Exploring appetitive, metabolic and ketotic effects and weight-loss potential of Dapagliflozin in patients with Type 2 Diabetes Mellitus and Obesity, with concomitant dietary intervention

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Declaration

I, Dr Petra Hanson, declare that all the work in this Thesis is mine, except for the following:

- Urinary analyses were carried out by Catherine Darby
- Serum and plasma analyses were carried out by Gemma Reidy

The material from this Thesis has not been published before. This Thesis has not been submitted in any previous application for any degree.

Abstract

Background: Type 2 Diabetes Mellitus (T2DM) is closely associated with obesity and increased metabolic risk. Dapagliflozin, the first-in-class of the Sodium Glucose-Like Co-Transporter 2 (SGLT2) inhibitor agents, licensed for use in patients with T2DM, has been demonstrated both on evidence from randomized controlled trials and real-world studies, to be associated with secondary weight-loss and improved cardiovascular outcomes. There have been relatively few studies exploring the metabolic effects of Dapagliflozin and how they relate to weight loss. Moreover, the long-term metabolic adaptations to SGLT2 inhibitors are unknown.

Aims: Our primary objective was to execute detailed phenotyping (metabolic changes, glycosuric response, natriuretic response, changes to appetite, and body composition) in participants with T2DM and obesity, treated with Dapagliflozin therapy and concomitant dietary intervention. We also explored the ketotic potential of Dapagliflozin.

Methodology: Participants underwent detailed metabolic studies, including indirect calorimetry (energy expenditure measurements), quantification of body fat, blood and urine samples analyses and appetite assessments in Metabolic Research Unit located at University Hospitals of Coventry and Warwickshire (UHCW), before, during and after 12-month therapy with Dapagliflozin.

Results: Dapagliflozin therapy resulted in a significant weight loss at 12 months (8.4 kg, $p<0.001$) driven by a fat loss (8.9 kg, $p<0.001$), reduction in leptin and insulin levels and reduction in insulin resistance, increase in glucagon levels, glycosuria and urinary volume. There were no changes in appetite, basal metabolic rate, adiponectin levels, fasting glucose and natriuresis. The rise in ketone levels was significant numerically but not clinically, indicating that SGLT2 inhibitor therapy alone does not cause significant ketosis.

Conclusion: Long term treatment with Dapagliflozin leads to significant metabolic changes in patients with T2DM and obesity which are sustained over 12 months and represent mechanisms that could reverse metabolic syndrome.

Abbreviations

ARB:	Angiotensin receptor blocker
Alt:	Alanine transaminase
BMI:	Body Mass Index
BP:	Blood pressure
CI:	Confidence interval
CKD:	Chronic Kidney Disease
CPAP:	Continuous positive airway pressure
CV:	Cardiovascular
DKA:	Diabetic ketoacidosis
DPP4:	Dipeptidyl peptidase 4
EDTA:	Ethylenediaminetetraacetic acid
EE:	Energy Expenditure
eGFR:	Estimated glomerular filtration rate
ESRD:	End stage renal disease
euDKA:	Euglycaemic diabetic ketoacidosis
GLP1:	Glucagon like peptide 1
GORD:	Gastro oesophageal reflux disease
HbA1c:	Glycosylated haemoglobin
HDL:	High density lipoprotein
HF:	Heart failure
HMRU:	Human metabolism research unit
HOMA-IR:	Homeostatic Model Assessment of Insulin Resistance
HOMA %B:	Homeostasis Model of beta-cell insulin secretory function
LDL:	Low density lipoprotein
MACE:	Major adverse cardiovascular events
MI:	Myocardial infarction
mmHg:	Millimetre of Mercury
MRI:	Magnetic resonance imagining

mTORC1:	Mammalian target of rapamycin complex
NASH:	Non-alcoholic steatohepatitis
NHE3:	Sodium-hydrogen exchanger isoform 3
NSAID:	Non-steroidal anti-inflammatory drug
OA:	Osteoarthritis
OSA:	Obstructive sleep apnoea
PCOS:	Polycystic ovary syndrome
PE:	Pulmonary Embolus
RAAS:	Renin angiotensin aldosterone system
RPM:	Revolutions per minute
RRT:	Renal replacement therapy
SD:	Standard deviation
SE:	Standard error
SGLT2:	Sodium glucose like cotransporter 2
SST:	Serum separator collection tubes
T1DM:	Type 1 Diabetes Mellitus
T2DM:	Type 2 Diabetes Mellitus
TSH:	Thyroid stimulating hormone
UACR:	Urinary albumin to creatinine ratio
UHCW:	University Hospitals of Coventry and Warwickshire
UK:	United Kingdom

Chapter 1: Introduction

Chapter 1: Introduction

In this chapter, I will set the context to my research work in patients with Type 2 diabetes mellitus (T2DM) and introduce my research project focusing on metabolic adaptations following Sodium Glucose Co-Transporter 2 (SGLT2) inhibitor therapy.

I will review literature on whole-body adaptations following SGLT2 inhibitor therapy, specifically the effects mediated by enhanced glucose excretion (glycosuria) and enhanced sodium excretion (natriuresis). This chapter is concluded with my research aims and objectives.

1.1 Context of the research

1.1.1 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder characterised by hyperglycaemia that results from impaired insulin secretion, insulin resistance or both (Krentz and Bailey, 2001). According to World Health Organisation the prevalence of diabetes (both type 1 and 2) was 422 million in 2014, 8.5% of the entire world population (WHO, 2020). In the UK, the prevalence is 3.9 million and on average, 90% of the patients have T2DM (DiabetesUK, 2020).

Insulin resistance syndrome (also known as metabolic syndrome) is seen in most patients with T2DM, and consists of obesity, glucose intolerance, hypertension, dyslipidaemia, endothelial dysfunction, atherosclerotic cardiovascular disease, hyperinsulinaemia and insulin resistance (DeFronzo, 2010). Additionally, patients with diabetes have chronic sodium retention, and resulting water retention could partly explain the link between diabetes, hypertension and congestive cardiac failure (O'Hare and Corrall, 1988).

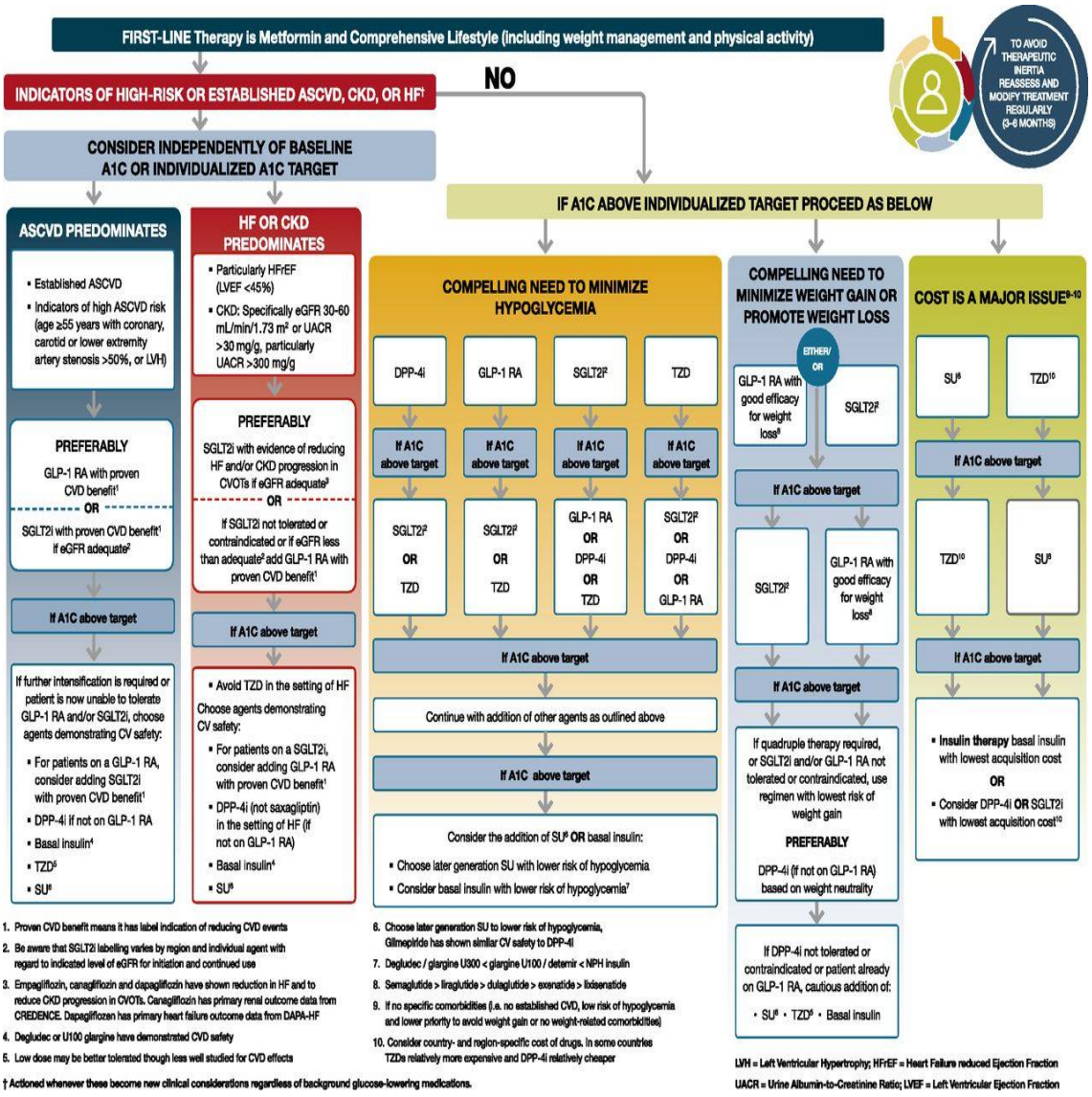
Patients with T2DM have increased microvascular and macrovascular complications, with the latter representing the primary cause of mortality for these patients (DeFronzo, 2010). Life expectancy of patients with T2DM is reduced on average by 5 to 10 years, and the leading cause for this is cardiovascular mortality (Krentz and Bailey, 2001).

Improved glycaemic control in patients with T2DM leads to a reduction in microvascular complications (Stratton et al., 2000), however, tight glycaemic control with insulin does not significantly alter risk of macrovascular complications in these patients (UKPDS, 1998, Gerstein et al., 2008, Heller, 2009, Duckworth et al., 2009). On the other hand, each component of insulin resistance syndrome is known to be associated with increased risk of cardiovascular disease (Bonadonna et al., 1990, Bajaj and Defronzo, 2003) and treatment that addresses components of insulin resistance syndrome could lead to a reduced risk of macrovascular complications in these patients. However, the relationship between insulin resistance syndrome and macrovascular complication is more complex, as evidenced by cardiovascular outcomes of thiazolidinediones, a class of antidiabetic medication designed to improve insulin resistance. Despite improved insulin resistance, this class of medication (the only medication from its class available in the UK is pioglitazone) has clear warning against its use in patients with heart failure.

Traditionally, treatment for diabetes was focused on glucose lowering but not on other metabolic risk factors, such as obesity, hypertension and insulin resistance. It is unsurprising that treatment of diabetes with the older agents, such as insulins or sulphonylureas, did not alter patients' risk of cardiovascular disease to a great extent. (Abdul-Ghani et al., 2016). The newer classes of antidiabetic medication, sodium glucose co-transporter (SGLT) 2 inhibitors and glucagon like Peptide Hormone 1 (GLP1) analogues, acts as glucose lowering agents but also address multiple components of insulin resistance syndrome, as well as having effects on renal handling of sodium. The latest guideline (shown in Figure 1.1) for treatment of T2DM reflects the new evidence and recommends medications that have evidence for not only glucose lowering, but overall cardiovascular risk reduction. The most recent network meta-analysis by Palmer et al. (2021) confirmed that both SGLT2 inhibitors and GLP1 analogues reduced cardiovascular and renal adverse outcomes in patients with T2DM.

Figure 1. 1: Glucose-lowering medication in type 2 diabetes: overall approach

(American Diabetes Association, 2020)



ASCVD, atherosclerotic cardiovascular disease; CKD, chronic kidney disease; CV, cardiovascular; CVD, cardiovascular disease; CVOTs, cardiovascular outcomes trials; DPP-4i, dipeptidyl peptidase 4 inhibitor; eGFR, estimated glomerular filtration rate; GLP-1 RA, glucagon-like peptide 1 receptor agonist; HF, heart failure; SGLT2i, sodium–glucose cotransporter 2 inhibitor; SU, sulfonylurea; TZD, thiazolidinedione

1.1.2 Sodium-glucose co-transporter

Currently 6 types of SGLTs are recognised (Wright et al., 2011); in the this review I will focus on SGLT1 and SGLT2 subtypes. SGLT2 is a high capacity low affinity membrane protein, which couples with 1 molecule of sodium and 1 molecule of glucose; whereas SGLT1 is a high affinity low capacity transporter, which couples with 2 molecules of sodium and one glucose molecule (Wright et al., 2011). Figure 1.2 shows the structure of SGLT (Image and description of an image are from Faham et al. (2008)).

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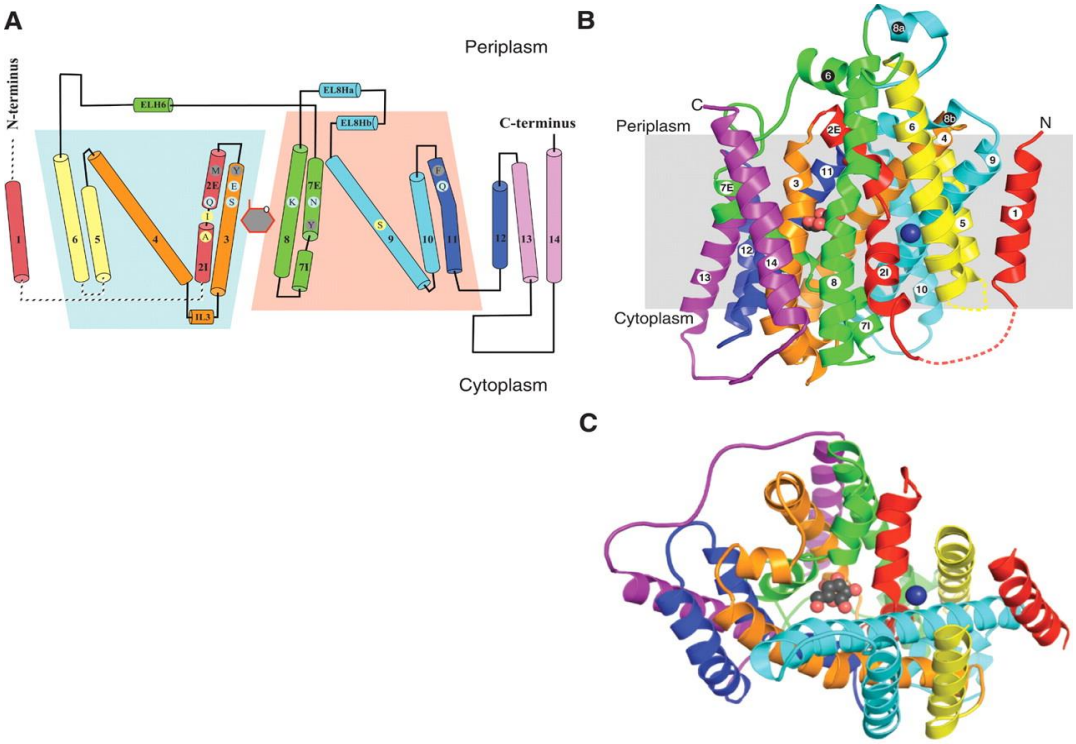
Figure 1. 2:Structure of a vSGLT (bacterial homolog of SGLT coded by the SgLs gene of Vibrio parahaemolytics)



(A)Topology. The structure is coloured as a rainbow from the N terminus (red) to the C terminus (purple). The blue and red trapeziums represent the inverted topology of TM2 to TM6 and TM7 to TM11. The grey hexagon with red trim represents the galactose. Residues involved in sugar recognition, gate residues, and a proposed Na+ site are shown in cyan, grey, and yellow circles. (B) Structure viewed in the membrane plane. The colouring scheme and numbering of α helices is the same as in Fig. 1A. Bound galactose is shown as black and red spheres for the C and O atoms. The proposed Na+ ion is coloured as a blue sphere. (C) Structure viewed from the intracellular side.

SGLT2 is mainly found in kidney (early proximal tubule), but in small amounts in brain, liver, thyroid gland, muscle and heart; SGLT1 is found mostly in the small intestine, but also in kidney (late proximal tubule), brain, heart, intestine, trachea, testis and prostate (Nishimura and Naito, 2005).

In a healthy person kidneys filter roughly 180 L of plasma every 24 h, containing around 160-180 g of glucose, almost all of which is reabsorbed (Diamant and Morsink, 2013). As a result, in a non-diabetic person with a plasma glucose levels between 4-10 mmol/L the urine should not contain any glucose (Wright et al., 2011), but as the plasma concentration exceeds 11.1 mmol/L, glucose starts appearing in the urine and then gets excreted proportionally to its plasma level.

SGLTs are responsible for reabsorption of most of the glucose filtered by the glomerulus. The relative contributions of these receptors were thought to be 90% and 10% of the renal glucose reabsorption for the SGLT2 and SGLT1 respectively (Chao and Henry, 2010, Wright et al., 2011). However, this relationship was proven to be more complex as SGLT2 inhibitors were found to block only up to 50% of renal glucose reabsorption in vivo (Komoroski et al., 2009) and a study by Nagata et al. showed both types of SGLT are working at a different capacity depending on the glycaemic conditions (Nagata et al., 2012). Hummel et al. (2011) proposed that in fasting human subjects SGLT2 would work at 50% capacity and Nagata et al. (2012) found that hypoglycaemia resulted in SGLT1 being dominant receptor for the renal glucose reabsorption. These findings could explain why the risk of hypoglycaemia with SGLT2 inhibitors is minimal, as the SGLT1 is contributing to most of the renal glucose reabsorption when blood glucose concentration drops.

Interestingly, increased plasma glucose in patients with T2DM leads to an increased glucose renal reabsorption, further worsening their hyperglycaemia (List et al., 2009). It has been reported that there is 20% increase in renal glucose reuptake in diabetic patients compared to healthy subjects with the equivalent plasma glucose level (Vallon, 2015).The likely mechanism for this

is upregulated expression of SGLT2 in diabetic patients (Rahmoune et al., 2005).

Such an increase in SGLT2 expression will also lead to enhanced reabsorption of sodium, contributing to increased sodium retention seen in patients with diabetes. Changes in sodium and glucose concentration, both osmotically active particles, trigger changes in water distribution, and will impact on the renin-angiotensin-aldosterone system (RAAS) and other homeostatic mechanisms. It is not clear whether RAAS is downregulated to offset the sodium retention, or increased to offset fluid loss caused by osmotic effects of increased glycosuria in patients with poorly controlled T2DM.

1.1.3 SGLT2 inhibitor

There are many commercially available SGLT2 inhibitors. These include Dapagliflozin, Empagliflozin, Canagliflozin and Ertugliflozin which are available in the UK, and Ipragliflozin, Luseogliflozin and Tofogliflozin available worldwide. Sotagliflozin is a combined SGLT1 and SGLT2 inhibitor. In this review I will focus on Dapagliflozin.

SGLT2 inhibitors prevent reabsorption of glucose and sodium, and thus promote urinary glucose excretion (glycosuria) and urinary sodium excretion (natriuresis). The changes in glucose and sodium excretion following SGLT2 inhibitor therapy depend on many factors and are responsible for the therapeutic effects seen with this class of antidiabetic medication.

SGLT2 inhibitors decrease plasma glucose in an insulin independent way (List et al., 2009). A study on healthy volunteers found that SGLT2 inhibitors act in a dose-dependent manner, with maximum urinary excretion of glucose being 18-62 grams per day (Komoroski et al., 2009). In patients with T2DM, the amount of glucose excreted ranges between 50 and 120g per day when taking SGLT2 inhibitors over the period of 12 weeks (List et al., 2009, Merovci et al., 2014, Devineni et al., 2012, Sha et al., 2014). Bolinder et al. (2014) looked at changes in urinary glucose excretion in patients with T2DM taking SGLT2

inhibitors for 102 weeks and found that the level of glucose excreted was stable (115 mmol/L) at 102 weeks. However, his study used spot urine glucose measurements as an estimate of overall renal glucose excretion rather than 24-hour measurements (and hence mmol/L were used rather than grams per day). Currently, there is no long term follow up data (beyond 12 weeks) on 24-hour urinary glucose excretion with SGLT2 inhibitors.

Similarly, the evidence on long term effect of SGLT2 inhibitors on urinary sodium excretion is mixed, with studies showing no effect on natriuresis at 2 months (List et al., 2009), as well as ongoing natriuresis at 6 months (Kawasoe et al., 2017). Acute natriuresis (within day 1 to 5) following initiation of SGLT2 inhibitor therapy has been well documented in several studies (Sha et al., 2014, Tanaka et al., 2017). It is possible that most of the sodium loss occurs in the first few days following treatment, and then compensatory mechanisms, such as activation of renin angiotensin aldosterone system, prevent further sodium losses. However, long term treatment with SGLT2 inhibitors lead to plasma volume reduction that is sustained (Reed, 2016), which means that the initial sodium deficit is unlikely to be replaced.

Our incomplete understanding of changes to sodium excretion following SGLT2 inhibition is also influenced by our current measurement of sodium handling. Monitoring sodium balance with 24-hour urinary sodium measurements does not provide full picture of sodium homeostasis. Titze et al. (2002) showed that 24-hour sodium excretion does not equal to 24-hour sodium ingestion in healthy volunteers. In this study sodium gain (via food) exceeded sodium loss but did not lead to corresponding weight gain. This was a first study that suggested that sodium can be accumulated in osmotically inactive form (in an interstitial fluid), highlighting a more complex explanation to sodium handling.

It has been suggested that SGLT2 inhibitors can affect interstitial fluid volume without affecting intravascular volume (Verma and McMurray, 2018). This has been supported by Karg et al. (2018) who measured sodium concentration in the skin of patients with T2DM using MRI, and found that treatment with SGLT2 inhibitor Dapagliflozin reduced skin sodium concentration in these

patents. Our understanding of changes in overall body sodium levels following SGLT2 inhibitors is still incomplete, but it is clear that 24-hour urinary measurements should be complemented with other techniques, such as sodium assessment with ^{23}Na-magnetic resonance imaging techniques.

1.1.4 Measurements of energy expenditure, fat mass and appetite

Accurate measurement of energy expenditure is a key aspect of metabolic studies. There are many methods for measuring energy expenditure, such as direct calorimetry, indirect calorimetry (metabolic chambers, metabolic carts, doubly labelled water) and non-calorimetric techniques, such as heart rate and ventilation monitoring. Direct calorimetry is based on the assumption that energy spent is dissipated as hear and that total energy expenditure can be measured by quantifying heat production (Jequier, 1986). This means that all heat transfers have to be captured and measured, such as radiation, convection, conduction and heat loss (Webb, 1980). Even though this provides accurate results, it is technically challenging and also cannot be used to measure acute changes in energy expenditure (Jequier, 1986). Newer armband devices are able to monitor skin temperature and heat flux from the skin surface and may provide better methods for direct calorimetry in the future.

Indirect calorimetry assesses metabolic rate by measuring amount of oxygen consumption and carbon dioxide production and calculating the amount of energy released during oxidation (Ravussin et al., 1982). The detailed description of indirect calorimetry is in the methodology section. The main limitation of indirect calorimetry performed in a sealed chamber is that subjects are confined to a small space and thus energy expenditure in a free-living space and effects of exercise cannot be measured reliably. However, this method provides accurate estimation of energy expenditure. Additionally, this method provides information about carbohydrate, protein and fat oxidation.

Metabolic carts (also known as hoods) are alternative methods of indirect calorimetry as the amount of oxygen and carbon dioxide exchange is measured. The main advantage of this method is the fact that subjects are more mobile but most studies using hoods measure metabolic rates for hours, rather than days (Lam and Ravussin, 2016). Doubly labelled water uses water with a known amount of labelled non-radioactive isotope of hydrogen and oxygen, which can be measured (and turnover rates quantified) when they leave body as water (measure in urine) and carbon dioxide (Lam and Ravussin, 2016). With known respiratory quotient the ration of gas exchange can be used to estimate energy expenditure. This technique is useful for longer studies and for assessing energy expenditure in free living environment (Rochon et al., 2011).

Non-calorimetric techniques encompass physical activity logs, pedometers and accelerometers, heart rate monitoring and ventilation monitoring (V VO_2, and VCO_2 (Lam and Ravussin, 2016). The main advantage of these techniques is the non-invasiveness and ability to monitor energy expenditure in a free-living environment, however, the accuracy of these measurements is lower than for the calorimetric techniques.

Fat mass assessment can be done with bioelectric impedance machines, densitometry studies (Dual energy X-ray Absorptiometry (DEXA) scans, whole body densitometry BodPod), computed tomography (CT), and magnetic resonance imaging (MRI) and spectrometry (Baum et al., 2016). Bioelectric impedance machines are widely available, however, cannot assess regional compartments of body fat and their characteristics (Mulasi et al., 2015). Densitometry scans provide differentiation of bone, fat and lean soft tissue, are relatively quick and easy to perform but do not provide localisation of fat mass. CT provides determination of visceral and subcutaneous fat mass, as well as liver fat mass, however, exposes individual to radiation (Baum et al., 2016). MRI provides the same information as CT scan without the dangerous radiation. The main disadvantage of MRI in assessing body fat content is the time, cost and acceptability to obese population,

Appetite measurement relies on subjective analysis of a subject's appetitive sensations using a visual analogue scale. Visual analogue scales (VAS) are validated 100 mm scales routinely used for assessment of appetite (Sepple and Read, 1989) and consists of questions about motivation to eat degree of hunger. A more detailed description is in the methodology section. The main limitation of VAS is the validity and reproducibility of appetitive assessment (Lam and Ravussin, 2016). A variety of external factors, such as environment, sensory stimulation or habit influence subject's responses (Mattes et al., 2005). Appetite is influenced by gut peptides, such as ghrelin and GLP-1, as well as adipocytokine leptin and glucose levels. Inclusion of these parameters improve accuracy of appetitive measurement as they are influenced by external events to a lesser degree (Lam and Ravussin, 2016).

1.2 A whole-body adaptation to SGLT2 inhibitor therapy- a literature review

1.2.1 SGLT2 inhibitors and effects mediated by glucose excretion

1.2.1.1 SGLT 2 inhibitors and changes in plasma glucose, insulin and glucagon

Human metabolism is likely to undergo changes in response to glucose deficit induced by SGLT2 inhibitors by various mechanisms, such as adaptation of hormonal levels, appetite, energy expenditure and fuel selection (Ferrannini et al., 2014).

Treatment with SGLT2 inhibitors leads to a reduced plasma glucose levels, translating to a reduction of HbA1c. The observed reduction in HbA1c ranges from 0.66% to 0.8% (Vasilakou et al., 2013, Vallon, 2015). A comprehensive study by Ferrannini et al. (2014) found that SGLT2 inhibition in patients with T2DM led to a reduction in both fasting and post prandial blood glucose levels, with corresponding reduction in insulin secretion and improved insulin sensitivity. This occurred despite 25% increase in endogenous glucose production, which represented the likely compensatory mechanism for induced glycosuria. Beta cell function was estimated to be improved as there was 25% more insulin released for the equivalent level of plasma glucose, despite the overall drop in insulin output (Ferrannini et al., 2014).

Similar trend of rise in endogenous glucose production despite lowered overall fasting plasma glucose was also observed in diabetic patients taking SGLT2 inhibitors in a study by Merovci et al. (2014). Such rise in an endogenous glucose production in patients taking SGLT2 inhibitors was not expected, and was labelled as ‘glucose paradox’ by Cefalu (2014). The rise in endogenous glucose production could be partly explained by the observed increase of the glucagon response (Ferrannini et al., 2014).

Similar finding of an increased glucagon level following SGLT2 inhibition was reported by Bonner et al., who also found presence of SGLT1 and SGLT2 on pancreatic alpha cells (Bonner et al., 2015). Such a rise in plasma glucagon

and decrease in plasma insulin concentration could represent the mechanism for the glucose paradox, and potentially could explain the rare euglycaemic ketoacidosis, a side effect of SGLT2 inhibitors. However, this theory is yet to be tested as no study measured levels of ketones together with changes in glucagon and insulin ratios following SGLT2 inhibition. Additionally, as reduction in insulin and increase in glucagon is likely to be seen in all patients on SGLT2 inhibitors, this would not explain why only 0.3% of patients develop euglycaemic ketoacidosis (Wiviott et al., 2019).

Ferrannini et al. (2014) suggested that the increase in endogenous glucose production exactly balanced the urinary glucose loss, but Merovci et al. (2014) argued that this increase was not sufficient to neutralise the glucose loss in urine. Further studies are needed to elucidate the exact relationship between the amount of endogenous glucose production and urinary glucose loss.

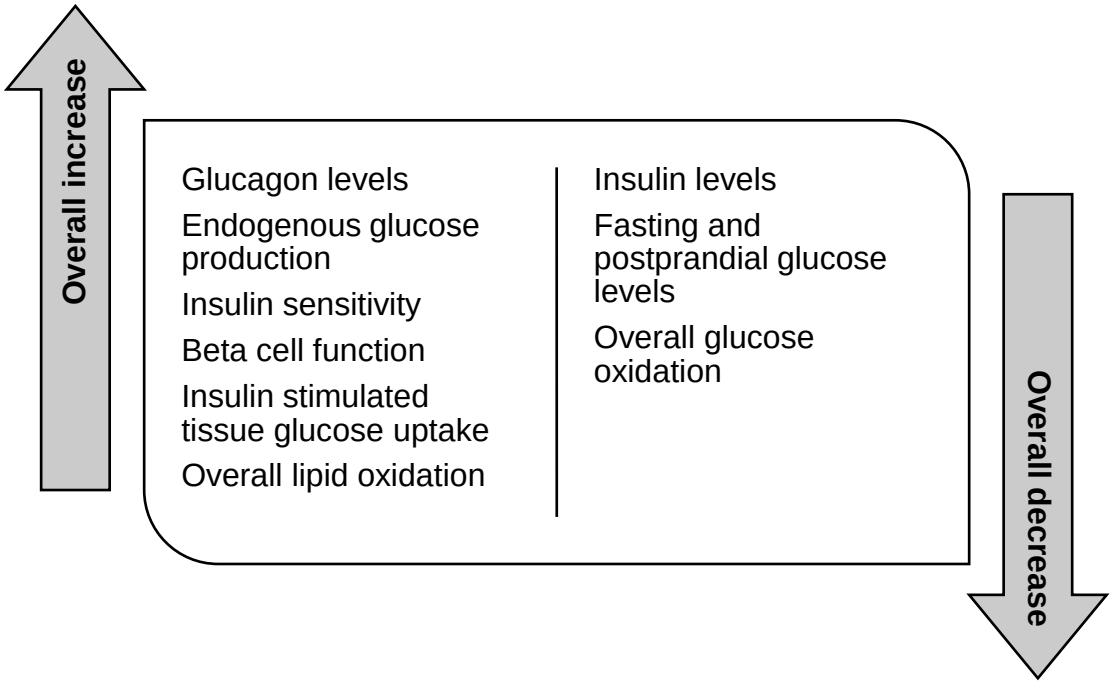
Glucotoxicity refers to a concept of chronic hyperglycaemia which worsens insulin resistance and beta cell dysfunction in patients with T2DM (Rossetti et al., 1990). Merovci et al. (2014) found that by reducing fasting plasma glucose, SGLT2 inhibitors improved insulin stimulated tissue glucose uptake, and as such improved insulin sensitivity and directly antagonised the effects of glucotoxicity. Additionally, fat loss observed with SGLT2 inhibitor therapy also leads to increased insulin sensitivity and thus represent additiona(Ferrannini et al., 2014)l mechanism for improved insulin sensitivity (Kurinami et al., 2018).

In addition to improved insulin sensitivity, SGLT2 inhibitors also led to a long-term decrease in glucose oxidation and rise in lipid oxidation (Ferrannini et al., 2014). It was suggested that if these compensatory mechanisms (increased endogenous glucose production and decreased glucose oxidation) did not occur, the observed drop in glucose would be 50% rather than 12% (Ferrannini et al., 2014).

In conclusion, the overall effect of SGLT2 inhibitors on glucose and associated hormones is a fall in insulin and rise in glucagon, together with improved insulin sensitivity of tissues (summarised in Figure 1.3, adapted from Ferrannini et al. (2014), Cefalu et al. (2014) and Merovci et al. (2017)). However, as Cefalu

(2014) highlighted, currently there is not enough high quality data evaluating the whole body metabolic adaptation to treatment with SGLT2 inhibitors, specifically reasons behind discrepancy between calories lost in urine (urinary glucose loss) and total weight loss, as well as impact of changes in insulin and glucagon and glycosuria on appetite controlling hormones. SGLT2 inhibitors are often prescribed on a long-term basis, and it is important to provide evidence for the long term (longer than 6 months) effects on insulin, glucagon, glycosuria and energy expenditure.

Figure 1. 3: Changes in insulin, glucagon, glucose, insulin sensitivity and substrate oxidation induced by SGLT2 inhibitor therapy



1.2.1.2 SGLT2 inhibitors and changes in body weight and energy expenditure

Weight loss is a crucial part of management of patients with T2DM and it has been shown that even a 5% weight loss improves insulin sensitivity, glycaemic control, dyslipidaemia and cardiovascular health (Maggio and Pi-Sunyer, 1997).

Weight loss is well recognised secondary effect of SGLT2 inhibitors. Many studies provide detailed insight into the weight loss potential of SGLT2 inhibitors. The reported range of weight loss with SGLT2 inhibitors is wide, starting from 0.14 kg to 3.2 kg at 24 weeks (Ferrannini et al., 2010, Bailey et al., 2012, Bailey et al., 2010, Yang et al., 2016, Wilding et al., 2012, Strojek et al., 2011, Rosenstock et al., 2012, Jabbour et al., 2013, Mathieu et al., 2015, Matthaei et al., 2015a, Matthaei et al., 2015b, Henry et al., 2012, Rosenstock et al., 2014). The difference in weight loss between these studies was likely due to different doses of SGLT2 inhibitor, combination treatment with other antidiabetic mediation, and different populations of patients with T2DM (level of diabetic control, BMI etc).

SGLT2 inhibitors also lead to a sustained weight loss beyond 24 weeks in patients with T2DM. The large cardiovascular outcome trials on Empagliflozin, Canagliflozin and Dapagliflozin provided comprehensive long-term data (from 2.4 to 4.2 years of median follow up) on weight loss (Zinman et al., 2015, Neal et al., 2017, Wiviott et al., 2018, Perkovic et al., 2019). These are summarised in Table 1.1.

Table 1. 1: Weight loss in large cardiovascular outcome trials of SGLT2 inhibitors, from (Zinman et al., 2015, Neal et al., 2017, Wiviott et al., 2019)

SGLT2 inhibitor	Length of follow up (years)	Number of patients	Weight loss (kg)
Empagliflozin	3.1	7 020	-1.98
Canagliflozin	2.4	10 142	-1.6
Dapagliflozin	4.2	17 160	-1.8

In summary, the long-term weight loss potential of these 3 SGLT2 inhibitors (in patients with T2DM and preserved renal function) is just under 2 kg, with no significant difference in weight loss potential between the various SGLT2 inhibitors (Molugulu et al., 2017).

A recent meta-analysis of 55 randomised controlled trials showed all SGLT2 inhibitors resulted in weight loss, mean weight loss ranging from 0.44 kg to 2.37 kg for the different classes of SGLT2 inhibitors (Cai et al., 2018), however, the doses, and types of SGLT2 inhibitors were different in each trial, and there was heterogeneity in the length of patient follow up, giving smaller weight loss values that described above.

Another meta-analysis from 2019 evaluated 11 studies (in total 6336 patients) of patients with chronic kidney disease and T2DM who were treated with SGLT2 inhibitor, and found that the average weight loss was 1.42 kg (Toyama et al., 2019). The lower overall weight loss seen in Toyama's study could be due to impaired glycosuric potential of the SGLT2 inhibitors as a result of impaired renal function.

It is possible that most of the weight loss occurs within the first 6 months of treatment, as Neeland et al.(2016) found that patients with T2DM who took Empagliflozin lost 1.7 kg at 12 weeks, followed by 1.9 kg at 24 weeks. This is similar to the weight loss (1.98 kg) seen with Empagliflozin at 3 years (Zinman et al., 2015). On the contrary, Bolinder et al. (2014) found that weight loss potential of SGLT2 inhibitors did not stop at 24 weeks, as patients taking

SGLT2 inhibitor Dapagliflozin lost 4.54 kg from their total body weight at 102 weeks, compared with 2.08 kg at 24 weeks. However, the study by Bolinder et al. involved only 182 patients at 24 weeks, which was further reduced to 140 patients at 102 weeks.

Given the glycosuric mechanism of action and minimal risk of hypoglycaemia, SGLT2 inhibitors were also tested in non-diabetic population, in order to assess their weight loss potential. There was a 2.8 kg weight loss following initiation of Canagliflozin 100 mg (Bays et al., 2014) and 3 kg weight loss following 10 mg of Dapagliflozin at 12 weeks (Ramírez-Rodríguez et al., 2018) in non-diabetic population. It is important to note that SGLT2 inhibitors are not licensed for weight loss in non-diabetic population. However, the weight loss observed was similar to that seen in clinical trials in patients with diabetes, suggesting that SGLT2 inhibitors lead to weight loss despite normoglycaemia.

Kurinami et al. (2018) assessed clinical factors associated with weight loss in diabetic patients taking SGLT2 inhibitors, and found that greater fat reduction was observed in smokers and in patients with HbA1c higher that 7.7%. This was most likely due to increased renal glucose loss in patients with higher HbA1c. Additionally, higher doses of SGLT2 inhibitors were associated with the bigger weight reduction, but only dapagliflozin had a significant trend for dosage and weight changes (Cai et al., 2018). Interestingly, SGLT2 inhibitor induced weight reduction is not associated with patients' age, gender, BMI or diabetes duration (Cai et al., 2018).

Weight loss potential of SGLT2 inhibitors is enhanced when combined with glucagon like peptide 1 (GLP1) analogue, another class of antidiabetic medication. Studies found that the weight loss of such a combination therapy ranges between 3 and 5 kg (Frías et al., 2016, Mearns et al., 2015). This is most likely as a result of GLP1 analogue suppressing appetite and thus preventing the possible hyperphagia that could be induced by SGLT2 inhibitors.

There are two proposed mechanisms for the observed weight loss. First one is the loss of calories due to increased urinary glucose excretion (Cai et al.,

2018), leading to a fat loss. Vallon and Thompson (2017) proposed that if there was no reabsorption of filtered glucose in a healthy subject (around 160 g), energy lost via kidneys would be roughly 30% of daily energy expenditure. This would mean that in a patient with diabetes who loses around 80 g of glucose as a result of SGLT2 inhibition, this could equate to 15% of daily energy expenditure.

Bolinder et al. (2014) found that weight loss in patients taking Dapagliflozin was mainly due to reduction in total fat mass observed throughout the 102 weeks of follow up (2.8 kg fat loss in treatment group versus 1.46 kg fat loss observed in placebo group, with no changes in lean mass). Merovci et al. (2014) found that chronic treatment with SGLT2 inhibitors increased lipid oxidation and decreased glucose oxidation (which will lead to fat loss), suggesting that this shift compensated for the negative energy balance induced by SGLT2 inhibitors. Similar finding was observed by Ferrannini et al. (2014).

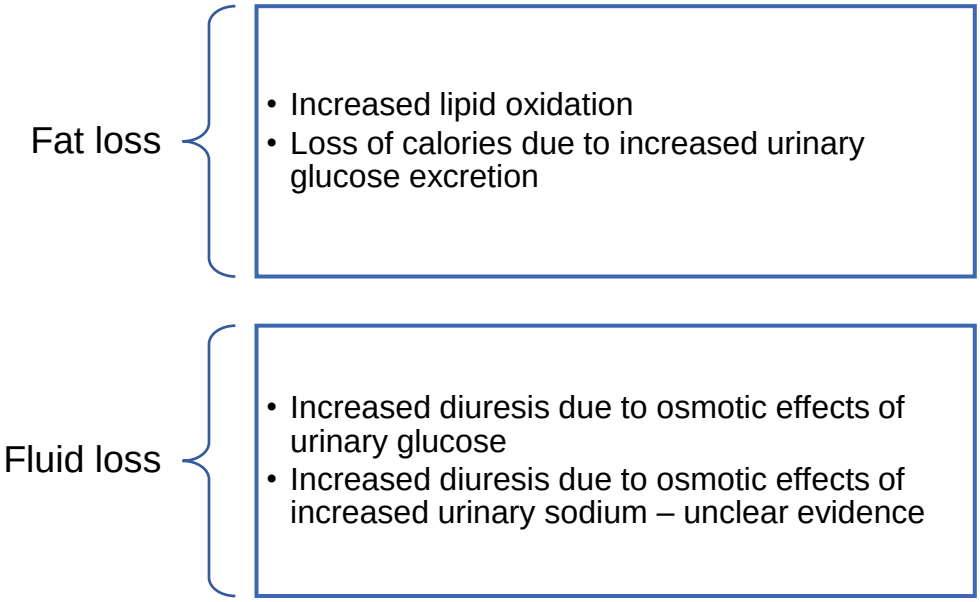
Second possible mechanism for weight loss is a fluid loss. List et al. (2009) demonstrated small dose-related increases in 24-h urine volumes (range 107–470 ml above baseline of 1.8–2.2 l) in patients treated with Dapagliflozin. Lambers Heerspink et al. (2013a) found that Dapagliflozin resulted in 7.3% reduction in plasma volume compared to placebo at 12 weeks. This was thought to be as a result of a osmotic diuresis due to a combination of enhanced sodium excretion and increased urinary glucose excretion (Lambers Heerspink et al., 2013a). However, Sha et al. (2014) showed that this hypothesis may not be true, as Canagliflozin did not change the 24-h total urinary excretion of sodium at week 1 or week 12 as compared with placebo. Further studies are clearly needed to explain the changes in 24-hour urinary sodium in patients taking SGLT 2 inhibitors.

Interestingly, Sha et al. (2014) found that Canagliflozin reduced plasma volume by 5.4% and increased urinary volume by 161 ml from baseline at week 1, but this reduction in plasma volume and increase in urinary volume was attenuated and not statistically different compared to placebo at week 12. However, the study observed statistically significant increases in haematocrit

and it was likely that small reduction in plasma volume was sustained at 12 weeks of treatment.

Most likely explanation for observed weight loss is a combination of fluid and fat loss. Bailey et al. (2010) found that patients taking Dapagliflozin experienced early weight loss, which was followed by a progressive reduction in body weight with decreased waist circumference. Lambers Heerspink et al. (2013a) also found that weight loss (3kg at 12 weeks) was biphasic, with initial steep fall followed by a gradual continuous reduction. It is likely that fluid loss contributes to the weight loss seen in the first week, and fat loss is responsible for the weight reduction seen in following weeks (Sha et al., 2014). Summary of the proposed mechanisms for weight loss is shown in Figure 1.4.

Figure 1. 4: Summary of suggested mechanism for weight loss with SGLT2 inhibitors



Despite the currently proposed theories, the mechanism for the observed weight loss is not fully understood. With SGLT2 receptors blocked, around 50% of the renal glucose (around 80 g) will be reabsorbed by SGLT1 receptors in patients with normoglycaemia, with more glucose lost in urine in patients with hyperglycaemia. As a result, the observed urinary glucose loss of 80-120 g of glucose/day in patients with diabetes could translate into caloric deficit between 320-480 kcal/day, leading to a weight loss between 5.5-7kg in 3-4

months (Cai et al., 2018), which is more than currently observed weight loss. Possible explanation for this discrepancy is upregulation of SGLT1 receptors, contributing to more glucose reabsorption, changes in food intake (Cai et al., 2018) or metabolic adaptations leading to reduced resting energy expenditure (Busetto et al., 2021).

It is well known that following weight loss human metabolism will adapt (by a reduction in a resting energy expenditure as well as increased hunger) to compensate for the weight loss in order to get back to the biological set point (Johannsen et al., 2012). The reduction in anorectic hormones and increase in orexigenic hormones leads to increased hunger and has been well described following weight loss (Busetto et al., 2021). Mathematical models exist to predict changes to body weight and composition following reduction in food intake or an increase in energy expenditure. Chow and Hall proposed two theories to model dynamics of human weight change, with the first theory being a stable fixed set point and second one of an invariant manifold, whereby a continuum of body weights exist, consistent with the same food intake and energy expenditure (Chow and Hall, 2008). However, human experiments need to be done to verify these theories and mathematical models.

Weight loss that is less than predicted from the amount of excreted urinary glucose was also reported by other studies (Ferrannini et al., 2010, Bailey et al., 2010). One of the possible explanations could be that following SGLT2 inhibition, total energy expenditure reduces to compensate for the negative energy balance, as seen following weight loss as part of metabolic adaptation discussed above. This was supported by a metabolic study in mice that showed that SGLT2 inhibitors led to an acute reduction of activity of brown adipose tissue and thus reduced energy expenditure (Chiba et al., 2016). However, a recent study in mice provided conflicting information about changes to the energy expenditure following SGLT2 inhibition. Lee et al. (2021) found that SGLT2 inhibition resulted in an increase of energy expenditure by increased thermogenesis, increased fatty acid oxidation in brown fat and reduction in the size of hypertrophied adipocytes. However, both of these studies were done in mice.

On the other hand, resting energy expenditure was unchanged following SGLT2 inhibition in people living with T2DM (Ferrannini et al., 2014). This study also suggested that changes in appetite induced by SGLT2 inhibition (discussed in next section), resulting in higher energy intake, could provide an alternative explanation for the observed discrepancy between expected and observed weight loss.

1.2.1.3 SGLT2 inhibitors and effects on appetite regulation

The hypothesis of altered appetite regulation following initiation of SGLT2 inhibitor was first investigated in 2012. Devenny et al. (2012) provided evidence that SGLT2 inhibitor resulted in compensatory hyperphagia in rodents, which reduced the potential for weight loss. The changes in food intake in these rodents occurred after one week, and therefore the proposed mechanism was an adaptation to chronically increased glucose excretion rather than a direct action of the SGLT2 inhibitors on appetite control (Devenny et al., 2012). This hypothesis was further supported by Nagata et al. (2013) who observed similar trend for increased food consumption in mice treated with SGLT2 inhibitor.

Napolitano et al. (2014) showed no difference in weight loss in patients with obesity treated with SGLT2 inhibitor compared to patients treated with placebo, despite the increased calorie loss due to increased urinary glucose excretion, postulating that SGLT2 inhibitors could increase appetite. None of these research groups evaluated the impact of appetite regulating hormones on the observed hyperphagia, however, Moran (2010) postulated that hypothalamic neurons have the ability to detect changes in hormonal and nutrient levels, such as changes in glucose, insulin and glucagon, which would further link the appetite control and increased urinary excretion induced by SGLT2 inhibitors.

A conflicting evidence was provided by Tahara et al. (2013), Terami et al. (2014) and Salim et al. (2016) who found no effect of SGLT2 inhibitor on food intake in rodents. A more recent study supported this hypothesis that SGLT2 inhibitor did not lead to increase food consumption in rodents, and in fact improved appetite regulating hormones (reduction in ghrelin) in rodents with T2DM, which prevented the compensatory hyperphagia following increased renal glucose loss (Tahara et al., 2018). In this study, SGLT2 inhibitors did not have any effect on leptin levels.

The likely mechanism for this action is a change in appetite stimulating hormone ghrelin. Patients with T2DM have increased levels of appetite

stimulating hormone ghrelin (Zuo et al., 2013). Tahara et al. (2016) found that SGLT2 inhibitors reduced the levels of appetite stimulating hormone ghrelin, thus improving their appetite control. Tahara et al. (2018) went further and found that SGLT2 inhibitor driven improvement in appetite-regulating hormones can be due to improving postprandial arteriovenous plasma glucose difference.

There is clearly a need for more detailed evaluation of any effects of SGLT2 inhibitors on appetite in humans, including the effects on appetite regulating hormones, such as leptin and ghrelin.

1.2.1.4 SGLT2 inhibitors and ketotic effects

Treatment with SGLT2 inhibitors leads to lipolysis and decreased glucose oxidation (Merovci et al., 2014) which results in increased ketone production. Historically, presence of ketones in patients with T1DM has been recognised as one of the hallmarks of a severe diabetic complication, diabetic ketoacidosis (DKA). DKA affects patients with T1DM, and consists of the triad of ketosis, hyperglycaemia and acidosis. The pathophysiology of DKA involves elevation of ketone levels to above 3.0 mmol in an insulin-deficient individual, alongside high blood glucose levels and falling pH (less than 7.30). In a healthy (non-diabetic) individual nutritional ketosis (such as in low carbohydrate diets) would be defined as ketone values above 0.5 mmol (Volek et al., 2015). There is no upper limit to nutritional ketosis, but it is generally accepted that nutritional ketosis will lead to blood ketone levels between 0.5 and 5mmol (Evans et al., 2017).

More recently euglycaemic DKA (euDKA) has been described in patients with diabetes. EuDKA can affect patients with both T1DM and T2DM, and is characterised by presence of ketones, acidosis and normal blood sugar level. It is a rare side effect of treatment with SGLT2 inhibitors, and has been first described in patient with T2DM and Prader-Willi syndrome on low carbohydrate diet by Hayami et al. (2015) and in two patients with T2DM following elective surgery (Peters et al., 2015). As it is a potentially dangerous side effect of SGLT2 inhibitors, a prompt testing for urinary or plasma ketones, together with plasma pH is important in patients who present with malaise, abdominal pain, vomiting or respiratory distress. The risk of developing euDKA is much higher in patients with T1DM and therefore it is important to establish the correct diagnosis for a patient who is started on SGLT2 inhibitor (Peters et al., 2015).

Increased glucagon levels observed following SGLT2 inhibition, together with increased lipolysis drive ketone production and have been postulated to drive development of euDKA (Vallon and Thomson, 2017). This could be further amplified in states of volume depletion that occur following period of illness or

hospitalisation, as hormones such as cortisol and noradrenaline will increase lipolysis and ketogenesis.

Interestingly, a large prospective trial of SGLT2 inhibitor empagliflozin did not find any difference in rates of ketoacidosis in patients with T2DM treated with active medication or placebo over the course of 3 years (Zinman et al., 2015), suggesting that the association between the SGLT2 inhibitor and development of euDKA is more complex. Meta-analysis of 4 cardiovascular outcome trials assessed 38 723 patients on SGLT2 inhibitors and showed that euDKA is a very rare event, with the risk of 0.17% (number needed to harm 595) (McKee et al., 2020).

On the contrary, possibly beneficial effects of mild ketosis driven by SGLT2 inhibitors have been described by Ferrannini et al. (2016). His team proposed a hypothesis of a beneficial effect of a mild hyperketonemia, mainly driven by beta-hydroxybutyrate replacing free fatty acids as a fuel in the heart. Beta-hydroxybutyrate is used as a substrate by brain, kidney and heart (Balasse and Féry, 1989). Beta-hydroxybutyrate is more efficient than free fatty acids as a substrate for energy as it requires less oxygen in the process of oxidation and thus improves cardiac efficiency (Sato et al., 1995).In hearts of patients with T2DM free fatty acids are used as a substrate to oxidation much more than in healthy controls, further increasing the amount of oxygen that is needed for oxidation by the muscle (Iozzo et al., 2002). If beta-hydroxybutyrate levels are increased following SGLT2 inhibition, they can be used as a substrate for oxidation in preference to free fatty acids, reducing overall oxygen consumption by the organ. Such a “thrifty substrate hypothesis” was suggested by Ferrannini et al. (2016) to partly explain the observed improved cardiovascular outcomes, such as improvement in heart failure and cardiovascular mortality. It is important to highlight that despite the plausible sounding hypothesis, there has been no human studies assessing the effect of beta-hydroxybutyrate infusion on myocardial function, and beta-hydroxybutyrate was not measured in any of the large cardiovascular outcome trials investigating the SGLT2 inhibitors.

There is a clear distinction between the potentially beneficial level of increased ketone production and progression to ketoacidosis which can be life threatening. The dose-response relationship between SGLT2 inhibitors and level of ketone production is not known, as well as the time course needed for the ketonemia to develop. Additionally, factors that can affect ketogenesis, such as low carbohydrate diets, have not been studied in detail relation to SGLT2 therapy in patients with T2DM. Low carbohydrate diets are known to increase the risk of developing euDKA in patients on SGLT2 inhibitors, however, the risk is higher in those who adhere to very strict low carbohydrate diets of less than 500kcal (Fukuyama et al., 2020). The effect of SGLT2 inhibitor combined with low carbohydrate diet (less than 100g of carbohydrate) without calorie restrictions has not been studied yet.

1.2.1.5 SGLT2 inhibitors and changes in lipids

The two main circulating lipids are triglycerides and cholesterol. Dyslipidaemia refers to abnormal levels of lipids, such as triglycerides, low-density lipoprotein (LDL) cholesterol or high-density lipoprotein (HDL) cholesterol. In clinical practice, three main categories of dyslipidaemia exist, which are hypercholesterolaemia, hypertriglyceridaemia and mixed hyperlipidaemia (Wass et al., 2014).

In patients with T2DM there are not only changes in lipid levels, but also changes in lipoprotein structure, its metabolism and turnover, all of which are associated with increased cardiovascular risk (Vergès, 2015).

There is a clear evidence to support the effect of SGLT2 inhibitor on lipid profile, including LDL cholesterol, HDL cholesterol, and triglycerides. First meta-analysis of studies published prior to 2015 found that SGLT2 inhibitor therapy (regardless of dose and type) increased HDL cholesterol (by 0.07 mmol/L) compared to placebo (Zaccardi et al., 2016). The review also identified that only Canagliflozin reduced triglyceride levels and increased LDL level (by 0.13mmol/L). The findings were based on 7829 patients, treated with different doses of SGLT2 inhibitor therapy. It is possible that no observed effect of Dapagliflozin and Empagliflozin on LDL levels and triglycerides was due to fewer studies available on those two agents.

In a subsequent meta-analysis of 34 randomised controlled trials (with total of 9154 patients) looking at SGLT2 inhibitors at their highest doses (Dapagliflozin 10mg, Empagliflozin 25mg and Canagliflozin 300mg), Stogaard et al. (2016) concluded that SGLT2 inhibitor therapy led to HDL and LDL increase (by 0.05 mmol/L and 0.09 mmol/L) and serum triglycerides decreased (by 0.09 mmol/L). There was a small difference between individual SGLT2 inhibitors, with Canagliflozin having larger effect on HDL and LDL cholesterol, as well as triglycerides (Storgaard et al., 2016). The latest meta-analysis (including 18 684 patients) found very similar effect of SGLT2 inhibitor on lipid profile, with increase in LDL and HDL by 0.09 mmol/L and 0.10 mmol/L(Zhang et al., 2018).

The mechanism underlying the increased LDL cholesterol and decreased triglycerides levels following therapy with SGLT2 inhibitors was investigated by Basu et al. (2018) who found reduced clearance of LDL cholesterol from the circulation, as well as greater lipolysis and faster clearance of triglycerides. However, this study was done in mice, limiting generalisability of the findings.

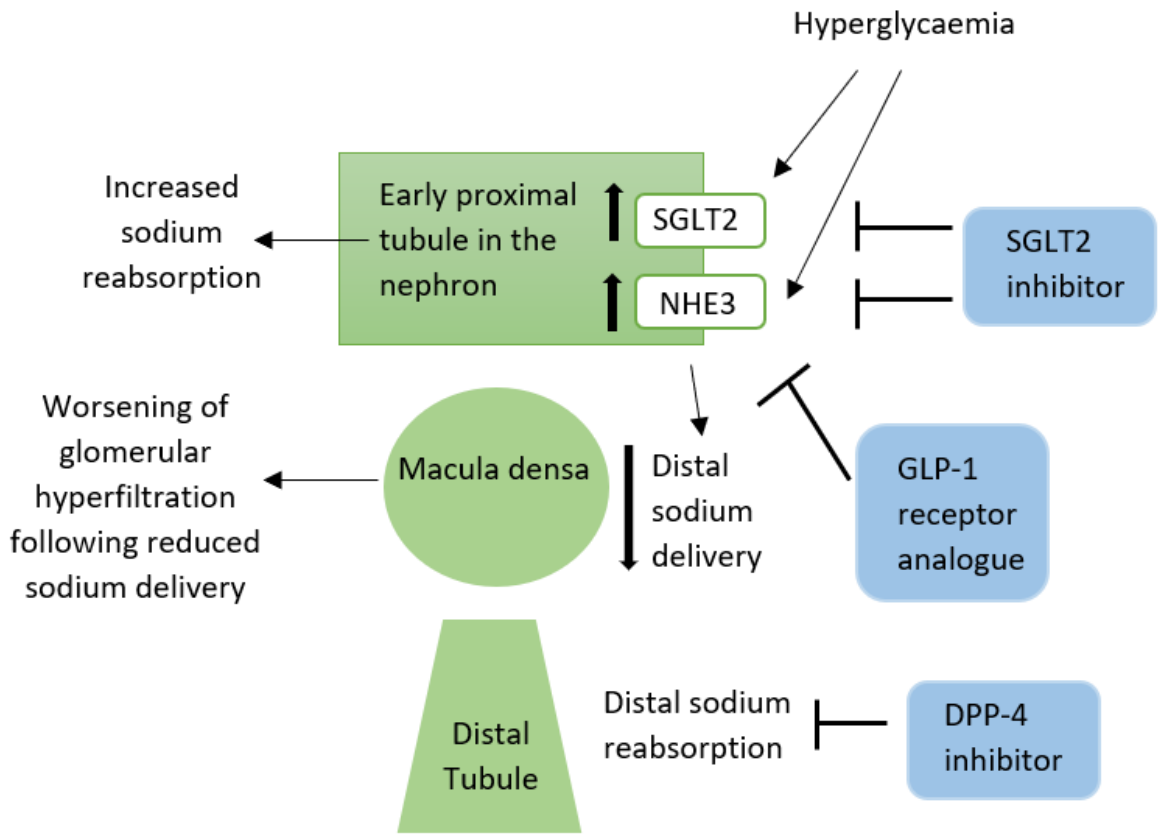
The clinical significance of the above discussed changes in lipids is questionable. With the treatment targets set by American Diabetes Association for LDL cholesterol to be less than 2.6 mmol/L for primary prevention of cardiovascular disease (2004), changes between 0.05 to 0.1 mmol/L will not provide any meaningful cardiovascular benefits.

1.2.2 SGLT2 inhibitors and effects mediated by sodium excretion

1.2.2.1 Natriuretic effect of antidiabetic medication

Three classes of antidiabetic drugs (GLP1 analogue, DPP-4 inhibitors and SGLT2 inhibitors) exert natriuretic effects that contribute to their ability to lower blood pressure (Kawasoe et al., 2017, Gutzwiller et al., 2004, Lovshin et al., 2017). As Packer (2018) pointed out, despite their similarity in lowering blood pressure and promoting natriuresis, these classes of drugs have different effects on the risk of macrovascular events and progression of renal disease. GLP-1 receptor agonists reduce atherosclerotic thromboembolic events, but not risk of heart failure, and have some effect on reducing the decline in renal function but no benefit on prevention of end stage renal disease; SGLT2 inhibitors decrease the risk of heart failure and cardiovascular death (mainly driven by reduction in heart failure death and hospitalisation), reduce the decline in renal function as well as risk of adverse renal events; and DPP-4 inhibitors have no benefit on cardiovascular or renal adverse events (Packer, 2018). This could be partly explained by their different mechanism of action, as shown in Figure 1.5 (adapted from Packer (2018)).

Figure 1. 5: Natriuretic mechanism of action of antidiabetic agents



SGLT2, sodium-glucose co-transporter-2; NHE3, sodium-hydrogen exchanger isoform 3; GLP-1, glucagon-like peptide-1; DPP-4, dipeptidyl peptidase4. Following hyperglycaemia, there is upregulation of SGLT2 and NHE3 receptors leading to increased sodium reabsorption, which leads to reduced sodium delivery to macula densa. This then causes afferent arteriolar vasodilatation and glomerular hyperfiltration.

1.2.2.2 Natriuretic Effects of SGLT2 inhibitors

The natriuretic (and diuretic) potential of SGLT2 inhibitors is affected by many factors, such as glomerular filtration rate and number of nephrons, as well as hyperglycaemia. Study by Layton and Vallon (2018) showed that acute administration of SGLT2 inhibitors in diabetic rats with normal kidney function led to natriuresis, but the natriuretic effect was reduced in chronic inhibition, possibly due to reduced hyperglycaemia and a drop in glomerular filtration rate (GFR). On the other hand, in non-diabetic rats, both acute and chronic SGLT2 inhibitors administration led to significant natriuresis (Layton and Vallon, 2018).

Short term effects of SGLT2 inhibitors on natriuresis and urine volume in patients with diabetes were investigated by Tanaka et al. (2017) who found that increased natriuresis and urinary volume occurred only on day 1 and returned back to baseline from day 2 onwards. However, this study only assessed first 5 days of SGLT2 inhibitor therapy. Study by Sha et al. (2014) showed that urinary volume is increased in the first week following SGLT2 inhibition, but returns back to normal at week 12. This study also found that 24-hour urinary sodium excretion did not change at week 1 and 12 following SGLT2 inhibition. Both studies assessed SGLT2 inhibitor Canagliflozin, but Tanaka et al. (2017) used lower dose 100mg and assessed 13 patients with diabetes, whereas Sha et al. (2014) used 300mg on 18 patients with diabetes.

Ferrannini (2017) found that the natriuretic response to SGLT2 inhibitors differs in fasting versus post prandial conditions. Acute administration of SGLT2 inhibitor increased natriuresis in both states, however, chronic administration (4 weeks) of SGLT2 inhibitor increased natriuresis in post prandial conditions (by 35% compared to baseline), but in fasting conditions level of sodium excretion returned to baseline. The observed difference was likely due to plasma glucose levels, suggesting that the natriuresis is influenced by glucose levels. However, Kawasoe et al. (2017) showed that inhibiting SGLT2 receptors in diabetic patients with preserved renal function resulted in increased urinary volume and increased sodium excretion both at

two weeks and at six months, providing conflicting evidence to the above discussed studies.

In diabetic rats with reduced kidney function (modelled by reduced number of nephrons) there was a reduced natriuretic response to acute administration of SGLT2 inhibitors, but following chronic inhibition there was an increase in natriuresis and diuresis (but not glucosuria) (Layton and Vallon, 2018). Furthermore, in rats with reduced kidney function the glycosuric effect was reduced, and the resulting hyperglycaemia further increased natriuretic and diuretic potential of SGLT2 inhibitors (Layton and Vallon, 2018). This also fits with findings by Ferrannini (2017) discussed above, who found that chronic SGLT2 inhibition increased natriuresis in post prandial conditions (with hyperglycaemia) but not in fasting conditions. This could explain why SGLT2 inhibitors reduce cardiovascular risk even in diabetic patients with reduced kidney function. Whilst the glucose lowering potential is diminished, the sodium lowering potential is increased.

In conclusion, there is a limited evidence for the long-term (4 weeks and longer) natriuretic effect of SGLT2 inhibitors. On the other hand, increase in urinary volume and urinary sodium excretion has been well documented following acute SGLT2 inhibitor therapy, however it is not clear whether the effect persists for the first day or the first 2 weeks as studies provide conflicting evidence. More clinical studies are needed in order to determine the long-term effects of SGLT2 inhibitor on urinary volume and urinary sodium, as well as the mechanisms underlying these changes. Enhanced natriuresis is also likely to explain the renal protective effects of SGLT2 inhibitors, discussed in the section below.

1.2.2.3 SGLT2 inhibitors and renal protective effects

Patients with diabetes have increased risk of developing chronic kidney disease (diabetic nephropathy). Diabetic nephropathy affects around 40% of patients with T2DM (System USRD, 2018). Diabetic nephropathy is classified as persistent urinary albumin to creatinine ration of ≥30mg/g and/or sustained estimated glomerular filtration rate (eGFR) <60ml/min/1.73m^2 (Alicic et al., 2017). Diabetic kidney disease is a chronic and progressive condition, with most patients experiencing ongoing reduction in eGFR, worsening albuminuria and glomerular hyperfiltration (Reboldi et al., 2018).

Current management of diabetic nephropathy targets known risk factors, such as hypertension, diabetes control, smoking and obesity. Until recently, renin-angiotensin-aldosterone inhibitor (RAAS) and angiotensin receptor blocker (ARB) were the only classes of medication known to provide benefit in preventing decline in eGFR and albuminuria in patients with diabetic nephropathy. In the last 5 years there has been increasing amount of evidence for renal protective effect of SGLT2 inhibitors.

The first evidence for the renal protective effect of SGLT2 inhibitors in patients with T2DM came from three major cardiovascular outcome trials (investigating Empagliflozin, Canagliflozin and Dapagliflozin) (Cherney et al., 2016, Neal et al., 2017, Wiviott et al., 2018). However, despite the large size of the trials, renal protection was not a primary outcome (but as secondary or exploratory outcomes), and only 30% of patients had diabetic nephropathy. Nevertheless, these three trials provided the initial evidence that SGLT2 inhibitors offer renal protection to patients with T2DM.

Given a large proportion of patients with T2DM develops chronic kidney disease, it was important to provide answers regarding renal protection of SGLT2 inhibitors among patients with impaired renal function. Toyama et al. (2019) meta-analysed 27 studies (with 7 363 patients with T2DM who had chronic kidney disease) and found that SGLT2 inhibitors reduced risk of composite renal outcome (doubling serum creatinine, end stage kidney disease, renal or cardiovascular death) by 29%. Such a protective effect of

SGLT2 inhibitors on patients with T2DM and CKD was further supported by CREDENCE trial, a first dedicated renal outcome trial in patients with established diabetic nephropathy (eGFR 30-90), which found that SGLT2 inhibitor (Canagliflozin) reduced primary outcome by 30% (Perkovic et al., 2019).

Another trial investigating renal outcomes of patients on SGLT2 inhibitors was DAPA-HF, assessing the impact of Dapagliflozin in patients with established heart failure, regardless of diagnosis of diabetes (McMurray et al., 2019). This was the first trial that included non-diabetic patients. Composite renal outcome was a secondary outcome, and hence not powered to detect difference. Nevertheless, there was 29% risk reduction in composite renal outcome, but has not reached statistical significance. All of the above discussed literature shows that SGLT2 inhibitors exhibit renal protective effects, both in patients with T2DM with normal kidney function, and reduced kidney function, as well as emerging evidence for renal protection in patients who do not have T2DM.

The latest trial was DAKA-CKD, a multicentre, double-blind, placebo-controlled, randomised trial involving people with chronic kidney disease with and without diabetes (Wheeler et al., 2021). The primary composite renal outcome showed that dapagliflozin reduced risk, regardless of presence of diabetes. This trial had to be stopped early due to the overwhelmingly positive outcomes (Wheeler et al., 2021).

Table 1.2 provides a summary of available evidence from the cardiovascular and renal trials, adapted from Sridhar et al. (2020).

Table 1. 2: Renal outcomes in SGLT2 inhibitor therapy: summary of evidence from cardiovascular and renal trials

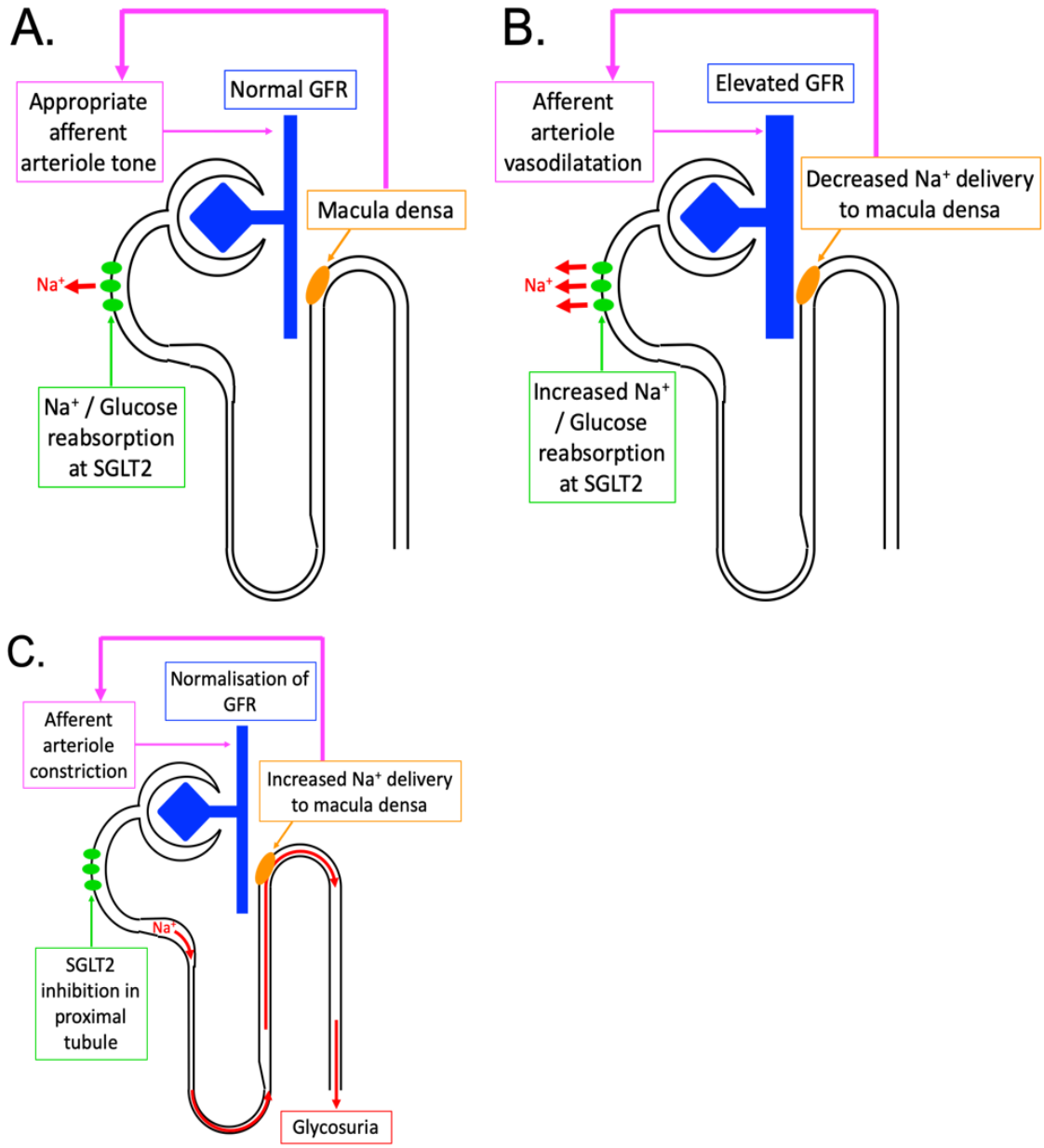
Trial	Baseline eGFR	Outcomes
EMPA-REG	30-44mL/min/1.73m^2:7.7% 45-59ml/min/1.73m^2:17.8% 60ml/min/1.73m^2 and above: 74.5%	Worsening nephropathy (UACR above 300mg/g): Risk reduction by 39%, p<0.001 Doubling serum creatinine: Risk reduction by 44%, p<0.001 Renal Replacement Therapy (RRT) initiation: Risk reduction by 55%, P=0.04
CANVAS	Mean eGFR 76.5 mL/min/1.73m^2 Patients with eGFR above 30 ml/min/1.73m^2 included	Progression of albuminuria: Risk reduction by 27% Composite outcome of 40% reduction in eGFR, need for RRT, death from renal cause: Risk reduction by 40%
DECLARE- TIMI 58	Mean eGFR 85.2 ml/min/1.73m^2 Patients with eGFR>60 mL/min/1.73m^2 included	Cardiorenal composite outcome (decline of≥ 40% in eGFR to <60 mL/min/1.73m^2, ESRD, or death from renal of CV causes): Risk reduced by 24% Excluding death from CV causes, renal specific: Risk reduced by 47%
CREDENCE	Mean eGFR 56.2 mL/min/1.73m^2 Patients with eGFR 30-90 ml/min/1.73m^2 and UACR >300-5000mg/g included	Primary outcome (composite of ESRD, doubling of serum creatinine, death from renal or CV causes): Risk reduction by 30% ESRD: Risk reduced by 32%
DAPA-HF	Mean eGFR 66 mL/min/1.73m^2 41.8% had T2M	Composite renal outcome (≥50% sustained decline in eGFR/ESRD/renal death): Risk reduction 29%
DAPA-CKD	Mean eGFR 43 mL/min/1.73m^2 68% had T2DM	Composite renal outcome risk reduction by 36% in patients with T2DM and 50% in patients without T2DM

UACR: urine albumin creatinine ratio, ESRD: end stage renal disease

1.2.2.4 Mechanisms underlying renal protection

It is well known that hyperglycaemia causes transient increase in GFR (and glomerular hypertension) in patients with diabetes (Vallon et al., 2003), which over time leads to gradual decline in renal function. SGLT2 inhibitor therapy normalises glomerular pressure and leads to a transient drop in GFR, followed by improvement of GFR after first year (Wanner et al., 2016, Neal et al., 2017). The explanation for reduction of intraglomerular pressure following SGLT2 inhibitor therapy lies in improved tubuloglomerular feedback, whereby increased sodium delivery to macula densa (following reduced reabsorption by blocked SGLT2) leads to vasoconstriction of an afferent arteriole, reducing the blood flow and intraglomerular pressure. This is shown in Figure 1.6, adapted from Cherney et al. (2014a). It is important to highlight that the current mechanistic explanation refers to diabetic patients with renal hyperfiltration (increased GFR), and not to patients with reduced GFR. Additionally, the effects of SGLT2 inhibitors on efferent arteriole remain unknown.

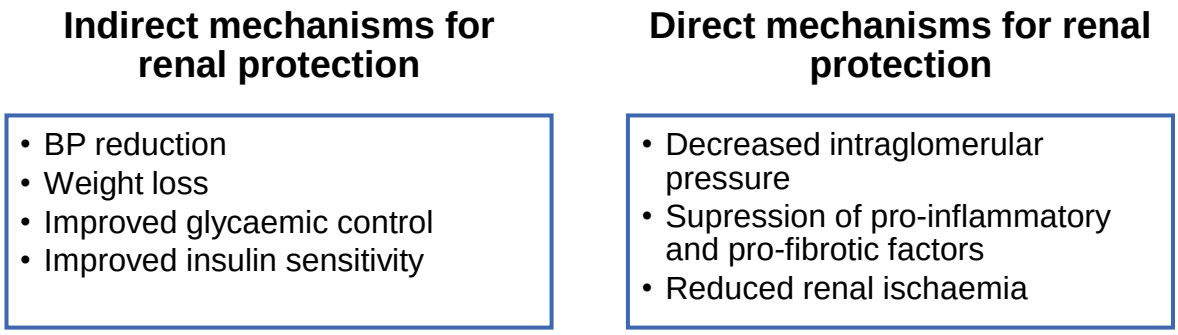
Figure 1. 6: A potential mechanism of action for renal protection following initiation of SGLT2 inhibitor (decreased intraglomerular pressure)



A. Normal tubuloglomerular feedback in a nephron of a healthy individual, whereby macula densa (specialised cells lining the wall of the distal convoluted tubule) detects sodium in the urine and influences tone of afferent arteriole, thus affecting glomerular filtration rate. B. Hyperfiltration in a nephron of a patient with diabetes, where due to increased sodium and glucose reabsorption there is less sodium detected by macula densa, which leads to afferent arteriole vasodilatation, and elevated glomerular filtration rate. C. Following initiation of SGLT2 inhibitor, there is less sodium (and less glucose) reabsorption and more sodium detected by macula densa, which leads to afferent arteriole vasoconstriction and normalisation of glomerular filtration rate.

Other possible mechanisms, such as reduction in renal oxygen consumption and renal ischaemia (van Raalte and Cherney, 2018) as well as reduction in pro-inflammatory factors (Yaribeygi et al., 2019) have been suggested to contribute to renal protection by SGLT2 inhibitors. Sridhar et al. (2020) summarised the direct (acting via kidney) and indirect mechanisms (not acting via kidney) which were suggested to explain the renal protection seen following SGLT2 inhibitor therapy. These are summarised in Figure 1.7, adapted from Sridhar et al. (2020). It is not known to what degree each of these mechanisms contribute to the observed renal protection, and whether the contribution changes depending on patient's starting GFR.

Figure 1. 7: Possible mechanisms of action for renal protection by SGLT2 inhibitors



1.2.3 SGLT2 inhibitors and effects mediated by combination of natriuresis and glycosuria

1.2.3.1 Blood pressure changes

Hypertension is defined as 'the average of 2 or more diastolic blood pressure (BP) measurements on at least 2 subsequent visits is ≥90 mm Hg or when the average of multiple systolic BP readings on 2 or more subsequent visits is consistently ≥140 mmHg' (Carretero and Oparil, 2000). More than 60% of patients with T2DM are hypertensive (Colosia et al., 2013) and many patients with T2DM are on multiple antihypertensive agents, struggling to achieve good control.

Most antidiabetic medications do not produce an antihypertensive effect. Among the newer classes of antidiabetic medications, DPP4 inhibitors have neutral effect on BP, GLP1 analogues produce slight reduction and SGLT2 inhibitors lead to a significant reduction of BP (Grossman and Grossman, 2017).

A reduction in blood pressure following SGLT2 therapy in patients with T2DM has been well documented in several meta-analyses, with reduction between 2.46 and 3.76 mmHg in systolic BP and reduction between 1.46 and 1.83 mmHg in diastolic BP (Mazidi et al., 2017, Baker et al., 2017). The blood pressure lowering effect of SGLT2 inhibitors persist in patients with chronic kidney disease, despite the fact that the glucose lowering potential is reduced in these patients (Kohan et al., 2014).

Many factors affect the extent to which SGLT2 inhibitors reduce blood pressure. Sakai et al (2016) found bigger reduction in blood pressure in patients with higher BMI, but this could have been due to higher baseline readings in this subgroup. Similar finding was found by Sjöström et al. (2015b) who showed that higher BP at baseline was linked with higher BP reduction following SGLT2 inhibitor therapy.

Higher doses of some SGLT2 inhibitors also impact on the BP reduction. Significant dose-response relationship is seen with canagliflozin (Zaccardi et al., 2016) , but not with empagliflozin (Tikkanen et al., 2015).

Even though the reduction in blood pressure is modest, the changes in central aortic pressure may be much bigger (Abdul-Ghani et al., 2016). Striepe et al. (2017) found that following 6 weeks treatment with SGLT2 inhibitor central aortic pressure reduced by 5.14 mmHg. Takenaka et al. (2018) also found that central aortic pressured reduced by 5 mmHg after 6-month treatment with SGLT2 inhibitor, however this did not differ from the reduction in brachial blood pressure. Low number of patients in the trial could explain no difference between central and brachial pressure differences.

1.2.3.2 Mechanisms for reduction in blood pressure

Scheen (2019) summarised the three main mechanisms that are currently proposed as explanation for the reductions in in both systolic and diastolic blood pressures: weight loss, osmotic diuresis and reduction in arterial stiffness.

Weight loss is not the major contributor to the BP reduction. Sjöström et al. (2015a) estimated (using linear regression) that 2 kg weight loss induced by SGLT2 inhibitor Dapagliflozin contributed to 28% of the overall systolic BP reduction and 24% of the diastolic BP reduction. Controversy exists about the long term osmotic and natriuretic effect of SGLT2 inhibitors. Yasui et al. (2018) found that SGLT2 inhibitor Empagliflozin produced transient diuresis, but within 4 weeks of treatment both urine volume and urinary sodium returned to normal. However, the blood pressure lowering effect persists long term, not keeping with purely diuretic effect. There is not a clear evidence about what happens to urinary sodium and urinary volume following a long-term therapy with SGLT2 inhibitors, as most studies assessed 24-hour urinary collection for a maximum of 12 weeks. While there is no long-term data on sodium excretion there is long-term data that shows that there is a reduction in plasma volume (as measured by haematocrit) (Lambers Heerspink et al., 2013b, Sha et al.,

2014). The early natriuresis is attenuated by the loss of volume from plasma, with those who have excess sodium (such as patients with T2DM and congestive cardiac failure) being possibly protected from sodium and fluid overload.

Cherney et al. (2014b) suggested that SGLT2 inhibitor therapy could reduce aortic stiffness, which was further supported by evidence from patients treated by empagliflozin, canagliflozin and dapagliflozin (Striepe et al., 2017, Ramirez et al., 2019, Solini et al., 2017). Reduction in arterial stiffness consequently lowers BP, and likely contributes to the reduced cardiovascular risk discussed later on in this section.

1.2.3.3 Cardiovascular risk

In 2008, the United States Food and Drug Administration stated that all new antidiabetic therapies in T2DM should demonstrate no unacceptable increase in cardiovascular risk (FDA, 2020). This requirement was introduced following finding that rosiglitazone increased risk of myocardial infarction by 43% and increased risk of death from cardiovascular cause by 64% (Hiatt et al., 2013).

As a result, all new antidiabetic medications need to be tested in cardiovascular outcome trial which evaluates their safety and focus on cardiovascular outcomes (and specifically look at cardiovascular death, non-fatal MI and non-fatal stroke, combined to MACE, major adverse cardiovascular events). There have been three large, high quality cardiovascular outcome trials investigating Empagliflozin, Canagliflozin and Dapagliflozin in patients with T2DM. I will summarise and appraise these below, using critical appraisal skills programme (CASP) (2018). Following on from these, there were other trials investigating cardiovascular effects of SGLT2 inhibitors in selected populations, such as T2DM and chronic kidney disease, or patients with heart failure, regardless of T2DM.

EMPA-REG OUTCOME trial assessed 7 020 patients with T2DM, out of which more than 99% (6 964) had established cardiovascular disease (defined as history of myocardial infarction, multivessel coronary artery disease, single vessel coronary artery disease not revascularized, unstable angina and history

of stroke or peripheral artery disease) (Zinman et al., 2015). Patients were randomised to receive placebo or empagliflozin, and were followed up 3.1 years (median follow up time). This trial addressed a clearly focused issue, but due to the population studied, it was applicable only to those patients with T2DM who had a previous cardiovascular event. Moreover, the population studied did not involve those who had a poor control of their condition (defined as HbA1c more than 10%) and also those who had BMI more than 45 kg/m^2. The assignment of patients to treatment was well randomised and the sequence allocation was concealed from both patients and researchers. The length of the trial was event driven (the aim was to get at least 691 patients who will have a primary event) and intention to treat analysis was performed. All the treatment groups were similar at the start of the trial, with a similar dropout rates between placebo and active treatment (29.3% discontinued of placebo and 23.4% discontinued Empagliflozin). Both groups were treated equally, as after 12 weeks of treatment, investigators were encouraged to adjust glucose lowering therapy.

The primary outcome of EMPA-REG was well defined as a composite major adverse cardiovascular event (MACE): cardiovascular death, non-fatal MI and non-fatal stroke. There was a statistically significant 14% reduction in MACE (with confidence interval between 0.74-0.99, suggesting risk reduction varied between 26% and 1%), driven by 38% reduction in cardiovascular mortality with no significant decrease in nonfatal myocardial infarction or stroke (Zinman et al., 2015). Interestingly, Empagliflozin resulted in statistically significant 35% reduction in hospitalisation for heart failure without affecting hospitalisation for unstable angina (Zinman et al., 2015). The results of this study can only be applied to a population of patients with T2DM who had a prior cardiovascular event and have HbA1c between 7% and 10%, as well as BMI less than 45 kg/m^2. In this trial, benefits of SGLT2 inhibitor clearly exceed harms, as serious adverse events such as DKA or amputations were occurring at similar rates to placebo.

CANVAS trial assessed 10 142 patients with T2DM, 65.6% (6 656) of whom had established cardiovascular disease (defined as documented symptomatic

atherosclerotic disease), and 34.4% (3 486) had multiple risk factors (defined as age ≥50 years with two or more of: diabetes duration ≥10 years, systolic blood pressure >140mmHg while on antihypertensive treatment, current daily smoking, micro- or macroalbuminuria, or HDL <1mmol/L) (Neal et al., 2017). Additionally, the inclusion criteria were HbA1c between 7-10.5% (similar to EMPA-REG). However, there was no limit for BMI and hence patients with BMI higher than 45 kg/m^2 could be included. The length of follow up was also event driven (aim for 688 cardiovascular events), with a median follow-up time of 2.4 years. Methodologically this study was very similar to EMPA-REG, with double blinding, and intention to treat analysis. Groups allocated to placebo or Canagliflozin were similar, with 10.9% withdrawing from the canagliflozin group and 14% of patients withdrawing from the placebo group.

Results were similar to EMPA-REG as there was a statistically significant reduction of 14% in MACE (with confidence interval between 25% and 3% of risk reduction), as well as statistically significant 33% reduction in hospitalization for hearth failure (Neal et al., 2017). The generalizability of the findings from this study is better than from EMPA-REG as both patients with and without prior cardiovascular event were studied. However, increased rate of lower limb amputation in the Canagliflozin group (6.3 vs 3.4 per 1000 patient years) was an important consideration in prescribing practice driven by this study.

DECLARE study looked at 17 160 patients with T2DM, 40.6% of whom (6 974) had established cardiovascular disease (defined as ischaemic heart disease, cerebrovascular disease or peripheral artery disease) and 59.4% (10 186) had multiple risk factors (defined as men aged ≥55 years or women aged ≥60 years with one or more of: hypertension, dyslipidaemia, or use of tobacco) (Wiviott et al., 2018). The median follow-up was 4.2 years.

In this trial, there was a bigger proportion of patients with T2DM who did not have established cardiovascular disease, making this trial different from the two previously discussed studies. However, only patients over the age of 40 were included, with a wide range of diabetic control (HbA1c 6.5-12%).

Methodology was very similar, with double blinding and intention to treat analysis. The groups were comparable at the start of the treatment.

DECLARE study looked at two primary outcomes, both of which were clearly defined. The first was MACE, and the second was combination of cardiovascular death and hospitalisation for heart failure. There was 7% risk reduction in MACE (with confidence intervals between 16% risk reduction to 3% risk increase), and this was not statistically significant (Wiviott et al., 2018). The likely reason was that there were only 40.6% of patients with established cardiovascular disease, as compared to the other two large trials discussed above with majority of patients having established cardiovascular disease. However, there was statistically significant 17% risk reduction in the second primary outcome (confidence intervals were between 27% risk reduction to 1% risk reduction). This was driven by 27% reduction in the risk for hospitalisation of heart failure.

CREDENCE study was the first dedicated renal outcome trial in patients with established diabetic nephropathy (eGFR 30-90), which also assessed cardiovascular risk in these patients (Perkovic et al., 2019).There were 4401 patients enrolled and randomised to receive placebo or Canagliflozin 100mg. The median follow-up was 2.6 years. Patients were included if they had T2DM (HbA1c between 6.5 and 12%), age 30 or more and chronic kidney disease, defined as urinary albumin-to-creatinine ratio >300 to 5000, (with albumin measured in milligrams and creatinine in grams) and eGFR 30 to <90 ml per minute per 1.73 m^2 of body-surface. The trial was randomised, double blinded and intention to treat analysis was performed. Cardiovascular risks (both MACE and risk of cardiovascular death and hospitalisation for heart failure) were defined as a secondary outcome. There was 20% risk reduction in MACE (confidence interval between 33% and 5% risk reduction) and 31% risk reduction in combination of cardiovascular death and hospitalisation for heart failure (confidence intervals between 43% and 17% risk reduction). There was 39% risk reduction in hospitalisation for heart failure. Interestingly, the increased risk for amputation found in CANVAS study was not confirmed as

there was no difference in the risk of amputation between placebo and Canagliflozin.

SGLT2 inhibitors also reduce cardiovascular risk in patients without T2DM. DAPA-HF randomly assigned 4744 patients with heart failure to either placebo or Dapagliflozin (McMurray et al., 2019). Unlike in previous trials, only 42% of the patients in each trial group had T2DM. Patients who were 18 or older and had an ejection fraction of 40% or less, and New York Heart Association (NYHA) class II, III, or IV symptoms were eligible.

The primary composite outcome was worsening of heart failure (hospitalization or an urgent visit resulting in intravenous therapy for heart failure) or death from cardiovascular causes. The study was double blinded and intention to treat analysis was performed. The median follow-up was 18.2 months. The main outcome from this study was a significant risk reduction of 26% in primary composite outcome (with confidence intervals between 35% risk reduction and 15% risk reduction). This was a first study that assessed cardiovascular effects of SGLT2 inhibitors in patients without T2DM and provided significant shift in approach to treatment of heart failure.

The latest cardiovascular outcome trial VERTIS CV assessed relatively new SGLT2 inhibitor Ertugliflozin (Cannon et al., 2020). There were 8246 patients with T2DM and established cardiovascular disease, who were following randomisation to either placebo or Ertugliflozin (various doses) followed up for a median length of 3.5 years. This study was very similar to the above discussed studies, with double blinding, as well as intention to treat analysis. Primary outcome was MACE, and there were many secondary outcomes. The results of this trial were surprising, as there were no significant differences in MACE except for reduced risk of heart failure hospitalisation. The risk was reduced by 30%, and confidence interval ranged from 46% risk reduction and 10% risk reduction. The results of the study highlighted the beneficial class effect of SGLT2 inhibitors on heart failure. The main characteristics of these studies are in Table 1.3.

Table 1. 3: Summary of cardiovascular outcome trials for SGLT2 inhibitors

Trial	Participants	Median follow-up (years)	Outcomes
EMPA-REG	6 964 eCVD All have T2DM	3.1	Significant 14% reduction in MACE Significant 35% reduction in HHF
CANVAS	6 656 eCVD 3 486 MRF All have T2DM	2.4	Significant 14% reduction in MACE Significant 33% reduction in HHF
DECLARE	6 974 eCVD 10 186 MRF All have T2DM	4.2	Non-significant 7% reduction in MACE Significant 27% reduction in HHF
CREDENCE	4401 with CKD out of which 2220 eCVD All have T2DM	2.6	Significant 20% reduction in MACE Significant 39% reduction in HHF
DAPA-HF	4744 with HF 42% had T2DM	1.5	Significant 26% reduction in worsening of heart failure and death from cardiovascular cause
VERTIS-CV	8246 eCVD All have T2DM	3.5	Non-significant 3% reduction in MACE Significant 30% reduction in HHF

eCVD (established cardiovascular disease), MACE (major adverse cardiovascular events), HHF (hospitalisation for heart failure), MRF (multiple risk factors), HF (heart failure)

1.2.3.4 Potential mechanisms for the observed cardiovascular benefit

Abdul-Ghani et al. (2016) pointed out that SGLT2 inhibitors were not likely to slow down the atherosclerotic process as there was no beneficial effect on nonfatal stroke and nonfatal MI, as well as no effect in patients with unstable angina. Moreover, the protective effect of SGLT2 inhibitors on cardiovascular mortality is seen very quickly, not keeping in with the rate with which

atherosclerotic changes would be seen (Abdul-Ghani et al., 2016). Similarly, improved glycaemic control is unlikely to contribute to the protective effect. As many studies demonstrated, intensive glucose control failed to decrease cardiovascular events in UKPDS (UKPDS, 1998), ACCORD study (Gerstein et al., 2008) and ADVANCE study (Patel et al., 2008).

Verma (2019) summarised the currently proposed protective mechanism comprising natriuresis, osmotic diuresis, reduction in inflammation and oxidative stress, reduction in arterial stiffness, blood pressure and body weight. All of these mechanisms are likely to lead to a reduced cardiac preload, reduced afterload, attenuation in cardiac fibrosis and improved myocardial energy production (Verma, 2019).

1.2.3.4.1 Reduced preload

Diuretic effect (both osmotic and natriuretic) of SGLT2 inhibitors leads to volume contraction and thus reduced preload. It has been suggested that 50% of the cardiovascular benefit seen following SGLT2 inhibitor therapy was secondary to its haemodynamic effects, specifically volume contraction, reflected by the reduced plasma volume seen in the large cardiovascular outcome trials (Inzucchi et al., 2018).

SGLT2 inhibitors do not produce compensatory tachycardia following reduction in plasma volume (Wan et al., 2018). This is as a result of their sympathoinhibitor action, further contributing to cardiovascular protective effect (Wan et al., 2018).

The natriuresis induced by SGLT2 inhibitors further helps to reduce plasma volume, and directly reduces the tissue sodium overload that has been well documented in patients with diabetes (Karg et al., 2018). Moreover, as Hallow et al. (2018) suggested SGLT2 inhibitors (unlike other diuretics) lead to twice bigger reduction in interstitial fluid compartment compared to plasma volume, reducing tissue congestion to a greater extent that reducing circulating plasma

volume, which is particularly beneficial for patients with congestive cardiac failure.

1.2.3.4.2 Reduced afterload

Reduction in blood pressure and arterial stiffness leads to reduced afterload (Verma and McMurray, 2018). Aortic stiffness and central aortic pressure are independent predictors of cardiovascular mortality (Cruickshank et al., 2002) and reduction in both could contribute to the observed protective effect of SGLT2 inhibitors.

Importance of circadian rhythm (with higher BP in the morning and drop in the evening) in cardiovascular health has been well documented (Chen and Yang, 2015) and there is a clear link between disturbed circadian rhythm of blood pressure and higher cardiovascular mortality (Kario et al., 2001). Patients with T2DM and hypertension are known to have blunted circadian BP changes (de la Sierra et al., 2009). SGLT2 inhibitor therapy restores circadian rhythm of blood pressure both in animal studies (Rahman et al., 2017) and patient studies (Baker et al., 2017), providing another potential mechanism for improved cardiovascular outcomes.

1.2.3.4.3 Changes in cardiac fibrosis

SGLT2 inhibitors have beneficial effect on left ventricular remodelling, as evidenced by reduced cardiac fibrosis (Lee et al., 2019) and reduced left ventricular mass (Connelly et al., 2019) in animal studies, as well as reduced left ventricular mass in human studies (Verma et al., 2019).

1.2.3.4.4 Improved myocardial energy production

SGLT2 therapy increases lipid oxidation and decreases glucose oxidation, leading to increased ketone production (Merovci et al., 2014, Ferrannini et al., 2014). However, the change from glucose to fat as a substrate for oxidation for myocardium would increase oxygen demand of the myocardium, and consequently worsened cardiovascular outcome (Ferrannini, 1988). Hence, this mechanism is also unlikely to explain observed benefits. However, ketone

body oxidation may lead to improved cardiac muscle efficiency (Cotter et al., 2013) and this could contribute the protective cardiovascular effect.

1.2.3.4.5 Other possible mechanisms

SGLT2 inhibitors increase uric acid excretion, and reduce overall plasma uric acid level (Chino et al., 2014). High levels of uric acid are associated with hypertension and vascular damage (Feig et al., 2008). This could be partly responsible for improved cardiovascular outcome, even though its effects would not be seen in such a rapid time scale. Moreover, as Badve et al. (2020) recently showed, urate lowering treatment with allopurinol did not have any effects on slowing down decline in eGFR, and as such the positive cardiovascular effects of lowering uric acid levels remain to be explored.

Direct inhibition of sodium-hydrogen exchange by SGLT2 inhibitor can also contribute to cardiovascular benefit. A study in mice found that SGLT2 inhibitor leads to reduced cardiac cytosolic sodium concentration and this can be protective, as increased myocyte sodium concentration was found to be associated with myocyte injury and fibrosis (Uthman et al., 2018). However, it is unclear whether such sodium changes would happen in human myocytes.

Epicardial fat can contribute to coronary artery disease as it produces molecules that affect cardiac function as well as contribute to atherosclerosis (Baker et al., 2006). Sato et al. (2018) found that 6-month therapy with SGLT2 inhibitors led to a reduction in epicardial fat in patients with diabetes, providing yet another possible mechanism for protective cardiovascular effects.

In conclusion, there are many possible reasons why SGLT2 inhibitors reduce risk of cardiovascular death and reduce hospitalisation from heart failure. While many studies were undertaken in patient population, some are animal-based studies and some are ex vivo studies, making it more difficult to translate the findings into patient population. Future research needs to focus on mechanisms underlying cardiovascular protection of SGLT2 inhibitors, for example imaging in vivo sodium shifts with whole body MRI.

Table 1.4 summarises the potential mechanisms for the cardiovascular benefit of SGLT2 inhibitors.

Table 1. 4: Summary of potential mechanisms for improved cardiovascular outcomes in patents taking SGLT2 inhibitors

Reduced preload	Diuretic effect leading to volume contraction Reduction in interstitial fluid compartment due to reduction in skin sodium levels
Reduced afterload	Reduction in brachial systolic and diastolic blood pressure Reduction in central aortic pressure Reduction in aortic stiffness Restoration of circadian rhythm of blood pressure
Changes in cardiac fibrosis	Reduced cardiac fibrosis Reduced left ventricular mass
Improved myocardial energy production	Increased ketone body oxidation
Other mechanisms	Increased uric acid excretion, and reduce overall plasma uric acid level Reduced cardiac cytosolic sodium concentration Reduction in epicardial fat

1.3 Research questions

1.3.1 Primary Objectives

1. To execute detailed phenotyping (metabolic changes, glycosuric response, natriuretic response, changes to appetite, and body composition) in human participants with Type 2 Diabetes Mellitus and obesity, treated with Dapagliflozin therapy and concomitant dietary intervention, with evaluation of independent predictors of weight-loss
2. To explore the ketotic potential of Dapagliflozin, thereby to provide important insight into a potential uncommon side-effect of euglycaemic ketoacidosis and development of preventive strategies

1.3.2 Primary Outcome measures

1. Accurate measures of 24-hour metabolic profile from whole-body calorimetry (including resting energy expenditure, thermic effect of food and activity-related expenditure)
2. BodPod measures of body composition, including monthly weights
3. Visual analogue assessments of appetite, including accurate measurements of fasting serum leptin
4. Measures of urinary glucose excretion, urinary sodium excretion and urinary protein excretion
5. Accurate measurements of fasting serum levels of glucagon, insulin, plasma and ketone levels

1.4 Chapter summary

In summary, in this chapter I have set the context of my research work in patients with T2DM and outlined my research work on the metabolic adaptations following therapy with SGLT2 inhibitors. I have critically reviewed all the available evidence about metabolic adaptations mediated by enhanced glucose and sodium excretion following SGLT2 inhibitor therapy and stated my research objectives.

Chapter 2: Methodology

Chapter 2: General Methodology

In this chapter I will describe methodology used to provide answers to my primary objectives. The research work consisted of the 12-month observational study, assessing the metabolic effects of SGLT2 inhibitors. The rationale for this study design was to generate new evidence on metabolic adaptations following long term therapy with SGLT2 inhibitor.

2.1 Participant selection and enrolment

All patients with T2DM who attended Obesity out-patient clinic and were suitable candidates for initiation of Dapagliflozin (on the basis of clinical reasoning and within its license) were eligible to participate in the observational study. The predefined inclusion and exclusion criteria are summarised in the Table 2.1.

Table 2. 1: Inclusion and Exclusion criteria

Inclusion criteria	Exclusion criteria
Adult patient (age >18 years) with established diagnosis of T2D	Any pre-existing use of drugs within the SGLT2-inhibitor or DPP4-inhibitor classes
Clinical need for initiation of Dapagliflozin based on inadequate glycaemic control	Children or female patients who are pregnant or breast-feeding
Decisional capacity and ability to provide informed consent	Elderly patients >75 years (due to lack of clinical data on efficacy of therapies)
Not on injectable therapies including insulin and GLP1 agents	Evidence of moderate to severe heart failure
Pre-existing metformin and SU use is acceptable	Any concomitant use of loop diuretic therapies or pioglitazone (as per licensing for Dapagliflozin)
Obesity (BMI >30Kgm^{-2})	eGFR<60mmol/l (either at the time of initiation of Dapagliflozin or during the 12-month treatment period with Dapagliflozin)
Renal function, eGFR>60mmol/l	History of severe or recurrent urinary- or genital-tract infections
	History of bladder cancer

Following identification of a patient in the Diabetes/Obesity out-patient clinic as a potential candidate for enrolment into this study (including justification of), each potential participant was provided with an information sheet outlining details of the study (provided in appendix).

All potential participants were given a few days to read the information sheet, and then invited to re-attend WISDEM to have any questions answered by the Principal Investigator (PI) or a member of the research team. If the potential participant was agreeable at this time, informed consent for enrolment into the study was signed. It was made clear at that time that each participant was free to withdraw their consent at any time, and that this would not adversely affect their ongoing clinical management in any way. Following informed consent (provided in appendix), each participant was invited for their baseline HMRU study.

At the time of informed consent, each participant was assigned a personal identification number (PIN) unique to this study. All samples and all electronic data related to each participant were labelled with this unique PIN, with no personally-identifiable data. This way, confidentiality of each participant was ensured throughout. The initial consent form with personal data was kept in a locked filing cabinet in HMRU, to which only the PI and research team members had an access.

This was an open-label non-randomized observational study on Dapagliflozin, with no control or placebo arm, and no randomization process. Following baseline study, participants were seen at month 1,2,3,4,5,6,7,8,9,10 and 12 for assessment of their weight, appetite, fasting blood test and 24-hour urinary collection, and on month 3,6 and 12 for assessment of body composition. The aim was to enrol 16 participants. This study did not have a control group due to many factors, such as the intensity of follow-up and time commitment needed from patients and staff. This limited the overall size of the study as well as the time frame in which this study had to be executed.

2.2 Body composition assessment

Body composition tracking system (BOD POD® Life Measurement Inc, Concord, California, USA) is a whole-body air-displacement plethysmography which uses whole body densitometry to determine body composition (LMi, 2004). It is based on determination of body weight (in kg) and body volume (in litres). BODPOD uses air instead of water to measure body volume (based on the principle of Boyle's Law). It is a reliable and a valid technique for measuring body composition in obese individuals (Fields et al., 2002). An alternative to using BODPOD is a DEXA scan, however, this was not available at our research unit.

Participants had to wear a swimming cap and underwear only, in order to minimise interference from clothing. The actual test takes about 10 minutes and all participants had to have a minimum of two consecutive tests. If the body composition results varied significantly between the first and second

reading, third measurement was undertaken. BODPOD was operated by a trained health care practitioner. Figure 2.1 provides a picture of BODPOD.

Participants were weighed with standard scales that were located in the HMRU. Participants were asked to remove shoes and any external layers of the clothing prior to the measurement. Waist circumference was measured at the level of the umbilicus, with hip circumference measurement taken at the level of the iliac crest. Both measurements were recorded in cm.

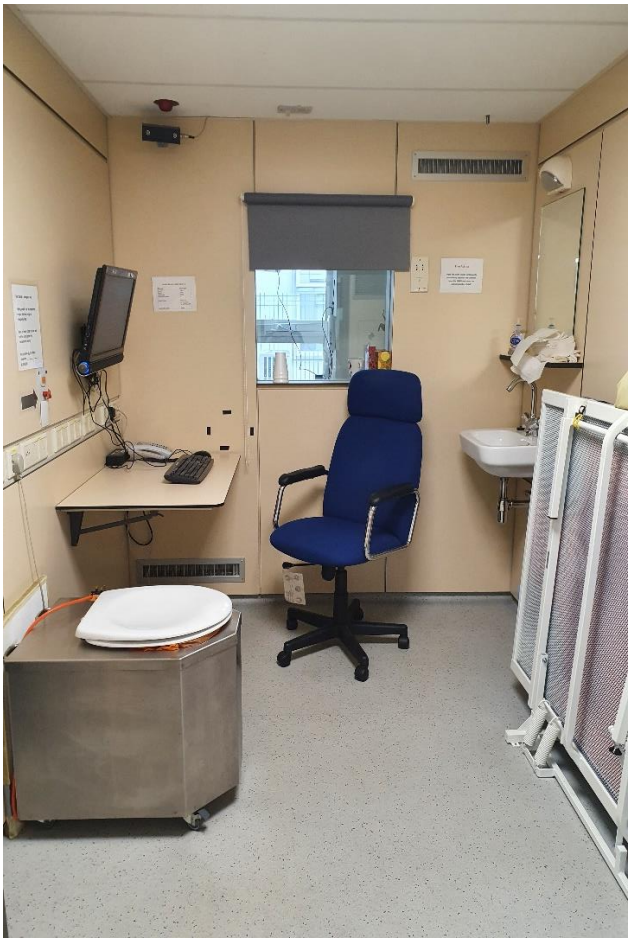
Figure 2. 1: BODPOD

2.3 Indirect calorimetry measurement

Whole body indirect calorimetry allows indirect measurement of 24 hour (or longer) energy expenditure (Brown et al., 1984). Measurements normally take place in an airtight respiratory chamber, which is part of open circuit ventilated indirect calorimeter allowing constant monitoring of oxygen and carbon dioxide. The method was described by Ravussin et al. (1982) and involves using thermomagnetic oxygen analyser (Magnos 2T, full scale range 19-21%) and infrared carbon dioxide analyser (Uras 2T, full scale range 0 to 1%). Calculation of rates of change of oxygen and carbon dioxide volume enables accurate estimation of energy expenditure.

The airtight respiratory chamber at Human Metabolism Research Unit (HMRU) has a large window, 2 airtight compartments where exchange of food, containers and other items can occur. Communication was enabled via intercom, but if intercom was not in use, chamber was sound proof. Air conditioning system-maintained temperature at 24ºC. Blood samples were obtained through a small opening in the door, whereby participant would put their arm through a plastic bag with a whole in to minimise air escaping, before the small port in the door was opened. Activity was recorded with Doppler radar. Figure 2.2 shows HMRU chamber.

Figure 2. 2: HMRU chamber



Overall daily energy expenditure has three main components: basal metabolic rate, diet induced thermogenesis, and the energy expenditure linked to physical activity (Westerterp, 2004). For this study, we measured overall 24-h energy expenditure, overnight resting metabolic rate from midnight to 6 AM, postprandial energy expenditure between 1:30 PM and 3:30 PM, exercise induced energy expenditure from 4:30 PM to 5:30 PM and energy expenditure during temperature drop, between 10:30 AM and the time of temperature reversal, which differed between participants, based on how the lower temperatures were tolerated. Temperature drop was used to induce activity in brown adipose tissue (van Marken Lichtenbelt et al., 2009) and thus increase resting metabolic rate during that time.

2.4 24-hour HMRU metabolic study

Following recruitment and informed consent, each participant was invited to attend the HMRU for a baseline detailed metabolic study. Each HMRU study commenced at 8AM with measurement of body composition in the BodPod. Lean body mass obtained from this analysis was used to predict 24-hour resting energy expenditure. Katch-Mcardle formula was used for calculation of basal metabolic rate (Basal Metabolic Rate = 370+ (21.6 x Lean Body Mass (kg)) (Katch and McArdle, 1975). Basal metabolic rate was then multiplied by 1.25 to get resting metabolic rate, equivalent of someone with limited mobility, such as inpatient. The calculated estimated metabolic rate was then used to adjust portions of standard meals for each participant in order to match the caloric requirements. Table 2.2 provides standard menu. Vegetarian option was available.

Table 2. 2: Standard menu for HMRU stay

Breakfast
Gloriously nutty muesli
Skimmed milk
Lunch
Lentil and smoked bacon soup/Spicy lentil soup
Rapeseed oil
Cheddar red onion croutons
Evening meal
M&S Beef bolognaise pasta bake/ Four cheese ravioli
Rapeseed oil
Snacks/drinks
Mixed dried fruit
Skyr yogurt
PhD Whey protein powder vanilla (to be made up with milk as below)
Skimmed milk

A fasting blood sample was taken and divided between two EDTA (Ethylenediaminetetraacetic acid, which binds calcium in the blood and keeps blood from clotting) bottles for plasma and two SST (serum separator collection tubes, containing a gel that separate the clot from the serum in the whole blood specimen) bottles for serum. Plasma and serum were sent to the hospital laboratory for assessment of baseline metabolic parameters. Second EDTA tube with plasma was immediately spun at 3.5 RPM, 4ºC for 10 min. SST tube with serum was left to stand for 30 min and then spun at 3.5 RPM, 4ºC for 10 min. Following centrifugation serum and plasma were stored at -20°C, for analysis of glucagon, insulin, glucose, ketones, leptin and adiponectin. Each participant then entered the metabolic chamber at 9AM for a 24-hour metabolic study to measure energy expenditure profile in real time. Prior to entry into the chamber, each participant was shown around the chamber by the HMRU nurse or HMRU member of staff, for orientation purposes.

Following chamber entry, participants were given breakfast at 9:30 AM. At 10.30 AM a standard temperature drop commenced to enable assessment of metabolic response to a cold environment. The standard temperature setting for metabolic studies in HMRU is 24°C (thermo-neutrality). To generate a temperature dose-response curve, between 10.30 AM and 1:30 PM, the temperature of the chamber was reduced gradually down to 15°C, in 1.5°C decrements each 30 minutes. From 1:30 PM and for the rest of the HMRU study, the temperature within the chamber was raised back to 24°C. This was based on preliminary studies (unpublished) that have been conducted in HMRU using the same temperature drop protocol using electromyogram data, to demonstrate that at these temperatures, participants do not shiver.

However, if participants were unable to tolerate temperature drop, it was reversed and researcher recorded the time of reversal and temperature at the time of reversal.

Blood samples were taken at thermoneutrality before the temperature drop at 10:30 AM and at 15°C at 1:30 PM for comparison. Heart rate and blood pressure were recorded at pre-defined times through an automated device to

allow assessment of cardiovascular response to cold exposure. Participants were required to document any shivering activity (the goal is to stimulate non-shivering thermogenesis during the cold-exposure part of the study).

Standard lunch was provided at 1:30 PM. Following the post-prandial period from the standard lunch, there was a blood test at 4:00 PM, then a standard activity protocol at 4:30 PM involving stepping for 15 minutes at a rate of one full-step up and down per second. This enabled assessment of standard activity-related energy expenditure. Following this at 5:00 PM, blood sample was taken and then a standard evening meal provided. There was a standard snack provided at 8:00 PM, and participants were requested to sleep from 10:30 PM with wake-up at 7:00 AM the next morning. The next morning, standard breakfast was provided at 7:30 AM following a morning blood test.

Perception of appetite was measured during regular intervals at 8:30AM, 10:30AM, 1:30PM, 4:00 PM, 5:00 PM, 7:30 PM, 10:15 PM, and 7:30 AM on the second day of the study through use of a visual analogue scale. Visual analogue scales are validated 100 mm scales routinely used for assessment of appetite (Sepple and Read, 1989). Participants were asked to make a vertical mark on each line that best matched their level of hunger, fullness, satiety and appetite (with question: “How much do you think you can eat?”). Scores were determined by measuring the distance from the left side of the line to the marked area (Parker et al., 2004).

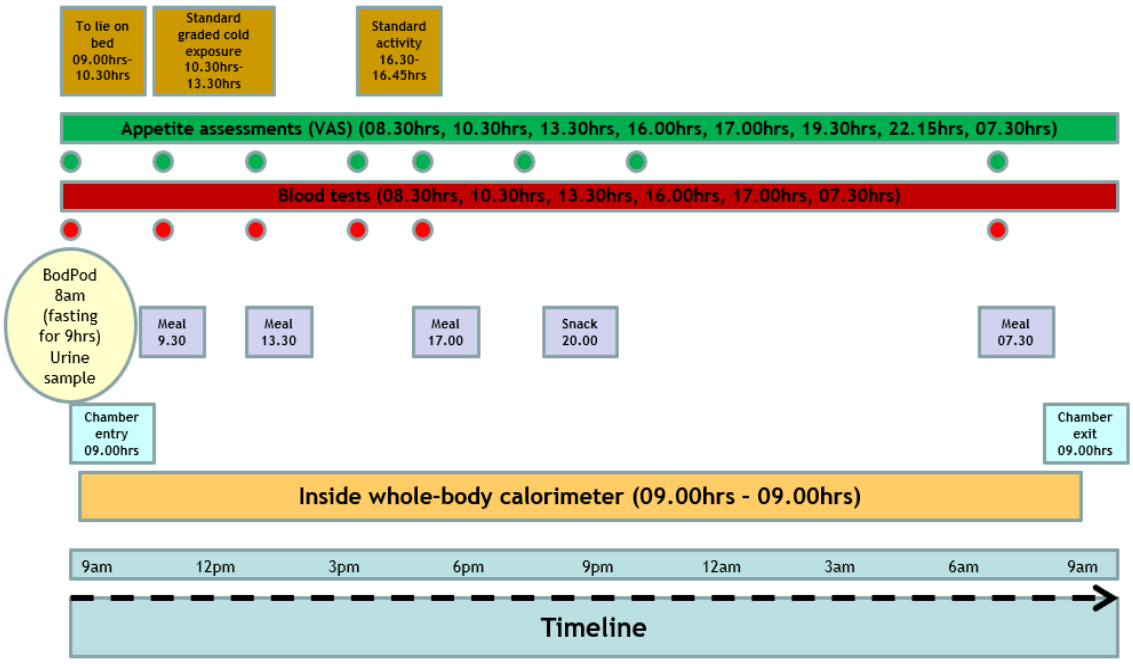
A 24-hour urine sample was collected for analysis of glycosuria, urine protein and urine sodium.

Participants were requested to exit the chamber at 09.00 AM following day, and start Dapagliflozin. Patients were informed that if they become unwell in any way (or become dehydrated) to contact lead researcher and pause SGLT2 inhibitor.

The HMRU protocol outlined above was repeated at 12-months. All participants were on Dapagliflozin therapy for the whole duration of the study. This enabled accurate assessment of any temporal changes in key appetitive, metabolic, endocrine, glycosuric and natriuretic parameters with Dapagliflozin

therapy between baseline and month 12. Figure 2.3 shows the timeline for 24h HMRU study.

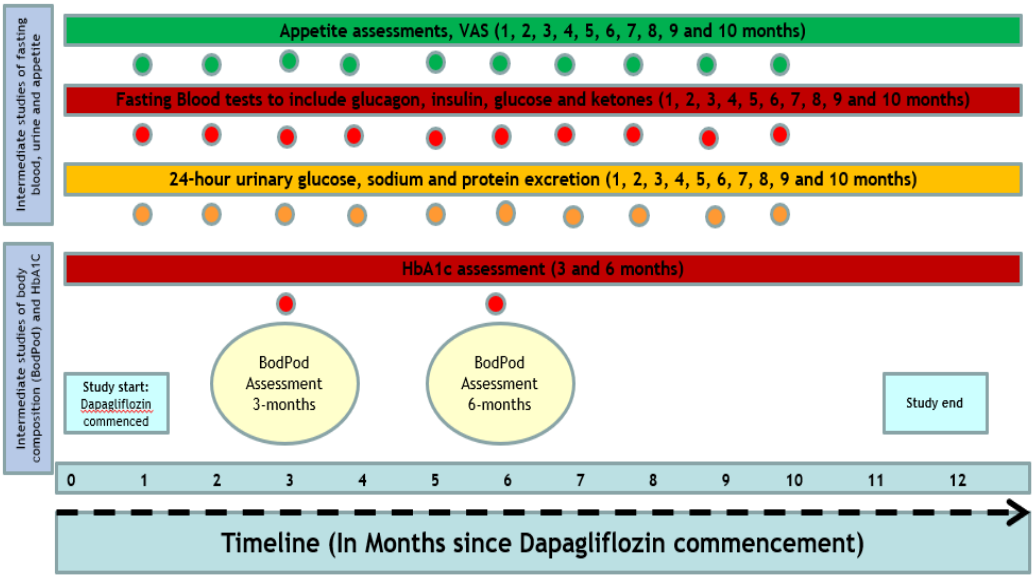
Figure 2. 3: Timeline for HMRU study



2.5 Intermediate studies

Intermediate studies were executed at 3 and 6-months for body composition assessment of lean and fat mass. Participants attended at month 1,2,3,4,5,6,7,8,9 and 10 for assessments of 24-hour urinary glucose, sodium and protein excretion, appetite measures (standard visual analogue score) and fasting blood tests. Appetite was measured at the same time interval (usually between 8-9AM) on each study visit. Analytes included fasting insulin, glucagon, glucose, ketones, leptin and adiponectin. Figure 2.4 shows a timeline for intermediate studies.

Figure 2. 4: Timeline for intermediate studies



2.6 Exploration of the ketotic potential of Dapagliflozin

Fasting plasma levels of glucagon, ketones, glucose and insulin were analysed at baseline, and at 1, 2,3,6 and 12-months following initiation of Dapagliflozin therapy. Fasting plasma glucagon and insulin levels were compared at baseline and following 12 months of therapy with Dapagliflozin. This assessment of the ketotic potential of Dapagliflozin is highly topical currently. The effects of Dapagliflozin on fat (versus carbohydrate) metabolism and ketone body formation is important both from the perspective of potential for Diabetic Keto-Acidosis (DKA) development, and for the wider physiological effects of subsequent ketone body metabolism (particularly regarding cardiac metabolism and effects on cardiovascular functioning). This aspect of the protocol therefore provided insight into the wider physiological effects of Dapagliflozin regarding its ketotic potential. The study design provided sequential assessments of this process to determine the temporal nature of ketosis following initiation of Dapagliflozin therapy, with concurrent assessments of other key phenotypic, appetitive, metabolic and endocrine factors.

2.7 Dietary Intervention

Each participant had an input provided from Specialist Weight Management dietician (based at the WISDEM Centre, UHCW) for the dietary aspects of this study. Additionally, each participant received ongoing dietary advice from the researcher, who evaluated carbohydrate consumption each month. Specifically, all participants were provided with an advice on how to adhere to a low carbohydrate diet (less than 100 g). Specialist dietitian provided dietary advice implemented on a one-to-one basis for each participant, as part of normal obesity service. The dietary intervention was based on a low-carbohydrate diet (less than 100g). This diet consisted of protein and fat (mainly from mono and polyunsaturated fats), with encouragement of 400g vegetables a day (5 portions per day), and water to be taken to replenish any water that is lost from carbohydrate-rich foods such as pasta. Participants were provided with on overview how to estimate carbohydrate content of foods and with alternatives for carbohydrate containing foods. Records of food intake (carbohydrate content) was encouraged throughout the study. Participants were asked to report the estimated amount of carbohydrate (less than 100 g, 100-300g, more than 300g) consumed during the monthly appointments. Researcher provided input on how to adhere to the low carbohydrate diet during each monthly review.

2.8 Biochemical assay details

All participants had fasting blood tests taken at baseline, month 3 and 12, which were sent to hospital laboratory for immediate analysis as part of safety assessment as well as a clinical assessment. The analysis included the following variables: Haemoglobin, platelet count, leukocyte differential count, creatinine, urea, sodium, potassium, bilirubin, alkaline phosphatise, alanine transaminase, albumin, eGFR, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, HbA1c. All of these were measured in the hospital laboratory.

All participants had a fasting blood test each month (except for month 11), which was divided into EDTA and SST tubes for plasma and serum respectively, and centrifuged as described above in the 24-hour HMRU metabolic study section. All samples were transferred into cryovials (4 cryovials for plasma and 4 cryovials for serum) and immediately put in the HMRU freezers (temperature -20°C). The samples were then transferred to the UHCW human tissue lab freezer which stores samples at -80°C.

Samples from baseline, month 1,2,3,6 and 12 were analysed for these markers: insulin, glucagon, glucose, ketones, leptin and adiponectin. Analysis was performed in the UHCW laboratory with these kits. Table 2.3 provides information about assays used for analysis of blood samples.

Table 2. 3: Assays used for analysis of blood samples

Chemical measured	Principle of the assay	Assay range	Supplier
Insulin	Sandwich enzyme immunoassay	15.6-500 pmol/L	R&D systems (R&Dsystems, 2020b)
Glucagon	Direct sandwich technique	1.54-124 pmol/L	Mercodia (MERCODIA, 2020)
Leptin	Sandwich enzyme immunoassay	15.6-1000 pg/mL	R&D systems (R&Dsystems, 2020c)
Adiponectin	Sandwich enzyme immunoassay	3.9-250 ng/mL	R&D systems (R&Dsystems, 2020a)
Glucose	Enzymatic	0.11-41.6 mmol/L	Roche (Roche, 2009)
Ketones (D-3-Hydroxybutyrate)	Enzymatic	0.1-3.2 mmol/L	Randox (RANDOX, 2020)

Urine samples were collected in bottles that had 5 thymol crystals added to stop glucose decomposition. Thymol, in combination with low temperatures slows down decomposition of glucose in 24 hour urine collections (MCL, 2020). Urine collections were kept in the fridge and were transferred for analysis immediately following receipt of the sample. 24-hour urine collections were used for analysis of glucose, sodium and protein. The assay used was Glucose HK Gen. 3 by Roche (Roche, 2009). The lower limit of detection of

glucose is <0.11 mmol/L (19.82 mg/L). The test principle is a UV test and enzymatic reference method with hexokinase is used (hexokinase catalyses the phosphorylation of glucose to glucose-6-phosphate by ATP (Kunst et al., 1984).

2.9 Statistical Analysis

The total sample size required to detect an expected standardized difference Δ at two-sided significance level α and power 1-β, is given by the following expression (Machin et al., 2008).

$$N = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2}{\Delta^2} + \frac{Z_{1-\alpha/2}}{2}.$$

Where $Z_{1-\alpha/2}$ and $Z_{1-\alpha/2}$ are percentage points of the Normal distribution, which for 5% significance and 80% power are given by $Z_{1-\alpha/2} = 1.96$ and $Z_{1-\beta} = 0.84$, and standardized difference $\Delta = (d_t - d_0)/sd$ for group mean d_t at time t and group mean at baselined d_0, and standard deviation of the difference *sd*. An approximate expression is given by $N \approx 2 + 8/\Delta^2$ for 80% power at the 5% level.

The study was planned based on clinically significant expected change in mean weight (weight loss) of 4kg, at time-point t. The standard deviation of the participant weight in this population is unknown, but we expected a large to moderate sized standardized mean difference, which suggests values for in the range 0.5 to 1. This gives sample sizes N (number of data pairs) as shown in Table 2.4.

Table 2. 4: Potential sample sizes for the study

Standardized Difference	Power (%)	N	Power (%)	N
0.5	80	33	90	44
0.6	80	24	90	31
0.7	80	18	90	23
0.8	80	14	90	18
0.9	80	12	90	15
1.0	80	10	90	13

Choosing an intermediate effect size of 0.7 to 0.8, suggests that a sample size of 18 individuals will provide 80% power to detect a change in weight of 4 kg for a modest effect size of 0.7 and 90% power for the larger effect size of 0.8. In summary, recruiting 18 individuals into the study will provide reasonable power to detect a clinically significant effect in weight loss if it exists. Power calculation for the other research outcomes was not done.

Participants' characteristics, such as weight, body composition, urinalysis, appetite and various metabolic parameters from plasma and serum, were measured at multiple time points in order to assess temporal variation. Energy expenditure was measured at the baseline and 12 months. All collected outcome variables were continuous. Data were ordered by study participant (i.e. the time sequence of measurements for an individual) to facilitate modelling, prior to a graphical exploratory data analysis (Diggle et al., 2002). Graphical data display guided the choice of a statistical model used for analysing the data. Quantile-quantile (Q-Q) plots were used to test the assumption that all data were normally distributed. Q-Q plots enable graphical representation of comparing two probability distributions by plotting their quantiles against each other (Wilk and Gnanadesikan, 1968). Data are approximately normally distributed if they are close to the straight line on the

graph (Wilk and Gnanadesikan, 1968). Where data were not close to the straight line on the graph, natural log-transformation was used. Data from hormonal parameters were analysed both not transformed and log-transformed, as hormonal levels are widely recognised as not normally distributed. Parametric tests were used for analysis. Q-Q plots are shown in appendix.

In order to account for the correlation between repeated observations made on participants, a linear mixed-effects (repeated measures) regression model was used to quantify and draw inferences on the participant data (Beaumont, 2012). The mixed-effects model included a random intercept for each participant, to model the temporal correlations, and fixed effects for time. This model assumes data are approximately normally distributed. Mixed model enables a more efficient estimation of the fixed effects (time) and yields more robust statistical tests compared to analyses of variances such as ANOVA (Arnau et al., 2012). For data where natural log transformation was used, mixed-effect model was run on both non transformed and log-transformed data.

For measures where only two time points were available, paired student t-test was used, on the basis of assumption that data residuals are approximately normally distributed. Residuals are the difference between the observed and the fitted model data. The assumptions required for regression and most of the parametric methods used are that the residuals (and not the observed data) are approximately normally distributed.

Additionally, for measures where a marked difference was expected between baseline and month 1 (urine, sodium and protein urinary excretion) t-test was used to analyse difference in the first month, followed by mixed model to assess changes over 12 months.

Data were analysed on a complete case basis; i.e. missing data were assumed to be missing at random (Wikipedia, 2020, Little and Rubin, 2014). However, for a graphical display of changes in weight over time, missing data were

replaced with imputed data (linearly interpolated), as well as original data to visually display any difference that missing data could have made.

SPSS version 26 and 27 was used for all analyses. Graphs were created in Excel, using scatter plot.

Supervisors and a statistician advised on the calculation of sample size and statistical analyses used throughout the study.

2.10 Ethical approval

The 12-month observational study was approved by Research Ethic Committee in September 2017 (IRAS 229929, REC 17/EM/0330). Local approval by Research and Development department at UHCW was obtained in October 2017. This study was sponsored by University of Warwick (SC.80/16-17).

Chapter 3: Changes in energy expenditure, body composition and appetite

Chapter 3: Changes in energy expenditure, body composition and appetite

In this chapter I will describe baseline characteristics of participants, the changes to estimated and measured energy expenditure (over 24-hours, overnight, post-prandial, after exercise and during temperature drop), body composition (weight, muscle mass and fast mass) and self-reported appetite over 12 months.

3.1 Descriptive statistics of participants at baseline

There were 18 patients who participated in the 12-month observational study. Majority of patients (17/18) completed all 12 months of the study, with only 1 patient missing the final appointment. The mean age of participants was 51 years. There were 7 female and 11 male participants. The mean weight of participants was 129.4 kg, and mean BMI was 46.1 kg/m^2. Mean year of diagnosis of T2DM was 2014. The mean baseline HbA1c was 53.9 mmol/mol, which would indicate relatively good diabetic control. Mean value of total cholesterol was 4.4 mmol/L, triglyceride 2.8 mmol/L, alanine aminotransferase (ALT) 28.6 IU/L, blood pressure 137.8/82.5 mmHg and heart rate of 77.2. Table 3.1 summarises the demographic information about the recruited participants, as well as their comorbidities, medication and anthropometric data.

Two patients did not want to undergo 24-h stay in HMRU, and as a result did not have energy expenditure measured. However, they participated in the monthly follow-up visits.

3.1.1 Adverse events during the study

During the 12-month study, two participants experienced urinary tract infection, which responded to a short course of antibiotics. There were 8 episodes of thrush (fungal infection of genital tract), all responding to treatment

with topical antifungals or oral fluconazole. There were no serious adverse events in this study.

Table 3. 1: Descriptive characteristics of participants

Participant	Age (yrs.)	Sex	Ethnicity	Medical Conditions	Medications	Height (cm)	Weight (kg)	BMI (kg/m^2)	T2DM diagnosis
1	43	F	Mixed	T2DM, Hypercholesterolaemia	Metformin, Atorvastatin, NSAIDS	163	132	49.7	2017
2	48	M	White British	T2DM, Asthma, GORD	Metformin, Seretide, Zantac, Antihistamine	175	133.2	43.5	2015
3	54	M	White British	T2DM, GORD, L Knee OA, previous Osteomyelitis, Hypercholesterolaemia	Metformin, Omeprazole, Timodine cream	169	139.6	48.9	2013
4	65	F	White British	T2DM, OSA, Epilepsy, Psoriatic arthritis, OA, Fibromyalgia	Metformin, Pravastatin, Lansoprazole, Tegretol, Amitriptyline, Oxybutynin, Tramadol	156	135.4	55.6	2010
5	60	M	Black British Caribbean	T2DM, HIV, Hypertension, Hypercholesterolaemia	Metformin, Amlodipine, Antiretroviral medication	168	108.1	38.3	2017
6	30	F	White British	T2DM, Microcytic anaemia, Anxiety, Fibromyalgia, Migraine, PCOS, Bipolar disorder	Metformin, propranolol, Zapain, Domperidone, Esomeprazole, Gabapentin, Quetiapine, Mirena coil	166	137	49.7	2017
7	52	M	White British	T2DM, Multiple PE's, OSA on CPAP, COPD, Carotid sinus hypersensitivity with pacemaker, depression, hypothyroidism, hypertension, hypercholesterolaemia	Metformin, Warfarin, Thyroxine, Omeprazole, Ramipril, Rosuvastatin, Ventolin, Steroid inhaler, Venlafaxine	167	100.8	36.1	2014
8	48	M	White British	T2DM, Angina	Metformin, Aspirin, Atorvastatin, Bisoprolol, GTN, Omeprazole, Orlistat	182	134.9	40.7	2017
9	49	M	White British	T2DM, OSA, Hypertension, Asthma,	Metformin, Atorvastatin, Doxazosin, Losartan, becotide, Ventolin	171	135	46.2	2012
10	59	F	Black British Other	T2DM, Abdominal hernia, OSA, COPD	Metformin, Carnipine, Simvastatin, Atenolol	154	99.05	41.8	2007
11	51	M	Indian	T2DM, OSA, asthma, GORD	Metformin, Ramipril, Inhalers(steroid)	170	190.8	66.0	2013
12	54	M	Indian	T2DM, OSA, hypogonadotropic hypogonadism	Metformin, Propranolol	171	118.9	40.7	2014
13	34	F	White British	T2DM, Right ovary cyst	Metformin	167	119.5	42.8	2018
14	54	M	White British	T2DM, OSA, OA	Metformin	175	185.2	60.5	2008
15	60	F	Asian	T2DM, hypothyroidism, asthma	Metformin, thyroxine, domperidone, lansoprazole, Gaviscon, Seretide, Ventolin	151	90	39.5	2015
16	42	F	White British	T2DM	Metformin	158	130.4	52.2	2016
17	59	M	White British	T2DM, hypothyroidism, previous MI, hypertension, OSA, NASH	Metformin, thyroxine, aspirin, amitriptyline, atorvastatin, omeprazole, bisoprolol, felodipine, losartan, ISMN	177	112.3	35.8	2014
18	57	M	White British	T2DM, mild HF, hypertension	Metformin, aspirin, atorvastatin, bisoprolol, ramipril, amlodipine, doxazosin	173	127	42.4	2015

NSAIDs: non-steroidal anti-inflammatory agents, GORD: gastro oesophageal reflux disease, OA: osteoarthritis, OSA: obstructive sleep apnoea, PE: pulmonary embolus, PCOS: polycystic ovary syndrome, CPAP: continuous positive airway pressure, NASH: non-alcoholic steatohepatitis, MI: myocardial infarction, HF: heart failure

Participants were asked to report on the estimated amount of carbohydrate consumed on a daily basis. However, most participants found that the carbohydrate consumption varied between weeks, and therefore this information was not used for analysis as the reported values were not consistent. The information reported by participants is below.

Table 3. 2: Estimated amount of carbohydrates reported by participants

Participant	Carbohydrate diary
1	not consistent
2	not consistent
3	not consistent
4	not consistent
5	more than 300 g
6	more than 300 g
7	less than 100g
8	less than 100g
9	not consistent
10	not consistent
11	more than 300g
12	100-300 g
13	more than 300g
14	100-300g
15	low carb 100g
16	100-300 g
17	100- 300 g
18	less than 100g

Additionally, participant 3 was started on additional therapy (GLP 1 analogue) at month 4 as a result of poor glycaemic control. However, results from this participant were included in the study.

3.2 Changes in energy expenditure

3.2.1 Overall energy expenditure

Participants had their 24-hour energy expenditure measured prior to initiation of Dapagliflozin and then again after 12 months of taking Dapagliflozin via indirect calorimetry measurements in HMRU. Estimated energy expenditure was calculated based on participants muscle mass.

16 participants had baseline 24-hour indirect calorimetry, but only 14 participants attended one-year follow-up for 24-hour indirect calorimetry. Out of 14 participants, one had to leave at midnight during a follow-up study (thus overall energy expenditure was only for the length of 14.5 hours). One participant had to leave at 6 am during both study days, and as such the length of indirect calorimetry was 20.5 hours. For the remaining participants, the length of stay in HMRU was between 22.5 and 23 hours.

One participant did not attend HMRU follow-up but had body composition assessment at month 12, which enabled calculation of estimated energy expenditure. Table 3.3 provides information about the actual (measured) total energy expenditure and estimated (calculated based on muscle mass) total energy expenditure at baseline and at one-year follow-up.

Table 3. 3: Total energy expenditure (EE), both measured and estimated

Participant	Baseline measured EE (kcal)	Follow-up measured EE (kcal)	Baseline estimated EE (kcal)	Follow-up estimated EE (kcal)
1	2190	2221	1715	1777
2	2988	2915	2333	2258
3	3289	2838	2357	2258
4	2478	2521	1969	1302
5	1883	1835	2144	2128
6	2580	2526	2056	2050
7	2450		2220	
8	3211		2512	2396
9	3254	2900	2515	2576
10	1929	1989	1640	1707
11	3152	3206	2074	2342
12	2885	2381	2069	2177
13	1816	1920	2134	2120
14	3665	3262	2461	2698
15	2649	2363	1701	1753
16	2828	2944	2323	2412

The mean measured total energy expenditure was 2685 kcal (SD 574 kcal) at baseline and 2559 kcal (SD 466 kcal) at one-year follow-up. The mean estimated total energy expenditure was 2134 kcal (SD 289 kcal) at baseline and 2130 kcal (SD 367 kcal) at follow-up.

Paired sample t-test was used to analyse the difference between the measured energy expenditure at the baseline and one-year follow-up, and estimated energy expenditure between the baseline and one-year follow-up. Numbers were rounded to the nearest whole calorie.

There was no statistical difference between measured energy expenditure at baseline and one-year follow-up, however, there was a trend for decrease in the measured energy expenditure at month 12, with a reduction of 126 kcal (SD 223 kcal) and p=0.055. One possible reason why this did not reach significance was that only 14 participants out of 18 attended both 24-hour HMRU studies.

There was no statistical difference in the estimated total energy expenditure between the baseline and one-year follow-up, with a decrease of 3 kcal (SD 213 kcal) and p=0.954. Table 3.4 provides summary of the paired samples t-test analyses.

Table 3. 4: Paired student t-test results for measured total energy expenditure and estimated total energy expenditure

Energy expenditure	The difference in means between one year and baseline	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
Measured total EE (kcal)	-126	223	-255	3	14	0.055
Estimated total EE (kcal)	-3	213	-121	115	15	0.954

SD: standard deviation, CI: confidence interval

3.2.2 Energy expenditure overnight, post-prandial, post-exercise and during temperature drop

Overnight energy expenditure was calculated as a sum of calories between midnight and 6 AM. As a result of one patient leaving at midnight during a follow-up study, these results were not included for calculation of overnight energy expenditure, leaving 13 patients with paired data for overnight energy expenditure.

Post-prandial energy expenditure was taken as sum of calories between 1:30 PM and 3:30 PM, in order to coincide with time of lunch (1:30 PM). Paired data for post-prandial energy expenditure were available for 14 patients.

Exercise-induced energy expenditure was taken between 4:30 PM and 5:30 PM, in order to capture the effect of stepping exercise at 4:30 PM. Energy expenditure during temperature drop was taken between the time temperature drop was started and the time when participant asked for reversal of the temperature. This was at different time points as some participants tolerated drop to 15ºC, whereas others asked to reverse temperature at 21ºC.

Equivalent sections were taken for each participant (for example energy expenditure between 10:30 AM and 12:30 AM for baseline and follow-up) in order for measures to be comparable. Table 3.5 summarises energy expenditure overnight, post-prandial, after exercise and during temperature drop at baseline and follow-up.

Table 3. 5: Energy expenditure overnight, post-prandial, after exercise and during temperature drop at baseline and follow-up

Participant	Baseline overnight EE (kcal)	Follow up overnight EE (kcal)	Baseline post-prandial EE (kcal)	Follow up post-prandial EE (kcal)	Baseline EE post-exercise (kcal)	Follow up EE post-exercise (kcal)	Baseline EE during temp drop (kcal)	Follow up EE during temp drop (kcal)
1	489	479	253	266	226	242	352	374
2	631	704	333	318	327	281	440	393
3	727	664	364	327	331	265	513	454
4	508	549	297	303	229	210	207	211
5	541	506	238	216	179	199	129	127
6	611	616	304	279	205	236	389	394
7	609		280		235		280	
8	763		352		288		427	
9	657	624	363	314	336	297	511	406
10	471	482	226	216	152	149	225	211
11	733	788	369	390	266	271	415	392
12	563	534	269	244	253	261	216	203
13			322	340	270	257	337	367
14	848	724	376	349	410	369	432	405
15	598	533	309	275	242	226	342	290
16	635	634	315	353	278	287	304	338

The mean overnight energy expenditure was 616 kcal (SD 108 kcal) at baseline and 603 kcal (SD 98 kcal) at one-year follow-up. The mean post prandial energy expenditure was 310 kcal (SD 49 kcal) at baseline and 299 kcal (SD 52 kcal) at one-year follow-up. The mean after exercise energy expenditure was 265 kcal (SD 69 kcal) at baseline and 254 kcal (SD 52 kcal) at one-year follow-up. The mean energy expenditure during temperature drop was 344 kcal (SD 117 kcal) at baseline and 326 kcal (SD 100 kcal) at one-year follow-up.

Paired student t-test was used to analyse difference between energy expenditure overnight, post-prandial, after exercise and during temperature drop between baseline and follow-up. Despite a trend for reduction in energy expenditure in each of the categories, there was no statistically significant

difference between overnight energy expenditure (difference -13kcal, SD 54 kcal, p=0.384), no difference between post-prandial energy expenditure (difference -11 kcal, SD 26 kcal, p=0.147), no difference between post exercise exergy expenditure (difference -11 kcal, SD 29 kcal, p=0.172) and no difference between energy expenditure during temperature drop (difference -18kcal, SD 39 kcal, p=0.110). Table 3.6 provides summary of the paired student t-test.

Table 3. 6: Paired student t-test results for changes in energy expenditure overnight, post-prandial, after exercise and during temperature drop

Energy expenditure	The difference in means between one year and baseline	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
Overnight EE (kcal)	-13	54	-46	19	13	0.384
Post-prandial EE (kcal)	-11	26	-25	4	14	0.147
Post-exercise EE (kcal)	-11	29	-27	5	14	0.172
EE during temp drop (kcal)	-18	39	-40	5	14	0.110

3.3 Body composition changes

3.3.1 Weight and body composition changes at one year

There were 18 participants who had recorded body weight at baseline and 17 participants who had body weight recorded at month 12. The mean weight at baseline was 129.4kg (SD 25.4 kg) (based on 18 participants) and 123 kg (SD 24.2 kg) (based on 17 participants) at one-year follow-up. Paired sample t-test was used to analyse difference between baseline and one-year follow-up and found that there was a statistically significant weight loss of 8.1 kg (SD 11.3 kg), p=0.009 at one-year follow-up. This calculated difference was bigger than observed difference as a result of missing data for one participant at month 12.

The mean muscle mass at baseline was 62.7 kg (SD 10.3 kg) and 63.6 kg (SD 10.5 kg) at one-year follow-up. There was no statistically significant difference between muscle mass (1.1, SD 4.1 kg, p=0.306) between baseline and one-year follow-up.

The mean fat mass at baseline was 69 kg (24.4 kg) and 61.2 kg (SD 23.3 kg) at follow up. There was a statistically significant fat loss of 9.9 kg (SD 10.4 kg), p=0.002. The calculated fat loss was bigger than the observed fat loss due to missing data at month 12.

The mean fat percentage at baseline was 51.3% (SD 9.9%) and 47.9% (SD 11%) at one-year follow-up. There was a statistically significant reduction of 4.5% (SD 4.7%) p=0.002. The calculated difference was bigger than the observed difference due to missing data at month 12.

The mean waist circumference at baseline was 134.2 cm (SD 20.4 cm) and 130.9 cm (SD 21 cm) at one year follow up. There was no statistically significant difference in the waist circumference, even though a trend for reduction was observed with reduction of 4.8 cm, SD 10.5 cm and p=0.079.

The mean hip circumference at baseline was 132.7 cm (SD 20.9 cm) and 130.1 cm (SD 18.3 cm) at one-year follow-up. There was no statistically

significant difference between hip circumference (mean -4.6 cm, SD 13.8, p=0.192).

The changes (based on paired student t-test analysis) between baseline and one-year follow-up are summarised in Table 3.7.

Table 3. 7: Body composition changes between baseline and one-year follow-up

Follow up-baseline	Mean	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
Weight changes (kg)	-8.1	11.3	-13.9	-2.3	17	0.009
Muscle mass changes (kg)	1.1	4.1	-1.1	3.2	16	0.306
Fat mass changes (kg)	-9.9	10.4	-15.5	-4.3	16	0.002
Fat percentage changes (%)	-4.5	4.7	-7.0	-2.0	16	0.002
Waist changes (cm)	-4.8	10.5	-10.1	0.6	17	0.079
Hip changes (cm)	-4.6	13.8	-11.6	2.5	17	0.192

3.3.2 Temporal changes in body weight

There were 9 missing weights during the 12-month study. 4 participants missed 1 visit, 1 participant missed 2 visits and 1 participant missed 3 visits. Table 3.8 shows all available data for weights. The mean starting weight was 129.4 kg (SD 25.9 kg), with the mean weight at 6 and 12 months 124.2 kg (SD 26.2 kg) and 123 kg (SD 24.2 kg) respectively. One participant failed to attend follow-up at month 12 and this affected the mean weight loss at 12 months, given his weight at month 10 was 83 kg. If this data point was included (and assumption made his weight stayed 83 kg at month 12), mean weight loss at 12 months would have been 120.7kg.

Table 3. 8: Complete data set for available weights (kg)

Participant	Baseline	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9	Month 10	Month 12
1	132	132.8	131.2	132.7	132.6	132.4	135.8	134.4	133.6	132.6	134.6	135.4
2	133.2	132.6	130.2	127.6	129	128.6	128.7	130.6	130.4	132.6	128.4	127.4
3	139.6	138.4	140.2	134.7	131.4	129.4	124.2	124	123.8	123.4		120.6
4	135.4	133.8	134.4	131.9	132.8	132.6	128	126.5	129.6	130.7	131.6	132.4
5	108.1	105.6	105	104.7	104.8	105.4	104.1	104.8	104.4	104	106.4	106.7
6	137	136	134.2	132.7	131.3	132	130	131	132	132.2	131.6	131.9
7	100.8	96	94.2	88.4	88.8	88.8	84.2	83.5	84.8	83.8	83	
8	134.9	135.2	132.4	127.4	127.2	119.6	119.7				100	96.7
9	135	134.6	134.4	127.9	126.8	127.2	123.6	127.2	126.4	126.4	124.8	124.6
10	99	99.2	101.8	99.3	99.8	99			98.3	98	99.2	98.2
11	190.8	187.8	187.4	188.1	191	190.2	190.9	189.6	190.2	188	187	188.9
12	118.9	118.6	116.8	112.4		112.8	111	111.4	111.6	113.2	113.6	113.5
13	119.5	119.2	117.6	118.5	117.4	119.2	121.5	121.4	122.4	120.6	120.2	119.7
14	185.2	182.2	183.8	179.6	179.6	180.4	176.1	176.8	173	167.4	167.8	164.1
15	90	88	90.2	90.4	91.6	91.4	91.2	92.2	93.2	92.6	93	94
16	130.4	128.4	126.6	125.5	123.6	122	121.8	120	121.8	121.4	121.8	119.3
17	112.3	113.4	110.8	109.5	111	111.4	110	114.4	114.6	114.8		113
18	127	125	121.6	117.5	116.6	114.6	111	110.2	108.8	105.8	104.6	103.9

Figure 3.1 shows cumulative changes to weight loss. Figure 3.2 shows percentage (%) cumulative weight loss.

Figure 3. 1: Cumulative weight loss (kg), with bars representing standard error of the mean

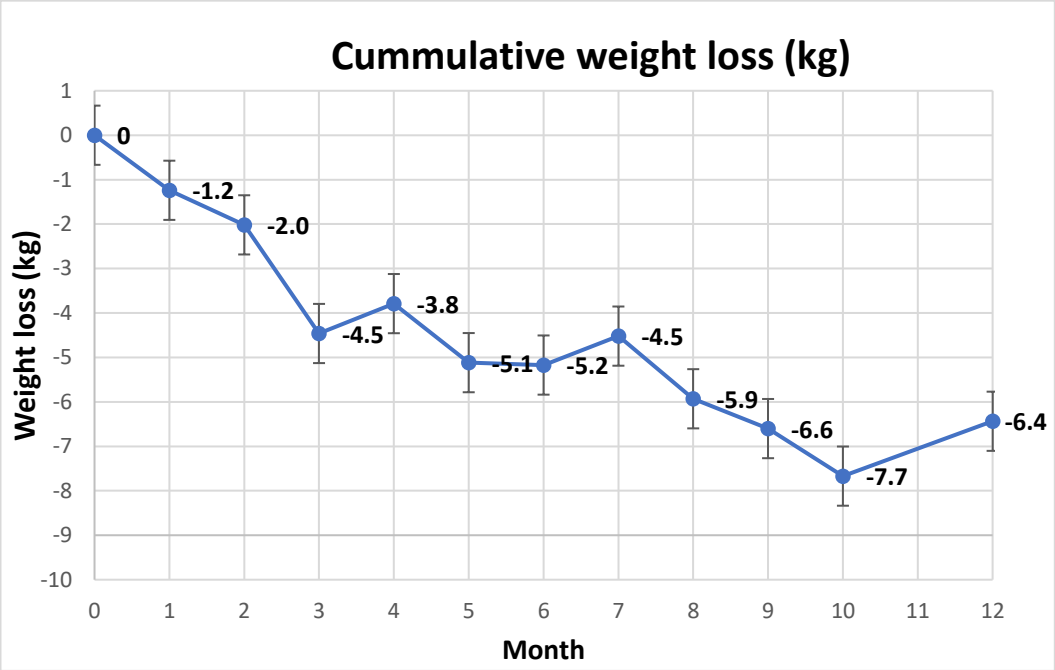
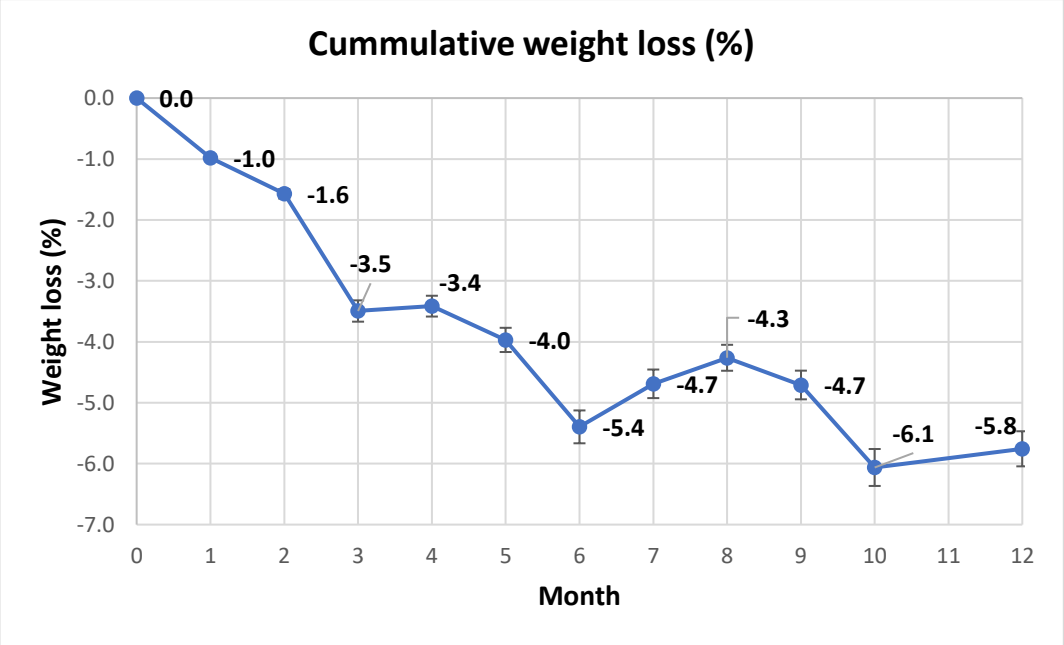


Figure 3. 2: Cumulative weight loss (%), with bars representing standard error of the mean



Median weight at baseline was 131.2 kg. Participants were divided into 2 groups based on their starting weight (90-131.2 kg, 131.2-199 kg). Figure 3.3 shows weight changes over time for each group, implying that those with smaller starting weight had smaller weight loss, however this difference was not seen when all incomplete data sets were removed, as shown in Figure 3.4.

On the other hand, there was a difference in weight loss based on starting weight when linear interpolation was used to predict missing data, as shown in Figure 3.5.

Figure 3. 3: Cumulative weight loss changes stratified by starting weight, with bars representing standard error of the mean

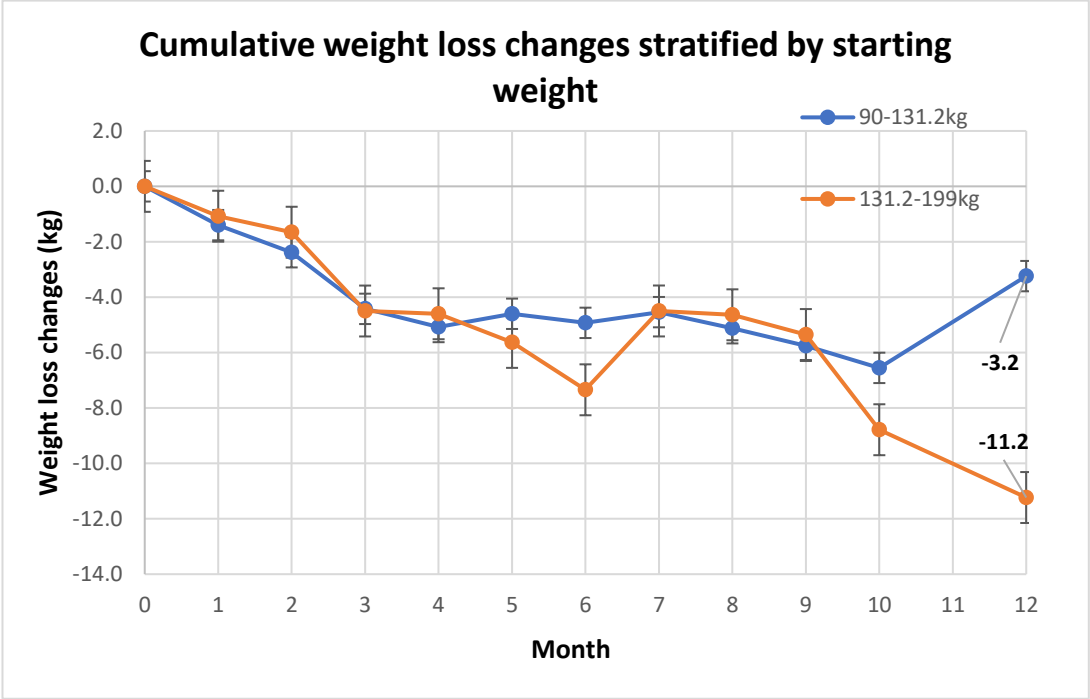


Figure 3. 4: Cumulative weight loss changes stratified by starting weight with incomplete data sets removed, with bars representing standard error of the mean

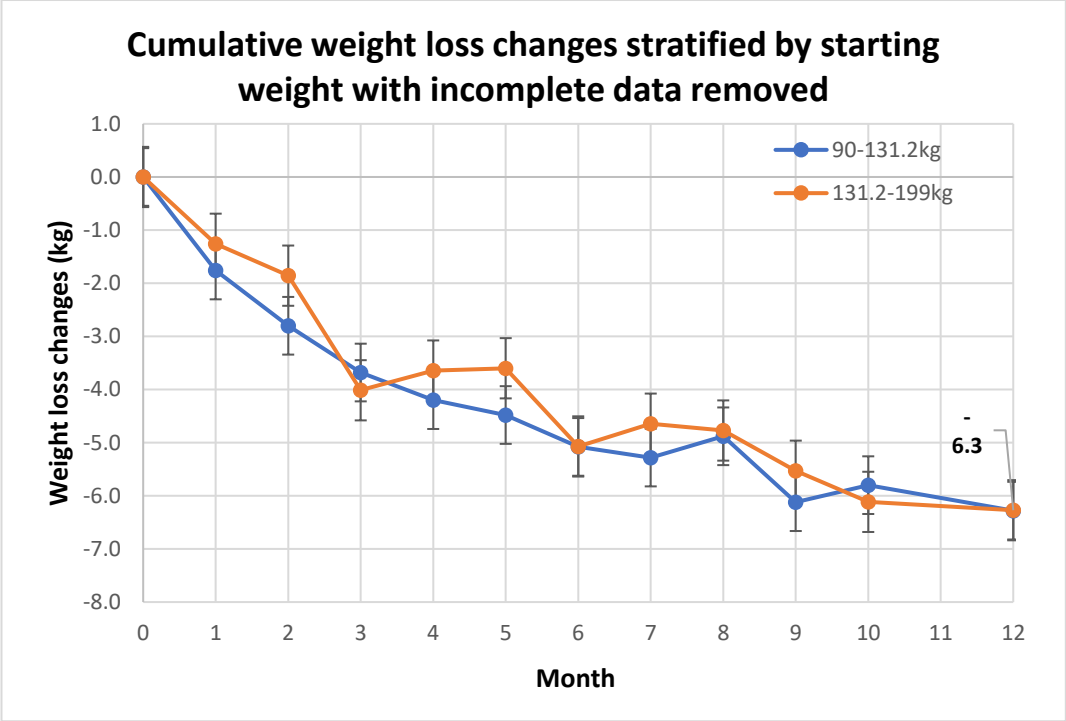
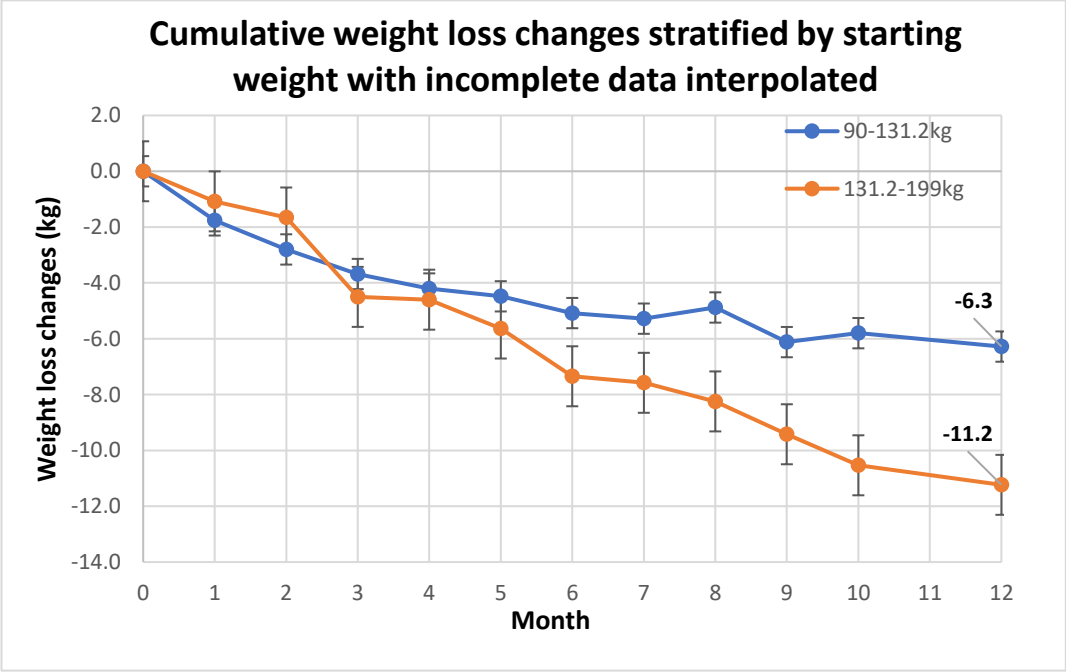


Figure 3. 5: Cumulative weight loss changes stratified by starting weight with incomplete data points interpolated, with bars representing standard error of the mean



In order to determine whether starting HbA1c had any impact on the cumulative weight loss, data were divided into two groups based on the median HbA1c of 49 mmol/mol (<50 mmol/mol and ≥50 mmol/mol). Figure 3.6 shows weight changes over time with all data included, implying that those with HbA1c less than 50 mmol/mol had smaller weight loss, however this difference was reduced when all incomplete data sets were removed, as shown in Figure 3.7.

The difference in the cumulative weight loss was apparent again when linear interpolation was used to predict missing data, as shown in Figure 3.8.

Figure 3. 6: Cumulative weight loss changes stratified by starting HbA1c, with bars representing standard error of the mean

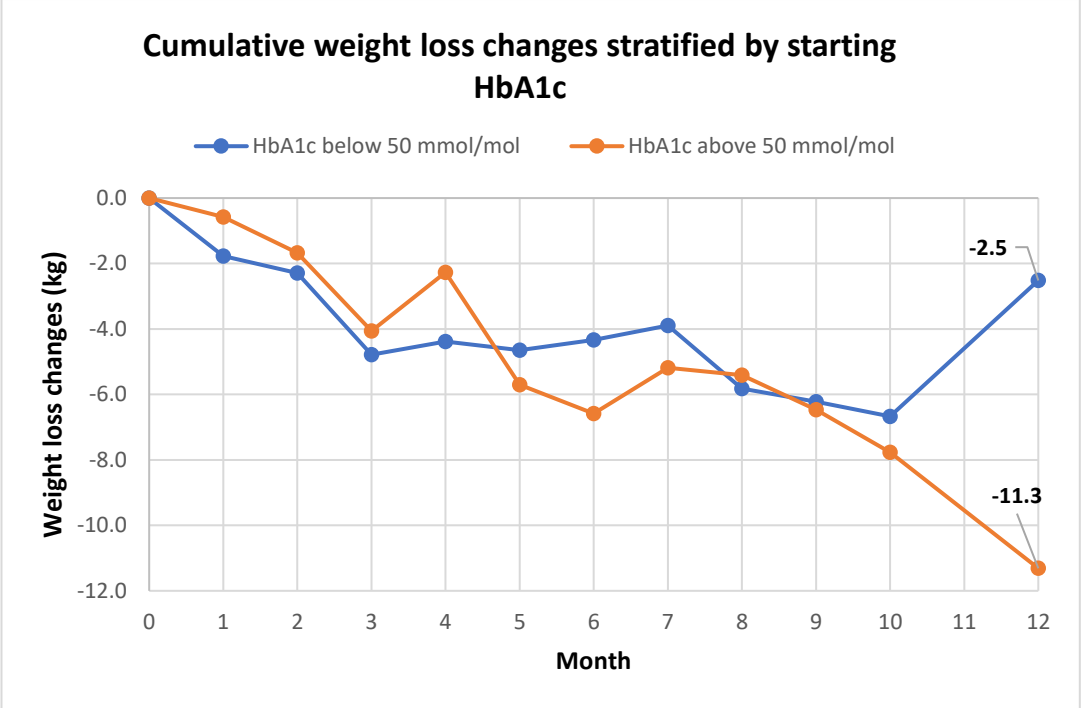


Figure 3. 7: Cumulative weight loss changes stratified by starting HbA1c with incomplete data sets removed, with bars representing standard error of the mean

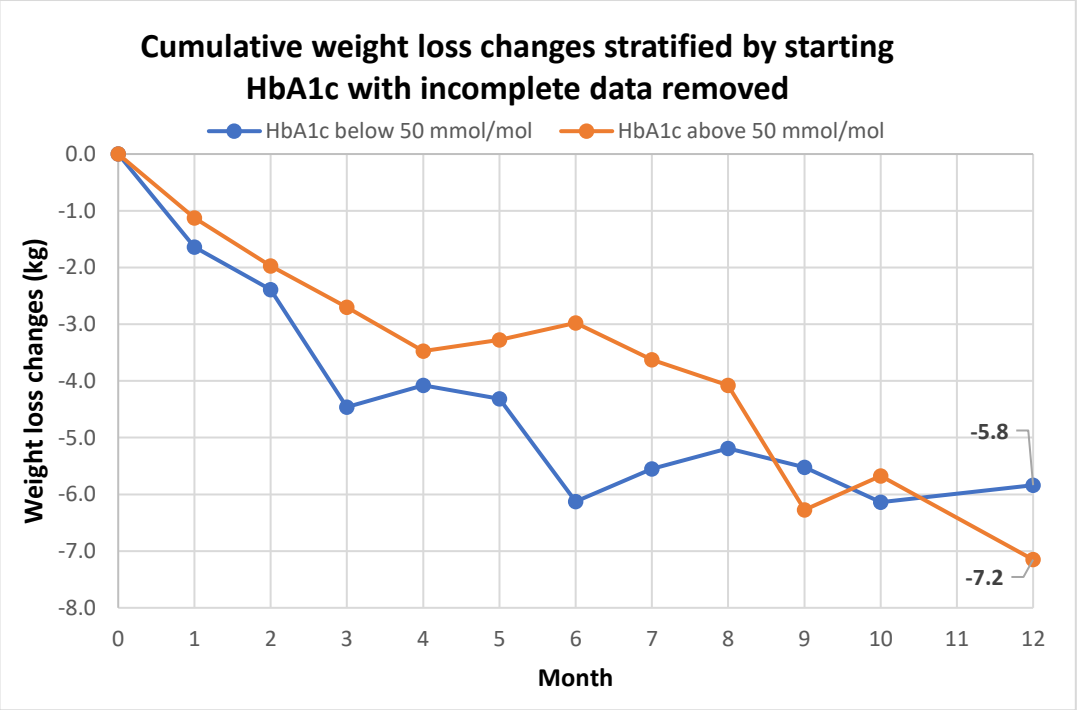
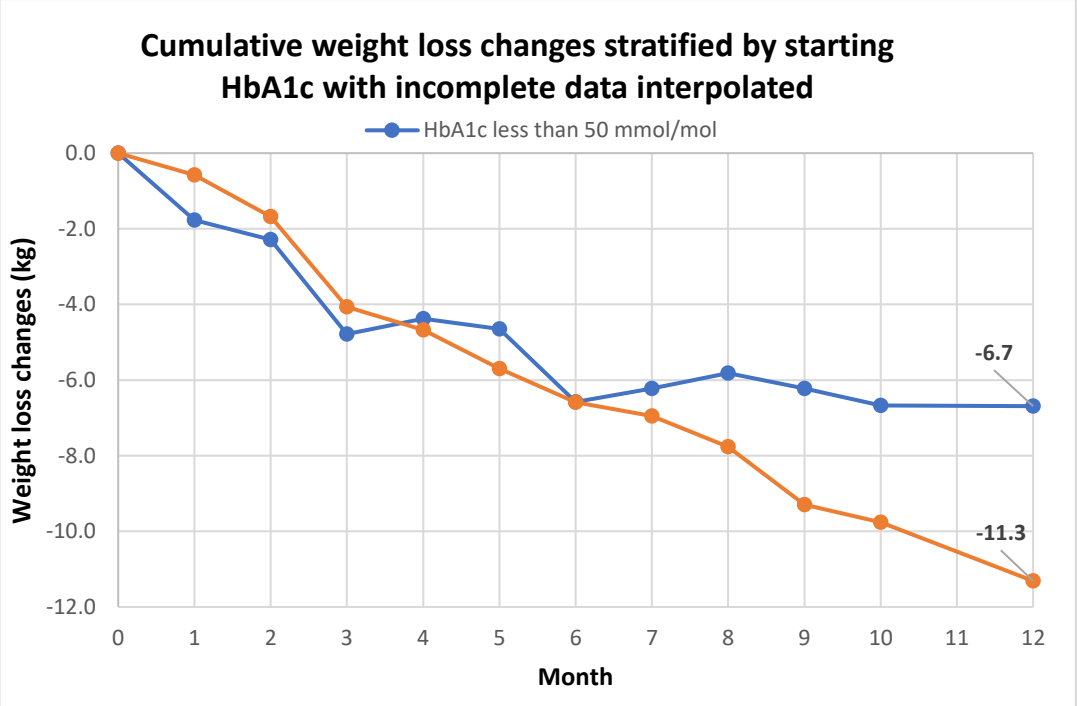


Figure 3. 8: Cumulative weight loss changes stratified by starting HbA1c with incomplete data points interpolated, with bars representing standard error of the mean



Independent student t-test was used to assess whether there was a statistically significant difference in the weight loss between the two groups based on starting HbA1c and starting weight. There was no statistically significant difference in weight loss based on the starting HbA1c (p=0.224) and starting weight (p=0.240). Table 3.9 provides summary of the independent t-tests.

Table 3. 9: Independent student t-test results for difference in weight loss between groups based on starting characteristics

Groups	Weight loss difference (kg)	Lower 95% CI	Upper 95% CI	Sample size	P value
HbA1c more than 50 mmol/mol compared to HbA1c less than 50 mmol/mol	6.6	-4.4	17.6	17	0.224
Starting weight more than 131.2kg compared to starting weight less than 131.2 kg	6.6	-4.9	18.1	17	0.240

Mixed effect model was used to assess changes in weight over time. Fitting the mixed effect model gave an estimate for the intercept (starting weight) of 129.1 kg, SE of 6 kg and a monthly change -0.7 kg (SE 0.1 kg) (i.e., weight loss), resulting in mean weight of 120.7 kg at 12 months follow up (Y= 129.1-X* 0.7), giving a total predicted weight loss of 8.4 kg at 12 months. This weight loss was statistically significant, with p< 0.001. Table 3.10 provides summary of the results for fixed effect model.

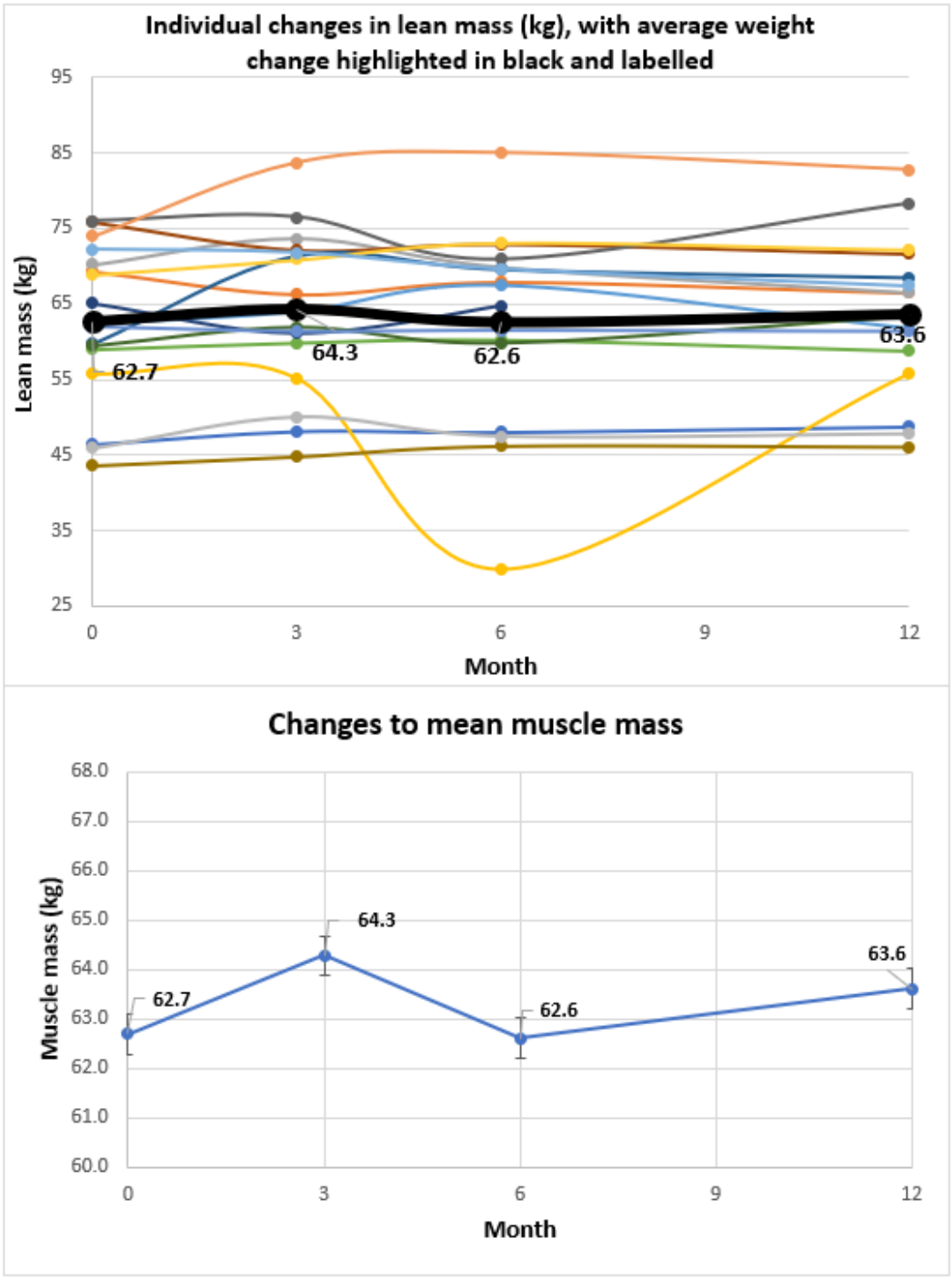
Table 3. 10: Estimates of Fixed Effects, where dependent variable is weight

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	129.1	6.0	<0.001	116.4	141.8
Month	-0.7	0.1	<0.001	-0.9	-0.6

3.3.3 Monthly muscle mass changes

Seventeen participants underwent body composition analysis in the BODPOD machine for analysis of muscle mass at baseline, month 3, and 6, and sixteen patients underwent body composition analysis at month 12. The mean muscle mass at baseline was 62.7 kg (SD 10.3 kg), with little change at month 6 and 12 (62.6 kg (SD 13.2 kg) and 63.6 kg (SD 10.5 kg) respectively). Figure 3.9 shows individual muscle mass changes over 12 months as well as the mean muscle mass changes.

Figure 3. 9: Individual changes in lean mass and changes to mean muscle mass over 12 months, with bars representing standard error of the mean



There was no significant change in muscle mass during 12 months, with an intercept 63.1 kg (SE 2.6 kg) and gradient of change 0.03 kg (SE 0.1 kg), p=0.762. Table 3.11 provides summary of the results for fixed effect model.

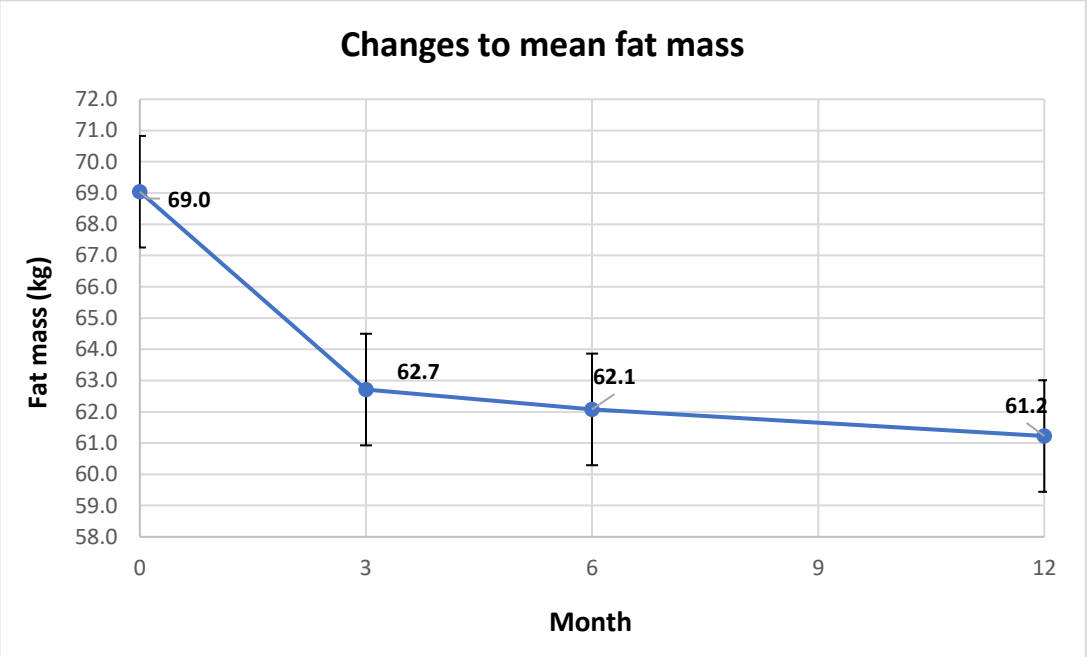
Table 3. 11: Estimates of Fixed Effects, where dependent variable is muscle mass (Lean mass)

Parameter	**Estimate**	**SE**	**P value**	**95% Confidence Interval**	
				Lower Bound	Upper Bound
Intercept	63.1	2.6	<0.001	57.6	68.7
Month	0.03	0.1	0.762	-0.2	0.3

3.3.4 Monthly fat mass changes

Seventeen participants underwent body composition analysis in the BODPOD machine for analysis of fat mass. The data for fat mass at month 12 was missing for one participant. The mean fat mass at baseline was 69 kg (SD 24.4 kg), with reduction to 62.7 (SD 22.4 kg), 62.1 (SD 25.8kg) and 61.2 (SD 23.3 kg) at month 3, 6 and 12. Figure 3.10 shows changes to mean fat mass during 12 months.

Figure 3. 10: Changes to mean fat mass over 12 months, with bars representing standard error of the mean



Mixed effect model showed that this reduction was statistically significant ($p<0.001$), with an intercept of 67.1 kg (SE 5.9 kg) and gradient of -0.7 kg (SE 0.2 kg), with a mean of 8.9 kg fat loss at 12 months ($Y=67.1-0.7*X$). Table 3.12 provides summary of the results for fixed effect model on fat mass changes.

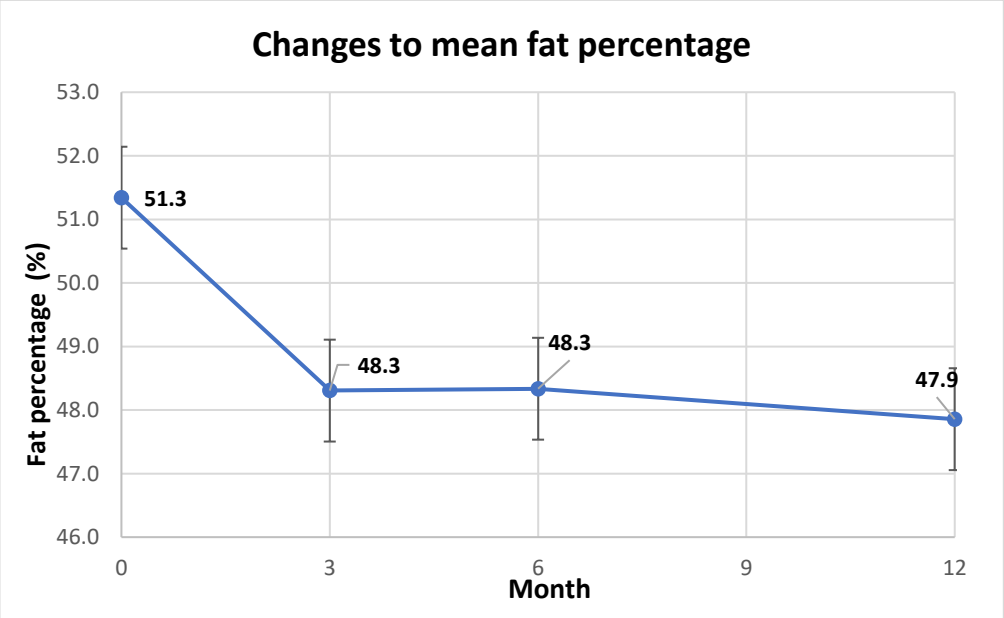
Table 3. 12: Estimates of Fixed Effects, where dependent variable is fat mass

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	67.1	5.9	<0.001	54.7	79.5
Month	-0.7	0.2	<0.001	-1.1	-0.4

3.3.5 Monthly fat percentage changes

Seventeen participants underwent body composition analysis in the BODPOD machine for analysis of fat percentage. The data for percentage at month 12 was missing for 1 participant. The mean fat percentage was 51.3% (SD 9.9%) at baseline, with reduction to 48.3% (SD 13.3%) and 47.9% (SD 11%) at months 6 and 12. Figure 3.11 shows changes to average fat percentage during 12 months.

Figure 3. 11: Changes to mean fat percentage over 12 months, with bars representing standard error of the mean



Mixed model regression analysis revealed that the intercept was 50.5% (SE 2.7%), with the gradient of -0.3% (SE 0.1%). Changes were statistically significant, with predicted 12-month average loss of 3.6% (y=50.5-0.3*x),

p=0.002. Table 3.13 provides summary of the results for fixed effect model on fat percentage changes.

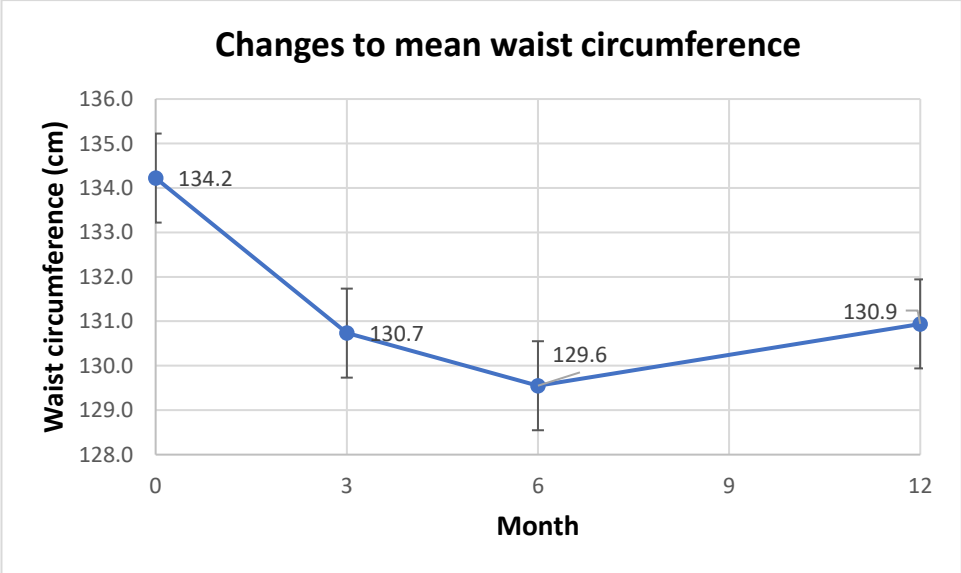
Table 3. 13: Estimates of Fixed Effects, where dependent variable is fat percentage

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	50.5	2.7	<0.001	44.8	56.1
Month	-0.3	0.1	0.002	-0.5	-0.1

3.3.6 Monthly waist circumference changes

Eighteen participants had waist circumference measured at baseline. Data for waist circumference at month 3 were missing for 3 participants, at month 6 missing for 2 participants and at month 12 was missing for 1 participant. The mean waist circumference at baseline was 134.2cm (SD 20.4cm), with reduction to 130.7 cm (SD 23.4cm), 129.6 cm (SD 21.6 cm) and 130.9 cm (SD 21.0 cm) at month 3,6 and 12. Figure 3.12 provides graphical representation of changes to mean waist circumference over 12 months.

Figure 3. 12: Changes to mean waist circumference, with bars representing standard error of the mean



Mixed effect model showed that this reduction was statistically significant (p=0.014), with an intercept of 133 cm (SE 4.9 cm) and gradient of -0.4 cm (SE 0.1 cm), with a mean of 4.8 cm reduction at 12 months (Y=133-0.4*X). Table 3.14 provides summary of the results for fixed effect model on waist circumference changes.

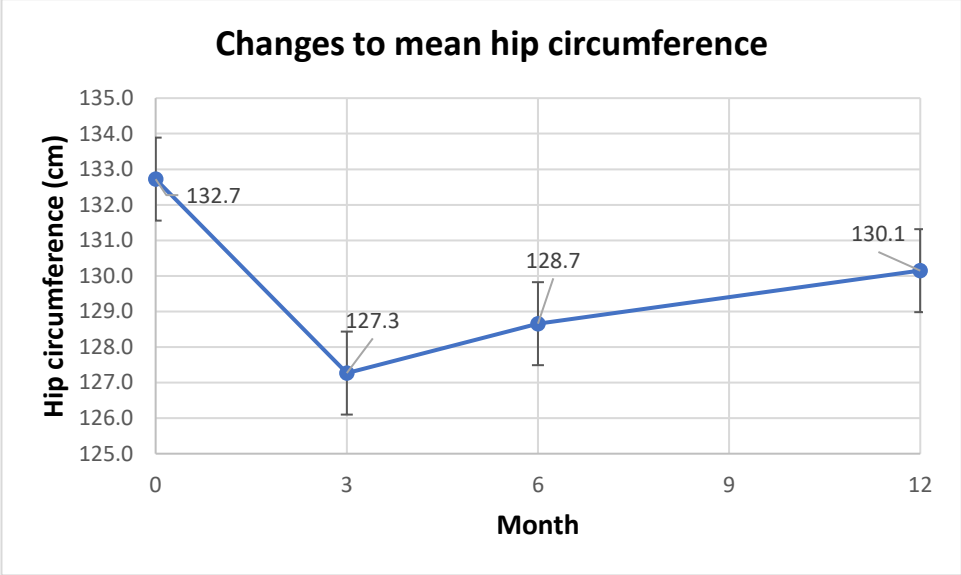
Table 3. 14: Estimates of Fixed Effects, where dependent variable is waist circumference

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	133.0	4.9	<0.001	122.7	143.3
Month	-0.4	0.1	0.014	-0.7	-0.1

3.3.7 Monthly hip circumference changes

Eighteen participants had hip circumference measured at baseline. Data for hip circumference at month 3 were missing for 3 participants, at month 6 missing for 2 participants and at month 12 missing for 1 participant. The mean hip circumference at baseline was 132.7 cm (SD 20.9cm), with reduction to 127.3 cm (SD 20.3cm), 128.7 cm (SD 21.2 cm) and 130.1 cm (SD 18.3 cm) at month 3,6 and 12. Figure 3.13 shows changes to mean hip circumference over 12 months.

Figure 3. 13: Changes to mean hip circumference during 12 months, with bars representing standard error of the mean



Mixed effect model showed that this reduction was not statistically significant (p=0.059), with an intercept of 132 cm (SE 4.8 cm) and gradient of -0.4 cm (SE 0.2 cm), with a predicted mean reduction of 4.8 cm at 12 months (Y=132-0.4*X). Even though the effect of time did not reach statistical significance, there as a trend for a reduction in overall hip circumference over 12 months. Table 3.15 provides summary of the results for fixed effect model on hip circumference changes.

Table 3. 15: Estimates of Fixed Effects, where dependent variable is hip circumference

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	132.0	4.8	0.000	122.0	141.9
Month	-0.4	0.2	0.059	-0.7	0.0

3.4 Changes in appetite

3.4.1 Perceived changes in appetite during the 12-month study

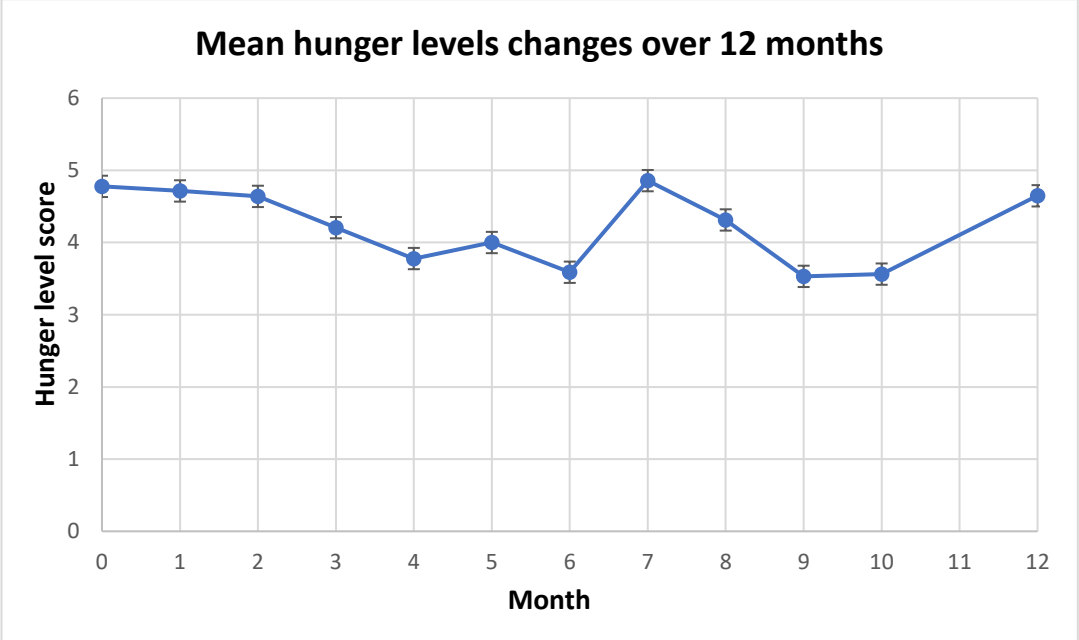
Participants were asked to complete visual analogue appetite scale (assessing hunger levels, satiety, fullness and overall appetite control) during each month of their morning visit (participants were fasted). In total, there were 198 visual analogue scales for appetite completed. This was less than the expected 216 as a result of some participants missing follow-up appointments or arriving to appointments following food consumption.

There were no statistically significant differences between participants perceived fasting hunger levels, fasting satiety scores, fasting fullness levels and overall appetite following fasting during the 12 months study.

3.4.1.1 Hunger levels

Participants were asked to score hunger on a scale between 0 and 10, where 0 is not hungry at all and 10 is I have never been hungrier. Numbers were rounded to one decimal place. The mean hunger score at baseline was 4.8 (SD 2.4) which decreased to mean score of 4.6 (SD 2.3) at month 12. Figure 3.14 shows the mean changes to hunger levels each during 12 months.

Figure 3. 14: Changes to mean hunger levels during 12 months, with bars representing standard error of the mean



There was no statistically significant difference in perceived hunger levels during the study. Mixed effect model showed that there was a trend for a reduction in hunger levels, however, the reduction was not significant (p=0.076), with an intercept at 4.5 (SE 0.5) and gradient of -0.1 (SE 0.04), with a predicted hunger reduction of 1.2 at 12 months (Y=4.5-0.1*X). This would represent only 12% reduction in perceived hunger levels. Table 3.16 provides summary of the results for fixed effect model on hunger level changes.

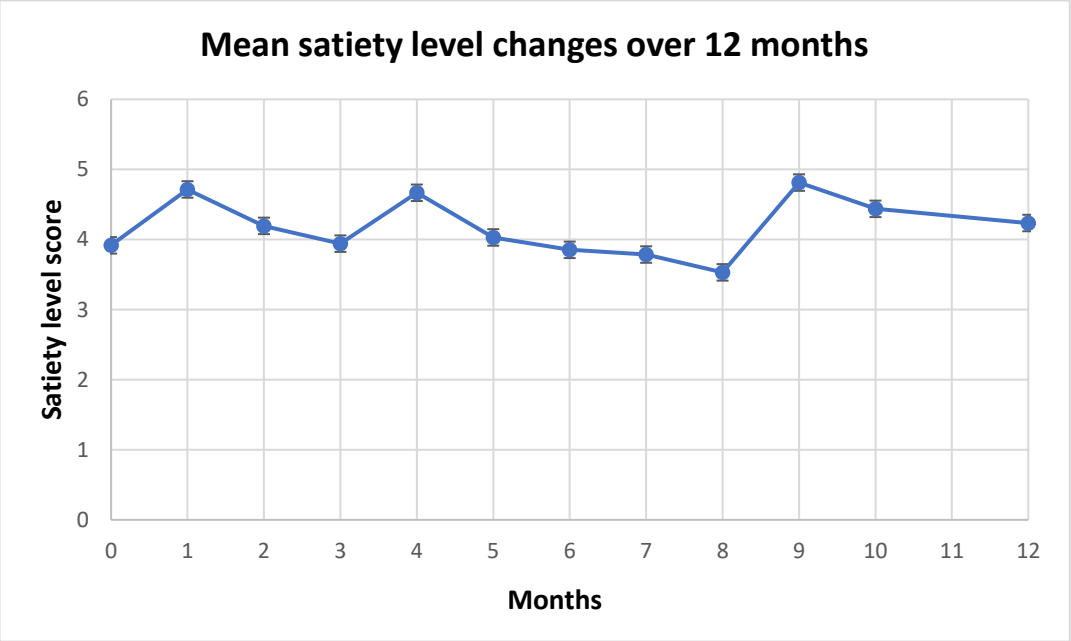
Table 3. 16: Estimates of Fixed Effects, where dependent variable is hunger score

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	4.5	0.5	<0.001	3.5	5.6
Month	-0.1	0.04	0.076	-0.1	0.01

3.4.1.2 Satiety score

Participants were asked to score satiety on a scale between 0 and 10, where 0 is I am completely empty and 10 is I cannot eat another bite. Numbers were rounded to one decimal place. The overall mean satiety score was 3.9 (1.8) at baseline and 4.2 (2.6) at month 12. Figure 3.15 shows the mean changes to satiety levels each during 12 months.

Figure 3. 15: Mean satiety level changes over 12 months, with bars representing standard error of the mean



Mixed effect model showed that there was no trend for a change in satiety scores, with an intercept at 4.1 (SE 0.5) and gradient of 0.01 (SE 0.04), p=0.717. Table 3.17 provides summary of the results for fixed effect model on satiety score changes.

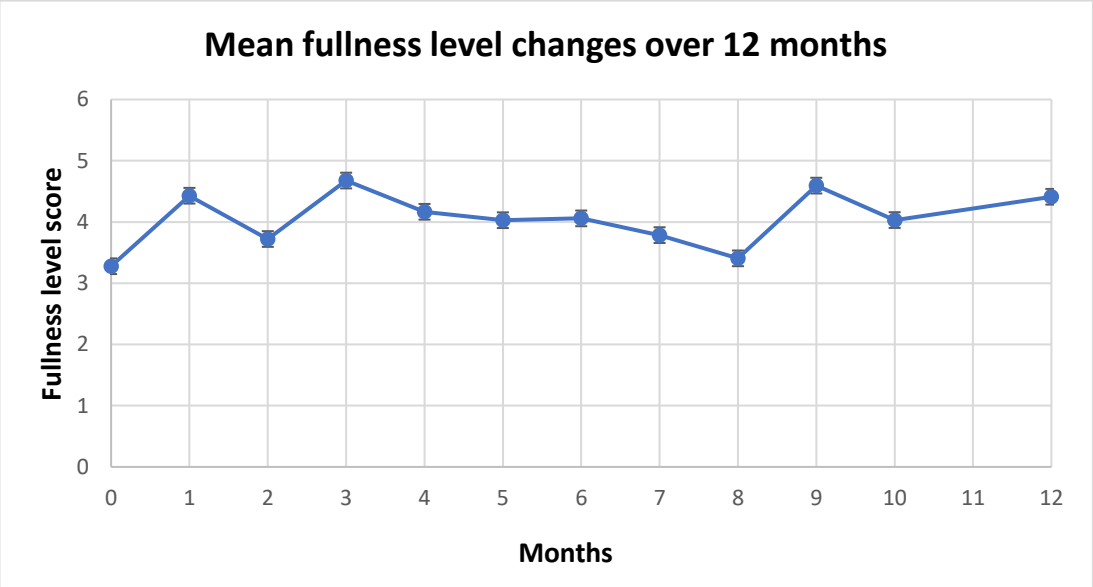
Table 3. 17: Estimates of Fixed Effects, where dependent variable is satiety score

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	4.1	0.5	<0.001	3.1	5.2
Month	0.01	0.04	0.717	-0.1	0.1

3.4.1.3 Fullness score

Participants were asked to score fullness on a scale between 0 and 10, where 0 is I am not at all full and 10 is I am totally full. Numbers were rounded to one decimal place. The overall mean fullness score was 3.3 (SD 2.1) and 4.4 (2.7) at month 12. Figure 3.16 shows the mean changes to satiety levels each during 12 months.

Figure 3. 16: Changes to mean fullness levels over 12 months, with bars representing standard error of the mean



Mixed effect model showed that there was no trend for a change in fullness scores, with an intercept at 3.8 (SE 0.6) and gradient of 0.04 (SE 0.04),

p=0.278. Table 3.18 provides summary of the results for fixed effect model on fullness level changes.

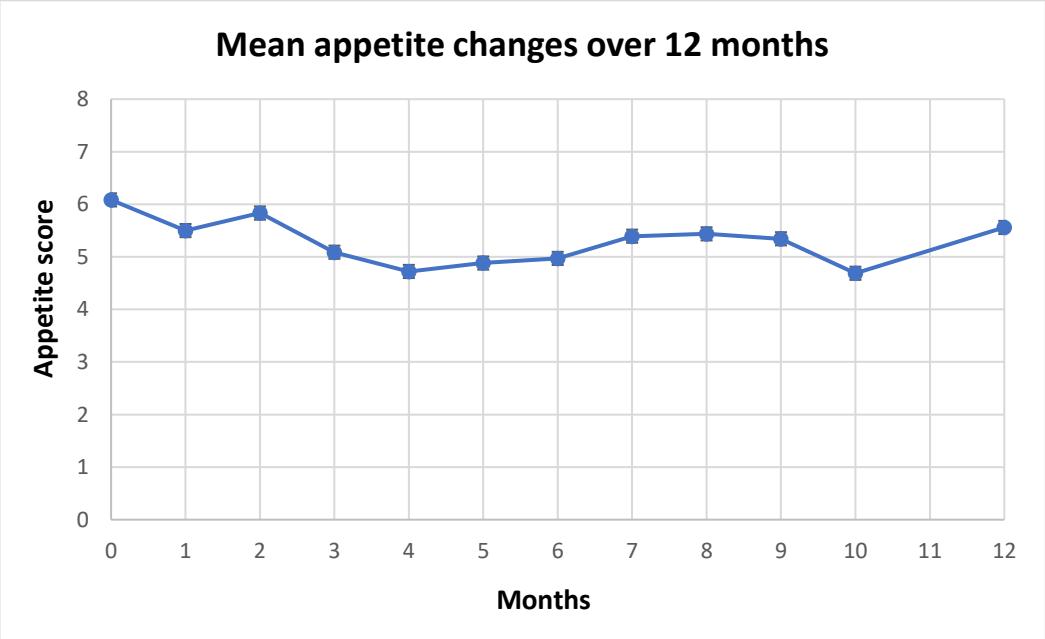
Table 3. 18: Estimates of Fixed Effects, where dependent variable is fullness score

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	3.8	0.6	<0.001	2.7	4.9
Month	0.04	0.04	0.278	-0.03	0.1

3.4.1.4 Appetite score

Participants were asked to score appetite on a scale between 0 and 10, where 0 is I can eat nothing at all and 10 is I can eat a lot. Numbers were rounded to one decimal place. The overall mean appetite score was 6.1 (SD 2) at baseline and 5.6 (SD 2.6) at month 12. Figure 3.17 shows the mean changes to satiety levels each during 12 months.

Figure 3. 17: Changes to mean appetite score over 12 months, with bars representing standard error of the mean



Mixed effect model showed that there was no trend for a change in appetite scores, with an intercept at 5.5 (SE 0.5) and gradient of -0.05 (SE 0.03), p=0.144. Table 3.19 provides summary of the results for fixed effect model on appetite changes.

Table 3. 19: Estimates of Fixed Effects, where dependent variable is appetite score

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	5.5	0.5	<0.001	4.5	6.5
Month	-0.05	0.03	0.144	-0.1	0.0

3.4.2 Perceived changes in appetite during the 24-hour HMRU studies

Participants were asked to complete visual analogue appetite scale (assessing hunger levels, satiety, fullness and overall appetite control) at prespecified time points during their 24-hour HMRU study, both at baseline and after 12 months of being on Dapagliflozin. The time points were 10:30 AM, 1:30 PM, 4:00 PM. 5:00 PM, 7:30 PM, 10:15 PM and 8:45 AM the following day.

The reason for assessing appetite during these time intervals was to see how appetite changes during the day, and provide a holistic assessment of appetite changes with Dapagliflozin in relation to meal consumption. Overall, there was no statistically significant difference between hunger levels, satiety score, fulness score and overall appetite during each of the prespecified time points at baseline and month 12. Tables with results from the paired student t-test are provide below.

Table 3. 20: Hunger level changes between time points at one year and baseline

Time	Mean difference in hunger levels between one year and baseline	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
10:30 AM	0.3	3.3	-2.1	2.6	10	0.817
1:30 PM	0.6	2.1	-0.7	2.0	12	0.334
4:00 PM	-0.2	1.6	-1.4	1.1	9	0.766
5:00 PM	0.7	2.5	-1.1	2.4	10	0.429
7:30 PM	2.6	3.0	0.3	4.9	9	0.029
10:15 PM	0.4	3.6	-2.7	3.4	8	0.778
8:45 AM	-0.8	2.3	-3.6	2.0	5	0.477

Table 3. 21: Satiety score changes at one year and baseline

Time	Mean difference in satiety levels between one year and baseline	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
10:30 AM	1.1	3.6	-1.5	3.7	10	0.357
1:30 PM	0.4	1.8	-0.8	1.5	12	0.497
4:00 PM	0.6	1.5	-0.5	1.7	9	0.243
5:00 PM	0.9	1.9	-0.5	2.3	10	0.171
7:30 PM	-0.6	3.3	-3.1	2.0	9	0.625
10:15 PM	0.3	3.0	-2.2	2.7	8	0.818
8:45 AM	1.4	0.9	0.3	2.5	5	0.025

Table 3. 22: Fullness score changes at one year and baseline

Time	Mean difference in fullness levels between one year and baseline	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
10:30 AM	0.8	2.3	-0.8	2.4	10	0.300
1:30 PM	0.6	2.6	-1.0	2.2	12	0.448
4:00 PM	0.9	2.0	-0.7	2.4	9	0.225
5:00 PM	0.5	2.5	-1.3	2.3	10	0.545
7:30 PM	0.6	2.5	-1.3	2.6	9	0.49
10:15 PM	0.4	2.9	-2.1	2.8	8	0.728
8:45 AM	1.8	1.5	0.0	3.6	5	0.053

Table 3. 23: Appetite changes at one year and baseline

Time	Mean difference in appetite between one year and baseline	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
10:30 AM	0.2	2.9	-1.9	2.2	10	0.873
1:30 PM	0.5	1.6	-0.5	1.6	12	0.278
4:00 PM	-1.1	2.5	-3.0	0.8	9	0.214
5:00 PM	0.4	3.0	-1.7	2.5	10	0.678
7:30 PM	0.8	2.3	-1.0	2.6	9	0.344
10:15 PM	-1.0	2.4	-3.0	1.0	8	0.286
8:45 AM	-0.2	2.5	-3.3	2.9	5	0.866

3.5 Chapter summary

In summary, there was no statistically significant difference in measured and calculated energy expenditure between baseline and month 12. Additionally, there was no difference in energy expenditure overnight, after lunch, after exercise and after temperature drop between baseline and month 12. There was a statistically significant reduction in body weight at month 12, which was driven by a reduction in fat mass with no changes in muscle mass. The reduction in fat mass was accompanied by a statistically significant reduction in waist circumference over the period of 12 months, with a trend for reduction in hip circumference (which did not reach significance). Participant did not report any changes in appetite during the 12-month study.

Chapter 4: Changes in urinary excretion and cardiovascular parameters

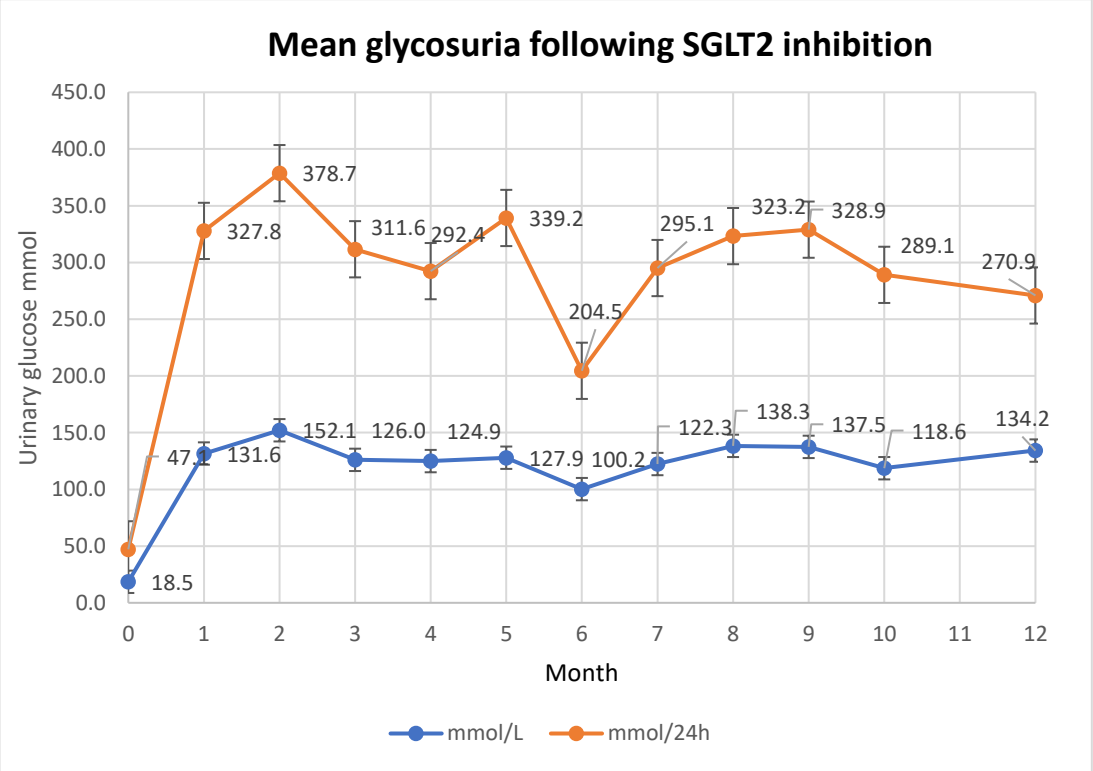
Chapter 4: Changes in urinary excretion and cardiovascular parameters

In this chapter I will describe Dapagliflozin induced changes in urinary excretion and cardiovascular markers over 12 months. I will describe changes in urinary glucose (glycosuria), urinary sodium (natriuresis), urinary protein (proteinuria), urinary volume and changes in cardiovascular parameters, such as blood pressure and heart rate.

4.1 Changes in glycosuria

Out of 216 urinary collections there were 19 urinary samples missing, as well as 17 samples that were not complete (either collected for less than 24 hours or analysed more than 4 days after collection). This reflects the fact that it was not always possible for participants to do 24-hour urinary collections. All available samples were analysed. Data are presented as amount of urinary glucose per 24 hour (mmol/24 hours) and also urinary glucose per litre (mmol/L). Figure 4.1 provides a graphical representation of average changes to urinary glucose levels in 24 hours (orange line) and urinary glucose level per litre (blue line).

Figure 4. 1: Mean glycosuria at baseline and following SGLT2 inhibition, with bars representing standard error of the mean



The mean baseline for 24-hour urinary glucose was 47.1 (SD 175.4) mmol. The mean baseline for urinary glucose per litre was 18.5 (SD 63.4) mmol. As expected, after 1 month of SGLT2 inhibitor therapy, there was a marked increase in 24-hour urinary glucose by 294.7 (SD 298) mmol to 327.8 mmol (59 g), and a marked increase in urinary glucose per litre by 127 (SD 71.9) mmol to 131.6 mmol (23.6 g). Paired student t-test was used to analyse the difference seen between baseline and month 1. Data from 4 participants were missing for 24-hour urinary glucose collection for month 1. Data from 2 participants were missing for urinary glucose excretion per litre at month 1. The data used for paired student t-test are summarised in Table 4.1.

Table 4. 1: Changes in urinary glucose (both 24-hour collection and per litre) between baseline and month 1

Urinary glucose	Mean	N	SD	SE
Baseline glucose per 24 hours (mmol/24h)	60.5	14	198.4	53.0
Month 1 Glucose per 24 hours (mmol/24h)	327.8	14	332.4	88.8
Baseline glucose per litre (mmol/L)	20.8	16	67.1	16.8
Month 1 glucose per litre (mmol/L)	131.6	16	75.1	18.8

SD: Standard deviation, SE: Standard error

There was a statistically significant difference between baseline and follow-up at month 1 urinary glucose excretion per 24 hours (increase of 247.5 mmol/24 h, SD 165.7, p<0.001) and between baseline and follow-up at month 1 urinary glucose excretion per litre (increase of 108.5 mmol/l, SD 67.4, p<0.001). This is summarised in Table 4.2.

Table 4. 2: Difference between month 1 and baseline 24-hour urinary glucose excretion and urinary glucose excretion per litre

Urinary glucose	The difference in means between month 1 and baseline	SD	Lower 95%CI	Upper 95%CI	Sample size	P value
Per 24 hours (mmol/24h)	267.3	183.5	161.3	373.2	14	<0.001
Per litre (mmol/L)	110.8	71.3	72.8	148.8	16	<0.001

SD: Standard deviation, CI: Confidence interval

Mixed effect model was used to assess the effect of time on changes in urinary glucose excretion per 24 hours during the 12 months. There was no statistically significant difference in 24 h urinary glucose excretion between each month from baseline to month 12, with intercept (baseline starting value) at 234.1 mmol, and an estimate of an effect of time (how much change occurs per month, gradient of change) 5.7 mmol, p=0.122, summarised in Table 4.3.

Table 4. 3: Estimates of Fixed Effects, where dependent variable is 24-h urinary glucose, from baseline to month 12

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	234.1	46.2	<0.001	139.8	328.3
Month	5.7	3.7	0.122	-1.5	12.9

SE: Standard error, CI: Confidence interval

From the graph and paired student t-test analysis it was clear that there was a marked change in urinary glucose excretion (both per litre and in 24 hours) from baseline to month 1, after which elevated values were seen. Mixed effect model was also applied to urinary glucose excretion per 24 hours data from month 1 to month 12, to assess whether there is any difference between months whilst participants were on Dapagliflozin. When mixed effect model was applied to data from month 1 to month 12, there was no significant effect of time on glycosuria, with intercept of 329.4mmol and effect of time was -5.9 mmol, p=0.113, shown in Table 4.4.

Table 4. 4: Estimates of Fixed Effects, where dependent variable is 24-h urinary glucose, from month 1 to month 12

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	329.4	46.3	<0.001	234.7	424.2
Month	-5.9	3.7	0.113	-13.3	1.4

SE: Standard error, CI: Confidence interval

Mixed effect model was applied to assess the effect of time on changes in urinary glucose excretion per litre during the 12 months. There was statistically significant (p=0.003) difference in urinary glucose excretion per litre during the study, with an intercept at 92.2mmol and monthly increase of 3.9mmol, resulting in urinary glucose of 139 mmol per litre at month 12 (y=92.2+3.9*time), summarised in Table 4.5.

Table 4. 5: Estimates of Fixed Effects, where dependent variable is urinary glucose per litre, from baseline to month 12

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	92.2	13.9	<0.001	64.0	120.3
Month	3.9	1.3	0.003	1.3	6.4

SE: Standard error, CI: Confidence interval

Mixed effect model was also applied to data from month 1 to month 12, to assess whether there is any difference in urinary glucose excretion per litre between months whilst participants were on Dapagliflozin. There was no

significant difference between month 1 and 12, with intercept at 130.4 mmol and monthly decrease of 0.7mmol, p=0.578. This is shown in Table 4.6. This would suggest that the significant difference seen in the mixed model analysis when all data from baseline to month 12 were included was driven by the difference between baseline and month 1.

Table 4. 6: Estimates of Fixed Effects, where dependent variable is urinary glucose per litre, from month 1 to month 12

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	130.4	13.8	<0.001	102.2	158.5
Month	-0.7	1.2	0.578	-3.1	1.7

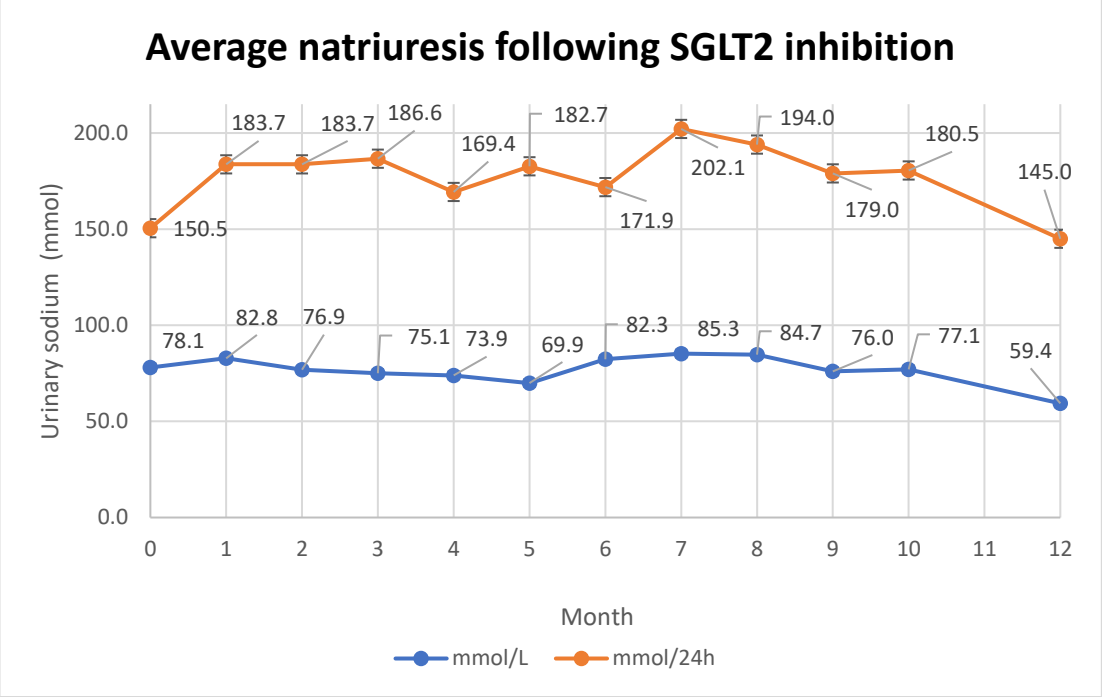
SE: Standard error, CI: Confidence interval

Data for urinary glucose were log-transformed and re-analysed with paired student t-test and mixed effect model. Using log-transformed data in the mixed-model analysis has in impact on the interpretation of the parameters. The standard model y = a + bx (additive model) changes to log(y) = a + bx. If log is to base e, then this can be written as y = exp(a)exp(bx), so the model becomes multiplicative rather than additive. The regression coefficients (gradients) from the model are multipliers not additions. The results from mixed effect models and paired student t-test from log-transformed data are shown in appendix. Analysis from both the non-transformed and log-transformed data shows that there is a significant difference between urinary glucose (both per litre and per 24 hours) between baseline and month 1, and no difference in urinary glucose after therapy with Dapagliflozin has been initiated.

4.2 Changes in natriuresis

Data are presented as amount of urinary sodium per 24 hour (mmol/24 hours) and also urinary sodium per litre (mmol/L). Figure 4.2 shows monthly changes to mean natriuresis in 24 hours (orange line) and mean natriuresis per litre (blue line).

Figure 4. 2: Monthly changes to 24-hour natriuresis and natriuresis per litre, with bars representing standard error of the mean



When all available data were included, the mean baseline for 24-hour urinary sodium was 150.5 (SD 70.3) mmol (3.4 g). The mean baseline for urinary sodium per litre was 78.1 (SD 33.2) mmol (1.8 g). After 1 month of SGLT2 inhibitor therapy the 24-hour urinary sodium was 183.7 (SD 121.5) mmol (4.2 g) and urinary sodium per litre was 82.8 (SD 31.5) mmol (1.9 g).

Paired student t-test was used to analyse the difference between baseline data and month 1 data. Data from 2 participants were missing for month 1 urinary sodium per litre (due to participants not providing any urinary collection). Data from 4 participants were missing for month 1 24-hour urinary sodium collection (due to 2 participants not providing full 24-hour collection and 2 participants not providing any urinary collection).

The data used for paired student t-test are summarised in Table 4.7. There was no statistically significant difference between baseline and first month urinary sodium excretion per 24 hours (increase of 30.6 mmol/24 h, SD 102.4, p=0.283) and no statistically significant difference between baseline and first month urinary sodium excretion per litre (decrease of 0.03 mmol/l, SD 35.1, p=0.988). This is summarised in Table 4.8.

Table 4. 7: Changes in urinary sodium (both 24-hour collection and per litre) between baseline and month 1

Urinary sodium	Mean	N	SD	SE
Baseline mmol/24h	153.1	14	62.1	16.6
Month 1 mmol/24h	183.8	14	116.3	31.1
Baseline mmol/L	82.8	16	32.2	8.0
Month 1 mmol/L	82.8	16	31.5	7.9

Table 4. 8: Difference between 24-hour urinary sodium excretion and urinary sodium excretion per litre between month 1 and baseline

Urinary sodium	The difference in means between month 1 and baseline	SD	Lower 95%CI	Upper 95% CI	Sample size	P value
Per 24 hours (mmol/24h)	30.6	102.4	89.8	-28.5	14	0.283
Per litre (mmol/l)	0.0	35.1	18.7	-18.7	16	0.998

Mixed effect model was used to assess the effect of time on changes in urinary sodium excretion (both per 24 hours and per litre) during the 12 months. There was no statistically significant difference in 24 hours urinary sodium excretion during the length of the study, with an intercept of 172.4 mmol, and monthly change of 0.6 mmol, p=0.727, summarised in Table 4.9.

Table 4. 9: Estimates of Fixed Effects, where dependent variable is 24-hour urinary sodium excretion per 24h hours

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	172.4	17.6	<0.001	136.6	208.1
Month	0.6	1.6	0.727	-2.6	3.7

SE: Standard error, CI: Confidence interval

Similarly, mixed effect model showed there was no difference between sodium excretion per litre during the 12 months, with intercept of 80.3 mmol, monthly decrease of 0.8 mmol, p=0.131, summarised in Table 4.10.

Table 4. 10: Estimates of Fixed Effects, where dependent variable is urinary sodium excretion per litre

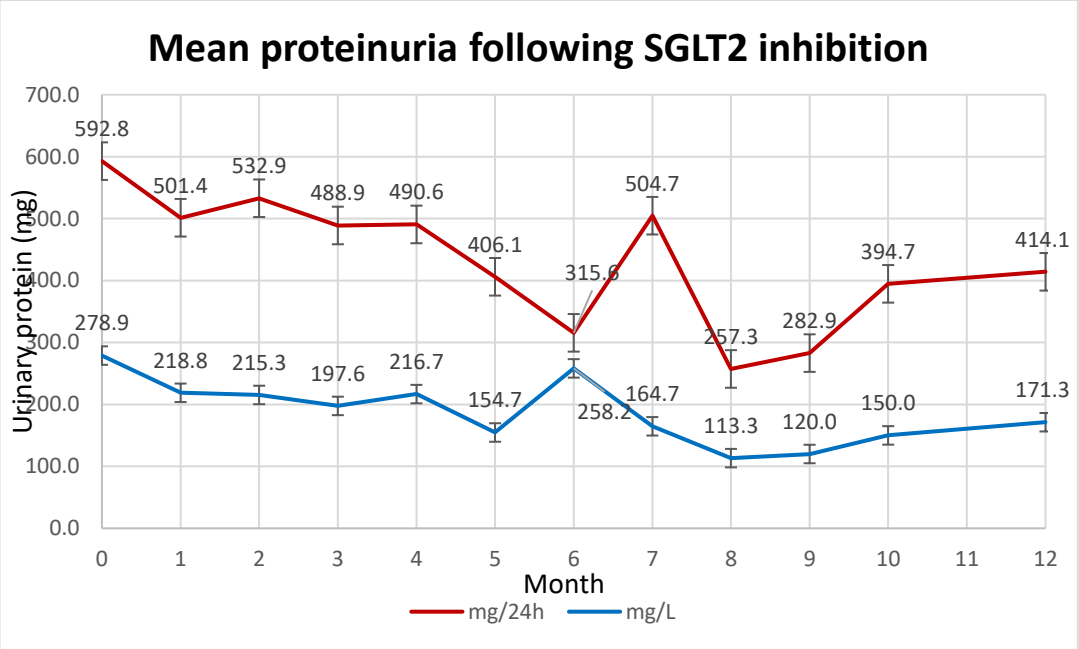
Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	80.3	6.0	<0.001	68.0	92.6
Month	-0.8	0.5	0.131	-1.7	0.2

SE: Standard error, CI: Confidence interval

4.3 Changes in proteinuria

Data are presented as amount of urinary protein per 24 hour (mg/24 hours) and also urinary protein per litre (mg/L). Figure 4.3 shows monthly changes to mean proteinuria in 24 hours (red line) and mean proteinuria per litre (blue line).

Figure 4. 3: Monthly changes to 24-hour proteinuria and proteinuria per litre, with bars representing standard error of the mean



When all available data were included, the mean 24-hour urinary protein was 592.8 (SD 1228.5) mg at baseline. The mean urinary protein per litre was 278.9 (SD 486.3) mg/L at baseline. At month 12 the mean 24-hour urinary protein was 414.1 (SD 548.9) mg and the mean urinary protein per litre was 171.3 (SD 244.6) mg. This means that the mean protein excretion of participants was at the level of significant proteinuria (more than 300 mg/24 hours) as defined by KDIGO guidelines, suggesting at least stage G1 of CKD (2020).

Paired student t-test was used to analyse the difference between baseline data and month 1 data. Data from 2 participants were missing for month 1 urinary protein per litre (due to participants not providing any urinary collection). Data

from 4 participants were missing for month 1 24-hour urinary protein collection (due to 2 participants not providing full 24-hour collection and 2 participants not providing any urinary collection).

The data used for paired student t-test are summarised in Table 4.11. There was no statistically significant difference between month 1 and baseline urinary protein excretion per 24 hours (decrease of 162.1 mg/24 h, SD 653.4, p=0.370) and no statistically significant difference between month 1 and baseline urinary protein excretion per litre (decrease of 86.3 mg/L, SD 261.1, p=0.206). This is summarised in Table 4.12.

Table 4. 11: Changes in urinary protein (both 24-hour collection and per litre) between baseline and month 1

Urinary protein	Mean	N	SD	SE
Baseline mg/24h	663.6	14	1385.2	370.2
Month 1 mg/24h	501.4	14	820.0	219.2
Baseline mg/L	305	16	511.3	127.8
Month 1 mg/L	218.8	16	299	74.7

SE: Standard error, SD: Standard deviation

Table 4. 12: Difference between 24-hour urinary protein excretion and urinary protein

Urinary protein	The difference in means between month 1 and baseline	SD	Lower 95%CI	Upper 95% CI	Sample size	P value
Per 24 hours (mg/24h)	-162.1	653.4	215.1	-539.4	14	0.370
Per litre (mg/l)	-86.3	261.1	52.9	-225.4	16	0.206

SD: Standard deviation, CI: Confidence interval

Mixed effect model was used to assess the effect of time on changes in urinary protein excretion (both per 24 hours and per litre) during the 12 months. There was a trend for reduction, but no statistically significant difference in 24 hours protein excretion during the length of the study, with an intercept at 546.9 mg, and monthly change of -6.6 mg, p=0.297, summarised in Table 4.13.

Table 4. 13: Estimates of Fixed Effects, where dependent variable is 24-hour urinary protein excretion

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	546.9	207.0	0.017	111.7	982.1
Month	-6.6	6.3	0.297	-19.0	5.8

SE: Standard error, CI: Confidence interval

Similarly, mixed effect model showed there was a trend for reduction, but no difference between urinary protein excretion per litre during the 12 months, with intercept of 247.3 mg, and monthly decrease of 4.9 mg, p=0.089, summarised in Table 4.14.

Table 4. 14: Estimates of Fixed Effects, where dependent variable is urinary protein excretion per litre

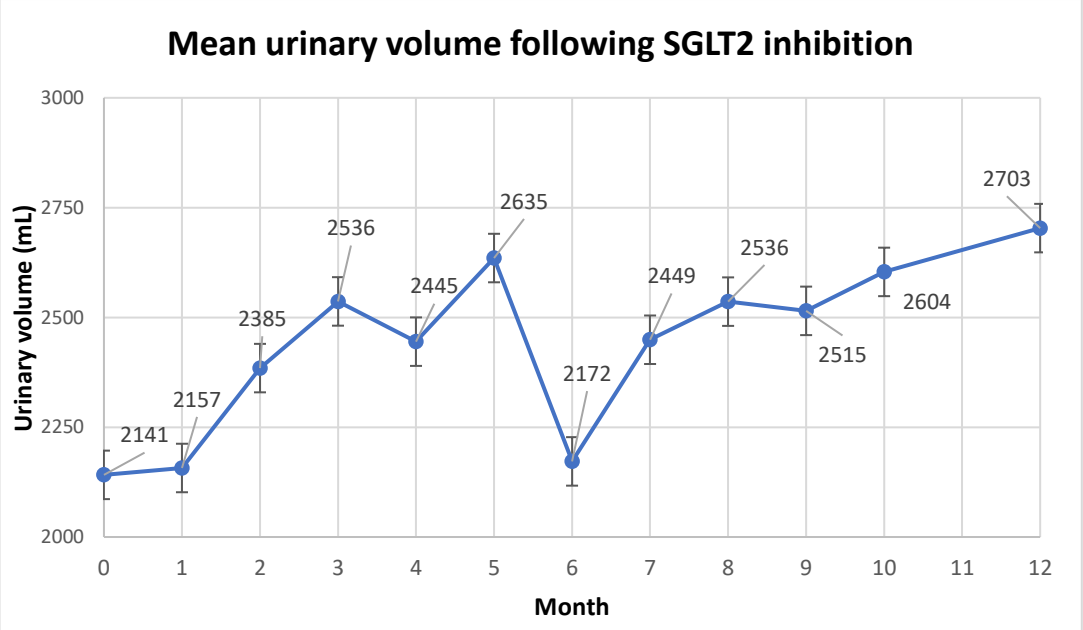
Parameter	**Estimate**	**SE**	**P value**	**95% Confidence Interval**	
				Lower Bound	Upper Bound
Intercept	247.3	87.9	0.012	62.6	432.0
Month	-4.9	2.8	0.089	-10.5	0.8

SE: Standard error, CI: Confidence interval

4.4 Changes in urinary volume

There was an increase in total urinary volume during the 12-month study. The mean volume was 2141 (SD 792) ml at baseline and 2703 (SD 904) ml at month 12. Figure 4.4 shows the changes in mean volume over time, recorded in ml.

Figure 4. 4: Monthly changes to mean urinary volume in ml, with bars representing standard error of the mean



Paired student t-test was used to analyse the difference between baseline data and month 1 data. Data from 4 participants were missing for month 1.

The data used for paired student t-test are summarised in Table 4.15. There was no statistically significant difference between month 1 and baseline urinary volume (increase of 215.9 ml, SD 996.2, p=0.432). This is summarised in Table 4.16.

Table 4. 15: Changes in urinary volume between baseline and month 1

Urinary volume (ml)	Mean	N	SD	SE
Baseline	1941.3	14	695	185.7
Month 1	2157.1	14	1102.7	294.7

SE: Standard error, SD: Standard deviation

Table 4. 16: Summary of paired sample t-test analysis

	The difference in means between month 1 and baseline	SD	Lower 95%CI	Upper 95% CI	Sample size	P value
Urinary Volume (ml)	215.9	996.2	-329.3	791.1	14	0.432

SD: Standard deviation, CI: Confidence interval

Mixed model analysis was used to assess the impact of time on changes in urinary volume. There were 193 urinary collections that were included. There was a significant effect of time on urinary volume, with p=0.021, intercept of 2233 ml and monthly increase of 38 ml, enabling to have an equation to predict changes of urinary volume over time as y=2233+38*time (increase of 456 ml in 12 months). This is summarised in Table 4.17.

Table 4. 17: Estimates of Fixed Effect of time, where dependent variable is urinary volume (recorded in ml, rounded to the nearest ml)

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	2233	203	<0.001	1802	2625
Month	38	17	0.021	5	72

SE: Standard error, CI: Confidence interval

In order to find out whether the amount of urinary volume correlates with glycosuria, Pearson correlation was undertaken. We found that there was no significant correlation between overall urinary volume and urine glucose concertation, suggesting that the osmotic effect of urinary glucose does not fully explain the increased amount of urinary volume over time. The statistics is summarised in Table 4.18.

Table 4. 18: Pearson Correlation between urinary volume and urinary glucose (both per litre and per 24 hours) during the 12 months

Variable	Statistics	Urinary volume	Urinary glucose/24h	Urinary glucose/l
Urinary volume	Pearson Correlation	1	0.512	0.547
	Sig. (2-tailed)		0.089	0.066
Urinary glucose/24h	Pearson Correlation	0.512	1	.966**
	Sig. (2-tailed)	0.089		0
Urinary glucose/l	Pearson Correlation	0.547	.966**	1
	Sig. (2-tailed)	0.066	0	
** Correlation is significant at the 0.01 level (2-tailed).				

4.5 Changes in cardiovascular parameters

Blood pressure and heart rate were measured at baseline and at month 12 follow-up, during both 24-hour studies. There were 8 measurements of blood pressure and heart rate, at these times: 8:30 AM, 10:30 AM, 1:30 PM, 4:00 PM, 5:00 PM, 7:30 PM, 10:15 PM and 8:15 AM following day. Mean blood pressure and heart rate reading was taken for both baseline and follow-up HMRU visits. However, in subjects who did not undergo 24-hour HMRU study only one reading was available. Data were missing for some of the measures in 5 participants (as a result of participants self-monitoring and not recording some of the values, or only recording some of the values). Table 4.19 provides mean readings for blood pressure, heart rate at baseline and follow-up.

Table 4. 19: Mean cardiovascular parameters at baseline and follow-up

Participant	Systolic BP baseline (mmHg)	Systolic BP follow up (mmHg)	Diastolic BP baseline (mmHg)	Diastolic BP follow up (mmHg)	Heart rate baseline	Heart rate follow up	Initial Cardiovascular risk score (%)
1	122	133	74	97	94	87	2.9
2	137	137	93	90	91	94	9.3
3	116	112	72	74	81	85	10.2
4	154	146	75	70	75	72	17.7
5	164	118	97	62	69	70	14.7
6	127	113	78	74	83	83	3.5
7	124		81		85		35.4
8	153	158	88	87	88	94	18
9	158	142	94	85	61	68	12.4
10	157	173	92		55	56	18.8
11	137	141	87	85	77	72	18.4
12	138	137	85	88	86		16.8
13	141	138	86	85	78	78	2.7
14	122	122	71	69	61	56	10.9
15		136		62		91	
16	129	124	78	83	93	77	3.6
17	135	150	74	88	69	70	17.9
18	129		78		67		16.2
Mean	137.8	136.3	82.5	79.9	77.2	76.9	13.5
SD	14.6	16.3	8.4	10.7	12.0	12.2	8.2

SD: Standard deviation

Paired student t-test was used to compare mean readings at baseline and follow-up. There was no significant difference in systolic blood pressure (mean difference -3.1 mmHg, SD 15.1, p=0.444) diastolic blood pressure (mean difference -1.1 mmHg, SD 12.7, p=0.758) and heart rate (mean difference -0.9 BPM, SD 6, p=0.571) between baseline and one year follow up study. Table 4.20 summarises these findings.

Table 4. 20: Changes in cardiovascular parameters between follow-up and baseline, based on paired student t test

Cardiovascular parameter	The difference in means between month 12 and baseline	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
Systolic Blood Pressure (mmHg)	-3.1	15.1	-11.4	5.3	15	0.444
Diastolic Blood Pressure (mmHg)	-1.1	12.7	-8.4	6.3	14	0.758
Heart rate (BPM)	-0.9	6.0	-4.4	2.5	14	0.571

SD: Standard deviation, CI: Confidence interval

4.6 Chapter summary

In summary, there was a significant increase in glycosuria at month 1 following initiation of therapy with SGLT2 inhibitors. Thereafter, there were no changes in glycosuria but the level of glycosuria was maintained. There were no changes in natriuresis following initiation of therapy with SGLT2 inhibitors. Similarly, there was no difference in proteinuria following SGLT2 inhibitor initiation, however, there was a trend for reduction which did not reach significance. The amount of urinary volume was significantly increased during the 12 months of therapy with SGLT2 inhibitors. Finally, there were no difference between heart rate, systolic and diastolic blood pressure at baseline and month 12.

Chapter 5: Metabolic markers

Chapter 5: Metabolic markers

In this chapter I will describe changes in metabolic parameters obtained for routine clinical care at baseline and after 12 months of Dapagliflozin therapy. The effect of haemolysis, lipaemia and icterus on the analyses of blood samples will be described, as well as Dapagliflozin induced changes in insulin, glucagon, adiponectin, leptin, glucose and ketones by comparing levels at baseline, month 1,2,3,6 and 12.

5.1 Baseline and follow-up metabolic parameters for routine clinical care

Fasting blood samples for metabolic parameters (HbA1c, Total cholesterol, Triglycerides, Alt, TSH, Creatinine, Urea and Testosterone in men) were taken at the beginning (prior to starting Dapagliflozin, month 0) and at follow-up at month 12, and immediately analysed at hospital laboratory (as part of a normal clinical care). Some of the samples taken at month 12 haemolysed and as a result values were not available for month 12. For the participants whose blood samples haemolysed at month 12, blood test results from month 10 were used, if available. If results from month 10 were not available, no data were entered (this affected results in two participants).

Table 5.1 provides information about individual participant's metabolic parameters.

Table 5. 1: Metabolic parameters for individual participants

Participant	Baseline HbA1c (mmol/mol)	F/U HbA1c (mmol/mol)	Baseline total cholesterol (mmol/L)	F/U total cholesterol (mmol/L)	Baseline triglycerides (mmol/L)	F/U triglycerides (mmol/L)	Baseline Alt (IU/L)	F/U Alt (IU/L)	Baseline TSH (mU/L)	F/U TSH (mU/L)	Baseline Creatinine (µmol/L)	F/U Creatinine(µmol/L)	Baseline Urea (mmol/L)	F/U Urea (mmol/L)
1	64	70	3.2	4.5	1	0.9	19	14	2.3	2	94	74	8.1	6.3
2	48	47	5.1	4.3	1.7	2.3	34	19		1.9	81	89	5.1	5.6
3	94	115	7.3	7.7	11.1	5.1	19	22	3.2		59	67	5	7
4	47	42	4.5	4.7	1.8	2	11	16	2.7	2.9	56	54	6.7	5.3
5	49	47	3.8	3.4	0.9	0.8	26	28	2.5	2	106	112	7	6.6
6	46	46	3.8	4.3	1.5	2	53	55	2	1.8	48	50	4.1	4.8
7	39	39	4.1	4.3	2.9	2.8	16	13	3.3		79	62	5.4	6.5
8	66	44	4.3	2.6	4.3	1.4	26	25	1.9	1.3	67	64	3.5	3.7
9	42	39	4.1	4.4	3.9	4.4	33	32	0.5	0.6	73	78	5	5.6
10	45	46	4	4.2	1.2	1.2		24	1.5	2.8	71	72	8.4	4.8
11	47	47	5.2	5.1	0.8	0.6	40	32	2.1	1.9	74	75	4.6	5.5
12	61	44	3.3	3.5	0.8	1	38	29	3.6	3.5	65	72	5.1	4
13	70	74	5.8	4.7	7	4	21	30	1.5	1	60	54	3.5	2.8
14	57		4.9	4.4	2.8	1.9	19	14	1.5	2.5	82	68	5.4	6.4
15	43	47	4.8	4.7	1	2.2	19	27	0.5	0.7	72	82	4.7	7.3
16	60	54	4.6		2.1		18		1.9	1.9	50	48	3.4	4.3
17	56	58	3.7	3	4.1	3	62	90	2.1	1.7	105	83	11.3	6.9
18	40	36	2.5	2.7	1.1	0.7	21	15	2	1.8	64	63	2.5	4.6

F/U: follow up

Paired student t-test was used to compare metabolic parameters at baseline and follow-up. There was no statistically significant difference between any of the readings. Table 5.2 provides mean at baseline and one year, as well as summary of the results from paired student t-test.

Table 5. 2: Paired student t-test comparing metabolic parameters

Metabolic parameter	Mean baseline (SD)	Mean one year (SD)	The difference in means between one year and baseline (SD)	Lower 95% CI	Upper 95% CI	Sample size	P value
HbA1c (mmol/mol)	53.9 (14.1)	52.7 (19.0)	-1.3 (9.2)	-6.0	3.4	17	0.569
Total cholesterol (mmol/L)	4.4 (1.1)	4.3 (1.2)	-0.1 (0.7)	-0.5	0.2	17	0.52
Triglycerides (mmol/L)	2.8 (2.7)	2.1 (1.3)	-0.7 (1.8)	-1.6	0.2	17	0.132
Alt (IU/L)	28.6 (14.0)	28.8 (19.4)	0.3 (9.8)	5	-5.5	16	0.92
TSH (mU/L)	1.9 (0.8)	1.9 (0.8)	0 (0.50	0.3	-0.3	15	0.943
Creatinine (µmol/L)	72.6 (16.7)	70.4 (15.5)	-2.2 (9.9)	-7.1	2.8	18	0.368
Urea (mmol/L)	5.5 (2.1)	5.4 (1.3)	0 (1.9)	-1.0	0.9	18	0.921

SD: Standard deviation, CI: Confidence interval

All blood tests measuring hormones were analysed using non-transformed and log transformed data. Analyses from log-transformed data are shown in appendix if the results are the same as from non-transformed data.

5.2 Sample analysis for haemolysis, lipaemia and icterus

The degree of haemolysis, lipaemic and icterus in a blood sample affects analysis of various metabolic parameters. This degree of haemolysis, icterus and lipaemia was therefore tested on all stored blood samples that were subsequently analysed for metabolic parameters. The limits of haemolysis, icterus and lipaemia for each of the analytes are provided in the table 5.3 below. The indices do not have any units.

Table 5. 3: Haemolysis, icterus and lipaemia indices

(Roche, 2007)

Analyte	Haemolysis	Icterus	Lipaemia
Glucose	1000	60	1000
D-3-Hydroxybutyrate	1000	N/A	N/A
Insulin	1000	N/A	N/A
Glucagon	500	N/A	N/A
Leptin	NA	N/A	1000
Adiponectin	N/A	N/A	N/A

N/A: not affected

If analysis of a blood sample shows that haemolysis, icterus or lipaemia parameters are above the cut off for any of the above-mentioned analytes, the result is likely inaccurate and cannot be used in the analysis.

All blood samples were analysed for the degree of haemolysis, icterus and lipaemia and the indices are provided in the tables below. The indices do not have units and relate to the colour and opaqueness of the sample.

The main findings were that a sample from participant 16 at month 12 was haemolysed to a level that it affected glucagon interpretation and sample from participant 3 at month 2 was lipaemic to a level that it affected glucose interpretation. As a result, glucose results for participant 3 month 2 and glucagon results for participant 16 month 12 could not be used for analyses.

Table 5. 4: Haemolysis indices

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	5	13	18	2	0	4
2	26	16	12	12	10	8
3	0	0	33	0	37	9
4	38	2	3	5	21	3
5	15	10	12	16	27	9
6	8	6	13	5	10	9
7	0	15	24	30	37	
8	4	13	0	39	16	27
9	24	35	26	65	13	8
10	251	44	15	17	93	6
11	9	28	5	20	34	315
12	10	14	18	37	18	10
13	207	6	9	6	11	9
14	11	14	9	11	19	395
15	7	5	7	8		10
16	1	7	7	13	10	**643**
17	8	28	13	19	24	91
18	7	8	7	7	8	11

Sample that has haemolysed to the extent that prevented glucagon analysis is highlighted in bold

Table 5. 5: Icterus indices

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	0	0	0	0	0	0
2	1	1	1	1	1	1
3	0	0	0	0	0	1
4	0	0	0	0	0	0
5	0	0	0	0	1	1
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	1	1	2	2	1
10	0	0	0	0	1	1
11	0	1	1	1	1	1
12	0	1	0	0	0	0
13	1	1	1	1	1	1
14	1	0	0	1	1	0
15	1	1	1	1	1	0
16	1	1	1	1	1	1
17	1	1	1	1	1	1
18	1	1	1	1	1	1

Table 5. 6: Lipaemia indices

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	47	47	62	57	49	31
2	32	72	60	40	31	29
3	472	559	**1279**	382	36	42
4	42	100	82	143	52	31
5	39	40	57	86	40	30
6	34	66	70	102	74	26
7	176	51	23	52	96	
8	240	334	394	59	41	17
9	168	137	84	36	29	20
10	28	59	29	23	17	14
11	28	24	12	11	24	19
12	26	23	51	26	41	22
13	102	33	47	24	69	42
14	38	107	70	37	21	28
15	29	32	22	113	33	117
16	24	20	11	20	9	26
17	51	24	37	19	49	61
18	17	14	12	7	17	11

Sample that has been lipaemic to the extent that prevented glucose analysis has been highlighted in bold.

5.3 Changes in insulin, glucagon, adiponectin and leptin levels

5.3.1 Insulin

Participants had insulin levels measured from a fasting blood test taken at month 0,1,2,3,6 and 12. The range of insulin detectable with our assay was 15.6 to 500 pmol/L. Two of the participants (participant 6 at month 6, participant 9 at month 1) had insulin level that exceeded the maximum detectable amount on the assay (above 500 pmol/L) and therefore the maximum value was entered for the purpose of the statistical analyses.

Similarly, some of the participants (participant 11 at month 12 and participant 12 at all of the time points) had insulin levels that were below the minimum detectable amount (less than 15.6 pmol/L) and therefore the minimum detectable value was used for statistical analyses. One participant (participant 7) was not fasted at month 1 and therefore the insulin value was not used in analysis. Additionally, participant 7 failed to attend month 12 appointment and therefore blood test is missing.

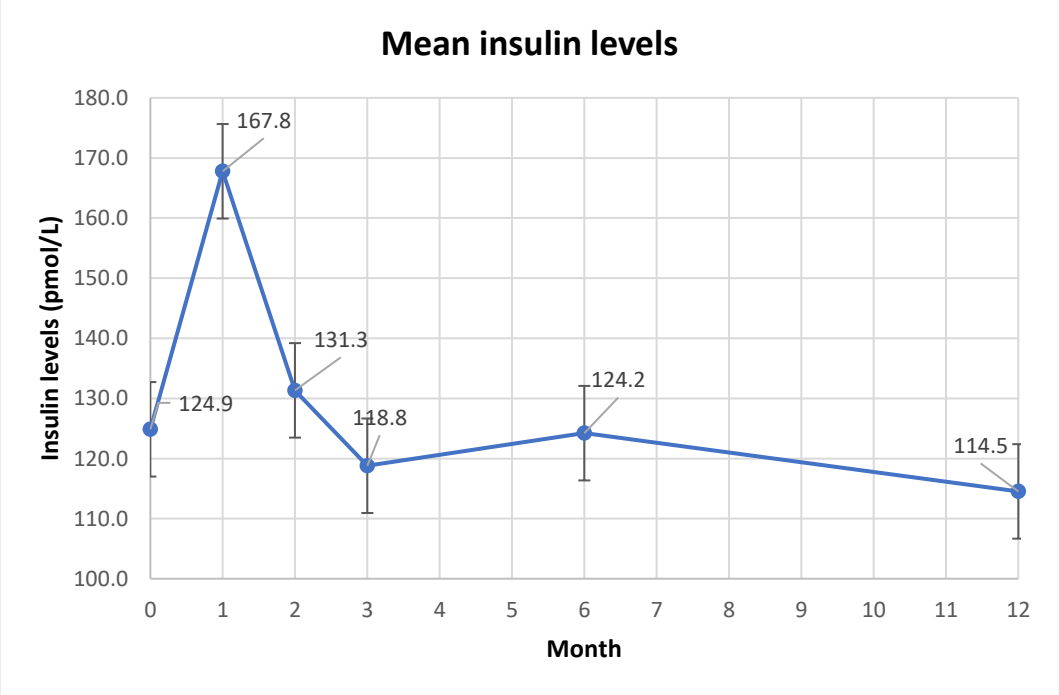
The mean insulin level was 124.9 (SD 69) pmol/L at baseline and 114.5 (86.7) pmol/L at month 12. Table 5.7 shows the insulin values for each participant (with those who were above/below detectable value highlighted) and Figure 5.1 provides graphical representation of the mean insulin levels over time.

Table 5. 7: Participants' insulin levels over time

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	98.2	151.3	136.7	233.9	120.7	127.8
2	121.4	234.5	83.1	122.8	138.5	335.3
3	168.9	97.7	120.0	103.1	84.0	65.5
4	166.9	134.8	140.8	144.0	124.2	152.6
5	93.0	185.3	246.7	120.2	90.6	78.2
6	255.9	254.3	303.7	226.2	**500.0**	271.8
7	66.4		61.5	53.3	48.3	
8	76.1	133.5	87.8	73.1	98.2	40.9
9	263.3	**500.0**	206.1	149.9	137.3	197.0
10	94.6	250.2	114.5	69.3	55.2	58.6
11	213.2	109.0	141.3	131.3	116.0	**15.6**
12	**15.6**	**15.6**	**15.6**	**15.6**	**15.6**	**15.6**
13	53.1	122.9	119.7	89.0	71.5	108.1
14	138.2	168.6	120.7	135.2	138.5	101.8
15	55.9	95.7	121.6	114.7	100.9	138.8
16	137.4	114.2	94.6	119.2	120.4	50.6
17	147.8	132.5	172.7	165.3	221.1	115.8
18	81.5	152.2	76.9	72.5	54.9	73.0

The insulin values that have been below or above the detection range are highlighted in bold

Figure 5. 1: Mean insulin levels over time (bars represent standard errors)



There was a sharp increase in the mean insulin levels at month 1. Paired student t-test was used to compare insulin means at baseline and month 1. There was no statistically significant rise between month 1 and baseline, with difference of 39.5 pmol/L, p=0.069. The paired student t-test is shown in Table 5.8

Table 5. 8: Paired student t-test comparing insulin levels at month 1 and baseline

Metabolic parameter	The difference in means between month 1 and baseline	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
Insulin (pmol/L)	39.5	83.4	-3.4	82.4	17	0.069

SD: Standard deviation, CI: Confidence interval

However, when data was log-transformed and re-analysed using paired t-test, the difference became statistically significant. The mean log insulin at month 1 as 4.93, and the mean log insulin at baseline was 4.68, which when transformed back is equal to a mean insulin at month 1 139.5 pmol/L and at baseline 107.5 pmol/L, giving an increase of 32 pmol/L, p=0.045. This is shown in Table 5.9. When using t-test on log transformed data, the mean difference between the parameters cannot be obtained by direct reversal of the log value (mean(A) does not equal to exp(mean(log(A)). Instead, means of both of the log transformed values can be reversed back (using EXP function) and the difference between them is the actual mean.

Table 5. 9: Paired student t-test comparing log-transformed insulin levels at month 1 and baseline

Metabolic parameter	The difference in means between month 1 and baseline	SD	Lower 95% CI	Upper 95% CI	Sample size	P value
Insulin (pmol/L)	0.26	0.49	0.01	0.51	17	0.045

SE: Standard error, CI: Confidence interval

Mixed effect model (using non transformed data) showed that there was a trend for reduction in insulin over 12 months with an intercept at 138.3 (SE

16.3) pmol/L and gradient (monthly changes) of -2.4 (SE 1.4) pmol/L, however, this did not reach statistical significance (p=0.097). Table 5.10 provides summary of the results of fixed effect model analysis on insulin levels. Intercept refers to the mean baseline level of insulin. This is used in all the tables in the chapter referring to mixed model analysis. P value is provided for both the intercept (the mean baseline level) and the monthly changes and indicates whether the starting level and monthly changes are significant.

Table 5. 10: Estimates of Fixed Effects, where dependent variable is insulin level

Parameter	**Estimate**	**SE**	**P value**	**95% Confidence Interval**	
				Lower Bound	Upper Bound
Intercept	138.3	16.3	<0.001	104.5	172.1
Monthly changes	-2.4	1.4	0.097	-5.2	0.4

SE: Standard error, CI: Confidence interval

However, when mixed effect model was applied to log-transformed data, the reduction in insulin over time reached statistical significance. At month 12, the insulin level was calculated to be 83 pmol/L (EXP 4.8* EXP (12*-0.03)), p=0.002. This is summarised in Table 5.11.

Table 5. 11: Estimates of Fixed Effects, where dependent variable is log-transformed insulin level

Parameter	**Estimate**	**SE**	**P value**	**95% Confidence Interval**	
				Lower Bound	Upper Bound
Intercept	4.77	0.15	<0.001	4.46	5.08
Monthly changes	-0.03	0.01	0.002	-0.05	-0.01

SE: Standard error, CI: Confidence interval

5.3.2 Glucagon

Participants had glucagon levels measured from a fasting blood test taken at month 0,1,2,3,6 and 12. The range of glucagon detectable with our assay was 1.54 to 124 pmol/L. Some of the participants (participant 2 at month 0 and month 1, participant 3 at month 1 and month 3, participant 4 at month 0,1,2 and 3, participant 6 at month 0,1,2, and 3, participant 15 at month 2 and participant 16 at month 12) had glucagon levels that were below the minimum detectable amount (less than 1.5 pmol/L) and therefore the minimum detectable value was used for statistical analyses and graphical representation.

Additionally, participant 13 had a very high glucagon level (42.2 pmol/L) at month 2. This was 10 times higher than other values of this participant and therefore was considered an outlier and was not included in the graph and statistical representation. Participant 7 was not fasted at month 1 and also did not attend for month 12, and therefore these values are missing. Additionally, blood test for participant 16 month 12 was grossly haemolysed and could not be accurately measured, and therefore was also removed.

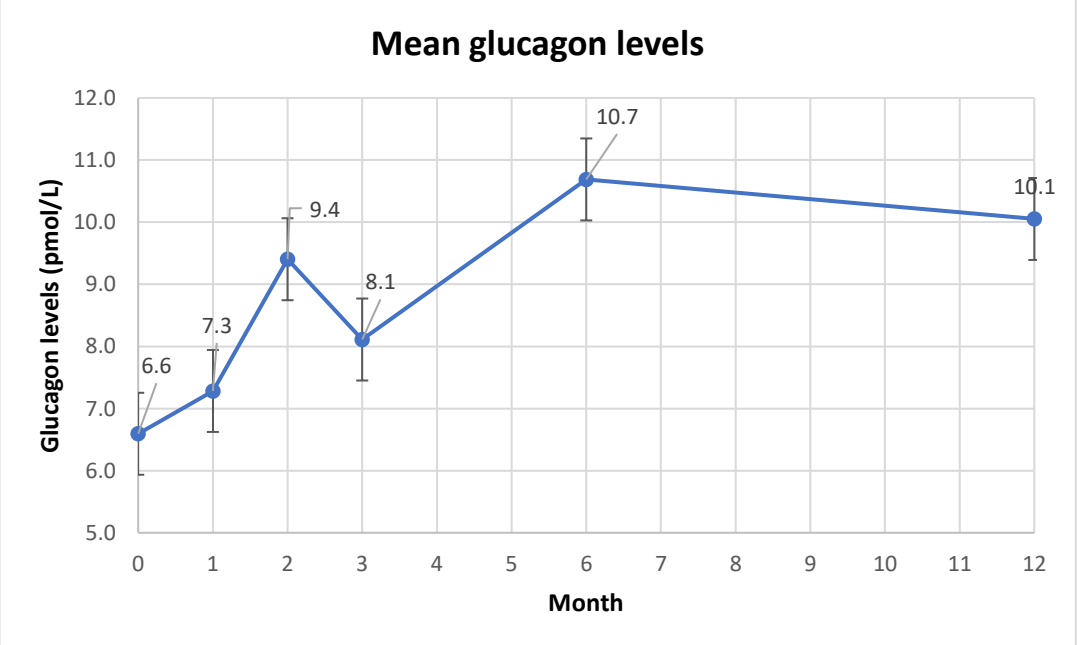
The mean glucagon level was 6.6 (SD 5.7) pmol/L at baseline and 10.6 (7.6) pmol/L at month 12. Table 5.12 shows the glucagon values for each participant (with those who were below detectable value and the outlier value highlighted) and Figure 5.2 provides graphical representation of the mean glucagon levels over time.

Table 5. 12:Participants' glucagon levels over time

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	1.8	8.5	5.3	8.4	10.2	10.2
2	**1.5**	**1.5**	4.0	7.6	31.6	33.3
3	1.6	**1.5**	2.0	**1.5**	3.1	4.3
4	**1.5**	**1.5**	**1.5**	**1.5**	2.2	1.9
5	4.1	5.1	5.6	11.4	9.6	9.3
6	**1.5**	**1.5**	**1.5**	**1.5**	4.2	5.0
7	6.8		9.8	7.6	5.2	
8	7.4	10.4	8.7	19.9	20.1	13.9
9	9.9	13.3	18.0	12.1	18.9	14.3
10	4.4	5.9	5.1	4.5	3.9	3.2
11	23.1	12.8	12.3	7.3	10.5	9.2
12	13.9	17.4	15.7	14.8	27.5	14.8
13	3.7	5.8	**42.2**	9.3	7.8	17.3
14	13.5	14.2	11.8	7.2	9.8	9.0
15	2.5	6.9	**1.5**	18.8	3.9	11.9
16	8.9	10.3	11.6	6.0	12.6	
17	8.5	3.7	6.9	3.3	8.4	6.0
18	4.1	5.3	5.6	3.3	3.1	5.9

The glucagon level that is below the detection range, as well as the outlier value, are highlighted in bold

Figure 5. 2: Mean glucagon levels over time (bars represent standard errors)



Mixed effect model showed that there was a statistically significant increase in glucagon over time with an intercept at 7 (SE 1.2) pmol/L and gradient of 0.4 (SE 0.1) pmol/L, p=0.003, resulting in calculated glucagon of 11.8 pmol/L at month 12. Table 5.13 provides summary of the results of fixed effect model analysis on glucagon levels.

Table 5. 13:Estimates of Fixed Effects, where dependent variable is glucagon level

Parameter	**Estimate**	**SE**	**P value**	**95% Confidence Interval**	
				Lower Bound	Upper Bound
Intercept	7.0	1.2	<0.001	4.6	9.5
Monthly changes	0.4	0.1	0.003	0.1	0.6

SE: Standard error, CI: Confidence interval

Statistical analyses from log-transformed data provided the same results and are provided in the appendix.

5.3.3 Adiponectin

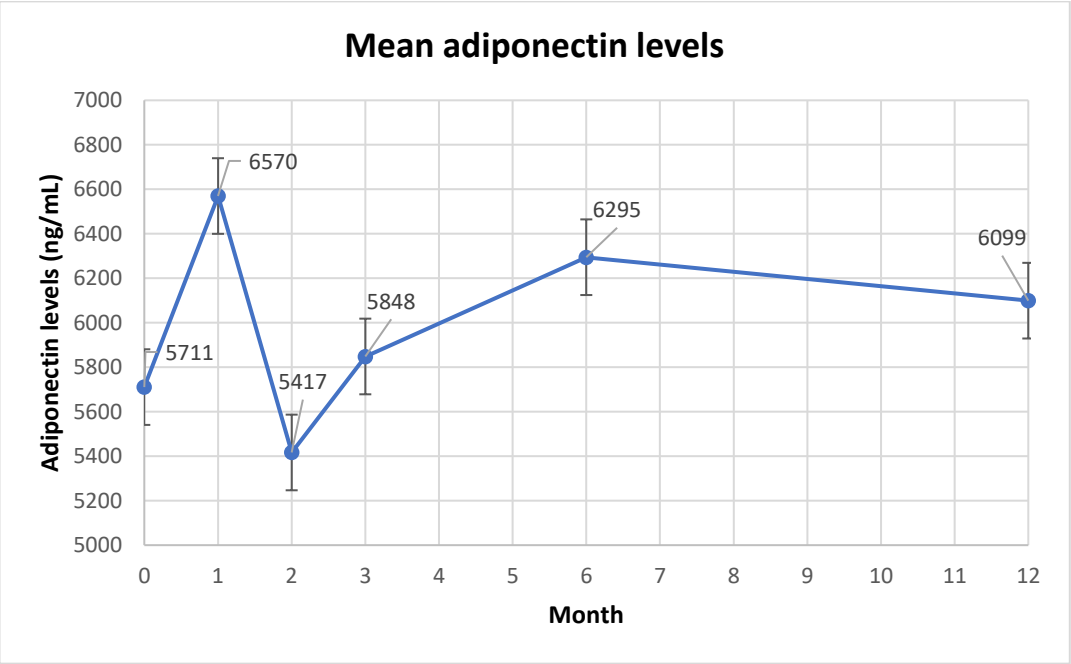
Participants had adiponectin levels measured from a fasting blood test taken at month 0,1,2,3,6 and 12. The range of adiponectin detectable with our assay was 3.9 to 250 ng/mL (this was range of diluted samples). Participant samples were diluted 1:100 as recommended by manufacturers. Results produced were then multiplied by 100 to deduce the original sample concentration.

As adiponectin levels are not affected by fasting status, blood result from non-fasted participant 7 at month 1 was included. The mean adiponectin level was 5711 (SD 4275) ng/mL at baseline and 6099 (3653) ng/mL at month 12. Table 5.14 shows the adiponectin values for each participant and Figure 5.3 provides graphical representation of the mean adiponectin levels over time.

Table 5. 14:Participants' adiponectin levels over time

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	6537	14442	8597	5491	10962	8576
2	4023	4096	4184	6659	5245	5357
3	2260	3657	2244	2787	2923	3826
4	4034	4450	4425	3778	3825	3663
5	4356	5887	3163	5044	4015	3712
6	3558	4904	2953	3952	2256	2718
7	2519	2801	3895	3654	4999	
8	1340	1181	1338	1606	2180	3335
9	3305	5055	3115	3998	3668	4152
10	6801	7859	7571	6795	12805	7454
11	6261	6578	4738	5375	7315	5163
12	3088	3733	3497	3052	3372	3469
13	6863	6576	3076	4895	5273	4918
14	9446	10808	6576	11616	6337	6947
15	18288	17254	20515	18401	18411	14048
16	13098	10764	10564	10244	11792	13876
17	1973	2039	2015	2963	2900	2254
18	5043	6171	5037	4960	5025	10219

Figure 5. 3: Mean adiponectin levels over time (bars represent standard errors)



Mixed effect model showed that there was no statistically significant change in adiponectin over time with an intercept at 5923 (SE 944) ng/mL and gradient of 11 (SE 37) ng/mL, p=0. 761.Table 5.15 provides summary of the results of fixed effect model analysis on adiponectin levels.

Table 5. 15: Estimates of Fixed Effects, where dependent variable is adiponectin level

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	5923	944	<0.001	3939	7907
Monthly changes	11	37	0.761	-62	85

SE: Standard error, CI: Confidence interval

Statistical analyses from log-transformed data provided the same results and are provided in the appendix.

5.3.4 Leptin

Participants had leptin levels measured from a fasting blood test taken at month 0,1,2,3,6 and 12. Participant 7 was not fasted at month 1 and therefore this result was not included. The range of leptin detectable with the assay was 15.6-1000 pg/mL (this was range of diluted samples). Participant samples were diluted 1:100 as recommended by manufacturers. Results produced were then multiplied by 100 to deduce the original sample concentration.

Some of the participants (participant 1 at all measured time points, participant 6 at month 1 and participant 11 at month 0) had leptin values that exceeded the maximum detectable amount on the assay (above 1000 pg/mL in the diluted form) and therefore the maximum value was entered for the purpose of the calculation. These values are highlighted in the table below.

Additionally, sample from participant 3 at month 2 was grossly lipaemic and it is known that grossly lipaemic samples may show lower results. However, the degree of reduction is not known and the sample was therefore still included in the final analysis. Mean leptin levels at baseline (month 0) was 51713 (SD 32021) pg/mL and 42880 (SD 26005) pg/mL at month 12. Table 5.16 shows individual leptin values at the specified time points.

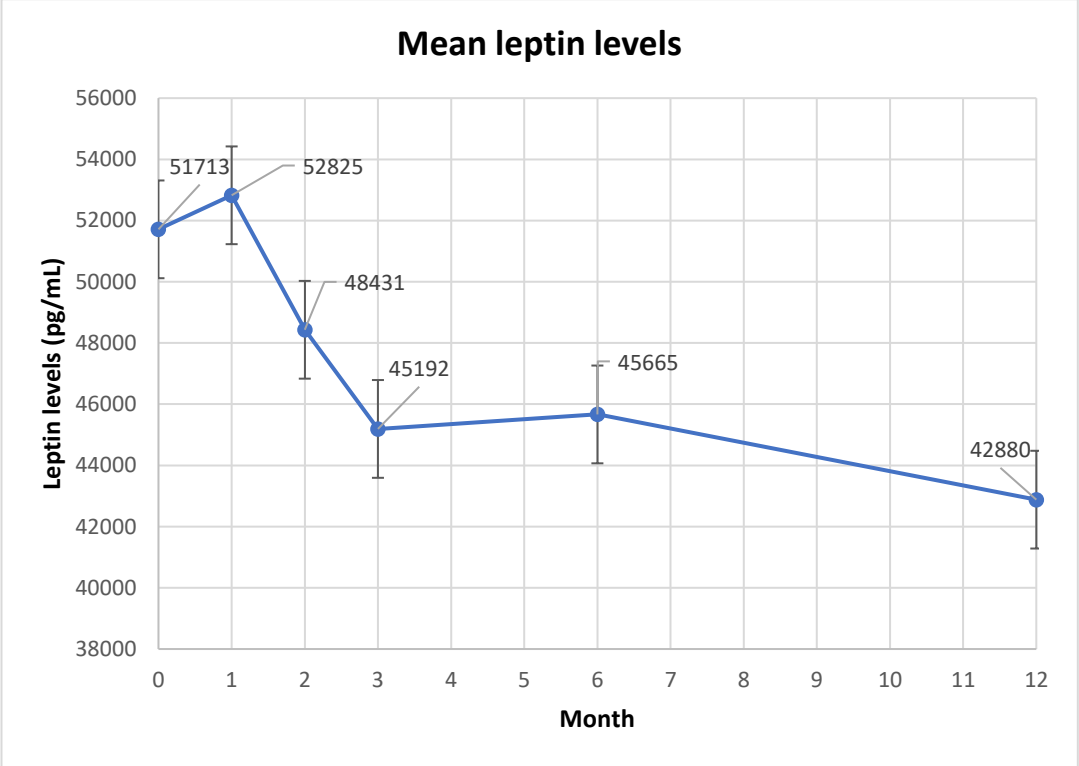
Table 5. 16: Leptin levels at prespecified time points

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	**100000**	**100000**	**100000**	**100000**	**100000**	**100000**
2	22168	23006	21448	20182	30188	18971
3	16033	20907	18933	12970	11101	20143
4	89848	72332	82990	70799	67817	80147
5	13575	18528	17573	16186	18035	17756
6	80726	**100000**	64128	68339	67175	63929
7	10536		14492	10579	11581	
8	38149	46353	39330	44787	32814	16330
9	34661	23483	29631	28427	21396	21329
10	62681	59707	46061	40749	61075	50150
11	**100000**	90700	99743	65898	86093	77824
12	55794	51773	37374	32705	40421	29336
13	34541	44545	41993	48413	39780	39850
14	85763	76875	72496	68252	51833	36114
15	40089	55372	77589	64149	82883	54038
16	92931	74133	70975	78488	62733	59908
17	29915	16861	19937	23542	22063	20663
18	23424	23455	17069	18983	14985	22480

The leptin levels that were above the detection range are highlighted in bold

Graphical representation of the changes to the mean leptin levels over time are shown in Figure 5.4.

Figure 5. 4: Changes to the mean leptin levels over time (bars represent standard errors)



Mixed effect model showed that there was a reduction in leptin levels over time, with an intercept at 50482 (SE 6571) pg/mL and gradient of -852 (SE 215) pg/mL, p<0.001, resulting in calculated leptin level of 40258 pg/mL at month 12. Table 5.17 provides summary of the results of fixed effect model analysis on leptin levels.

Table 5. 17: Estimates of Fixed Effects, where dependent variable is leptin level

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	50482	6571	<0.001	36652	64312
Monthly changes	-852	215	<0.001	-1279	-424

SE: Standard error, CI: Confidence interval

Statistical analyses from log-transformed data provided the same results and are provided in the appendix.

Leptin is produced by an adipose tissue. We have shown that there was a significant reduction in leptin over time and given the significant reduction in fat mass (discussed in chapter 3), we have done Pearson correlation to assess whether changes in fat mass were correlated to changes in leptin. Table 5.18 shows that there was a significant correlation between fat mass reduction and reduction in leptin levels.

Table 5. 18: Pearson correlation between fat mass and leptin levels

Statistics	**Leptin**	**Fat mass**
Pearson Correlation	1	.978
Sig. (2-tailed)		0.022

5.4 Changes in glucose and ketone levels

5.4.1 Glucose levels

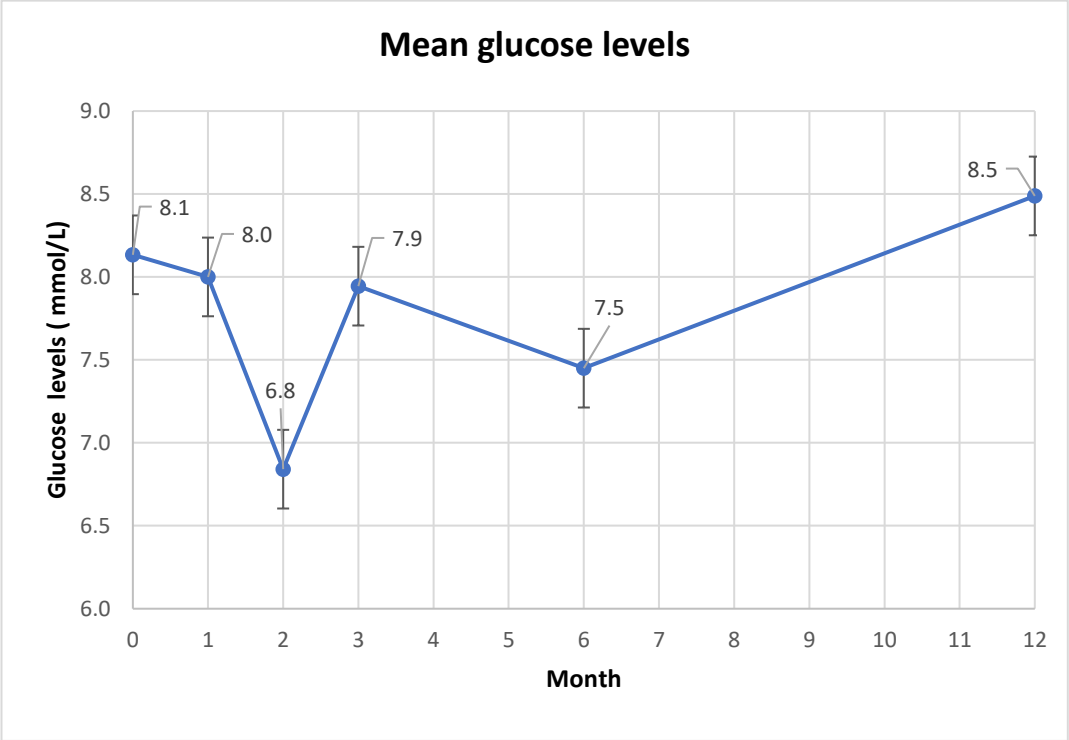
Participants had glucose levels measure from a fasting blood test taken at month 0,1,2,3,6 and 12. The technical limit of the assay (limit of detection) was 0.11-41.6 mmol/L. However, glucose cannot be accurately analysed if the sample is haemolysed (haemolysis limit 1000 mmol/L) or lipaemic (lipaemic limit 1000).

One participant had grossly lipaemic sample at month 2 (1279) and therefore glucose value at that month was excluded as it would be an inaccurate result. One participant was not fasted at month 1 and therefore this result was also excluded. The mean glucose level was 8.1 (SD 3.6) mmol/L at baseline and 8.5 (4.0) mmol/L at month 12. Table 5.19 shows glucose values for each participant and Figure 5.5 provides graphical representation of the mean glucose levels over time.

Table 5. 19: Participants' glucose levels over time

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	10	8.4	8.2	12.1	11.6	19.9
2	6.4	7.3	5.5	5.6	6.5	8.8
3	20.6	11.1		24.3	14	17.2
4	7.4	5.8	6.3	6.5	6.1	6.9
5	5.2	6.8	6.5	5.6	5.6	5.1
6	6.1	6.1	6.1	6.2	7.6	6.8
7	4.3		5	4.8	5.3	
8	9.2	11.9	8	7.7	7.8	6.3
9	6.9	9.7	6	5.9	6.1	6.4
10	6.2	7.2	6.5	5.3	6.5	6.6
11	8.3	7	8.3	8.5	8	7.6
12	7.2	5.9	5.9	5.7	6	6.3
13	10	10.1	8.3	10	9	10.6
14	8.3	8.4	8.5	8	7	6.9
15	5.5	5.9	6.6	5.8	6	6.5
16	8.3	6.7	6.1	6.7	7.5	8.3
17	10.4	10.6	8.5	8.6	8	8
18	6.1	7.1	6	5.7	5.5	6.1

Figure 5. 5: Mean glucose levels over time (bars represent standard errors)



Mixed effect model showed that there was no statistically significant change in glucose over time with an intercept at 7.7 (SE 0.7) mmol/L and gradient of 0.02 (SE 0.04) mmol/L, p=0.531. Table 5.20 provides summary of the results of fixed effect model analysis on glucose levels.

Table 5. 20:Estimates of Fixed Effects, where dependent variable is glucose level

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	7.7	0.7	<0.001	6.3	9.2
Monthly changes	0.02	0.04	0.531	-0.06	0.1

SE: Standard error, CI: Confidence interval

Statistical analyses from log-transformed data provided the same results and are provided in the appendix.

5.4.2 Ketone levels

Participants had ketone (D-3-Hydroxybutyrate) levels was measured from a fasting blood test taken at month 0,1,2,3,6 and 12. The technical limit of the assay was 0.1-3.2 mmol/L. The linearity and variation in the assay are validated from 0.1 mmol/L, so the variation at levels lower than 0.1 mmol/L is likely higher than the 3.5% for the rest of the assay. As all results were obtained within the same analytical run, those below 0.1mmol/L will be subject to the same degree of variation, so assumption can be made that any trends observed are consistent (If the analyses were to be used clinically, it would be worth validating the assay at those lower levels in-house to define the degree of variation expected).

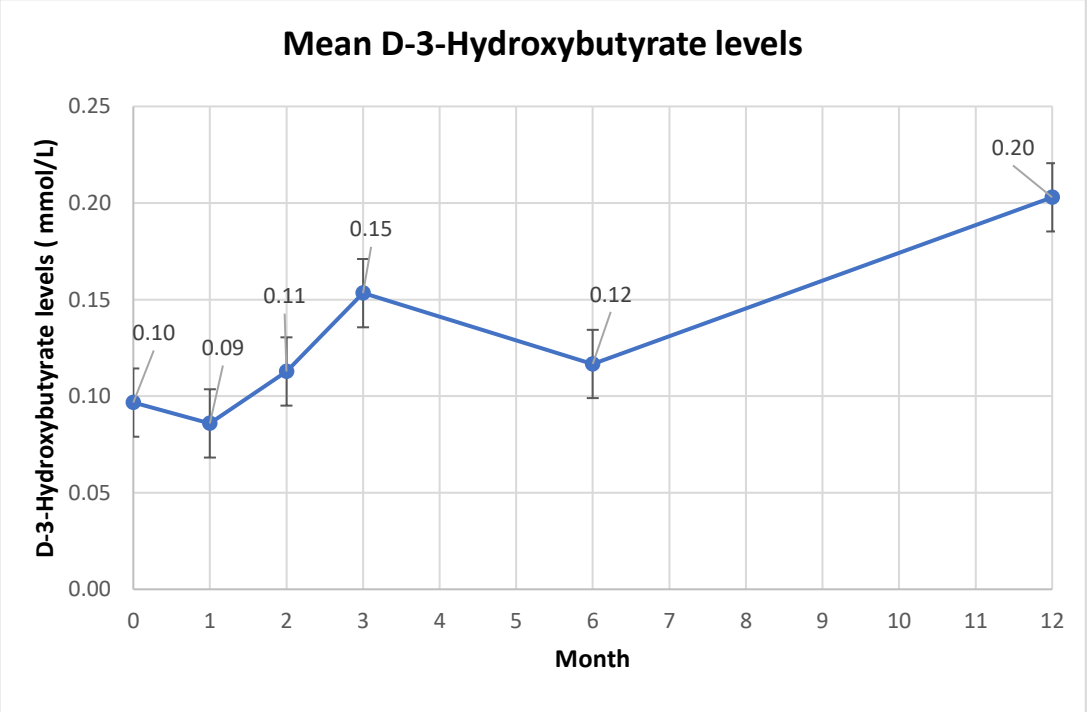
The values from 0.01 mmol/L are reported, with values less than 0.01 mmol/L represented by 0.01 mmol/L, and highlighted in the table. Participant 7 was not fasted at month 1 and therefore that sample was not analysed. The mean D-3-Hydroxybutrate level was 0.1 (SD 0.06) mmol/L at baseline and 0.2 (SD 0.23) mmol/L at 12 months. Table 5.21 shows D-3-Hydroxybutyrate values for each participant and Figure 5.6 provides graphical representation of the mean D-3-Hydroxybutyrate levels over time.

Table 5. 21:Participants' D-3-Hydroxybutyrate levels over time

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	0.12	0.13	0.06	0.07	0.06	0.15
2	0.10	**0.01**	0.11	0.11	0.08	0.05
3	**0.01**	**0.01**	0.27	1.26	0.20	0.48
4	0.07	**0.01**	0.03	0.03	0.10	0.06
5	0.12	0.03	**0.01**	**0.01**	0.05	0.20
6	0.08	0.03	0.08	0.06	0.04	0.13
7	0.13		0.07	0.21	0.34	
8	**0.01**	0.02	**0.01**	0.11	0.08	0.08
9	0.09	0.10	0.04	0.13	0.16	0.11
10	0.16	0.08	0.07	0.05	0.08	0.05
11	0.07	0.07	0.07	0.07	0.06	0.21
12	0.05	0.22	0.10	0.07	0.10	0.06
13	0.11	0.12	0.15	0.10	0.15	0.97
14	0.20	0.07	0.16	0.04	0.10	0.38
15	0.24	0.11	0.03	0.07	0.04	0.15
16	0.06	0.31	0.56	0.12	0.08	0.2
17	0.06	0.10	0.08	0.07	0.08	0.09
18	0.06	0.04	0.13	0.18	0.30	0.08

Ketone values less than 0.01 highlighted in bold

Figure 5. 6: Mean D-3-Hydroxybutyrate levels over time (bars represent standard errors)



Mixed effect model showed that there was statistically significant change in D-3-Hydroxybutyrate over time with an intercept at 0.09 (SE 0.02) mmol/L and gradient of 0.009 (SE 0.003) mmol/L, p=0.025. Table 5.22 provides summary of the results of fixed effect model analysis on D-3-Hydroxybutyrate levels.

Table 5. 22: Estimates of Fixed Effects, where dependent variable is D-3-Hydroxybutyrate level

Parameter	**Estimate**	**SE**	**P value**	**95% Confidence Interval**	
				Lower Bound	Upper Bound
Intercept	0.09	0.02	<0.001	0.04	0.15
Monthly changes	0.009	0.003	0.025	0.001	0.016

SE: Standard error, CI: Confidence interval

5.5 Insulin resistance and beta cell function

Insulin resistance was calculated using HOMA-IR calculator (DTU, 2004). The highest insulin value that could be used for calculation using this formula was 400 pmol/l, the lowest insulin value that could be used for calculation was 20 pmol/l. In two cases insulin values were above 500pmol/l and therefore the value of 400 pmol/l was used for calculation. Similarly, in 7 cases the value was less than 15.6 pmol/l and the value of 20pmol/l was used of calculation.

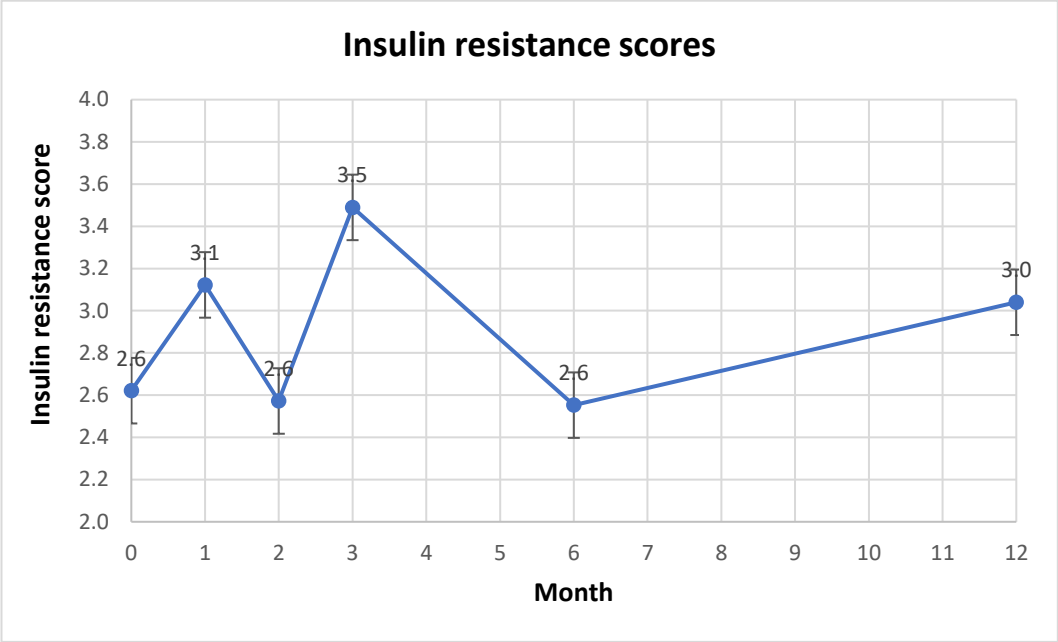
In some participants it was not possible to calculate this index, as one of the input variables (either fasting insulin, or fasting glucose) was missing. The mean insulin resistance index was 2.6 (SD 2) at baseline and 3 (SD 2.9) at month 12. Table 5.23 shows insulin resistance scores for each participant and Figure 5.7 provide graphical representation of the mean insulin resistance scores over time.

Table 5. 23: Participants' insulin resistance scores over time

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	2.1	3.1	2.8	5.1	2.7	5.7
2	2.4	4.6	1.6	2.3	2.7	6.6
3	8.4	2.2		22.7	2.1	2.1
4	3.3	2.6	2.7	2.8	2.4	3
5	1.7	3.6	4.7	2.3	1.7	1.5
6	4.8	4.7	5.6	4.3	**7.5**	5.2
7	1.2		1.2	1	0.9	
8	1.6	3	1.8	1.5	2	0.8
9	5	**7.9**	3.9	2.8	2.6	3.8
10	1.9	4.8	2.2	1.3	1.1	1.2
11	4.3	2.2	2.9	2.7	2.4	**0.4**
12	**0.4**	**0.4**	**0.4**	**0.4**	**0.4**	**0.4**
13	1.2	2.6	2.5	1.9	1.5	2.4
14	2.8	3.4	2.8	2.8	2.7	2
15	1.1	1.9	2.4	2.2	2	2.7
16	2.8	2.3	1.8	2.4	2.4	1.1
17	3.2	2.9	3.5	3.4	4.4	2.4
18	1.6	3	1.5	1.4	1	1.4

The highlighted values show insulin resistance scores were the highest (400 pmol/l) and lowest (20 pmol/l) insulin cut off values were used.

Figure 5. 7: Mean insulin resistance scores over time (bars represent standard errors)



Mixed effect model showed that there was no statistically significant change in insulin resistance over time with an intercept at 3.1 (SE 0.4) and gradient of -0.1 (SE 0.1), p=0.302. Table 5.24 provides summary of the results of fixed effect model analysis on insulin resistance.

Table 5. 24: Estimates of Fixed Effects, where dependent variable is degree of insulin resistance

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	3.1	0.4	<0.001	2.2	3.9
Month	-0.1	0.1	0.302	-0.2	0.1

SE: Standard error, CI: Confidence interval

However, when insulin resistance scores were log-transformed and then re-analysed using mixed effect model, the difference in insulin resistance over time became statistically significant. The calculated insulin resistance score at month 12 was EXP 0.9*EXP (-0.02*12) = 1.8, p=0.014. This is summarised in Table 5.25.

Table 5. 25: Estimates of Fixed Effects, where dependent variable is log-transformed of insulin resistance score

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	0.91	0.15	<0.001	0.60	1.21
Month	-0.03	0.01	0.014	-0.05	-0.01

SE: Standard error, CI: Confidence interval

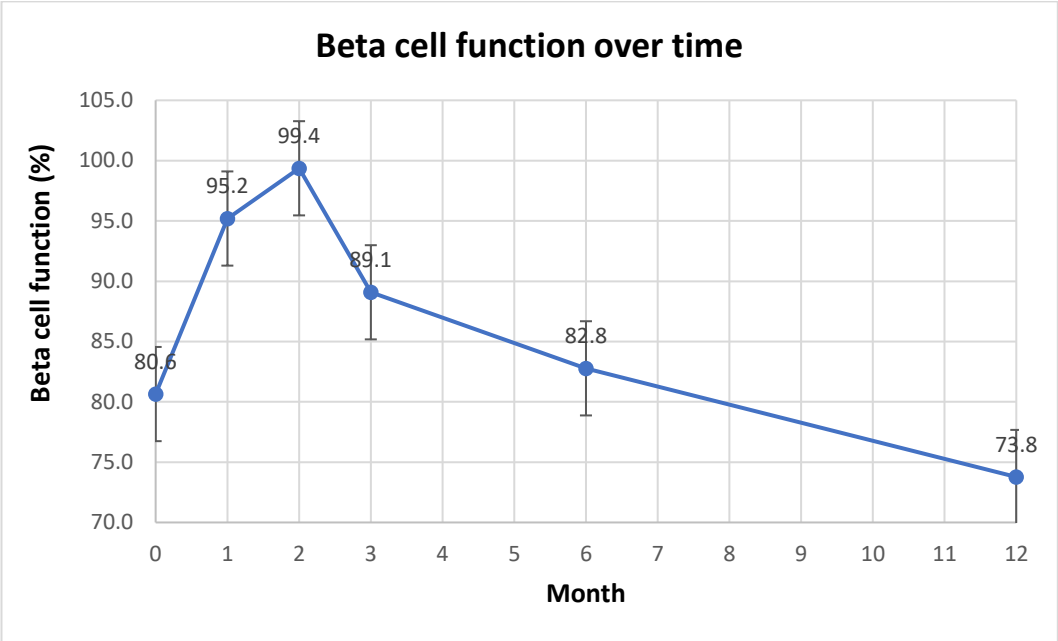
Beta cell function was assessed by HOMA-IR calculator. The mean beta cell function was 80.6% (SD 47.5%) at baseline and 73.8% (SD 47.3%) at month 12. Table 5.26 shows beta cell function scores for each participant and Figure 5.8 provide graphical representation of the mean insulin resistance scores over time.

Table 5. 26: Participants' beta cell function scores over time (%)

Participant	Month 0	Month 1	Month 2	Month 3	Month 6	Month 12
1	39	73.6	71.2	57.6	35.5	17
2	103.3	130.3	106.3	134.2	110.1	125
3	21.6	32.4		10.8	19.4	11.3
4	99.2	134	118.1	113.2	115	105.6
5	128.2	124.7	166.1	132.4	108.9	118.3
6	191.1	190.3	215.7	170.1	**180.9**	164.4
7		148.8	104.6	103	79.1	
8	37.5	37	53.9	50.8	61.3	50
9	156.5	**124.2**	169.1	139	123.3	145.5
10	92.2	140	96.3	101	57.8	58.5
11	97.2	80.8	71.4	64.8	66	**21.1**
12	**23.5**	**35.1**	**35.1**	**37.7**	**34**	**30.8**
13	24.6	45.5	63.2	36.2	37.3	37.9
14	70.2	79.8	60.9	73.8	95.9	79.1
15	81.1	102.2	97.6	119.8	102.7	110.3
16	69.9	90.7	95.1	93.5	76.3	33.9
17	50.1	44.5	79.6	75.4	106.4	65.9
18	85.8	100	85	90.2	80.1	79.5

The highlighted values show beta cell function scores were the highest (400 pmol/l) and lowest (20 pmol/l) insulin cut off values were used.

Figure 5. 8: Mean beta cell function scores over time (bars represent standard errors)



Mixed effect model showed that there was statistically significant reduction in beta cell function over time with an intercept at 91.9 (SE 10.1) % and gradient of -1.3 (SE 0.4) %, p=0.002. Table 5.27 provides summary of the results of fixed effect model analysis on degree of beta cell function.

Table 5. 27: Estimates of Fixed Effects, where dependent variable is degree of beta cell function

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	91.9	10.1	<0.001	70.6	113.1
Month	-1.3	0.4	0.002	-2.2	-0.5

SE: Standard error, CI: Confidence interval

Statistical analyses from log-transformed data provided the same results and are provided in the appendix.

5.6 Chapter summary

In summary, there was no significant difference between routinely measured metabolic parameters (such as HbA1c, TSH or Urea) at baseline and 12 months of Dapagliflozin therapy. There was a statistically significant reduction in insulin levels and a statistically significant increase in glucagon levels during the 12 months of therapy with Dapagliflozin. Adiponectin levels did not change significantly, however, there was a statistically significant reduction in leptin levels. Glucose levels remained stable with no significant changes over 12 months. Ketone levels significantly increased during the 12 months; however, the increase was minimal. There was statistically significant reduction in insulin resistance and a significant reduction in beta cell function over 12 months.

Chapter 6: Discussion

Chapter 6: Discussion

In this chapter I will provide summary of the primary outcome measures, I will discuss how my findings compare to the existing evidence, their limitations and clinical significance.

6.1 Accurate measurements of 24-hour metabolic profile

In our study energy expenditure was measured via indirect calorimetry and has been reported as a 24-hour energy expenditure, post-prandial energy expenditure, overnight energy expenditure, post-exercise energy expenditure and temperature drop induced energy expenditure. Additionally, 24-hour energy expenditure was also estimated based on participant's muscle mass. We observed a numerical reduction (126 kcal) between 24-hour energy expenditure at baseline and at 1-year follow-up. However, this did not reach statistical significance. There was no difference in the estimated energy expenditure between baseline and 1-year follow-up, driven by the fact that there was no difference in muscle mass between baseline and 1-year follow-up. There was no difference in overnight, post-prandial, post-exercise, temperature drop induced energy expenditure between baseline and 1-year therapy with Dapagliflozin. This finding was surprising given the findings from previous meta-analysis that weight loss leads to reduction in total energy expenditure (Astrup et al., 1999) and the fact that the degree of weight loss influences the degree of metabolic slowing, despite the preservation of fat free mass (Johannsen et al., 2012).

Our findings of no changes in energy expenditure following SGLT2 inhibitor therapy are similar to the previously reported study by Ferrannini et al. (2014), who assessed how tissue substrate utilization adapted to glycosuria induced by SGLT2 inhibitors, and whether the glycosuria-induced energy deficit altered resting energy expenditure. This study found that resting and post-prandial energy expenditure was maintained after 4 weeks of therapy with SGLT2 inhibitor, however there was an increase in lipid oxidation, with parallel

reduction in glucose oxidation. The chronic (4 weeks) response to SGLT2 inhibitor therapy was different to the acute (1 dose administration) response, in which lipid oxidation was not altered but non-oxidative glucose disposal was reduced. Ferrannini et al. (2014) measured resting and post-prandial energy expenditure via indirect calorimetry (same method as used in our study), however, the calorimetry was performed only for periods of 30 mins at fixed intervals, and did not measure full 24-hour energy expenditure, and therefore could not provide information about changes in energy expenditure during 24 hours. Nevertheless, the study provided important insight into changes in substrate oxidation and maintenance of resting and post-prandial energy expenditure among participants taking SGLT2 inhibitors for 4 weeks. There have been no other studies up to date investigating the changes in 24-hour energy expenditure in human participants following therapy with SGLT2 inhibitors.

Several studies investigated the impact of SGLT2 inhibitors on energy expenditure in mice. Devenny et al. (2012) assessed changes in energy expenditure in mice following administration of Dapagliflozin (38 days) and did not find any difference in energy expenditure (measured by indirect calorimetry).

Conflicting evidence was provided by Xu et al. (2017) who found that SGLT2 inhibitor Empagliflozin (16 weeks) enhanced energy expenditure in mice (measured by indirect calorimetry). Additionally, this study found that Empagliflozin promoted heat production, enhanced fat utilisation and browning, and reduced insulin resistance. The observed increase in energy expenditure could be explained by increase in the activation of brown adipose tissue as Xu et al. (2017) found that Empagliflozin significantly upregulated mRNA level of brown fat-selective genes.

Chiba et al. (2016) studied changes in energy expenditure following the acute response to Dapagliflozin (18 hours) and found that SGLT2 inhibition acutely supressed oxygen consumption and energy expenditure by reduction in brown adipose tissue generated thermogenesis. Chiba et al. (2016)

suggested that this reduction in energy expenditure may try to compensate for the negative energy balance induced by the calories lost in urine. It is important to highlight that these were findings in mice and similar findings were not replicated in human participants.

Many of the above-mentioned studies investigated brown adipose tissue. Brown adipose tissue is a multilobular, mitochondria rich organ which produces heat through non-shivering thermogenesis (Goldenberg et al., 2013). Brown adipose tissue has been found to be present in humans, and its presence influenced by age, sex and BMI (Pfannenberg et al., 2010, Lee et al., 2010, Ouellet et al., 2011). Brown adipose tissue activity is believed to play an important role in human energy expenditure, and has been reported to be significantly lower in overweight or obese male subjects than in lean male subjects (van Marken Lichtenbelt et al., 2009). Additionally, brown adipose tissue activity was found to be induced by a cold exposure and negatively correlated with change in distal skin temperature (van Marken Lichtenbelt et al., 2009).

In our study, we measured energy expenditure that occurred during temperature drop. Temperature drop was used as a trigger to increase activity of brown adipose tissue. It is important to highlight that we did not measure volume of brown adipose tissue in our participants and therefore temperature drop was used as a hypothetical stimulus, as we could not ascertain presence of brown adipose tissue in our participants. Nevertheless, we did not observe any difference in the energy expenditure during temperature drop at baseline and at 1-year follow-up.

In this study we found that a chronic (1 year) administration of Dapagliflozin did not result in statistically significant reduction in energy expenditure. This is an important finding as it provides evidence that total energy expenditure does not change despite the calorific deficit and weight loss induced by SGLT2 inhibitors. One possible hypothesis for unaltered energy expenditure could be that the reduced energy expenditure following weight loss is compensated by

an increased energy expenditure following activation of brown adipose tissues following SGLT2 inhibition, however, this is very hypothetical at this stage.

An important area of future research remains the effect of SGLT2 inhibitors on the activity of brown adipose tissue in human, as the current evidence exists only from mice studies.

6.2 Changes in body composition

We have found a significant reduction in body weight, which was driven by a statistically significant reduction in fat mass, with no changes in lean mass. The reduction in body weight (8.4 kg at month 12) was bigger than previously reported in other studies, including the large cardiovascular outcome trials. This could be due to the fact that participants in our study were more obese and younger that participants in the larger cardiovascular outcome trials. Additionally, improved diet and increased physical activity could play role in the observed weight loss, as participants had frequent follow-up and 24/7 support available from the research doctor.

In our study weight loss continued throughout the 12 months of therapy with Dapagliflozin and did not reach plateau as previously described by Cai et al. (2018). However, the observed rate of fat mass reduction and weight loss was highest in the first 3 months, and slowed down thereafter. This could fit the hypothesis that energy losses induced by SGLT2 inhibitors will be over time offset by other factors (such as increased appetite) in order to reach a new stable energy balance (Thomas and Cherney, 2018).

We did not observe any statistically significant changes in lean mass, which was similar to previously reported study whereby SGLT2 inhibitor treatment produced a non-significant reduction of 0.4 kg in lean mass (Bolinder et al., 2014). A potential explanation for no change in lean mass could be the fact that participants increased their physical activity.

Assuming 1 gram of glucose equates to 4 kcal of energy and 1 kg of fat equates to 7700 kcal of energy (Sha et al., 2014), we can calculate the estimated fat loss based on the amount of glucose lost in urine in participants taking Dapagliflozin. The mean amount of glucose lost in urine of 18 participants in this study was 305.6 mol per 24 hours, which is 55.1 g per day. This would mean that on average participants lost 220.4 kcal per day in urine. Assuming that the amount of glucose lost in urine was constant and that participants did not increase their calorific intake, the amount of fat loss

experienced per month (based on 28 days in a month) would be 0.8 kg, and 9.6 kg in 12 months. This does not take into account the amount of fluid lost and the likely homeostatic mechanisms that will try to offset the weight loss, but the rough figure of 9.6 kg is close to the 8.4 kg observed in this study.

The acute weight loss induced by SGLT2 inhibition (and resulting glycosuria) has been attributed to the depletion of hepatic glycogen and water loss, with the fat loss (with reduction in visceral and subcutaneous deposits) responsible for the weight loss in long-term (Bolinder et al., 2012). In our study, we observed ongoing increase in urinary volume, so it is possible that the water loss contributed not only to the acute, but also the long-term weight loss.

On a molecular level, shift in substrate utilisation occurs (as described in the previous section), which results in reduced glucose oxidation and enhanced lipolysis with ketogenesis (Ferrannini, 2017). The enhanced lipolysis could explain the significant fat loss observed in people treated with SGLT2 inhibitors. Interestingly, weight loss occurs in patients with impaired renal function, in whom SGLT2 inhibitors have reduced potential for glycosuria due to impaired glomerular filtration rate (Cherney et al., 2018). Thomas and Cherney (2018) hypothesised that weight loss is therefore not dependent purely on the amount of glycosuria, but on the magnitude of glycosuria per nephron.

6.2.1 Factors affecting weight loss

6.2.1.1 Low carbohydrate diet

One of the possible explanations for larger than previously observed weight loss with Dapagliflozin in our study was the combination of Dapagliflozin therapy with a low carbohydrate diet. Low carbohydrate diet is a common dietary intervention popularised by Atkins diet. Atkins diet, originally published in 1973, and then again in 1992 and 2002 has been one of the most popular low carbohydrate diet (RC., 1998).

There is no clear definition for low carbohydrate diet. Some studies define low carbohydrate diet as less than 45% of total energy intake from carbohydrates (Naude et al., 2014), other studies refer to less than 50g of carbohydrate (Accurso et al., 2008) per day. However, as was pointed out by van Wyk et al. (2016) in a critical review, low carbohydrate diets which are strict (less than 50g of carbohydrates per day) are difficult to achieve long term. For this reason Churuangsuk et al. (2020) suggested that low carbohydrate diet can be any diet where carbohydrate intake is between 50 g/d and 130 g/d, or between 10% and 40% of energy from carbohydrate.

Data from observational studies on low carbohydrate diets showed weight loss of 8.3% at 8 weeks (Larosa et al., 1980) and 10.3% at 6 months (Westman et al., 2002). Foster et al. (2003) conducted the first randomised controlled trial to evaluate efficacy of low carbohydrate diet (which also consisted of high protein and high fat), compared to high carbohydrate, low fat, calorie restricted diet. 63 obese non diabetic patients were involved in this 1-year study. The low carbohydrate diet consisted of 20g of carbohydrate in the first 2 weeks, which was very slowly increased thereafter, but no information was provided on what was the final amount of carbohydrate patients were consuming at the end of the study. Moreover, there was a high dropout rate as only 43% of patients in the low carbohydrate group and 39% of patients in high carbohydrate calorie restricted group completed the study at 1 year.

Results of this study showed better weight loss in the low carbohydrate group in the first 3 and 6 months, following which the difference between groups disappeared. Interestingly, urinary ketones were also measured and were elevated in the low carbohydrate group for only first 3 months, potentially implying noncompliance with low carbohydrate diet after 3 months. This could be due to the fact that adherence to low carb programme can be difficult long term. Moreover, the study concluded that the initially greater weight loss at 3 and 6 months in the low carbohydrate group was due to lower overall calorie consumption rather than ketosis, as no relationship was seen between ketosis and weight loss (Foster et al., 2003).

Hession et al. (2009) systematically reviewed 13 randomised controlled trials of low carbohydrate diet (less than 60g of carbohydrate) compared to low fat calorie reduced diet and found that low carbohydrate diets lead to better weight loss than low fat calorie reduced diets at 6 months, and similarly effective at 1 year. Given that weight loss was similar between the two diets at 1 year, it is possible that long term continuation of low carbohydrate diet (with less than 60g of carbohydrate per day) is unrealistic among patients with obesity.

Rolland et al. (2009) investigated weight loss potential of low carbohydrate diet compared to very low-calorie diet. In this randomised controlled trial, 72 obese patients were randomised to either low carbohydrate diet (with less than 40g of carbohydrate and between 800 and 1500 kcal) or very low-calorie diet (consisting of less than 550 kcal). There was a significant difference at 9 months, with 15.1 kg weight loss among patients in the very low-calorie diet compared to 1.9 kg weight loss in the low carbohydrate diet group. It was hypothesised that this difference was due to lower energy intake in the very low-calorie group. Veldhorst et al. (2009) described that in people who adopt a low-carbohydrate diet there was an acute increase in resting energy expenditure, driven by increased rate of gluconeogenesis. However, long term low-carbohydrate diet was associated with a reduced metabolic rate as various metabolic compensations took place (such as changes in substrate utilisation) (Winwood-Smith et al., 2017).

From the current literature there is mixed evidence about effectiveness of low carbohydrate diet, partly due to varying amount of carbohydrates (20-60g), different amount of overall calorie consumption and length of follow up. A recent meta-analysis by Goldenberg et al. (2021) showed that following low carbohydrate diet (less than 120 g of carbohydrate) resulted in a weight loss of 3.46 kg at 6 months, with the weight loss diminishing at 12 months. Interestingly, very low carbohydrate diets were less effective than low carbohydrate diets, most likely due to diet adherence. Sustainability of low carbohydrate diet is well recognised challenge. In our study, we found that no participant was able to sustain diet with less than 100 g of carbohydrate, and

therefore it was unlikely that low carbohydrate diet per se was a significant factor in the observed weight loss.

The contribution of low carbohydrate diet to the observed weight loss in this study is unclear as none of the subjects were able to adhere to the diet long term. However, most of the participants reported improved diet and the frequent follow-up with the research team would have provided greater support compared to the standard of care. In future studies, alternative method of logging dietary diaries, such as digital photo diary, may provide a more accurate record.

6.2.1.2 Other possible factors affecting observed weight loss

Some studies suggested that therapy with SGLT2 inhibitors increase hunger and sugar intake, resulting in increased calorie intake (Devenny et al., 2012, Horie et al., 2018). Thomas and Cherney (2018) hypothesised that increased dietary adherence (to prevent this secondary increased intake) may help with achieving larger weight loss.

In our study, participants had frequent follow-ups and therefore could have experienced better support with adhering to their diet. Moreover, all participants had an access to the study researcher 24/7 and support was available for the duration of the entire study. Therefore, it was more likely that overall greater dietary adherence rather than low carbohydrate diet was an important factor in achieving the observed weight loss.

Kurinami et al. (2018) showed that weight reduction is proportional to blood glucose levels, with higher reduction observed in those with higher blood sugar levels. We observed a greater weight loss in those patients who had HbA1c above 50 mmol/mol compared to those whose HbA1c was below 50 mmol/mol. However, the difference seen in our study was not statistically significant.

On the other hand, evidence suggested that BMI, race, gender or background therapy did not influence weight loss induced by SGLT2 inhibitors (Zaccardi et al., 2016). In our study there was a numerical difference between weight loss experienced by participants, with those whose weight was above 131.2 kg experiencing greater weight loss than those whose starting weight was below 131.2 kg. However, this difference also did not reach statistical significance. Another important factor that affects weight loss is appetite and this will be discussed below.

6.3 Appetite changes

There were no changes in participants' self-reported appetite during the 12 months study. This is surprising, because with long term glycosuria one would expect a compensatory response to increase calorie consumption, as occurs in other forms of calorie deficit. Devenny et al. (2012) showed that this was the case in mice treated with Dapagliflozin. Moreover, Dapagliflozin was shown to increase appetite for sugar-rich foods among patients with T2DM (Horie et al., 2018). The dose of Dapagliflozin was 5 mg (half of the dose used in our study) and diet history questionnaire was completed after 3 months. Interestingly, there was no difference in total calorie intake or the proportions of the major nutrients (Horie et al., 2018), unlike in Devenny et al.(2012) study where overall 30% increase in calorie intake was observed. The 30% increase in energy intake was not replicated in later rodent studies which showed only 4 kcal increase (4%, which was not significant) in energy intake in rodents taking Ipragliflozin (Yokono et al., 2014).

In our study we measured appetite, rather than nutrient and calorie intake and it is possible that despite the reported no change in appetite, participants altered their total intake. The strength of our appetite measurement was that it occurred on a monthly basis and consisted of 4 different measures of appetite (hunger, satiety, fullness and overall appetite). Additionally, it was a validated instrument (shown in appendix). The important finding was that we observed the same results (of no change) in each of the categories.

Evidence shows that appetite can be reduced with ketogenic diets (Gibson et al., 2015). Such a nutritionally induced ketosis is defined by the serum level of beta-hydroxybutyrate of 0.3 mmol/L and more (Gibson et al., 2015). Literature suggests that ketosis is not likely to occur when carbohydrate intake is more than 100 g (Mullins et al., 2011), with carbohydrate intake between 50 and 70 g likely to induce ketosis that will prevent feeling of hunger that follows weight loss (Deemer et al., 2020).

In our study participants were asked to consume no more than 100 g of carbohydrate, however, they were unable to stick to this long term. This was reflected by the plasma ketone levels, which were below 0.3 mmol/l, and thus suggesting no ketosis. This could explain why no effect on appetite was seen. However, increase in appetite would be expected following weight loss (Gibson et al., 2015) and participants in our study did not report any increase in appetite. There is currently no evidence for a threshold of the level of ketones needed for appetite suppression (Deemer et al., 2020) and it is possible that even a slight increase from 0.1 to 0.2 mmol/l could play a role in appetite control. More evidence is clearly needed to investigate the relationship between elevated ketone levels and appetite control as no study objectively measured long term food intake of participants on ketogenic diets and therefore not enough evidence exists to prove that elevated ketone levels lead to a direct reduction of appetite stimulating hormones (Deemer et al., 2020).

6.4 Changes in urinary glucose, sodium, protein and volume

We have shown a significant increase in urinary glucose that was sustained over the 12-month duration of the study. The mean value of glycosuria over the 12 months was 305.6 mol per 24 hours (which is 55.1 g of glucose lost in urine per day), similar to glycosuria reported in other studies (Merovci et al., 2014, Sha et al., 2014).

Our study is the first study that measured 24-hour urinary glucose excretion beyond 12 weeks as other studies measured the amount of glycosuria beyond 12 weeks by a spot urine measurement (Bolinder et al., 2014). The previously reported amount of glycosuria ranged from 50 to 120 g of glucose per day (List et al., 2009, Merovci et al., 2014, Devineni et al., 2012, Sha et al., 2014). In our study most patients had very well controlled diabetes with HbA1c below 50 mmol/mol and this could explain why the amount of glycosuria observed in our study was on the lower end of the range observed in other studies.

Unlike glycosuria, the amount of sodium excreted in urine did not change after initiation of Dapagliflozin. We have measured natriuresis at one-month intervals and it is probable that changes occurred in the first few days following initiation of Dapagliflozin, with subsequent homeostatic mechanisms preventing a long-term sodium loss.

As discussed in the first chapter, the evidence on natriuresis following SGLT2 inhibition is mixed, with reports on natriuresis lasting from one day only (Tanaka et al., 2017), to 2 weeks as well as 6 months (Kawasoe et al., 2017). On the other hand, Sha et al. (2014) did not find any changes to natriuresis at 12 weeks. The latest study (DAPASALT) by Scholtes et al. (2020) showed no difference in natriuresis among 14 patients with T2DM treated with Dapagliflozin on a controlled standardised diet of 150 mmol sodium per day at day 2-4 or day 12-14.

Brady and Hallow (2018) hypothesised that SGLT-dependent pathways (both SGLT1 and SGLT2 receptors) are responsible for reabsorbing 25 g of sodium (4-6 % of the total filtered sodium load). It is possible that after inhibiting SGLT2 receptor more sodium gets reabsorbed via SGLT1 receptor, however it is not clear whether SGLT1 receptor in the late proximal tubule reabsorbs all the sodium or whether other mechanisms play a role in preventing long term sodium loss.

From the latest study by Scholtes et al. (2020) it is clear that there is no increase in natriuresis from day 2 onwards following SGLT2 inhibitor initiation.

The acute changes induced by the prevention of sodium reabsorption at SGLT2 receptor need to be investigated to understand what homeostatic mechanisms prevent ongoing natriuresis. Previous studies clearly showed reduction in haematocrit (Sano and Goto, 2019) and plasma volume in patients treated with SGLT2 inhibitors (Dekkers et al., 2019) . In order to further our understanding of sodium dynamics in human body, new techniques, such as sodium MRI, need to be refined in order to measure sodium changes induced by SGLT2 inhibitors.

We did not observe any significant changes in proteinuria in our study, unlike previously reported reductions in albuminuria following SGLT2 inhibitor therapy (Cherney et al., 2017). This could be due to the fact that most of participants in our study did not have any significant proteinuria. There was also no difference in renal function between baseline and the 12-month follow-up. This differs from other studies that reported slower decline in renal function among people treated with SGLT2 inhibitors compared to control group of patients (Cherney et al., 2017), with the most recent one, DAPA-CKD, showing that Dapagliflozin reduces decline in renal function among patients with chronic kidney disease, regardless of presence or absence of diabetes (Heerspink et al., 2020).

In our study, the changes to the renal function were not assessed as an outcome and the study was not powered to detect any changes in renal function given the study sample and length of follow up, as well as the fact that most participants had normal kidney function.

In our study participants had a sustained increase in urinary volume following treatment with Dapagliflozin (456 ml increase at 12 months). This is contrary to previously published evidence that showed that increase in urinary volume following SGLT2 inhibitor initiation was transient and normalised after one day, despite ongoing glycosuria (Tanaka et al., 2017, Yasui et al., 2018). We have shown that the increase in urinary volume was not significantly correlated with the changes in urinary glucose, suggesting that increased urinary volume is not purely driven by osmotic diuresis.

Tanaka et al. (2017) suggested that following SGLT2 inhibition kidneys improve its ability to concentrate urine and reduce free water clearance. The preservation of water and thus prevention of long-term diuresis following SGLT2 inhibitor would make sense from a physiological perspective and Marton et al. (2021) suggested a possible mechanism for the water conservation. This hypothesis is based on the concept of aestivation, defined by “a series of evolutionary conserved metabolic switches that allow organisms to survive arid or hot conditions with restricted water availability” (Loomis, 2010). This very interesting hypothesis suggests that therapy with SGLT2 inhibitor trigger aestivation like response, due to the loss of energy and solutes in urine. The key changes that occur in aestivation are an increase in the production or urea that leads to improved ability to conserve water, and reduction in metabolic rate together with a switch to endogenous source of energy and building blocks for increased urea production (Marton et al., 2021). These changes will reduce energy expenditure and promote water conservation.

Previous studies showed that urea-driven urine concentration limits osmotic diuresis in cases of high solute excretion (Rakova et al., 2017), and Dapagliflozin has been shown to induce compensatory urea-driven water reabsorption in diabetic rats (Chen et al., 2016) .

These water conserving mechanisms are energy expensive –and on top of already energy negative state induced by SGLT2 inhibitors, where is the energy coming from? Marton et al. (2021) suggested that basal metabolic rate is reduced and body uses some of its fuel source (from triglycerides or protein) to build urea osmolytes via intensification of the hepatic urea cycle. The generation of urea not only needs energy but also nitrogen, and both can come from dietary protein or endogenous protein storage in skeletal muscle (Marton et al., 2021).

Even though the above discussed hypothesis is logical and backed up by various evidence, our study generated findings that do not support it. We have found that there was no change in metabolic rate, no change in lean mass and

a sustained increase in urinary volume following treatment with Dapagliflozin. There was also no change in urea during the study.

We did find a significant reduction in fat mass and this could be driven by the increased energy loss in the form of glycosuria, as well as increased energy needed to produce more urea solutes. Additionally, the fact we did not see any changes in the muscle mass could be explained by the fact that participants had a sufficient exogenous protein intake, and thus did not have to catabolise endogenous protein. Lastly, the increased urinary volume could be explained by the fact that the urea concentrating mechanisms were not able to fully offset the glucose-driven osmosis and other mechanisms leading to increased urinary loss.

In order to test the above-mentioned hypothesis future studies will need to focus not only on changes in metabolism, body composition and urinary volume, glucose and sodium, but also on the urea osmolyte production and its supply to the kidney. Moreover, future research should investigate the mechanism of sodium handling in health and in patients with diabetes, as it is likely this underlies the protective mechanism of SGLT2 inhibitors. One potential technique of measuring sodium dynamics could be with sodium MRI, which enables identification of sodium in skin and subcutaneous tissues.

6.5 Changes in glucose, glucagon, insulin, insulin resistance, ketone bodies, leptin and adiponectin

6.5.1 Glucose

In our study fasting glucose levels, as well as HbA1c, did not change during the 12 months therapy with Dapagliflozin. The starting mean HbA1c was 53.9 mmol/mol and the mean fasting plasma glucose was 8.1 mmol/l. In previous studies SGLT2 inhibitor therapy was found to produce a significant reduction in HbA1c (0.6-0.9%) and reduction in fasting plasma glucose by 1-2 mmol/l (Zaccardi et al., 2016). However, in patients with better control of diabetes, smaller reductions in HbA1c were observed (Abe et al., 2018) .

The mechanism of action of SGLT2 inhibitor is glucose dependent, and in those with lower plasma glucose smaller amount of glucose is filtered by the kidney. The glucose-lowering potential is dependent on renal function, as those with higher glomerular filtration rates will filter more glucose than those with a lower glomerular filtration rate (Cherney et al., 2018). In our study, all participants had preserved renal function and therefore this did not affect the amount of glucose filtered and excreted.

6.5.2 Glucagon

We have shown that level of glucagon significantly increased during the course of 12 months treatment with Dapagliflozin. Similar findings of increased glucagon levels in patients treated with SGLT2 inhibitor therapy were reported by other studies (Ferrannini, 2017, Ferrannini et al., 2014, Bonner et al., 2015). The rise in glucagon is a compensatory mechanism that follows reduction in plasma level of glucose triggered by SGLT2 inhibitors (Alatrach et al., 2020), as well as the direct action of SGLT2 inhibitor at the alpha cells (Bonner et al., 2015). However, study by Wang et al. (2017) found that in rodent models of T2DM, Dapagliflozin decreased glucagon signalling and reduced expression of the glucagon receptor, effectively reducing glucagon levels. It is possible

that this difference was due to the fact that it was a rodent study and that regulation of glucagon signalling differs between human and rodents.

A recent study by Alatrach et al. (2020) confirmed findings by Wang et al. group and showed that SGLT2 inhibitors do not act directly on the pancreatic alpha cells and thus cannot cause rise in glucagon via that route. By preventing the drop in plasma glucose concentration after SGLT2 inhibitor therapy, glucagon levels did not rise, as would be otherwise expected if SGLT2 inhibitor acted directly on the alpha cells (Alatrach et al., 2020). However, Saponaro et al. (2020) found that there is a heterogeneity in SGLT2 expression and its regulation among humans which could impact on glucagon secretion from alpha cells following SGLT2 inhibition.

In our study we did not see any drops in fasting plasma glucose levels and so other factors, such as ongoing glycosuria and compensatory mechanisms must play role in triggering the rise in glucagon. The relative glucagon time course (steady increase) differs from the time course of glycosuria which showed sharp rise that was sustained long term. This could be due to ongoing metabolic changes at the level of kidney and liver, and the differing contribution that each has for glucose homeostasis.

6.5.3 Insulin

We have observed a statistically significant reduction in insulin levels during the 12-month therapy with Dapagliflozin. This is similar to previously published studies that found small but significant reduction in insulin levels following SGLT2 inhibition (Merovci et al., 2014, Martinez et al., 2018, Ferrannini et al., 2014). On the other hand, Jobori et al. (2017) found that despite a small reduction in insulin at day 1, at day 14 insulin returned to baseline. The drop in insulin levels is thought to be secondary to the drop in plasma glucose after initiation of SGLT2 inhibitor.

In our study we did not see any changes in fasting glucose levels during the 12 months study, most likely due to already well controlled plasma glucose. However, between baseline and month 1 we observed a trend for insulin increase, which reached statistical significance. This could have been as a result of raised endogenous glucose production, which was possibly more than the amount of glucose lost in urine at month 1. However, with the observed stable fasting glucose levels it is unclear what is driving this transient rise.

6.5.4 Endogenous glucose production

The rise in glucagon and reduction in insulin are important factors in regulating endogenous glucose production (Cherrington, 1999). The rise in endogenous glucose production has been well documented following SGLT2 inhibition, and it has been suggested that it was the rise in glucagon and drop in insulin levels that were responsible for the increase in endogenous glucose production. (Ferrannini et al., 2014, Merovci et al., 2014).

However, Alatrach et al. (2020) showed that the original hypothesis was incorrect. SGLT2 inhibition resulted in increased endogenous glucose production among patients with T2DM, despite keeping the levels of glucagon, insulin and glucose at fasting level (Alatrach et al., 2020). This was the first study that provided evidence against the role of glucagon and insulin in changes to the endogenous glucose production that occurs in SGLT2 inhibition. Sasaki et al. (2017) showed that SGLT2 inhibition promotes renal gluconeogenesis and it is possible that a significant portion of gluconeogenesis occurs in kidney (and thus is independent of hormonal regulation, unlike hepatic gluconeogenesis).

6.5.5 Insulin resistance and beta cell function

The level of insulin resistance and beta-cell function were measured with HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) and HOMA %B created by Oxford university (DTU, 2004). The mathematical model was originally introduced by Matthews et al. (1985) and uses individuals fasting plasma glucose and insulin concentration to estimate insulin sensitivity and level of beta-cell function, as percentages of a normal reference population.

Even though there is no absolute cut off value to interpret HOMA-IR values (as they depend on the population of interest) it is generally accepted that a value of 2.5 and above is an indicator of insulin resistance in adults (Muniyappa et al., 2008). The level of insulin that HOMA2 calculator accepts is between 20 to 400pmol/L. In our study, some values were below and above this range.

The mean HOMA-IR was 2.6 and 3 at baseline and 12 month-follow up. However, statistical analysis showed that there was a statistically significant reduction in insulin resistance (calculated from 2.5 to 1.8) over 12 months of Dapagliflozin therapy. This was likely driven by the significant amount of fat loss seen in our participants. Given differing results between raw data and statistical analysis as well as the small sample size, the significance of this result needs to be questioned. It is important to bear in mind that the HOMA-IR calculator was designed for large scale clinical trials and population studies. This uncertainty of the effect of SGLT2 inhibitor on insulin resistance is reflected in the mixed evidence.

Ferrannini et al. (2014) found that insulin sensitivity was significantly improved following acute (1 day) but not following a chronic (4 weeks) administration of SGLT2 inhibitor. Improved insulin sensitivity of peripheral tissues was shown by Merovci et al. (2014), where after 2-week treatment with SGLT2 inhibitor there was increased insulin-mediated tissue glucose disposal, as well as reduction in fasting plasma glucose levels, despite a paradoxical rise in endogenous glucose production.

On the other hand, Latva-Rasku et al. (2019) did not show any changes to insulin sensitivity (both whole body and at the level of muscle) among 14 patients with T2DM after treatment with SGLT2 inhibitor for 8 weeks. The participants in this study were more obese than those in previous studies, and it is possible that this impacted the response to insulin sensitivity.

It is possible that following a rapid correction of hyperglycaemia there is a marked improvement in insulin sensitivity, and after a period of time of improved glycaemic control, insulin sensitivity stabilises, however, it is not clear how long the improved insulin sensitivity lasts for. Additionally, it is possible that the degree of pre-existing insulin resistance influences the degree to which insulin resistance changes post treatment with SGLT2 inhibitors.

In our study we found that there was a significant reduction in beta cell function from 80.6% (SD 47.5%) to 73.8% (SD 47.3%). This could be secondary to reduced insulin requirements and therefore less insulin needed to stabilize blood glucose.

This is contrary to previously published evidence. Ferrannini et al. (2014) found both acute and chronic SGLT2 inhibitor treatment improved beta cell function (as insulin secretion was enhanced by 25% in the context of plasma glucose levels, even though the overall insulin levels were reduced), possibly as a result of reduced glucose toxicity following treatment with SGLT2 inhibitor, as well as reduced beta cell workload.

Takahara et al. (2015) found that 4 weeks of SGLT2 inhibitor improved beta cell function among 10 diabetic patients, with the positive effect on beta cell function persisting after SGLT2 inhibitor was discontinued (for one week). Polidori et al. (2014) studied the effect of SGLT2 inhibitor on 359 patients for 6 and 12 months and found that treatment improved model-based beta cell function long term. The studies provide a strong evidence that SGLT2 inhibitor have a positive protective effect on beta cells, a key role in management of T2DM.

It remains unclear why in our study beta cell function reduced. However, the precision of measurement of beta cell function with using HOMA-IR calculator in small samples is not known.

6.5.6 Leptin and adiponectin

In our study we observed a statistically significant reduction in leptin levels and no significant changes in adiponectin levels among patients with T2M and obesity treated with Dapagliflozin.

Leptin and adiponectin are adipocytokines produced by adipocytes (D'Souza A et al., 2017). Role of leptin is to inhibit food intake and facilitate glucose and lipid metabolism (Farooqi and O'Rahilly, 2014). In people with obesity leptin levels are elevated due to the increased fat mass (D'Souza A et al., 2017), leading to leptin resistance, interfering with glucose metabolism and worsening insulin resistance. Reduction of leptin levels in leptin resistant individuals may improve their leptin sensitivity and thus reverse the negative metabolic consequences triggered by leptin resistance.

Adiponectin regulates glucose production and reduces insulin resistance (Ouchi et al., 2000). Adiponectin in inversely correlated to adiposity and basal plasma insulin (Bouassida et al., 2010), suggesting that higher adiponectin levels have a positive impact on metabolic status.

A recent meta-analysis showed that therapy with SGLT2 inhibitors leads to a significant reduction in leptin (290 pg/ml) (Wu et al., 2019). The meta-analysis included 10 studies with 3 to 6 months follow-up. In our study the reduction in leptin was 852 pg/ml per month, which is much bigger increase in leptin than reported in Wu at al. study (2019). One possible explanation for such a marked difference is the significant reduction in fat mass observed in our study.

The results for adiponectin changes are less clear. The levels of adiponectin observed in our study were similar to previously reported adiponectin levels among people with T2DM and obesity (Frühbeck et al., 2019). Imbeault et al.

(2007) showed that adiponectin concentration increased after a significant weight loss (8-9% weight loss). This has been further supported by Lin et al. (2007) who showed that increases in adiponectin was negatively correlated with changes in body weight and fat mass. However, some studies did not show rise in total adiponectin levels following a significant weight loss and it has been suggested that this could be due to a redistribution of adiponectin oligomers which is not apparent with the total adiponectin concentration is measured (Bobbert et al., 2005).

Bonnet and Scheen found that SGLT2 inhibitors may influence levels of adiponectin as in 3 out of 5 studies there was a significant rise in adiponectin following SGLT2 inhibitor treatment (Bonnet and Scheen, 2018). Meta-analysis by Wu et al. (2019) concluded that SGLT2 inhibitors produce a significant increase in adiponectin (300 ng/ml). The observed changes are likely to be driven by weight loss, however, other factors will contribute as Garvey et al. (2018) found that increase in adiponectin following SGLT2 inhibitor therapy was independent of weight loss as well as glycaemic benefit.

Fruhbeck et al. (2019) suggested that rather than absolute values of leptin and adiponectin, it is the ratio of adiponectin to leptin that is an important predictor of metabolic disease. Fruhbeck's study showed that ratio of less than 0.5 indicates a severe increase in cardiometabolic risk, as there was a significant negative correlation between the adiponectin (measured in micrograms/ml) to leptin (measured in ng/ml) ratio and serum amyloid concentration, a marker of adipose tissue dysfunction (Frühbeck et al., 2019). In our study the starting ratio was 0.11 (adiponectin 5710 ng/ml and leptin 51 713 pg/ml) with end of study ratio 0.14 (adiponectin 6099 ng/ml and leptin 42880 pg/ml), with a non-significant difference (p=0.161) suggesting that patients had a high risk of metabolic disease which did not change significantly during the study.

It remains unclear what other factors (additional to fat loss) affect adiponectin levels and whether there is a specific percentage of fat loss needed in order to produce a significant change in adiponectin.

6.5.7 Ketone bodies

We have shown a significant sustained increase in ketone bodies (beta-hydroxybutyrate) during the 12-month therapy with Dapagliflozin. However, the increase is unlikely clinically significant (from 0.1 to 0.2 mmol/l).

Previous evidence shows that SGLT2 inhibition triggers sustained lipolysis and ketogenesis (Ferrannini, 2017). Daniele et al. (2016) found that Dapagliflozin increased plasma ketone concentration from 0.05 to 0.19 mmol/l, a rise similar to the one observed in our study. Jobori et al. (2017) assessed the changes in ketone levels in 15 patients with T2DM following initiation of SGLT2 inhibitors and found a significant rise in ketone bodies at day 1 and day 14 (from 0.53 mmol/l to 0.67 mmol/l), unlike in non-diabetic patients treated with SGLT2 inhibitor. It is important to point that the starting level of fasting ketones (prior to commencement of SGLT2 inhibitor) was 0.53 mmol/l which is 5 times more than the baseline level in our cohort. Furthermore, participants in Jobori et al. (2017) study had worse diabetes control (HbA1c 61.7 mmol/mol) and were relatively lighter BMI of 31.1 kg/m^2 compared to 46.1 kg/$m^{2.}$

Ketone bodies (acetoacetate, acetone and beta-hydroxybutyrate) are used as energy substrate when body has limited supply of carbohydrates. Newman and Verdin (2014) showed that synthesis of ketone bodies is regulated by insulin and glucagon, as well as mTORC1 (mammalian target of rapamycin complex). mTORC1 is activated by abundant glucose (Howell and Manning, 2011). Insulin and activation of mTORC1 inhibits formation of ketone bodies, with glucagon having the opposite effect (Newman and Verdin, 2014). We and others have shown that SGLT2 inhibitors produce rise in glucagon, which could explain the significant (albeit small) rise in ketone bodies.

It has been suggested that ketone bodies (beta-hydroxybutyrate) are more than just a metabolite, due to their signalling roles (Newman and Verdin, 2014). Ketone bodies act as ligands for two surface receptors, are an

endogenous inhibitor of histone deacetylases, and the downstream products of ketone metabolism also have signalling roles (Newman and Verdin, 2014). Elevation of ketone body concentration up to 3 mmol/l is not clinically harmful (Cotter et al., 2013). On the other hand, the rise in ketone levels has been suggested to explain some of the cardiovascular protective effects, as ketone bodies may be a better energy substrate for the heart (Ferrannini, 2017). Elevated ketone production triggered by SGLT2 inhibition could be also reno-protective, as found by Tomita et al. (2020) who found that ketone bodies reverse renal damage by blocking mTORC1 signalling, which if activated, leads to impaired renal lipolysis and subsequent damage. Suppression of appetite is another possible positive effect of mildly elevated beta-hydroxybutyrate that was discussed in the previous section on appetite.

However, if the ketone level rises above 3 mmol/l, such as in unwell patient with T1DM, diabetic ketoacidosis (DKA) can develop. Some sources suggest that DKA occurs due to the acidic nature of ketones (Laffel, 1999) , but this is incorrect. Acetoacetate and beta-hydroxybutyrate are conjugate bases and acetone is neither an acid nor a base (Green and Bishop, 2019). It is the process of ketogenesis (adipose tissue lipolysis, fatty acid activation, beta oxidation, co-enzyme A synthesis) that will produce hydrogen ions (protons) and thus can lead to acidosis under very high rates of lipid catabolism (Green and Bishop, 2019).

The pathophysiology of DKA is complex, but the key features are hyperglycaemia triggered by lack of insulin, increased ketogenesis and resulting increased production of hydrogen protons, osmotic diuresis and dehydration, acute kidney injury, followed by inability of kidney to compensate for the increased acid load created as a by-product of ketogenesis (Green and Bishop, 2019). A very rare side effect of SGLT2 inhibitor therapy is a euglycaemic DKA (euDKA) with its hallmarks of normoglycaemia with ketosis and acidosis- described in more detail in the introduction chapter.

We have shown that despite the rise in glucagon to insulin ration there was not a clinically worrying rise in ketones, and therefore this is unlikely to be the

main contributor to development of euDKA. On the other hand, dehydration and subsequent renal impairment with reduced ability to compensate for acid base disturbances will be an important contributor to development of euDKA.

6.6 Limitations

Our study had several limitations. The sample size was small and we did not have a control group. In order to overcome this, participants acted as their own controls. The changes we were measuring in this study (weight loss, glycosuria) have been shown extensively in short term placebo-controlled studies. Our aim was to investigate metabolic adaptations over 12 months in a great detail and it would have been difficult to recruit additional 18 participants to act as controls. Moreover, the intensity of the physiological measurements and commitment of staff and patients limited the overall size that could have been achieved in the time limits of this project. This was also a reason why the power to detect differences in some of the measurements was not calculated.

Additionally, participants also did not adhere to a low carbohydrate diet and it was therefore not possible to draw any meaningful conclusions regarding the effect of this diet on the observed results.

Additionally, there has been a failure of Tissue Bank freezers during the first year of this project. The freezers stopped working on 29th of June 2018 and this was not detected till 5th of July 2018. This meant that 36 out of 216 of samples were defrosted and kept at fridge temperature of 8.2 ºC for 5 days. Following this, they were frozen to -80 ºC again.

In order to investigate how this incident could have affected results, we looked at previously published studies assessing the stability of hormones and biomarkers of interest. The evidence suggested that glucagon, insulin, leptin, adiponectin, glucose and ketones are stable with 1 freeze thaw cycle (Cegla et al., 2017, Flower et al., 2000, Schraw et al., 2008, Chen et al., 2013, Gislefoss et al., 2017, Zander et al., 2014).We have therefore included all the samples in the final analyses.

6.7 Novel findings

Sasaki et al. (2019) published findings of a 12-month study assessing metabolic changes in obese patients with T2DM which showed that treatment with SGLT2 inhibitor led to a significant reduction in fat mass, increase in urinary glucose excretion, increase in urinary volume and increase in ketones, however, unlike in our study, this study was done in diabetic patients with BMI less than 35 kg/m^2. Our study produced several novel findings, such as the long-term effect of SGLT2 inhibitors on urinary sodium excretion, levels of leptin, insulin, glucagon and adiponectin, as well as the longitudinal data on detailed metabolic changes including energy expenditure and body composition in patients with T2DM and class 3 obesity.

Conclusion

This is a first study providing highly detailed phenotypic assessment of changes induced by a long-term therapy with Dapagliflozin. We have shown that 12-month therapy with Dapagliflozin resulted in significant metabolic changes among patients with obesity and T2DM. The significant changes that occurred were weight loss, fat mass loss, reduction in leptin levels, insulin levels and insulin resistance, increase in glucagon, beta-hydroxybutyrate, glycosuria and urinary volume. The important negative findings were that there was no change in appetite, basal metabolic rate, adiponectin, fasting glucose and natriuresis.

One possible implication for future clinical practice includes combining SGLT2 inhibitor therapy with a strict dietary intervention combined with a frequent follow-up, which will support patients with dietary adherence.

It remains unclear how SGLT2 inhibitor induced metabolic changes link to the cardiovascular and renal protection observed in this class of medication. In order to link the effects mediated by changes in glucose and sodium future studies need to measure dynamics of sodium using whole body assessments rather than relying on urinary sodium. One such technique could be highly specialised sodium MRI techniques that enable identification and quantification of sodium in its active and inactive state. Our group is hoping to explore this in future research studies.

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Appendix 1. 1: Protocol for the HMRU study

Clinical Study Protocol

Drug Substance	Dapagliflozin
Study Code	ESR-16-12140
Edition Number	003
Date	*23rd of October, 2017*

Exploring appetitive, metabolic and ketotic effects and weight-loss potential of Dapagliflozin in patients with Type 2 Diabetes Mellitus and inadequate glycaemic control, with concomitant dietary intervention

Sponsor: *University of Warwick, UK*

The following Amendment(s) and Administrative Changes have been made to this protocol since the date of preparation: N/A

Amendment No.	Date of Amendment	Local Amendment No:	Date of Local Amendment

Administrative Change No.	Date of Administrative Change	Local Administrative Change No.	Date of Local Administrative Change

PROTOCOL SYNOPSIS for PART 1, HMRU STUDY

Exploring appetitive, metabolic and ketotic effects and weight-loss potential of Dapagliflozin in patients with Type 2 Diabetes Mellitus and inadequate glycaemic control, with concomitant dietary intervention

Principal Investigator

Dr. Thomas M Barber

Study site(s) and number of subjects planned

Study Site: **Human Metabolism Research Unit**, University Hospitals Coventry and Warwickshire, Clifford Bridge Road, Coventry, CV2 2DX, UK.

Number of Subjects Planned for part 1, the HMRU study: **16**

Number of Subjects Planned for part 2, the survey**: 250.** Separate ethics approaval will be sought for part 2 of the study.

Study period		**Phase of development**
Estimated date of first subject enrolled	Q4, 2017	N/A
Estimated date of last subject completed	Q2, 2019	N/A

Study design

Observational study

Objectives

Primary Objectives:	**Outcome Measures:**
To execute detailed phenotyping (glycosuric response, appetitive and metabolic changes, caloric intake, sleep quality and fat mass) in human participants with T2D treated with Dapagliflozin therapy and concomitant dietary intervention, with evaluation of independent predictors of weight-loss	*Accurate measures of 24-hour metabolic profile from whole-body calorimetry (including resting energy expenditure, thermic effect of food and activity-related expenditure)* *Measures of urinary glucose excretion* *BodPod measures of body composition* *Measures of sleep quality (including apnoea-hypopnoea index) through portable sleep machine* *Visual analogue assessments of appetite, including ingestion of standard buffet meals*
To explore the ketotic potential of Dapagliflozin, thereby to provide important insight into a potential uncommon side-effect of euglycaemic ketoacidosis and development of preventive strategies	*Accurate measurements of fasting serum levels of glucagon and insulin* *Accurate measurements of urinary and plasma ketone levels*

Secondary Objective:	**Outcome Measure :**
To explore effects of Dapagliflozin on human brown adipose tissue, including metabolic response to standard cold exposure, and volume of brown adipose tissue	*Assessment of metabolic response to a standard and gradual drop in ambient temperature within a whole-body calorimeter. Delta change in energy expenditure (Kcal/minute) during the temperature drop will be used as a measure of metabolic response* *Measurement of changes in brown adipose tissue volume (at baseline versus following 12-months of Dapagliflozin therapy) through use of IDEAL MRI scans*

Target subject population

Adult patients (over the age of 18 years) with a confirmed diagnosis of Type 2 Diabetes Mellitus and obesity (BMI > 30Kgm^{-2}), with inadequate glycaemic control (HbA1C 8-10%), naive to injectable therapies (insulin and GLP1 agents), DPP4 and SGLT2 inhibitor agents. The target subject population will also be good candidates for treatment with an SGLT2 inhibitor based on clinical scenario, and will not have any preclusions for initiation of Dapagliflozin (including eGFR > 60mmol/l, no concomitant use of loop diuretics or pioglitazone, and under the age of 75 years).

Duration of treatment

12 months

Investigational product, dosage and mode of administration

Dapagliflozin, 10mg once daily, oral administration

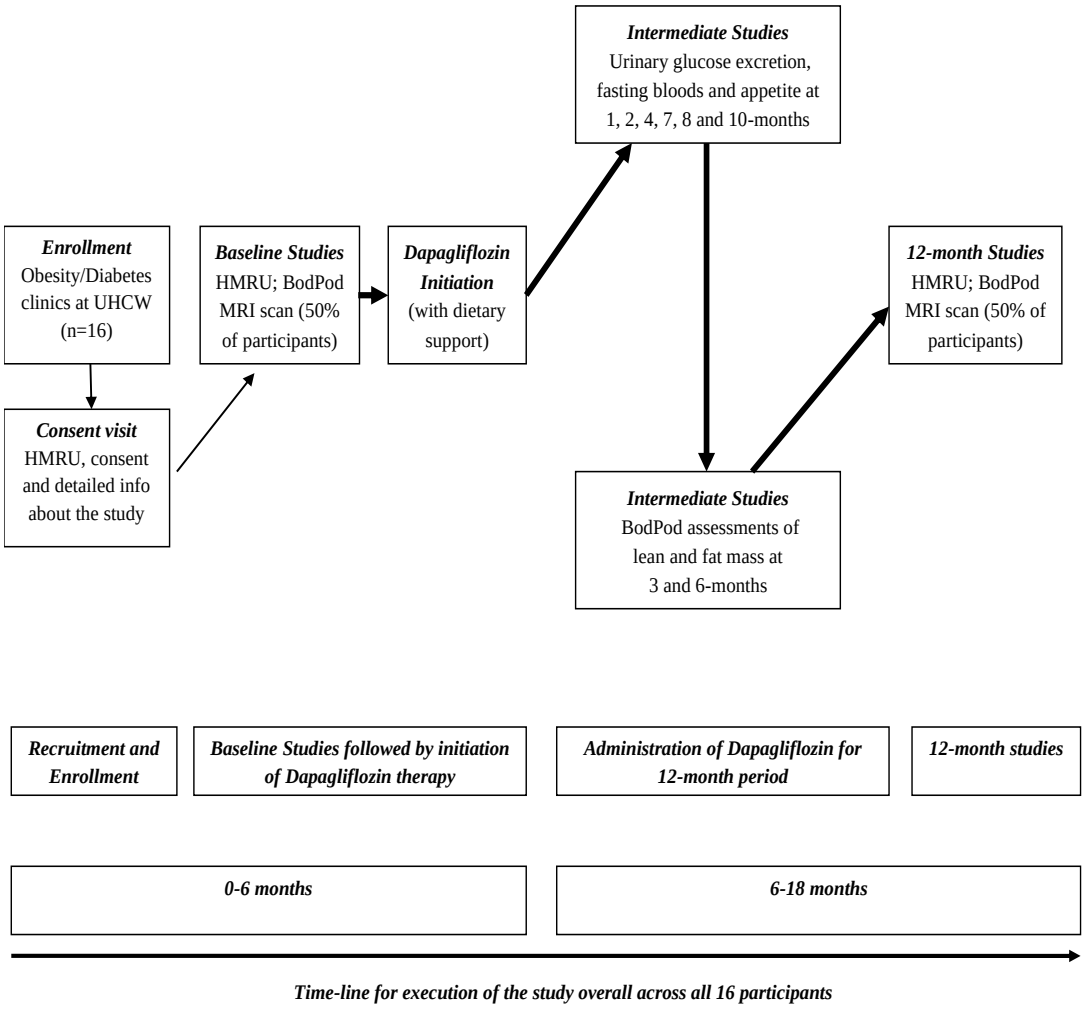
Statistical methods

This study will generate prospective data on use of Dapagliflozin in Type 2 Diabetes Mellitus in one of the most highly-phenotyped studies to date. The prospective nature of this study design will enable subsequent data analyses to explore independent predictors of response to Dapagliflozin therapy (through correlation of baseline data with markers of successful responses such as weight-loss, using multivariate analyses). These analyses will provide useful data to facilitate future prescribing of Dapagliflozin in T2D: evidence-based prescribing based on predicted clinical, biochemical, metabolic and appetitive responses.

All data analyses will be performed in SPSS. Statistical analyses will be predominantly through use of paired-sample t-tests, with comparison of baseline data with those data obtained following 12-months therapy with Dapagliflozin. Each participant will act as their own control given the observational nature of this study, thereby limiting confounding factors, and facilitating a focused and detailed phenotypic study in n=16 participants. Other analyses will include Pearson correlations between fasting glucagon:insulin ratios, urinary and plasma ketone levels, and glycaemic and weight-loss responses to Dapagliflozin therapy to provide invaluable insight into the ketotic potential of Dapagliflozin, and the possible role of ketosis in its glycaemic and secondary weight-loss effects. Such data will also provide a basis for future development of preventive strategies for avoidance of development of ketoacidosis in patients with T2D treated with Dapagliflozin.

Study Design

Figure 3: Summary of protocol design with timings of HMRU and intermediate studies



Appendix 1. 2: Patient Information Sheet

Department of Endocrinology
WISDEM Centre
University Hospital
Clifford Bridge Road
Walsgrave
Coventry
CV2 2DX

www.uhcw.nhs.uk

Version 2

Exploring appetitive, metabolic and ketotic effects and weight-loss potential of Dapagliflozin in patients with Type 2 Diabetes Mellitus and inadequate glycaemic control, with concomitant dietary intervention

Investigator: Dr. Thomas M. Barber

(t.barber@warwick.ac.uk)

PATIENT INFORMATION SHEET

Invitation

You are being invited to take part in a research study. Before you decide if you want to take part, you may wish to consider two things: firstly, why the research is being done and secondly what you would have to do. Please take time to read the information below and talk it over with someone else if you want to. If anything is not clear or you would like to know more please ask.

Thank you for your time, and for considering our study.

What is the study, and why have I been chosen?

Type 2 Diabetes Mellitus (T2D) is closely associated with weight-gain and obesity. Weight-loss is a key strategy for most patients with T2D. Unfortunately, traditional therapies for T2D promote weight-gain, which worsens control of blood glucose and health in general. Dapagliflozin, a new medication licensed for use in T2D, is associated with weight loss. This is thought to be due to promotion of glucose excretion by kidneys, translating to a calorie deficit of 250-300Kcal per day. There have been relatively few studies reported in the literature exploring the appetitive and metabolic effects of Dapagliflozin.

You have been invited to participate as you have T2D and you are a candidate for initiation of therapy with Dapagliflozin. This drug is already used in practice but there have are no observational studies about its effects on various metabolic parameters.

The purpose of this study is to look at the different effects of Dapagliflozin on **a)** body composition (i.e. lean body mass, body fat mass, BMI), **b)** metabolic profiles (markers in blood tests, metabolic rate), and **c)** functioning of fat tissue.

The decision relating to the management of your condition has been made independently of this study. Your addition to this study would be to observe the effects of Dapagliflozin mentioned above.

Do I have to take part?

No. It is for you to decide whether you wish to take part. If you do decide to take part you will be given this information sheet to keep. You will later be contacted, and asked to sign a consent form. Even if you decide to take part you can withdraw at any time. If you decide to withdraw, you do not need to tell us why and it will not change your treatment in any way.

What will happen to me if I take part?

Once you decide you would like to have more information and participate in the study, you will be invited to come to HMRU for detailed information in order to provide written consent, and we will check the inclusion/exclusion criteria for the study. Afterwards you will be invited for 4 study visits over a period of 1 year. A flowchart of participant involvement in the study is included at the end of this document.

Study Visit 1

You will first be asked to attend our Human Metabolism Research Unit (HMRU) at University Hospital, Coventry (UHCW), at 8am, having had your last meal at 8pm the previous day. We will take a brief medical history from you, and perform some measurements including height, weight, blood pressure, waist (tummy), and hip and neck circumference which will take approximately 40 minutes to complete. An estimate of how much fat you have in your body will also be done with the use of the BOD POD®, which is an egg-shaped chamber that you will need to sit inside for a few minutes for the measurement to be completed; this will take around 10 minutes to complete.

We will also take a blood and urine sample after an overnight fast (we would ask that you not eat or drink anything except for water for 12 hours prior to your morning appointment). The fasting is important so that we can assess

your blood without food interfering with the results. **Please note: It is important that you take your usual medications on the morning of your visit.** Measurements from these tests would include blood fats, sugars, and other factors related to metabolism and metabolic health. Part of all blood samples collected in the course of the study will be stored in a freezer for future analysis.

You will, at this point, also take part in a 24 hour stay for continual measurement of your energy expenditure in our specially-designed 'whole body calorimeters' or 'metabolic chamber' within the HMRU. You will enter the metabolic chamber at **8.50am**, in order that the experiment will begin and you will already be in the metabolic chamber, at 9am. This process measures the gases that you breathe in and out to calculate your energy expenditure (the energy that you use). The metabolic chamber is a small, self-contained room equipped with a bed, desk, chair, computer with internet access, TV, toilet, sink and curtains. The door is air-tight but can be opened from the inside or outside at any time if the participant wants to leave the chamber (although this would mean that the measurement of energy expenditure is no longer valid). There are two identical rooms next to each other and both rooms will be occupied at most times. The metabolic chamber is fairly small, so it is possible you may feel a little claustrophobic and isolated, but you will be able to converse with the investigator and/or participant in the other metabolic chamber through an intercom system throughout the study. You will be shown around the HMRU and metabolic chambers, prior to taking part in the study and have an opportunity to ask any questions you may have.

Standard meals will be provided (breakfast, lunch and evening meal, and two snacks in the mid-afternoon and evening), calculated to your calorific requirement. Throughout the study, blood tests will be taken at 10:30am, 1:30pm, 4pm, 5pm, 7:30am (fasting) the next morning. We will also collect your urine throughout your stay. During your overnight stay, you will have your sleep quality monitored using sleep monitoring apparatus. This monitors the rise and fall of your chest, as well as your levels of oxygen overnight; using a non-invasive sensor that slips over your finger (this is totally painless and is not invasive).

Following the above, we will also perform an MRI scan of your body to look at the composition of your body fat, in particular a type of fat tissue called 'Brown Fat', which we know has implications for metabolic health. This involves no radiation whatsoever, and as such is not in any way harmful. It will involve lying still for an extended period of time (around 1 hour) while the scan is performed. Please note, owing to the availability of the scanner, it may not always be possible to perform this on the same day. If this is the case, you will not have to stay with us for this; you can come back when we have arranged a date/time with you as soon as possible. We will try, wherever possible though, to undertake this on the same day.

After the above is completed, you will then leave and will continue on Dapagliflozin, medication for T2D.You will be seen by a specialist dietitian following your 1st study visit.

Study Visit 2

You will then attend after 3 months for a second visit in the HMRU in the morning, having had your last meal at 8pm the previous day. We will measure parameters of your metabolic health as above. This will include the measurement in BOD POD®, but there will be no 24 hours study in the HMRU and no MRI scan. This visit should take no more than 45 min. After this visit, you will again continue your therapy with Dapagliflozin for a further 3 months. After this, you will be invited back for a third visit similar to above.

Study Visit 3
You will then attend for third visit in the MHRU to again measure parameters of your metabolic health as above, and we will assess how these have or have not changed. This will be a morning visit, having had your last meal at 8pm the previous day. The visit will be exactly the same as 'Study Visit 2' and will involve all of the same tests and samples. This visit will take no longer than 45 minutes.

Study Visit 4

For your final visit you will attend at 12 months following the initiation of Dapagliflozin therapy. This will involve measuring parameters of your metabolic heath as in above study visit 1-3, and additionally 24 hour study in the HMRU and an MRI scan to assess the composition of your body fat, as in Study visit 1. You will be invited to come along at 8am, having had your last meal at 8pm the previous day.

On completion of 'Study Visit 4' this will complete your full 12 month involvement in the study, and you will **continue your usual medication** under the continued care of your medical team. Please note you may be contacted from time to time by telephone between visits to arrange details of the study.

During this 12 months study you will be invited for a simple fasting blood test, appetite assessment and urine collection at months 1, 2,4,7,8 and 10. These will be short morning appointments, not taking longer than 15 minutes, and will be arranged according to your availability.

It is important to note that we are simply observing your progress on a drug you are already taking as part of the management of your condition.

You are not testing an unlicensed drug, or a drug in which safety profiles are not known. The decision relating to your management remains the decision of your usual medical team.

What are the advantages/disadvantages of taking part?

Your participation will allow us to observe, in detail, the effects of Dapagliflozin on body composition, metabolic health, and fat tissue functioning. This drug is licensed for its use in T2D, **Please note, your participation in the study does NOT change your medical management.**

You will have a detailed assessment of your metabolic profile, as outlined, including blood tests for various metabolic markers including cholesterol. You will also have measurements including blood pressure and body fat estimation as well as estimation of energy expenditure. Abnormal results such as high cholesterol will be sent to your GP for further action. You will have to spend 24 hours during visit 1 and 4 in the metabolic chamber during the study which

may be inconvenient for you. The collection of blood samples is inconvenient but not expected to cause you any harm.

Your stay in the calorimeter, MRI scan (there is no radiation involved in this scan), and collection of urine samples are perhaps a little inconvenient, but do not pose any risk to health. Participants with tattoos can undergo MRI scanning. If you have any metal parts in your body (for example pacemaker) you will not be asked to undergo MRI scanning. MRI is not harmful in pregnancy but you will not be able to take part in this study if you are pregnant. A separate MRI information sheet is provided.

Will I receive any payment for taking part in the study?

You will not be reimbursed for your time in this study. However, if you incur any travelling or parking expenses as a result of participating in this study through attending for blood tests or the more detailed metabolic studies then these will be fully reimbursed.

Will my taking part in this study be kept confidential?

All the information which we collect about you during the course of the study will be kept strictly confidential. Any information that leaves the hospital/university will not have your name and address so that it cannot be traced to you (i.e. will be fully anonymised). In addition, participants will be given coded study numbers, and no names will be used for the storage of samples and data. All information collected during the course of the study will be accessible only to the researchers participating in the study and will be kept on hospital/university computers that can only be assessed by the study researchers using a password. Your GP may be notified of your participation in the study with your consent. The study samples will be stored according to relevant regulations for up to 10 years, in the tissue bank at the University Hospital, Coventry (UHCW). These samples will be analysed in the laboratory and results used for study purposes. These samples and the results of these will not be shared with any individuals outside of the study (with the exception of the below, in that we would share a clinically significant result with yourself, and your GP will receive results of the routine blood tests as normal). At the end of the study, samples will continue to be stored in the UHCW human tissue lab as further assays may be performed on the samples at a later date (again, outlined as a separate tick-box in the signed consent form). Once the samples have reached their agreed storage time (10years), the samples will be destroyed accordingly, based on guidelines and ethical practice applied by the UHCW human tissue lab.

What will happen with the results of the research?

If any of your results have clinical relevance for you (such as raised cholesterol) then we would inform you of these. Your General Practitioner (GP) would receive results of the routine tests. The blood samples for research measurements will be anonymised with a unique patient identification code. The link between a participant's unique identification code and their other data collected during this study will be stored in a secure location within the University Hospital, Coventry. Only the Chief Investigator or a member of his research team will have access to this database. If necessary, analysis of study data may require the assistance of statistical expertise from the University of Warwick – in this event all data will be anonymised to patient identification codes as above, with no personal information transferred.

Depending on the findings of the research the results are likely to be presented in scientific congresses and published in scientific journals, which would be available for public perusal. Your specific personal details will ***not*** appear in the presentations or journals. At the end of the study a general letter on the findings of the study will be sent to subjects involved in the study.

Please note all of your personal and study data will be the responsibility of the chief investigator. All analyses of data will be anonymised to personal identification codes as referenced above. All data will be kept for 10 years as is standard for most studies at the University of Warwick. Confidentiality rules will continue to apply throughout this period, and data will be anonymised if given to any third parties such as statisticians. After 10 years, all data will then be destroyed.

What will happen if I don't want to carry on with the study?

You may withdraw from the study at any time. You may also withdraw your permission relating to the use of any future data and samples. It may not be possible to withdraw samples and data already collected and anonymised, as they may be used but no further samples/data will be collected. All data are totally anonymised. A decision to withdraw from the study will ***not*** influence your treatment either with your GP or at the hospital clinics in any way.

What if there is a problem?

This study is covered by the University of Warwick's insurance and indemnity cover. If you have an issue, please contact the Chief Investigator of the study, Dr Barber (t.barber@warwick.ac.uk). You can also contact PALS (Patient Advice and Liaison Service).

Who should I contact if I wish to make a complaint?

Any complaint about the way you have been dealt with during the study or any possible harm you might have suffered will be addressed. Please address your complaint to the person below, who is a senior University of Warwick official entirely independent of this study:

Head of Research Governance
Research & Impact Services
University House
University of Warwick
Coventry
CV4 8UW
Email: researchgovernance@warwick.ac.uk
Tel: 024 76 522746

Who is organising and funding the research?

This study will be performed at University Hospital (UHCW) in Coventry. Patients will be recruited from this site. The chief investigator is Dr Tom Barber. Commercial funding in the form of a research grant has been obtained, though this study is fully investigator designed and implemented, with no input from funders on the design of the study.

Who has reviewed the study?

Before any research is carried out, it has to be thoroughly checked by an ethics committee. The committee makes sure that the research is appropriate to do in accordance to Good Clinical Practice (GCP) principles, regulations and guidelines. This study has been reviewed and approved by The Leicester South Research ethics Committee which is entirely independent of any hospital trust. It has also been sent to an independent reviewer prior to ethical approval.

Thank you for taking time to read this sheet and for considering participating in this research study. If you decide to participate you will be given a copy of the information sheet and a signed consent form to keep. Please ask any questions if you need to.

Contact for further information:

Dr. Thomas M. Barber – Associate Professor & Honorary Consultant in Endocrinology and Diabetes
Clinical Sciences Research Laboratories
University Hospital Coventry & Warwickshire NHS Trust
Clifford Bridge Road
Coventry, CV2 2DX.
Email: t.barber@warwick.ac.uk

Dapagliflozin Study: Flowchart of Subject Visits to HMRU

Attends WISDEM/OCDEM for out-patient clinic and identified as a potential candidate for the study.
Information sheet provided if they are interested in taking part
Dapagliflozin started on the day of clinic appointment

↓

Consent Visit (Month 0) Discussion with potential candidate regarding the study.
Further information about HMRU and the study provided
Informed consent gained (consent form signed) if interested in taking part.

↓

Study Visit 1 (Month 0, within 2 weeks of starting dapagliflozin): Full metabolic assessment as described above. Measurements taken (height, weight etc), BOD POD body composition measurement. Fasting blood and urine tests taken as described.
Participant enters HMRU metabolic chamber at 8.50 (study begins at 9am), with standard lunch, dinner and supper with two snacks.
Blood tests taken at 10:30am,1:30pm, 4pm, 5pm, and 7:30am (fasting) next morning.
Urine samples collected throughout study.
IDEAL-MRI scan performed on leaving calorimeter in first 8 patients.
Participant leaves and continues on Dapagliflozin.
Patient is seen by the dietitian within the next 4 weeks

↓

Fasting Blood test, urine collection and appetite assessment at months 1 and 2
This is a morning appointment lasting no longer than 15 min

↓

Study Visit 2 (Month 3): This will be 30-45 min visit. We will measure parameters of your metabolic health as above, including measurement in BOD POD®, but there will be no 24 hours study in the HMRU and no MRI scan. Fasting blood tests and urine sample will be taken.
Participant leaves to continue current management plan.

↓

Fasting Blood test, urine collection and appetite assessment at month 4
This is a morning appointment lasting no longer than 15 min

↓

Study Visit 3 (Month 6): This will be 30-45 min visit. We will measure parameters of your metabolic health as above, including measurement in BOD POD®, but there will be no 24 hours study in the HMRU and no MRI scan. Fasting blood tests and urine sample will be taken.

↓

Fasting Blood test, urine collection and appetite assessment at months 7, 8, 10
This is a morning appointment lasting no longer than 15 min

↓

Study Visit 4 (Month 12): This will be a 24 hour study, identical to study visit 1.
Fasted participant enters HMRU metabolic chamber at 8.50 (study begins at 9am), with standard lunch, dinner and supper with two snacks.
Blood tests taken at 8:00am, 10:30am,1:30pm, 4pm, 5pm, and 7:30am (fasting) next morning.
Urine samples collected throughout study.
IDEAL-MRI scan performed on leaving calorimeter in first 8 patients.
All patients to continue with current management as according to usual medical team.
Return to care of usual medical team.

↓

End of involvement.
Summary of findings letter sent to participants after total conclusion of study

Above: *Photograph showing the environment within the Metabolic Chambers/Calorimeters*

HMRU

Below: *Participant in the BOD POD®*

Appendix 1. 3: Informed Consent From

Consent form for participants recruited into the 'Metabolic Response to Dapagliflozin Study' based at HMRU, University Hospitals Coventry and Warwickshire
Principle investigator: Dr. Thomas Barber

Please Insert initials

1. I have read the information sheet on the above project and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being performed and any foreseeable risks involved. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the research team, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I understand that I will be informed about the results of my medical blood tes if any of these have clinical implications for my health. ☐

5. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

6. I give consent for my General Practitioner to be informed of my involvement in this study and for him/her to be informed of the results of the investigations carried
out during this study. ☐

7. I agree that the samples I have given and the information gathered about me can be looked after and stored in the University Hospitals Coventry for use in future studies with relevant ethical approval, as described in the information sheet. ☐

I understand that some of these ethically approved projects may be carried out in the future by researchers other than the study team, including researchers working for commercial companies. However when samples are passed to other investigators, no information that would allow personal identification will be shared.

8. I know how to contact the research team if I need to. ☐

9. I give consent for the Principle Investigator or a member of the research staff Working in the Human Metabolic Research Unit at the University Hospitals Coventry ☐
to re-contact me at a later date regarding my possible involvement in future studies

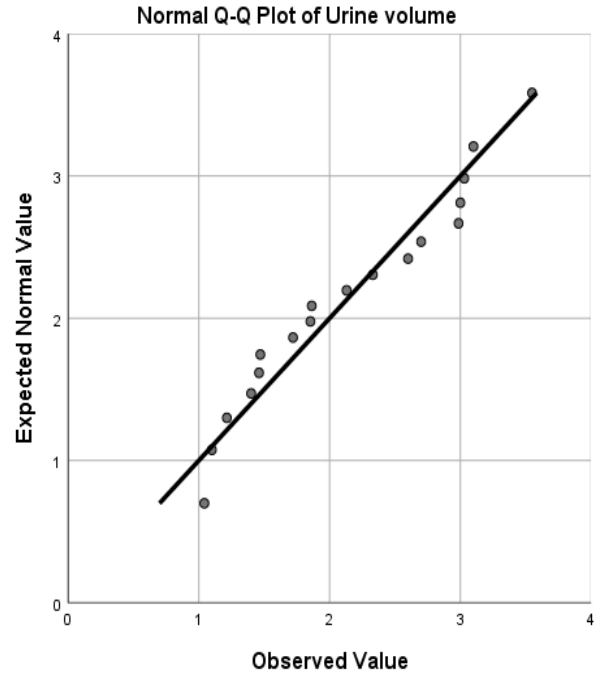
……	…….	…….
Name of participant (BLOCK CAPITALS)	Date	Signature

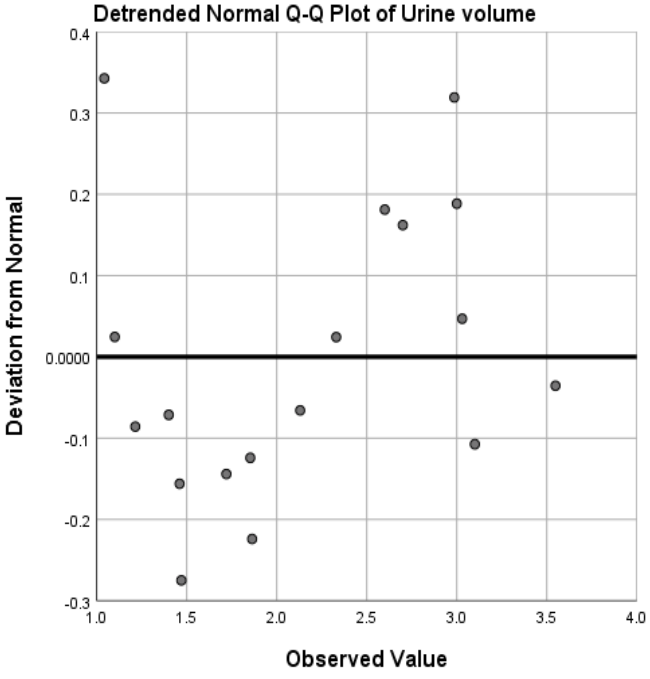
……..	…….	……..
Name of person taking consent (if different from researcher)	Date	Signature

…….	…….	…….
Name of researcher	Date	Signature

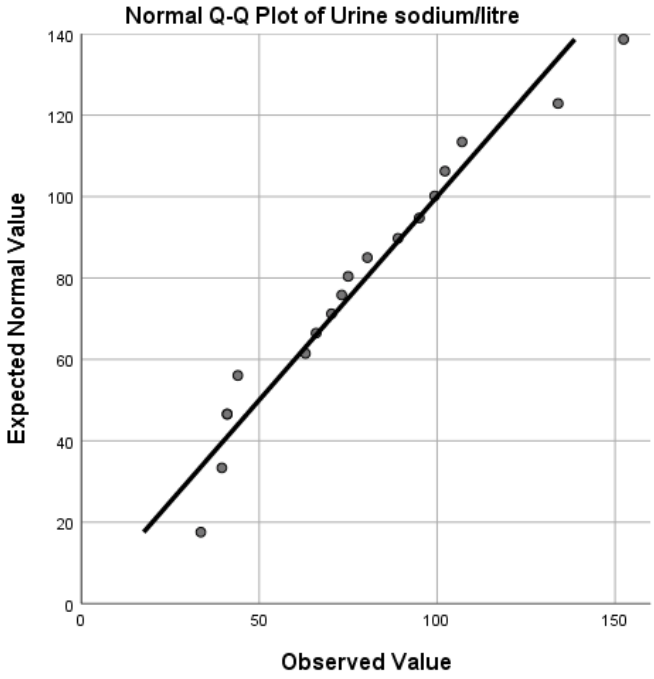
Appendix 1. 4: Q-Q plots for assessment of normal distribution

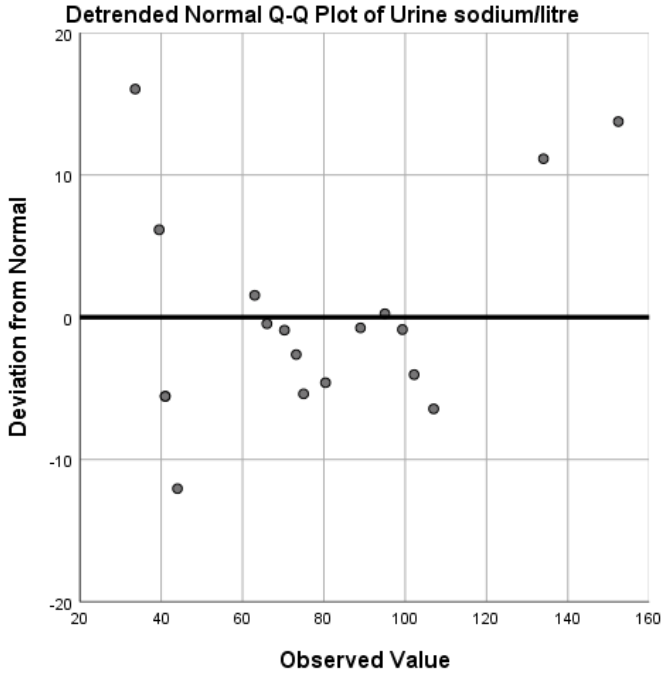
Urine Volume Q-Q plot



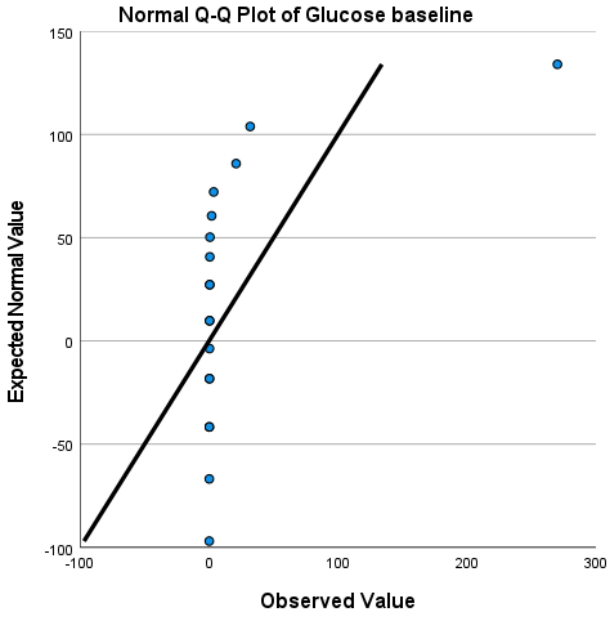


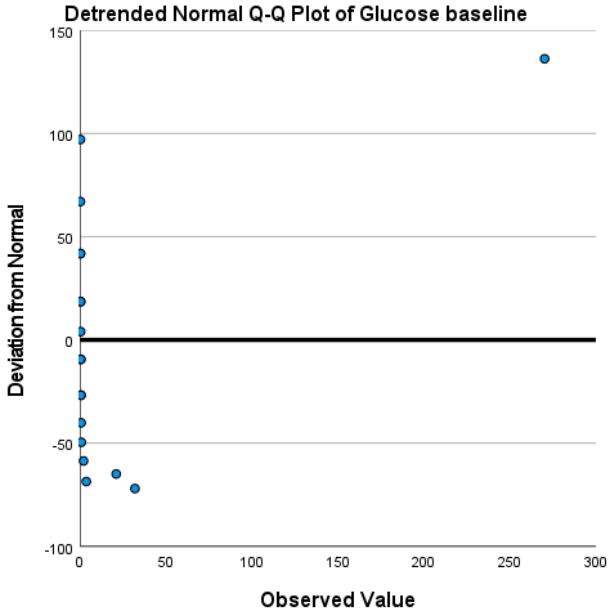
Urine sodium per litre Q-Q plot



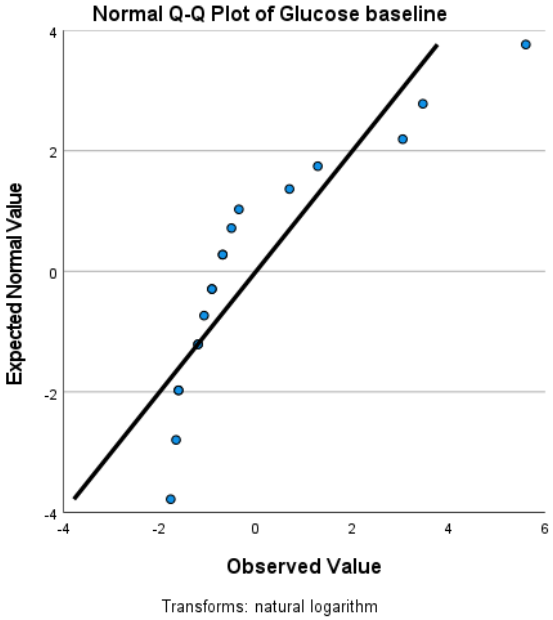


Urine glucose per litre Q-Q plot

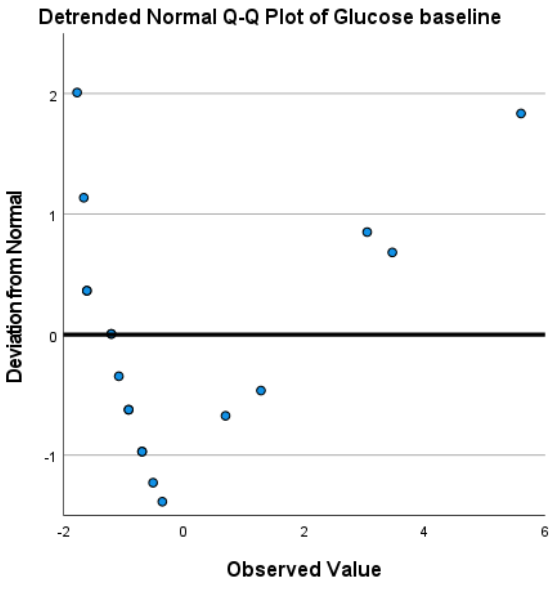




Urine glucose per litre Q-Q plot after logarithmic transformation

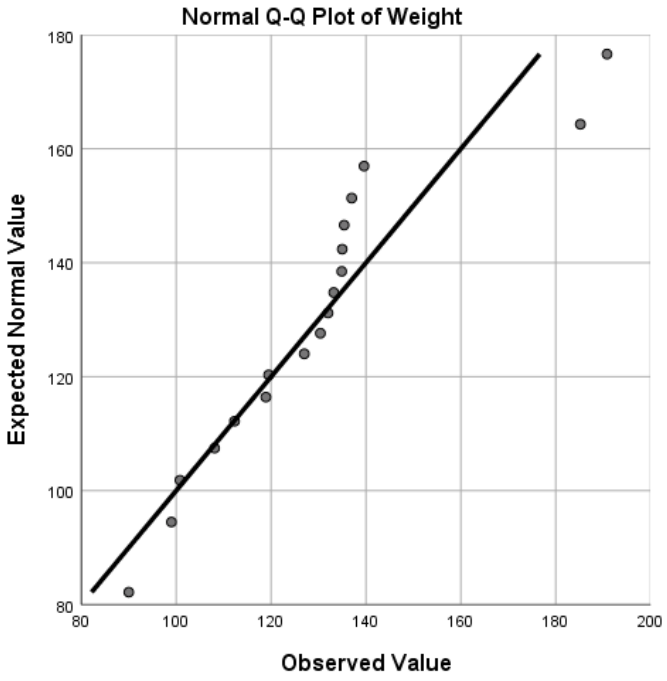


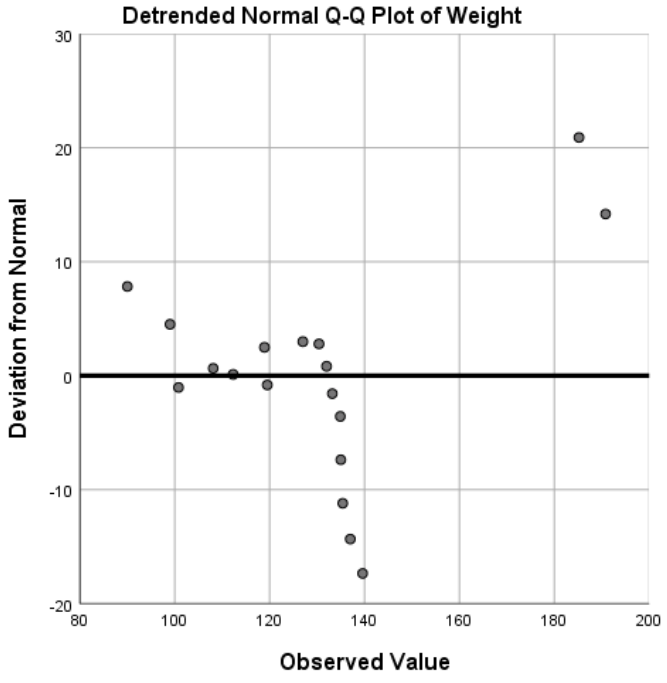
Transforms: natural logarithm



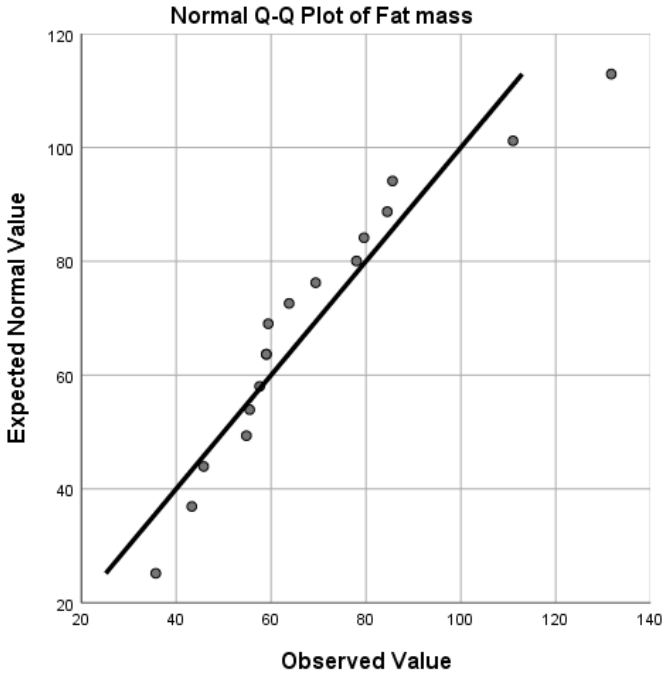
Transforms: natural logarithm

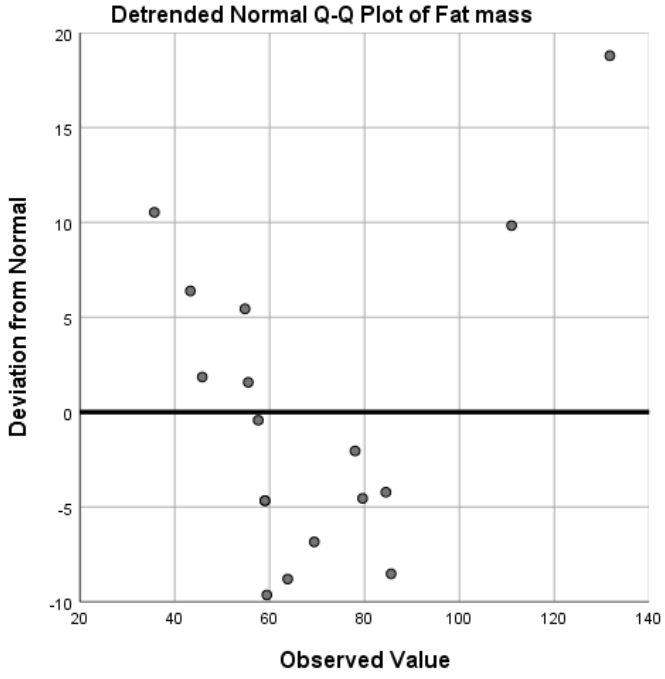
Weight Q-Q plot



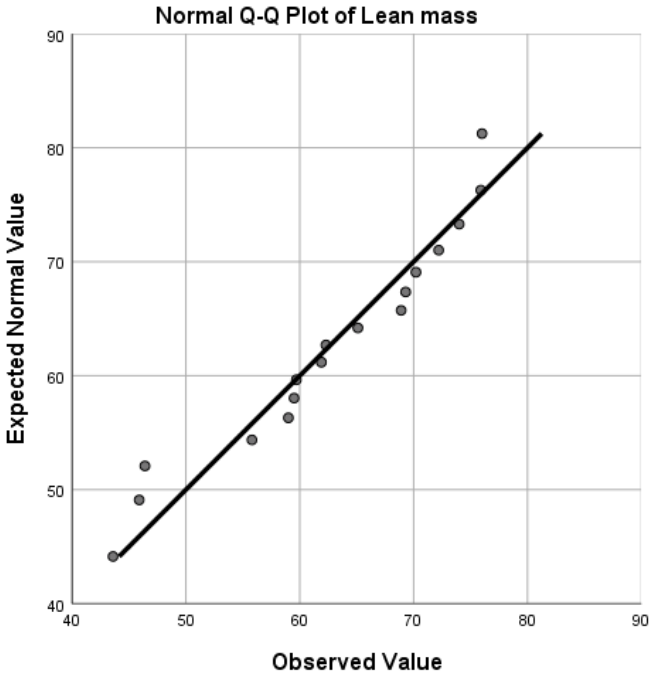


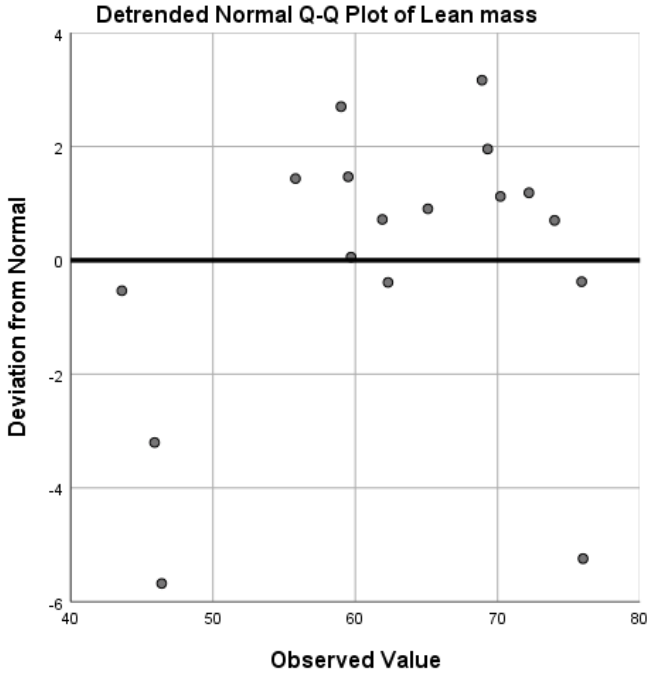
Fat mass Q-Q plot



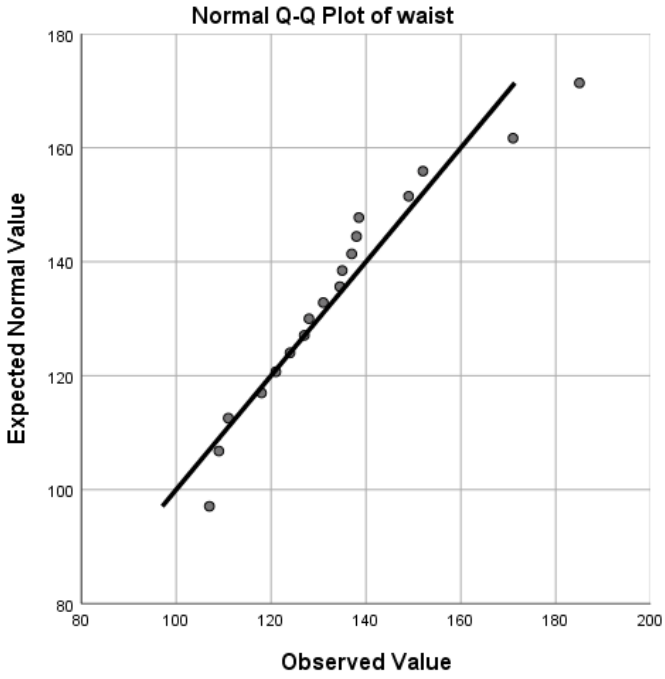


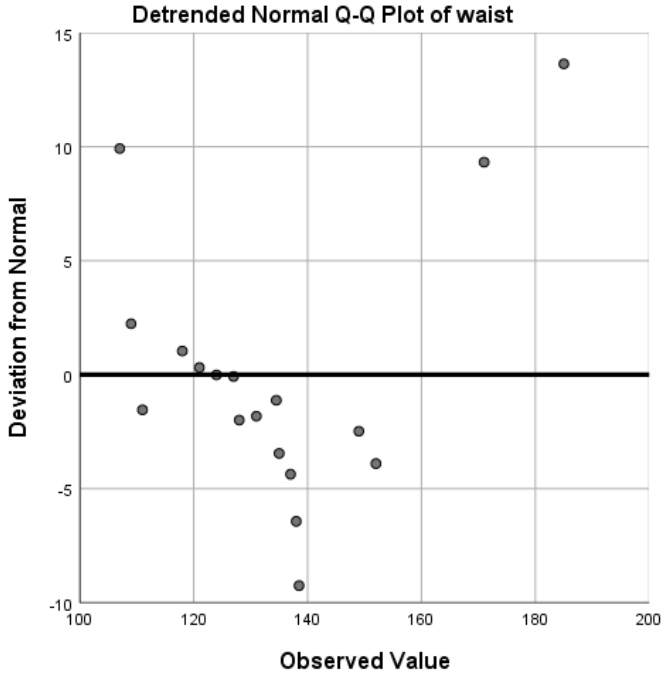
Lean mass Q-Q plot



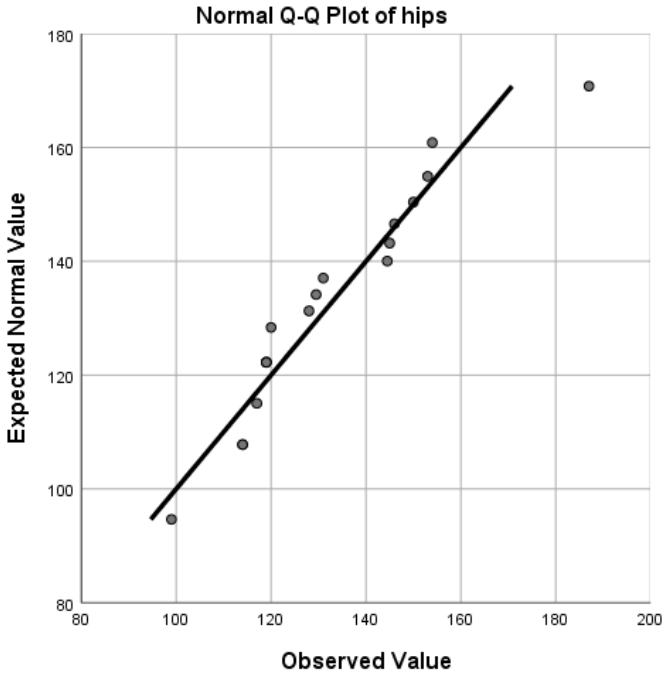


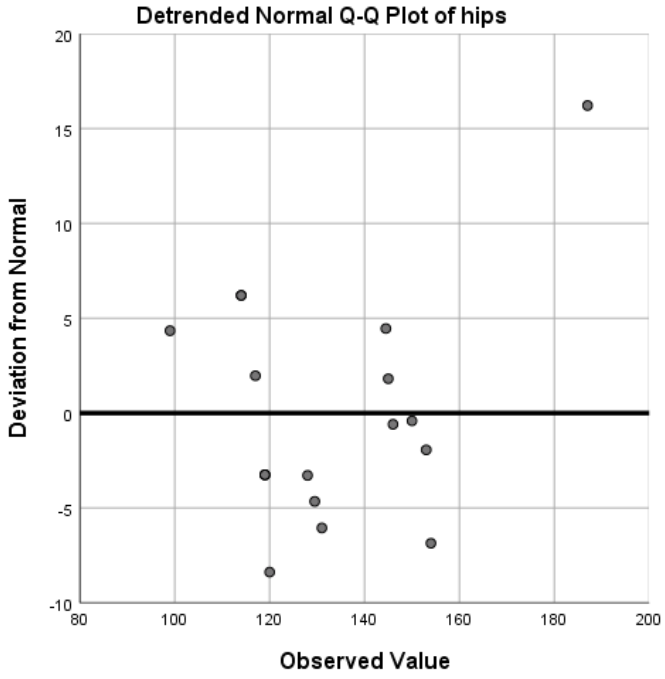
Waist Q-Q plot



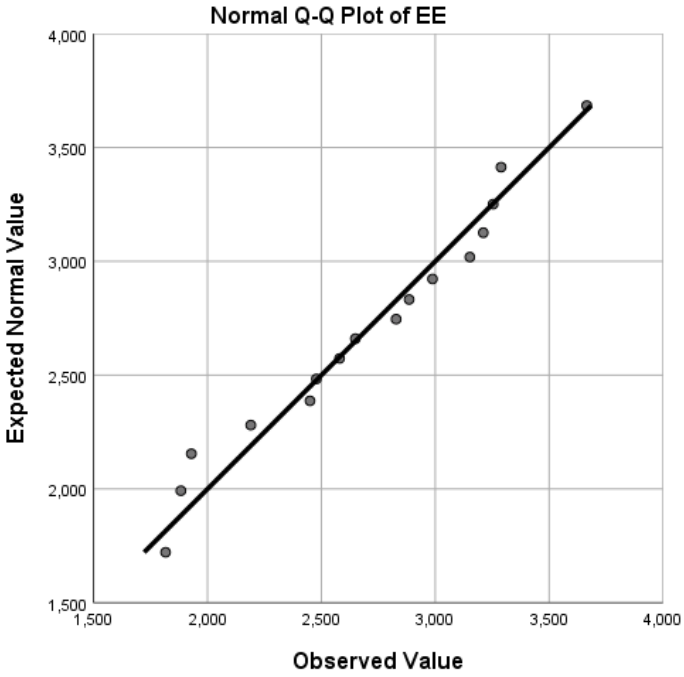


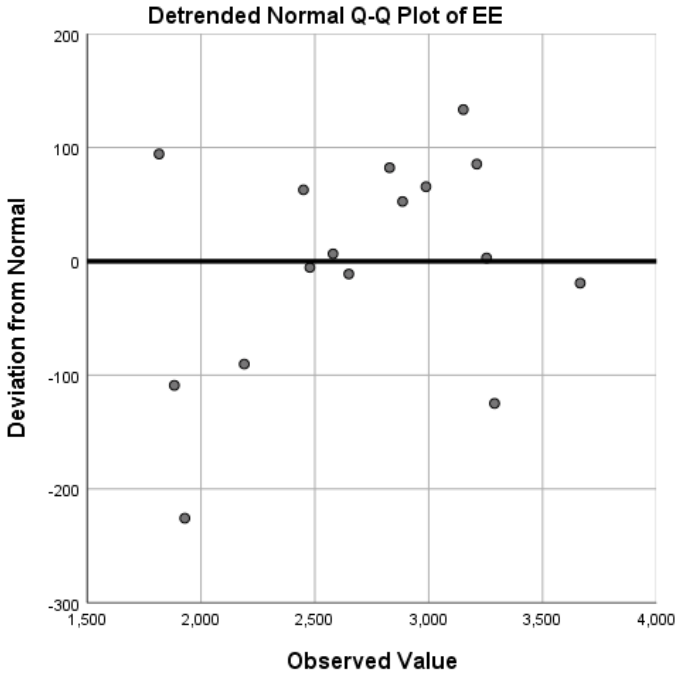
Hips Q-Q plots



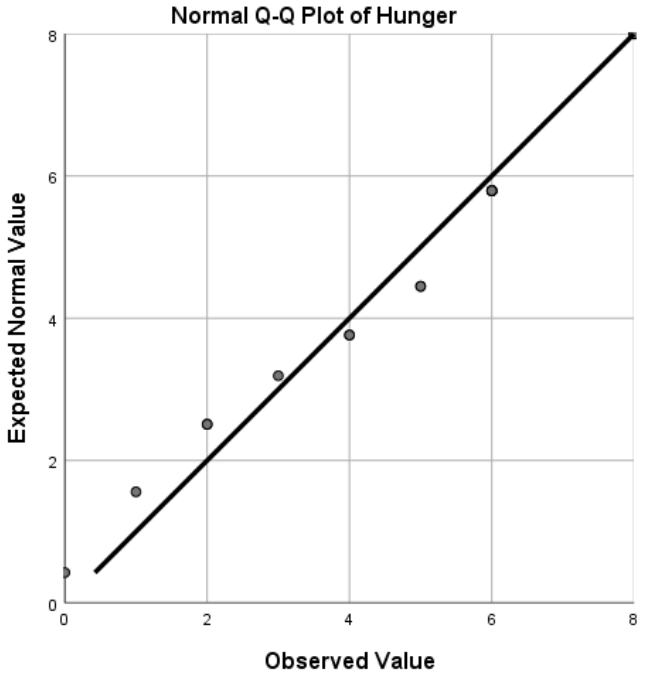


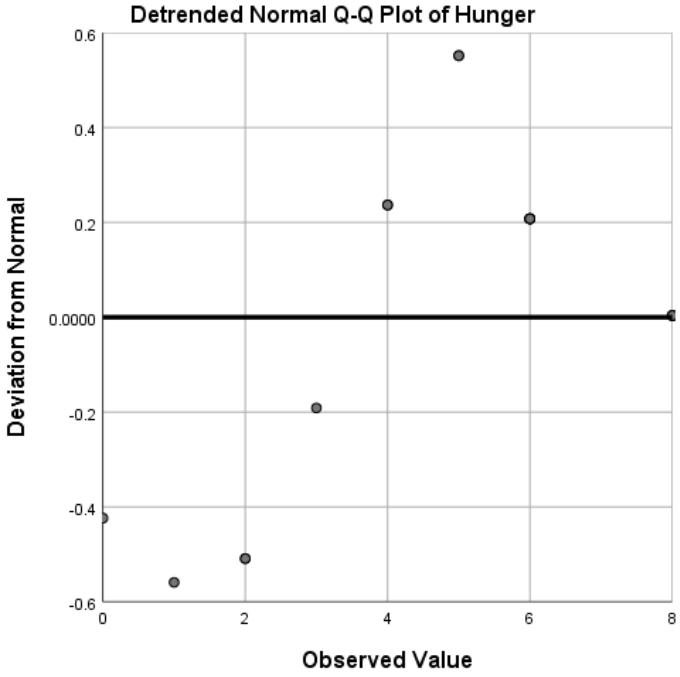
Energy expenditure Q-Q plots



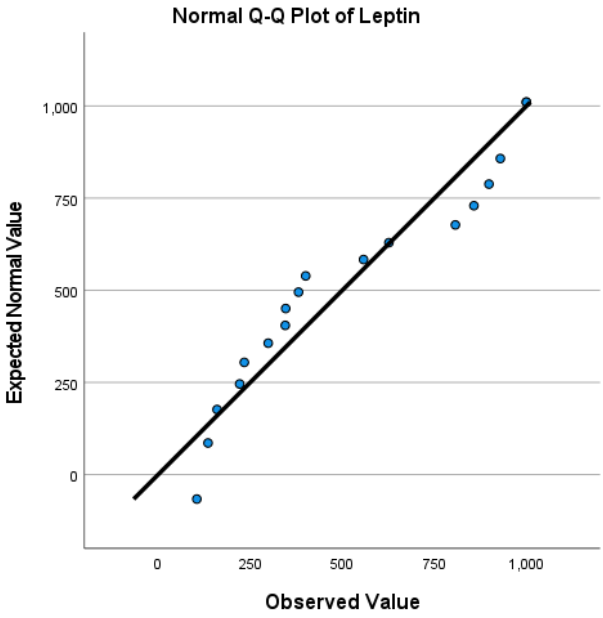


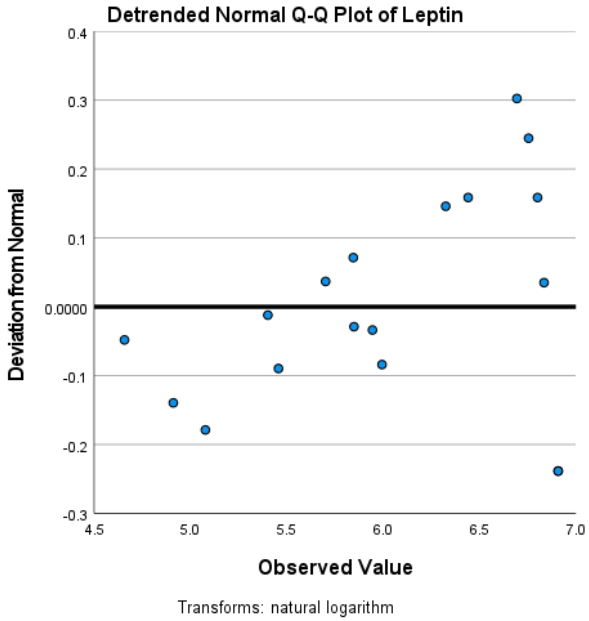
Hunger Q-Q plots





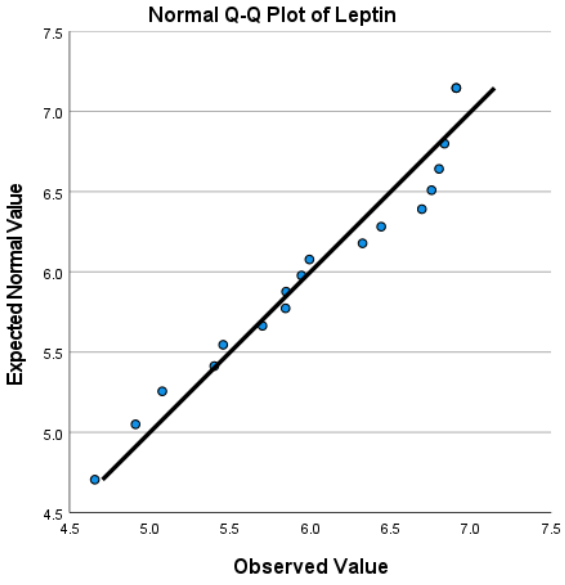
Leptin Q-Q plots



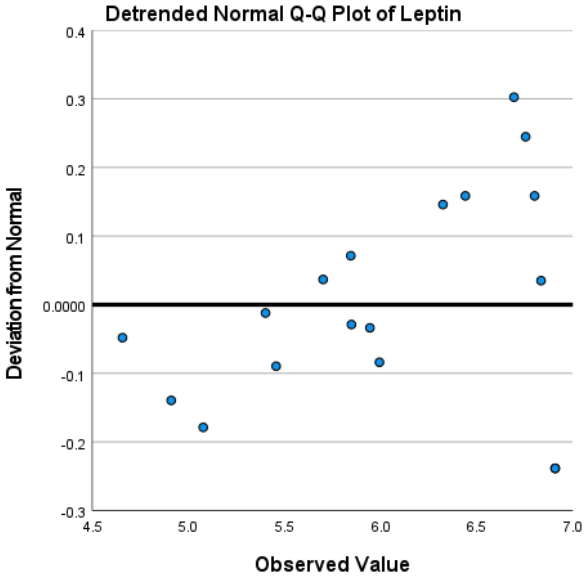


Transforms: natural logarithm

Leptin Q-Q plots after logarithmic transformation

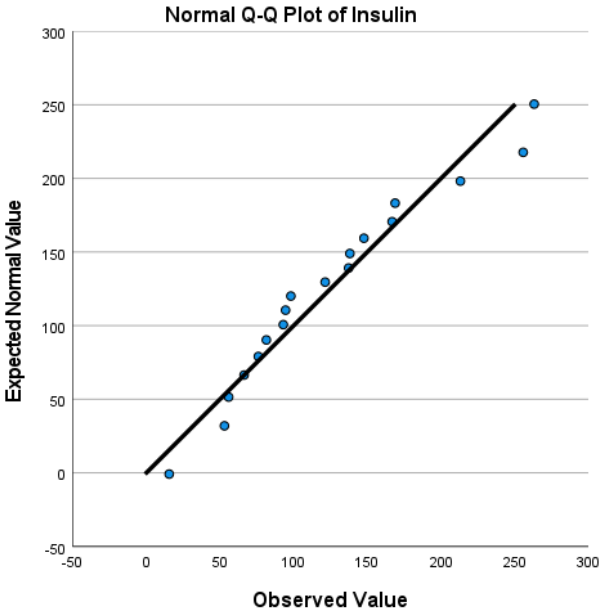


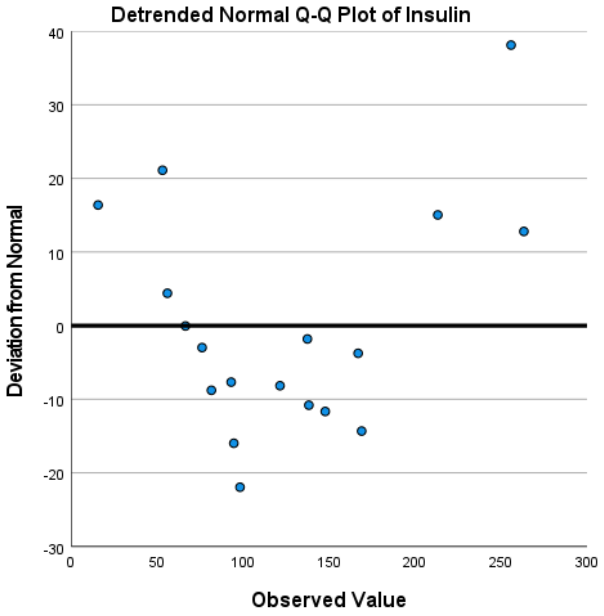
Transforms: natural logarithm



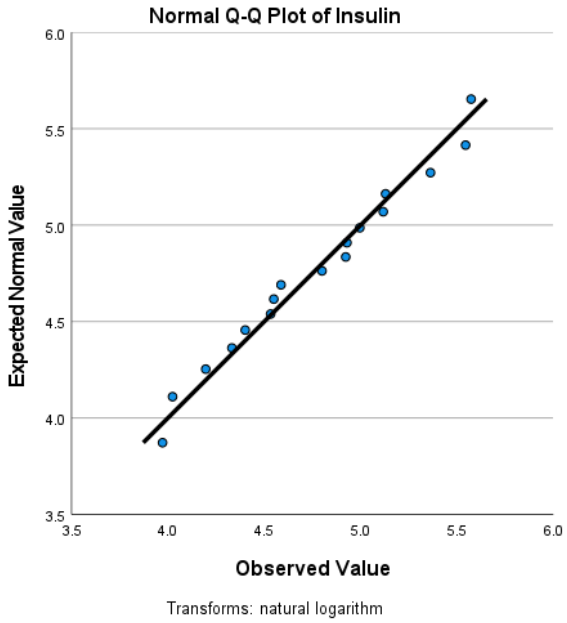
Transforms: natural logarithm

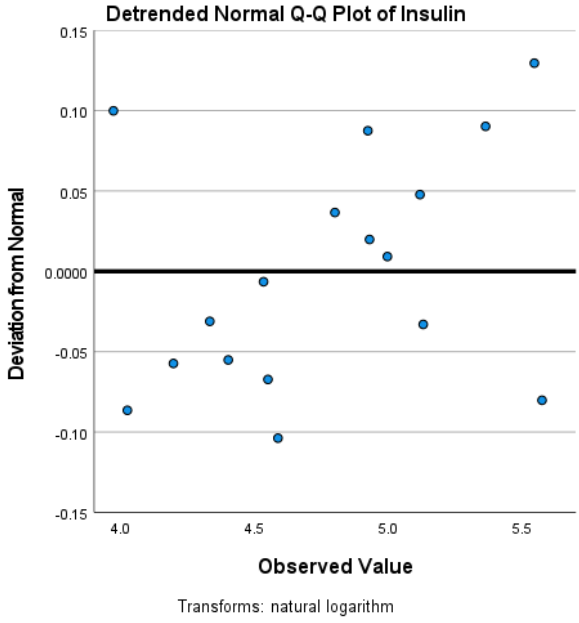
Insulin Q-Q plots



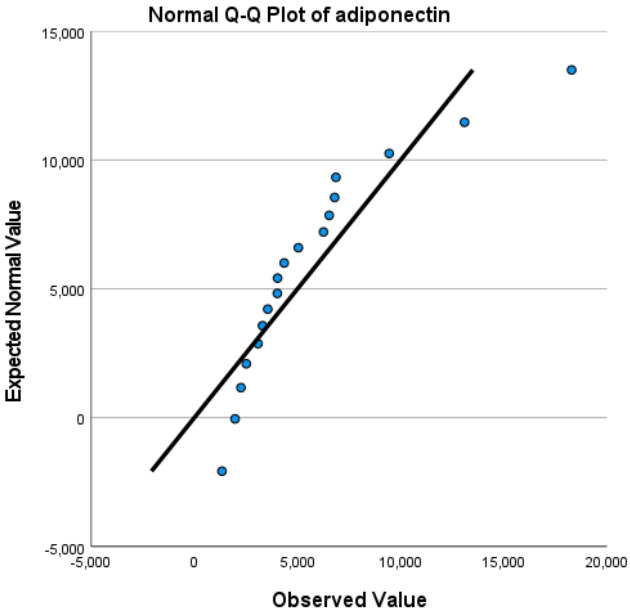


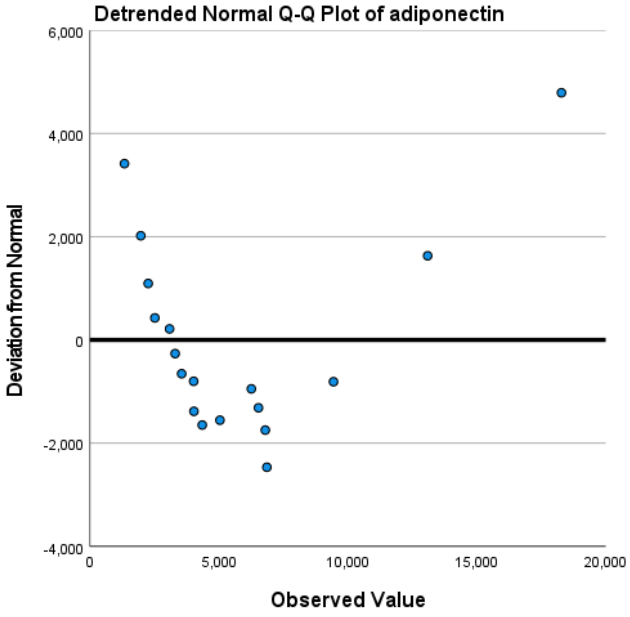
Insulin Q-Q plots after logarithmic transformation



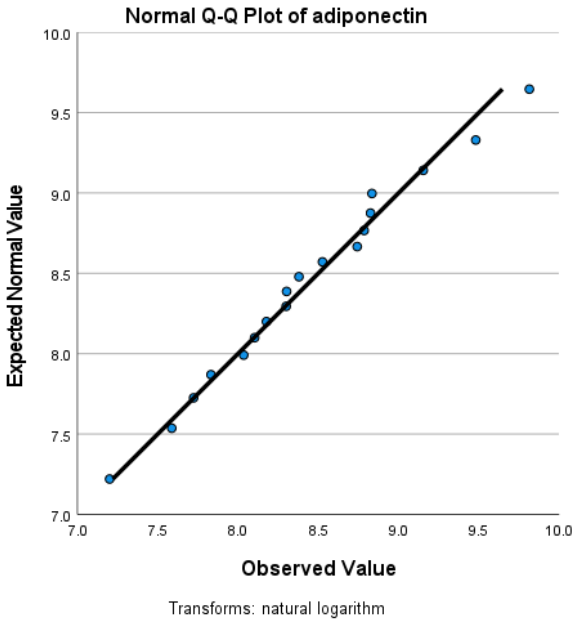


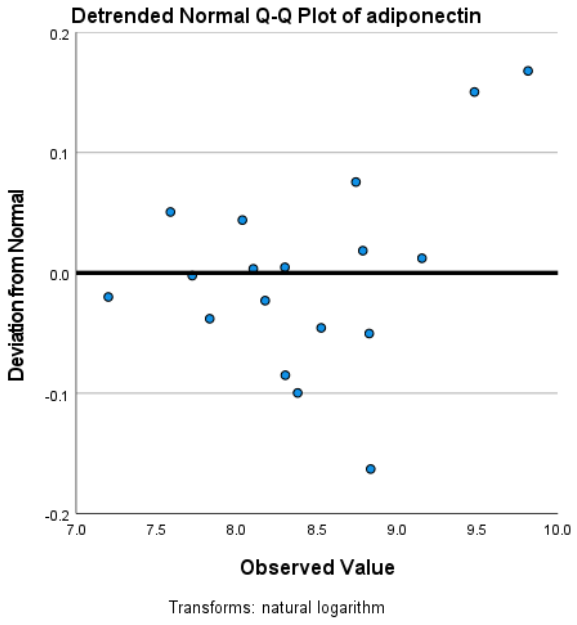
Adiponectin Q-Q plots



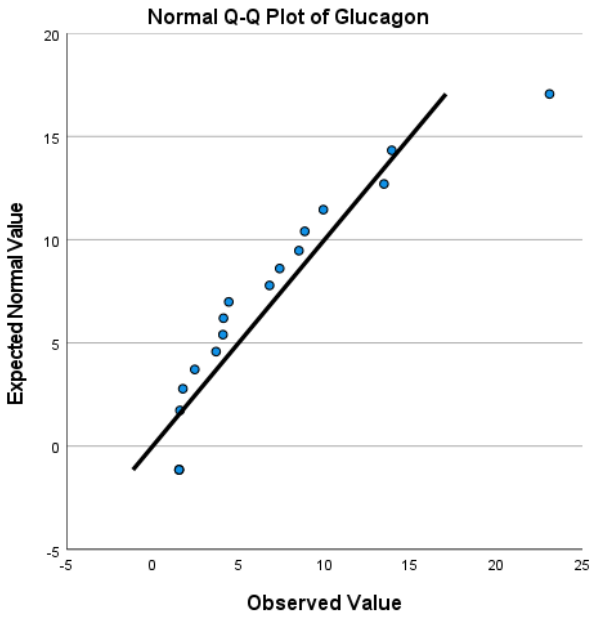


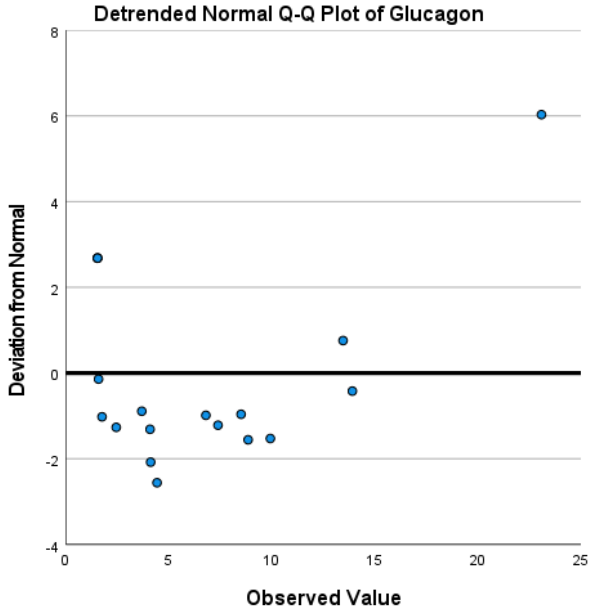
Adiponectin Q-Q plots- after logarithmic transformation



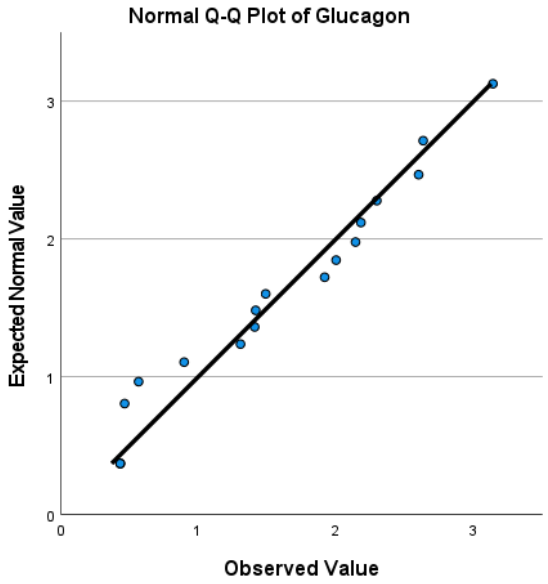


Glucagon Q-Q plots

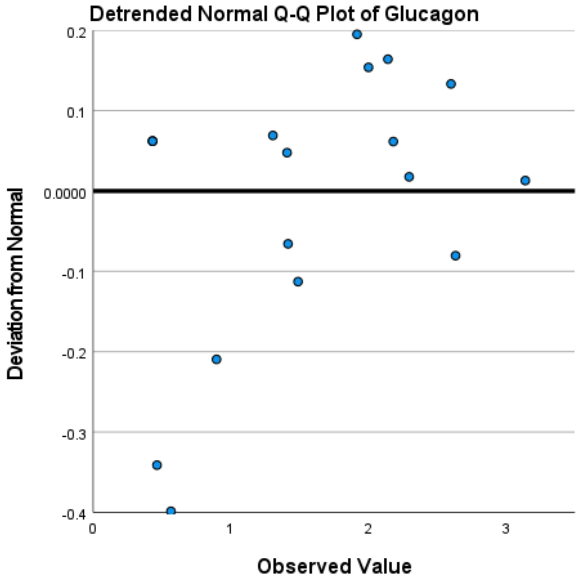




Glucagon Q-Q plots after logarithmic transformation

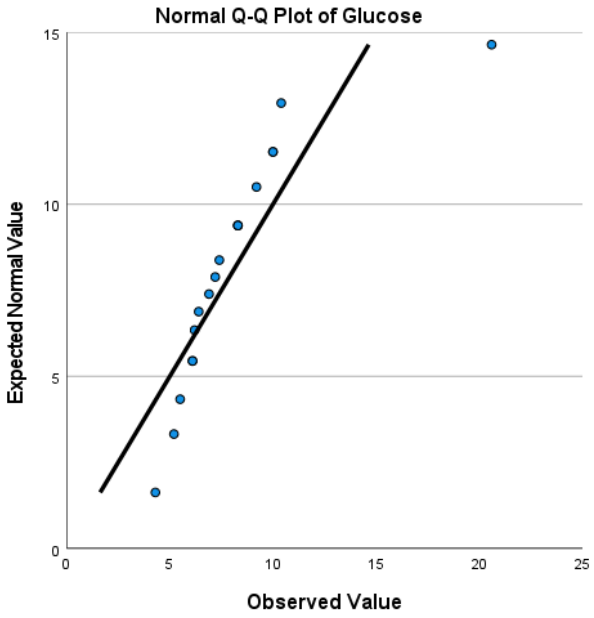


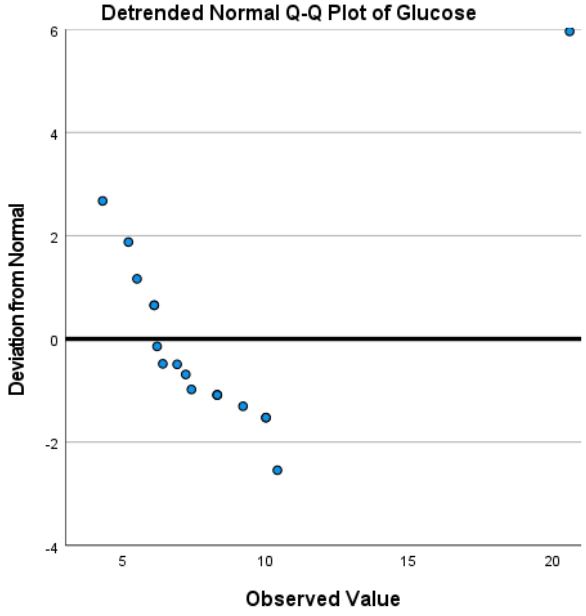
Transforms: natural logarithm



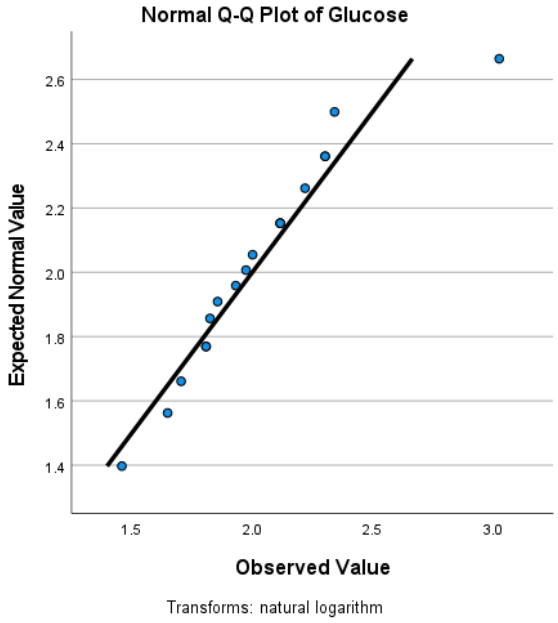
Transforms: natural logarithm

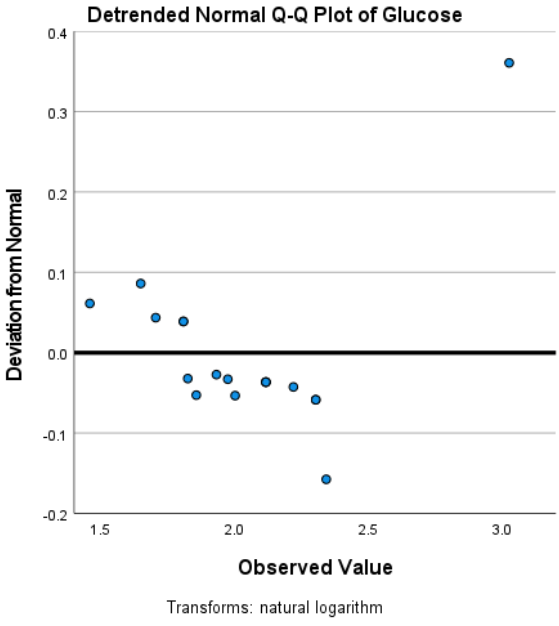
Glucose Q-Q plots



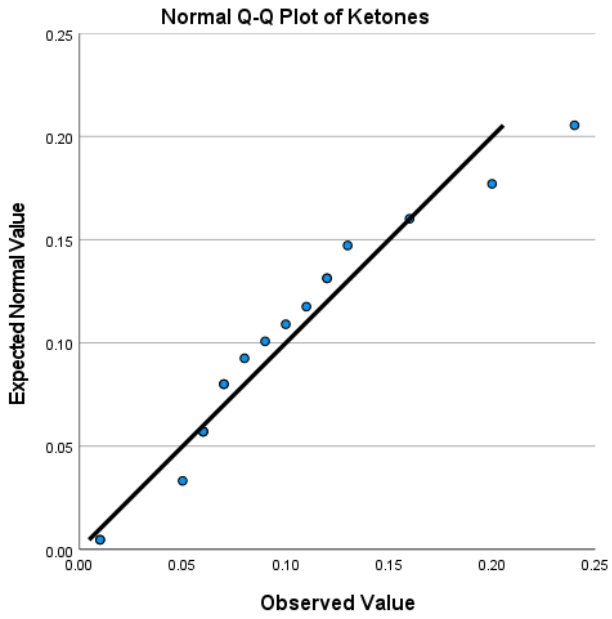


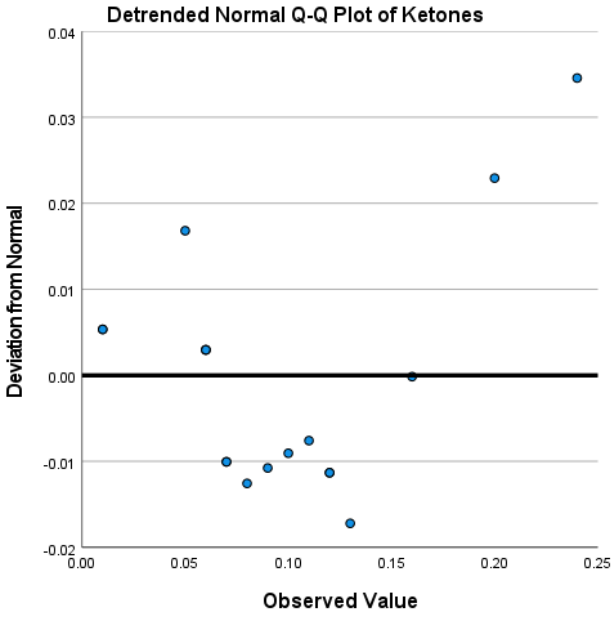
Glucose Q-Q plots after logarithmic transformation



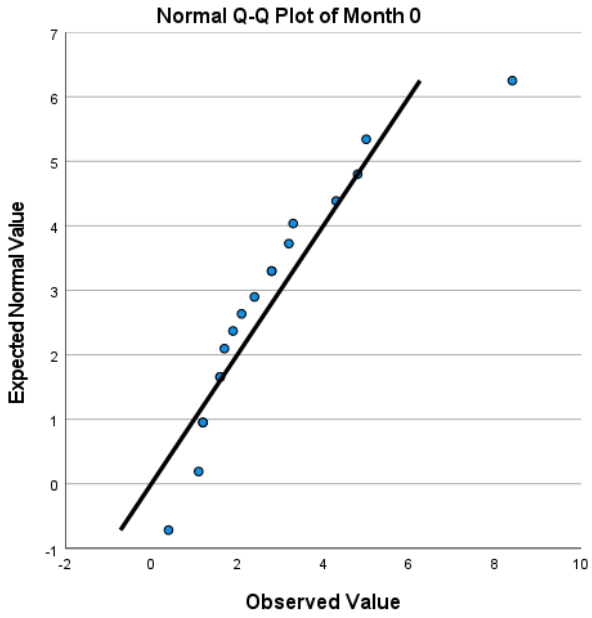


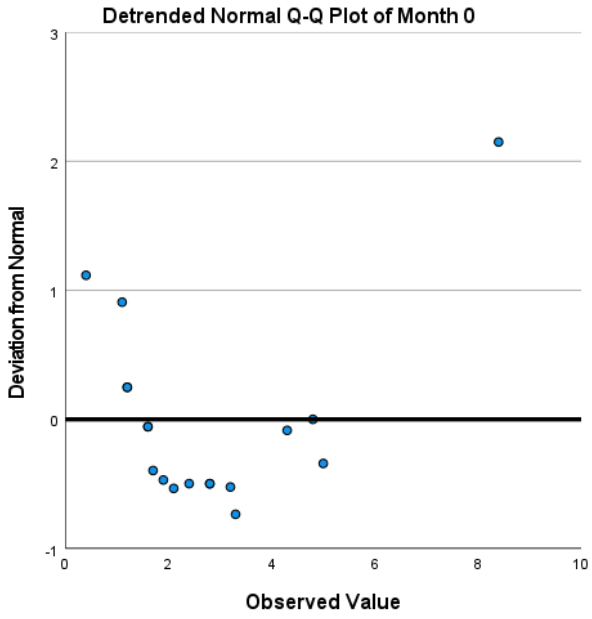
Ketones Q-Q plots



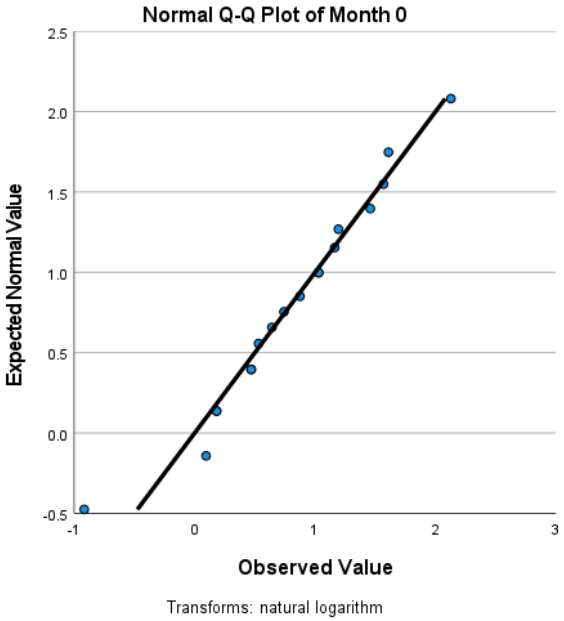


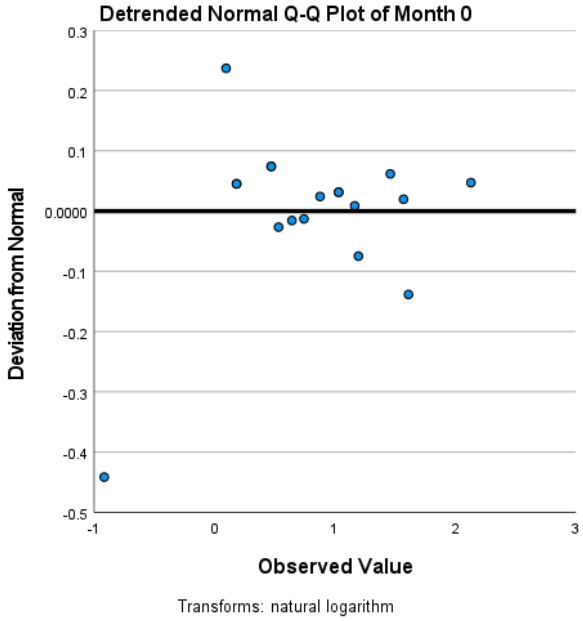
HOMA-IR scores Q-Q plots



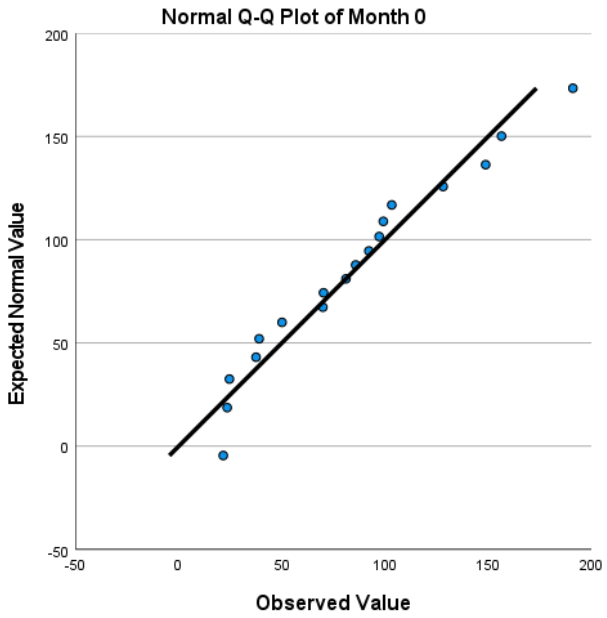


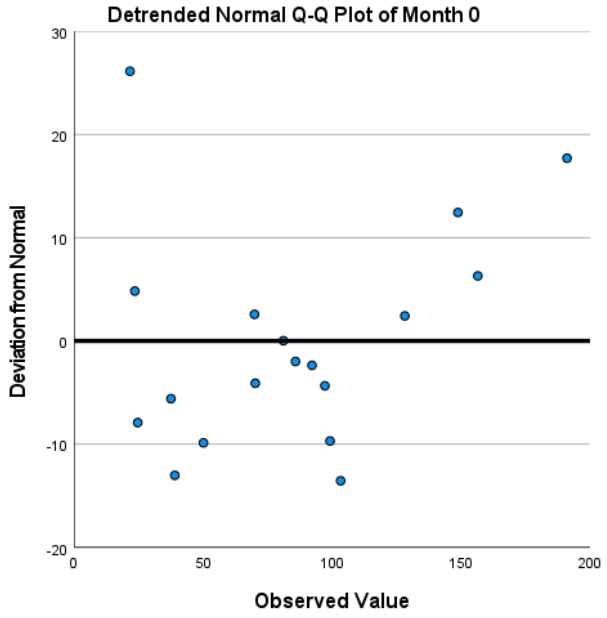
HOMA-IR scores plots after logarithmic transformation



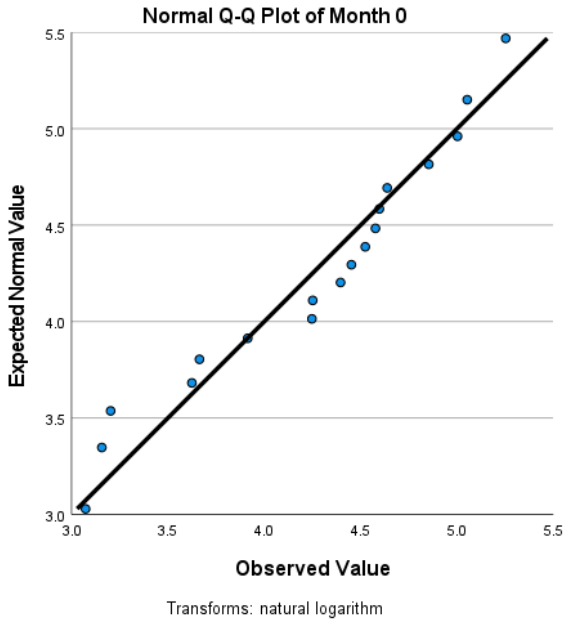


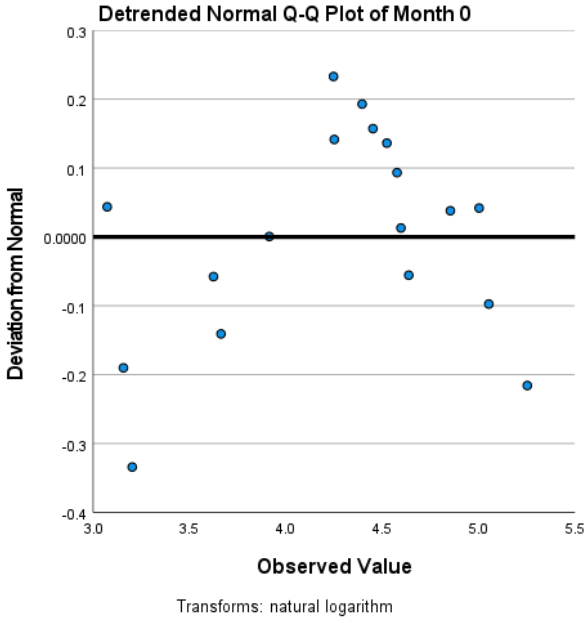
Beta cell function scores Q-Q plots





Beta cell function Q-Q plots after logarithmic transformation





Appendix 1. 5: Visual Analogue Questionnaire

Please note that all scales were 100 mm when used in the study but due to formatting requirements of the Thesis changed slightly.

100 mm Visual Analogue Scale

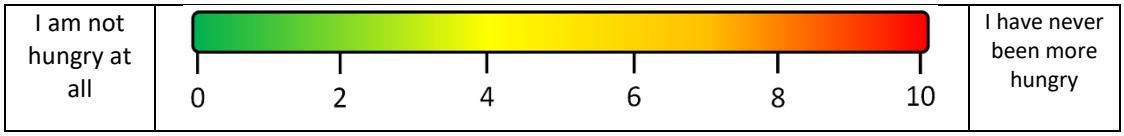
Patient Study number:

Date: **Time:**

Please mark with a pen on each scale depending on how you feel at this moment.

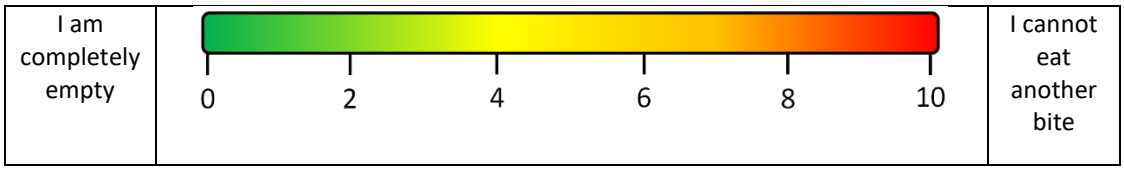
Hunger

How hungry do you feel?



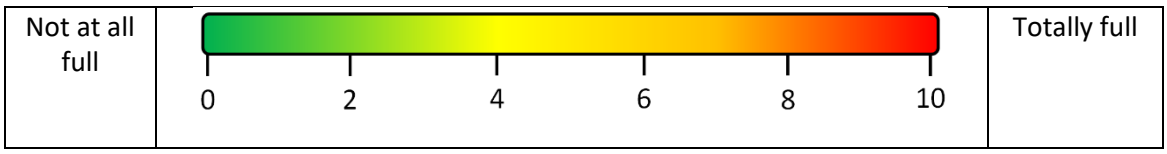
Satiety

How satisfied do you feel?



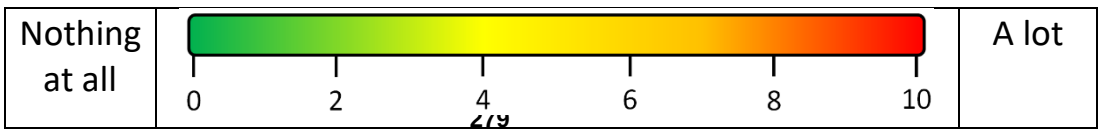
Fullness

How full do you feel?



Appetite

How much do you think you can eat?



Appendix 1. 6: Site approval letter (scanned first page and signature page)

Study Title: Exploring appetitive, metabolic and ketoic effects and weight-loss potential of Dapagliflozin in patients with Type 2 Diabetes Mellitus and inadequate glycaemic control, with concomitant dietary intervention.
Reference: 17/EM/0330

AGREEMENT FOR A PARTICIPATING SITE IN A CLINICAL STUDY

SPONSORED BY THE UNIVERSITY OF WARWICK

This Agreement dated 20th October 2017 is between

University of Warwick, whose administrative

offices are University House, Kirby Corner Road, Coventry CV4 8UW,

(referred to as "Warwick")

AND

University Hospital Coventry and Warwickshire NHS Trust

(referred to as "the Participating Site")

Which are collectively referred to as the "Parties" or individually referred to as a "Party"

Study Acronym	Effects of Dapagliflozin with dietary change on appetite, metabolism and ketosis in T2D
Protocol Title	Exploring appetitive, metabolic and ketotic effects and weight-loss potential of Dapagliflozin in patients with Type 2 Diabetes Mellitus and inadequate glycaemic control, with concomitant dietary intervention.
REC Ref No	17/EM/0330
IRAS ID No.	229929
Sponsor No	University of Warwick SC.80/16-17
Chief Investigator	Dr Thomas Barber

Study Title: Exploring appetitive, metabolic and ketoic effects and weight-loss potential of Dapagliflozin in patients with Type 2 Diabetes Mellitus and inadequate glycaemic control, with concomitant dietary intervention.
Reference: 17/EM/0330

SIGN OFF
Signed by the duly authorised representatives of the Parties on the date stated at the beginning of this Agreement.

SIGNED ON BEHALF OF THE UNIVERSITY OF WARWICK

Dr Sybille Kubis-Waller | Research Support Manager | [redacted] | 1st November 2017

Name | Position | Date

SIGNED ON BEHALF OF PARTICIPATING SITE

Ceri Jones | Head of R&D | [redacted] | 25th October 2017

Name | Position | Date

Appendix 1. 7: Ethics Approval Letter

Copy of the letter

NHS
Health Research Authority

Dr Thomas M Barber
Associate Professor in Clinical Endocrinology and Diabetes
University of Warwick
Clinical Sciences Research Institut
University Hospital
Clifford Bridge Road, Coventry
CV22 5PX

Email: hra.approval@nhs.net

19 September 2017

Dear Dr Barber

Letter of HRA Approval

Study title:	**Exploring appetitive, metabolic and ketotic effects and weight-loss potential of Dapagliflozin in patients with Type 2 Diabetes Mellitus and inadequate glycaemic control, with concomitant dietary intervention**
IRAS project ID:	**229929**
REC reference:	**17/EM/0330**
Sponsor	**University of Warwick**

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England

The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read *Appendix B* carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The document *"After Ethical Review – guidance for sponsors and investigators"*, issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

In addition to the guidance in the above, please note the following:

- HRA Approval applies for the duration of your REC favourable opinion, unless otherwise notified in writing by the HRA.
- Substantial amendments should be submitted directly to the Research Ethics Committee, as detailed in the *After Ethical Review* document. Non-substantial amendments should be submitted for review by the HRA using the form provided on the HRA website, and emailed to hra.amendments@nhs.net.
- The HRA will categorise amendments (substantial and non-substantial) and issue confirmation of continued HRA Approval. Further details can be found on the HRA website.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

IRAS project ID	229929

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **229929**. Please quote this on all correspondence.

Yours sincerely

Kevin Ahmed
Assessor

Telephone: 0207 104 8171
Email: hra.approval@nhs.net

Copy to: *Mrs Jane Prewett, Sponsor Contact, University of Warwick*
Mrs Ceri Jones, R&D Contact, University Hospital of Coventry and Warwickshire

Appendix A - List of Documents

The final document set assessed and approved by HRA Approval is listed below.

Document	*Version*	*Date*
Contract/Study Agreement template	1	30 March 2017
Costing template (commercial projects)	1	11 April 2017
Covering letter on headed paper [Response letter to the REC]	1	07 September 2017
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only)	1	26 July 2016
GP/consultant information sheets or letters	1	27 July 2017
IRAS Application Form [IRAS_Form_12092017]		12 September 2017
Letter from funder	1	11 April 2017
Letter from sponsor	1	27 July 2017
Other [updated clinical trials policy]	2	31 July 2017
Other [Patient information sheet updated]	2	04 September 2017
Other [Generic MRI information sheet and consent]	1	07 September 2017
Participant consent form	2	04 September 2017
Participant information sheet (PIS)	1	27 July 2017
Referee's report or other scientific critique report	1	16 November 2016
Research protocol or project proposal	2	04 September 2017
Summary CV for Chief Investigator (CI)		
Summary CV for student		
Validated questionnaire [Visual Analouge Scale]	1	01 July 2017
2017.09.19 17-0330 FIFO		19 September 2017

IRAS project ID	229929

Appendix B - Summary of HRA Assessment

This appendix provides assurance to you, the sponsor and the NHS in England that the study, as reviewed for HRA Approval, is compliant with relevant standards. It also provides information and clarification, where appropriate, to participating NHS organisations in England to assist in assessing and arranging capacity and capability.

For information on how the sponsor should be working with participating NHS organisations in England, please refer to the, *participating NHS organisations, capacity and capability* and *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* sections in this appendix.

The following person is the sponsor contact for the purpose of addressing participating organisation questions relating to the study:

Name: Mrs Jane Prewett
Tel: 02476522746
Email: sponsorship@warwick.ac.uk

HRA assessment criteria

Section	HRA Assessment Criteria	Compliant with Standards	Comments
1.1	IRAS application completed correctly	Yes	No comments
2.1	Participant information/consent documents and consent process	Yes	No comments
3.1	Protocol assessment	Yes	No comments
4.1	Allocation of responsibilities and rights are agreed and documented	Yes	This is a non-commercial single site study taking place in the NHS where that single NHS organisation's partner University is the study sponsor. Therefore no study agreements are required.
4.2	Insurance/indemnity arrangements assessed	Yes	Where applicable, independent contractors (e.g. General Practitioners) should ensure that the professional indemnity provided by their medical defence organisation covers the

Section	HRA Assessment Criteria	Compliant with Standards	Comments
			activities expected of them for this research study
4.3	Financial arrangements assessed	Yes	External study funding has been secured from Astrazeneca
5.1	Compliance with the Data Protection Act and data security issues assessed	Yes	No comments
5.2	CTIMPS – Arrangements for compliance with the Clinical Trials Regulations assessed	Not Applicable	No comments
5.3	Compliance with any applicable laws or regulations	Yes	No comments
6.1	NHS Research Ethics Committee favourable opinion received for applicable studies	Yes	No comments
6.2	CTIMPS – Clinical Trials Authorisation (CTA) letter received	Not Applicable	No comments
6.3	Devices – MHRA notice of no objection received	Not Applicable	No comments
6.4	Other regulatory approvals and authorisations received	Not Applicable	No comments

Participating NHS Organisations in England

This provides detail on the types of participating NHS organisations in the study and a statement as to whether the activities at all organisations are the same or different.

This is a non-commercial single site study taking place in the NHS where that single NHS organisation's partner University is the study sponsor. Therefore there is only one site type involved in the research.

If this study is subsequently extended to other NHS organisation(s) in England, an amendment should be submitted to the HRA, with a Statement of Activities and Schedule of Events for the newly participating NHS organisation(s) in England.

The Chief Investigator or sponsor should share relevant study documents with participating NHS organisations in England in order to put arrangements in place to deliver the study. The documents should be sent to both the local study team, where applicable, and the office providing the research management function at the participating organisation. For NIHR CRN Portfolio studies, the Local LCRN contact should also be copied into this correspondence. For further guidance on working with participating NHS organisations please see the HRA website.

If chief investigators, sponsors or principal investigators are asked to complete site level forms for participating NHS organisations in England which are not provided in IRAS or on the HRA website, the chief investigator, sponsor or principal investigator should notify the HRA immediately at hra.approval@nhs.net. The HRA will work with these organisations to achieve a consistent approach to information provision.

Confirmation of Capacity and Capability

This describes whether formal confirmation of capacity and capability is expected from participating NHS organisations in England.

The HRA has determined that participating NHS organisations in England that are participating in the study **are not expected to formally confirm their capacity and capability to host this research,** because this is a non-commercial single site study taking place in the NHS where that single NHS organisation has Joint Research Office arrangements in place with the study sponsor.

- The HRA has informed the relevant research management offices that you intend to undertake the research at their organisation. However, you should still support and liaise with these organisations as necessary.
- The document "Collaborative working between sponsors and NHS organisations in England for HRA Approval studies, where no formal confirmation of capacity and capability is expected" provides further information for the sponsor and NHS organisations on working with NHS organisations in England where no formal confirmation of capacity and capability is expected, and the processes involved in adding new organisations. Further study specific details are provided in this *Appendix B* (*Participating NHS Organisations* and *Agreement* sections).

Principal Investigator Suitability

This confirms whether the sponsor position on whether a PI, LC or neither should be in place is correct for each type of participating NHS organisation in England and the minimum expectations for education, training and experience that PIs should meet (where applicable).
A Principal Investigator should be appointed at study sites. GCP training is not a generic training expectation, in line with the HRA statement on training expectations.

HR Good Practice Resource Pack Expectations

This confirms the HR Good Practice Resource Pack expectations for the study and the pre-engagement checks that should and should not be undertaken
As a non-commercial single site study taking place in the NHS where that single NHS organisation's partner University is the study sponsor, it is unlikely that letters of access or honorary research contracts will be applicable, except where local network staff employed by another Trust (or University) are involved (and then it is likely that arrangements are already in place). Where arrangements are not already in place, network staff (or similar) undertaking any of the research activities listed in A18 or A19 of the IRAS form (except for administration of questionnaires or surveys), would be expected to obtain an honorary research contract from one NHS organisation (if university employed), followed by Letters of Access for subsequent organisations. This would be on the basis of a Research Passport (if university employed) or an NHS to NHS confirmation of pre-engagement checks letter (if NHS employed). These should confirm enhanced DBS checks, including appropriate barred list checks, and occupational health clearance. For research team members only administering questionnaires or surveys, a Letter of Access based on standard DBS checks and occupational health clearance would be appropriate.

Other Information to Aid Study Set-up

This details any other information that may be helpful to sponsors and participating NHS organisations in England to aid study set-up.
The applicant has indicated that they intend to apply for inclusion on the NIHR CRN Portfolio.

Appendix 1. 8: Abstract submitted for American Diabetes Association Conference 2019

Abstract category: **314-LB** in category **22-B Obesity–Human**.

Dapagliflozin induced Weight Loss and Metabolic Effects in conjunction with a Low Carbohydrate Diet

Dr Petra Hanson, Prof Paul O'Hare, Prof Harpal Randeva, Dr Thomas M Barber

Type 2 Diabetes Mellitus (T2DM) is closely associated with obesity. Lifestyle modification, including dietary modification, leads to weight loss, a crucial part of management of patients with T2DM. Evidence shows that treatment with Dapagliflozin leads to weight loss. However, there is a lack of in-depth studies exploring the body composition changes induced by Dapagliflozin in patients following low carbohydrate diet.

Our aim was to undertake a detailed assessment of the appetitive and metabolic effects of Dapagliflozin in patients with T2DM. Participants had detailed metabolic studies, including indirect calorimetry (energy expenditure measurements), appetite assessment and quantification of body fat using BodPod in the Human Metabolism Research Unit; University Hospitals of Coventry and Warwickshire (UHCW), UK.

The study duration is for 12 months; interestingly, preliminary data from 10 participants who completed 6 months follow up showed statistically significant weight loss of 5.7 kg at month 6. There was significant decrease in fat mass observed at month 3 (6.3kg) with no difference in muscle mass. There was no difference in energy expenditure before and after 12 months treatment with dapagliflozin. There was no change in self-reported appetite. These results indicate that low carbohydrate diet improves Dapagliflozin's potential for weight loss, with fat loss in particular.

Appendix 1. 9: Abstract submitted for International Society for Magnetic Resonance in Medicine 2021

Sodium NMR relaxation times of human skin as potential biomarkers for Type 2 Diabetes Mellitus

Daria V. Fomina[1,2], Christopher J. Philp[1], Thomas Meersmann[1], Galina E. Pavlovskaya[1,2]

[1]Sir Peter Mansfield Imaging Centre, School of Medicine, University of Nottingham, Nottingham UK

[2]NIHR Nottingham Biomedical Research Centre,
University of Nottingham, Nottingham UK

Elnur G. Sadykhov[3], Olga S. Pavlova[3,4], Nikolay V. Anisimov[4], Alexander M. Makurenkov[3], Yury A. Pirogov[3]

[3] Faculty of Physics, Lomonosov Moscow State University, Moscow, Russia

[4]Faculty of Fundamental Medicine, Lomonosov Moscow State University, Moscow, Russia

Petra Hanson[5,6], Harpal S. Randeva[5,6], J. Paul O'Hare[5,6], Thomas M. Barber[5,6]

[5]Warwick Medical School, University of Warwick, Coventry, UK

[6]Warwickshire Institute for the Study of Diabetes Endocrinology and Metabolism, University Hospitals Coventry and Warwickshire, Coventry, UK

Synopsis

Skin plays an important role in sodium regulation in the human body. As sodium interaction with macromolecules in biological tissue results in a bi-exponential T_2 relaxation, a sensitive characterization of the molecular environment of the sodium ions can be made. This allows us to investigate sodium relaxation times, T_{2short} and T_{2long}, in human skin samples from patients with and without Type 2 Diabetes Mellitus (T2DM) using high-resolution ^{23}Na MRS at high field 9.4 T. We find that there is a significant elongation of T_{2long}

in T2DM patients. This could be due to the altered skin structure of patients with T2DM.

Introduction

In biological tissue, sodium (^{23}Na) experiences bi-exponential T_2 relaxation with components T_{2long} and T_{2short} which account for 40% and 60%, respectively. This is due to the restricted and anisotropic motion created by different macromolecules, which creates conditions not only for dipole-dipole, but also for quadrupole relaxation mechanisms. Loss of tissue anisotropy and decrease of the sodium environment's viscosity will lengthen sodium spin-spin relaxation time. Recently, a new paradigm has appeared that sodium regulation is performed by skin in the human body, along with the kidneys.[1] Skin dermis is enriched with glycosaminoglycans (GAGs) in the extracellular matrix. GAGs provide a high density of negatively charged sites to bind sodium cations and make it osmotically inactive.[2] Type 2 Diabetes Mellitus (T2DM) was chosen as a clinical model to investigate ^{23}Na relaxation in human skin. It is estimated that 463 million patients worldwide have diabetes, and 90% of the cases account for T2DM.[3] This work aims to demonstrate that T_{2short} and T_{2long} of sodium in human skin may serve as a biomarker for the presence of T2DM and provide more insight into the disease's development.

Methods

We studied 13 human skin samples from 6 control patients and 10 samples from 6 patients with T2DM *in vitro*. The skin samples were obtained from the abdomen and foot/leg.The necessary ethics permits were in place for this study. High resolution ^{23}Na MRS, in particular the Carr-Purcell-Meiboom-Gill (CPMG) method, was applied to measure sodium relaxation times in an ultra-high field (UHF) 9.4T spectrometer (Avance III, UltraShield 400WB Plus, Bruker, USA) at a temperature 37°C. The Fourier Transforms of ^{23}Na echoes were integrated in the frequency domain and the integrals were divided by a value of the receiver gain. Assuming a slow exchange between bound sodium in the skin and sodium in the extracellular fluid of the tissue, the CPMG echo train was fitted with bi-exponential decay with offset to obtain T_{2short} and T_{2long} (**Error! Reference source not found.**). Constraints were applied for T_{2short} to b e less than 5 ms, following the literature.[4] The data were analysed for normality

using Kolmogorov-Smirnov and Jarque-Bera tests. After this, an unpaired two-tailed t-test was used to establish a difference between the two groups of patients.

Results

No significant difference was found for T_{2short} and T_{2long} between the abdomen and foot/leg skin samples in the control group of patients (**Error! Reference s ource not found.**a). Therefore, the two skin locations were combined into one control group. Regardless of age, weight, sex of the patients and other pre-existing conditions, T2DM patients had significantly longer sodium T_{2long} than control patients (diabetic: 18.37±1.55 ms, control: 13.69±1.08 ms, $P<0.001$) (**Error! Reference source not found.**b). T_{2short} was found to be indifferent to the presence of the disease (diabetic: 0.83±0.08 ms, control: 0.86±0.13 ms).

Discussion

The main finding of our work can be explained in two ways: (a) there are fewer GAGs in the extracellular matrix of skin in diabetic patients compared to the control group; (b) extracellular volume fraction is increased in the skin of patients with T2DM. In the former case, macromolecule maintenance is shifted towards proteoglycan degradation. In the latter case, sodium balance between intra- and extracellular compartments is disturbed. Both of these situations are indicators of tissue pathology that potentially opens a new insight into the development of Diabetes Mellitus. Our results also show that skin from the abdomen and foot/leg has similar viscous properties.

Conclusion

Sodium T_{2long} in human skin can serve as a biomarker for the presence of T2DM. The data of relaxation measurements on ^{23}Na can guide a therapeutic intervention to reduce drugs' related side effects [5] for T2DM patients. It could also be monitored in the skin non-invasively during treatment to control its efficiency. Proposed methodology and obtained results may help to monitor the efficiency of intervention in patients with other diseases involving sodium homeostasis such as chronic kidney disease and cystic fibrosis.

Acknowledgements. GEP and TM thank the Medical Research Council for funding (Grant No. MC_PC_15074). We would like to thank Sean James from the Arden Tissue Bank at UHCW for his help and support in the coordination

and collection of the skin biopsy samples. The reported study was also funded by Russian Foundation for Basic Research and Royal Society according to the research projects No. 19-29-10015 and 20-52-10004.

Appendix 1. 10: Statistical analyses with log-transformed data for urinary glucose

Urinary glucose data were log-transformed and data re-analysed. Paired student t-test from log transformed data showed statistically significant difference between baseline and month 1. (The difference between month 1 and baseline in urinary glucose per litre was 81.2 mmol, and 144.6 mmol).

Appendix Table 1.10.1: Results from paired student t-test using log-transformed data

Urinary glucose	The difference in means between month 1 and baseline	SD	Lower 95%CI	Upper 95%CI	Sample size	P value
Per 24 hours (mmol/24h)	4.29	3.02	2.55	6.03	14	<0.001
Per litre (mmol/L)	4.30	2.43	3.00	5.59	16	<0.001

Using mixed model analysis of log-transformed data, the difference in urinary glucose excretion per 24 h between baseline and month 12 was statistically significant. At month 12, the urinary glucose per 24 hours will be EXP 3.4 * EXP (12*0.23), which is 472.4 ml. This is summarised below.

Appendix Table 1.10.2 Estimates of Fixed Effects from log-transformed data, where dependent variable is 24-h urinary glucose, from month 0 to month 12

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	3.4	0.3	<0.001	2.76	3.94
Month	0.23	0.04	<0.001	0.16	0.31

SE: Standard error, CI: Confidence interval

Mixed model effect was also applied on the log-transformed data from month 1 to month 12 to see if the difference occurred during the therapy with Dapagliflozin. There was no statistically significant difference in the 24 h urinary glucose excretion between month 1 and month 12. This is summarised below.

Appendix Table 1.10.3: Estimates of Fixed Effects from log-transformed data, where dependent variable is 24-h urinary glucose, from month 1 to month 12

Parameter	Estimate	SE	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	5.23	0.21	<0.001	4.80	5.65
Month	0.02	0.02	0.47	-0.03	0.06

SE: Standard error, CI: Confidence interval

Data for urinary glucose per litre were log-transformed and re-analysed with mixed effect model. The difference in urinary glucose excretion per litre between baseline and month 12 was statistically significant. At month 12, the urinary glucose per litre will be EXP 2.96 * EXP (12*0.19), which is 189.1ml. This is summarised in Appendix Table 1.10.4.

Appendix Table 1.10.4: Estimates of Fixed Effects from log-transformed data, where dependent variable is urinary glucose per litre, from month 0 to month 12

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	2.96	0.25	<0.001	2.47	3.46
Month	0.19	0.03	<0.001	0.13	0.25

SE: Standard error, CI: Confidence interval

Mixed model effect was also applied on the log-transformed data from month 1 to month 12 to see if the difference occurred during the therapy with Dapagliflozin. There was no statistically significant difference in the urinary glucose excretion per litre between month 1 and month 12. This is summarised in Appendix Table 1.10.5.

Appendix Table 1. 10.5: Estimates of Fixed Effects from log-transformed data, where dependent variable is urinary glucose per litre, from month 1 to month 12

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	4.57	0.18	<0.001	4.21	4.93
Month	0.00	0.02	0.931	-0.04	0.04

SE: Standard error, CI: Confidence interval

Appendix 1. 11: Statistical analyses with log-transformed glucagon data

Using mixed model analysis of log-transformed data, there was a statistically significant difference in glucagon during the duration of the study. At month 12, glucagon level was EXP 1.65* EXP (12*0.05), which is 9.4 pmol/L. This is summarised below.

Appendix Table 1.11: Estimates of Fixed Effects from log-transformed data, where dependent variable is glucagon level

Parameter	**Estimate**	**SE**	**P value**	**95% Confidence Interval**	
				Lower Bound	Upper Bound
Intercept	1.65	0.16	<0.001	1.31	1.99
Monthly changes	0.05	0.01	<0.001	0.03	0.08

SE: Standard error, CI: Confidence interval

Appendix 1. 12: Statistical analyses with log-transformed adiponectin data

Using mixed model analysis of log-transformed data, there was no statistically significant difference in adiponectin during the duration of the study. This is summarised below.

Appendix Table 1.12: Estimates of Fixed Effects from log-transformed data, where dependent variable is adiponectin level

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	8.47	0.14	<0.001	8.18	8.77
Monthly changes	0.01	0.01	0.184	0.00	0.02

SE: Standard error, CI: Confidence interval

Appendix 1. 13: Statistical analyses with log-transformed leptin data

Using mixed model analysis of log-transformed data, there was a statistically significant difference in leptin during the duration of the study. At month 12, leptin level was EXP 10.62* EXP (12*-0.02), which is 32 305 pg/ml. This is summarised below.

Appendix Table 1.13: Estimates of Fixed Effects from log transformed data, where dependent variable is leptin level

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	10.62	0.16	<0.001	10.29183	10.95575
Monthly changes	-0.02	0.00	<0.001	-0.02682	-0.00812

SE: Standard error, CI: Confidence interval

Appendix 1. 14: Statistical analyses with log-transformed glucose data

Using mixed model analysis of log-transformed data, there was no statistically significant difference in fasting glucose during the duration of the study. This is summarised below.

Appendix Table 1.14: Estimates of Fixed Effects from log transformed data, where dependent variable is glucose level

Parameter	**Estimate**	**SE**	**P value**	**95% Confidence Interval**	
				Lower Bound	Upper Bound
Intercept	2.00	0.07	<0.001	1.85	2.14
Monthly changes	0.002	0.003	0.647	-0.01	0.01

SE: Standard error, CI: Confidence interval

Appendix 1. 15: Statistical analyses with log-transformed beta cell function data

Using mixed model analysis of log-transformed data, there was no statistically significant difference in fasting glucose during the duration of the study. This is summarised below.

Appendix Table 1.15 : Estimates of Fixed Effects from log transformed data, where dependent variable is degree of beta cell function

Parameter	Estimate	SE	P value	95% Confidence Interval	
				Lower Bound	Upper Bound
Intercept	4.39	0.14	<0.001	4.09	4.68
Month	-0.02	0.01	0.001	-0.04	-0.01

SE: Standard error, CI: Confidence interval