21% p≤0.05) post surgery (Figure 3.3.2 B). In this surgery 71.4% of the participants achieved resolution in 3 or more components, and the remaining participants (28.5%) attained resolution in 2 components of their cardio-metabolic disease. At 6 months post BPD, there was an increase in the proportion of individuals with; triglycerides ≥ 1.7 mmol/L (21% to 34%; p≤0.05) and HDL < 1.29 mmol/L (85% to 100%; p≤0.05), while there was a decrease in proportion of individuals with; BP ≥ 130/85 mmHg (57% to 28%; p≤0.05) and CRP > 3 mg/L (57% to 28%; p≤0.05) (Figure 3.3.2 C). In this surgery 28.5% of the participants had resolution in 3 or more components, 35.7% achieved resolution in 2 components, and 28.5% reached resolution in 1 component surgery. While 7.1% showed no resolution in any components of the cardio-metabolic disease.

As all the participants in this study were obese at 6 months post surgery (BMI ≥ 30 kg/m^2), the criterion for central obesity was fulfilled. Additionally all these participants were in diabetes remission at 6 months as well, therefore glucose and BMI were not included in the analysis of the cardio-metabolic disease prevalence post surgery.


A
SG
Prevalence (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
Triglycerides
HDL
BP
CRP
Baseline
6 months

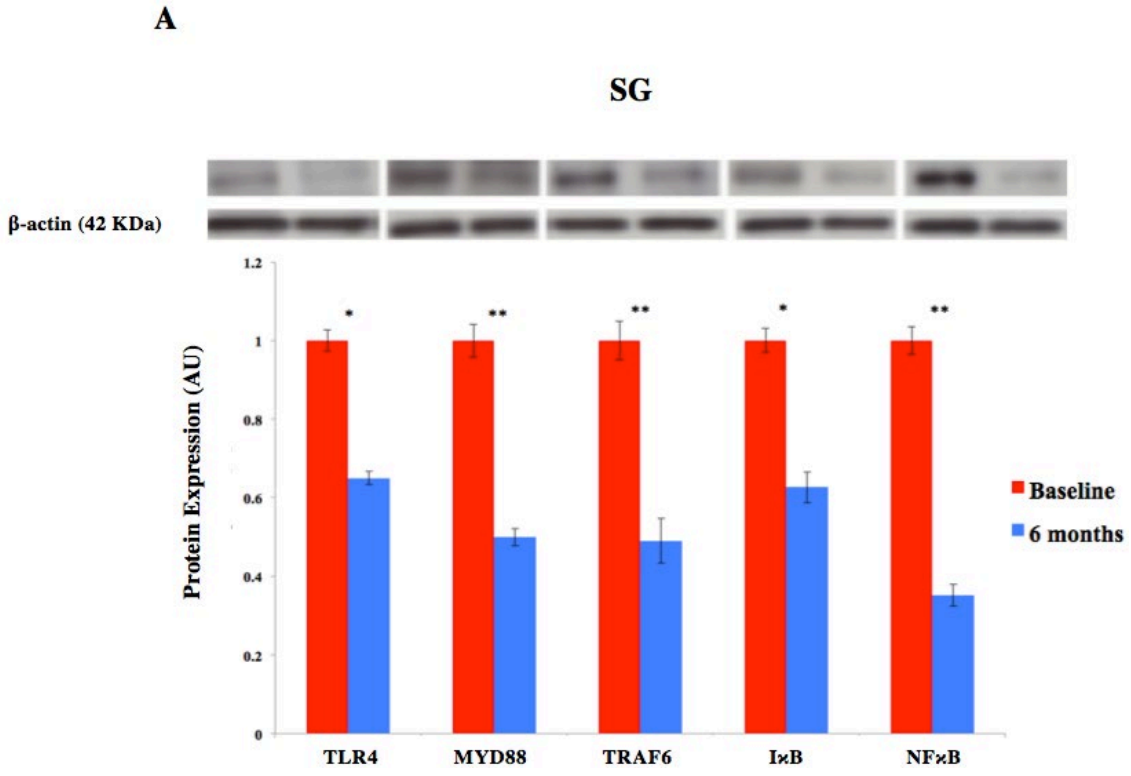


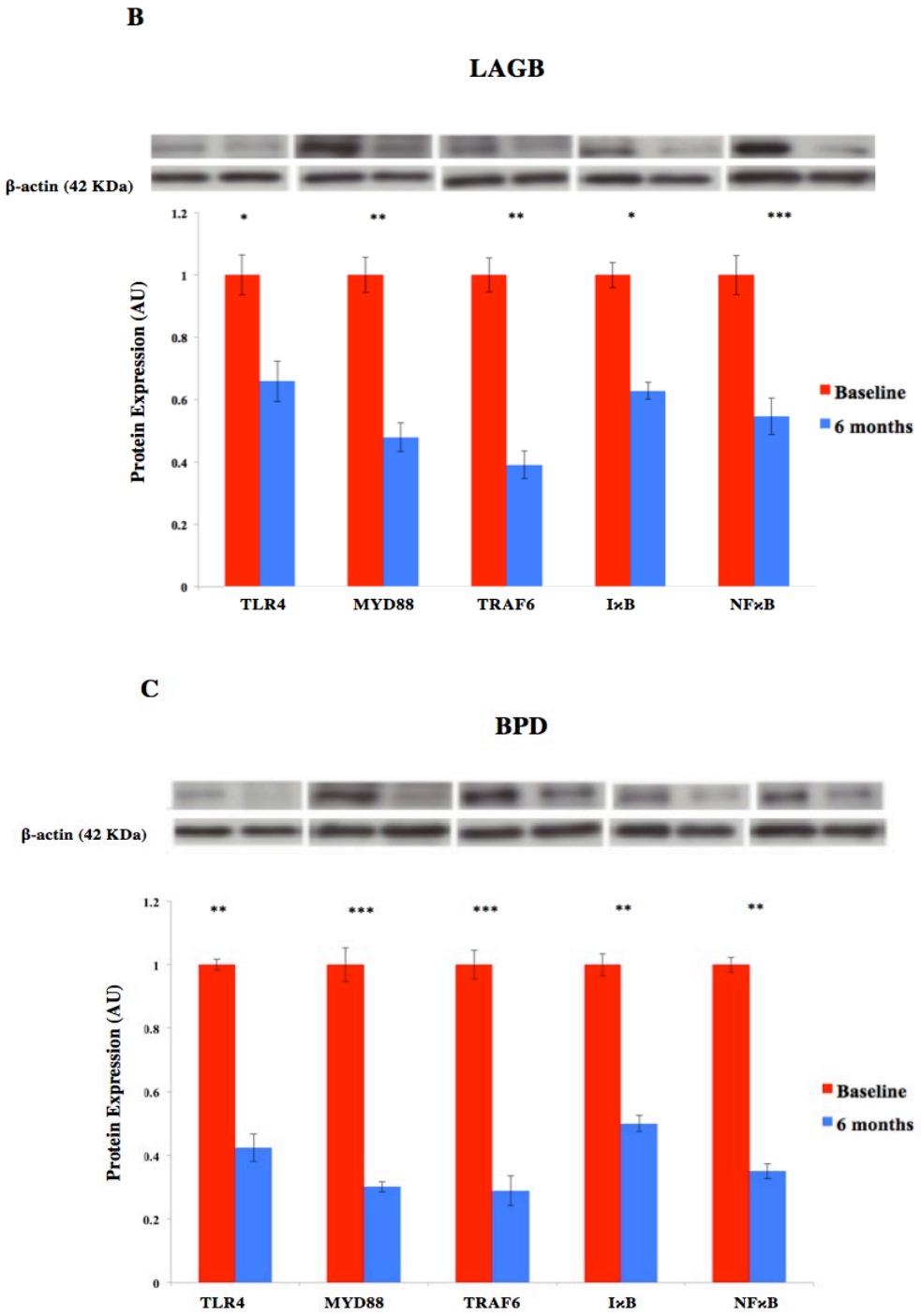
3.3. 2 Prevalence of cardio-metabolic components over time due to the effects of surgery.

Percentage of participants with each cardio-metabolic component; triglycerides, HDL, BP and CRP, at baseline and 6 months post- (A) Sleeve gastroctomy - (B) laparoscopic adjustable gastric banding, - (C) and biliopancreatic diversion. (* Represents p-value: * $p \leq 0.05$)

3.3.3 All inflammatory markers in the MYD88-dependent pathway significantly reduced in SG, LAGB and BPD at 6 months.

The protein expression of all five inflammatory markers examined at 6 months reduced significantly in comparison to baseline in SG surgery, however MYD88 (p=0.005), TRAF6 (p=0.004), and NFκB (p=0.012) had a significantly greater reduction than TLR4 (p=0.03) and IκB (p=0.04) (Figure 3.3.3 A). Participants that underwent the LAGB surgery were observed to significantly reduce five markers of inflammation post-surgery in this group. The inflammatory markers were TRAF6 (p=0.007), which had the greatest significant reduction, followed by MYD88 (p=0.003), NFκB (p=0.0004), IκB (p=0.02), and finally TLR4 (p=0.01) (Figure 3.3.3 B). Similar to the other two surgeries every inflammatory marker in the MYD88-dependent pathway was reduced after BPD, MYD88 (p=0.0007) and TRAF6 (p=0.001) achieved the greatest significant reduction, followed by NFκB (p=0.009), TLR4 (p=0.01), and IκB (p=0.002) respectively (Figure 3.3.3 C).





3.3. 3 Protein expression of inflammatory markers in MYD88 pathway pre-and post-surgery.

Western blot analysis was used to measure the following markers; TLR4 (molecular weight: 95 KDa), MYD88 (molecular weight: 33 KDa), TRAF6 (molecular weight: 58 KDa), IκB (molecular weight: 35 KDa), NFκB (molecular weight: 60 KDa), and β-actin (42 KDa). Statistical analyses were performed to determine the difference in protein expressions of each marker at baseline and 6 months post- (A) Sleeve gastroctomy – (B) laparoscopic adjustable gastric banding (C) and biliopancreatic diversion. (* Represents p-value: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

3.3.4 Effects of bariatric surgery on LPS, CRP and IL-6

The findings from assessment of systemic LPS concentrations determined that there was some apparent reduction in SG and BPD across these surgeries but did not achieve significance, whilst no change was observed in LAGB participants at 6 months post-surgery. Inflammatory marker IL-6 appeared increased in SG and LAGB post-surgery but this did not achieve significance, however, there was a significant increase in the BPD participants at 6 months post-surgery. CRP concentration remained unchanged from baseline to 6 months post-surgery in SG and LAGB participants, while this concentration significantly reduced in the BPD participants.

	SG N=14			LAGB N=14			BPD N=14		
	Pre Mean (SE)	Post Mean (SE)	*p*.value	Pre Mean (SE)	Post Mean (SE)	*p*.value	Pre Mean (SE)	Post Mean (SE)	*p*.value
LPS (EU/ml)	17.84(1.43)	16.12(1.86)	.474	14.94(1.01)	14.68(1.22)	.826	16.19(2.33)	13.84(1.57)	.372
IL-6 (pg/mL)	1.66(.06)	1.80(.07)	.097	1.55(.34)	1.74(.08)	.264	1.64(.05)	1.92(.13)*	.048
CRP (mg/L)	4.94(.71)	4.45(.91)	.294	5.63(1.06)	5.80(1.05)	.808	11.65(1.87)	7.83(1.30)*	.028

***P≤0.05**

3.3. 4 LPS, IL-6 and CRP concentrations at pre (baseline) and post (6 months) stratified by SG, LAGB and BPD.

Variables are presented as means and standard error. Within group statistics (pre-post) was measured by two-tailed paired t-test. (* Represents p-value: * p≤0.05).

3.3.5 IL-6 inversely correlates with HDL post BPD, but not in the other two surgeries.

There were no correlations identified between inflammatory marker IL-6 and any of the weight markers in all three surgeries. There was however a significant negative correlation between IL-6 and HDL cholesterol in the BPD participants, which was not observed in SG and LAGB participants.

	SG N=14		LAGB N=14		BPD N=14	
Variable (Δ)	**r**	***p*.value**	**r**	***p*.value**	**r**	***p*.value**
Body weight (kg)	-.422	.196	-.473	.237	-.083	.846
BMI (kg/m^2)	-.384	.243	-.532	.175	-.024	.995
Fat mass%	-.130	.720	-.281	.500	-.302	.510
HDL Cholesterol (mmol/L)	.262	.436	.125	.769	-.745	.034*
Triglyceride (mmol/L)	-.333	.316	-.100	.814	.300	.470

***P≤0.05**

3.3. 5 Correlation between Δ IL6 with Δ weight markers and lipid markers in all three surgeries.

Pearson's correlation coefficient analysis was preformed using absolute change (Δ) variables (pre-post surgery percentage change) in each surgery. The correlation was between inflammatory marker IL-6 with weight markers (body weight, BMI, fat mass) and lipid markers (HDL cholesterol and triglycerides). (* Represents p-value: * p≤0.05)

3.3.6 Low-grade inflammation persists in BPD at 6 months post-surgery.

There was a positive correlation between inflammatory/cardiovascular marker CRP and waist to hip ratio (WHR) in the BPD, this correlation was not observed in the other two types of surgeries.

	SG N=14		LAGB N=14		BPD N=14	
Variable (Δ)	**r**	***p*.value**	**r**	***p*.value**	**r**	***p*.value**
Body weight (kg)	.091	.768	-.046	.875	.366	.242
BMI (kg/m^2)	.093	.762	-.026	.931	.476	.118
Fat mass (%)	.061	.852	-.369	.194	.314	.347
WHR (cm)	.035	.913	-.208	.495	.633	.037*

***P≤0.05**

3.3. 6 Correlation between Δ CRP with Δ anthropometric weight markers in all surgeries.

Pearson's correlation coefficient analysis was preformed using absolute change (Δ) variables (pre-post surgery percentage change) in each surgery. The correlation was between inflammatory/ cardiovascular marker CRP with weight markers (body weight, BMI, fat mass and WHR). * Represents p-value: * p≤0.01)

3.4 Discussion

This study was designed to investigate the effects of different bariatric surgery procedures on metabolic outcomes of morbidly obese women. The main findings of this study indicate that the number of improvement in cardio-metabolic components, and the resolution in this disease was higher in SG and LAGB at 6 months post-surgery. All three surgeries resulted in significant reduction of their MYD88-dependent inflammatory pathway in abdominal SAT. However, there was a persistence of mild inflammation due to circulating LPS and IL-6 at 6 months post- BPD. Therefore this can explain the lack of improvements that were observed in the number of cardio-metabolic components and the resolution of this disease in the BPD participants in comparison to the SG and LAGB participants.

The effects of the three surgical interventions in this study resulted in significant reduced adiposity and T2DM remission at 6 months, however, unexpectedly, these reductions were not in line with significant improvements in other cardio-metabolic components unanimously post all surgeries. It appears at 6 months SG and LAGB participants had a more rapid improvement in their systolic blood pressure and lipid profile (triglycerides and triglyceride/HDL ratio) and therefore more improved cardio-metabolic outcomes. In contrast, post-BPD participants still appear to be at cardiovascular risk compared to the other two surgeries based on the findings of three specific lipid markers; triglycerides, triglyceride/HDL ratio, and HDL. These markers are routinely used to predict the increased risk in development of cardiovascular disease in adults (Gaziano, J. M. *et al.*,1997, Cooney, M. T. *et al.*,2009, Salazar, M. R. *et al.*,2012), and since there is an increase in triglycerides and triglyceride/HDL ratio and a significant decrease in HDL levels, these participants continue to be at risk, despite reaching normal LDL and total cholesterol levels at 6 months post-surgery (Arsenault, B. J. *et al.*,2011).

Similar to lipids, CRP is regarded as a marker of inflammation and plays a major role in cardiovascular disease assessment and prediction(Albert, M. A.,2000). Therefore, in the present study, CRP concentration was measured for this cohort in line with other pro-

inflammatory markers. These findings indicate that CRP remained unchanged in SG and LAGB and significantly reduced in BPD post-surgery, however as this value is still greater than 3 mg/L, it is considered to be above normal and therefore these participants are at moderate-high risk of cardiovascular disease at 6 months post-surgery (Ridker, Paul M,2003, Abdellaoui, A. *et al.*,2007). In the BPD group there was a positive correlation between CRP concentration and WHR, which is in line with the results reported by other studies (Selvin, E. *et al.*,2007, J., Choi *et al.*,2013). This finding highlights the continued presence of low-grade inflammation in the BPD participants, as they still remain in the high BMI range (BMI≥30 kg/m^2) at 6 months. The lack of change in CRP concentration in LAGB and especially in SG at the 6 months could be due to the duration of the observation period, with some studies reporting that less than 6 months is too early to observe a major reduction in CRP concentration after SG (van Dielen, F. M. *et al.*,2004, Iannelli, A. *et al.*,2011). An additional factor that may influence these findings may arise due to the percentage of excess body fat or weight loss achieved at 6 months, with SG and LAGB groups attaining an average of 17% and 20% excess weight loss respectively, this might not be significant enough to promote a reduction in this marker. One particular study has determined that SG participants had significant reduction in CRP concentrations only after an excess weight loss of more than 40% at 6 months post-surgery (van Dielen, F. M. H. *et al.*,2004), and as such for the present study it might be more accurate to measure CRP levels at 1 year postoperatively.

As a surrogate to CRP, IL-6 a pro-inflammatory marker with a strong association with coronary heart disease was also measured (Danesh, J. *et al.*,2008, 2012). The finding for the current study suggest that IL-6 levels in SG and LAGB did not change due to surgery at the points measured, while the BPD participants achieved a significant increase in this marker at 6 months post-surgery. Since it has been reported that 25% of IL-6 is secreted from adipose tissue (Mohamed-Ali, V. *et al.*,1997), and all of our 42 participants had significant fat mass reduction post-surgery, it was anticipated that IL-6 would decrease in all surgeries. It was especially surprising that IL-6 was significantly increased in the BPD participants as CRP, which is produced due to IL-6 stimulation in the liver was significantly reduced in these participants. The increase in IL-6 levels in this current study could have been due to a number of possibilities. Firstly, lengthy

surgical duration (Anderson, Blaire *et al.*,2013), followed by the prolonged healing process which are all due to more invasive nature of surgeries, such as BPD compared to others is likely to promote an inflammatory state during the first 6 months post-surgery. Secondly, accelerated weight loss can lead to elevated intracellular fatty acid concentrations, which can cause inflammatory lesions in the liver post-weight loss. Severe form of these inflammatory lesions, as well as, liver failure have been observed in BPD participants especially post-surgery (Grimm, I. S. *et al.*,1992). A third possible explanation can be the accelerated weight loss in SG and BPD is comparable to starvation status that occurs in anorexia nervosa participants. Many studies have reported that inflammatory markers were elevated in the malnourished environment of anorexia nervosa, and post-feeding, these markers return back to normal levels (Opara, E. I. *et al.*,1995, Allende, L. M. *et al.*,1998, Nakai, Yoshikatsu *et al.*,1999). Therefore, at approximately 12 months of recovery as the body weight in parallel with metabolic instability improves, it is then most likely that IL-6 would reduce.

Furthermore a fourth scenario could be that translocation of bacterial DNA and pro-inflammatory LPS into the blood of severely obese participants may cause persistent increase in IL-6 levels and other inflammatory markers in some of these participants independent of the significant weight reduction they have achieved via bariatric surgery (Ortiz, Sergio *et al.*,2014). As LPS can exert its damaging effects through activation of TLR-4 and the MYD88-dependent pathway (Yang, Y. *et al.*,2016) causing up-regulation of IL-6 production and secretion, the inflammatory status of LPS, and the markers in this pathway (TLR-4, MYD88, TRAF6, IκB and NFκB) was investigated. This current study showed that LPS levels remained unchanged in LAGB and only slightly reduced in SG and BPD, however these concentrations still remained high enough to up-regulate inflammatory markers such as IL-6 (Li, Yang *et al.*,2016).

The findings of MYD88-dependent pathway have shown significant reduction in all the markers in this pathway in all three surgeries. With both LPS and IL-6 remaining at levels that represent the presence of inflammation, in contrast to the results of MYD88-dependent pathway, it can be therefore assumed that LPS is up-regulating IL-6 through the MYD88-independent pathway also known as the Toll/interleukin-1 receptor-

domain-containing adapter-inducing IFN-β (TRIF)-dependent pathway (Yang, Y. *et al.*,2016).

The final explanation for the IL-6 increases in all the participants, specifically the BPD group, is the dyslipidaemia that occurs as the result of this surgery. These current findings show a significant negative correlation between HDL cholesterol and IL-6 in BPD and not in the other two surgeries. This could arise due to lipid metabolism being affected by IL-6 mechanism through inhibiting lipoprotein lipase activity and lipolysis stimulation, leading to triglycerides and free fatty acids accumulation, and therefore resulting in low-grade inflammation (Sola, E. *et al.*,2009).

Post SG and LAGB surgeries there was a significant decrease in the prevalence of the number of components in cardio-metabolic risk factors, and an increase in the number of participants that have had resolution of their cardio-metabolic disease post these two surgeries. However in BPD participants the prevalence of half of the number of these components were significantly increased, while fewer participants were in cardio-metabolic resolution at 6 months post-surgery in comparison with the other two surgeries. Although rapid weight loss is one of the beneficial outcomes of the BPD surgery, the prolonged inflammatory status and the disrupted lipid metabolism during the initial 6 months post-surgery, can put these participants at further risk of cardiovascular disease.

There are a number of study limitations to this study including; 1) the participants of this study were not placed on a certain dietary regiment and had a relatively sedentary lifestyle pre and post-surgery. 2) This cohort included only European women with T2DM, therefore the results of the surgeries might be different in other ethnicities and men. 3) Weight loss post-bariatric surgery often has a nonlinear pattern in the first year, and rebounds subsequently; therefore long-term data is required to determine whether the cardio-metabolic effects of these different types of bariatric surgeries are maintained in this population.

3.5 Conclusions

This study has demonstrated that equal resolution of inflammation in adipose tissue was achieved post all surgeries, however systemic pro-inflammatory markers appear to persist in BPD participants at 6 months. The following factors such as; technical complexity of the BPD surgery and the operation being time consuming, metabolic disturbances such as dyslipidaemia and malnutrition, in addition to endotoxin activity post-operatively, appears to extends the inflammatory state irrespective of the extensive weight loss that occurs with BPD procedure. Therefore, as BPD participants may still be at risk of cardiovascular disease, participants selected for this surgery need to be monitored closely for markers of inflammation at least for the first 6 months post-surgery.

CHAPTER 4:

Effects of Bariatric surgery on FGF-19 & FGF-21 levels in obese T2DM women

4.1 Introduction

Lipid and glucose metabolism is governed by a number of metabolic hormones, which are secreted from adipose tissue and various other organs such as the pancreas, as well as the gastrointestinal and adrenal glands. These hormones form a combined network for the regulation of substrate utilization and energy balance in response to nutritional status. In the state of obesity, the abnormal secretion and the dysfunction in these metabolic hormones is a major contributing element in the development of obesity related cardio-metabolic disease.

Playing an important role in the regulation of these metabolic hormones are two members of the FGFs superfamily. FGF-19 and FG-F21 have been identified as being involved in various cellular activities such as metabolic regulation. The FGF heparin-binding proteins function mainly through autocrine or paracrine activity, however FGF-19 and FGF-21 display unique structural properties, allowing them to function as endocrine hormones different to other FGFs. FGF- 19 is synthesized in the ileum, (-15 mouse ortholog) which is regulated by bile acids, and also regulates glucose and lipid metabolism (Potthoff, M. J. *et al.*,2011). Previous studies have highlighted the effect of FGF-19 on metabolism, work with obese mice has shown that administration of human FGF-19 to such mice induces a significant dose-dependent decrease in BMI, blood glucose, triglyceride, and insulin sensitivity (Fu, L. *et al.*,2004). This negative correlation between obesity and FGF-19 has been further confirmed in human studies, whereby obese participants have been shown to have a significantly lower FGF-19 level in comparison to the healthy control participants (Gallego-Escuredo, J. M. *et al.*,2015).

FGF-21 regulation within insulin target tissues mainly appears to be active in the liver. Its role in the liver appears to support improvement of metabolic functions by promoting lipid metabolism, insulin sensitivity, and energy expenditure (Angelin, B. *et al.*,2012, Owen, B. M. *et al.*,2015). As this hormone increases during obesity, it has been suggested that obesity coupled by insulin resistance may induce a FGF-21 resistant state (Fisher, ffolliott M. *et al.*,2010). Additionally, studies have shown that patients with cardiovascular disease exhibit significantly elevated FGF-21 levels, which has a

positive correlation with risk factors such as adverse lipid profile and CRP (Chow, W. S. *et al.*,2013, Shen, Y. *et al.*,2013).

Various studies have demonstrated that FGF-19 and FGF-21 are potentially involved in the cardio-metabolic recovery post bariatric surgery (Kyrou, I. *et al.*,2017, Patton, A. *et al.*,2017). It has been suggested that post-bariatric alterations in the metabolism of bile acids and possibly in the gut microbiome, stimulate an elevation in circulating FGF-19, which then leads to the regulation of glucose and lipid metabolism and body adiposity (Kuipers, F. *et al.*,2014, Ryan, K. K. *et al.*,2014, Owen, B. M. *et al.*,2015). However this behaviour of FGF-19 post bariatric surgery is not unanimously supported, with at least one study stating that an increase in FGF-19 levels is stimulated more post bariatric surgery regardless of surgery type (Gomez-Ambrosi, J. *et al.*,2017); whilst another study suggests that changes in FGF-19 levels are surgery dependent (Martinez de la Escalera, Lucia *et al.*,2017). Mice studies have also indicated that FGF-21 might be involved in preventing increased hunger and weight regain post-bariatric surgery (Morrison, Christopher D. *et al.*,2016). In humans FGF-21 has been labelled as the marker of metabolic stress (Crujeiras, A. B. *et al.*,2017), with most studies reporting a reduction in this hormone after specific bariatric surgeries (Fjeldborg, K. *et al.*,2017, Gomez-Ambrosi, J. *et al.*,2017).

Currently, there is limited insight on the changes in both FGF-19 and FGF-21 across bariatric surgery types, and the associated influence this may have on anthropometric and biochemical biomarkers.

Therefore, the aims of this study were to:

1. Investigate the effects of SG, LAGB and BPD on FGF-19 and FGF-21 levels at 6 months post surgery.

2. Define the changes in FGF-19 and FGF-21 levels in participants at high, medium and low risk of cardio-metabolic disease within each surgery.

3. Compare the effects of FGF-19 and FGF-21 on cardio-metabolic risk factors, by using glucose and lipid profile markers, within all three surgeries.

4.2 Subjects and Methods

For this study the same cohort along with anthropometric and biochemical measurements as chapter 3, section 3.2 were used.

4.2.1 Cardio-metabolic disease criteria

Cardio-metabolic disease was characterised by using IDF standards (Alberti, George *et al.*,2006). A high-risk group was defined as participants that had resolution in one component, medium risk group had resolution in 2 components, and low risk group had resolution in 3 or more components of cardio-metabolic disease post surgery.

4.2.2 Quantitation of inflammatory markers

The concentrations of CRP were assessed through ELISA (DCRP00, R&D Systems), with the assay detection range of 0.8-50 ng/mL, coefficient of variance of 3.8% intra-assay, and 7% inter assay precision.

4.2.3 FGF-19 and FGF-21 Serum levels

Serum FGF-19 (pg/mL) was measured by using ELISA kit (DF1900, R&D Systems). Measurements were preformed in duplicates based on the manufacturers instructions. This assay has a detection range of 15.6-1000 pg/mL, and a coefficient of variation of 4.5 % for intra-assay and 5.5 % inter-assay precision. Serum FGF-21 (pg/mL) was measured by using ELISA kit (DF2100, R&D Systems). Following the manufacturers instructions measurements were preformed in duplicates. The detection range of this

assay is 31.3-2000 pg/mL, and the coefficient of variation of the intra-assay is 2.9% with 6.4% inter-assay precision.

4.2.4 Statistical Analysis

Data were reported as mean and SE. Shapiro-Wilks test was applied to normalise the data. Within surgery group statistical significance (pre to post) was determined by paired two-tailed t-test or one-way ANOVA (if parametric) and Kruskal-Wallis test (if non-parametric) was used for the comparison of between surgery groups. Welch's t-test was used for high, medium and low risk group comparison. Pearson correlation analysis was done between changes in variables (Δ), which were calculated as percentage absolute change [((post surgery-pre surgery)/pre surgery) x100]. Multivariate linear regression (forward model) was preformed for ΔFGF-19 and ΔFGF-21 as dependent variables with the variables (Δ) that had shown significant correlation with ΔFGF-19 and ΔFGF-21 as independent variables. All statistical analysis was preformed by using SPSS 24.0.

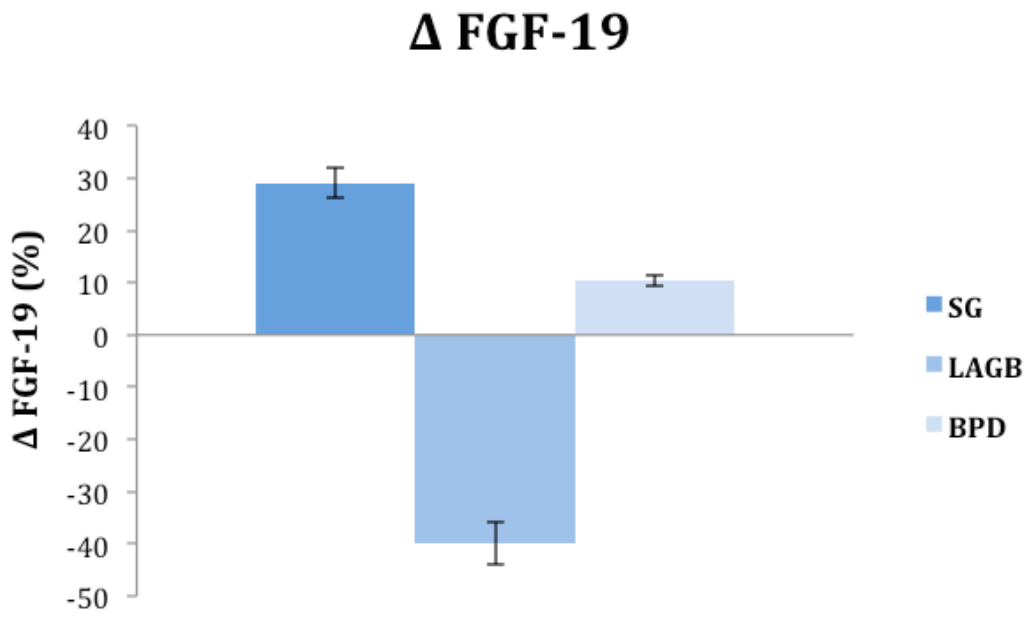
4.3 Results

4.3.1 Body composition and metabolic variables

The results in this section are same to that of chapter 3, section 3.3.1.

4.3.2 SG caused an increase in FGF-19 level post surgery

Percentage absolute change in FGF-19 increased by 29% in the SG, LAGB decreased by 39% and BPD had the least increase with 10%. The percentage change in pre-to post FGF-19 was not significant in any of the three surgeries, however there was significant difference between SG and LAGB in FGF-19 absolute change (p=0.004).

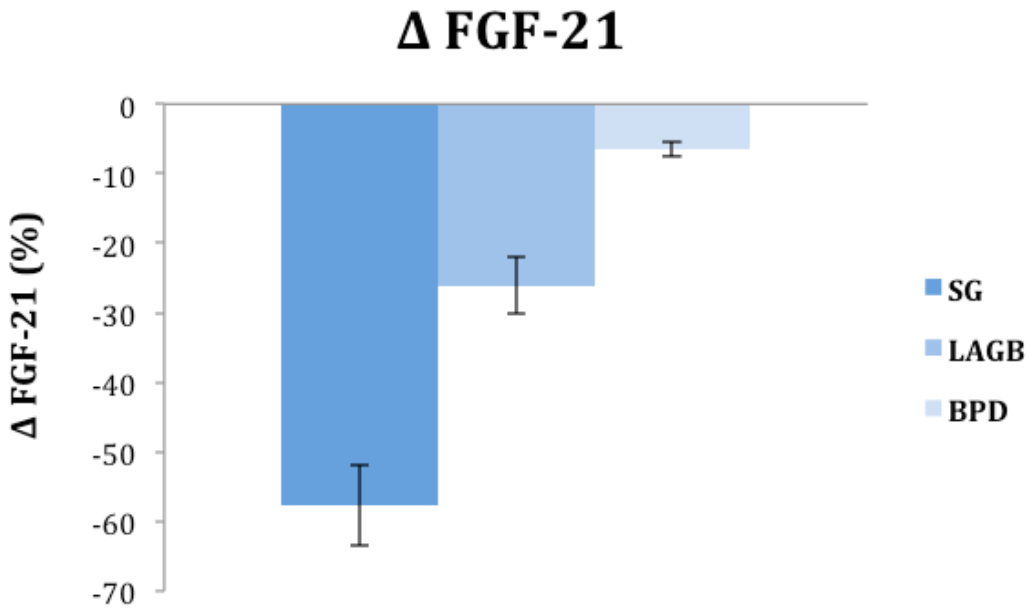


4.3. 1 Effect of SG, LAGB and BPD serum FGF-19 absolute change pre- to post surgery.

Paired two-tailed t-test was used for the within group comparison of pre to post surgery levels, while ANOVA test determined the significant difference in serum FGF-19 between SG and LAGB groups (p=0.004) SG: sleeve gastrectomy, LAGB: laparoscopic adjustable gastric banding, biliopancreatic diversion.
(* Represents p-value: ** $p \leq 0.01$)

4.3.3 SG caused a decrease in FGF-21 levels post surgery

Percentage absolute change in FGF-21 decreased by 57% in the SG, LAGB decreased by 26% and BPD had the least decrease with 6%. The percentage change in pre-to post FGF-21 was not significant in any of the three surgeries, however there was significant difference between SG and BPD in FGF-21 absolute change (p=0.050).

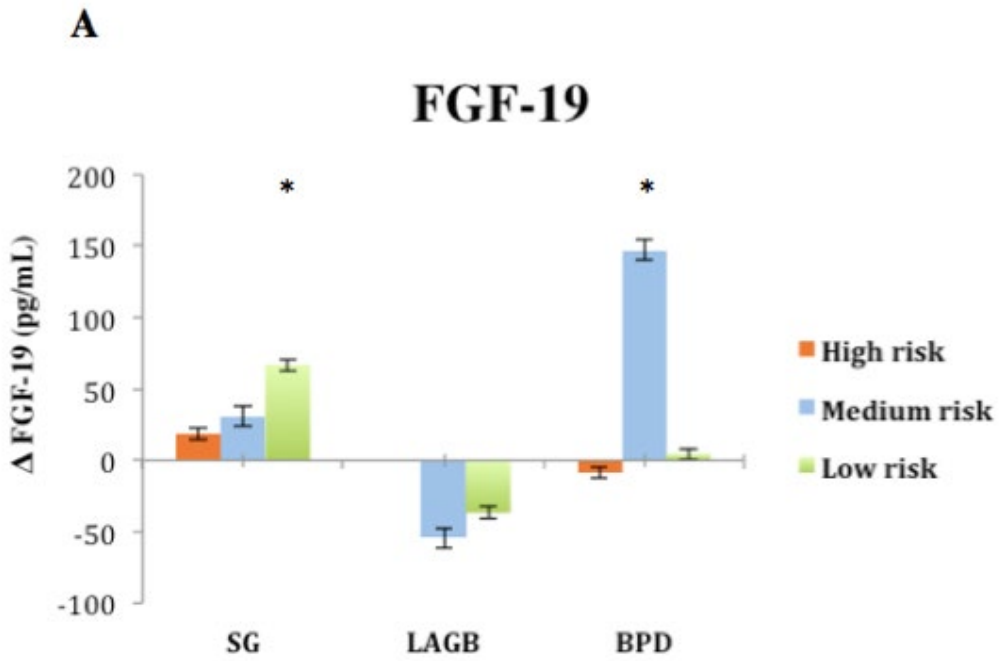


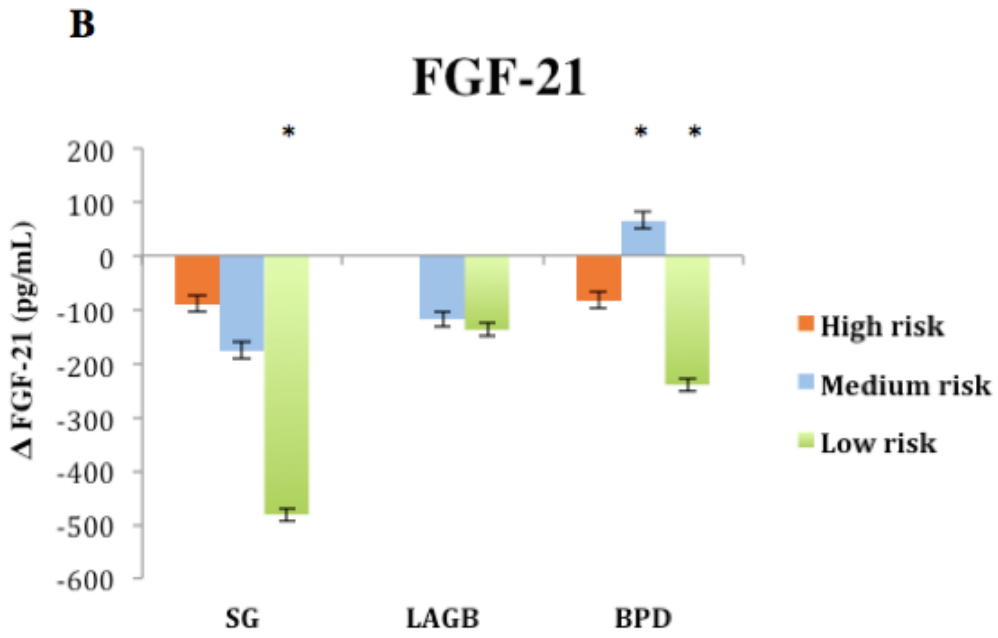
4.3. 2 Effect of SG, LAGB and BPD on serum FGF-21 absolute change pre- to post surgery.

Paired two-tailed t-test was used for the within group comparison of pre to post surgery levels, while ANOVA test determined the significant difference in serum FGF-21 between SG and LAGB groups. (p=0.050) SG: sleeve gastrectomy, LAGB: laparoscopic adjustable gastric banding, biliopancreatic diversion. (* Represents p-value: * $p \leq 0.05$)

4.3.4 In SG participants, significant increase in FGF-19 and significant decrease in FGF-21 was in the low risk group.

In the SG group there was an overall increase in FGF-19 concentration, however, there was a significant increase in FGF-19 in the individuals who are at low risk of cardio-metabolic risk at 6 months post surgery. FGF-19 decreased in LAGB participants in both medium and low risk groups. In the BPD group, FGF-19 had decreased in the high-risk group, while there was a significant increase of this hormone in the medium risk group (4.3.4 A). There was an overall decrease in FGF-21 in the SG participants; however, there was a significant decrease in the low risk participants in this group. LAGB participants had a decrease in FGF-21 in both medium and low risk participants, although this was not significantly different between these two risk groups. In the BPD group, FGF-21 was significantly increased in the medium risk group; while this hormone was significantly decreased in the low risk group at 6 months post surgery (4.3.4 B).





4.3. 3 FGF-19 and FGF-21 concentrations absolute change pre- to post surgery stratified by cardio-metabolic risk.

These figures illustrate the Δ FGF-19 **(A)** and Δ FGF-21 **(B)** concentrations, which are divided into SG participants at low cardio-metabolic risk (n= 6 of 14), medium cardio-metabolic risk (n=4 of 14) and high cardio-metabolic risk (n=4 of 14). LAGB was divided into low risk (n= 10 of 14) and medium risk (n=4 of 14). BPD was divided into low risk (n=4 of 14), medium risk (n= 5 of 14) and high risk (n=4 of 14) groups. Welch's t-test was used for the comparison of high, medium and low risk groups. (* Represents p-value: * $p \leq 0.05$)

4.3.5 FGF-19 correlation with anthropometric and metabolic variables

Percentage change in pre-to post FGF-19 was positively correlated with HDL in the SG group. There was also a positive correlation between FGF-19 and triglyceride/HDL ratio in the LAGB group, while this hormone had a negative correlation with BMI, plasma insulin and HOMA-IR in the BPD group.

Δ FGF-19

Surgery	SG		LAGB		BPD	
Variable ((Δ)	r	P value	r	P value	r	P value
Body weight	-0.320	0.265	0.097	0.778	-0.507	0.092
BMI	-0.381	0.179	0.077	0.823	-0.605*	0.037
Fat mass %	-0.456	0.101	0.039	0.910	-0.105	0.758
WHR	-0.097	0.740	0.095	0.794	-0.391	0.234
Systolic BP	-0.175	0.567	0.424	0.295	-0.411	0.239
Diastolic BP	-0.523	0.067	-0.645	0.061	-0.124	0.734
MAP	-0.405	0.170	-0.306	0.424	-0.077	0.832
HbA1c	-0.320	0.264	0.331	0.350	-0.398	0.255
Plasma glucose	-0.040	0.892	0.231	0.495	-0.178	0.579
Plasma insulin	-0.216	0.458	0.339	0.308	-0.712*	0.014
HOMA-IR	-0.252	0.385	0.405	0.217	-0.741**	0.009
Total cholesterol	-0.160	0.586	0.081	0.812	-0.302	0.339
LDL-Cholesterol	0.058	0.843	0.083	0.808	-0.200	0.533
HDL-Cholesterol	0.597*	0.024	0.398	0.225	-0.117	0.717
Triglycerides	-0.396	0.161	0.578	0.063	-0.056	0.863
Triglyceride/HDL	-0.432	0.123	0.627*	0.039	0.022	0.946
CRP	-0.536	0.072	-0.163	0.631	-0.137	0.688

4.3. 4 Correlation between FGF-19 and anthropometric and metabolic variables.

Pearson correlation analysis was used to determine the correlation between surgical-induced changes in pre-to post FGF-19 concentration and the changes in other variables. BMI, body mass index; WHR, waist to hip ratio; MAP, mean arterial pressure; HbA1c, glycated haemoglobin; HOMA-IR, Homeostatic assessment model of insulin resistance; CRP, C-reactive protein. (* Represents p-value: * $p \leq 0.05$, ** $p \leq 0.01$)

4.3.6 FGF-21 correlation with anthropometric and metabolic variables

Percentage change in pre-to post FGF-21 was positively correlated with body weight, BMI and WHR, additionally this hormone has a positive correlation with plasma glucose in the SG group. FGF-21 correlates negatively with diastolic blood pressure in LAGB. In the BPD group, FGF-21 is positively correlated with fat mass percentage and HDL.

FGF-21

Surgery	SG		LAGB		BPD	
Variable ((Δ)	r	P value	r	P value	r	P value
Body weight	0.732**	0.007	0.261	0.412	-0.386	0.193
BMI	0.783**	0.003	0.291	0.359	-0.537	0.058
Fat mass %	0.422	0.196	0.243	0.447	-0.599*	0.040
WHR	0.614*	0.044	0.510	0.109	-0.201	0.532
Systolic BP	0.436	0.207	0.511	0.160	-0.296	0.406
Diastolic BP	0.309	0.386	-0.652*	0.041	-0.274	0.444
MAP	0.418	0.229	0.290	0.417	-0.292	0.412
HbA1c	0.140	0.681	0.256	0.448	-0.076	0.823
Plasma glucose	0.762**	0.004	0.538	0.071	-0.289	0.338
Plasma insulin	0.131	0.686	0.327	0.300	-0.146	0.650
HOMA-IR	0.336	0.286	0.521	0.083	0.028	0.931
Total cholesterol	0.366	0.241	0.017	0.959	-0.257	0.397
LDL-C	-0.085	0.792	0.034	0.917	-0.156	0.611
HDL-C	0.013	0.968	-0.345	0.273	-0.505*	0.048
Triglycerides	0.171	0.595	0.080	0.805	0.236	0.438
Triglyceride/HDL	0.163	0.614	0.007	0.983	0.366	0.218
CRP	0.354	0.316	-0.204	0.525	-0.426	0.191

4.3. 5 Correlation between FGF-21 and anthropometric and metabolic variables.

Pearson correlation analysis was used to determine the correlation between surgical-induced changes in pre-to post FGF-21 concentration and the changes in other variables. BMI, body mass index; WHR, waist to hip ratio; MAP, mean arterial pressure; HbA1c, glycated haemoglobin; HOMA-IR, Homeostatic assessment model of insulin resistance; CRP, C-reactive protein. (* Represents: P-Value: * $p \leq 0.05$, ** $p \leq 0.01$

4.3.7 FGF-19 predicted by HOMA-IR in BPD participants

The model that best predicted the change in FGF-19 concentration included only the changes in HOMA-IR as predictive variable. Based on this model 61% of the BPD surgical-induced change in FGF-19 can be predicted by the changes in HOMA-IR.

Variable	r^2	β	P value
Model	0.615		0.007**
ΔHOMA-IR		0.784	0.007**

4.3. 6 Multiple liner regression analysis with ΔFGF-19 as dependent variable in the BPD group.

Variables that were excluded in the forward model were: ΔBMI and Δ plasma insulin. Values are presented as adjusted r^2 (r^2), standardized coefficients (β) and associated p values, absolute change in homeostatic assessment model of insulin resistance (HOMA-IR). (* Represents p-value: * $p \leq 0.05$, ** $p \leq 0.01$)

4.3.8 FGF-21 predicted by HDL cholesterol in BPD participants

The model that best predicted the change in FGF-21 concentration was the change in HDL cholesterol. Based on this model 41% of the changes in the BPD surgical-induced change in FGF-21 can be predicted by the change in HDL.

Variable	r^2	β	P value
Model	0.412		0.033*
ΔHDL		-0.642	0.033*

4.3. 7 Multiple linear regression analysis with ΔFGF-21 as dependent variable in the BPD group.

Variables that were excluded in the forward model were: Δfat mass%. Values are presented as adjusted r^2 (r^2), standardized coefficients (β) and associated p values, absolute change in high-density lipoprotein (HDL). (* Represents p-value: * $p \leq 0.05$, ** $p \leq 0.01$)

4.4 Discussion

This study addressed the impact of FGF-19 and FGF-21 status pre and post surgery, and whether the type of surgery may have influenced a change in these hormones. The findings determined that FGF-19 was altered by surgery type, with the highest FGF-19 concentration in SG participants, who also shown to have the best cardio-metabolic outcomes at 6 months. Concurrently FGF-21 was also affected by surgery type, with the lowest concentration observed in SG participants that were in low risk of cardio-metabolic disease post surgery. Additionally, the results of this study indicated that potentially FGF-19 status could be used as a marker for the prediction of metabolic improvement in glucose, while FGF-21 can be utilised as marker for prediction of lipid status post BPD surgery.

In this study, FGF-19 concentration increased as an effect of SG and BPD, whilst it decreased as the result of LAGB surgery. Studies have suggested that LAGB surgical procedure does not apply the gastrointestinal anatomy changes, which is needed in order to elicit an effect for this particular endocrine hormone to increase (Pournaras, Dimitri J. *et al.*,2012, Lips, M. A. *et al.*,2014). Interestingly as BPD participants had the most substantial weight loss in comparison to SG participants, it was expected for FGF-19 concentration to be higher in these participants, however this was not the case. The possible underlying reasons for the apparent disconnect between FGF19 concentration in BPD patients could be due to the gastrointestinal rearrangement, which takes place as part of the surgery. The physical separation of bile acids from nutrients (Albaugh, VL *et al.*,2016) can change nutritional absorption and therefore the intestinal microenvironment resulting in altered FGF-19 concentration.

Bile acids are synthesized from cholesterol in hepatocytes, and are highly regulated by FXR activation, which results in stimulation of FGF-19 transcription in the ileum. There might be a possibility that BPD surgery has an effect on bile acid transport since reduced surface area of ileum, as a result of this surgery, which would lessen the exposure and absorption of bile acids. It is predicted that BPD simulates the effects of

blocking intestinal bile acid absorption through the use of bile acid sequestrants, which increases the cholesterol into bile acids conversion (Einarsson, K. *et al.*,1991, Root, C. *et al.*,2002) and, therefore reduces total cholesterol levels post surgery (Scopinaro, N.,2012). These effects disrupt hepatic bile acid synthesis leading to bile acid sequestrants causing reduced FGF-19 concentration in fasted or fed state (Brufau, G. *et al.*,2010, Beysen, C. *et al.*,2012). This mechanism may in effect be in the present study as well, since BPD participants have had the most significant reduction in total cholesterol and the least increase in FGF-19 in comparison with SG.

Secondly, bile acid antimicrobial activity can affect the composition and quantity of gut microbiota (Merritt, M. E. *et al.*,2009, Islam, K. B. *et al.*,2011, Li, T. *et al.*,2015). This means that there is a close integrated relationship between bile acids and gut microbiota, in that bile acids affect the colonisation and development of intestinal bacteria, while bacteria get involved in the biotransformation of bile acids (Li, T. *et al.*,2014). This equilibrium between gut microbiota and bile acid pool size gets disrupted post BPD surgery, due to increased bile acid flow into the colon and the gut microbiota overgrowth. In such environment, metabolic effect of bacteria results in deconjugation of bile acids and secondary bile acids production (Tomkin, Gerald H. *et al.*,2016). Hepatocytes reabsorb majority of these unconjugated bile acids, however, some manage to affect the intestinal barrier permeability and enter the systemic circulation (Kaska, L. *et al.*,2016), resulting in high plasma bile acid concentration. Importantly, the secondary bile acid compounds produced by these bacteria are potent FXR antagonists thus lowering the FXR-FGF-19 expression (Sayin, Sama I *et al.*,2013, Flynn, Charles Robb *et al.*,2015).

Although the mentioned mechanisms affect the bile acid transport and synthesis, which blunts the increase of FGF-19 at 6 months post BPD, it is possible that the bile acid sequestrants actively play a role in improving glycaemia (Chen, L. *et al.*,2012, Prawitt, J. *et al.*,2014). The activity of these bile acid sequestrants appears to be influenced by increased glucose uptake or increased secretion of glucagon-like peptide 1 (GLP-1), which arises with elevated bile acids or free fatty acids signalling within the intestine (Hofmann, A. F.,2011, Prawitt, J. *et al.*,2014). Therefore, there is a possibility that the reduced bile acid or nutrient uptake in the ileum contributes to the GLP-1 increase, post

BPD surgery (Valverde, I. *et al.*,2005, Astiarraga, B. *et al.*,2013). Additionally glucose metabolism is further improved post BPD surgery due to the exclusion of the longer intestinal limb from the alimentary passage followed by concentrated bile acid transition and extreme caloric restriction, which collectively result in a greater impact on improvement of HOMA-IR (Kaska, L. *et al.*,2016). In line with these findings, in the present study BPD participants have had the most significant improvement in their HbA1c, and a similar significant improvement in their HOMA-IR in comparison with SG. Additionally, similar to what has been reported in other malabsorptive bariatric procedures (Gerhard, Glenn S. *et al.*,2013), the results of this study indicates that in BPD participants changes in FGF-19 is a predictor of HOMA-IR status post surgery. Therefore, this endocrine hormone can potentially be a predictor of T2DM improvement in this category of participants.

The increase in FGF-19 concentrations in SG and BPD participants seems to be concurrent with the decrease of FGF-21 concentrations in the same groups. However, it was surprising that FGF-21 had the least decrease in the BPD group as they had the most substantial weight loss and glucose homeostasis improvement compared to SG participants. There are a few mechanisms that may explain this observation. Firstly, the mechanical changes to the gastrointestinal anatomy, which results in the extreme calorie restriction post BPD triggers both amplified postprandial insulin followed by increased free fatty acid and glucagon secretions. These " fed and fasted signals" activate hepatic PPAR α, which in return could activate the hepatic FGF-21 expression, and therefore, can maintain the elevated levels of this hormone (A., Lips Mirjam *et al.*,2014) at 6 months post BPD. In this environment an increase in glucagon stimulates the increase in lipolysis (Perea, A. *et al.*,1995, Duncan, Robin E. *et al.*,2007), resulting in dyslipidaemia (hypertriglyceridemia and low HDL cholesterol) (Ryden, M. *et al.*,2017), which may explain the observations in this study of BPD participants at 6 months post surgery. The maintained elevated FGF-21 concentration is beneficial as this hormone has an inhibitory effect on lipolysis, which can help to retain the distribution of lipids between adipose tissue and liver thus preventing lipotoxicity caused by continuous increase of free fatty acids (Ge, Xuan *et al.*,2012) after BPD.

Additionally, in this study the small reduction in FGF-21 levels (6%), followed by the significant decrease in BMI at 6 months post BPD could indicate a partial normalisation of FGF-21 sensitivity, therefore, stimulating further secretion of FGF-21 and maintaining the increased levels of this hormone. The normalization of FGF-21 sensitivity has also been reported after other malabsorbitive and restrictive surgeries (D., Haluzíková *et al.*,2013, Gómez-Ambrosi, Javier *et al.*,2017).

In the present study, the participants that were still at high risk of cardio-metabolic risk at 6 months post BPD had the least reduction in their FGF-21 concentration, and the most reduction in this hormone was in the low risk group.

Mounting evidence from animal and human-based research indicates that increased FGF-21 levels are associated with cardio-metabolic disease (Kharitonenkov, Alexei *et al.*,2005, Kharitonenkov, A. *et al.*,2007, Zhang, X. *et al.*,2008, Gallego-Escuredo, J. M. *et al.*,2015). One study has demonstrated that increased FGF-21 concentration can independently be a risk factor for cardio-metabolic disease. Furthermore, it was shown that this association was significantly more effective than the impact of individual components of this disease (Zhang, X. *et al.*,2008). This FGF-21 and cardio-metabolic risk factor trend was observed in SG and LAGB as well.

The effect of FGF-21 on cardio-metabolic disease, especially its regulatory role in lipid metabolism has been further demonstrated by genetic studies. Genetic association analysis on a white European hypertensive cohort was preformed in order to analyse whether polymorphisms in the FGF-21 signalling pathway may be associated with cardio-metabolic risk. The findings of this previous study showed that few single nucleotide polymorphisms in genes reside in the FGF-21 pathway (FGFR1, FGFR2, FGFR3, and KLB), which are associated with LDL, HDL, and triglycerides (Kaess, B. M. *et al.*,2010). In the present study, although genetic analysis was not undertaken, regression analysis showed that 41% of the changes in HDL could be predicted by FGF-21 change in the BPD participants. Therefore FGF-21 could be used as a marker for cardio-metabolic improvement, in specific, for a more in depth lipid profile assessment at least for the first 6 months post BPD surgery, as this group may still be at risk of this disease in comparison with SG and LAGB groups.

The limitation of this study was the small sample size in regards to the grouping of the participants based on the cardio-metabolic risk factor.

4.5 Conclusions

Bariatric surgery types impact endocrine hormones in different ways. At 6 months post surgery, SG resulted in a higher increase in FGF-19 concentration, specifically in participants that were at low risk of cardio-metabolic, in comparison to BPD surgery. Additionally in the SG group, especially the low risk participants had a significantly lower FGF-21 concentration than the BPD group. The lack of significant increase in FGF-19 and decrease in FGF-21 post BPD at 6 months post surgery in combination with micro and macro-nutrient deficiencies can still place these participants at risk of cardio-metabolic disease despite the significant weight loss that these participants had. Therefore, emphasizing the lack of suitability of BPD, especially when procedures such as SG can achieve significant weight loss along with higher resolution of cardio-metabolic components without the long-term complication rates and nutritional malabsorption.

Chapter 5:

Effect of liraglutide on adipose tissue angiotensinogen protein in mature adipocytes

5.1 Introduction

The RAS has been well documented in human physiology for its role in regulating blood pressure along with electrolyte and fluid balance. Within RAS the protein AGT, as a key precursor to derive all bioactive angiotensin peptides, is primarily synthesised in the liver and undergoes enzymatic cleavages to produce other components of this system (Lavoie, J. L. *et al.*,2003). It has also been shown that all components of RAS, including derivatives of AGT are produced in adipose tissue and secreted from mature adipocytes (Engeli, Stefan *et al.*,2003, Gustavo-Salom *et al.*,2013). This has led to studies examining the role of RAS as an important influence in the pathogenesis of obesity related cardio-metabolic disease (Must, A. *et al.*,1999, Schmieder, R. E. *et al.*,2007). Studies to date have shown that with increasing weight gain increased AGT secretion occurs from mature adipocytes. This production of AGT and ANG II, the active metabolite, are considered a relevant contributing source in the development of hypertension (Harte, et al, 2005). Studies in mice have also shown that excess secretion of adipose specific AGT contributes to as much as a 30% increase in plasma ANG II levels, with mice developing hypertension and insulin resistance. While adipose-specific AGT knockout mice appear to lead to a 25% decrease in AGT plasma levels, demonstrating the endocrine effect of adipose AGT (Kalupahana, N. S. *et al.*,2012). Beyond blood pressure changes adipose specific AGT also appears to reduce lipolysis and stimulate lipogenesis whilst inhibiting adipogenesis and promoting production of pro-inflammatory adipocytes (Kalupahana, N. S. *et al.*,2012). These effects of adipocyte AGT synthesis influences and contributes significantly to the systematic RAS, which is important in developing obesity related hypertension.

To date it is known that various nutrients, such as elevated salt intake and high fat diets (Serazin-Leroy, Valérie *et al.*,2000) as well as hormones (Harte, A. *et al.*,2005) can regulate adipose AGT expression. In addition, the inflammatory mediator, LPS may also play an important role in inducing adipose AGT expression in obesity related hypertension (Gao, Meng *et al.*,2014), although studies to date have been limited to explore this concept in human metabolism. A prior study of spontaneously hypertensive rats has shown that pre-treatment with LPS results in elevated AGT mRNA levels in

adipose tissue. Furthermore LPS can induce pro-inflammatory cytokines such as TNF-α, as the hypertensive rats were noted to increase their TNF-α mRNA levels in adipose tissue. Overall, this implied that LPS might induce TNF-α to affect AGT gene in adipose tissue of hypertensive rats (Nyui, N. *et al.*,1997).

GLP-1 agonist liraglutide used as T2DM therapy, has similarly been demonstrated to have the capability to exert metabolic effects on RAS components of various tissues. Studies have shown that liraglutide appears to decrease renal tissue angiotensin II concentration in T2DM, which results in protection from the kidney damage (Skov, J. *et al.*,2016). Whilst in rodent models of T2DM with pulmonary disorder, liraglutide appears to have a significant effect in increasing lung tissue ACE2 expression, which leads to improvements of the cardiopulmonary complications, associated with diabetes (Romaní-Pérez, Marina *et al.*,2015). Perhaps less well studied are the metabolic effects of this medication on adipose specific AGT.

Prior molecular studies have also shown that liraglutide can influence inflammation and adipogenesis, however these effects appear dependant on the origin and the differentiation stages of adipocytes. Studies with mature adipocytes, have identified liraglutide to affect inflammation through reducing TNF-α and elevating adiponectin expression (El Bekay, Rajaa *et al.*,2016); whilst in obese stem cells it also elevates adiponectin expression (Cantini, G. *et al.*,2015). In addition, other studies have shown that liraglutide appears to have an anti-adipogenic effects on adipose stem cells derived from obese patients (Cantini, G. *et al.*,2015) causing a reduction in adipogenesis and lipogenesis-related genes, while increasing lipolytic gene expression (El Bekay, R. *et al.*,2016). Whilst rodent adipocyte cells studies using 3T3-L1 has shown that liraglutide causes reduced apoptosis, while stimulating adipocyte differentiation and proliferation in pre-adipocytes of these cells (Challa, T. D. *et al.*,2012).

The primary effect of liraglutide on insulin sensitivity appears to be cell type dependent as well. It has also been reported that in 3T3-L1 adipocytes (Gao, H. *et al.*,2007) and pre-adipocytes (Yang, J. *et al.*,2013), treated with GLP-1 causes enhanced glucose uptake and insulin sensitivity, whereas in human adipocyte stem cells it appears to have an inhibitory effect on glucose uptake. Although these studies demonstrate the

beneficial effect of liraglutide on RAS and its components in other tissues, the influence of this drug on AGT, specifically in human adipocytes has not been explored in detail.

As such, the aims of this study were to:

1. Investigate the potential *in vitro* effects of different concentrations of liraglutide with and without the insult of LPS on AGT of lean and obese human SAT mature adipocytes.
2. Evaluate whether liraglutide interferes with cellular proliferation of human lean and obese mature adipocytes.
3. Assess the role of liraglutide on improving insulin sensitivity

5.2 Subjects and Methods

5.2.1 Subjects

SAT samples were obtained from 3 lean female participants (mean age ± SD: 35 ± 3 years; BMI ± SD: 21.7 ± 2.4 kg/m^2), and 3 obese female participants (mean age ± SD: 32 ± 2 years; BMI ± SD: 43 ± 4.2 kg/m^2). All human tissues were obtained through elective surgery in accordance with ethical committee approval, at University Hospital Coventry and Warwickshire. Lean participants on endocrine, T2DM, or antihypertensive therapy were excluded. Additionally obese participants affected by insulin resistance, infections, or on cancer and steroid therapies were also excluded.

5.2.2 Isolation and culture of primary adipocytes

The isolation of adipocytes from abdominal SAT was collected as previously reviewed (Rodbell, M.,1964). Adipogenesis was induced in the primary preadipocytes for 14 days. Mature adipocytes were treated with different concentrations of liraglutide (10 nM, 100 nM) which was gifted by Novo Nordisk, LPS (10 ng/ml, 100 ng/ml) (Sigma L2630), and different combinations of liraglutide and LPS: (LPS 10 ng/ml + Liraglutide 10 nM), (LPS 10 ng/ml + Liraglutide 100 nM), (LPS 100 ng/ml + Liraglutide 10 nM), and (LPS 100 ng/ml + Liraglutide 100 nM) for the following lengths of time: 24, 48 and 72 hours, with fresh treatment exposure every 24 hours. Adipocytes in untreated media were used as controls.

5.2.3 Cell count

Seeded adipose SAT cells were treated for 24, 48 and 72 hours, then trypsinized and counted by the hemocytometer. The mean of cell number was attained by performing four replicates for each point of every experiment.

5.2.4 Glucose uptake measurement

Seeded cells were grown to confluence, and glucose uptake assay method was performed as previously described (Liu, Fang *et al.*,2001). SAT cells were grown to confluence. These cells were washed with PBS and incubated at 37 °C for 3 hours with KHB with BSA (0.01%) and glucose (5 mmol/L) and for insulin and serum starvation. At 37 °C, cells were incubated for 30 minutes with KHB either without glucose or BSA, and further treated with DMSO (control) or 100nM of insulin (Sigma # 19278) in the presence of 10nM or 100nM of liraglutide. 2-deoxy-[^{3} H]D-glucose [1 μCi/μl) (PerkinElmer) was then added and cells were incubated for 10 minute. Finally cells were washed and harvested, and cell lysate was then transferred to scintillation and radioactivity was then measured.

5.2.5 Protein isolation, immunoprecipitation and western blotting

Following treatments, cells were harvested for protein extraction and quantified (Jaubert, A. M. *et al.*,1995). Immunoprecipitation was preformed by the use of SureBeads, Protein A magnetic beads (Bio-Rad #161-4013), based on the manufacturers standard protocol. AGT antibody was added to the magnetic beads and re-suspended and rotated in room temperature. The magnetized and washed beads containing the antibody were then added to liraglutide/LPS samples and incubated and rotated in room temperature. The beads were then washed, magnetized, and centrifuged. Residual buffer was removed and the cells were incubated in laemmli buffer for 10 minutes at 70 °C. Finally, the cells were magnetized and the eluent was ready for the western blot.

For protein analysis, primary antibody AGT 1:5000 (ab103549) was utilised for western blot method as previously described (Alhusaini, Saif *et al.*,2010). This procedure was replicated at least three times for each sample for all the time points.

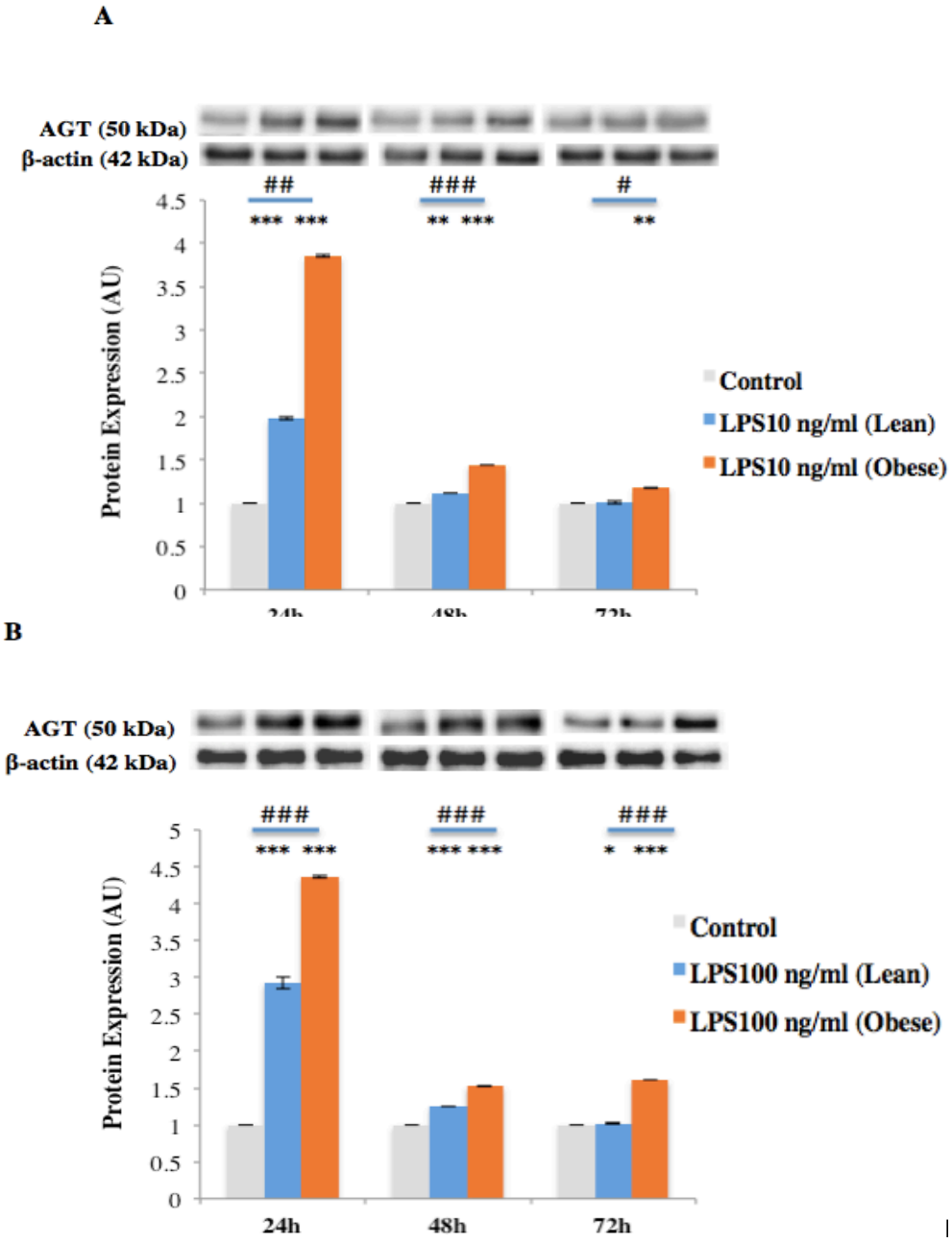
5.2.6 Statistical analysis

The data was reported as mean and SE. Shapiro-Wilks test was applied to normalize the data. Two-tailed paired sample t-test was used for the statistical analysis of between two groups and one-way ANOVA was used for multiple comparisons. All statistical analyses were preformed by using SPSS 24.0 software.

5.3 Results

5.3.1 AGT protein expression in cultured mature SAT lean and obese adipocytes

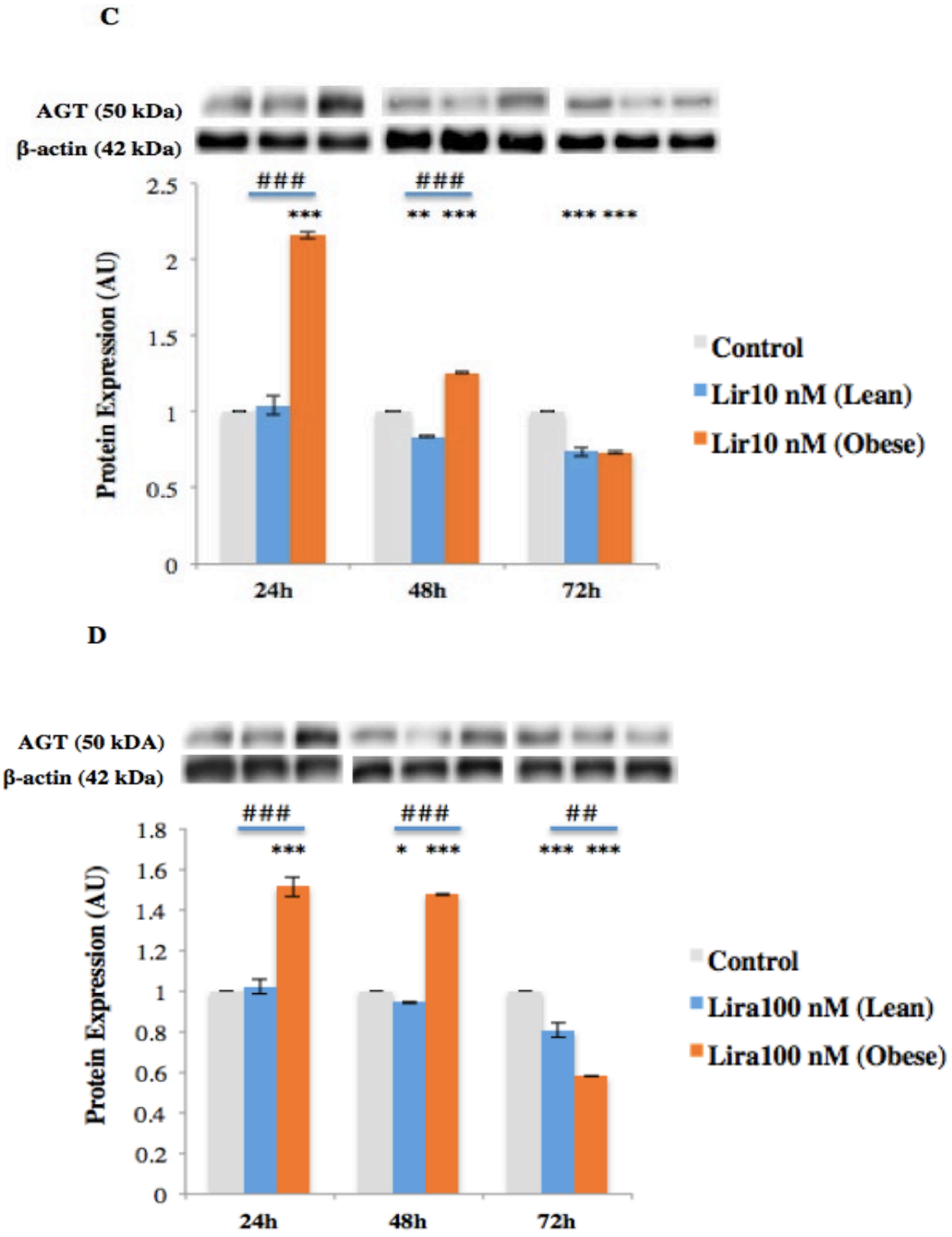
AGT protein expression was significantly higher in both lean and obese adipocytes at 24 hours post treatment with LPS 10 ng/mL ($p<0.05$), however, obese adipocytes seemed more sensitive to the insult of LPS than lean adipocytes. This elevated AGT expression was significantly reduced at 48 and 72 hour treatments in lean and obese adipocytes in comparison to the 24 hour treatment (Figure 5.3.1.1 A). The effect of LPS 100 ng/ml on AGT expression similarly to LPS 10 ng/ml is more dependent on time rather than the dose used (Figure 5.3.1.1 B).



5.3.1. 1 Angiotensinogen (AGT) protein expression in lean and obese adipose tissue treated with different concentrations of lipopolysaccharides over 24, 48 and 72 hours.

The graph shows the expression of AGT (molecular weight 50 kDa) in mature SAT adipocytes of lean and obese, treated with; **A)** LPS 10 ng/ml, **B)** LPS 100 ng/ml, three different time points. The absence of treatment was taken as the control. The data is expressed as mean ± SEM (lean n=3, obese n=3); comparison between lean/obese and control group was analysed by t-test (* Represents p-value: *≤0.05, **≤0.01, ***≤0.001). The comparison between lean and obese groups was performed by ANOVA analysis (# Represents p-value: #≤0.05, ##≤0.01, ###≤0.001).

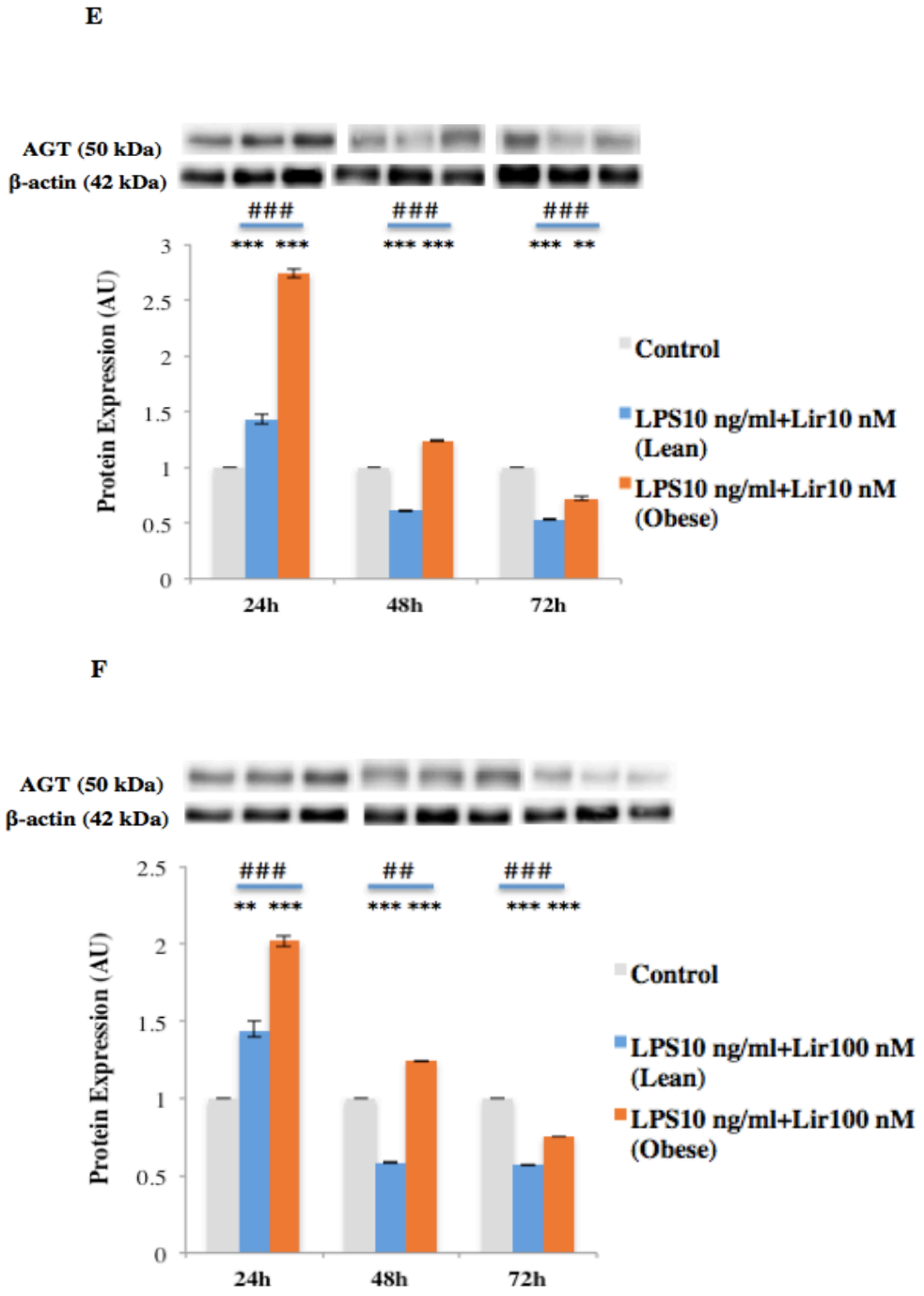
The 24 hour treatment of liraglutide 10nM exerted a significant increase in AGT expression in obese adipocytes, while this treatment did not have any effect on the lean adipocytes in comparison to the control. However, despite the early rise in AGT expression, 72 hour treatment significantly reduced this expression in the adipocytes of lean and obese compared to the control (Figure 5.3.1.2 C). The effect of liraglutide 100 nM on AGT expression similarly to the lower dose of liraglutide is more dependent on time rather than the dose used (Figure 5.3.1.2 D).



5.3.1. 2 Angiotensinogen (AGT) protein expression in lean and obese adipose tissue treated with different concentrations of liraglutide over 24, 48 and 72 hours.

The graph shows the expression of AGT (molecular weight 50 kDa) in mature SAT adipocytes of lean and obese, treated with; **C)** Liraglutide 10 nM, **D)** Liraglutide 100 nM, over three different time points. The absence of treatment was taken as the control. The data is expressed as mean ± SEM (lean n=3, obese n=3); comparison between lean/obese and control group was analysed by t-test (* Represents p-value: *≤0.05, **≤0.01, ***≤0.001). The comparison between lean and obese groups was performed by ANOVA analysis (# Represents p-value: # ≤ 0.05, ## ≤ 0.01, ### ≤ 0.001).

Treatment with LPS 10 ng/ml + Liraglutide 10nM in obese adipocytes maintained an increase in AGT expression that was time dependent, with a significant increase at 24 and 48 hours and a significant reduction at 72 hours in compared to the control. The effect of this treatment on lean adipocytes significantly increased the AGT expression at 24 hours, while this expression was significantly reduced at 48 and 72 hours in comparison to the obese adipocytes and control (Figure 5.3.1.3 E). Treatment with LPS 10 ng/ml + Liraglutide 100nM in obese and lean adipocytes on AGT expression had a similar time dependent effect as in LPS 10 ng/ml + Liraglutide 10nM (Figure 5.3.1.3 F).

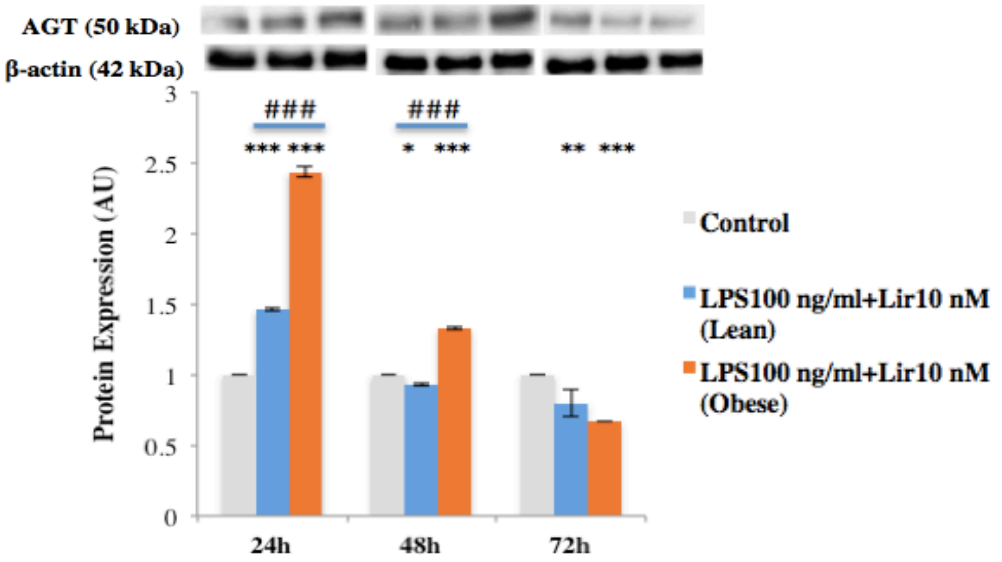


5.3.1. 3 Angiotensinogen (AGT) protein expression in lean and obese adipose tissue treated with different concentrations of lipopolysaccharides and liraglutide over 24, 48 and 72 hours.

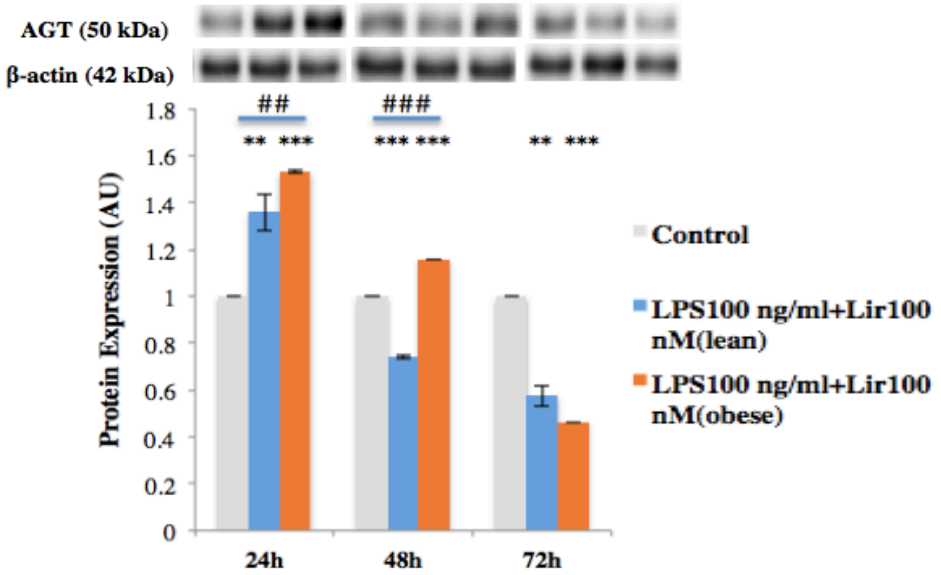
The graph shows the expression of AGT (molecular weight 50 kDa) in mature SAT adipocytes of lean and obese, treated with; **E)** LPS 10 ng/ml + Liraglutide 10 nM, **F)** LPS 10 ng/ml + Liraglutide 100 nM, over three different time points. The absence of treatment was taken as the control plus vehicle. The data is expressed as mean ± SEM (lean n=3, obese n=3); comparison between lean/obese and control group was analysed by t-test (* Represents p-value: *≤0.05, **≤0.01, ***≤0.001). The comparison between lean and obese groups was performed by ANOVA analysis (# Represents p-value: # ≤ 0.05, ## ≤ 0.01, ### ≤ 0.001).

Compared to the control, AGT expression in obese adipocytes significantly elevated at 24 and 48 hours, and was significantly reduced at 72 hours with LPS 100 ng/ml + Liraglutide 10nM treatment. While the effect of this treatment on lean adipocytes caused a significant increase in AGT expression at 24 hours and reduced significantly with 48 and 72 hours compared to the control (Figure 5.3.1.4 G). The effect of LPS 100 ng/ml + Liraglutide 100nM treatment on AGT expression in obese and lean adipocytes were time dependent similar to the LPS 100 ng/ml + Liraglutide 10nM treatment (Figure 5.3.1.4 H).

G



H

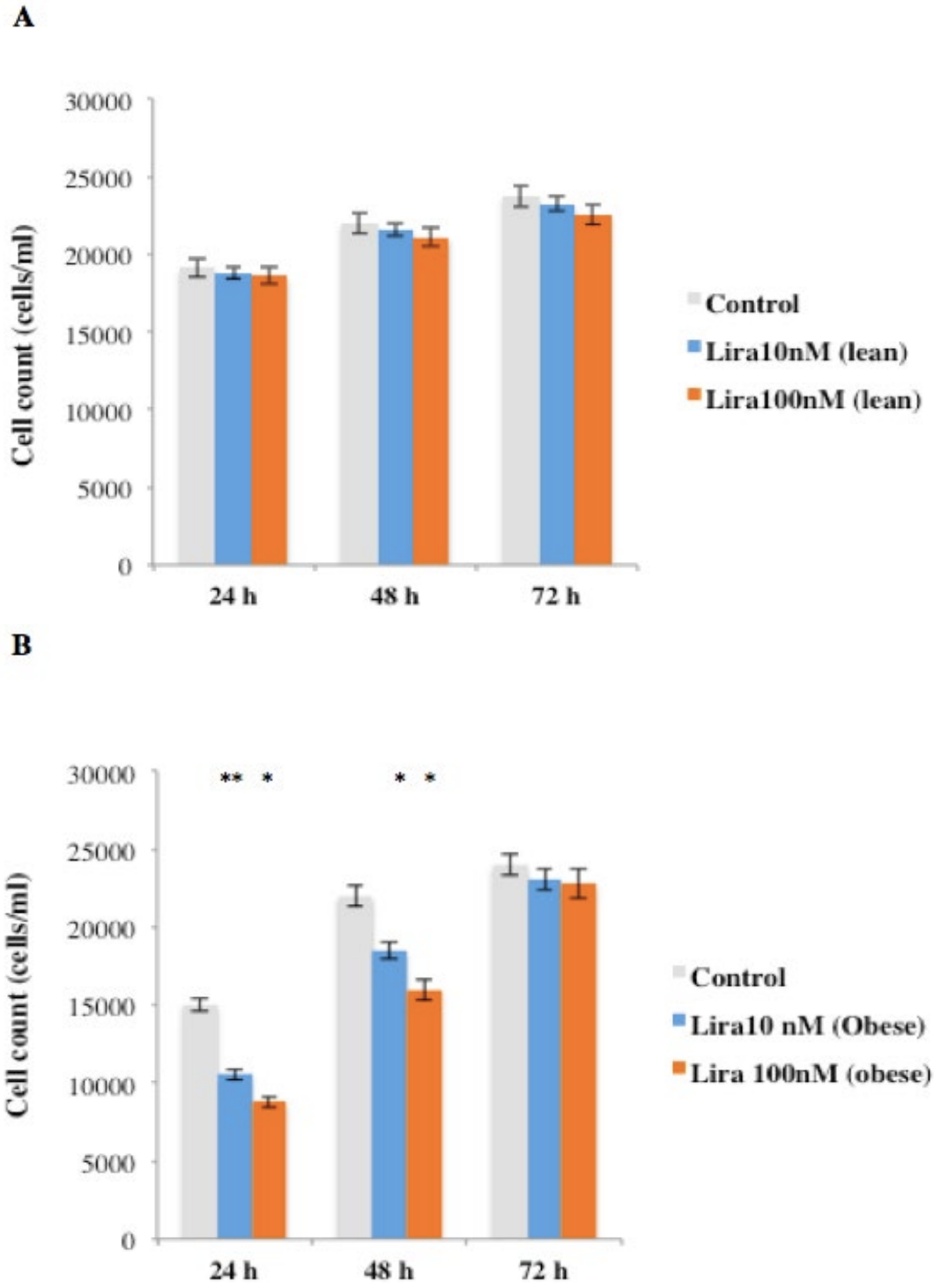


5.3.1. 4 Angiotensinogen (AGT) protein expression in lean and obese adipose tissue treated with different concentrations of lipopolysaccharides and liraglutide over 24, 48 and 72 hours.

The graph shows the expression of AGT (molecular weight 50 kDa) in mature SAT adipocytes of lean and obese, treated with: **G)** LPS 100 ng/ml + Liraglutide 10 nM, **H)** LPS 100 ng/ml + Lira 100 nM, over three different time points. The absence of treatment was taken as the control. The data is expressed as mean ± SEM (lean n=3, obese n=3); comparison between lean/obese and control group was analysed by t-test (* Represents p-value: *≤0.05, **≤0.01, ***≤0.001). The comparison between lean and obese groups was performed by ANOVA analysis (# Represents p-value: # ≤ 0.05, ## ≤ 0.01, ### ≤ 0.001).

5.3.2 Liragutide stimulates proliferation in obese mature adipocytes

Liraglutide at 10 nM and 100 nM did not alter lean adipocytes cell count over time in comparison with the control (Figure 5.3.2.1A). However, in the obese adipocytes liraglutide at both concentrations reduced cell proliferation significantly at both 24 and 48 hour time points, however, by 72 hours proliferation rate appeared to increase in these cells with no significant difference with the control (Figure 5.3.2.1 B).

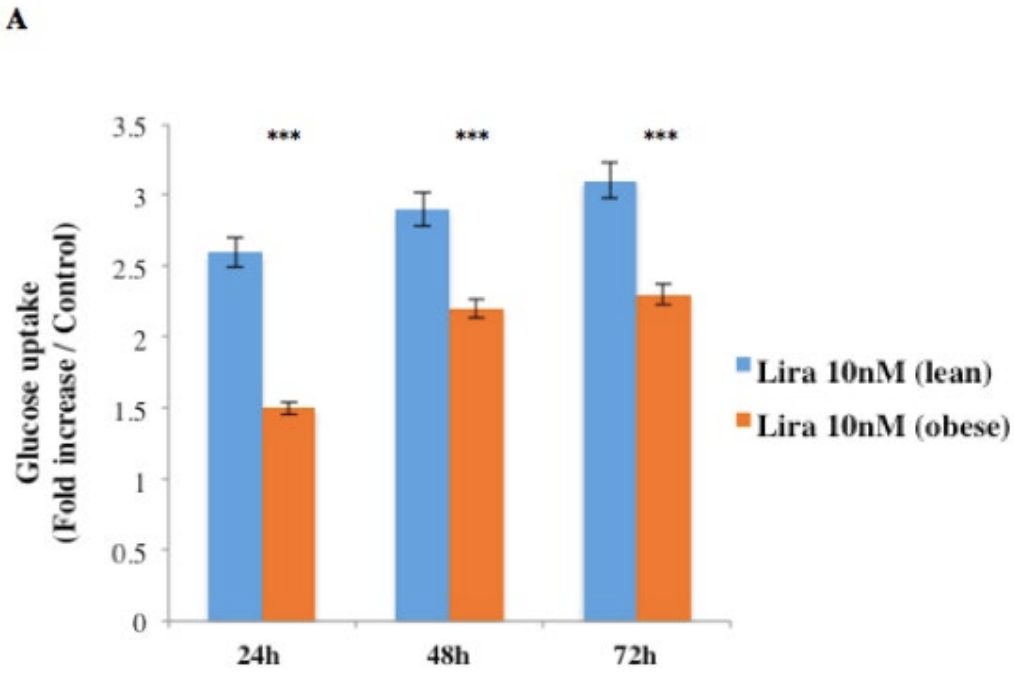


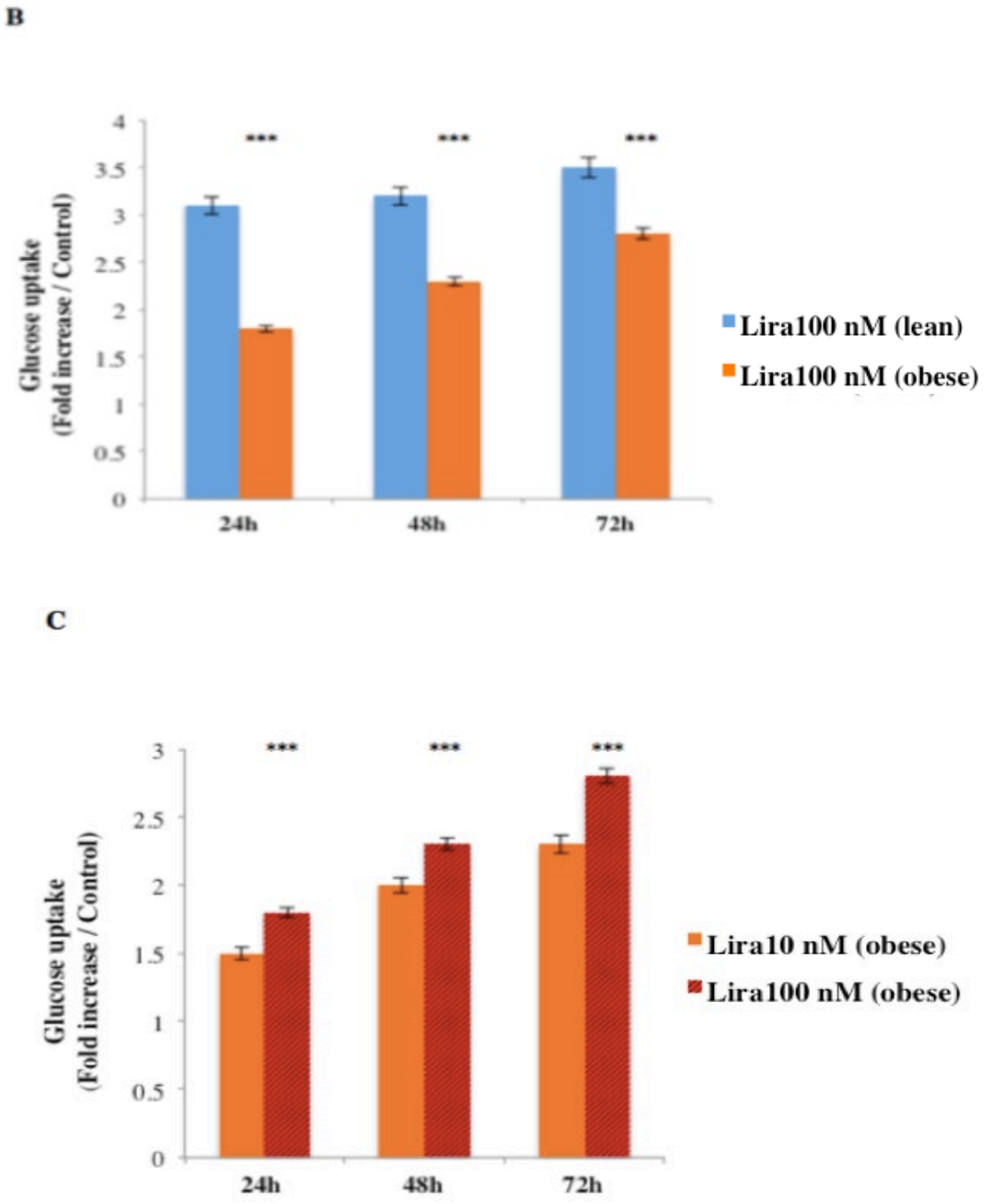
5.3.2. 1 Liraglutide inhibited obese adipocyte proliferation.

Figure **A** and **B** represent liraglutide at 10 nM and 100 nM concentrations having an effect on cell count of lean and obese mature adipocytes, post 24h, 48h and 72 hours of treatment. The absence of treatment is represented as control. The cell count was performed by hemocytometer and the results are expressed as mean ± SEM (lean n=3, obese n=3); comparison between lean/obese and control group was analysed by t-test (* Represents p-value: *≤0.05, **≤0.01, ***≤0.001).

5.3.3 Liraglutide increases glucose uptake

Stimulating with 100nM of insulin in the presence of 10nM (Figure 5.3.3.1 A) and 100nM (Figure 5.3.3.1 B) concentrations of liraglutide caused a significantly higher increase in glucose uptake in lean compared to the obese mature adipocytes treated for 24, 48, and 72 hours. Glucose uptake in obese adipocytes treated with 100nM of liraglutide is significantly higher than adipocytes treated with 10nM, in all treatment periods (Figure 5.3.3.1 C).





5.3.3. 1 Liraglutide increased glucose uptake post insulin stimulation.

Glucose uptake was performed in lean and obese mature SAT adipocytes after stimulation with insulin 100nM, in the presence of **A)** liraglutide 10nM and **B)** Liraglutide100nM. **C)** Comparison of glucose uptake between liraglutide 10 nM and 100 nM in obese adipocytes. Results are expressed as means ± SEM (lean n=3, obese n=3) of glucose uptake fold increase versus the adipocytes without insulin treatment (control). The significant difference between lean/obese adipocytes versus the control has been evaluated by ANOVA analysis (* Represents p-value: *≤0.05, **≤0.01, ***≤0.001).

5.4 Discussion

This study investigated the effects of a GLP 1 agonist, liraglutide on the impact of inflammatory insults, cell proliferation and insulin sensitivity in differentiated mature adipocytes from obese and lean individuals. The immediate findings of this study highlighted that AGT expression was increased by LPS, which was not substantially mitigated by the acute (24 hours) co-treatment with liraglutide, in both lean and obese adipocytes. However as a consequence of longer treatment time, liraglutide did appear to reduce AGT expression overall. Additionally, in the obese adipocytes, liraglutide also appeared to initially reduce proliferation to a state less than the control, which only to returned to control levels by 72 hours. The lean and obese adipocytes subjected to liraglutide were observed to enhance insulin sensitivity, although this was more pronounced in the lean individuals than the obese subjects; highlighting the existence of partial insulin resistance within the obese adipocytes. Clearly the effects of liraglutide on Adipose-specific AGT, as well as regulating body weight via altering adipocyte proliferation, and, enhancing insulin sensitivity collectively plays an important role in making this drug beneficial for obesity related hypertension.

In the present study, the LPS-induced endotoxemia effected AGT expression in both lean and obese mature adipocytes in a dose and time dependent manner. The 24-hour treatment with high dose of LPS stimulated significant increase in AGT levels, while the lowest expression was with the 72 hour treatment. It was expected that with longer exposure to LPS especially at a high dose, AGT expression would increase. However, mouse studies have shown that although adipocytes are extremely sensitive to LPS, pre-adipocytes are significantly more responsive to LPS treatment especially at lower doses of LPS (20 ng/ml), whereas mature adipocytes are not greatly reactive to even the highest doses (2000 ng/ml) (Berg, A. H. *et al.*,2004). Additionally longer treatment time of these mature primary adipocytes with LPS has proven to be even less effective (Hoareau, Laurence *et al.*,2010).

The effect of Liraglutide treatment without the presence of LPS on AGT expression in obese adipocytes showed a dose and time dependent change. The 24-hour treatment with high dose of liraglutide led to a significant up-regulation, while the 72-hour treatment resulted in a significant down-regulation of AGT expression in obese adipocytes. These findings could be due to the effect that liraglutide has on adipocyte proliferation. With the 24 hour treatment, liraglutide does not initiate proliferation and appears to acts as an inhibitor, while by 72 hour treatment, it appears to lose this inhibition, therefore the proliferation of mature adipocytes returns to that as of the control cells. This effect may in part be explained through the involvement of PPARγ, a key adipocyte differentiation and proliferation regulator. Studies have shown that GLP-1 (100nM) treatment increases the expression of PPARγ, in SAT (El Bekay, R. *et al.*,2016). PPARγ also as a human AGT promoter has been shown to regulate the activity of RAS and AGT in the same tissue (Safonova, I. *et al.*,1997, szer, Tamás *et al.*,2010, Gustavo-Salom *et al.*,2013). Therefore, it is possible that activating PPARγ by liraglutide requires a longer treatment period (72-hours), in order to result in a significant down-regulation of AGT expression in obese adipose tissue or adipocytes.

The delay in stimulating proliferation at 24 hours can also be due to the specific doses used in the present study. The dose range (10-100nM) of liraglutide is compatible to 1.2 -1.8 mg subcutaneous injections used in diabetic patients (Watson, E. *et al.*,2010, Jiang, J. *et al.*,2011). However a higher dose of 3.0 mg, which is recommended for obesity with co-morbidities such as hypertension (NICE,2017), may stimulate *in-vitro* obese adipocyte proliferation with the 24-hour treatment. In addition, this behaviour of liraglutide at 24 hours on proliferation in the present study may be specific to mature adipocytes. As liraglutide has been reported to have varying effects on differentiation and proliferation between species (rodent versus human) (Yang, J. *et al.*,2013, El Bekay, R. *et al.*,2016) and even between cell types (pre-adipocytes versus mature adipocytes) (Challa, T. D. *et al.*,2012, Cantini, Giulia *et al.*,2015).

Obese mature adipocytes treated with liraglutide in the presence of LPS resulted in AGT expression to be up-regulated less than when these cells were treated with LPS alone at 24 hours, which would appear to be as a result of liraglutide treatment. The

high dose of liraglutide was significantly more effective at down-regulation of AGT expression at 72 hours even with the high dose of LPS present.

Lean mature adipocytes treated with liraglutide did not result in any significant effect on AGT expression, nor did it have any significant effect on cell proliferation. Additionally, liraglutide with the presence of LPS mainly appeared to represent similar effects of that of LPS treatment alone on AGT expression in the lean adipocytes, where AGT expression was up-regulated at 24 hours, and reduced as the effect of LPS subsided with longer exposures. Studies have reported that obese adipocytes contain more GLP-1 receptor than the lean adipocytes (Vendrell, Joan *et al.*,2011, El Bekay, R. *et al.*,2016), which might explain the lack of effect that liraglutide had on lean adipocyte AGT expression in the present study.

In this study, similar to other GLP-1 agonists, liraglutide stimulated glucose uptake in the obese and lean mature adipocytes. Although it can be speculated that the gradual increase in glucose uptake observed in lean adipocytes is mainly due to the origin of these cells and not the effect of liraglutide as such, as lean are more insulin sensitive than the obese adipocytes (Horowitz, Jeffrey F. *et al.*,2001). It appears that in the obese mature adipocytes liraglutide enhances insulin sensitivity, with the higher response at the 72 hour treatment, which may also relate to the rate of proliferation at this treatment time. Changes in insulin are known to directly effect AGT and blood pressure, with genetic studies noting that the AGT gene is directly associated with insulin sensitivity and resistance, and therefore changes in insulin resistance appear relevant to changes in AGT expression (Guo, X. *et al.*,2005, Underwood, Patricia C. *et al.*,2013). There maybe a possibility that in addition to liraglutides' effect on changing proliferation that led to AGT down-regulation, reducing insulin resistance also contributes to the AGT reduction with the 72 hour treatment, in the obese adipocytes.

There are a number of limitation to this study including: 1) the low number of lean and obese SAT samples. 2) Only adipocytes from women were used and therefore the effects on men could not be directly determined by this study. 3) SAT samples were obtained from non-menopausal women, therefore the results might differ in menopausal women.

5.5 Conclusions

In-vitro, liraglutide appears to have a different impact depending on the origin and type of adipocytes. In mature adipocytes, 72 hour treatment with liraglutide 100 nM is capable of overcoming the inflammatory insult of LPS even at high dose, it is also effective at reducing insulin resistance and altering proliferation, all of which play a critical role in reducing AGT expression in obese adipose tissue. Therefore, these studies contribute to the beneficial effect of this multifunctional peptide, and the effect it has in adipose tissue in order to reduce obesity related hypertension.

CHAPTER 6:

Biomarker assessment to detect T2DM status pre and post remission using urinary based analysis

6.1 Introduction

An important factor to reduce the impact that T2DM has on individuals is early detection of non-diabetic hyperglycaemia, also known as the "pre-diabetes" state. Many studies suggest that the long-term complications of T2DM have already started to develop during the pre-diabetic stage, as the risk of cardiovascular disease is 2-3 fold higher in pre-diabetics in comparison to those with normal glucose levels (Pratley, R. E.,2013). As obesity is clearly the single most important and relevant risk for progression to T2DM (Gillett, M. *et al.*,2012), screening for pre-diabetes in overweight and obese individuals could be a cost effective approach to health care disease prevention. It has been estimated that NHS could save £132 million per year over 10 years with the early detection and prevention of this disease (diabetes.org,2014). However, there is a worldwide inconsistency regarding which test is appropriate for diagnosis of pre-diabetes and, which diagnostic thresholds is more suitable (Barry, Eleanor,2017).

Current accurate tests rely on a physician and/or allied healthcare professional to conduct an invasive blood based test to diagnose T2DM status, which may include fasting plasma glucose test, oral glucose tolerance test, and HbA1c test. These current tests also carry limitations, both in detection sensitivity and accuracy (Barry, E. *et al.*,2017). Furthermore there is reluctance by participants to undertake an invasive blood collection by needle use, as well as the need to provide a fasted sample with confirmation of diabetes status through oral glucose tolerance test and repeated sample acquisition. Therefore, there is a need for a convenient, non-invasive, and cost effective test that would act as an indicator of T2DM and stratify individuals, allowing only those with determined need to undergo further investigations. The use of urine as an alternative method to assess diabetes risk is seen as more acceptable and patients often provide such samples for other tests both at home or in primary care centres.

In recent year there has been significant development in analytical procedures for identifying VOCs in urine, which has created the opportunity for participants' urine to

be used as a screening and detection tool in different types of cancer (Arasaradnam, R. P. *et al.*,2014, Khalid, Tanzeela *et al.*,2015, Mazzone, Peter J.,2015, Navaneethan, U. *et al.*,2015) and infectious diseases (Guernion, N. *et al.*,2001, Banday, K. M. *et al.*,2011). This same methodology to use urinary VOCs as a non-invasive early detection-screening test could be used to detect T2DM. This method of detection may encourage those at risk to seek suitable diagnosis for any disease they are likely to have, something they may not have otherwise undertaken. This insight could therefore, result in an earlier diagnosis and more importantly better prognosis for participants. Beyond the use of VOCs to detect T2DM status, it could also be further considered as a factor to assess whether T2DM remission has occurred following lifestyle, medical or surgical weight loss (Rubino, F. *et al.*,2016, Schauer, P. R. *et al.*,2016).

Currently the most successful way to achieve T2DM remission appears to be through surgery. As surgery is expensive, many healthcare systems have eligibility criteria in place to ensure that the majority of participants' achieve a successful outcome. Predicting who will achieve the best outcome is challenging, however, most bariatric clinics worldwide have a preoperative weight loss requirement, which participants must meet in order to be eligible for surgery. This requirement is put in place as it increases the probability of weight loss maintenance post-surgery (Ali, M. R. *et al.*,2007, Alger-Mayer, S. *et al.*,2008, Solomon, H. *et al.*,2009). It may also improve the status of diabetes and other co-morbidities post-surgery, however the evidence on this is inconsistent (Brethauer, S.,2011, Ochner, C. N. *et al.*,2012). Therefore a predictive test that would identify the participants likelihood of improving their T2DM status post bariatric surgery would be beneficial in supporting the preoperative weight loss criteria, and identifying individuals that may need more support post-operatively to sustain good health outcomes. Urinary VOCs may suggest a means for such a medical test due to their potential alignment to metabolic health risk markers.

Therefore the aims of this study was to investigate whether,

1) Urinary VOCs can act as a convenient, non-invasive indicator of T2DM status and any change within the same individual over time.

2) Urinary VOCs be a predictive test that can be used as a pre-bariatric surgery indicator of post-surgery health and a means to stratify appropriate post care support.

6.2 Subjects and Methods

A cohort of 42 Caucasian adult women participants with morbid obesity (BMI ≥ 35 kg/m^2) and T2DM, who underwent different types of bariatric surgery; LAGB (n=14), SG (n=14) or BPD (n=14), were recruited.

6.3 Study Design

The participants of this study were recruited and had surgery in the Obesity clinic, Prague, Czech Republic. The urine was collected in standard universal sterile specimen containers and frozen at – 80 °C within a 2 hour of collection with subsequent batch analysis undertaken pre-surgery and 6 months post-surgery.

6.4 Anthropometric and biochemical measures

All measurements were performed at baseline and at 6 months post surgery. Blood was taken from all participants after a 10 hour fast. HbA1c, serum glucose and lipids were measured using the Cobas 6000 analyzer. HOMA-IR was used to quantify insulin resistance by the following equation: HOMA-IR= fasting glucose (mmol/L) x fasting insulin (μU/L) / 22.5 (Matthews, D. R. *et al.*,1985). LDL cholesterol was calculated by using the Friedwald formula (Friedewald, W. T. *et al.*,1972). Height and weight were measured via standardised protocol. BMI was calculated as weight (in kilograms) / height^2 (meters), and percentage excess weight loss was calculated by using the following equation: (preoperative weight - postoperative weight) / (preoperative weight

- ideal body weight) x 100. Body fat mass was measured using the bioimpedance method (Tanita TBF-300; Tanita corporation).

Blood pressure data, which is presented as systolic over diastolic values, is the average of three BP readings with 5-minute resting period in between; these readings were taken by using an automated device. MAP was calculated by using: [(2diastolic BP + systolic BP) / 3] (Brzezinski., Walter A.,1990).

6.5 Chemical Analysis and Instruments

6.5.1 Field Asymmetric Ion Mobility Spectrometry (FAIMS)

A Lonestar (Owlstone Ltd, UK) FAIMS unit was used to analyse the headspace of pre and post-operative urinary VOC samples from all participants. The FAIMS device used a 63Ni ionization mechanism. Unlike traditional analytical techniques, FAIMS is capable of separating gas molecules at both atmospheric pressure and room temperature. Upon ionisation, the sample is passed through an oscillating electric field with an asymmetric high voltage waveform. Ions "zigzag" through the field and only ion species with no overall transverse motion pass through without deionising, based on properties of the waveform and the ion species. Certain properties of the waveform are then manipulated to identify the different ion species present in the analyte.

6.5.2 FAIMS Analysis

Urine samples were defrosted overnight to 5°C. For each sample a 5ml aliquot was pipetted into a 20ml glass sample bottle. This was then placed into an Owlstone ATLAS At-Line Sampling module attached to the Lonestar FAIMS unit. The ATLAS module heats the sample to 40°C, and introduces the headspace of the sample into the FAIMS for analysis. Four full sweeps of the dispersion field strength were carried out in succession for each sample. Before the first sample and between each sample, an air blank was run for three sweeps to allow any residue from the previous sample or other experiments to clear from the machine. No data was collected for these sweeps.

6.5.3 Data Analysis

Each sample yielded eight 512 × 51 matrices, four positive and four negative ion counts across the tested compensation voltages and dispersion field strengths, for a total of 208,896 variables per sample. As an initial step in the analysis of this data, the matrices were transformed using a discrete wavelet transformation. The levels of the transform corresponding to the two smallest-bandwidth passes were discarded to eliminate noise. Wavelet transformation is a common step in signal processing, and can help in separating structure from noise in an image, compressing the informative variables into fewer dimensions and consequently reducing the dimensionality of the data. After transforming the data, there were 52,224 variables per sample. Similarly to Fourier transforms, wavelet transforms create a representation of an image in an alternative basis space. However, in comparison to Fourier transforms, wavelet transforms capture information about both the size (or wavelength) of structures in the image, and information about where those structures occur. This helps to concentrate the useful information in the image into fewer predictors, which helps when selecting relevant features for classification tasks. To assess the ability of FAIMS analysis of urine samples in predicting diabetes status post-surgery, HOMA-IR was considered as a classification task. For HOMA-IR, the cohort was divided into two classes. Binning the data was necessary due to the small sample size (both FAIMS and indicator data were available for 25 participants) and the high dimensionality of the FAIMS data (52,224 features), which hampered regression analysis. To perform the binning for this indicator, participants were divided into those with an indicator value which was less than or equal to the median value of the cohort (the “Low” group for that indicator), and those with a value above the median value of the cohort (the “High” group for that indicator). Participants who lacked data for this indicator were excluded from further analysis. FAIMS data was available for 25 participants, and indicator data was available from at least 24 participants for each indicator.

6.5.4 Statistical analysis

This analysis was followed by the MWW test to rank features according to how well they separate the cohort into High and Low classes. The top number features were then taken forward and used to train a classification model, with n and the classification model selected using a separate cross-validation analysis. Four learning algorithms were considered: radial SVM, sparse logistic regression, logistic regression, and random forests. To assess the performance of HOMA-IR, 10-fold cross validation was used to produce predicted classes for each participants, with both feature selection and model creation being carried out in the cross-validation. Classification performance was assessed by using the Receiver Operating Characteristic (ROC) curve, which is defined by the area under the curve (AUC).

6.6 Results

6.6.1 Body composition and metabolic variables

Clinical and anthropometric data obtained from 42 obese-T2DM women participants before undergoing bariatric surgery and 6 months after were collected and analysed (Table 6.6.1). All participants had significant reduction in all weight indices, except waist to hip ratio (WHR). Participants showed improvements in their blood pressure, with systolic and mean arterial pressure (MAP) markers significantly reduced. There was a significant improvement in HbA1c, plasma glucose, plasma insulin, HOMA-IR, and all participants were in T2DM remission at 6 months post surgery. Although lipid markers overall reduced; only total cholesterol and triglycerides were shown to significantly reduce ($P<0.01$).

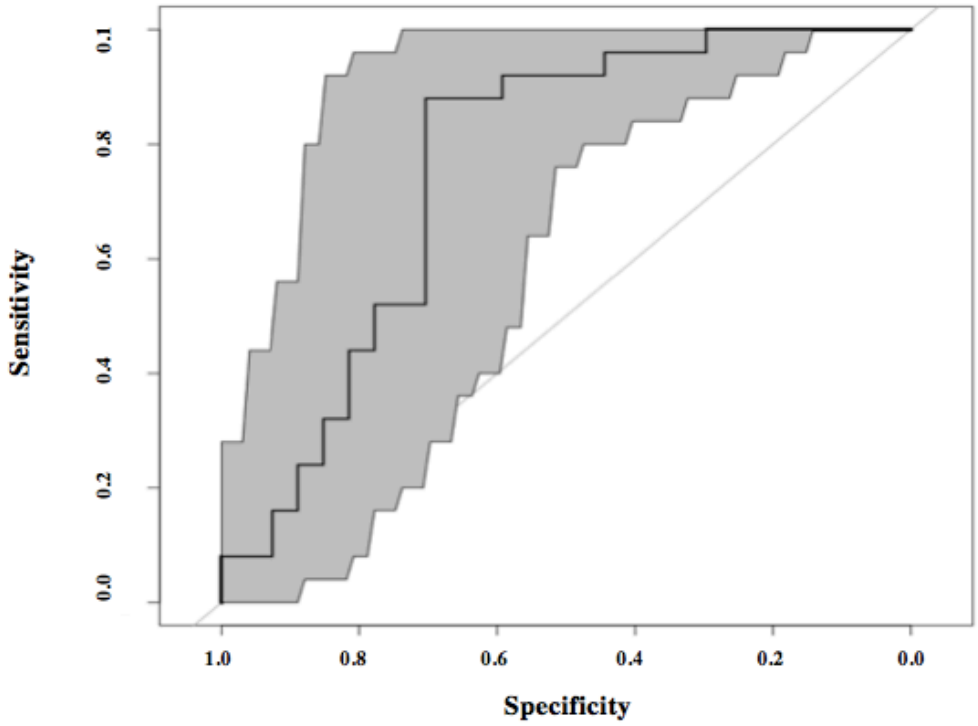
	N=42		
	Pre Mean (SE)	Post Mean (SE)	P-value
Age (years)		52.46±8.2	
Excess weight loss (%)		22.37 (1.44)	
Body weight (kg)	118.72 (3.02)	103.32 (2.51)***	<0.001
BMI (Kg/m^2)	43.61 (1.05)	37.84 (0.90)***	<0.001
Fat mass (%)	49.17 (0.54)	45.32 (0.63)***	<0.001
WHR (cm)	0.90 (0.01)	0.88 (0.01)	0.248
Systolic bp (mmHg)	128.97 (2.21)	122.44 (2.32)*	0.013
Diastolic bp (mmHg)	79.17 (1.92)	76.23 (1.5)	0.202
MAP	95.78 (1.80)	91.61 (1.49)*	0.039
HbA1c (mmol/mol)	54.37 (1.58)	44.77 (1.48)***	<0.001
Plasma glucose (mmol/L)	8.93 (0.35)	7.16 (0.23)***	<0.001
Plasma insulin (mmol/L)	27.90 (2.46)	16.63 (1.44)***	<0.001
HOMA-IR	11.25 (1.16)	5.34 (0.53)***	<0.001
Total Cholesterol (mmol/L)	4.88 (0.12)	4.38 (0.13)***	<0.001
LDL Cholesterol (mmol/L)	2.88 (0.14)	2.73 (0.11)	0.321
HDL Cholesterol (mmol/L)	1.06 (0.04)	0.99 (0.046)	0.072
Triglyceride (mmol/L)	1.77 (0.15)	1.41 (0.09)**	0.007
Triglyceride / HDL ratio	1.88 (0.20)	2.04 (0.53)	0.773
C-Reactive Protein (mg/L)	6.96 (0.75)	5.90 (0.63)	0.090

6.6. 1 Anthropometric and metabolic variables at pre (baseline) and post (6 months) bariatric surgery.

Continues variables are represented as mean and standard error. BMI-body mass index; WHR-waist to hip ratio; MAP-mean arterial pressure; HbA1c-glycated haemoglobin; HOMA-IR- Homeostatic assessment model of insulin. LDL-low density lipoprotein; HDL-high density lipoprotein.(*Represents P-Values: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

6.6.2 Volatile organic compounds differentiated between pre and post-operative urine

The findings from the urine VOC analysis identified the change between pre and post-operative VOCs, with sensitivity of 0.72 (95% CI: 0.51-0.88) and specificity of 0.70 (95% CI: 0.50-0.86). The AUC was 0.76 (95% CI: 0.62-0.9).

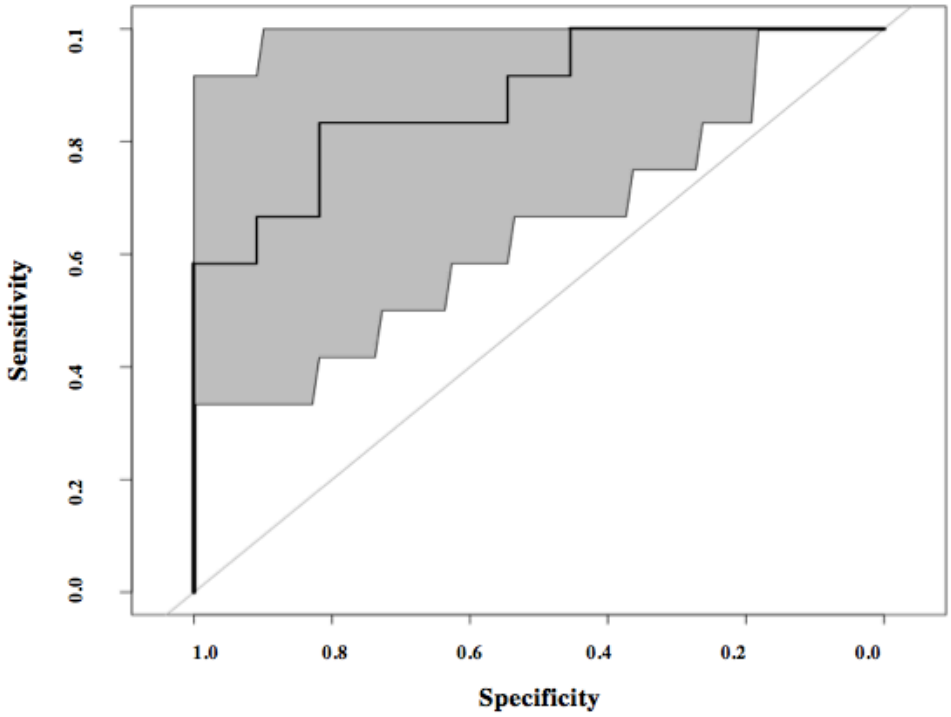


6.6. 2 Receiver operator characteristic (ROC) curve for pre and post operative urine.

Receiver operator characteristic (ROC) curve analysis illustrates the accuracy for identifying pre operative VOCs verses 6 months post operative urinary VOCs, which was established by using a classification algorithm.

6.6.3 Identification between pre- operative VOCs and post-operative T2DM status

Urinary VOCs highlight the change between pre-operative VOCs and the participant's T2DM status at 6 months post-surgery, with the sensitivity of 0.83 (95% CI: 0.52-0.98) and specificity of 0.82 (95% CI: 0.48-0.98). The AUC=0.88 (95% CI: 0.74, 1.00).

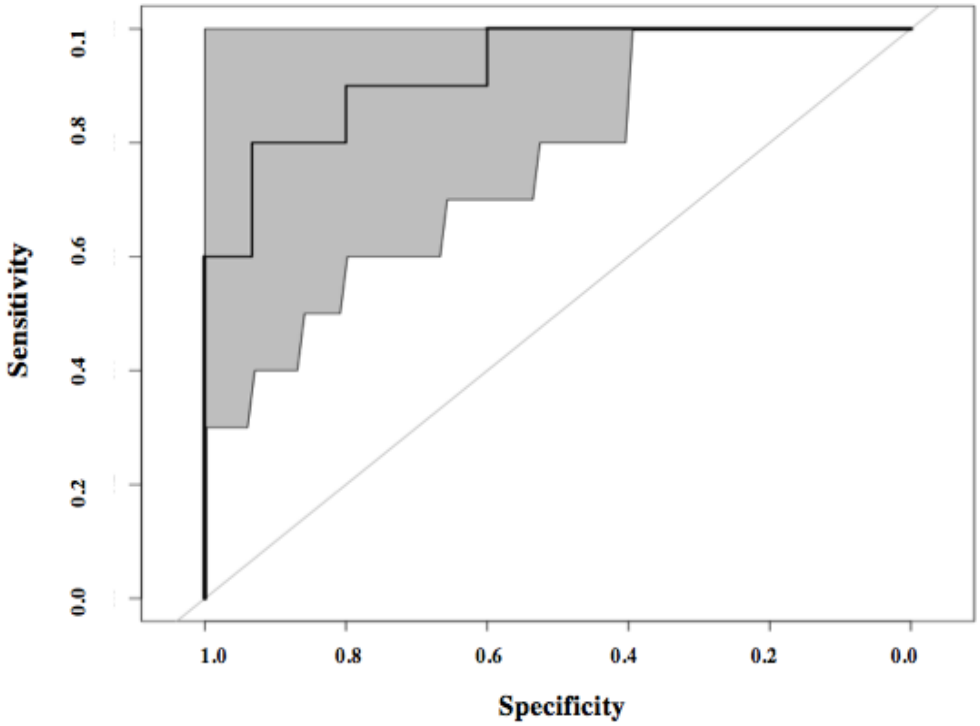


6.6. 3 Receiver operator characteristic (ROC) curve for pre-operative VOCs and post operative T2DM status.

(ROC) curve analysis illustrates the accuracy for identifying between pre-operative VOCs and post-operative HOMA-IR status, by using a classification algorithm.

6.6.4 Identification between post-operative volatile organic compounds and post-operative T2DM status

Further analysis indicated the urinary VOCs at 6 months post-surgery identifying the T2DM status of the participants at 6 months post-surgery with sensitivity of 0.80 (95% CI: 0.44-0.97) and specificity of 0.80 (CI: 0.52-0.96). The AUC= 0.93 (95% CI: 0.83-1.00).



6.6. 4 Receiver operator characteristic (ROC) curve for post-operative VOCs and post operative T2DM status.

(ROC) curve analysis illustrates the accuracy for identifying the between post-operative VOCs and post-operative HOMA-IR status, by using a classification

6.7 Discussion

The findings of this study indicate that urinary VOCs could firstly act as a non-invasive indicator of T2DM status, but also be a factor to determine probable health outcome following bariatric surgery. It is known that urinary VOC profiles differ with health conditions, which is also confirmed in this study with regards to alignment to T2DM. This distinction was demonstrated due to assessment of VOCs pre- and post- bariatric surgery and the correlation that urinary VOCs have with T2DM status at 6 months post bariatric surgery.

The current data suggests a method to provide a highly correlated VOC profile to T2DM status as a convenient, non-invasive and cost effective test for T2DM that would identify individuals that are in need of further investigations much earlier than it is currently occurring. The results of this study suggest that VOC analysis of urine samples could fulfil this need, leading to earlier diagnosis and better prognosis. This would also reduce the cost to healthcare system, as less co-morbidity would occur with early diagnosis.

Additionally with the significant correlation between pre-surgery urinary VOCs and diabetic status at 6 months post-surgery, which was observed in this study, can reveal an exciting potential to use VOC analysis as part of the selection criteria for individuals undergoing bariatric surgery. Utilised alongside the current weight loss criteria most hospitals have in place, VOC profiles could identify those participants who, despite losing the required weight, may need more assistance post-surgery to improve their diabetic status.

Whilst the potential of VOC analysis for diagnosis of T2DM has been discussed before, the main focus of this study is the analysis of VOCs emanating from exhaled breath, and not the use of FAIMS technology (Phillips, M. *et al.*,2004, Minh Tdo, C. *et al.*,2012, Kim, iI-Doo *et al.*,2015, Das, S. *et al.*,2016). Many studies use techniques that are time consuming, expensive, and require highly trained specialist staff, such as gas

chromatography-mass spectrometry. Several researchers have used Electronic Nose (eNOSE) technology to detect T2DM in breath samples (Arasaradnam, R. P. *et al.*,2011, Sabeel, T. M. A. *et al.*,2013, Seesaard, Thara *et al.*,2016), however this technique suffers from poor intra-device repeatability, limited temporal stability and poor chemical selectivity (Boyle, Billy *et al.*,2018). FAIMS technology does not suffer from these problems and is highly sensitive, measuring parts per trillion rather than parts per billion (Khalid, Tanzeela *et al.*,2015), as it measures the physical composition of the analyte as opposed to the chemical composition. This results in it being much less susceptible to adverse interactions between the analyte and the sensor, and thus provides more consistent measurements over time. Urinary VOC analysis using FAIMS technology has previously been shown to successfully detect colorectal cancer in individuals (Khalid, Tanzeela *et al.*,2015), as does the results of this study with regards to T2DM.

Whilst these results reveal the potential for the use of VOCs in the future of T2DM diagnosis and bariatric surgery, the limitation was that the cohort of participants in this study consisted only of post-menopausal female Caucasians. As such, this work may benefit from a future study with a more diverse and greater number of participants.

6.8 Conclusions

This study has demonstrated that the unique chemical fingerprint of VOCs present in urine can be used to identify individuals with T2DM, as well as to predict the T2DM status following bariatric surgery. This highlights the potential of using urine as a screening tool to identify individuals who are at risk of T2DM, as well as to identify those that may benefit from further assistance to improve diabetic status following bariatric surgery in the future.

Chapter 7: Conclusions

This current thesis explored and evaluated the effects that different types of bariatric surgery on cardio-metabolic improvements as well as disease resolution, inflammatory markers and endocrine hormones in women with obesity and cardio-metabolic comorbidities. Additionally this thesis sought to consider a way to monitor such changes as a predictive tool using urinary VOCs as a detection tool.

A prior literature review into the outcomes of different bariatric surgeries indicated the effectiveness of the three procedures as treatments for obesity and obesity related cardio-metabolic disease. Specifically studies have reported that BPD which is a combination of restrictive and malabsorpative procedures, results in a greater weight loss than compared with restrictive surgeries such as SG and LAGB. However, BPD patients are often reported to have metabolic related complications (Anderson, Blaire *et al.*,2013, Roslin, Mitchell *et al.*,2018). Therefore since white adipose tissue plays a fundamental role in inflammation and metabolic health in obesity, it was hypothesised that various types of bariatric surgery impact adipose tissue inflammatory markers differently, which can directly influence the improvement in cardio-metabolic disease, despite the amount of weight loss post-operation. Therefore, initial studies in chapter 3, analysed the inflammatory MYD88-dependent pathway in abdominal SAT (pre and post-surgery) from bariatric patients as a mechanism for resolution of inflammation. Whilst all participants across the three surgery types led to significant weight loss, with BPD surgery resulting in the highest weight loss, the improvement in cardio-metabolic components, and the disease resolution was lower in these participants in comparison with the individuals that underwent the other two surgeries, SG and LGB. Further investigation of circulating endotoxin and pro-inflammatory/cardiovascular marker such as IL-6, revealed continued inflammation at 6 months post BPD. The elevated IL-6 concentration explained the subsequent dyslipidaemia, and therefore the lack of improvement in the cardio-metabolic components observed at 6 months post-surgery. This study offers an insight into the fact that all aspects of BPD surgery (surgical procedure and time, recovery time, accelerated weight loss, post-surgical malnutrition) maintained an inflammatory response in their abdominal SAT for at least the first 6 months post-surgery. As a result of this the individuals that underwent BPD surgery could still be at risk of cardiovascular disease posed by adipose tissue.

Subsequent studies explored the effects of SG, LAGB and BPD surgeries on endocrine hormones that govern cardio-metabolic disease recovery post-surgery. Although there are studies supporting the involvement of FGF-19 and FGF-21 in cardio-metabolic recovery post bariatric surgery (Kyrou, I. *et al.*,2017, Patton, A. *et al.*,2017), there exists conflicting data on the behaviour of FGF-19 post-bariatric surgery, in addition, to the lack of research on the effect of LAGB on these hormones. Therefore, the aims in chapter 4 of these studies were firstly to define the effects of SG, LAGB and BPD on FGF-19 and FGF-21 concentrations pre and post-surgery, and evaluate the changes in these endocrine hormones based on the cardio-metabolic risks at 6 months post surgery. Secondly, by using glucose and lipid markers, the effects of FGF-19 and FGF-21 were compared with cardio-metabolic risk factors. The findings from these studies demonstrated that the highest post-operative FGF-19 and the lowest FGF-21 concentrations were noted in SG participants, these subjects were also observed to have the lowest cardio-metabolic risk at 6 months post-surgery. Both FGF-19 and FGF-21 reduced in LAGB participants, this unexpected effect on FGF-19 could have been due to the lack of gastrointestinal anatomy changes in this surgery. Despite the amount of high weight loss in BPD, FGF-19 increase and FGF-21 decrease was not more than observed in the SG participants. As such these studies may offer a potential novel prospect for the use of FGF-19 status post-surgery as a marker for the prediction of metabolic improvement in glucose, while FGF-21 could be used as a marker of lipid status prediction at 6 months post BPD surgery.

Collectively the findings from Chapters 3 and 4 of this thesis emphasized that despite achieving great weight loss, BPD could be considered as an unsuitable and somewhat harmful procedure. As this surgery type leads to lack of significant improvement in FGF-19 and FGF-21, in addition to persistence of an inflammatory status, with less improvements in cardio-metabolic components, lower disease resolution than other surgeries; whilst it may still place patients at risk of cardiovascular disease at 6 months post surgery. Although unlike BPD surgery, SG surgery does not result in as much weight loss in comparison to BPD in the first 6-month post-surgery, it is more beneficial for the cardio-metabolic disease resolution, as well as improvements in FGF-19 and FGF-21 endocrine hormone.

Besides bariatric surgery consideration to reduce cardio-metabolic risk and inflammation, evidence suggests liraglutide may have positive effects on white adipose tissue metabolism and inflammation, which would appear to enhance this anti-diabetic medication as an effective anti-obesity agent as well. Studies with liraglutide have shown this medication has modest impact on reducing blood pressure in obese-hypertensive individuals. In addition, *in-vitro* studies have shown the metabolic effects of liraglutide on RAS, and its components in various tissues as well (Harte, A. *et al.*,2005). Secreted by mature adipocytes, adipose specific AGT influences and contributes to the systemic RAS, therefore plays an important role in the development of obesity related hypertension and insulin resistance. Furthermore, as endotoxin can induce AGT expression this adds weight to inflammation's influence on hypertension. As such in these studies it has been hypothesised that liraglutide may exert its antihypertensive influence by firstly mediating a direct effect on AGT, and reducing the insult of endotoxin LPS on obese mature adipocytes. Secondly liraglutide can enhance insulin sensitivity in adipose tissue. Therefore, this thesis, in, explored the role of liraglutide in adipocyte, which down-regulated AGT alone and in response to LPS given chronically. In addition, liraglutide initially appeared to reduce cell proliferation with acute treatment, whilst this effect was observed to lessen over a longer duration. Liraglutide also appeared to enhance insulin sensitivity in lean and obese mature adipocytes although this effect was reduced in adipocytes from obese patients.

Ultimately early detection and diagnosis of T2DM is greatly beneficial at reducing the onset of additional obesity related cardio-metabolic disease, and therefore reducing mortality rates. Review of current early detection methods revealed that current tests appear invasive, time consuming and often unreliable due to lack of appropriate sensitivity. Urinary VOCs have been successfully used for the detection and screening of diseases such as different cancers and infection diseases. Therefore chapter 6 investigated whether urinary VOCs could be utilized as a convenient, non-invasive indicator of T2DM status, as well as detecting any change in this status over a period of time within the same individual. Additionally whether urinary VOCs have the potential of being used as a predictive test that can be used as a pre-bariatric surgery indicator of the post-surgery health outcome, for a more appropriate post care support and treatment method. The findings from VOC profiling of urine headspace indicated that urinary

VOCs could be used with good reliability and specificity in detection of T2DM status. Urinary VOCs showed the capability of differentiating between pre-and post bariatric VOCs, as well as post-bariatric T2DM status was predicted with the use of pre-bariatric VOCs. Therefore this unique chemical fingerprint has the potential to be used as a convenient, non-invasive, cost effective tool for screening and detection of T2DM, and as a predictive test for the outcome of T2DM status post-bariatric surgery.

7.1 Limitations

There are several limitations in these studies, in that the current cohort although clinically well characterised, only examined white Caucasian females therefore the effect that ethnicity and gender may have had on the surgical and tissue responses could not be examined. Ethnicity is important, as we know for a given BMI South Asian subjects would be at higher cardio-metabolic risk, which is also comparable for male participants compared with pre-menopausal women. These studies were also not able to directly extract adipocytes to culture cells from the bariatric patients' pre and post-surgery due to the logistical of collecting appropriate sample sizes. As such, the cell culture studies utilised a group of lean and obese individual's adipocyte cells, which evaluated cell insulin sensitivity prior to any treatment and analysis. However the use of lean and obese derived adipocytes cells did allow the differences in insulin sensitivities to highlight how this altered cellular function responses. This would not necessarily have been observed in the bariatric adipose tissue samples, as despite the loss in weight the tissue remained inflammatory.

7.2 Future work

This thesis has highlighted a few areas that may be useful to study in the future. In regards to the liraglutide study it would be useful to design a larger range of liraglutide concentrations with longer series of treatment times, in order to analyse the effect of this medication on adipogenesis. It may also be beneficial to compare the effect of this medication in different human cell types such as; mature adipocytes versus pre-adipocytes, subcutaneous adipocytes versus visceral adipocytes, obese adipocytes versus morbidly obese adipocytes. This would allow studies to evaluate depot and cellular differences in response to medication on RAS. Additionally investigating into vital transcription factors that strongly regulates differentiation such as PPARα and adipocyte differentiation-related protein (ADRP) and fatty acid binding protein 4 (FABP4), could provide a further insight into the effect of liraglutide on these mechanisms.

7.3 Final conclusions

In conclusion, this thesis investigated the impact of various treatment types from invasive bariatric surgery to pharmaceutical therapies such as liraglutide on the insult of endotoxin LPS and adipose tissue inflammation, and therefore, obesity related cardio-metabolic risk. This thesis further investigated the potential of utilizing VOCs as biomarkers for detection of T2DM status.

The findings of these studies highlighted:

1. SG in comparison to BPD is more suitable for the improvements of endocrine hormones FGF-19 and FGF-21 and for obesity related cardio-metabolic disease resolution, post surgery.
2. Liraglutide subsides LPS inflammatory effect and down-regulates adipose specific AGT expression, while enhancing insulin sensitivity in obese mature adipose tissue in a time dependent manner.
3. Urinary VOCs have the potential of being used as a non-invasive, cost effective, early detection test for T2DM and possibly as a predictive test for the outcome of T2DM status post bariatric surgery.

The initial aims that were set for this thesis were met according to the findings established. In regards to the bariatric studies, the findings of this thesis adds a new dimension to the numerous limitations of the BPD surgery, and although the rate of BPD performance has reduced worldwide, this study highlights the importance of keeping a close observation and follow-up of patients that have to undergo this procedure, especially for the first 6 months post-operation.

This study sets the foundations for understanding the mechanisms involved in the beneficial effect of liraglutide on hypertension. Since liraglutide is already used for the treatment of T2DM and obesity, the potential use of this medication as an anti-hypertensive therapy as well, could be useful for obese-T2DM hypertensive individuals.

Liraglutide in a form of a monotherapy for obese individuals could reduce the burden of multi-medication use, which may be more cost effective and time efficient for patients. Furthermore, the simplicity and the non-invasiveness of urinary VOCs as an early detection test for T2DM, could encourage far more individuals to get tested, resulting in early detection and management of disease, and delaying the onset of severe disease complications that would reduce life expectancy. Additionally, utilizing VOCs as a predictive test for the outcome of T2DM post bariatric surgery can provide the opportunity for selecting the patients that would benefit from further assistance in improving their diabetic status following bariatric surgery.

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