Examining the effects of short-term energy restriction on liver lipid, metabolism and inflammatory status in severely obese adults.

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Abstract

A pre-operative energy restrictive diet is common practice prior to bariatric surgery in the United Kingdom. A review of current practice found variability in form, duration and application of the diet used, with little evidence for efficacy beyond patients successfully undergoing surgery. Imperative for best practice was to provide evidence of the efficacy of dietary approaches both in terms of the original aim of the diet to reduce liver size and improve access to enable laparoscopic surgery, but also to investigate the wider effects of the diet on this population.

A clinical trial was designed to compare two very low energy dietary approaches applied over two weeks before bariatric surgery, using a) a food-based diet, which is standard practice at the Royal Derby Hospital, and comparing this to b) an alternative meal replacement approach with products supplied by LighterLife. The diets were designed to offer similar energy intakes, 800kcal/d, but with varying macronutrient composition and format.

Both diets achieved comparable body weight loss (median -3.4%) and excess body mass index loss (median -1.79kg/m^2). The trial found similar results from both diets for the original aim of the diet, which is to enable surgery. Surgeons' visual assessment of the liver at the time of surgery for operative difficulty was associated to histologically assessed levels of steatosis, with lower levels of steatosis associated to less operative difficulty. Patient evaluation of the diets revealed no significant difference in outcomes between diets, and overall satisfaction of the diets was very high, with 92% and 85% reporting that they were satisfied or very satisfied with their diet respectively. Both diets produced a favourable change in circulating lipids, inflammatory markers and glucose (pooled median LDL -0.4mmol/L, Glucose-0.2mmol/L, CRP -3.6mg/L, IL6 -0.4pg/mL, ELF -0.8). Findings including histology and gene expression data suggest a dynamic lipid environment in both liver and omental adipose tissue post-diet, concurrent with insulin sensitivity, but suggest a less dynamic environment in subcutaneous adipose tissue.

Overall the diets could not be separated in terms of effects on circulating biomarkers, Non-Alcoholic Fatty Liver disease outcomes, operative difficulty, gene expression levels, or patient acceptability. Negative associations with diabetes status were found for steatosis and inflammation post-diet and warrant further investigation. Also, childhood weight status was found to be linked to the level of improvement in insulin sensitivity and requires further work. Limitations suggest additional work should incorporate reporting or recording of physical activity levels, pre-diet assessment of liver condition, diabetes duration and habitual dietary intake. Work should also confirm findings and corroborate suppositions regarding physiological changes observed from mRNA transcript expression levels, into protein and onto functional levels.

This work informs the format and application of a short-term pre-operative diet in severe obesity. It has established that pre-bariatric surgery patients were satisfied with either format of VLED when applied in clinical practice. It has also extended the existing evidence base on the effects of a short-term VLED, specifically the potential beneficial effects on progressive features of NAFLD, and adipose tissue inflammatory status, which could be of value in the management of the disease.

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Disclaimer

Work which has been carried out by people other than myself has been highlighted throughout this thesis. In summary;

1. The survey of practice

I completed all collection, collation and analysis of data. Mr Paul Leeder and Dr Iskandar Idris assisted in writing and formatting the journal article for publication.

2. Design and development of the clinical trial

Professor Michael Larvin, Professor Guru Aithal, Professor Ian Macdonald, Dr Garry Tan, Dr Andrew Bennett and I, were all involved in the design of the clinical trial. I completed all the study documents and regulatory paperwork/applications.

3. Selection and recruitment

A nurse from Derby Hospitals Research & Development team completed the screening process. I completed all recruitment and consent of participants.

4. Collection of data/samples

I collected, processed and recorded all data and biological samples.

5. Blood analyses

All serum and plasma assays described were carried out by Sally Cordon, Karen Swift (University of Nottingham, School of Life Sciences) or Jane Grove (Nottingham Digestive Diseases Centre). ELF marker assays were carried out by an external laboratory, Iqur[1].

6. Tissue analyses

Processing, sectioning and staining of samples was carried out at Nottingham University Hospital NHS Trust, Pathology Department, Research and Development. All histological assessments were carried out by a single Hepatobiliary Histopathologist Dr P Kaye (as above). I carried out all RNA and Taqman work.

7. Statistics and analyses

I carried out all randomisations, statistical and analytical work.

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[1] iQur is an independent reference laboratory based at Windeyer Building, University College London

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Abbreviations

Adi	Adiponectin (assay)
AE	Adverse Event
ALT	Alanine aminotransferase
AST	Aspartate amino transferase
BHB	Betahydroxybutyrate
BOMSS	British Obesity and Metabolic Surgery Society
BMI	Body mass index
cc	Correlation coefficient
CI	Chief Investigator overall
CK-18	Cytokeratin-18
CRF	Case Report Form
CRP	C-reactive protein (high sensitivity assay)
CV	Coefficient of variation
DM	Type 2 diabetes
EFSA	European Food Safety Authority
ELF	Enhanced liver fibrosis (test)
EnR-Lin	Energy restriction – Liver lipid and inflammation
FD	Food-based diet
Fet	Fetuin-A (assay)
FM	Fat mass
GCP	Good Clinical Practice
Glu	Glucose (assay)
GGT	Gamma glutamyl transpeptidase
HA	Hyaluronic acid
HDL	High density lipoprotein
HOMA-IR	Homeostasis model assessment – insulin resistance
IL-6	Interleukin-6
IFG	Impaired fasting glucose
Ins	Insulin (assay)
IPA	Ingenuity Systems pathway analysis software

kcal	Kilocalories*
LED	Low energy diet
Lep	Leptin (assay)
LDL	Low density lipoprotein
MM	Muscle mass
MRP	Meal replacement plan
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NBSR	National Bariatric Surgery Registry
NEFA	Non-esterified fatty acids
NHS	National Health Service
OAT	Omental adipose tissue
P	Statistical significance of result
PIIINP	Amino-terminal pro-peptide of type 3 collagen
REC	Research Ethics Committee
SAE	Serious Adverse Event
SAT	Subcutaneous adipose tissue
TAG	Triacylglycerol/triglyceride
TNF	Tumour necrosis factor alpha
TIMP-1	Tissue inhibitors of metalloproteinase-1
VLCD	Very low calorie diet
VLED	Very low energy diet
VLDL	Very low density lipoprotein

* kcal is the unit of energy used throughout this thesis as the current convention in dietetic practice in the UK. 1kcal is equivalent to 4.184kJ

Chapter 1

Introduction

This thesis describes work undertaken to examine the effects of short-term very low energy diets in the severely obese population. The introduction examines the main subject areas individually,

1. Obesity and adipose tissue function,

2. Non-alcoholic fatty liver disease (NAFLD),

3. Clinical application of a very low energy diet.

Following this the overlapping areas of research are brought together and focussed on reversal of metabolic dysfunction with acute energy restriction. The introduction then outlines the objectives of the work that was undertaken, and how it addresses the gaps in current evidence.

Obesity

Aetiology

Body weight, and therefore obesity, is determined by interaction between genetic, external environmental and psychosocial factors, acting through the physiological regulation of energy intake and expenditure (Kopelman, 2007). For obesity to develop requires a positive energy balance, when energy intake is greater than expenditure, which promotes energy storage and body weight gain (WHO, 2000). There are many influences on body weight control, both internal and external. The physiological processes responsible for weight regulation include signalling mechanisms from the intestine and adipose tissue that are coordinated in the brain. These mechanisms influence eating behaviour, e.g. appetite control, and metabolism, e.g. energy storage (WHO, 2000). Single-gene defects causing obesity are rare in humans; however severe obesity is a feature of several inherited syndromes, with the most common Prader-Willi syndrome. Children presenting with this gene mutation develop a voracious appetite leading to severe obesity (Baqai and Wilding, 2015).

In considering the genetic contribution to obesity development, there is evidence from family studies that the propensity to develop obesity is partly inherited. Both body weight and body fat distribution have heritable influences, and can account for >20% of variation in body mass index (BMI) and waist-hip ratio. Pathway analyses support a role for the central nervous system in susceptibility to obesity and links to insulin secretion and action, energy metabolism, lipid biology and adipogenesis. As a consequence, this susceptibility could affect fat distribution and function, and pathophysiological mechanisms. 75 gene loci have been identified to date that could influence body weight, including the fat mass and obesity associated (FTO) gene which is thought to have the greatest influence of those identified so far. However, its presence is only related to a potential increase in weight of 1.5kg, and therefore it appears that even the most influential genotype, currently acknowledged, contributes little to the overall heritability of body weight (Locke et al., 2015, Shungin et al., 2015, Baqai and Wilding, 2015).

Defining obesity

Obesity in humans is most significantly characterised anatomically, by an increase in fat mass in relation to fat-free mass, as a consequence of a period of excessive energy intake

relative to expenditure. This fat mass expansion is seen in various adipose tissue depots throughout the body (Tan and Vidal-Puig, 2008, Ouchi et al., 2011).

Body weight can provide an indication of fat stores, but because body build and composition are extremely variable, measurement of body weight is not a direct indicator of adiposity. Alternative measurements such as BMI are used to classify weight status and related disease risk, although BMI does not account for differences in body fat distribution or muscle mass (WHO, 1995, WHO, 2008).

Body mass index (BMI)

The calculation of BMI is a widely accepted method used to aid the definition of health risks associated to weight status. It is calculated by dividing an individual's body weight measurement (in kilograms) by the square of their height (in metres) (WHO, 1995). BMI classifications are age and gender independent. They are used as a crude tool to define relative health risks, however, the interpretation of BMI categories in relation to risk may differ for different populations, and limitations to the use of BMI relate to the non-conformity of body weight as a representation of fat mass and distribution. For example, different ethnic groups will have alternative levels of associations between BMI and health risks due to percentage of body fat and body fat distribution across their population (WHO, 2008, WHO, 2004, WHO, 2014).

An example of the BMI classifications follow in Table 1, which shows the current BMI categories, as defined by the World Health Organisation (WHO), for a white European population. The "normal range" is associated with the lowest health risks.

Table 1 international classification of adult body weight according to body mass index (BMI)

(WHO, 1995, WHO, 2000, WHO, 2004, WHO, 2014, WHO, 2008)

Classification BMI	**(kg/m^2)**
Normal range	18.5–24.9
Overweight	25–29.9
Obesity I	30–34.9
Obesity II	35–39.9
Obesity III	40 or more

National Institute for Health and Clinical Excellence (NICE) guidance suggests that for the management of overweight and obesity in adults, BMI should be used to classify the degree of obesity in order to determine the health risks (NICE, 2006), but that this should be interpreted in conjunction with other anthropometric measures, such as waist circumference, in order to better identify risk (IC, 2011, WHO, 2008, NICE, 2014). An additional burden of disease can be categorised by high BMI and a high waist-circumference[2]. Both measures are used as the distribution and location of adipose tissue affects the risks associated (Lean et al., 1995, Ardern et al., 2004). Table 2 below from NICE 2014, for the health risks categorised by BMI and waist circumference.

Table 2 BMI and waist circumference classification, with associated health risks (NICE 2014)

Table 2	Waist circumference		
BMI classification	Low	High	Very high
Overweight	No increased risk	Increased risk	High risk
Obesity	Increased risk	High risk	Very high risk
For women, waist circumference of less than 80 cm is low, 80–88 cm is high and more than 88 cm is very high.			
For men, waist circumference of less than 94 cm is low, 94–102 cm is high and more than 102 cm is very high.			

[2] The upper level of waist circumference (94 cm for men and 80 cm for women) represents a threshold above which health risks are increased (Lean, Han *et al* 1995, Ardern, Janssen *et al* 2004)

Prevalence of obesity

Data from the Organisation for Economic Cooperation and Development (OECD) show the prevalence of obesity is increasing in adults worldwide(Figure 1) (OECD, 2014). In England, the prevalence of adult obesity rose from 14.9% to 25.6% between 1993 and 2014. An increase in overweight and obesity means that the proportion of adults in England with a healthy BMI (18.5 - 24.9) decreased between 1993 and 2014 from 41.0% to 32.7% among men, and 49.5% to 40.4% among women (HSCIC, 2015).

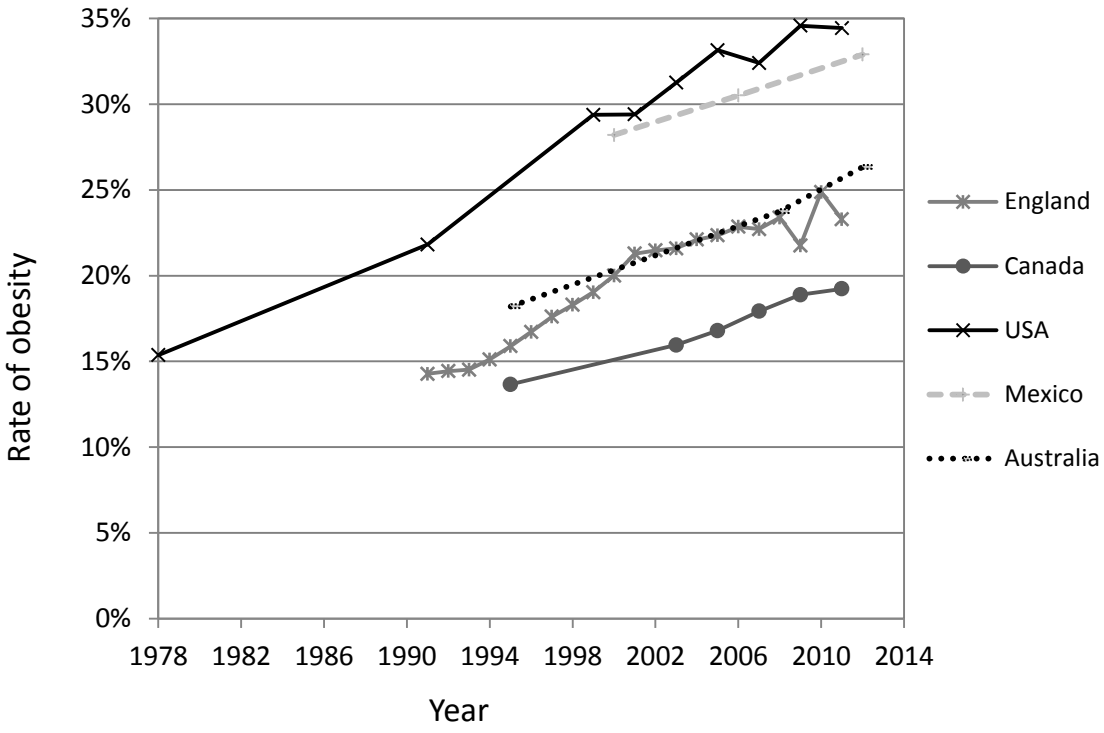


Figure 1 Trends in rates of obesity. Data from OECD Obesity Update 2014. Age- and gender-adjusted rates of obesity and overweight using the 2005 OECD standard population. Measured height and weight in Australia, England, Mexico and USA; self-reported data in Canada. Obese defined as BMI ≥ 30kg/m^2.

Prevalence of child excess weight and child obesity in England increased between 1995 and 2004. Levels then reduced or plateaued, but in 2013 there remained around 30% of children 2-15years with excess weight and 15% with obesity (PHE, 2015a). In an analysis by Ells *et al* (2015) looking specifically at the prevalence of severe obesity in children in England, they found a prevalence of 2-4% in 2012/13 (Ells et al., 2015). Some of the influence on severity of obesity in childhood and adolescence is related to parental BMI,

and the association is reported to increase into adolescence (Svensson et al., 2011). If this is followed through into adulthood as suggested by work by O'Connell *et al* (2010), earlier onset of obesity is likely to lead to reaching a greater BMI in adulthood, and therefore the proportion of adults with severe obese is likely to continue to increase (O'Connell et al., 2010). Alongside a risk of increasing body weight into adulthood, excess body weight in childhood is now consistently linked to adverse consequences such as metabolic dysfunction in childhood, premature mortality and morbidity in adulthood (Reilly and Kelly, 2011, Boney et al., 2005).

Health consequences of overweight and obesity

Obesity has been described as a chronic disease, with health and psycho-social consequences. The complications of obesity affect almost every body system, and the spectrum of obesity-related comorbidities include cardiovascular disease, Type 2 diabetes (DM), several cancers, obstructive sleep apnoea, and muscular skeletal problems, amongst others (Kopelman, 2007, Finer, 2015).

In an expert review by Muller et al (2012) they describe how characterization and defining the contribution of individual body components is essential to understanding the pathophysiology of obesity and obesity-related metabolic risks (Müller et al., 2012). For example considering metabolic health; increasing percentage fat mass (FM) adversely impacts metabolic and cardiovascular health, whereas increasing muscle mass (MM) reduces these health-related risks (Lang et al., 2015). Specifically, body composition, including whole body fat, skeletal muscle and liver fat, account for 35% of the variance in the risk of developing insulin resistance (IR), with liver fat having the greatest association (Müller et al., 2012, Lim et al., 2011, Taylor, 2013). Also, adipose tissue location, morphology and function play a role in the pathogenesis of obesity-related comorbidities (Hwang et al., 2015, Eriksson-Hogling et al., 2015, Cancello et al., 2013). For example in non-alcoholic liver disease (NAFLD), inflammatory progression to non-alcoholic steatohepatitis (NASH) has been linked to the severity of adipose tissue insulin resistance and worsening hyperinsulinemia (Bril et al., 2014).

Insulin resistance (IR)

As described above IR is implicated in a number of obesity-related conditions due to associated metabolic and physiological changes. For example it is a strong risk factor for development of Type 2 diabetes and cardiovascular disease (Frayn, 2001). IR is literally characterised by the resistance of metabolically active tissues to the anabolic actions of insulin. This is exhibited through impaired glucose disposal and enhanced lipolysis, resulting in hyperlipidaemia, hyperglycaemia, and in some individuals compensatory hyperinsulinaemia (Chawla et al., 2011).

The molecular causes of insulin resistance are not completely clear, but may be linked to an insulin signalling defect, glucose transporter defect, or lipotoxicity (Taylor, 2013). These mechanisms m to be most likely caused directly or indirectly by inflammation (Sears and Perry, 2015). This inflammation could be as a result of mediators including inflammatory cytokines, cellular stress or may be diet-induced via various dietary fatty acids and their derivatives (Sears and Perry, 2015). There may be evidence for an interaction between adipose tissue, liver and muscle in systemic IR, but the chronology is not wholly understood (Szendroedi et al., 2014, Snel et al., 2012).

Separately, accumulation of intramuscular lipids is associated with skeletal muscle IR (Perseghin et al., 1999), and hepatic IR is associated with hepatic lipid accumulation (Perry et al., 2014). As for the role of adipose tissue in development or maintenance of IR, it may well be linked to its potential as a source of lipids, its energy storage capacity (or lack of) and inflammatory status (0) (Kriketos et al., 2003). It is agreed that in obesity chronic IR results in reduced suppression of fat mobilization in adipose tissue, and the release of fatty acids, which promotes ectopic accumulation of lipids and lipid metabolites in muscle, liver and other organs (Snel et al., 2012).

Severe obesity

The number of people in the most severe grade of obesity, which is associated to the highest levels of health-related risks is increasing, and predicted to continue increasing. In adults the prevalence of severe obesity in the UK has increased since 1993 for both men and women. Currently the occurrence of severe obesity is much higher in women at around 3.5% (1.6% in men) (Foresight, 2007, PHE, 2015b, HSCIC, 2015). This inequity is also

observed in the proportion of women undergoing weight loss surgery for severe obesity, where between 74-81% of patients (2006-2013) are women (BOMSS, 2014c, BOMSS, 2011).

British Obesity and Metabolic Surgery Society (BOMSS) reported data in severe obesity suggest that depression, asthma and gastro-oesophageal reflux are more commonly reported by women (BOMSS, 2014c). In women of reproductive age severe obesity is associated with increased risk of preterm birth and babies that are large for gestational age, with risk increasing with BMI (Lutsiv et al., 2015). Being large for gestational age has been found to increase risk of developing metabolic dysfunction earlier in life and links back to lifelong effects from maternal obesity for the offspring (Boney et al., 2005).

54% of men and 41% of women who underwent bariatric surgery in the UK between 2011-2013 had four or more obesity related diseases, with multi-morbidity commonplace with BMI >40kg/m^2, and the number of comorbid conditions increasing with BMI (BOMSS, 2014c, Agborsangaya et al., 2015). Severely obese men are reported to commonly suffer with hypertension, obstructive sleep apnoea, DM, dyslipidaemia, atherosclerosis and liver disease (BOMSS, 2014c). Up to 91% of severely obese patients awaiting surgery are reported to have hypertension and 76% DM, within which 14% and 16% respectively were previously undiagnosed (Mostaedi et al., 2014). Also, supported by a recent study by Cordeiro *et al* (2015), NAFLD is universal in severe obesity, with presence of steatosis found in the majority of livers examined (98-100%), and steatohepatitis present in one-third (Cordeiro et al., 2015, Machado et al., 2006).

There is an inverse relationship between obesity and reported quality of life, with the poorest quality of life in those king obesity surgery, possibly associated to the poor functional status observed in three quarters of this population (van Nunen et al., 2007, BOMSS, 2014c). With the highest incidence of perceived weight discrimination in this obese category, compared to those who are overweight, weight discrimination is also reported to explain a substantial proportion of the association between obesity and psychological well-being (Jackson et al., 2015a, Jackson et al., 2015b).

In 2009, the Prospective Studies Collaboration published an analysis of 57 prospective international studies of the association between obesity and causes of mortality. The analysis revealed strong associations between severe obesity and a number of causes of death, with very severe obesity (class III) (BMI 40-50 kg/m2) reducing life expectancy by eight to ten years. People aged 35-59 years with class III obesity were five times more likely to die from ischaemic heart disease, six and half times higher risk of dying from stroke, and 22.5 times higher risk of dying from DM, than those with a BMI in the normal range (Prospective Studies, 2009).

Despite the relationship between extreme obesity and chronic multi-morbidity, weight loss of >5% body weight is associated with a reduction in multi-morbidity with improvements in health and functional status. Functional status is important as it contributes to quality-of-life, employment status and dependence upon carers (BOMSS, 2014c, Agborsangaya et al., 2015). However, a target weight loss of >15 kg is necessary to reverse the diagnosis of DM (Lean, 2015), and this has been adopted as the target for severe and complicated obesity in the national Scottish Intercollegiate Guidelines Network (SIGN) (SIGN, 2010). In considering other comorbidities, the degree of weight loss to realise improvements varies widely, and subsequent weight regain often reduces their durability (Rueda-Clausen et al., 2015).

Extreme obesity

Although bariatric surgery is currently accepted as the most effective management solution for severe obesity, patients with a BMI >60kg/m^2 may not be suitable for surgical intervention. In these extreme cases palliative management with a specialist multidisciplinary team is suggested, where significant support is provided to prepare and enable surgery as the long-term goal (Somers and Carter, 2015).

Adipose tissue

There are two main types of adipose tissue in humans, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT forms the bulk of adipose tissue in adult humans and its major metabolic role is in storage of chemical energy as lipids. In simple terms, at times of energy excess adipocytes store energy in the form of triacylglycerol (triglycerides or TAG), and then release non-esterified fatty acids (NEFA) when required, for example during fasting (Bjorndal et al., 2011, Frayn, 2010).

Alternatively, BAT utilises the chemical energy stored in TAG as heat for temperature homeostasis. Brown adipocytes contain a high density of mitochondria and are effective at uncoupling fatty acid oxidation to generate heat (Dodd et al., 2015). Brown adipose tissue is mainly found in newborn infants and was thought to diminish with age, although depots have been found in adults. Its primary role in thermogenesis (Virtanen et al., 2009), and its identification in adult humans has motivated the search for methods aimed at increasing its thermogenic activity as an approach to treat obesity. A related form of adipocytes are described as brite/beige, these adipocytes exist within WAT. Beige adipocytes dissipate energy as heat, but their abundance is diminished in obesity. Stimulating beige adipocyte development, or "WAT browning", increases energy expenditure and is being explored for its potential for combating obesity (Dodd et al., 2015). Recent work by Rachid *et al* (2015) found that after bariatric surgery and body mass reduction, there was an increase in detectable brown/beige adipose tissue (Rachid et al., 2015).

White adipose tissue (WAT)

The major white adipose tissue depots are abdominal subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). VAT surrounds the inner organs and can be divided into omental, mesenteric, retroperitoneal that surrounds the kidney, gonadal, and pericardial that surrounds the heart (Bjorndal et al., 2011, Spalding et al., 2008).

There are gender-specific differences in the relative distribution of adipose tissue and its contribution to total mass (Tchoukalova et al., 2008). There are also depot-specific disease associations, such as an association between visceral adipose tissue and IR, dyslipidaemia and NAFLD (Bjorndal et al., 2011, Tordjman et al., 2008, Rossi et al., 2011). This is one reason why measures of central, or visceral, adiposity such as waist circumference or waist-hip ratio, are pertinent to classify health risks (WHO, 2008).

Cell types present in WAT include; adipocytes, pre-adipocytes (pre-cursor adipocytes), fibroblasts, vascular cells and immune cells, such as macrophages and T-cells. Cells types, other than adipocytes, are generally grouped together and described as the stromal vascular fraction (SVF) (Ouchi et al., 2011, Balistreri et al., 2010).

Tissue expansion

WAT mass is a product of both adipocyte number and volume, and in obesity there may be hyperplasticity (increased number) and hypertrophy (increased volume). Currently there is insufficient evidence to support hyperplasticity, beyond recruitment of existing precursor cells, and it is believed that from adolescence adipocyte number is constant, even after weight loss, with only age-related decline in cell turnover leading to a reduction (Arner and Spalding, 2010, Spalding et al., 2008).

In a recent cohort study of overweight and obese subjects Eriksson-Hogling et al (2015) examined subcutaneous adipose tissue biopsies before and after weight loss through diet or post bariatric surgery. They suggest that adipocyte hypertrophy was associated with higher fasting plasma insulin, and that post-intervention improvement in IR (HOMA-IR) was more pronounced in individual's that showed initial hypertrophy. From their findings they suggest that subcutaneous adipose tissue morphology could predict improvements in insulin sensitivity following weight loss (Eriksson-Hogling et al., 2015).

Other adipose tissue constituent proportions have been found to vary in obesity. Angiogenesis (blood vessel formation) and extracellular matrix (ECM) remodelling are required for expansion, but have not always been found to be sufficient (van Tienen et al., 2011). Van Tienen *et al* (2011) found that both ECM and cytoskeletal remodelling were downregulated in obese subjects with Type 2 Diabetes (DM). Also, a concurrent increase in macrophage infiltration has been observed with some adipocyte hypertrophy, which may be as a result of cell damage (Virtue and Vidal-Puig, 2010, Ouchi et al., 2011).

Lipid flux

Lipogenesis (fed state)

TAG in adipocytes is produced from non-esterified fatty acids (NEFA), which may have entered the cell either from the breakdown of lipoprotein particles, for example very low density lipoproteins (VLDL) (endogenous pathway) or chylomicrons (exogenous pathway), or as a result of intracellular synthesis from glucose uptake. Levels of both glucose and NEFA are controlled through entry to the cell. Insulin regulates the uptake of glucose into adipocytes through movement of GLUT4 transporters to the cell membrane, and the

activity of lipoprotein lipase (LPL) to breakdown lipoproteins (Frayn, 2010). Carbohydrate response element binding protein (ChREBP) plays an important role in *de-novo* lipogenesis, responding to GLUT-4 mediated glucose uptake, which is determined by cellular insulin sensitivity (Herman et al., 2012).

Lipolysis (fasted state)

Liberation of NEFAs and glycerol from adipose tissue TAG stores is catalysed by lipase enzymes, and again this is affected by insulin, this time negatively. This regulation ensures there is not a futile cycle within the cell, and lipogenesis and lipolysis are not competing. Lipolysis is also regulated by adrenaline (circulating hormone) and noradrenaline (sympathetic nervous system stimulation) (Frayn, 2010). A small proportion (20% or less) of NEFA released by lipolysis are re-esterified in adipose tissue in the fasting state, and recent evidence suggests that re-esterification is positively related to BMI. The majority of NEFA are released into plasma bound to albumin, and circulate around the body being available for other tissues as an energy source for β-oxidation (Frayn, 2010, Bjorndal et al., 2011, Frayn and Humphreys, 2011).

Bioactive compounds

Adipose tissue is widely accepted as being more than just a storage vessel for excess energy, and plays a key role in metabolic regulation. It has been shown to produce a number of adipokines, which elicit paracrine or endocrine effects. In adipose tissue, adipocytes, pre-adipocytes and macrophages are all capable of releasing mediators with biological effects (Balistreri et al., 2010, Ouchi et al., 2011).

The secretory status of adipose tissue changes in obesity, with cellular composition changes, such as increasing macrophage concentration and adipocyte hypertrophy (Ouchi et al., 2011). In obesity the adipose tissue-derived adipokine, adiponectin, is down-regulated, which evidence suggests has a negative consequence, increasing IR and inflammation (Galic et al., 2010, Polyzos et al., 2010).

Another important adipokine, leptin, circulates at levels directly proportional to whole-body adiposity, and is established as a key metabolic regulator mediating actions on brain

function affecting feeding and thermogenesis (Velloso and Schwartz, 2011). The major physiological function of leptin is to signal states of negative energy balance and decreased energy stores. As fat mass depletes, plasma leptin concentrations fall, this stimulates appetite and suppresses energy expenditure. When fat mass increases, leptin levels increase and should suppress appetite, however, overnutrition associated with obesity is known to be accompanied by hyperleptinaemia (Rosenbaum and Leibel, 2014). The failure of elevated leptin levels to suppress feeding and mediate weight loss in common forms of obesity is described as leptin resistance. In the liver, leptin should suppress glucose production and de-novo lipogenesis, and induce fatty acid oxidation, however hyperleptinaemia has found to be associated to hepatic steatosis and NASH (Polyzos et al., 2011).

Inflammation

Increased expression of adipose-originated pro-inflammatory cytokines, mainly from the SVF, such as tumour necrosis factor alpha (TNFα) and interleukin-6 (IL6), have also been implicated in systemic IR, as well as influencing a systemic pro-inflammatory state (Balistreri et al., 2010, Galic et al., 2010).

It is suggested that apart from its direct effects on the insulin receptor, Fetuin-A (Fet) may also promote IR by propagating a pro-inflammatory state, as observed when Fet treatment augments pro-inflammatory cytokine mRNA and protein expression in adipocytes. Recent work by Pal *et al* (2012) suggests that NEFA induces this inflammatory response via an indirect association with Toll-like receptor 4 (TLR4), through interaction with Fet acting as the ligand that binds to the receptor (Pal et al., 2012, Trepanowski et al., 2015).

Macrophages form part of the innate immune response, and they are the dominant immune presence throughout human tissues, ready to defend against potential pathogens. They are able to secrete bioactive peptides, termed cytokines (Odegaard and Chawla, 2011, Chawla et al., 2011). Adipocyte death in white adipose tissue involves the recruitment of macrophages rather than controlled apoptosis, and because of this, in tissue which is hypertrophically expanded to excess, macrophages increase in number to manage damaged adipocyte recycling (Arner and Spalding, 2010, Odegaard and Chawla, 2011).

Alternative activated macrophages (M2) have a tissue repair and homeostasis profile, whereas classically activated macrophages (M1) have a pro-inflammatory profile. The M2 phenotype protects against IR through the direct suppression of inflammation as well as through positive homeostatic maintenance activities, including remodelling of adipose tissue to prevent hypoxia and clearance of apoptotic cells to prevent necrosis. M1 activation occurs in response to a number of factors, for example derived as a result of bacterial infection (Odegaard and Chawla, 2011).

Increased macrophage infiltration in obesity, as a response to adipocyte injury, has been proposed as a contributor to a pro-inflammatory state. In metabolically dysfunctional obesity, macrophages switch to a predominantly M1 state, influenced in part by either adipokine production or down-regulation, or NEFA dysregulation (Ouchi et al., 2011, Chawla et al., 2011). Overall the dramatic increase in M1s and their pro-inflammatory cytokines in obese adipose tissue cancels out the homeostatic functions of M2s resulting in increased inflammation (Odegaard and Chawla, 2011, Gregor and Hotamisligil, 2011).

Management of obesity

The main aim of management is to achieve and maintain weight loss. NICE guidance on the management of obesity includes acceptance that "*Preventing and managing overweight and obesity are complex problems, with no easy answers*." They go on to suggest public health initiatives and multicomponent interventions should be the treatments of choice, including weight management programmes with behaviour change strategies (NICE, 2006). NICE (2014) also suggest that weight management treatment should be decided on a person's individual preference and social circumstance, and the outcome of any previous treatments; considering the individual's level of risk, based on BMI and waist circumference, and any co-morbidities (NICE, 2014).

Lifestyle

The initial approach to intervention for weight loss recommended by NICE (2014) involves modification of an individual's physical activity and dietary intake. With adults encouraged to increase their physical activity, to aim for at least 30 minutes of moderate-intensity physical activity on 5 or more days a week, increasing to 45–60 minutes of moderate-intensity activity a day, for maintenance of weight loss.

The main requirement of a dietary approach for weight loss is that total energy intake should be less than energy expenditure. NICE (2014) recommends an initial intervention of a 600kcal/d deficit for sustainable weight loss. They suggest that more intense intervention could then be appropriate, for example a low-calorie diet (800–1600kcal/d). They only recommend a very-low-calorie diets (VLCD) (i.e. an intake of less than 800kcal/d in cases of clinical need, for example very-low-calorie diets as part of a multicomponent weight management strategy for people who are obese and who need to lose weight rapidly (NICE, 2014).

Drugs

Pharmacological treatment is only to be considered after dietary, physical activity and behavioural approaches have been trialled, where target weight loss has not been achieved or maintained (NICE, 2014). The only currently available drug treatment for weight loss is Orlistat, and can only be prescribed as part of an overall plan for managing obesity in adults who meet one of the following criteria (NICE, 2006):

- BMI of 28.0kg/m2 or more with associated risk factors
- BMI of 30.0kg/m^2 or more.

Orlistat is an inhibitor of gastrointestinal lipases, which exerts its therapeutic effect in the lumen of the stomach and small intestine by forming a covalent bond with the active serine site of the gastric and pancreatic lipases. The inactivated enzyme is therefore unavailable to hydrolyse dietary fat, in the form of triglycerides, into absorbable NEFA and monoglycerides, and there is consequential fat malabsorption and excretion (Roche, 2011).

Weight loss surgery

Bariatric surgery is recommended as a treatment option for adults with obesity if all of the following criteria are fulfilled (NICE, 2014):

- they have a BMI of 40kg/m^2 or more, or between 35kg/m^2 and 40kg/m^2 and other significant disease (for example, DM or high blood pressure) that could be improved if they lost weight
- all appropriate non-surgical measures have been tried but have failed to achieve or maintain adequate, clinically beneficial weight loss for at least 6 months
- the person has been receiving or will receive intensive management in a specialist obesity service
- the person is generally fit for anaesthesia and surgery
- the person commits to the need for long-term follow-up.

Bariatric surgery is also recommended as a first-line option (instead of lifestyle interventions or drug treatment) for adults with a BMI of more than 50 kg/m^2 when other interventions have not been effective (NICE, 2014). In severe obesity non-operative management of weight does not achieve sustainable weight loss, and surgery is a more clinically and cost-effective approach (Picot, 2009, Colquitt et al., 2009). Recent changes to the NICE guidance (2014) them recommending bariatric surgery for people with recent-onset DM, with a BMI over 30kg/m^2; which follows evidence of remission of DM in up to 80% of patients following bariatric surgery (BOMSS, 2014c).

Developments in surgery and medical devices, alongside increasing experience in minimally invasive surgery over the past two decades, has enabled the majority of bariatric cases to be performed laparoscopically. Conversion rates from laparoscopic to more invasive open surgery range from 0–5.7%, with the highest rates observed in more complex malabsorptive procedures (Eldar et al., 2011).

Types of surgery

Gastric banding, sleeve gastrectomy, and Roux-en-Y gastric bypass (RYGB) (*Figure 2*) are currently the most commonly performed bariatric procedures (ASMBS, 2015). The various bariatric procedures achieve different levels of weight loss; however the overall percentage

of excess weight loss (EWL) is reported to be as high as 70% long term (Eldar et al., 2011, BOMSS, 2014c).

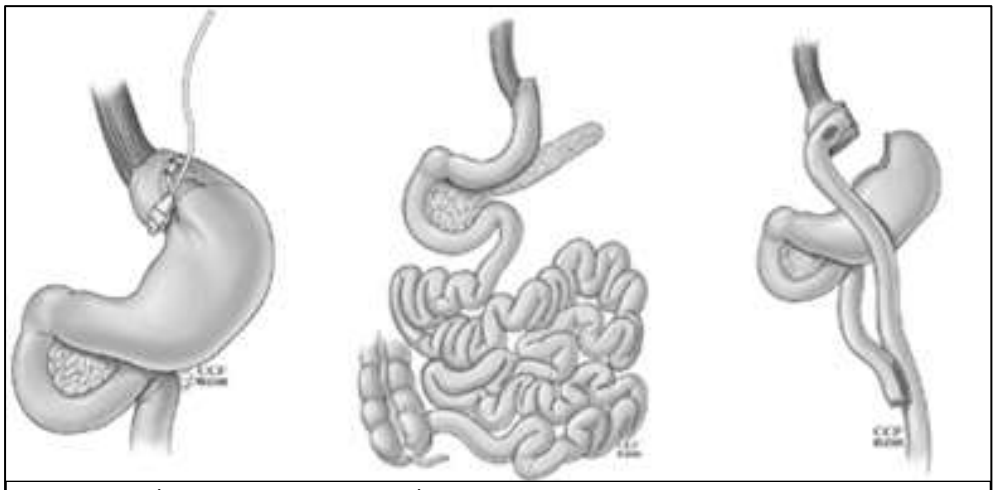


Figure 2 bariatric surgery procedures, from left to right, gastric banding, sleeve gastrectomy, and Roux-en-Y gastric bypass (adapted from Eldar et al 2011).

Gastric band - This approach involves the insertion of an inflatable band around the upper portion of the stomach, creating a small stomach pouch above the band. The size of the stomach opening is adjusted by filling the band with sterile saline, which is injected through a port placed under the skin. The band is adjusted over time, reducing or increasing the size of the opening into the lower stomach pouch. (ASMBS, 2015).

This procedure does not require dissection of the stomach or rerouting of the intestines. It also has the benefit of being completely reversible, and with the inflatable nature of the band, can be adjusted to manage outcomes. Of the three bariatric procedures it has the lowest rate of early postoperative complications. However, it has risks related to the insertion of a foreign body, including band slippage or erosion of the stomach. Although Band patients have the lowest risk for vitamin/mineral deficiencies postoperatively of the three procedures, long-term they have to adhere strictly to dietary guidance in order to avoid gastro-oesophageal reflux and dilation of the oesophagus through overeating (ASMBS, 2015).

Sleeve gastrectomy - This approach is performed by permanently removing around 80% of the stomach, leaving a tubular pouch. It does not require foreign objects to be inserted and there is no bypassing or re-routing of the intestine. However, it is a non-reversible procedure, and similarly to RYGB has the potential to induce long-term nutritional deficiencies which require long-term monitoring and maintenance (ASMBS, 2015).

Gastric bypass - The Roux-en-Y Gastric Bypass, also called gastric bypass, is currently considered the 'gold standard' in weight loss surgery. The components of the procedure include; creation of a small stomach pouch by separating a part of the stomach; division of the first portion of the small intestine; connecting the bottom end of the divided small intestine to the newly created stomach pouch; connecting the top portion of the divided small intestine to the small intestine further down. Digestive enzymes from the bypassed stomach continue to be released and are delivered into a lower portion of the small intestine (ASMBS, 2015).

This is a technically more complex operation than the gastric banding or sleeve and potentially could result in greater complication rates (ASMBS, 2015). The dissection of the stomach and reorganisation of the small intestine can lead to long-term vitamin/mineral deficiencies particularly deficits in vitamin B12, iron, calcium, and folate, and therefore requires adherence to lifelong dietary recommendations and vitamin/mineral supplementation (BOMSS, 2014b).

Proposed mechanisms of action

Historically bariatric procedures were classified by three categories: restrictive, malabsorptive, and combined. However the potential mechanisms of action are now thought to include changes in eating behaviour, energy expenditure, energy restriction and appetite. Consensus on mechanisms of action has not been reached, but it is likely that several factors contribute to the efficacy of the procedures (ASMBS, 2015).

Outcomes

Weight loss is still used as a convenient measure of the effectiveness of surgery. The degree of excess weight loss (EWL) is greatest after the RYGB 55-70%, followed by sleeve gastrectomy 55-60% and gastric banding 45-55% (BOMSS, 2014c).

However, weight loss is no longer described as the principal aim of bariatric surgery because of the positive postoperative health-related changes, including effects on many diseases associated with obesity. The National Bariatric Registry Report (2014) shows statistically significant rates of resolution within one year for major comorbidities after bariatric surgery including hypertension, Type 2 diabetes, dyslipidaemia and sleep apnoea. Also, they report that prior to surgery over 70% of men and women undergoing bariatric surgery had poor functional status i.e. they could not climb three flights of stairs without resting; and one year after surgery this rate had reduced below 30%. NBSR data also show resolution rates are maintained two years after surgery (BOMSS, 2014c). Despite a lack of data for long-term postoperative outcomes, the existing data demonstrate that 10-15years after surgery in adults and adolescents, there is durable weight loss and cardiometabolic benefits including prevention of Type 2 diabetes (Inge et al., 2017, Puzziferri et al., 2014).

Non-alcoholic Fatty Liver Disease (NAFLD)

NAFLD can be subdivided, based on the aetiology, and is traditionally categorized as 'primary' or 'secondary'. 'Primary' NAFLD occurs more commonly, and its pathogenesis is discussed and examined in this thesis. However, before formulating a diagnosis of primary NAFLD, other competing aetiologies of potential 'secondary' NAFLD should be ruled out. The term 'secondary' NAFLD is now being replaced, with an alternate nomenclature including causative factor and resultant pathology, e.g. total parenteral nutrition-induced, drug-induced steatosis/steatohepatitis (Musso et al., 2010).

Primary NAFLD, has been described as a leading cause of chronic liver disease in adults and children, and represents a continuum of disease states ranging from benign steatosis to inflammatory steatohepatitis (NASH) and fibrosis (cirrhosis) (Tiniakos et al., 2010). The pathogenesis of NAFLD and most particularly the mechanisms involved in disease progression remain poorly understood (Gambino et al., 2010). Pathophysiology in NAFLD has been related to lipid excess in the liver, resulting from dysregulation of lipolysis, *de*

novo lipogenesis and VLDL secretion, concurrent with hepatic IR (Fabbrini et al., 2010, Tiniakos et al., 2010).

Please note: For all following considerations the term NAFLD will refer only to primary aetiology.

Liver structure and function

Anatomy and description

The liver plays an important role in energy homeostasis, through both anabolic and catabolic pathways. It is positioned anatomically to receive blood directly from the gastrointestinal tract via the portal vein, which provides 70-80% of its blood supply. This is particularly relevant as dietary intake can directly influence the liver's structure and function (Liu et al., 2011, Fabbrini et al., 2010). Appendices page 268 for detailed diagrams of liver anatomy and structure.

Liver tissue is made up of a number of different cells; the parenchyma is composed predominantly of hepatocytes, but also includes macrophages (Kupffer cells), stellate and endothelial cells. Hepatocytes are arranged in hexagonal units or lobules, supplied with blood from sinusoidal (capillaries) infiltration through the tissue, and are the main site of metabolic processes within the liver (Frayn, 2010, Hernandez-Gea and Friedman, 2011). Stellate cells have two states, quiescent or active, and when activated they exhibit features that promote fibrogenesis (Marra et al., 2008, Friedman, 2000).

Hepatic metabolism

The capacity for an organism to adapt fuel oxidation to fuel availability, that is, to preferentially utilize carbohydrate and lipid fuels and to be able to rapidly switch between them is termed metabolic flexibility (Storlien et al., 2004). The ratio of hormones glucagon to insulin determines the use and storage of glucose and NEFA. When insulin levels are high (fed state) some glucose is utilised in energy metabolism and some is stored as glycogen in the liver or muscle, or its derivative is used in adipose tissue to enable TAG re-esterification. NEFA are converted to TAG in hepatocytes, and are then transported by lipoproteins for storage in adipocytes. When insulin levels decrease (fasting state) the stores of liver glycogen are liberated as glucose for use as fuel. In adipose tissue hormone

sensitive lipase activity increases, regulated by insulin and glucagon, and the TAG stored in adipocytes is released as NEFA (Laffel, 1999, Frayn and Humphreys, 2011).

Under normal metabolic conditions lipid content in hepatocytes is less than 5% of liver volume or weight. Hepatic lipid content, in the form of TAG, is derived from uptake of plasma NEFA released from hydrolysis of adipose tissue TAG, NEFA released from hydrolysis of circulating TAG (from lipoproteins); and *de novo* synthesis (lipogenesis). This input is balanced against usage for fatty acid oxidation and export within VLDL and bile secretion (Fabbrini et al., 2010, Marra et al., 2008, Choi and Ginsberg, 2011). High-density lipoprotein (HDL) is mainly synthesised in the liver, and a small amount in the intestine. HDL picks up lipid and protein constituents from chylomicrons and VLDL as these particles undergo lipolysis and returns them to the liver for recycling (Borén et al., 2012).

The numerous individual steps in hepatic lipid metabolism are coordinated through hormones, nuclear receptors (NR), intracellular signalling pathways and transcription factors. For example hepatic fatty acid synthesis is catalysed by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), where FAS is regulated by a number of nuclear receptors (NR) such as PPARα, PPARɣ and the bile acid receptor (farnesoid X receptor FXR) (Bechmann et al., 2011).

Insulin signalling plays an important role and influences de-novo lipogenesis on several levels, through induction of lipogenic genes, and activation of transcription factors such as sterol regulatory element-binding protein 1c (SREBP-1c). SREBP-1c and ChREBP work together, at a transcriptional level, to induce expression of FAS and ACC. Insulin, along with other mediators, also inhibits autophagy within hepatocytes, and represses lipid-degradation in the fed state (Bechmann et al., 2011). (Autophagy page 35)

VLDL, as well as conveying TAG, contains phospholipids, cholesterol, and specific proteins called apolipoproteins. ApoB-100 is the major structural apolipoprotein of VLDL and plays a role in lipoprotein assembly and secretion. TAG is packaged into VLDL particles, by conjugation to apoB-100 in a 5:1 TAG:cholesterol ratio. Once in the blood, TAG content of VLDL is progressively reduced by the action of LPL, eventually resulting in intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL) with relatively high cholesterol content; with ApoB-100 providing the ligand for LDL receptor-mediated endocytosis. VLDL

assembly is reliant on TAG and ApoB-100 availability, and is controlled by SREBP-1c, ChREBP, and the NRs, the liver X receptor (LXR), and farnesoid X receptor (FXR). FXR also activates LPL, responsible for clearance of lipoprotein lipids from plasma (Fon Tacer and Rozman, 2011, Borén et al., 2012, Choi and Ginsberg, 2011, Bechmann et al., 2011).

Ketones

When glucose levels drop due to depletion of glycogen stores, fatty acids become the major fuel for most cells and hepatocytes produce ketones, which can be used peripherally as an energy source. Ketone production during fatty acid metabolism provides an alternative metabolic substrate for most tissues which can spare glucose, and reduces protein breakdown. During an overnight fast ketones supply up to 6% of the body's energy requirements and up to 40% during a three day fast. Ketones are the collective term for, acetoacetate (AcAc), β-hydroxybutyrate (βHOB) and acetone. Ketogenesis includes; β-oxidation of fatty acids to acetyl CoA, formation of acetoacetyl CoA, conversion of acetoacetyl CoA to 3-hydroxy-3-methylglutaryl CoA (HMG CoA) and then to AcAc; and finally reduction of AcAc to βHOB (Laffel, 1999).

Ketosis is therefore predominantly a transient condition, with the most common physiological causes including fasting or a "ketogenic" low carbohydrate diet. However, ketosis can also be observed due to pathological causes such as endocrine disease, diabetes mellitus, toxic ingestion of ethanol or salicylates, or inborn errors of metabolism (Laffel, 1999, Sumithran and Proietto, 2008).

Aetiology and progression

Steatosis

The first stage in the development of NAFLD is the deposition of lipid within the hepatocytes, over and above normal physiological levels. Steatosis occurs when the uptake of fatty acids and synthesis of TAG exceeds the limitations of output, through oxidation or secretion. A minimum of 5% steatosis is used for the operational minimal definition of histological NAFLD in biopsy specimens, defined using low to medium-power evaluation of parenchymal involvement by steatosis (Kleiner et al., 2005). NAFLD is characterised by large, or macro, lipid droplets which displace cell contents (Tiniakos et al., 2010, Yerian, 2011).

There is a consistent association between central obesity, which reflects visceral adiposity, IR and steatosis. Taylor *et al* in their work on the reversibility of metabolic dysfunction in the "Twin-cycle hypothesis" suggest a timeline of DM development where excess energy intake alongside pre-existing muscle IR results in raised plasma glucose and basal insulin levels, which promotes hepatic lipid deposition. It is this lipid deposition that is predictive of DM onset as it results in hepatic IR and failure of the inhibition of gluconeogenesis (Taylor, 2013, Steven and Taylor, 2015).

By way of confirmation of the source of hepatic lipid, Donnelly *et al* (2005) demonstrated that around 60% of hepatic TAG stores in NAFLD come from NEFA derived from adipose, compared to only 25% from *de novo* lipogenesis (Donnelly et al., 2005). Further to this, McQuaid *et al* (2011) compared the fatty acid trafficking in lean and obese men, and found insufficiency in extraction of chylomicron-TAG by LPL in obesity. They suggested the remaining TAG-rich chylomicron remnants provide additional TAG to be deposited in the liver and thereby provide substrate for VLDL production (McQuaid et al., 2011).

A relationship between the progression of NAFLD and a pro-inflammatory state has been suggested. As hypothesised in the "multiple hits hypothesis", whereby the initial "hit" of lipid deposition is subsequently the target for further "hits" of inflammation, endoplasmic reticulum (ER) stress and oxidation (Chang and Chuang 2010, Hijona et al 2010, Lewis and Mohanty 2010, Tilg and Moshcen 2010). NASH has also been linked with reduction in whole-body insulin clearance, and compared to patients with steatosis, more severe adipose tissue IR (Bril et al., 2014).

Non-alcoholic steatohepatitis (NASH)

Steatosis is a key component of NASH, however it is not known if greater quantities of steatosis predicate development of NASH (Liu et al., 2010). NASH has been described as the next stage in NAFLD development after lipid accumulation. NASH is characterised by hepatocellular ballooning, where hepatocytes are enlarged, cell death, and lobular inflammation, with inflammatory cell infiltration and Kupffer cell aggregation. There may also be chronic portal inflammation, whose severity has been linked to features of IR (Tiniakos et al., 2010).

Fibrosis and Cirrhosis

Fibrosis is a potentially reversible restorative response characterised by accumulation of extra-cellular matrix following liver injury. However, if injury is sustained, such as in a chronic inflammatory state, liver parenchyma is replaced irreversibly by scar tissue in a process which can result in cirrhosis (Hernandez-Gea and Friedman, 2011).

Insulin Resistance (IR)

Development of NAFLD is strongly associated with hepatic IR, and patients with NAFLD almost universally have hepatic IR. It is thought that in NAFLD insulin is unable to alter hepatic glucose metabolism, however, the ability of insulin to activate lipogenesis ms to be intact (Perry et al., 2014).

Reviews of lipoprotein metabolism and kinetic studies suggest that subjects with IR have significantly increased production (up to +45%), and reduced catabolism of VLDL, resulting in an increase in the concentration of VLDL particles. Boren *et al* (2012), from their own modelling work, established that IR was associated with an abnormal concentration of lipoproteins, elevated VLDL production, and increase in plasma LDL (Borén et al., 2012, Choi and Ginsberg, 2011). In addition, a low HDL cholesterol concentration is observed in these subjects, primarily related to hyper-catabolism of its major structural protein, which is not compensated for by increased production (Borén et al., 2012). Non-HDL (all ApoB-containing lipoproteins excluding HDL) circulating levels have recently been posited as a new biomarker for NASH, and for discrimination of steatosis from NASH (Corey et al., 2012).

Lipotoxicity

Lipotoxicity was originally used to describe the toxic effects of excessive fatty acids on pancreatic beta cells, but is now used to describe cellular injury and death caused by a range of lipids (Neuschwander-Tetri, 2010). It has been posited that a surplus of fatty acids in non-adipocytes (ectopic lipid accumulation) may enter pathways leading to cell dysfunction through lipotoxicity. Metabolites of NEFA cause hepatocellular injury expressed as ER stress, inflammation and structural distortions such as ballooning, and also

lead to apoptotic cell death. The generation of lipotoxic metabolites of fatty acids typically occurs with accumulation of triglyceride droplets, resulting in the phenotype recognized as NASH (Neuschwander-Tetri, 2010, Alkhouri et al., 2009).

Endoplasmic Reticulum (ER) stress

ER is the essential site of protein, lipid and sterol synthesis, and discrepancies between demand and capacity lead to ER stress. It is accepted that a surplus of nutrients and energy stimulates synthetic pathways and may lead to overloading in the ER. Conditions like this, which impact on ER function can cause accumulation of unfolded proteins and prompt an intracellular response - the unfolded protein response (UPR) - in order to restore homeostasis. (Banhegyi et al., 2007, Fu et al., 2011, Schroder, 2008, Leclercq et al., 2011). Evidence for an activation of UPR in the steatotic liver has been provided in animal models of NAFLD, and Puri *et al* (2008) recently confirmed these findings in humans. Also Gregor *et al* (2009) provided evidence of activation of ER stress in the liver and adipose tissue of obese patients undergoing bariatric surgery (Gregor et al., 2009).

Inflammation

Inflammation is one of the key features of NAFLD progression. Damage to hepatocytes caused by lipotoxicity leads to the activation of Kupffer cells and intracellular signalling pathways, which all contribute to cytokine secretion responsible for the recruitment of inflammatory cells. Recruitment is also influenced by factors produced by activated hepatic stellate and sinusoidal endothelial cells; and inflammatory cells releasing fibrogenic factors, such as platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β1). It is suggested that circulating inflammatory mediators such as TNF-α and IL-6 affect hepatic IR through promotion of further inflammatory mediators. As a consequence this can lead to a vicious circle of worsening liver damage, through IR, maintenance of steatosis, further inflammation, and disease progression (Fabbrini et al., 2010, Paredes-Turrubiarte et al., 2015).

Autophagy

Singh *et al* (2009) investigated the role of autophagy in lipid homeostasis within hepatocytes *in vitro* and in mice, and concluded that there is an inter-relationship between autophagy and lipid metabolism. They suggest that autophagy regulates lipid content, and

where there is an abnormal increase in intracellular lipid, autophagy is inhibited (Singh et al., 2009).

Adipokines

Adipokines also play a role, especially those secreted directly into the portal circulation by visceral adipose tissue, by acting on liver parenchyma cells (Marra et al., 2008, Hernandez-Gea and Friedman, 2011). It has been hypothesised that although leptin may initially prevent hepatic steatosis, prolonged hyperleptinemia with leptin resistance may result in increased expression of suppressors of cytokine signalling (SOCS3), which ultimately affects hepatic de-novo lipogenesis, thereby resulting in IR and NAFLD (Polyzos et al., 2011).

Diagnosis and prognosis

NAFLD can be asymptomatic, and even in cirrhosis, liver function tests may only be mildly abnormal. Currently diagnosis of NAFLD is most likely to be an incidental finding during exploratory examination of the liver for other disease (Dowman et al., 2011b). Initial steatotic (fatty deposits) development is difficult to establish, but in patients with a diagnosis of steatosis the incidence of cirrhosis is reported to be low (0.6–3%) after 10 to 20 years follow up. In contrast, the rate of cirrhosis reaches 15–25% within 10 to 15 years in patients diagnosed with NASH (Fan et al., 2011, Yerian, 2011).

Progression of disease with sustained fibrogenesis leads to cirrhosis, which is not just the end-stage accumulation of scarring, but is characterized by structural distortion. Hepatic fibrosis may be largely asymptomatic, but progression to cirrhosis confers a risk of significant morbidity and mortality (Hernandez-Gea and Friedman, 2011, Younossi et al., 2011).

Prevalence

The prevalence of NAFLD increases with that of obesity, and has been found to be up to five times more likely to occur in obese subjects than those with a healthy BMI (Tiniakos et al., 2010, Yan et al., 2007). In a recent body composition study completed by Lang *et al* (2015) fat mass (FM) and muscle mass (MM) were obtained by dual energy X-ray

absorptiometry (DEXA) analyses. They found increasing levels of FM and ratio of FM to MM as BMI increased concurrent to increasing prevalence of steatosis (Lang et al., 2015).

In a systematic review completed by Vernon *et al* (2011), the prevalence of steatosis in the obese population ranged from 30-37%, and the prevalence of NASH in the obese population ranged from 10-56%, dependent on the source data (Vernon et al., 2011).

In severe obesity (BMI >40kg/m^2) prevalence of steatosis has been found to be universal (99-100%) and NASH 24–98% (mean 37%) (Machado et al., 2006, Cordeiro et al., 2015). When these high levels were found recently in a severely obese Brazilian cohort (100% steatosis and 30% steatohepatitis), they also found IR in 76% (HOMA-IR) and a positive relationship between IR and NAFLD severity (Cordeiro et al., 2015).

Assessment

Biopsy remains the gold-standard for diagnosis and the three recognised forms of NAFLD are separated based on their distinct histological features and prognoses (Yerian, 2011, Dowman et al., 2011b). Non-invasive assessments can also provide information on the presence of liver fat, NASH, liver function or level of fibrosis. Methods include ultrasound, magnetic resonance imaging and biochemical markers, such as the Enhanced Liver Fibrosis panel (ELF) and Cytokeratin-18 (CK18) (Dowman et al., 2011b, Guha et al., 2008).

Increased hepatocyte death, through apoptosis, is typically present in NASH, but absent in simple steatosis. CK18 is an intracellular filament protein in the liver, which is cleaved by caspases during apoptosis, resulting in caspase-cleaved CK18 fragments. Determination of CK18 fragment levels from blood plasma has been validated to be predictive of histological NASH (Feldstein et al., 2009, Linder, 2011). Further to this, Diab *et al* (2008) showed in a pilot study that CK18 levels were significantly higher in bariatric surgery patients with NASH (BMI 48kg/m^2), and confirmed there was no evidence to suggest a difference in CK18 levels between subjects with and without NAFLD or a borderline diagnosis (Diab et al., 2008).

Management and treatment of NAFLD

There are no current UK clinical guidelines for management of NAFLD, but primary interventions are aimed at improving metabolic variables that contribute to disease progression. This could include management of obesity, DM, hypertension and dyslipidaemia (Dowman et al., 2011a). The "*Guidelines for the diagnosis and management of non-alcoholic fatty liver disease"* (update 2010) were published in the Journal of Digestive Diseases in 2011. Intervention is suggested to correct and change the patients' behaviour and lifestyle, after evaluating the severity of the disease and comorbidities. Lifestyle treatments are aimed at weight loss, especially in obesity, increased activity levels, and an alteration of the diet composition to a low carbohydrate and low-fat balanced diet (Fan et al., 2011).

Clinical aims include improving IR and correcting metabolic disorders, according to clinical requirement. Interventions could include adopting drug treatments; for example use of angiotensin receptor blockers, insulin sensitiser (e.g. metformin) and statins, to treat hypertension, glucose and lipid metabolism disorders respectively. Also suggested in progressive disease is the use of liver protective and anti-inflammatory drugs to prevent and treat steatohepatitis and advanced fibrosis; although they state that at present there is not enough evidence to recommend the routine use of these types of drug in patients with NAFLD and NASH. (Fan et al., 2011).

Research, including Lim et al 2011 , have shown that in obese subjects, as little as 5% weight loss significantly reduces intrahepatic lipid content, and that after only 48hours of energy restriction subjects showed improvements in intrahepatic lipid levels. It has also been reported that 80% of the decrease in liver volume in response to acute energy restriction through a VLED is achieved after the first two weeks (Kirk et al., 2009, Colles et al., 2006). In a recent systematic review by Thoma *et al* (2012) they determined that there was consistent evidence that lifestyle interventions designed to reduce energy intake and/or increase physical activity will reduce intrahepatic lipid and improve insulin sensitivity in patients with NAFLD. They also stated that the degree of weight reduction is positively correlated to these improvements (Thoma et al., 2012).

Clinical application of acute energy restriction

Energy balance and weight loss

Early stage voluntary weight loss can be viewed as the time period directly following the start of a phase of negative energy balance, and is associated with rapid weight loss within the first few days/weeks (Heymsfield et al., 2011). The loss of body weight during this first phase reflects loss of fat and fat-free mass and will depend on the starting composition, but will include protein, water, minerals and electrolytes. Sources of metabolic fuel during this period include glycogen, protein and fat. Glycogen stores are drawn on first, followed by loss of protein and fat. Rates of adipose tissue loss are highly variable, ranging from rapidly mobilised visceral adipose tissue to minimal changes in bone marrow adipose tissue (Heymsfield et al., 2011).

Of concern during voluntary weight loss, especially rapid weight loss, is the depletion of fat-free mass which can be associated with adverse effects, such as loss of cardiac muscle. Christensen *et al* (2011) compared the effects of a very low energy diet (VLED) (415-554kcal/d), and a low energy diet (LED) (810kcal/d), in obese individuals, and observed changes over eight weeks. They found that weight loss was comparable between the two groups, but that the very low energy group showed a greater loss of fat-free mass (Christensen et al., 2011).

Appetite is considered the cornerstone for maintenance of energy homeostasis and body weight control, with increased appetite associated with an increase in energy intake resulting in body weight gain and if maintained obesity. Appetite communication is via chemical messages and there are a multitude of neurotransmitters that disseminate or terminate appetite impulses, co-ordinated through the hypothalamus. Co-existence and/or co-release of orexigenic and anorexigenic signalling supports an environment where subtle shifts result in hyperphagia or anorexia (Kalra et al., 1999). Satiety mechanisms, which should primarily constrain intake, have been described as taking place in a cascade. Signals can be neural, for example from stretch receptors in the gastrointestinal tract, hormonal including leptin, insulin, glucagon, and ghrelin, or gastrointestinal peptides such as cholecystokinin (CCK), glucagonlike peptide-1 (GLP-1), gastric inhibitory peptide (GIP), and peptide YY (PYY), among others. However, persistent stimulatory influences can override

inhibitory mechanisms resulting in excessive intake and weight gain (Tremblay and Bellisle, 2015).

Adaptive thermogenesis (AT) results in a decrease in resting energy expenditure and occurs alongside promotion of appetite in individuals following a hypo-energetic intake, resulting in less than anticipated weight loss. Tremblay et al (2013) suggest that short-term interventions (<6 weeks) produced twice as much reduction in energy expenditure (–28 kcal $kg^{-1}d$) than observed in longer-term interventions (> 6 weeks; −13 kcal $kg^{-1}d$). This metabolic adaptation observed in response to energy restriction has been found to be associated with decreased plasma leptin concentrations, suggesting a role for this adipokine in adaptive thermogenesis (Lecoultre et al., 2011). This is supported by work in leptin deficient individuals, where leptin replacement prevents the decrease in energy expenditure observed after weight loss (Galgani et al., 2010). Alongside this is the role of leptin in appetite control, as described previously, and accumulating evidence endorses the compromise of food intake control during energy intake restriction (Tremblay et al., 2013).

Very low energy diet (VLED)

Definition and comparison to other approaches

A very low energy diet (VLED) is defined as a daily energy intake of less than 800kcal by NICE (NICE, 2014), but definitions in research are not always clear, with some studies crossing the boundary into a low energy diet (LED) over 800kcal.
When considering the use of rapid weight loss versus a slower weight loss through a low energy diet, Purcell et al (2014) found a VLED over twelve weeks achieved greater weight loss than a LED over 36weeks. At follow-up at 144weeks there was also no significant difference in weight regain.

If comparing the use of a fasting or intermittent dietary intake as an alternative approach, a recent systematic review by Seimon et al (2015) concluded that intermittent fasting appears to produce similar but not superior effects to continuous energy restriction, and does not appear to attenuate other adaptive responses to energy restriction or improve weight loss efficiency. Seimon et al also highlight there is a lack of evidence and the reviewed publications were not necessarily powered to assess these outcomes .

Evidence for superior dietary format in terms of liquid, meal replacement or food-based diets is equivocal. However, the majority of dietary studies use a meal replacement approach, with products that replace normal foods and are nutritionally complete. The European Food Safety Authority recently issued guidance on the essential composition of total dietary replacement programmes. They recommend 75g high-quality protein, a minimum content of digestible carbohydrates of 30g/day, and a minimum amount of total fat of around 20g/day, providing a minimum energy content of 600kcal/day (EFSA, 2015). Commercial brands providing meal replacements include Nestle ®, LighterLife ® and Cambridge Weight Plan ®, and products include Modifast ® and Optifast ®. Use of these products aim to control dietary intake in free-living individuals to within tight ranges (Collins et al., 2011, Lewis et al., 2006, Colles et al., 2006, Van Nieuwenhove et al., 2011, Jazet et al., 2007).

One reason for the use of meal replacement diets is the control of macronutrient content. For example there is evidence from short and long-term studies that beside weight loss, carbohydrate restriction could differentially affect insulin response, glucose control and hepatic lipid content (Tay et al., 2015, Browning et al., 2011, Tendler et al., 2007). There is currently debate about restriction of carbohydrates, with disagreement about definitions and what should replace carbohydrate in the diet (Feinman et al., 2015). Increasing fat in the diet may encourage hepatic IR and lipid deposition (Van Der Meer et al., 2008, Jornayvaz et al., 2010), whereas ad libitum higher protein diets may slow weight regain following a VLED (Astrup et al., 2015), by encouraging satiety (Veldhorst et al., 2008).

A low carbohydrate ketogenic diet has been described as containing 20 to 50g/d carbohydrate (Feinman et al., 2015). It has been suggested that the clinical benefit of a ketogenic diet is in preventing an increase in appetite, during weight loss. This was observed during an eight-week ketogenic VLED, where participants without DM lost 13% of initial weight, fasting ketones (BHB) levels rose, and subjective measures of appetite were lower than during a period of ad libitum intake (Sumithran et al., 2013). Westman et al (2008) also found positive results in an obese cohort with DM, with the ketogenic diet resulting in weight loss, improved glycaemic control and elimination or reduction in diabetic medication. They suggest that a diet containing fewer carbohydrates was most effective for improving glycaemic control. In a recent systematic review Gibson et al (2015) suggest that ketosis appears to provide a plausible explanation for some suppression of

appetite, but it is unclear if other factors are involved e.g. type of macronutrient intake, or what level of ketosis is necessary to achieve this.

Outcomes

Just two days of VLED have been found to reduce glucose production in obese DM patients (Jazet et al., 2005), and reduce intra-hepatic TAG and improve hepatic insulin sensitivity in obese with IR (Kirk et al., 2009). An obese cohort that undertook a 30-day VLED achieved significant weight loss (10%). They improved total cholesterol, systolic and diastolic blood pressure and fasting glucose. When followed up at eighteen months it was even suggested that some of these beneficial changes were sustained (Jazet et al., 2007).

After six weeks of VLED a severely obese cohort had achieved a significant weight loss (8%), with reductions in liver lipid, subcutaneous and visceral adipose tissue area. Wahlroos et al (2007) also observed a significant association between the reduction in hepatic IR and liver lipid.

Lim et al (2011) took a small cohort of obese adults with DM (<4years duration) and followed them through eight weeks of VLED. Further to the improvements observed elsewhere in IR, they suggest that they observed a reversal of DM with normalisation of pancreatic function. They observed an early response in hepatic insulin sensitivity, after one week as hepatic lipid was depleted, and a later improvement of beta cell function as pancreatic lipid reduced (Lim et al., 2011). This research group then followed this up more recently to examine the effects of an eight week VLED in patients with longer duration DM, with 50% success in reversing metabolic dysfunction (Steven and Taylor, 2015).
In a longer study by Lean *et al* (2013) severely obese patients followed a VLED for twelve weeks, which was then followed with a twelve month weight maintenance programme. It was reported to have achieved and maintained a mean weight loss of 12.4kg, with DM reversal in one-third of participants, however one-third did not achieve weight loss maintenance of >5kg. The study authors suggest that the programme offered an acceptable approach that was successful in maintaining >15kg weight loss in one-third of their severely obese cohort (Lean et al., 2013).

Examining other VLED effects, a systematic review by Rolland *et al* (2013) found significant weight loss with VLED did not lead to detrimental hepatic or renal outcomes, however their review from 2011 suggested that VLED application would lead to transient reductions in

HDL (Rolland and Broom, 2011). A further systematic review completed by the same research group in 2012 examined VLED and morbidity, and found that short-term beneficial changes may extend into the longer term up to five years (Mulholland et al., 2012). However a caveat to each of these reviews is the paucity of evidence on which these conclusions are drawn.

A potential negative effect of VLED is the loss of lean mass, and specifically this could affect bone health. In severe obesity there is a less positive relationship between BMI and bone mineral density than observed when BMI generally increases, and it is suggested that severe obesity may be a risk factor for certain types of fractures (Mpalaris et al., 2015). Energy restriction is observed to induce hormonal changes that could negatively impact bone mass and acute energy restriction could be expected to have more rapid and extreme effects. However a recent systematic review could not confirm that short-term or acute energy restriction had a significant impact on bone mineral density. They did find a significant decrease in total body bone mineral density after dietary weight-loss interventions of six months in duration. The authors suggested that any decrease in bone density is small in comparison to benefits of weight loss, however, they also state that there was significant heterogeneity of effects among interventions (Zibellini et al., 2015).

In the treatment of obesity the use of VLEDs is recommended only with clinical supervision, and management of risks associated with fat-free mass and micronutrient depletion are countered by ensuring sufficient protein and micronutrient intake (NICE, 2014, Baker et al., 2009). NICE have expressed concerns over the application of VLEDs in severe obesity because of the lack of information on their use in patients with a BMI above 40kg/m^2. They have also expressed concern that VLEDs could encourage disordered eating or weight cycling, and asked for further evidence to differentiate between VLEDs that are liquid only and those that incorporate some solid foods to identify whether the liquid formulation or the energy reduction alone affect weight loss, quality of life, or subsequent disordered eating (NICE, 2014). In partial response to this, in a systematic review by da Luz *et al* (2015) they concluded that clinically supervised severe dietary energy restriction appeared beneficial for obese individuals with binge eating disorder, and would not necessarily trigger binge eating in those without binge eating disorder (da Luz et al., 2015).

The use of a VLED would m positive in obesity and severe obesity to achieve short-term weight loss and conceivable short to middle-term metabolic changes, and can be used as a

motivator to initiate behaviour change. However, subsequent weight regain can reduce the long-term benefits, and limit the significant health effects of sustained weight reduction (Rueda-Clausen et al., 2015), and more evidence is required to confirm both the format and efficacy of dietary approach (NICE, 2014).

Pre-operative diet and bariatric operative outcomes

Bariatric surgery is the most effective means of achieving long-term weight loss in the severely obese, and the majority of procedures are currently performed laparoscopically (BOMSS, 2014c). The left lobe of the liver overlies the upper stomach and gastro-oesophageal junction, and must be mechanically retracted to provide adequate anatomical access for surgery. A bulky liver is more difficult to retract, and if it is also fatty, it is more easily damaged by retraction. The most frequent cause for conversion to an open operation[◊] in bariatric surgery is excessive visceral fat and a bulky fatty left lobe of the liver (Schwartz et al., 2003).

In the pre-surgical setting rapid pre-operative weight loss facilitates the laparoscopic approach, by decreasing liver size and improving liver flexibility, reportedly without compromising immune function or wound healing, with few adverse effects (Lewis et al., 2006, Colles et al., 2006, Fris, 2004). In practice a short-term pre-operative energy restrictive "liver reducing" diet is promoted, which could vary in format, application and duration (Segaran and Wheeler, 2008). A pre-operative diet is encouraged in UK national guidelines and preferred to prescriptive pre-operative weight loss goals. Guidance suggests that patients are expected to lose weight during a short, supervised diet in order to make surgery technically feasible, and demonstrate engagement with the process (Mahawar et al., 2015, BOMSS, 2014a, Ochner et al., 2012). Additionally, patients have been shown to satisfactorily adhere to the strict intake of VLED during a limited period (2-4 weeks); and that use of this type of regime could be beneficial in the pre-surgical setting in terms of post-operative weight loss, operative times, and complication rates (Colles et al., 2006, Edholm et al., 2011), however the evidence is unclear.

[◊] with a large abdominal incision, as opposed to small incisions in the laparoscopic approach

Van Nieuwenhove *et al* (2011) completed a randomised, blinded multicentre study to examine the effect of a pre-operative 2-week VLED meal replacement regime (800kcal/d), in morbidly obese patients undergoing laparoscopic gastric bypass. Interpretation of their data suggests a reduced perceived complexity during the operation with patients on the VLED, but without a proven effect on operating time or intraoperative complications between groups. They also found non-compliance of just over 10% due to intolerance or non-adherence to the diet (Van Nieuwenhove et al., 2011).

Edholm *et al* (2011) examined changes in liver volume and intrahepatic fat, in morbidly obese subjects treated with LED, using a meal replacement plan providing a total energy content of 800–1,100kcal/d. The four-week pre-operative LED treatment was associated with a reduced liver volume by 12% and a decreased intrahepatic fat content by 40%; along with visible improvement in sharpness of liver shape and reduction in omentum thickness, with a consequential reduction in perceived operative complexity by the surgeon. They speculated that although both groups had similar body weight at the time of surgery the liver complexity scores were more favourable in the LED group, due to mobilisation of fat mainly from the liver and intestinal area, making this specific improvement of intra-abdominal anatomy a better operative environment. They also showed a good compliance to the LED regime, despite some complaints of fatigue, and most patients were reported as feeling a strong engagement in their upcoming bariatric treatment (Edholm et al., 2011).

However, as outlined in a recent review by Mahawar et al (2015) despite some evidence, it is not conclusive that pre-operative weight loss improves clinical safety or weight loss outcomes.

Integration of background topics

It is accepted that DM is a progressive disease, where despite glucose management half of patients will go on to require insulin replacement treatment (Turner et al., 1999). However, work designed to elucidate the mechanisms that underlie the pathophysiological progression, have resulted in evidence for the reversibility of metabolic dysfunction. Studies have described return of the first phase insulin response over eight weeks of a very low energy diet (VLED), and rapid resolution of fasting glucose associated with improved insulin sensitivity in the liver (Taylor, 2013, Lim et al., 2011, Steven and Taylor, 2015).

Furthermore, recent work has contradicted the accepted wisdom that reversal is only possible in those with a short duration of DM e.g. less than four years from diagnosis. Their findings suggest that up to 50% of patients with DM duration greater than eight years will be able to achieve fasting glucose levels equivalent to those without DM through eight weeks of very low energy diet. They suggest that in these cases a critical mass of beta cells in the pancreas are not permanently damaged and can be metabolically disinhibited as pancreatic lipid stores are depleted (Steven and Taylor, 2015). However his work does not include consideration of the influence of a systemic inflammatory state that has been observed in obesity; or the role of adipose tissue, which quantitatively and physiologically has a greater impact with increasing obesity. Cottam *et al* described the "chronic inflammatory hypothesis" for the morbidity associated with severe obesity over ten years ago. In a review they found that the level of inflammation directly related to the level of adiposity, increasing with severe obesity (Cottam et al., 2004). It has also been described how obesity-related IR is at least in part initiated by chronic inflammatory disease in adipose tissue (Xu et al., 2003). A review of the mechanisms thought to underlie this pathophysiology by Gregor and Hotamisligil (2011) suggests that the inflammatory trigger in obesity is metabolic, caused by the excess consumption of nutrients. They suggest that as a consequence adipose tissue cells initiate the inflammatory response (Gregor and Hotamisligil, 2011). This is then combined with adipokine dysregulation as described by Maury and Brichard (2010), where pro-inflammatory adipokines are overproduced in increasing adiposity, and anti-inflammatory adipokines decreased (Maury and Brichard, 2010); leading to a systemic inflammatory state.

However, despite population-based measures that indicate raised body mass index (BMI) and excess adiposity predisposes to comorbidities and increased disease risk, not all obese

people have metabolic dysfunction. Approximately one third of obese individuals are "metabolically healthy". Klöting *et al* observed that insulin sensitive compared with IR obese individuals have significantly lower visceral fat area. They found the strongest predictors of insulin sensitivity were macrophage infiltration in omental adipose tissue together with circulating adiponectin, indicating that adipose tissue location and dysfunction are associated with metabolic health (Klöting et al., 2010). Hwang *et al* prospectively followed a group of metabolically healthy obese over ten years, and confirmed that the state was transient with 65% converting to metabolically unhealthy obese. They concluded that conversion in the long-term was related to baseline visceral adipose tissue mass (Hwang et al., 2015).

A further theory which endeavours to explain this contradiction relates to a disposition for or against metabolic health in obesity, dependent on adipose mass early in life. The adipose tissue "expandability hypothesis" suggests that an individual has a limit for adipose tissue expansion. In obesity, adipocytes are recruited from a quantitatively fixed pool and then filled to capacity, with subsequent lipotoxicity and pathogenesis resulting from lipid-overflow and ectopic lipid deposition (Spalding et al., 2008, Virtue and Vidal-Puig, 2010, Arner and Spalding, 2010).

Therefore metabolic health in obesity may be related to adipose tissue location, function and inflammation, liver response to excess energy intake, lipid deposition, IR and function, together with the influence of chronic systemic inflammation.

Taylor et al describe the reversal of metabolic dysfunction initiated by reversal of the trigger, which is excess energy intake. They have demonstrated that acute energy restriction has an effect on liver lipid, circulating glucose and insulin. However evidence for reversal of the inflammatory state is not categorical in acute energy restriction (Solá et al., 2009). It has been suggested that the reversal or resolution in co-morbidities upon weight loss are related to the reduction in the severity of the pro-inflammatory state, however these data are based on comparison of obese subjects after months or years of weight loss. Cancello *et al* examined a cohort of severely obese patients three months post-bariatric surgery. They found reduction in macrophage number and an altered macrophage activity profile to an anti-inflammatory state (Cottam et al., 2004, Cancello et al., 2005). In contrast, after a VLED and short-term weight loss macrophage infiltration and activity may

not change or indeed may increase relative to energy restriction (Capel et al., 2009, Kovacikova et al., 2011).

This inconsistency could well be related to adipose tissue location, the preferential loss of visceral adipose tissue in acute energy restriction and the differential environments between visceral and subcutaneous adipose tissue. Chaston and Dixon (2008) completed a review to assess specificity of weight loss on adipose depots. They found no evidence that any weight loss intervention would preferentially target visceral fat, however their review suggested that modest weight loss, including rapid weight loss over a short period, may preferentially affect visceral fat; the effect being reduced as weight loss increases (Chaston and Dixon, 2008). Evidence provided by Harman-Boehm *et al* can be interpreted to suggest a differential inflammatory environment in subcutaneous and visceral adipose tissue. They found increased macrophage infiltration in visceral (omental) adipose tissue compared to subcutaneous, and that this correlated to BMI and features associated to metabolic dysfunction (Harman-Boehm et al., 2007).

Further to Taylor et al examination of liver lipid and its reversal, is the broader consideration of non-alcoholic fatty liver disease (NAFLD), with its potential progression through inflammation and onto fibrosis, and the effects of acute energy restriction on the disease (Tilg and Moschen, 2010, Steven and Taylor, 2015). However, beneficial effects that might be presumed to link to reduced liver lipid content have been harder to find. The Pennington CALERIE Team did not find associations between hepatic lipid loss and changes in biomarkers such as liver function tests, circulating cholesterol or markers of inflammation (Larson-Meyer et al., 2008).

A further related area for examination is the application of a short-term VLED in current clinical practice, as a pre-operative diet prior to bariatric surgery. Rapid pre-operative weight loss has been suggested to facilitate the laparoscopic approach to bariatric surgery, by decreasing liver size and improving liver flexibility by reducing stores of lipid, and reducing visceral adipose tissue depots enabling access to the upper stomach and oesophagus (Colles et al., 2006, Edholm et al., 2011, Fris, 2004). This reduction in liver size is achieved in practice with the use of a short-term pre-operative energy restrictive diet, or “liver reducing” diet, whose clinical use, style and format is promoted through guidance from the British Obesity and Metabolic Surgery Society (BOMSS) (Segaran and Wheeler,

2008). But, despite this guidance the use of pre-operative diets, their efficacy, gains or benefits are unclear (Mahawar et al., 2015, van Wissen et al., 2016). Also VLEDs are only recommended for use in specific clinical circumstances. The National Institute for Health and Care Excellence (NICE) suggesting that they should only be considered for people who are obese and who have a clinically assessed need to rapidly lose weight (NICE, 2014).

Objectives

As outlined above considerable work has already been undertaken examining the potential mechanisms and the roles for hepatic lipid deposition, inflammation and IR in metabolic dysfunction in obesity. However, lacking is a consolidation of concepts and examination of reversibility in relation to progressive NAFLD and adipose tissue dysfunction. As a pre-operative diet is currently recommended in clinical practice, there is also a need to examine the effects of the VLED within the severely obese population, so that effects and mechanisms can be observed. From this basis questions and gaps in knowledge were identified, and a plan of work developed.

1. *What dietary approaches are used pre-operatively in bariatric surgery, and how do they differ in format from the very/low energy diets used to affect metabolic improvement in Type 2 diabetes?* Prior to bariatric surgery patients are recommended to undertake a short-term energy restrictive "liver reducing" diet, and the first step for this work is to identify the format and duration of the diet used in practice in bariatric services in the UK. This then informs how this approach might differ to that used in the sphere of very/low energy diets affecting metabolic health, and provides evidence to support clinical decision making regarding the application and format of the pre-operative diet. This is explored in Chapter 3.

2. *Does the pre-operative diet have effects outside those of its original aim of enabling the laparoscopic approach to surgery, such as modification of circulating metabolic markers, and how are the diets' effects perceived by patients?* Recently research by Taylor et al (2015) has identified that reversal of metabolic dysfunction observed in DM, which impacts on an individual's ability to control fasting glucose, can be initiated through a short-term low energy diet. Therefore does a pre-operative diet in the severely obese population show any equivalent effect on fasting glucose? Further to this, the dietary approach used to produce the observable improvement in fasting glucose was a meal replacement plan would the type of dietary approach affect the outcome? To this end a comparison of two dietary approaches will be carried out, aiming to achieve a similar energy restriction in matched groups. The dietary approaches used will include a meal replacement diet which will be compared to an

alternative format identified from the survey of practice. This is a novel approach as a comparative study has not previously been completed. As well as anthropometric measures, circulating biomarkers will be examined pre and post diet to assess changes associated with application of the dietary approaches. These include metabolic markers such as glucose and other circulating markers used to assess contributors to any change such as inflammatory markers. In order to assess wider "perceived" effects of the diets, patients will be asked to self-report side-effects such as constipation, and evaluate the use of the dietary approaches. This is explored in Chapter 4.

3. *Are levels and features of NAFLD after the pre-operative diet different to the severely obese population norm?* Ethically a pre-diet liver biopsy is not possible in this clinical context as the patients will be undergoing major surgery post-diet. However, histological assessment of NAFLD, including examination for steatosis, portal and lobular inflammation, and fibrosis, at the time of surgery will be compared to reported levels in the severely obese population and other comparators, such as a lower BMI population. This question is examined in Chapter 5.

4. *Following a pre-operative diet, are surgeons' able to assess levels of liver steatosis and how does this relate to the ease of access or difficulty associated with the laparoscopic approach to bariatric surgery?* One of the aims of this work is to investigate the suggested benefits of the pre-operative diet. This will be achieved through an observational assessment at the time of surgery by the surgeons, following application of a pre-operative energy restrictive diet. Specifically this will be undertaken with patients undergoing weight loss surgery at Derby Hospitals NHS Foundation Trust. An assessment via liver biopsy will examine if the observed levels of NAFLD have any associations to the perceived level of operative difficulty. This is explored in Chapter 6.

5. *What are the mechanisms that contribute to observed biomarker changes and tissue environment following the pre-operative diet?* It has been suggested that liver lipid depletion is one step in the reversal of metabolic dysfunction, as has improvement in adipose tissue function alongside reduction in inflammation. Subcutaneous and visceral adipose tissue depots are described as having fundamentally different roles in metabolic functioning and may be affected differently by acute energy restriction. The liver, as described above, has an essential role in metabolism and has also observed to be affected by acute energy restriction. Therefore in order to explore this, biopsies of adipose tissue from visceral and subcutaneous depots, as well as from the liver, will be taken post-VLED at the time of surgery. These will be examined for genetic expression of metabolic, enzymatic and regulatory factors, and associations explored to other anthropometric and blood results. This will comprise elements outlined above including inflammatory markers and factors involved in lipid metabolism, cell morphology, cell death and molecular transport. This objective is examined in Chapter 7.

Chapter 2

General Methods

This chapter contains the general methods for investigations carried out in the randomised clinical trial. Each subsequent chapter also contains a brief methods section which serves to augment this information, particularly to highlight any specific methodological variables that may have affected outcomes specific to the chapter.

Randomised clinical trial design

Study summary

Title: Effects of short term energy restriction on liver lipid, metabolism and inflammatory status in severely obese adults.

Short title:	Effects of short term energy restriction on liver lipid
ISRCTN:	ISRCTN65605485.
Acronym:	EnR-Lin
NRES reference:	12/WM/0017
Trial Sponsor:	University of Nottingham

Funding Sources:

Schools of Life Sciences and Medicine, University of Nottingham

Biotechnology and Biological Sciences Research Council studentship

Meal replacement products provided by LighterLife

Trial personnel

Chief Investigator: Professor Ian Macdonald, University of Nottingham School of Life Sciences

Deputy Chief Investigator: Dr Iskandar Idris, University of Nottingham Medical School

Chief Investigator (October 2011-June 2012): Professor Michael Larvin, University of Nottingham School of Medicine

Co-Investigators:

Emma Baldry, University of Nottingham School of Life Sciences

Dr Andrew Bennett, University of Nottingham School of Life Sciences

Mr Paul Leeder, Consultant General Surgeon Royal Derby Hospital

Professor Guruprasad Aithal, NHS Director, NIHR Biomedical Research Unit: Nottingham Digestive Diseases Centre

Co-investigator (October 2011- June 2012): Dr Garry Tan, University of Nottingham School of Medicine

Feasibility

Prior to the finalisation of the protocol, a feasibility study was performed. A random sample of 62 patients awaiting Bariatric surgery at Derby Hospitals, whose first assessment appointments were carried out between April and June 2011, were assessed for suitability to be included in the project.

- 3 would be excluded as receiving insulin (2 of whom were receiving Pioglitazone)
- 2 would be excluded as receiving pioglitazone
- 2 may have been excluded due to reported alcohol intake
- 2 would be excluded from the LighterLife group as receiving Warfarin
- 1 would be excluded from the LighterLife group as diagnosed with bulimia
- 2 would be excluded as taking medications which could contribute to secondary NAFLD

Therefore 12 patients would not be eligible to be included in the study. Extrapolated, this would suggest 19% of patients would be ineligible per year, providing a possible participant pool of 324 patients per year.

Hypotheses

- A two week period of energy restriction in severely obese adults will result in levels of steatosis (NAFLD) and steatohepatitis (NASH) below those observed in the general population.
- A two week period of energy restriction in severely obese adults will produce changes in body weight, and proportional changes in metabolic and inflammatory markers.

Outcome measures

Primary outcomes

- Histological assessment of liver steatosis and NASH using the Clinical Research Network scoring system definitions (comparison between diets and against population norm)
- Body weight change (pre to post-diet and between diets)

Secondary outcomes

- Fasting blood changes (pre to post-diet and comparison between diets)
    - Lipids
    - Ketones
    - Glucose, insulin and insulin resistance
    - Inflammatory and bioactive markers such as adipokines
- Macronutrient composition during the diet
- Dietary compliance (comparison of reported intake, and estimated energy deficit, to weight loss outcomes)*****

***** energy intake and deficit reported in kcal

Sample size estimate

The sample size estimate was based on data from bariatric populations undergoing surgery. Colles et al (2006) measured relative changes in liver volume, visceral adipose tissue area and body weight during a 12 week very low energy diet and demonstrated a 24% reduction in liver volume on magnetic resonance imaging at the end of two weeks on a meal replacement plan of 456–680kcal/d. Taking this reduction in liver volume as a reduction in liver fat, this will be reflected in liver histology, and the level of steatosis. Given that 93% of bariatric patients may be expected to show NAFLD on biopsy at the time of their surgery (Ong et al., 2005), it was expected that 69% of the patients following a comparable pre-operative diet will show NAFLD on histology. For 80% power (at p=0.05) 98 participants would need to be randomised to see a statistically significant difference in the level of steatosis (Instat Graph Pad V2.0 software).

Study regime in the context of the patient care pathway

All participants were recruited from patients accepted onto the bariatric surgical programme at Derby Hospitals NHS Foundation Trust, and followed the standard care pathway for bariatric surgery patients at Derby Hospitals, up to the point where they are randomised into intervention groups when allocated a pre-operative assessment date, just prior to surgery. This standard care pathway includes full medical, social and diet history and clinical assessment by a bariatric surgeon.

A pre-intervention fasting blood test was taken at the time of the standard pre-operative assessment appointment, usually two-three weeks prior to surgery. This was part of routine blood tests taken. Weight and height measurements were also taken and meal replacement products also supplied at this visit if applicable.

Dietary observance was assessed by completion of food and fluid diaries during the period of intervention, which were than analysed using standard dietary assessment software, Microdiet version 2 (Downlee Systems UK); and using composition data for the meal replacement products from the supplier (LighterLife UK). Participants completed a short qualitative evaluation on completion of the diet (Appendices page244).

On the morning of the participants' operation they were re-weighed. When the participant was cannulated for anaesthesia a further fasting blood sample was taken. Intra-operatively, samples of subcutaneous and visceral adipose tissue, and liver biopsies were taken (at the discretion of the surgeon).

Participants' anthropometric and medical history was recorded on the Case Report Form, including current medications, allergies, weight history, including self-reported healthy/overweight as a child and physical activity levels.

Post-operatively, participants followed the standard care pathway for Bariatric Surgery.

Duration of study

Patient participation lasted a maximum of 5 weeks - including pre-intervention consent and tests, 2 weeks of diet, and post-intervention sampling/measurements.

Adverse event reporting

Participants were asked to contact the study team immediately in the event of any adverse event. All adverse events were recorded and closely monitored until resolution, stabilisation, or until it had been shown that the study intervention administration was not the cause. Any occurrence was assessed for seriousness, expectedness and causality. The Chief Investigator was informed immediately of any serious adverse events. For definitions Appendices page 230.

Ethical and regulatory aspects

Regulatory approvals

The trial was not initiated before the protocol received a favourable opinion from the National Research Ethics Service (NRES), and the respective National Health Service (NHS) Research & Development (R&D) department at Derby. Ethical approval was given by the West Midlands NRES on 6/2/12, reference 12/WM0017.

The study was accepted onto the Clinical Research Network portfolio on 21/2/12, which gave access to support within NHS sites, including the support of a Research Nurse and financial support for use of NHS facilities and services, for example histological assessment of biopsies by a Histopathologist. Reference CLRN ID 93152.

The study was registered on the www.controlled-trials.com, and a lay summary produced for their website. Reference ISRCTN65605485.

A major amendment was accepted by the Sponsor, ethics committee and NHS R&D in June 2012, for a change of study personnel, including a new Chief Investigator and Deputy Chief Investigator; and regulatory paperwork was updated.

Informed consent

The process for obtaining participant informed consent was in accordance with the ethics committee guidance, and Good Clinical Practice (GCP). In practice both the investigator, or nominee, and the participant signed and dated the Informed Consent prior to participation.

The participant received a copy of the signed and dated forms and the original was retained in the Trial Master File. A second copy was filed in the participant's medical notes.

Approved documentation

All documentation used in the context of the study received favourable opinion from the ethics committee. These documents were then given version numbers and prefixed with the Sponsors' reference 11123, (amendment to these documents was not allowed without consultation with the Sponsor, and ethics committee). Documents included:

- Participant information leaflet (PIL) (Appendices page 232)
- Informed consent form (Appendices page 240)
- GP letter (Appendices page 241)

Trial configuration

The study was designed for a prospective cohort, randomised to intervention.

Setting

Secondary care. Royal Derby Hospital.

Eligibility criteria

Patients attending the regional Bariatric Surgical Service at Derby Hospitals NHS Foundation Trust (Appendices page 226 for the Service's referral criteria). Adults (18 years old and over) already under the care of the Bariatric Service at Derby Hospitals NHS Foundation Trust. (There was no upper age limit for the service.)

Exclusion criteria

- Secondary NAFLD
- Diabetes mellitus treated by insulin, GLP-1 agonist or pioglitazone
- Excess alcohol intake (above the national recommendations Appendices page227)
- Pregnancy and lactation
- Exclusions recommended by LighterLife[3] (Appendices page229)
- Any other condition judged by the investigative team to be clinically significant

Description of interventions

Participants were assigned to one of two intervention groups to undertake an energy restrictive diet for two weeks pre-operatively:

a) The Derby Hospitals standard pre-bariatric surgical food-based diet (Appendices page 219).
b) Meal replacement diet using LighterLife nutritional supplements (Appendices page 223)

Both diets aim to offer approximately 800kcal/d.

[3] LighterLife provide the meal replacement products

Randomisation

As patients were allocated pre-operative appointments they were randomised to intervention. They were assigned using block randomisation with matched pairs for gender and Diabetes status, to one of two intervention groups. In this randomisation each newly recruited participant was allocated a unique trial reference number, Participant Trial Number. The randomisation of reference number to group was achieved using an online tool – Edgarweb http://www.edgarweb.org.uk/. This produced a randomised list of paired interventions, for food-based diet and meal plan.

Data collection and analysis

Demographics/clinical information

Demographic and clinical data were collected directly from the participant, from patient medical notes and electronic patient information systems, after informed consent. Data were recorded in the Case Report Form (Appendices page 218).

Information collected included:

- Age at initial consent
- Gender (male/female)
- Ethnicity
- Alcohol intake/abstinence
- Mobility/physical activity levels
- Self-reported overweight/obesity as a child
- Recorded body weight (previous 18 months)
- Diagnosis of diabetes/impaired glucose tolerance
- Diabetes/other medications
- Allergy/intolerance history

Anthropometric data were also recorded (next section). Adverse events were also documented (Appendix 6) and affirmations when samples were collected.

Anthropometry

On the first participant visit, at pre-operative assessment, height was measured using a Seca 213 stadiometer (Seca UK Ltd), with the participant standing upright, without shoes. The measurement was taken in metres (to 2 decimal places).

At both the pre-operative assessment visit and preceding their operation on the day of surgery, each participant's body weight was measured, using a Marsden MPMS-300 high capacity scale (accuracy 50g <100kg> 100g). Participants were asked to remove outdoor clothing, shoes and empty pockets. Measurements were taken in kilograms (to 1 decimal place).

Energy intake and requirements

Food diary

Food diaries recorded type of food/drink, time taken and quantity, based on measured actual amounts (quantities for portions were stipulated in each diet's guidance). Diaries were analysed using Microdiet version 2 (2005) (Downlee Systems Limited UK), which uses the McCance and Widdowson 6th Summary Edition as a reference database (FSA, 2002). Nutritional breakdown of dietary intake was produced for macronutrients, protein, fat and carbohydrate; and a range of micronutrients.

Physical activity and energy requirements

The use of predictive equations to estimate energy expenditure, and therefore requirement, in the severely obese population group is controversial, and most predictive equations in use produce inaccurate results (Horie et al., 2011). In Horie's efficacy tests, the Harris-Benedict equation using actual body weight, produced a concordance coefficient of 0.839 (accuracy x precision), and therefore this was used as a basis for one estimate of energy expenditure.

More accurate estimations of expenditure are based on direct assessment of fat mass and fat free mass, as separate contributors to energy expenditure/requirement, rather than using total body weight. However, as this study did not collect data on body composition, it was necessary to use modelling to estimate fat and fat free masses. Baseline fat mass

was estimated using a gender-specific equation provided by Dr Diana Thomas (Pennington Biomedical Research Centre), which was produced using data from the NHANES database(Blackburn, 2003). Fat-free mass was calculated as total body weight minus estimated fat mass. This estimation of fat/fat-free mass was then used in a weight loss predictor model that calculates energy deficit using energy densities for both types of tissue (Thomas et al., 2011, Heymsfield et al., 2011)[4].

$$ES = 1020 \frac{dFFM}{dt} + 9500 \frac{dFM}{dt}$$

ES = difference of rate of energy intake, dFFM = change in fat free mass, dt = time, dFM = change in fat mass

For total energy expenditure, the estimates above were multiplied by an estimation of physical activity, which was based on the use of a population model factorial approach (PAL), using an estimation of low, moderate or high lifestyle activity levels (UN, 2004).

[4] The weight loss predictor model can be accessed at the Pennington Biomedical Research Centre website: http://www.pbrc.edu/research-and-faculty/calculators/weight-loss-predictor/about/

Dietary evaluation

No previous questionnaire was located which collected all the data required on acceptability of the diet, physical and psychological effects experienced by the participants, in a short format. However the design and content used was informed by work completed by Colles et al (2006) and a review by Berkow et al (2010) on adherence and acceptability of dietary interventions(Berkow et al., 2010) (Colles et al., 2006). The potential negative effects of VLEDs are well known, with constipation rated as one of the most frequent (Colles et al., 2006). Capture of the frequency of these negative effects was a priority, to see if they were associated with outcomes or acceptability of the dietary interventions. Therefore a short dietary evaluation questionnaire was devised specifically for the study, which was incorporated into the food diary (Appendices page 244). A Likert scale of scoring was used to aid recording of participant perceptions while providing an objective measurement that could be used in analysis. Question format and content was informed by practical experience working with patients in weight management, and from patients descriptions of potential influences on adherence, however there was no direct patient involvement in the questionnaire design due to time restraints.

Participants were asked to rate their dietary experience on a categorical scale of 1-4, where 1 was a low or negative response, to 4 a high or positive response. Questions asked:

- The diet was easy to follow
- I enjoyed the foods/drinks taken
- I had regular food and/or drink
- I felt hungry
- The diet was a positive experience
- While on the diet I noticed changes in my weight or shape
- While on the diet I noticed changes in my health/wellbeing
    - Constipation
    - Bad breath
    - Feeling cold
    - Feeling energised
    - Feeling tired
    - Mood swings
- Overall satisfaction of the diet

Blood

Fasting blood samples were collected before and after the diet intervention.

Sample collection

Pre-diet samples were collected directly into vacutainers by venepuncture, by the specialist health care assistant.

Post-diet samples were collected by an anaesthetist using a syringe via cannula or with a needle into a vein, and dispensed into vacutainer tubes with lids removed. Lids were replaced and processing completed as indicated below (Becton and Dickinson, 2014). Samples were to be collected prior to anaesthesia initiation however some samples were taken post-anaesthetic and timing noted.

Plasma samples for immediate freezing: Each vacutainer was inverted 8-10 times then the tube was placed immediately on wet ice. The sample was then transferred to the laboratory and spun in a pre-chilled (4^0C) centrifuge (1000g, 10mins) within 15mins of collection. Samples were kept on ice at all times. If haemolysis was apparent details were recorded. Plasma was aliquoted into screw capped cryovials using a Gilson style pipette. Aliquots were frozen immediately in liquid nitrogen, transported on dry ice and then placed at -80^0C for long term storage in analysis-specific boxes.

Potassium oxalate and sodium fluoride were used as additives in the collection of plasma for glucose quantification; alongside pre-chilling vacutainers and centrifuging within 15minutes; this reduced post collection glycolysis (Bruns and Knowler, 2009). Tetrahydrolipostatin (THL) was added to samples for non-esterified fatty acid quantification; to avoid falsely high concentrations due to ongoing lipolysis after collection (Krebs et al., 2000); alongside ethylene glycol tetra acetic acid (EGTA) as an anticoagulant and glutathione as an antioxidant.

Fresh plasma sample for lipoprotein collection: Ethylene diamine tetra acetic acid (EDTA) was used as an anticoagulant in vacutainers to yield plasma. Each vacutainer was inverted 8-10 times then tube was placed immediately on wet ice. The sample was then transferred to the laboratory and spun in a pre-chilled (4^0C) centrifuge (1000g, 10mins) within 15mins

of collection. Samples were kept on ice at all times. If haemolysis was apparent details were recorded. 2-4ml of plasma was transferred into a 5ml, pre-chilled tube. The samples were stored and transported on wet ice.

Serum samples for immediate freezing: The tubes were kept vertical and transferred to the laboratory. They were allowed to coagulate for at least 60 minutes at ambient temperature (18-22°C), before the clot was released from sides of tube with a plastic spatula/pipette. Centrifuge settings: room temperature, 1000g, 10mins. 1.5ml aliquots of serum were transferred to screw capped cryovials. (If volume of serum collected was low repeated releasing clot and re-spun). All aliquots were frozen immediately in liquid nitrogen, transported on dry ice, and then placed at -80^{0}C for long term storage in analysis-specific boxes.

Analyses/Techniques

All serum and plasma assays described below were carried out by Sally Cordon, Karen Swift (University of Nottingham, School of Life Sciences) or Jane Grove (Nottingham Digestive Diseases Centre). ELF marker assays were carried out by an external laboratory, Iqur[5].

Reference ranges for clinical chemistry quoted, were taken from Derby Hospital NHS Foundation Trust reference ranges used in clinical practice.

Spectroscopy using Horiba clinical analyser

Analysis was completed using an ABX Pentra 400 Clinical Chemistry benchtop analyser (Horiba UK Ltd). This is an automated high-throughput system, which dispenses and homogenises reagent into prepared sample cuvettes. It completes parallel measurement of light absorbed at selected wavelengths depending on the chosen reagent, using reagent-specific calibrators/controls.

[5] iQur is an independent reference laboratory based at Windeyer Building, University College London

Triglyceride (TAG)

ABX Pentra Triglycerides CP quantitative *in-vitro* determination of TAG by colorimetry, protocol reference A11A01640. Assay using internal quality controls and calibrator. Detection limit 0.08mmol/L. Inter-assay repeatability CV (mean) 2.2%.

A colorimetry method was used, the principle of which is based on the interaction between an enzyme and substrate to form a coloured, light-absorbing complex by adding other reagent. Light absorbance was measured after ending the enzyme reaction. Calibration curves were compared and an estimate of the amount of product generated or consumed can be reached (Huang et al., 2006). This assay uses enzymatic determination of triglycerides according to the following reactions resulting in quinoneimine dye, levels of which can be measured at 540nm.

(Lipoprotein lipase)

Triglycerides + H2O → Glycerol + fatty acids

(Glycerokinase)

Glycerol + ATP → Glycerol-3-phosphate + ADP

(Glycerol-3-phosphate Oxidase)

Glycerol-3-Phosphate + O_2 → H_2O_2 + DHAP

(Peroxidase)

$2H_2O_2$ + 4-AAP + p-Chlorophenol → Quinoneimine + 4H2O

(DHAP = Dihydroxyacetone phosphate, 4-AAP = 4-aminoantipyrine)

Gamma-Glutamyl Transferase (GGT)

ABX Pentra GGT CP quantitative *in-vitro* determination of GGT, protocol reference A11A01630. Assay using internal quality controls and calibrator. The detection limit is 4 U/L. Inter-assay repeatability CV (mean) 1.2%.

This kinetic photometric test catalyses the transfer of glutamic acid to acceptors (glycylglycine). 5-amino-2-nitrobenzoate is released and measured at 405 nm. The increase in absorbance at this wavelength is directly related to the activity of GGT.

L-Gamma-glutamyl-3-carboxy-4-nitranilide + Glycylglycine

←GGT→

Gamma-glutamyl-glycylglycine + 5-Amino-2-nitrobenzoate

Aspartate Amino Transferase (AST)

ABX Pentra AST CP quantitative *in-vitro* determination of AST by colourimetry. Protocol reference A11A01629. Assay using internal quality controls and calibrator. The detection limit was 4 U/L. Inter-assay repeatability CV (mean) 1.7%.

This assay uses enzymatic determination of AST according to the following reactions, resulting in NAD+, levels of which can be measured at 260 nm.

AST

L-Aspartate + 2-Oxoglutarate <——> L-Glutamate + 2-Oxalacetate

MDH

Oxaloacetate + NADH + H+ <——> L-Malate + NAD+

(AST = Aspartate Aminotransferase, MDH = Malate Dehydrogenase, NAD = Nicotinamide Adenine Dinucleotide)

C-Reactive Protein (CRP)

ABX Pentra CRP CP quantitative *in-vitro* determination of CRP by latex-enhanced immune-turbidimetric assay, protocol reference A11A01611. Assay using internal controls and calibrators. The minimum interpretation limit equals 0.1mg/L. Inter-assay repeatability CV (mean) 2.0%.

Agglutination results when an antigen-antibody reaction occurs between CRP in a sample and anti-CRP antibody, which has been sensitized to latex particles. This agglutination was detected as an absorbance change, with the magnitude of the change being proportional to the quantity of CRP in the sample.

Glucose

ABX Pentra Glucose PAP CP enzymatic determination of glucose by colourimetry. Assay using internal quality controls and calibrator. Detection limit 0.01mmol/L. Inter-assay repeatability CV (mean) 2.0%. This assay uses enzymatic determination of glucose using the following reactions (Trinder method) resulting in quinoneimine dye, levels of which can be measured at 540 nm.

$$\text{Glucose} + O_2 \xrightarrow{\text{(Glucose oxidase)}} \text{Glucose acid} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4AAP} \xrightarrow{\text{(Peroxidase)}} \text{Quinoneimine} + 4H_2O$$

(4AAP = 4-aminoantipyrine)

Total cholesterol

ABX Pentra Cholesterol CP using ''CHOD-PAP'' (cholesterol esterase and peroxidase) enzymatic photometric assay. Assay protocol reference A11A01634. Assay detection limit 0.09mml/L. Assay using internal quality controls and calibrator. Inter-assay repeatability CV (mean) 1.6%.

Determination of cholesterol follows enzymatic hydrolysis and oxidation, where the colorimetric indicator is quinoneimine generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder's reaction).

Low density lipoprotein (LDL)

ABX Pentra LDL Direct CP assay, protocol reference A11A01638. The detection limit was 0.04mmol/L. Assay using internal quality controls and calibrator. Manufacturer's protocol followed. Inter-assay repeatability CV (mean) 3.3%.

This method was in a two reagent format and depends on the properties of a detergent. This detergent (Reagent 1) solubilizes only the non LDL lipoprotein particles. The cholesterol released is consumed by cholesterol esterase and cholesterol oxidase in a non-colour forming reaction. A second detergent (Reagent 2) solubilizes the remaining LDL particles and a chromogenic coupler allows for colour formation. The enzyme reaction with LDL in the presence of the coupler produces colour that is proportional to the amount of LDL cholesterol present in the sample.

The recommended cholesterol targets were a serum total cholesterol <4.0mmol/l and LDL cholesterol <2.0mmol/l (JBS, 2005).

High density lipoprotein (HDL)

ABX Pentra HDL Direct CP Accelerator Selective Detergent assay, protocol reference A11A01636. The detection limit equals 0.05mmol/l. Assay using internal quality controls and calibrator. Inter-assay repeatability CV (mean) 1.8%.

This was a two reagent format depending on accelerating the reaction of cholesterol oxidase with non-HDL non-esterified cholesterol and dissolving HDL selectively using a specific detergent.

In the first reagent, non-HDL non-esterified cholesterol is subject to an enzyme reaction and the peroxide generated is consumed by a peroxidase reaction with DSBmT yielding a colourless product. The second reagent consists of a detergent capable of solubilizing HDL specifically, cholesterol esterase and chromogenic coupler to develop colour for the quantitative determination of HDL.

(Accelerator + CO)

HDL, LDL, VLDL, → Non-Reactive LDL, VLDL,

Chylomicrons (DSBmT + Peroxidase) Chylomicrons

(HDL Specific Detergent)

HDL → HDL Disrupted

(Cholesterol oxidase)

HDL Cholesterol → Δ4 Cholestenone + H_2O_2

(Cholesterol esterase)

(Peroxidase)

H_2O_2 + DSBmT + 4-AAP → Colour Development

(4-AAP = 4-Aminoantipyrine, CO = Cholesterol Oxidase,
DSBmT = N,N-bis(4-sulphobutyl)-m-toluidine-disodium)

Non-HDL to HDL cholesterol ratio was calculated using the results for total cholesterol minus HDL, for the non-HDL total, against HDL.

Glycerol

Free Glycerol Reagent from Sigma was used for *in-vitro* quantitative determination. Product number F6428. The Free Glycerol Reagent measures free, endogenous glycerol using coupled enzyme reactions without an initial lipase hydrolysis. Glycerol is phosphorylated by adenosine-5'-triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine- 5'-diphosphate (ADP) in the reaction catalysed by glycerol kinase (GK). G-1-P is then oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Peroxidase (POD) catalyses the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance maximum at 540 nm. The increase

in absorbance at 540 nm is directly proportional to the free glycerol concentration of the sample. The Horiba ABX Pentra 400 Clinical Chemistry benchtop analyser was used to measure absorbance. Inter-assay repeatability CV (mean) 1.2%.

Glycerol Assay Enzymatic Reactions:

	(GK)	
Glycerol + ATP	→	G-1-P + ADP
	(GPO)	
G-1-P + O_2	→	DAP + H_2O_2
	(POD)	
H_2O_2 + 4-AAP + ESPA	→	Quinoneimine Dye + H_2O

Non-esterified fatty acids (NEFA)

The Wako NEFA-HR (2) reagent was used for an *in-vitro* enzymatic colorimetric method to quantitatively determine NEFA. NEFA is converted to Acyl-CoA; which is then oxidized and produces hydrogen peroxide. In the presence of peroxidase, this yields a blue purple pigment. NEFA concentration was obtained by measuring absorbance at 546nm. The Horiba ABX Pentra 400 Clinical Chemistry benchtop analyser was used to measure absorbance. Inter-assay repeatability CV (mean) 1.5%.

Other spectroscopy

Betahydroxybutyrate (βHOB)

A miniaturised enzymatic spectrophotometric assay; in-house design of the University of Nottingham, School of Life Sciences, was used for the quantitative determination of βHOB. The method was based upon the oxidation of βHOB to acetoacetate by the enzyme hydroxybutyrate dehydrogenase, alongside which the cofactor NAD^+ is reduced to NADH. NADH abundance was then measured at 340 nm. Assay readings taken using Tecan Sunrise™ microplate absorbance reader (Tecan, Switzerland) (Tecan, 2014). Inter-assay repeatability CV (mean) 11.2%.

(3-HBDH)

βHOB + NAD+ → Acetoacetate + NADH + H+

↓removed

Hydrazine→Hydrazone

Hydrazine was added to the reaction mixture to trap acetoacetate (forming the hydrazone) enabling the reaction to proceed quantitatively left → right.

(3-HBDH = 3-Hydroxybutyrate dehydrogenase,
NAD+ = Nicotinamide adenine dinucleotide)

ELISA

Each ELISA assay readings were taken using Tecan Sunrise™ microplate absorbance reader, which is a multichannel absorbance plate reader for 96-well plates (Tecan, Switzerland) (Tecan, 2014).

Tumour necrosis factor (TNF)

BD OptEIA human ELISA set (BD Biosciences), using Anti-Human TNF monoclonal capture antibody and Biotinylated[6] Anti-Human TNF monoclonal detection antibody, was used for quantitative determination of circulating TNF. Internal standards were used for the standard curve. Manufacturer's protocol followed. Absorbance read at 450nm with correction from 570nm. Inter-assay repeatability CV (mean) 10.7%.

The cytokine sandwich ELISA method used has highly-purified anti-cytokine antibodies (capture antibodies) which are coated onto plastic microwell plates. After plate washings, the immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples which were applied to the plate. After washing away unbound material, the captured cytokine proteins were detected by biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by an enzyme-labelled streptavidin stage. Following the addition of a chromogenic substrate, the level of coloured product generated by the

[6] covalently attached to biotin

bound, enzyme-linked detection reagents was measured spectrophotometrically using an ELISA-plate reader at an appropriate optical density (Becton and Dickinson, 2014).

Interleukin-6 (IL6)

BD OptEIA human ELISA set (BD Biosciences), using Anti-Human Il-6 monoclonal capture antibody and Biotinylated Anti-Human Il-6 monoclonal detection antibody, was used for quantitative determination of circulating Il-6. Internal standards used for the standard curve. Manufacturer's protocol followed. Absorbance read at 450nm with correction from 570nm. The principles of this ELISA assay were as described previously. Inter-assay repeatability CV (mean) 13.5%.

Fetuin-A

Quantikine Human Fetuin A Immunoassay (R & D Systems Europe Ltd), product reference DFTA00, was used to quantitatively measure circulating Fetuin A levels. Manufacturer's protocol followed. Internal standards used for a standard curve. A face mask was used to prevent contamination from saliva.

The principles of this ELISA assay were similar to that described previously. It employed a monoclonal antibody specific for Fetuin A (mouse) which has been pre-coated onto a microplate. Fetuin A present in samples is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Fetuin A is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of Fetuin A bound. The colour development is stopped and the intensity of the colour was measured at 450nm, with correction from 570nm. Inter-assay repeatability CV (mean) 7.2%.

Cytokeratin-18 (CK18) M30 & M65

Peviva M30 Apoptosense ELISA kit reference 10010, was used to quantitatively test for caspase cleaved CK18 as a marker of apoptosis in epithelial cells. This ELISA assay is based on an M30 monoclonal antibody, which specifically binds caspase-cleaved CK18 but does not bind native, uncleaved, CK18. The assay measures a neo-epitope formed by caspase-

cleavage of CK18 at Asp396. The minimum detectable concentration was 25U/L, defined as the concentration of M30 that corresponds to the absorbance being two standard deviations from the absorbance of the Standard (A) 0U/L. Internal standards and controls used. Inter-assay repeatability CV (mean) 6.3%.

M65 EpiDeath ELISA kit reference 10040 was used to quantitatively measure total soluble CK18 released from dead cells (necrotic and apoptotic). The M65 EpiDeath ELISA can be used in combination with the M30 Apoptosense ELISA, in order to quantify the cell death mode (apoptosis or necrosis) (Peviva, 2014)[7]. The M65 EpiDeath ELISA is a solid-phase sandwich enzyme immunoassay. This ELISA uses two anti-keratin 18 mouse monoclonal antibodies (M5 and M6) of the IgG type, to measure both intact and cleaved circulating keratin 18. The minimum detectable concentration in the M65 EpiDeath® ELISA was 25U/L, defined as the concentration of CK18 that corresponds to the absorbance being two standard deviations from the absorbance of the Standard (A) 0U/L. M65 inter-assay repeatability CV (mean) 9.2%. The figure below (Figure 3) shows the binding sites for the CK-18 assays, indicating how the results can be separated into those related to apoptosis and those related to necrosis.

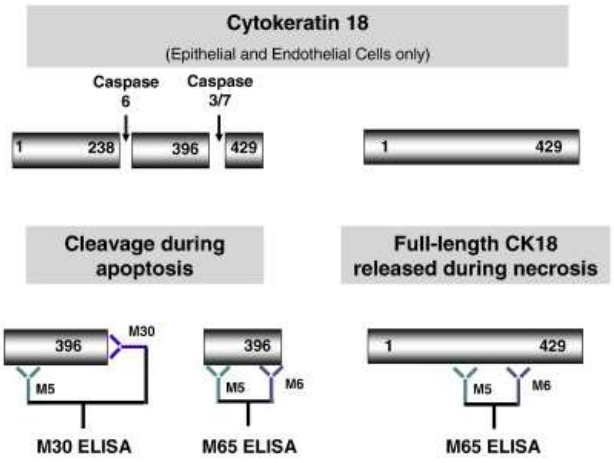


Figure 3 the M30 and M65 ELISAs detect caspase-cleaved and intact forms of cytokeratin 18 (CK18).
(Taken from Dean, Greystoke et al 2012)

The M30 assay detects a soluble CK18 neo-epitope exposed following caspase cleavage during apoptosis. The M65 assay detects full-length and caspase-cleaved soluble CK18 and is thus proposed as a measure of apoptosis and necrosis (Dean et al., 2012).

[7] "M30:M65 ratios" are only meaningful for samples that contain elevated levels of CK18 (i.e. >300U/L in the M65 EpiDeath® ELISA) PEVIVA. 2014. *Peviva AB* [Online]. Available: http://www.peviva.se/products.aspx 2014].

Enhanced liver fibrosis (ELF)

The ELF test (IQur) combines three serum biomarkers which have been shown to correlate to the level of liver fibrosis assessed by liver biopsy. These biomarkers include:

- Hyaluronic acid (HA)
- Procollagen III amino terminal peptide (PIIINP)
- Tissue inhibitor of metalloproteinase 1 (TIMP-1)

The test measures each of these markers by immunoassay, then uses an algorithm (-7.412 + (ln(HA)*0.681) + (ln(P3NP)*0.775) + (ln(TIMP1)*0.494)), to produce an ELF score (Guha et al., 2008).

Radioimmunoassays (RIA)

These assays use a fixed concentration of radiolabelled tracer antigen, which is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited. When unlabelled antigen is added to this, there is competition for binding sites on the antibody, and the amount of tracer bound to antibody reduces, as the concentration of unlabelled antigen increases. This is measured after separating antibody-bound from free tracer and counting one or the other fractions. A standard curve was set up with increasing concentrations of standard unlabelled antigen and from this curve the amount of antigen in unknown samples was calculated.

Adiponectin

Millipore human adiponectin RIA kit product reference HADP-61HK, using ^{125}I labelled murine adiponectin and a multi-species adiponectin rabbit antiserum, was used to measure circulating adiponectin. Manufacturer's protocol was followed, using internal standards and controls. The lowest level of human adiponectin that can be detected by this assay was 1ng/mL when using a 100µl sample size. Inter-assay repeatability CV (mean) 10.8%.

Leptin

Millipore human leptin RIA kit product reference HL-81K, using ^{125}I labelled human leptin and a human leptin antiserum, was used to measure circulating leptin. Manufacturer's protocol was followed, using internal standards and controls. The lowest level of leptin that can be detected by this assay was 0.437ng/mL when using a 100µL sample size. Inter-assay repeatability CV (mean) 8.4%.

Insulin

Millipore human insulin RIA kit product reference HI-14K, using ^{125}I labelled human insulin and a human insulin antiserum, was used to determine the level of circulating insulin. Manufacturer's protocol was followed, using internal standards and controls. The lowest level of insulin that can be detected by this assay was 2µU/mL when using a 100µL sample size. Inter-assay repeatability CV (mean) 7.5%.

Insulin resistance model

A model was used to estimate insulin resistance, based on circulating biomarkers, where direct measurement was not available.

Homeostatic model insulin resistance (HOMA-IR)

This model uses circulating fasting glucose (FPG) (mmol/l) and insulin (FPI) (mU/l) to estimate insulin sensitivity, and is calibrated to give a normal insulin resistance level at 1 (Wallace et al., 2004).

HOMA1 – IR = (FPI x FPG)/22.5

Outcomes from HOMA have been shown to correlate to the gold standard of insulin resistance assessment, the hyperinsulinemic euglycaemic glucose clamp, which directly measures insulin effects on glucose metabolism (Katz et al., 2000).

Tissue

Sample collection

Tissues samples were taken during surgery by the operating or assisting surgeon. The timing of the sampling was recorded e.g. at beginning/end of surgery.

Biopsy size:

- Subcutaneous adipose 5cc
- Liver 0.5-1cc
- Omental adipose 5cc

Once removed the tissue was placed onto a sterile petri dish, and divided using a disposable sterile scalpel. One liver tissue division was placed in formalin (4% buffered neutral formalin), at least 10x volume of tissue, and stored at room temperature. All other tissue sections were placed into cryovials and flash frozen in liquid nitrogen, before transportation on dry ice and storage in a liquid nitrogen tank (vapour phase).

Analyses/Techniques

RNA extraction[8]

Total RNA was obtained from frozen tissue, extracted with TriReagent (Invitrogen). A standard protocol was used as per manufacturer's instructions. A centrifugation separation step was included for adipose samples to remove lipids prior to RNA collection.

RNA concentration

RNA concentrations and the ratio of A260 to A280 (nucleic acids and proteins have absorbance maxima at 260 and 280nm) were determined with the use of a NanoDrop 2000

[8] Following MIQE checklist for Quantitative PCR Assays, BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L., VANDESOMPELE, J. & WITTWER, C. T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry,* 55**,** 611-22.

micro-volume spectrophotometer (Thermo Scientific), with acceptable 260:280 ratios indicating RNA purity ranging from 1.8-2.0.

RNA integrity

Integrity of the RNA was assessed by using a RNA 6000 Nano Chip Kit (Agilent technologies), used with an Agilent 2100 bioanalyzer. This assessment uses electrophoretic separation on microfabricated chips; RNA samples were separated and subsequently detected via laser induced fluorescence detection.

Reverse transcription (RT)

Affinity Script Multiple Temperature Reverse Transcriptase (Agilent Technologies) was used for reverse transcription from RNA to cDNA, following the standard protocol and manufacturer's instructions, using random primers (Invitrogen). RNAse out Recombinant Ribonuclease Inhibitor (Life Technologies) was also added to avoid RNA degradation. 500ng total RNA was used as a template for synthesis of first strand cDNA.

Real-time quantitative polymerase chain-reaction (RT-qPCR)

The template cDNA generated from the reverse transcription reaction was used as the basis for RT-qPCR using TaqMan® Universal PCR Master Mix (Life Technologies).

RT-qPCR measures mRNA amplification of a target sequence as it occurs, so that it is possible to estimate the starting concentration of nucleic acid. During amplification using a TaqMan® probe, accumulation of PCR products was detected directly by monitoring the increase in fluorescence of a reporter dye. During exponential amplification, when the fluorescence level breaches the background threshold, the cycle number was recorded for each sample; this is the CT value that was used in quantitation.

TaqMan gene array card

A custom TaqMan® Array Micro Fluidic Card was designed for each tissue (Life Technologies). This was a 384-well card preloaded with TaqMan® gene expression tests from Life Technologies inventoried assays, in format 96a with 95 target assays and a

mandatory manufacturing control. The manufacturer control gene which was automatically included on the card was Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 1 assay reference Hs99999905_m1 (Life Technologies).

Prior to running samples on the gene card Taqman® plates were run (details on page 80) against GAPDH housekeeper primers and probe to check amplification. At this point negative controls for non-template and negative reverse transcription were both used. Only when all samples showed amplification and negative results in the controls, were they used for the gene card.

A 7900HT Fast Real-Time PCR System (Life Technologies) was used following the standard protocol (Applied Biosystems Part Number 4400263). 110ng cDNA (from total RNA) was used per lane, in duplicate for each sample. TaqMan® Universal PCR Master Mix (Life Technologies) was used as recommended. The template provided with the card was used to set up the software (Sequence detection system (SDS) version 2.3); this defines passive reference, detectors and dyes.

Target genes were selected from background research and this list extended using IPA interactive pathways analysis software (Ingenuity Systems). This enables additional molecules to be added to a number of "focus" molecules through "Path Explorer" and "Connect" functions.

(Please see Appendices, for details of the target genes, network layouts and gene card designs)

Reference genes

Reference (housekeeper) genes were selected using Genevestigator version 4 software under a free-to-use academic licence at https://www.genevestigator.com/gv/ (Nebion AG, under exclusive license from the Swiss Federal Institute of Technology Zurich). The RefGenes service was used to search for stable genes in specific tissues and conditions, using a list of target genes as references (Hruz et al., 2011).

The reference genes selected:

hs.33719	RNA pseudouridylate synthase domain containing 3 (RPUSD3)
hs.490874	Metaxin 1 (MTX1)
hs.500013	DnaJ (Hsp40) homolog, subfamily B, member 12 (DNAJB12)
hs.503251	Protein phosphatase methylesterase 1 (PPME1)

Quantification

The comparative threshold cycle (CT) method was used to compare the gene expression in two different samples (sample A and sample B); with each sample related to a geomean of housekeeping genes prior to comparison. The three housekeepers were checked for normal distribution and correlation to each other (Appendices page 257 for correlation results). Targets' delta-CTs were also checked for normal distribution. Fold change was calculated as described by Schmittgen and Livak (2008), using $2^{-\text{delta CT}}$ (Livak and Schmittgen, 2001, Schmittgen and Livak, 2008).

Interpretation of comparative results

As expression levels were already normalised against control levels (housekeepers) prior to analyses their results could be negative or positive, dependent on the expression differential to the control. CT values that were larger indicate lower expression and later amplification, and lower CT values indicate higher expression and earlier amplification. ΔCT expression levels that were negative indicate expression of the transcript above that of the control and positive values indicate expression levels below that of the control. Therefore ΔCT values that were negative indicate expression levels higher than positive values. When correlations or association analyses were carried out, the direction of association has to take into account the inverse relationship between expression level and CT value.

Individual TaqMan assays

For individual genes (not included on the card) primers and probe were designed using Primer Express® version 2.0 (Applied Biosystems) and universal thermal cycling parameters followed, using the StepOne Plus RT PCR system with version 2.0 software (Applied Biosystems). Triplicates of each sample were analysed along with a non-template control

(water) and a negative reverse transcription sample (RT carried out omitting the transcriptase enzyme).

Carbohydrate response element binding protein (CHREBP)

There are 4 transcript variants:

(1) Represents the longest transcript and encodes the longest isoform (alpha). NCBI Reference Sequence: NM_032951.2

(2) This has an alternate in-frame splice site in the coding region, compared to variant 1, resulting in a shorter protein (beta). NCBI Reference Sequence: NM_032952.2

(3) This has an alternate in-frame splice site in the coding region, compared to variant 1, resulting in a shorter protein (gamma). NCBI Reference Sequence: NM_032953.2

(4) This has an alternate in-frame splice site in the coding region, compared to variant 1, resulting in a shorter protein (delta). NCBI Reference Sequence: NM_032954.2

Primers were designed for different variants:

1. CHREBP – a (alpha)

   Fwd: CCAGACAGCAACAAGACCGA

   Rev: TGCAGCGTGGTAGCTTTGC

   Probe: AGTGCCCAGCCCAGCCTCAAGGT

2. CHREBP – ad (alpha, beta, gamma and delta)

   Fwd: CCTACAGTGGCAAGCTGGTG

   Rev: TTGTTCAGGCGGATCTTGTCT

   Probe: AATTTCAAAGGCCTCAAGCTGCTCTGC

3. CHREBP – b (beta)

Variant 2 is the most recent variant to have been interpreted (Herman et al., 2012, Kursawe et al., 2012). Reverse primer and probe were based on sequences in common with other transcript variants:

Rev: CCTCCGCTTCACATACTGGA

Probe: AATTTCAAAGGCCTCAAGCTGCTCTGC

Fetuin-A

Fwd: TGAAATAGACACCCTGGAAACCA

Rev: TCGACAGCATGCTCCTTCAG

Probe: TGGACCCCACCCCTGT

Reference gene

Primers were designed to replicate those on the gene card (whose exact sequences were not available). GAPDH variant 1 was used for both tissues (Appendices page 256 for a diagrammatic representation of expression levels in different tissues).

GAPDH (variant 1)

Fwd: GCT CCT CCT GTT CGA CAG TCA

Rev: AAT CCG TTG ACT CCG ACC TTC

Probe: TTT GCG TCG CCA GCC GAG C

Quantification

This was determined after normalisation to GAPDH as the housekeeper (sample specific) by comparison of delta CT, calculating fold change as described by Livak and Schmittgen (2008) (Livak and Schmittgen, 2001, Schmittgen and Livak, 2008). Relative quantification was used in order to enable comparison between results from the gene array and individual Taqman® assays. Equality of assay efficiency was measured prior to carrying out final assays for all samples against GAPDH variant 1.

Histology

Non-alcoholic fatty liver disease assessment

Processing, sectioning and staining of samples was carried out at Nottingham University Hospital NHS Trust, Pathology Department[7], Research and Development.

Biopsies were paraffin embedded, sectioned (4μm) and stained with:

- H&E – stains nucleic acids
- Prussian Blue (Perls) - stains iron
- Picro-Sirius Red (PSR) – stains collagen fibres

Under a light microscope, assessment of tissue sections was carried out by a single Histopathologist Dr Philip Kaye, at Nottingham University Hospital NHS Trust. An adapted version for histological scoring of non-alcoholic fatty liver disease (NAFLD) was used (Table 3), based on NASH Clinical Research Network scoring system definitions (Promrat et al., 2004, Kleiner et al., 2005).

Table 3 histological scoring of non-alcoholic fatty liver disease (NAFLD), based on NASH Clinical Research Network scoring system definitions.

Item	Definition	Score
Steatosis Grade Low- to medium-power evaluation of parenchymal involvement by steatosis	<5%	0
	5%-33%	1
	>33%-66%	2
	>66%	3
Steatosis Location Predominant distribution pattern	Zone 3	0
	Zone 1	1
	Azonal	2
	Panacinar	3
Microvesicular steatosis Contiguous patches	Absent	0
	Present	1
Inflammation **Lobular inflammation** Overall assessment of all inflammatory foci	No foci	0
	<2 foci per 200× field	1
	2-4 foci per 200× field	2
	>4 foci per 200× field	3
Portal inflammation Assessed from low magnification	None	0
	Minimal	1
	Moderate	2
	Severe	3
Liver cell injury Ballooning degeneration/apoptosis /dropout cells	None	0
	Mild/Few	1
	Moderate	2
	Prominent ballooning/Many	3
Mega-mitochondria	Absent	0
	Present	1
Fibrosis **Stage**	None	0
	Perisinusoidal or periportal	1
	Mild, zone 3, perisinusoidal	1A
	Moderate, zone 3, perisinusoidal	1B
	Portal/periportal	1C
	Perisinusoidal and portal/periportal	2
	Bridging fibrosis	3
	Cirrhosis	4
Other findings	Visible on routine stains	
Mallory's hyaline	None	0
	Minimal	1
	Moderate	2
	Severe	3
Glycogenated nuclei	Contiguous patches	
	None	0
	Mild	1
	Moderate	2
	Severe	3

Additionally a NAFLD activity score was then calculated as the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2). NAS ranging from 0-8 (Kleiner et al., 2005).

Comparator liver tissue

In order to be able to compare liver biopsy results from this study to liver tissue from a range of individuals with different BMIs; liver tissue was used that had been collected as part of ongoing liver research (HRA ethics 04/Q2403/70[9]), provided from a range of patients who have undergone biopsy for disease management/diagnosis. Each biopsy was anonymised, however demographic and clinical data was collected for comparison purposes, such as gender, age, and Diabetes status. Biopsies were stored at the FRAME laboratory in vapour phase liquid nitrogen and used for gene expression studies after RNA extraction (as described above). Histological assessment for NAFLD was also carried out using a second, previously prepared, wax embedded biopsy, which was then processed and stained as described above.

Surgeon assessment

A visual analogue scale was devised with the assistance of the bariatric surgeons, specifically for the study, to assess perceived difficulty of the laparoscopic approach to surgery, based on the principles from Van Nieuwenhove's work in 2011 (Van Nieuwenhove et al., 2011). (Appendices page 259)

Perceived difficulty of the procedure for three categories related to surgical access and procedural efficacy, were noted on a 100mm visual analogue scale for which 100 and 0mm represented the highest and lowest degrees of difficulty, respectively.

The three categories were:

1. Rigidity of liver
2. Liver volume/size
3. Obstruction of gastro-oesophageal junction

9 Fund for the Replacement of Animals in Medical research (FRAME) Laboratory research HTA licence holder Associate Professor Andrew Bennett

Statistics and data handling

The data were analysed with IBM SPSS Statistics for WINDOWS version 22 (IBM Corporation).

Variable distribution was checked with Shapiro-Wilks test. As the majority of data were not normally distributed the statistical tests applied were for non-parametric data.

Categorical data were expressed as frequencies (and percentages). Crosstabular data (by category) were examined using Kendall's Tau C for non-parametric data. Continuous variables were summarized as median and range (for nonparametric data).

Changes within groups over the study period (related samples) were evaluated using the Wilcoxon signed rank test. Comparisons between groups (independent samples), for example comparing diet groups, were completed using Kruskal-Wallis test for non-parametric data. Correlations were examined using Spearman's Rho for non-parametric data.

The P value for statistical significance was 0.05. However, the Bonferroni correction was used to examine the statistical significance of results, where multiple tests were carried out on a data set, and required the probability of an α error (0.05) to be divided by the number of tests carried out, therefore reducing the cumulative error to below this threshold (Field, 2013). When this was carried out it is stated in the text with the level for statistical significance.

Randomisation

Gene card/Taqman

In order to avoid blocks of participants with the same intervention, gender or diabetes status on each card, a random list generated by http://www.random.org/ was used. Sample numbers were entered in numeric order into the software, which then randomly reordered them. Groups of 4 of this random list were then used for each card. This random order was also used when completing individual Taqman plates.

Recruitment

Over 25 months of recruitment, between May 2012 and June 2014, 196 potential participants were screened for eligibility (

Figure 4). 105 verbally consented, but 45 of these had to excluded due to inconsistent timetabling or transfer of surgery offsite. Of these 60 were randomised and started the trial, and 54 completed. Six participants were lost to follow-up; five due to operations being cancelled, where rescheduling did not allow patients to restart/continue the study, and one patient was started on insulin.

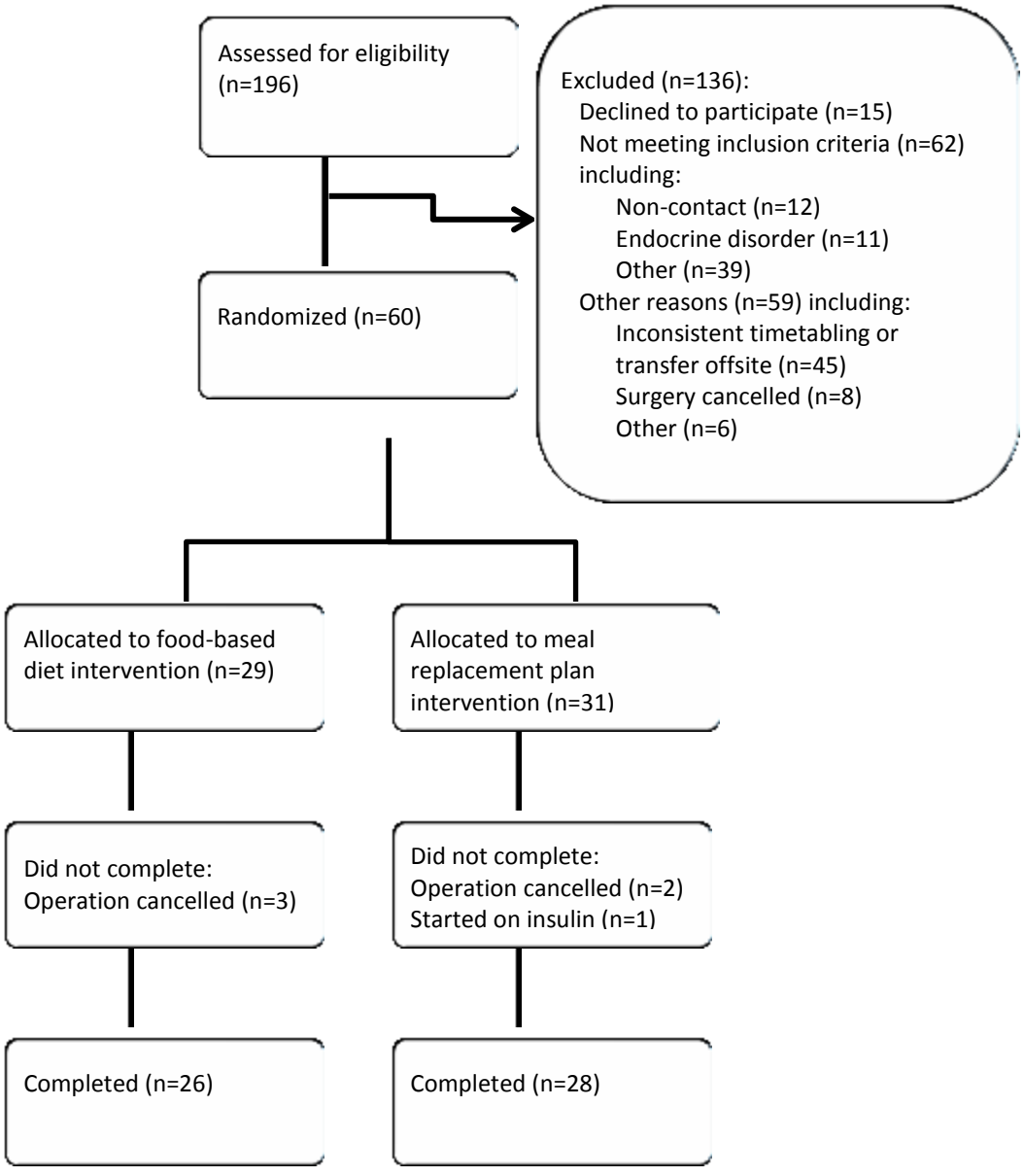


Figure 4 Consort-style flow diagram of recruitment, and progress through the phases of the parallel randomised trial of two groups:

(Moher et al., 2012)

Baseline demographics

Participants' body mass index ranged from 41.5-66.8kg/m^2, with a median of 50.7kg/m^2. 81.5% of participants were female, which is around the national average for patients undergoing bariatric surgery (BOMSS, 2011). Age ranged from 24 to 65 years, with median 45. 29.6% (n=16) had been diagnosed with Type 2 Diabetes (DM), and 56% (n=9) of those were taking Metformin▲. Only one participant was not white European. There was no statistical significant difference between diet groups for demographic features or diabetes prevalence♠. (Table 4)

Table 4 Body mass index (BMI), gender, age and diabetes status for the 54 participants that completed the study

Intervention	Number	Body Mass Index kg/m^2 (pre-diet)		Gender (%)	Age (years)		Type 2 Diabetes Prevalence (%)
		Median	Range	Female	Median	Range	
Food-based Diet	26	51.1	41.5-66.8	84.6	47	25-65	30.8
Meal Plan	28	50.1	41.5-62.9	78.6	42	24-59	28.6
Total	54	50.7	41.5-66.8	81.5	45	24-65	29.6

Diabetes and gender groupings

29 participants were considered to be euglycaemic (fasting plasma levels below 6.1mmol/L pre-diet), and 25 participants had previously diagnosed Type 2 Diabetes (DM) and/or impaired fasting glucose (IFG) (fasting plasma levels above 6.1mmol/L pre-diet) (Table 5). These groups are not significantly different between interventions Chi-Square 0.599. If these groups are further separated by gender this only leaves two males in the euglycaemic group. Therefore analyses have only been carried out by Diabetes status, and not by gender.

▲ No other Diabetes medications were reported as being taken

♠ Age Mann-Whitney U Test 0.245, BMI Kruskal-Wallis Test 0.691, Gender Chi-Square 0.568, DM status Chi-Square 0.860

Table 5 subgroups for diabetes status by diet group

Diabetes Status	Intervention	Number
Euglycaemia	Food-based Diet	13
	Meal Plan	16
IFG/Diabetes	Food-based Diet	13
	Meal Plan	12

Small numbers were also a problem when dividing the groups by dietary intervention and gender (male N=4 in the Food-based diet), therefore analyses have not been completed on dietary results by gender.

Childhood weight status

Participants self-reported if they had been overweight or obese as a child. 61% of all participants stated that they had been overweight or obese. This was 65% in the Food-based diet group, and 57% in the Meal plan group. There was no significant association between groups and weight trend (Chi-Square Test P=0.535).

Comparing the two groups for incidence of Type 2 Diabetes (DM) or impaired fasting glucose (IFG), no statistical difference was found between groups of reported child weight status (Chi-Square Test P=0.686).

Interpretation of demographics

There were no statistically significant differences between dietary intervention groups, for age, gender, body weight, BMI or incidence of Type 2 diabetes. This infers that there should be limited variation related to demographic differences in changes observed between diet groups pre to post intervention. Considering this, although gender proportions were not significantly different between dietary interventions, there was a large proportion of each group which was female, and this may be reflected in the results. When subdivided into diabetes status, the groups were also similar in proportions for those with Type 2 diabetes or impaired fasting glucose. However data were not collected on disease duration, which was an omission, as duration has an impact on insulin secretion capabilities due to progressive loss of beta cell function (Fonseca, 2009).

Adverse events

There were two adverse events reported, including one serious adverse event (mortality post-study participation i.e. post-operatively). One event occurred during the dietary phase of the intervention; a participant reported symptoms of shortness of breath while using physiotherapy equipment incorrectly. This incident was not related to the intervention. For the Serious Adverse Event, one participant, who had followed the Food-based diet, died as a result of complications related to their surgery that were not deemed to be related to the intervention.

Clinical trial limitations

There were a number of limitations to the clinical trial.

Unfortunately the trial under recruited and the sample size of 98 (49 per arm) was not met.

Prior to starting the intervention there was no typical diet recorded or any detailed monitoring of dietary habits, which makes conclusions related to changes observed difficult to place into a context of dietary changes from baseline. Also there were no pre-intervention adipose tissue samples, so that outcomes for adipose tissue can only be compared within the cohort for dietary interventions, or the two types of adipose tissue collected. No non-invasive measurement of hepatic lipid content was taken prior to interventions, for liver volume or stiffness, which could also have provided a baseline measurement for steatosis that would have allowed for more conclusive outcome appraisal than has been possible with the use of comparator tissue, given the variance when using an alternative cohort. Although some comparison can be made between liver biopsies from comparator tissue, and has been used to evaluate changes related to body mass index, it cannot replace the authority that would be provided in measuring the same cohort pre and post-diet.

Data were not available on duration of disease from diagnosis of Type 2 diabetes or impaired fasting glucose. This is important given the progressive nature of Type 2 diabetes, and the physiological changes as insulin resistance worsens, there is hyper-secretory compensation of insulin, and then progressive beta cell failure and insulin insufficiency. Also, having combined groups of participants with Type 2 diabetes and impaired fasting

glucose was not ideal for assessing outcomes in relation to insulin resistance, and if the population had been larger these groups would have been separated.

Limitations also include difficulties arising from NHS administrative procedures that prevented a number of consented patients from taking part, which if it had not happened would have improved the sample size, which unfortunately did not quite reach a sufficient level to power comparative analysis of diet-specific macronutrient effects on level of steatosis. Also, despite the protocol being prepared in collaboration with the Bariatric Service, constraints when deviations from standard practice occurred, affected the quality of sampling, for example the skill and willingness of the health professionals to take blood samples.

One major limitation was that no direct recording of habitual or atypical physical activity took place, and therefore although participants were asked to maintain levels of physical activity pre, during and post- intervention, there was no way to validate or exclude this as a variable that would affect the results.

Finally, the study participants were randomised to diet group, and at no time was personal preference allowed to influence this randomisation, however the investigator was not blinded to dietary protocol or allocated diet during the intervention. This did not affect application of the interventions or collection and interpretation of data.

Chapter 3

Investigation of the pre-operative diet in clinical practice

Introduction

This chapter aims to present data for the dietary approaches that were used pre-operatively in bariatric surgery, and examine if they differ in format from the very/low energy diets used to affect metabolic improvement in Type 2 diabetes.

In 2008 a consensus document was produced by dietitians for the British Obesity and Metabolic Surgery Society (BOMSS), outlining dietary principles, and three types of pre-bariatric surgery diet; 1) 1000kcal/d food diet, 2) Very low calorie diet (VLCD) liquid preparation (meal replacement), and 3) Milk and yoghurt (BOMSS dietary consensus document (Segaran and Wheeler, 2008)).

Beyond this consensus document, there was no up-to-date evidence regarding the use and nature of pre-operative restrictive diets in current practice across bariatric services. A survey was undertaken examining the pre-operative energy restrictive diets used in routine clinical practice in bariatric surgery centres around the UK, in order to report on current practice and provide evidence to inform clinical decision making.

The survey was published as "Pre-operative Dietary Restriction for Patients Undergoing Bariatric Surgery in the UK: Observational Study of Current Practice and Dietary Effects." Obesity Surgery 24(3): 416-421 (Baldry et al., 2014), details of which follow. (Appendix 17)

Methods

A cross sectional survey was undertaken of routine care across bariatric surgery services around the UK. Information was collected between September and November 2012 via internet and e-mail through an open request for information circulated to members of BOMSS. Services were asked to provide patient information material used in current practice, or in the past, for the application of the pre-operative diet.

Patient information material was examined and pre-operative diet data collated in the following categories:

- Service geographical location and whether private or public health provider
- Number of diets in use and/or diet choices
- Eligibility criteria for diet choice
- Description e.g. low carbohydrate, milk, meal plan
- Duration (days)
- Quoted energy intake (kcal/day)*****
- Multivitamin/mineral recommended
- Additional fluid recommended (quantity and description/day)
- Diabetes advice provided
- Carbonated drinks or alcohol allowed
- Diet rationale/description

Data were collected and compiled in a spreadsheet (Microsoft Excel, USA). The majority of information was qualitative and descriptive in nature.

Results

Information was provided by 28 services, including six private and 22 NHS services. This accounts for approximately one third of the services currently offering bariatric surgery in the UK (BOMSS, 2011). A total of 49 diets were in use at the time of the survey. The majority of services (61%) did not offer patients a choice of diet, but some offer up to six alternatives. Some choices were patient-based and related to patient food/drink preferences. However, information collected suggests that diet choices were primarily clinician-led, based on observed efficacy at the time of surgery, and adapted when results, such as a large/inflexible liver or obstruction to the gastro-oesophageal junction were observed.

***** kcal/d is the preferred energy unit used by dietetic services in the UK

Diet characteristics

Types of diet in use included low energy, low carbohydrate and liquid. 59% offered low energy/low carbohydrate food-based, 21% milk/yoghurt, 18% meal replacement (liquid) and 2% clear liquid (Figure 5). Clear liquid was the least popular approach, and consisted of clear soups and other fluids, used alone for patients with a BMI over 60kg/m^2, or for lower BMI patients, for a shorter period, following a low energy food-based diet.

Meal replacement (liquid) approaches were used by 18% of services. Commercially available products such as Optifast, Slimfast, Cambridge, LighterLife and supermarket/chemist branded products, were recommended by these services, and used either to completely replace normal foods, or in combination with normal foods. Twenty-one percent of services use milk/yoghurt diets. They range from unlimited quantities of low fat/low energy yoghurts with additional fluid including milk, to milk only, with additional fluids. The most commonly recommended diet consisted of three pints of semi-skimmed or skimmed milk with two low fat/sugar yoghurts daily.

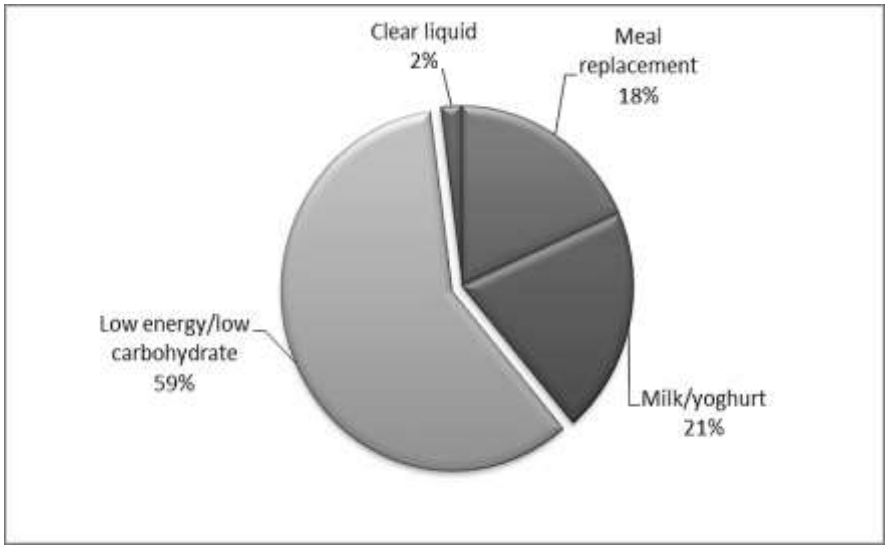


Figure 5 Types of diet used in clinical practice November 2012 (Percentage of total diets)

The most popular dietary option was food-based (59%); used predominantly to encourage patients to adapt eating behaviours and improve portion control. There was a wide variation in approaches, ranging from use of specific portions (grams per item/number per day) and exchange systems for macronutrients, to descriptive meal ideas.

Recurrent in the food-based diets was an emphasis on controlling carbohydrate intake, although recommendations range from no carbohydrate to over 90g/day (Figure 6). No services recommended an energy deficit level to be achieved, however a number of diets specified an energy intake and 23 of 25 that stipulated a quantity, aimed for an intake of 800-1000kcal/day.

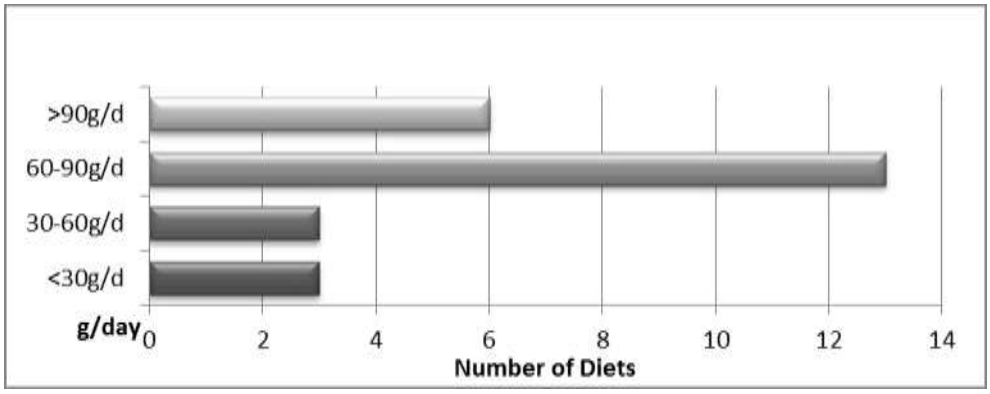


Figure 6 Estimated levels of carbohydrate allowance per day, by diet, based on nutritional analysis of food recommendations (estimated using Microdiet, UK 2005).

Diet duration was not standardised by approach, and varies between 7 to 42 days (Figure 7). This variation was clinician-led and based on BMI, with some diets followed for longer by patients with a higher BMI.

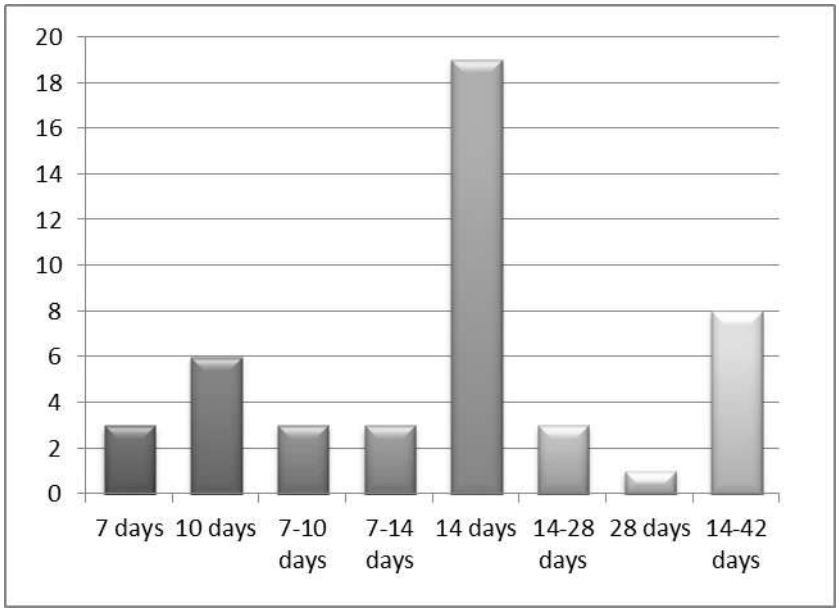


Figure 7 Diet duration by diet. Shown as number. Durations shown are number of days or ranges where specified by the services.

Patient Information

Patient information material varied in detail and structure, particularly regarding concurrent advice on medication and supplementation (Figure 8).

Multivitamin/minerals were recommended during all diet types, but not as standard, with 31% of services failing to include specific advice to take or omit supplements. Specific diabetes advice, (considered to be either to contact a diabetes specialist for advice, or information to adapt medication during the diet) was provided in 71% of diets.

Fluid recommendations were made with all diets, and ranged from an additional one litre of fluids/day to three and a half litres, dependent on the type of diet. For the food based diets the most common two approaches to fluid recommendations were for either an additional two litres of fluids, with examples such as water and sugar-free drinks, or for unlimited quantities of sugar-free drinks, with a milk and/or fruit juice allowance. It was unclear in half of diets whether alcohol and carbonated drinks were permissible.

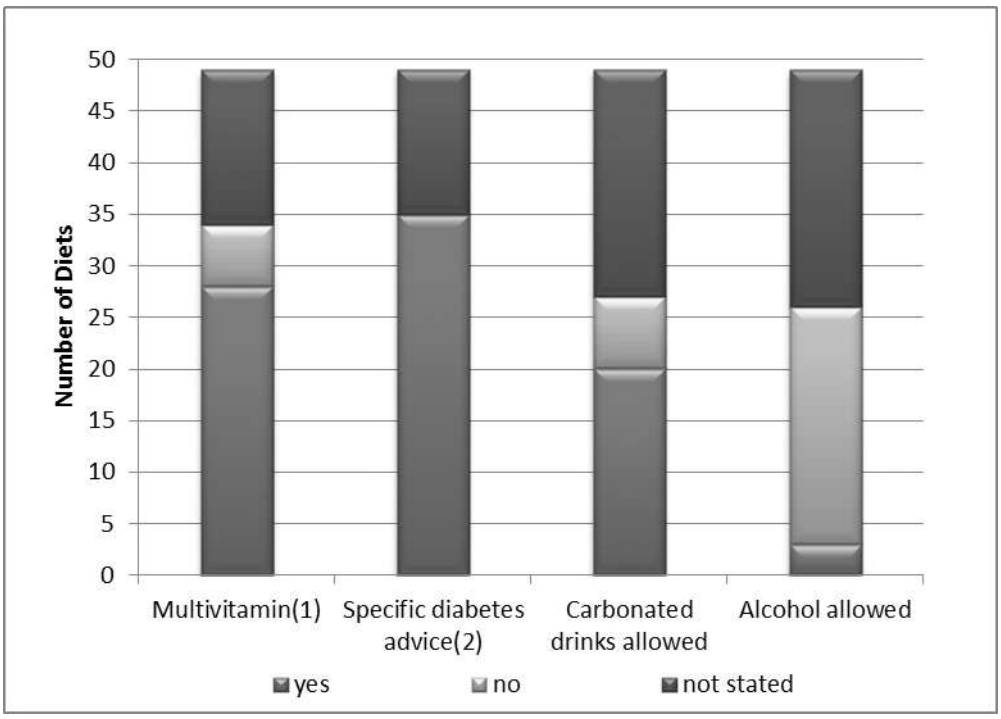


Figure 8 Patient information material recommendations during the per-operative diet. Use of a multivitamin/mineral, inclusion of carbonated drinks or alcohol, and whether to k advice if the patient has diabetes. Shown as count per total number of diets. (yes/no = specific advice, not stated = absence of specific advice either way) (1) Results include nutritionally complete meal replacement plans (2) Specific diabetes advice was considered to be either, to contact a diabetes specialist for advice, or information to adapt medication.

Services' evidence-base and rationale

Four services provided anecdotal evidence of efficacy, particularly in relation to modification of the energy intake level or carbohydrate portion size. Modifications were wholly clinician-led. There was no service-specific information regarding efficacy of dietary approaches in terms of clinical outcome measures, or for the comparison of approaches.

Three services provided evidence that their diets were actively being evaluated by their patients, including quality of patient experience and compliance. Compliance and cost were important factors in services' choice of diet, particularly in relation to the ease-of-use of milk/yoghurt diets and relative cost of meal replacement plan products in comparison to other approaches.

Discussion

Other than a consensus document produced in 2008 for BOMSS outlining dietary approaches, there has been no evidence detailing the pre-operative diets in use across bariatric services in the UK. This survey found that although services were broadly following the types of diet ascribed by the BOMSS document; interpretations and permutations differ, with the inevitable result that dietary composition and efficacy also vary.

Anecdotal evidence provided by services suggests that specification of the diet, in both form and duration, was related to encouraging patient compliance with respect to food/flavour preferences and financial cost. Services also suggest that diets were designed to enable effective application to a broad spectrum of patients in terms of body weight and co-morbidities in order to facilitate more effective patient management. Despite 27.5% of bariatric patients in the UK having Type 2 diabetes (BOMSS, 2011), it was surprising to find that nearly a third of services' dietary information did not provide specific signposting to diabetes guidance. Dietary choices appeared to be defined by clinicians based on observed efficacy at the time of surgery, in respect of liver volume and flexibility, and ease of access to the gastro-oesophageal junction (Colles et al., 2006, Edholm et al., 2011, Fris, 2004)

Findings also suggest that services rely on a fixed energy deficit irrespective of BMI, and that they depend on adaptation of duration in higher BMI patients, leading to diet length ranging from two to six weeks. However, the gains anticipated with longer duration diets may not be as beneficial as expected, as evidence indicates that 80% of liver volume reduction will occur in the first two weeks of a very low energy restrictive diet (Colles et al., 2006), and compliance to dietary restriction is known to reduce over time relative to severity of energy restriction (Del Corral et al., 2009). In addition, longer dietary restriction will have wider weight loss effects not focussed on the liver or visceral adipose tissue depots (Chaston and Dixon, 2008, Kirk et al., 2009).

In evaluation of the pre-operative rapid weight loss, which is supported by the short-term pre-operative diet, there has to be consideration of longer-term weight status and overall pre-operative weight loss; within which, evidence for clinical outcomes and improvement

of surgical complications, dependent on overall pre-operative weight loss, is not conclusive (Cassie et al., 2011, Kadeli et al., 2012).

As part of the preparatory care for patients enroute to bariatric surgery, bariatric services focus on modification of lifestyle and eating behaviour (NICE, 2006). Anecdotally, the use of a food-based diet was aimed at helping patients make healthy eating choices and adapting portion sizes (Segaran and Wheeler, 2008). In spite of this there was variability in the food-based approach, especially in terms of macronutrient composition, related to food group choices and portion sizes. The survey found a restriction in carbohydrates appears to be the predominant aim, but also indicates a wide-range of carbohydrate recommendations. There is debate in the literature around macronutrient composition of energy restrictive diets, and a detailed discussion on this topic was outside the remit of this survey. However, the level of carbohydrate intake may directly affect the level of liver lipid and therefore liver volume reduction (Browning et al., 2011). Also in wider terms, IR in obesity has been found to reduce in response to energy restrictive diets, with preferential results in the short-term found on a low carbohydrate diet (Kirk et al., 2009). The choice of macronutrients may therefore be important.

Only two studies in the literature examined the milk/yoghurt form of diet, which was found to be the second most popular approach offered. Despite claims of excellent compliance due to dietary simplicity, and clinicians' anecdotal evidence of efficacy, the attrition rate in the literature is reported to be around 20% (Barrett et al., 1999, Summerbell et al., 1998), double that of the meal replacement approach (Van Nieuwenhove et al., 2011). No direct evidence was provided to validate patients' compliance to the milk/yoghurt diet in this study.

Formula or meal replacement diets were used by 18% of bariatric services, and have been widely studied, providing the majority of evidence on efficacy of the pre-operative diet in the literature (Edholm et al., 2011, Fris, 2004, Colles et al., 2006, Lewis et al., 2006, Van Nieuwenhove et al., 2011). Non-compliance is only just over 10%, despite complaints of fatigue. Patients report a strong engagement in their upcoming bariatric treatment when following this type of diet (Edholm et al., 2011). Combining food and meal replacement approaches was seen in three services. Evidence suggests that compliance to partial use of a formula diet is more effective in reducing body weight than food-based alone, in part due

to improved compliance, and comparative composition of the formula to food-based meals (Shirai et al., 2013).

The survey also found variability in recommendations for the use of multivitamin/mineral supplements during the pre-operative diets. Supplementation is important, when the most popular pre-operative dietary approaches are unlikely to be micronutrient complete due to the limited nature of the food intake, and the bariatric population is known to have a prevalence of malnutrition and micronutrient deficiencies (Ernst et al., 2009, Schweiger et al., 2010). Nutritional deficiencies can be compounded longer-term when dietary intake is restricted post-operatively and with some surgical procedures that also influence uptake or availability of micronutrients (Damms-Machado et al., 2012, Thorell, 2011).

In considering fluid intake, all types of diet have recommendations for additional fluids, which varied with type of diet. In some cases it was unclear which types of fluids or quantities should be taken. Considering bariatric surgery candidates have a greater lifetime risk of alcohol use disorders (Heinberg et al., 2012), and the post-operative benefits of controlling intake of nutritionally poor/energy dense fluids (Olsen and Heitmann, 2009), advice regarding alcohol and carbonated drink intake might be expected to be universal; although this was not necessarily the case.

Some limitations to this current work need to be highlighted. Firstly, it should be acknowledged that data collection may not have been comprehensive, as the request for information was circulated via BOMSS, and as such, data was limited to members (and affiliates) of BOMSS. It is also possible that the duration of data collection (between September and November 2012) may not have allowed for all centres to receive or respond to the request for information. Evidence for application and changes to the dietary approach were collected in a narrative style, which allowed for flexibility in the responses but also interpretation of the services to the type and breadth of data supplied. It is acknowledged that factors outside those presented in this study may also affect clinical decision making, of choice and application of diet. However this information was not provided by services and therefore not presented in this study. Lastly, this survey examines only the short-term dietary period up to the day of surgery, where patients commonly fast and does not scrutinise the period just prior to surgery when oral carbohydrate treatment may be indicated (Awad et al., 2013).

In summary, this survey highlighted variability and lack of consensus in the form of pre-bariatric surgery diet used across different centres in the UK. Although services were broadly using the categories of diet outlined by BOMSS in 2008, there were wide variations in macro- and micro-nutrient composition, and duration of application, which will inevitably affect efficacy.

It was identified that further research was needed to compare outcomes for alternative diets in order to support best practice, particularly when considering the limitations in the breadth of evidence in the current literature. Further research should incorporate outcomes to enable evidence-based assessment of pre-operative dietary efficacy, effects on insulin sensitivity, diabetes management, and surgery completion such as liver flexibility and reduction in visceral adiposity. Consideration should also be given to the whole body approach to dietary effects encompassing wider criteria such as micronutrient status.

The objectives for this chapter were to identify the dietary approaches that were used pre-operatively in bariatric surgery, and how they differ from the very/low energy diets used to affect metabolic improvement in Type 2 diabetes.

As described above the survey identified the range of dietary approaches used in practice, and that the most common type of diet format was food-based. This format is different to the most predominant approach in research, which is to replace meals with pre-prepared nutritionally complete products. Dietary outcomes may be affected by format, but also dietary duration and energy intake deficit. The majority of pre-surgical diets aimed for low energy diets of 800-1000kcal/d, and were of only 14 days, whereas the diets used to effect reversal of diabetes are of longer duration, of six weeks and greater, and aim for very low energy up to 800kcal/d. This work has therefore highlighted discrepancies in format, energy intake and duration between the diets used in these two arenas, which will affect outcomes, and underlined the lack of evidence for the diets used in clinical practice.

Chapter 4

Outcomes of pre-operative diets for anthropometry, dietary evaluation and fasting blood

Introduction

The aim of this chapter is to examine if pre-operative diets have effects outside those of their original aim, of enabling the laparoscopic approach to surgery, including modification of circulating metabolic markers. In order to do this fasting blood samples were collected pre and post pre-operative diets, alongside collecting anthropometric data. In order to examine the perceived effects of the diet from the patient perspective a self-reported questionnaire was devised to elicit post-diet opinions, to include observations related to weight loss, satisfaction of the diet and any unwanted effects.

Methods

Clinical, demographic and anthropometric

Demographic and clinical data were collected directly from the participant, from patient medical notes and electronic patient information systems. At both the pre-operative assessment visit and preceding their operation on the day of surgery, each participant's body weight.

Blood collection

For some participants there were limitations to the total volume of blood collected. This was related to poor venous access. Where limited blood was collected not all aliquots were complete and this is the cause of the variation in sample numbers available for analyses. For each analysis the number of samples tested is included in the table of results.

Dietary intake and evaluation

All participants completed a food diary for the whole period of the dietary intervention. In addition at the end of the diet they were asked to rate their dietary experience on a categorical scale of 1-4, where 1 was a low or negative response, to 4 a high or positive response.

Questions asked:

- The diet was easy to follow
- I enjoyed the foods/drinks taken
- I had regular food and/or drink
- I felt hungry
- The diet was a positive experience
- While on the diet I noticed changes in my weight or shape
- While on the diet I noticed changes in my health/wellbeing
    - Constipation
    - Bad breath
    - Feeling cold
    - Feeling energised
    - Feeling tired
    - Mood swings
    - Overall satisfaction of the diet

Statistics and data handling

Data were not normally distributed and are presented as median and range. A Bonferroni correction was used were multiple variables were tested and the significance level is quoted with the results.

2-tailed tests were used throughout, however 1-tailed tests were used to check if the observed change in biomarkers had a significant association to the loss of body weight/excess BMI.

Results

Anthropometry

Weight change

Pre to post diet median body weight loss was 4.7kg, range 1.7-9.5kg. When calculated as a percentage of total body weight, this loss equates to a median loss of 3.4%, range 1.4-5.1% (Table 6). Results were not significantly different between diet groups, for body weight loss or percentage weight loss (P=0.494 and P=0.993 respectively).

Table 6 weight change pre to post diet, as percentage of total body weight

Intervention	Number	Weight Loss (%) Median	Weight Loss (%) Range
Food-based	26	3.6	2.0-5.0
Meal Plan	28	3.4	1.4-5.1
Total	54	3.4	1.4-5.1

Body mass index (BMI) change

BMI change pre to post diet was median -1.79kg/m^2, range -0.7 to -2.6kg/m^2. This change was not significantly different between the two diet groups (P=0.678♠). When calculated as excess BMI loss, which is change in BMI over the baseline of 25kg/m^2, this loss was median 6.4%, range 2.2-11.1%. Excess BMI loss was not statistically different between diet groups (P=0.965♠).

Gender and Diabetes specific weight/BMI change

Weight loss percentage and excess BMI loss were not significantly different between genders (P=0.174, P=0.367 respectively✺), or when separated into groups for those with Diabetes and IFG compared, to those with euglycaemia (P=0.400, P=0.842 respectively✺).

Physical activity

In interpreting results for weight loss, energy deficit, or outcomes related to these, consideration has to be made of physical activity levels. In assessing weight loss as an outcome there was no direct measurement of influences of physical activity. The study participants were asked to continue their usual lifestyle and physical activity, and self-report any changes. Physical activity should not therefore be an influential variable if participants have followed the guidance to maintain usual levels, however as this was not measured it should be considered as a potential variable when interpreting results.

♠ Kruskal-Wallis Independent Samples Test
✺ Mann-Whitney U-Test

Dietary intake

Energy intake

Intake levels and composition were all based on self-reported food diaries, and analysed as described in the Methods Section. The energy intake aim of both very low energy diets was around 800kcal/d (Table 7). The reported intakes had a larger range in the food-based diet, but median intakes were not statistically different between diet groups (P=0.311 Kruskal-Wallis Independent Samples Test) at 715kcal/d.

Daily energy deficit was calculated (as described in the Methods Section) using two models, Harris-Benedict (HB) and Fat Mass (FM), based on actual body weight loss. The results were not significantly different between diets within a model, (HB P=0.286, FM P=0.653 Independent samples Mann-Whitney U Test), but were significantly different between the two models for the same diet (Wilcoxon Signed Rank Test P=0.001). The FM model produced a much wider range of estimation of deficit, at the upper and lower end of the scale (Table 7).

Table 7 shows reported energy intake/day and estimated energy deficit using the Harris-Benedict equation and Fat Mass Model, for both dietary interventions.

Intervention		Energy Intake (kcal/d)	Harris-Benedict Estimated Energy Deficit (kcal/d)	Fat Mass Model Estimated Energy Deficit (kcal/d)
Food-based Diet	Median	714.9	1781	2145
	Range	476-1035	1091-2525	1338-3877
Meal Plan	Median	714.8	1883	2316
	Range	540-814	1151-2924	915-3877

Macronutrient intake

Macronutrient intake (energy source) differed between the two diets. The food-based diet had higher intake of carbohydrate (median 52.3% compared to 38.1%), whereas the meal plan had higher intakes of protein (median 36.5% compared to 29.9%) and fat (median 25% compared to 18.9%). All three macronutrients intakes were statistically different between groups (P<0.001 Mann-Whitney U-Test Bonferroni correction α error probability threshold 0.016) (Table 8).

Table 8 summary of macronutrient intake (percentage of total energy/d)

Intervention		**Fat (%)**	**Protein (%)**	**Carbohydrate (%)**
Food-based Diet	Median	18.9	29.9	52.3
	Minimum	13.7	22.2	38.7
	Maximum	32.5	36.9	60.2
Meal Plan	Median	25.0	36.5	38.1
	Minimum	19.3	32	34.2
	Maximum	33.6	39.2	42

Micronutrient intake

In considering the comparison of micronutrient intake between the two dietary approaches, it should be noted that the Meal plan was designed to be nutritionally complete, with vitamins and minerals added to the meal replacement products. The Food-based diet was not designed to be nutritionally complete but patients were advised to take a multivitamin/mineral supplement during (and after) the diet.

Taken exclusively on dietary intake, the two diets were statistically significantly different for all micronutrients (Table 9) except vitamin C (P= 0.762), and niacin (P=0.165 Mann-Whitney U Test (Bonferroni correction α error probability threshold 0.002)(Table 9).

Table 9 comparison of daily micronutrient intakes between the individual diets. (Daily Reference Nutrient Intake levels (RNI) can be found in the Appendices page 267).

		Sodium (mg)	Potassium (mg)	Calcium (mg)	Magnesium (mg)	
Food-based diet	Median	909.2	2107.5	611.2	173.4	
	Minimum	516.8	1254.4	316.9	108.3	
	Maximum	1837.7	4020.6	927.5	340.6	
Meal plan	Median	1778.0	3634.9	1361.2	423.1	
	Minimum	1254.4	2588.5	1045.5	306.1	
	Maximum	1928.9	3750.0	1504.5	458.7	
		Iron (mg)	**Zinc (mg)**	**Copper (mg)**	**Manganese (mg)**	
Food-based diet	Median	6.4	5.1	0.5	1.4	
	Minimum	4.0	3.2	0.3	0.7	
	Maximum	11.2	10.6	0.8	4.0	
Meal plan	Median	19.5	14.0	1.9	2.4	
	Minimum	14.4	10.5	1.5	1.8	
	Maximum	21.3	15.8	2.3	3.3	
		Vitamin C (mg)	**Niacin (mg)**	**Thiamin (mg)**	**Riboflavin (mg)**	**Vitamin B6 (mg)**
Food-based diet	Median	94.0	17.2	1.0	1.6	1.7
	Minimum	58.1	8.6	0.7	0.9	0.8
	Maximum	142.6	29.6	1.5	2.2	3.0
Meal plan	Median	95.4	19.0	1.5	2.8	2.3
	Minimum	69.3	13.5	1.0	2.2	1.7
	Maximum	104.0	20.0	2.6	3.3	2.5
		Iodide (ug)	**Folic Acid (ug)**	**Selenium (ug)**	**Vitamin B12 (ug)**	**Phosphorus (mg)**
Food-based diet	Median	129.5	202.0	38.0	4.0	1010.7
	Minimum	67.5	138.4	16.5	1.3	590.2
	Maximum	309.2	295.9	86.4	9.7	1228.6
Meal plan	Median	286.4	300.0	61.1	6.1	1295.9
	Minimum	211.7	224.2	44.3	3.9	915.4
	Maximum	351.8	336.6	78.4	7.2	1525.1

Fluid intake

Fluid intake, outside of fluid added to meal replacement products, was self-reported in the food diaries. The recommended fluid intake was a minimum of 2L/d. Median intakes for the Food-based diet 2.2L/d (range 344-4667ml/d) and for the Meal plan 2.5L/d (range 1375-5065ml/d). 33% of all participants reported taking less than 2L/d, with 35% on the Food-based diet and 31% on the Meal plan. There was no statistical significant difference in fluid intake between dietary groups P=0.213 (Independent samples Mann-Whitney U Test).

Diet evaluation

The majority of results showed no statistically significant relationship between dietary type and evaluation response. However results for constipation frequency and mood swings did show statistically significant results which differed between dietary interventions.

Constipation

73% of participants on the Food-based diet and 52% on the Meal plan did not have regular constipation. However, there was more self-reported constipation in the Meal plan group. Statistically a relationship was indicated between intervention type and constipation P=0.035 (Kendall's Tau-C Test).

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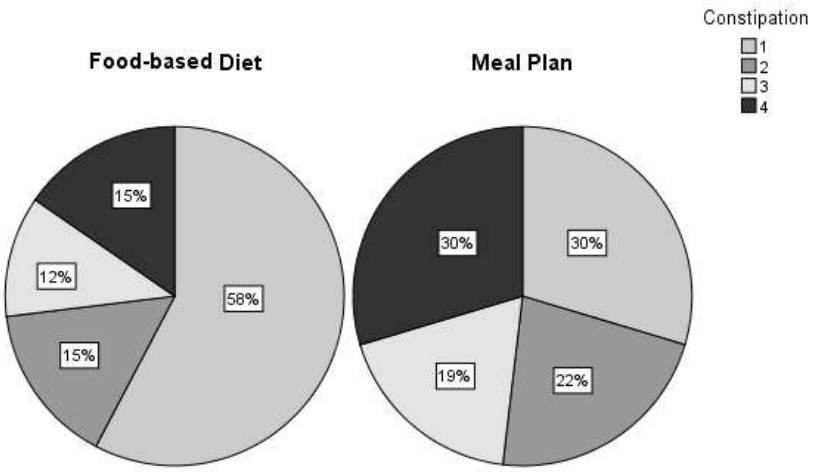


Figure 9 pie chart for dietary evaluation responses for "…noticed…constipation". Shown as percentage of responses, by diet group.

In order to discount fluid intake as a contributing factor to constipation occurrence, the relationship between occurrence of constipation and reported fluid intake was examined. There was no statistical significant difference in fluid intake between dietary groups P=0.213 (Mann-Whitney U Test). Also there was no significant association between constipation scores and fluid intake, above or below the recommend 2L/d P=0.767 (Kendall's Tau-C Test).

Mood swings

Mood swing occurrence was reported with more frequency in the Meal plan group, P=0.048 Kendall's Tau-C. Table 10.

Table 10 crosstab showing mood swing frequency against diet type. Higher frequency of mood swings was found in the group following the Meal Plan.

		Mood Swing Frequency Score None … Many times			
		1	2	3	4
Food-based Diet	**Count**	9	10	3	4
	% within Mood Swing	64.3%	66.7%	18.8%	50.0%
Meal Plan	**Count**	5	5	13	4
	% within Mood Swing	35.7%	33.3%	81.3%	50.0%

Easy to follow

Over 90% of participants found the diets easy to follow. No statistically significant relationship was found between responses and dietary intervention, P=0.169 Kendall's Tau-C. (Figure 10)

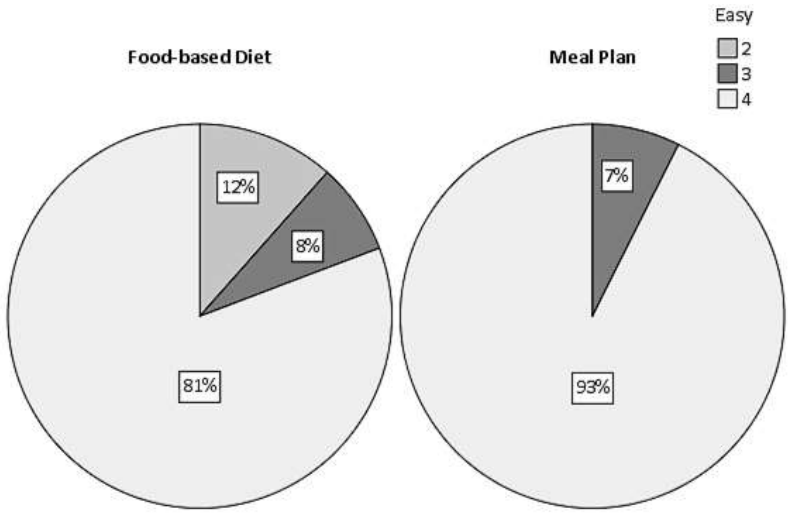


Figure 10 pie chart for dietary evaluation responses for "The diet was easy to follow". Shown as percentage of responses, by diet group.

Enjoyment

Around two-thirds of participants enjoyed some elements of their diet. No statistically significant relationship was found between responses and dietary intervention, P=0.549 Kendall's Tau-C. Figure 11 pie chart for dietary evaluation responses for "I enjoyed the food/drinks taken".

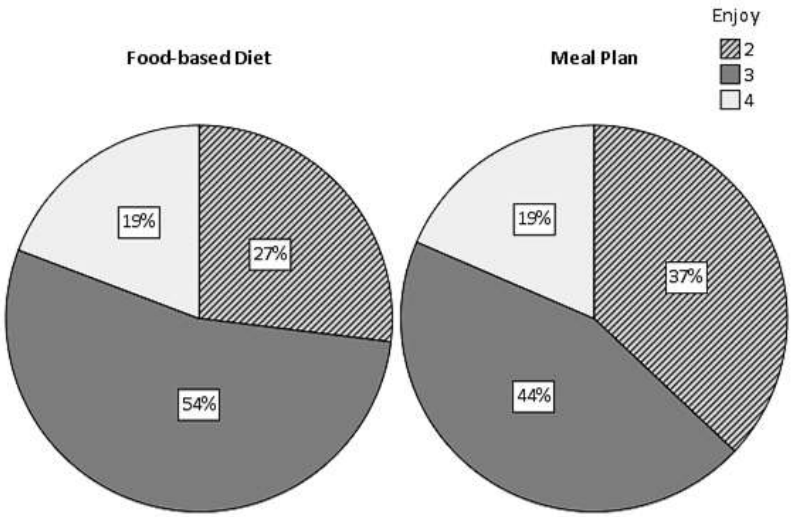


Figure 11 pie chart for dietary evaluation responses for "I enjoyed the food/drinks taken". Shown as percentage of responses, by diet group.

Hunger

Over half of all participants experienced regular hunger, with 19% and 15% always feeling hungry (Food-based diet and Meal plan respectively). No statistically significant relationship was found between responses and dietary intervention, P=0.710 Kendall's Tau-C. (Figure 12)

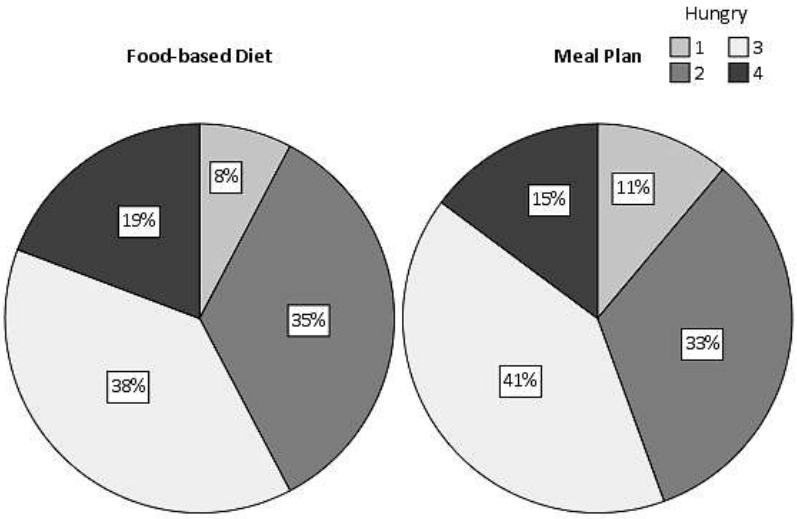


Figure 12 pie chart for dietary evaluation responses for "I felt hungry". Shown as percentage of responses, by diet group.

Positive experience

The majority of participants found their diet to be a positive experience (81% Food-based diet, 82% Meal plan). No statistically significant relationship was found between responses and dietary intervention,
P=0.627 Kendall's Tau-C.
(Figure 13)

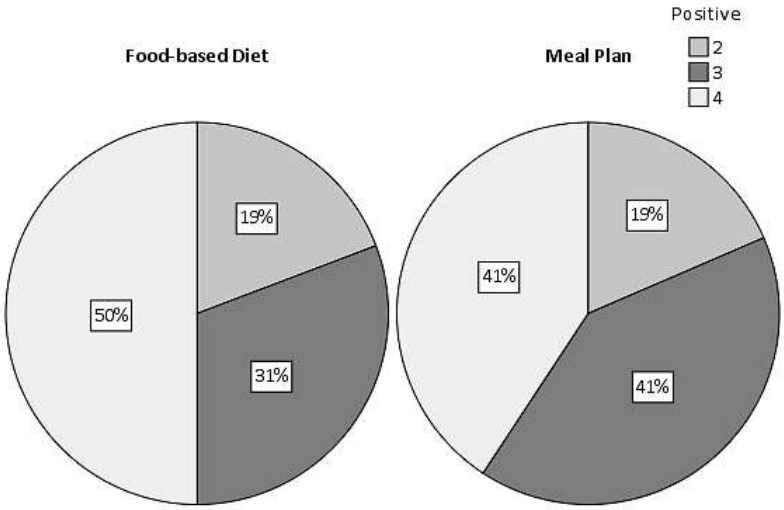


Figure 13 pie chart dietary evaluation responses for "The diet was a positive experience". Shown as percentage of responses, by diet group.

Regular food/drink

Responses indicate that the majority (84% and 92%, for the Food-based diet and Meal plan respectively) of participants ate/drank regularly. No statistically significant relationship was found between responses and dietary intervention, P=0.614 Kendall's Tau-C. Figure 14 pie chart for dietary evaluation responses for "I had regular food and/or drink".

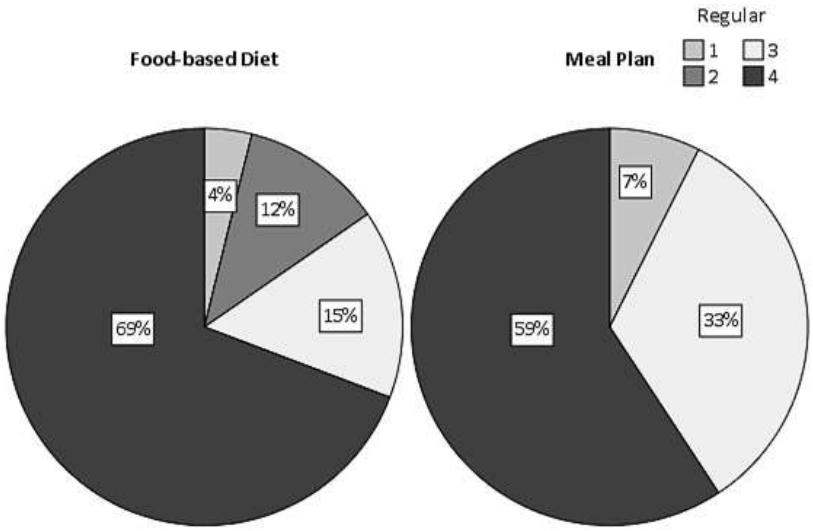
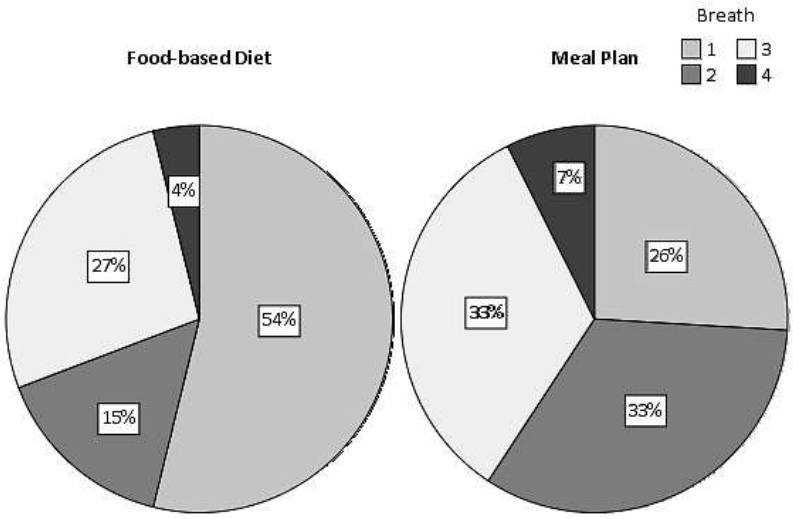


Figure 14 pie chart for dietary evaluation responses for "I had regular food and/or drink". Shown as percentage of responses, by diet group.

Bad breath

69% and 59% of participants had little or no awareness of bad breath (Food-based diet and Meal plan respectively). No statistically significant relationship was found between responses and dietary intervention, P=0.093 Kendall's Tau-C. Figure 15 pie chart for dietary evaluation responses for "…noticed…bad breath".

Figure 15 pie chart for dietary evaluation responses for "…noticed…bad breath". Shown as percentage of responses, by diet group.



Change in weight/shape

77% and 78% of participants noticed changes in their body weight/shape during the diet (Food-based diet and Meal plan respectively). No statistically significant relationship was found between responses and dietary intervention, P=0.227 Kendall's Tau-C. Figure 16 pie chart for dietary evaluation responses for "…noticed…changes in body weight/shape.

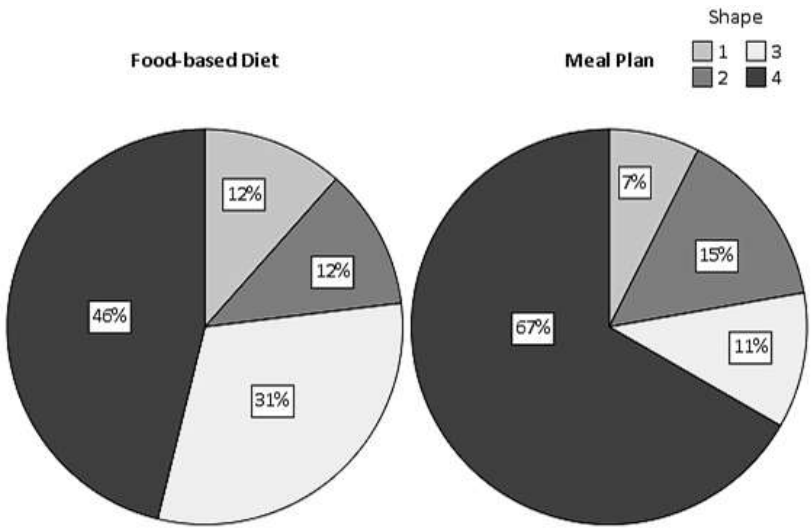


Figure 16 pie chart for dietary evaluation responses for "…noticed…changes in body weight/shape. Shown as percentage of responses, by diet group.

Energised/cold

No statistically significant relationship was found between responses and dietary intervention for feeling energised, P=0.887 Kendall's Tau-C (Figure 17). No statistically significant relationship was found between responses and dietary intervention for feeling cold, P=0.609 Kendall's Tau-C. (Figure 18).

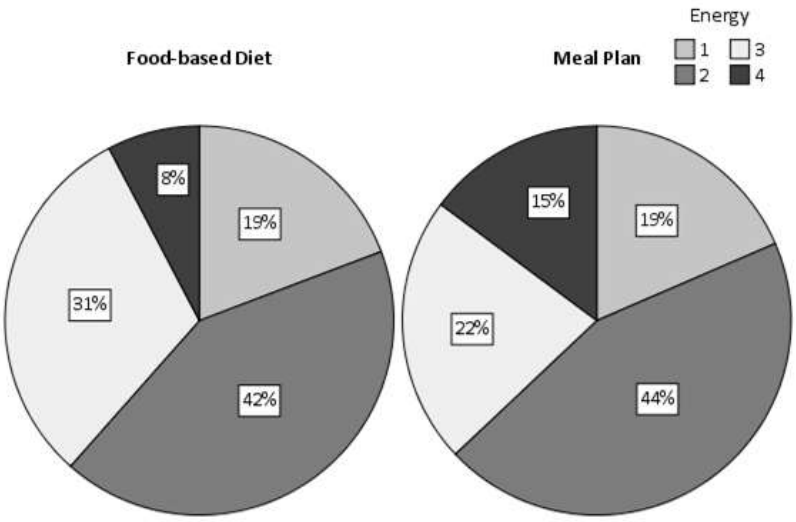


Figure 17 pie chart for dietary evaluation responses for "…noticed…feeling energised". Shown as percentage of responses, by diet group.

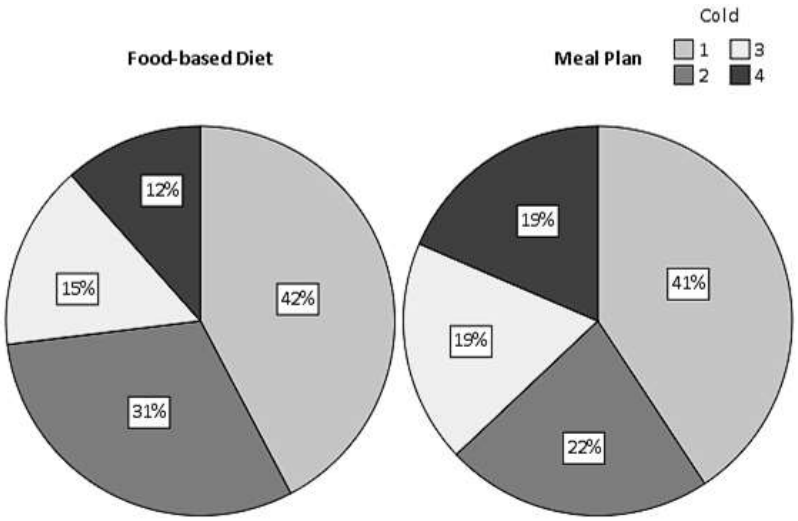


Figure 18 pie chart for dietary evaluation responses for "…noticed…feeling cold". Shown as percentage of responses, by diet group.

Tiredness

54% and 52% of participants reported regular tiredness (Food-based diet and Meal plan respectively). No statistically significant relationship was found between responses and dietary intervention, P=0.800 Kendall's Tau-C. Figure 19 pie chart for dietary evaluation responses for "…noticed…feeling tired".

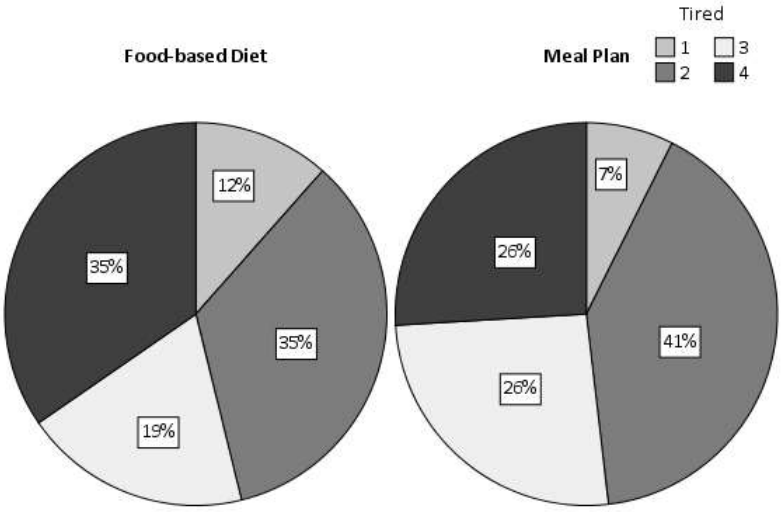


Figure 19 pie chart for dietary evaluation responses for "…noticed…feeling tired". Shown as percentage of responses, by diet group.

Satisfaction

Overall satisfaction of the diets was very high, with 92% and 85% reporting that they were satisfied or very satisfied with their diet. No statistically significant relationship was found between responses and dietary approach, P=0.675 Kendall's Tau-C. Figure 20 pie chart for dietary evaluation responses for "Overall satisfaction of the diet".

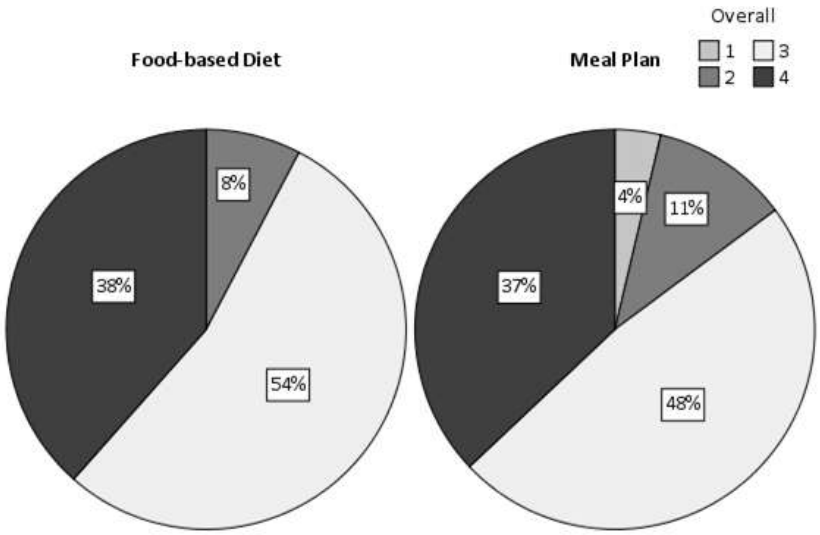


Figure 20 pie chart for dietary evaluation responses for "Overall satisfaction of the diet". Shown as percentage of responses, by diet group.

Hunger associations

In considering the effects of constipation a cross-tab was completed between hunger scores and constipation scores (Table 11) For pooled diets there was a significant negative relationship between constipation and hunger, with those reporting higher frequency of constipation also reporting less hunger P=0.002♠.

Table 11 cross-tab of constipation scores by hunger scores for pooled dietary groups.

Hunger (score) (Never 1-4 Always)		Constipation (score) (None 1-4 Many Times)			
		1	**2**	**3**	**4**
1	Count	1	1	1	2
2	Count	5	4	3	6
3	Count	11	3	3	4
4	Count	6	2	1	0

When results were split by diet group, this relationship was only observed in the Meal plan P=0.001, whereas the Food-based diet does not show a strong relationship P=0.199, most likely due to the low levels of constipation reported.

To investigate if body weight loss or reported intake had any relationship to reported hunger (self-reported hunger was scored, Never 1-4 Always), cross-tabs were completed, with results for body weight loss and reported intake divided into two groups, above and below the median in each group.

When diets were pooled there was a significant negative relationship between weight loss percentage achieved and hunger score P=0.030♠, with higher scores for hunger frequency in the lower weight loss group (Table 12). When split by diet group, this relationship remains, but was only significant for the Meal plan P=0.015, Food-based diet P=0.401♠.

♠ Kendall's Tau-C Test

Table 12 cross-tab of weight loss (%) groups against hunger scores, for pooled diet groups.

Weight loss (%)		Hungry (score) (Never 1-4 Always)			
		1	**2**	**3**	**4**
<= Median	Count	2	6	11	7
Median +	Count	3	12	10	2

When macronutrient intake was examined for fat, carbohydrate or protein (above or below median), against hunger scores, no significant associations were found. Neither were there any significant correlations found for macronutrient intake (% of total energy) against weight loss or excess BMI loss.

Body weight loss, energy deficit, intake reporting and hunger

Both dietary groups lost weight, but it was noticeable that there was a wide range of weight loss. Adaptive thermogenesis could have played a role in this variability, as well as hunger and non-compliance to the diet. This has been described in detail in a review by Tremblay *et al*, where the response of an individual to a short-term hypo-energetic diet can be a drop in metabolic rate (energy expenditure). They describe work which observed a reduction in energy expenditure of -28kcal per kg weight loss in an intervention of less than six weeks. They also describe an increase in the physiological drive to eat, which combined with reduced energy expenditure compromises weight loss to a lesser or greater extent. (Tremblay et al., 2013). Related to this was the observed drop in leptin which has been found to be independently related to the adaptive response, partly explaining a disproportionate reduction in energy expenditure on a hypo-energetic diet, and potentially explaining some of the increase in appetite also observed (Camps et al., 2015, Tremblay et al., 2013).

With this in consideration, the range of loss will be related to the level of energy deficit achieved. With the maximum loss 5% body weight or 9.5kg, and minimum 1.4% or 1.7kg; it is worth considering the question of participants' compliance to the diet. Thomas *et al* in their modelling work have suggested that alongside reduced energy expenditure an intermittent lack of adherence can contribute to the "plateau" of weight loss in longer term

dietary weight loss programs (Thomas et al., 2014), and even over the two weeks of this dietary intervention participants' readily admitted to non-compliance.

This was confirmed when examining the reported dietary intakes, where there was a wide range of results, from 476-1035kcal/d in the Food-based diet and 540-814kcal/d in the Meal plan. Some variance can be explained by the range of foods available and choices the participants' made. However, self-reported intakes are known to be unreliable, and more rigorous reporting methods than those used in this study produce underreporting of up to one-third (Rennie et al., 2007).

If the observed association between weight loss achieved and hunger is explored, an increase in intake might be expected to be observed with greater hunger, and was corroborated with the lower level of weight loss in this group. It can be interpreted from these results that those who reported more frequent hunger were unable to limit their intake as much as those that did not, resulting in reduced weight loss. Findings of non-compliance due to hunger may also be supported by an association between levels of circulating leptin and achieved weight loss, detailed in the following section page **Error! Bookmark not defined.**.

Fluid intake, constipation and hunger

Both dietary groups reported median fluid intakes above the recommended level of 2L/d. However as with other dietary intake there was a wide variation and in interpreting results self-reported fluid intake was likely to be subject to the same underreporting inaccuracies. This would then indicate that the majority (over 75%) meet the minimum recommendations.

Constipation was found to be of a higher frequency in the Meal plan, in contrast to the majority (n=15) in the Food-based diet not encountering any constipation. This frequency in the Meal plan was not related to fluid intake, but is unsurprising given the style of the diet and that it is recognised to have a low level of fibre. In practice this is counteracted by LighterLife in their regimes by the addition of normal foods or supplementary fibre packs (www.LighterLife.co.uk).

Blood results

Fasting blood samples were taken pre and post diet, and the results that follow are pooled for both diets, and diet specific. Change pre to post diet were compared between diet groups. None of the results for the circulating biomarkers examined, for change pre to post diet, were significantly different between diet groups (Kruskal-Wallis Test Bonferroni correction α error probability threshold 0.0026).

Pooled results for both diet groups indicate circulating biomarkers for Lep, IL6, CRP, Fet, ELF, LDL, HDL, non-HDL, NEFA, BHB, AST, CK18M30 all changed significantly pre to post diet. Whereas TAG, GGT, TNF, Adi, C18M65, Glu and Ins did not change significantly (Wilcoxon Signed Rank Test Bonferroni correction α error probability threshold 0.0026).

AST and CK18-M30 both increased significantly pre to post diet. GGT reduced pre to post diet, but this result was not significant. ELF reduced significantly pre to post diet. CK18-M65 results show a non-significant increase. (Table 13)

Lep, CRP, IL6 and FET, all reduced significantly pre to post diet. TNF and Adi did not change significantly. (Table 14)

Results for Glu and Ins reduce during both diets, but the results were not statistically significant. (Table 15)

Results for LDL, HDL, and Non-HDL reduce significantly pre to post diet (Table 18). NEFA and BHB rise significantly pre to post diet. TAG does not change significantly pre to post diet.

Diet-specific and pooled results

Table 13

Intervention		AST Pre	AST Post	Change pre to post diet	GGT Pre	GGT Post	Change pre to post diet	ELF Pre	ELF Post	Change pre to post diet
		(U/L)		(P value)	(U/L)		(P value)			(P value)
Food-based Diet	N	26	24	<0.001	26	24	0.092	25	24	<0.001
	Median	20.7	27.4		27.5	25.1		9.1	8.2	
	Range	21.3	30.6		54.6	110.4		3.4	3.0	
Meal Plan	N	28	28	<0.001	28	28	0.838	27	27	0.001
	Median	19.6	29.2		28.1	27.1		9.3	8.4	
	Range	23.7	46.4		79.8	87.5		2.1	3.6	
Total	N	54	52	<0.001	54	52	0.314	52	51	<0.001
	Median	20.3	28.0		27.5	26.4		9.2	8.4	
	Range	23.8	46.4		85.1	110.4		3.4	3.6	
Intervention		CK18M30 Pre	CK18 M30 Post	Change pre to post diet	CK18 M65 Pre	CK18M65 Post	Change pre to post diet			
		(U/L)		(P value)	(U/L)		(P value)			
Food-based Diet	N	25	25	0.019	25	25	0.600			
	Median	148.9	167.7		50.7	60.5				
	Range	334.4	281.3		456.7	358.2				
Meal Plan	N	28	28	0.011	28	28	0.017			
	Median	146.9	215.3		51.3	87.4				
	Range	416.9	326.9		315.8	465.8				
Total	N	53	53	0.001	53	53	0.032			
	Median	147.9	181.5		51.3	68.1				
	Range	427.1	333.5		466.8	473.0				

Circulating biomarkers of liver function and status, pre and post diet, for both diet interventions, and comparative total (pooled) results.

Data are median and range, with P values for the change pre to post diet♦.

N represents number of samples analysed per test.

♦ Bonferroni correction α error probability threshold 0.0026

Table 14

Intervention		Adi Pre	Adi Post	Change pre to post diet	Lep Pre	Lep Post	Change pre to post diet	TNF Pre	TNF Post	Change pre to post diet
		(ug/mL)		(P value)	(ng/mL)		(P value)	(pg/mL)		(P value)
Food-based Diet	N	26	24	0.415	26	24	<0.001	26	24	0.945
	Median	7.9	6.9		48.7	36.1		2.1	2.0	
	Range	10.2	12.8		102.1	74.2		2.7	2.2	
Meal Plan	N	28	28	0.776	28	28	0.001	28	28	0.970
	Median	7.1	6.9		47.5	39.9		2.0	2.0	
	Range	10.8	10.7		94.7	88.2		34.3	21.1	
Total	N	54	52	0.382	54	52	<0.001	54	52	0.924
	Median	7.4	6.9		48.1	37.6		2.1	2.0	
	Range	11.2	12.9		105.6	88.2		34.3	21.2	
Intervention		CRP Pre	CRP Post	Change pre to post diet	Fet Pre	Fet Post	Change pre to post diet	IL6 Pre	IL6 Post	Change pre to post diet
		(mg/L)		(P value)	(ug/mL)		(P value)	(pg/mL)		(P value)
Food-based Diet	N	26	24	0.007	26	24	0.008	26	24	0.175
	Median	8.2	5.1		554.4	543.0		3.7	3.7	
	Range	42.8	21.7		426.3	267.7		10.4	8.3	
Meal Plan	N	28	28	0.004	28	28	0.018	28	28	0.040
	Median	9.7	6.4		525.2	519.8		4.5	3.7	
	Range	29.1	21.8		336.9	329.9		42.6	25.4	
Total	N	54	52	<0.001	54	52	0.001	54	52	0.014
	Median	9.7	6.1		546.9	530.6		4.1	3.7	
	Range	43.6	21.8		426.3	360.5		42.7	25.4	

Circulating adipokines and markers of inflammation, for both dietary interventions, with comparative total (pooled) results. Data are median and range, with P values for the change pre to post diet♦.

N represents number of samples analysed per test.

♦ Bonferroni correction α error probability threshold 0.0026

Table 15 circulating levels of glucose and insulin, pre and post diet, for both dietary interventions, with comparative total (pooled) results. Data are median and range, with P values for the change pre to post diet♦. N represents number of samples analysed per test.

Intervention		Glu Pre	Glu Post	Change pre to post diet	Ins Pre	Ins Post	Change pre to post diet
		(mmol/L)		(P value)	(mU/L)		(P value)
Food-based Diet	N	26	25	0.128	26	24	1.000
	Median	6.0	5.7		19.8	17.5	
	Range	5.1	4.5		50.1	60.8	
Meal Plan	N	28	28	0.005	27	28	0.061
	Median	5.7	5.6		18.7	17.4	
	Range	11.3	6.0		113.3	53.2	
Total	N	54	53	0.003	53	52	0.202
	Median	5.9	5.7		19.0	17.4	
	Range	11.3	6.0		113.3	60.8	

♦ Bonferroni correction α error probability threshold 0.0026

Model of insulin resistance

HOMA-IR distribution was not statistically different between diet groups, pre-diet P=0.581, or post-diet P=0.398 (Kruskal-Wallis Independent Samples Test). Both diet groups show a reduction in HOMA-IR pre to post diet, and the Meal plan change was statistically significant P=0.021♦, whereas the Food-based diet group change was not P=0.909♦. (Table 16)

Table 16 HOMA-IR results pre and post diet, and change pre to post diet, for pooled diets and dietary interventions.

Intervention		**HOMA-IR Pre**	**HOMA-IR Post**	**HOMA-IR change**
Food-based Diet	Median	5.32	4.90	-0.08
	Minimum	2.13	0.74	
	Maximum	16.43	25.39	
Meal Plan	Median	4.62	4.49	-1.03
	Minimum	1.76	0.89	
	Maximum	30.56	11.06	

♦ Wilcoxon Signed Ranks Test

When the results for pooled diets pre to post intervention are split into groups for Diabetes status (as previously), the results indicate a drop in both the euglycaemic group , -0.36, and the DM/IFG group -1.14, however neither of these results was statistically significant (P=0.339, P=0.201 respectively♦). If separate dietary groups are divided by Diabetes status, all groups show a drop pre to post diet, but none of the groups show statistically significant changes (Food-based diet euglycaemia P=0.754, Food-based diet DM/IFG P=0.875, Meal plan euglycaemia P=0.098, Meal plan DM/IFG P=0.091♦).

To examine results related to the "adipose tissue expandability hypothesis" pooled dietary results were divided by childhood weight status. It is worth noting that the group that reports being overweight/obese as a child have higher results for minimum, median and maximum BMI pre and post diet, and HOMA-IR pre-diet. The two groups show a significant difference in distribution for BMI (pre-diet P=0.034, post-diet P=0.046), although they were not statistically different for BMI change (P=0.645), or HOMA-IR (Pre-diet P=0.156, Post-diet P=0.918) (all Mann-Whitney U Test). Both groups show a drop in HOMA-IR pre to post diet (results below), but only the group that were overweight/obese as children have a statistically significant result P=0.052, as opposed to those that reported being lean as a child P=0.986♦(Table 17), and this only remains significant in the Meal Plan group when separated by diet (Meal Plan P=0.005, Food-based diet P=0.910♦.

Table 17 BMI and HOMA-IR results pre and post diet by reported childhood weight status.

Child weight status		**BMI pre-diet**	**BMI post-diet**	**BMI change**	**HOMA-IR Pre**	**HOMA-IR Post**	**HOMA-IR change**
Lean	Median	47.3	46.2	-1.7	4.20	3.56	-0.20
	Minimum	41.5	39.6		1.76	2.04	
	Maximum	61.8	60.6		9.86	14.37	
Overweight	Median	51.9	50.3	-1.8	5.47	4.96	-1.03
	Minimum	43.1	41.1		1.91	0.74	
	Maximum	66.8	65.5		30.56	25.39	

♦ Wilcoxon Signed Ranks Test

Table 18

Intervention		NEFA Pre	NEFA Post	Change pre to post diet	TAG Pre	TAG Post	Change pre to post diet	BHB Pre	BHB Post	Change pre to post diet
		(mmol/L)		(P value)	(mmol/L)		(P value)	(mmol/L)		(P value)
Food-based Diet	N	26	25	<0.001	26	24	0.920	26	24	<0.001
	Median	0.5	1.0		1.3	1.5		0.2	1.0	
	Range	0.6	1.1		3.0	3.0		0.6	3.6	
Meal Plan	N	28	28	<0.001	28	28	0.001	28	28	<0.001
	Median	0.5	1.0		1.6	1.2		0.2	1.1	
	Range	0.9	1.1		2.4	2.4		1.2	4.1	
Total	N	54	53	<0.001	54	52	0.024	54	52	<0.001
	Median	0.5	1.0		1.3	1.4		0.2	1.1	
	Range	0.9	1.5		3.0	3.0		1.3	4.1	
		HDL Pre	HDL Post		LDL Pre	LDL Post		Non-HDL Pre	Non-HDL Post	
		(mmol/L)			(mmol/L)			(mmol/L)		
Food-based Diet	N	26	24	<0.001	26	24	<0.001	26	24	<0.001
	Median	1.3	1.0		3.6	3.1		4.3	3.7	
	Range	0.8	1.2		3.9	3.4		4.4	3.9	
Meal Plan	N	28	28	<0.001	28	28	<0.001	28	28	<0.001
	Median	1.1	0.9		3.5	3.1		4.2	3.8	
	Range	0.7	0.7		3.1	3.1		3.4	3.2	
Total	N	54	52	<0.001	54	52	<0.001	54	52	<0.001
	Median	1.2	1.0		3.5	3.1		4.3	3.8	
	Range	0.8	1.2		3.9	3.5		4.4	4.2	

Circulating biomarkers of lipids, pre and post diet, for both dietary interventions, with comparative total (pooled) results.

Data are median and range, with P values for the change pre to post diet♦.

N represents number of samples analysed per test.

♦ Bonferroni correction α error probability threshold 0.0026

Circulating lipids and lipid lowering medication

For completeness circulating lipids were analysed separated into two groups dependent on whether participants were taking a lipid lowering medication* or not. Of the cohort of 54 only six were taking these at the time of the study. The results pre to post diet remained significant and analogous in direction for NEFA, HDL, LDL and BHB, however Non-HDL results did not remain significant pre to post diet, and TAG results in the statin group did not change significantly whereas TAG in the non-statin group increased significantly. (Table 19)

Table 19 Wilcoxon Signed Rank test for circulating lipids pre to post diet change, grouped by lipid lowering medication use.

Biomarker Change (mmol/L)		HDL	LDL	Non-HDL	TAG	NEFA	BHB
No statin	Change (median)	-0.2	-0.5	-0.5	-0.2	0.5	0.7
	Change (range)	-0.6-0.3	-1.6-0.5	-1.7-0.6	-2.1-0.8	-0.6-1.2	0-4.1
	Asymp. Sig. (2-tailed)	<0.001	<0.001	0.021	<0.001	<0.001	<0.001
Statin	Change (median)	-0.2	-0.6	-0.6	0.1	0.7	1.4
	Change (range)	-0.3-0	-1.1-0.2	-1.5-0.2	-0.5-0.3	0.3-1.2	0.3-2.6
	Asymp. Sig. (2-tailed)	0.028	0.046	0.917	0.075	0.028	0.028

Body weight change and circulating biomarker change pre to post diet

In order to evaluate if significant changes observed in circulating biomarkers were associated to body weight loss achieved, Spearman's Rho Correlations were carried out.

Spearman's Rho Correlation results show significant associations between body weight loss and change in ELF, BHB, HDL, non-HDL, NEFA, TAG, CRP and Fet. Significant correlations were also found between excess BMI loss and change in BHB, LDL, non-HDL, NEFA, CRP and Fet. (Table 20 for full results)

* The statins (atorvastatin, fluvastatin, pravastatin, rosuvastatin, and simvastatin) competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, an enzyme involved in cholesterol synthesis, especially in the liver https://www.medicinescomplete.com/mc/bnf/current/PHP1623-statins.htm) accessed 11.9.14

Although there were significant change pre-post diet in Lep, IL6, AST, and CK18-M30, these were not correlated with body weight loss or excess BMI loss.

Table 20 Spearman's Rho correlation between change in body weight (%) or excess BMI loss (%) and change in circulation biomarkers.

		HDL Change	Non HDL Change	LDL Change	NEFA Change	ELF change
Excess BMI Loss (%)	Correlation Coefficient	-0.199	-0.292*	-0.237*	0.437**	-0.223
	Sig. (1-tailed)	0.079	0.018	0.045	0.001	0.058
Weight Loss (%)	Correlation Coefficient	-0.262*	-0.291*	-0.230	0.416**	-0.223
	Sig. (1-tailed)	0.031	0.018	0.050	0.001	0.058
		CRP Change	**Fet Change**	**BHB change**		
Excess BMI Loss (%)	Correlation Coefficient	0.266*	-0.315*	0.306*		
	Sig. (1-tailed)	0.028	0.011	0.014		
Weight Loss (%)	Correlation Coefficient	0.307*	-0.255*	0.387**		
	Sig. (1-tailed)	0.013	0.034	0.002		

**. Correlation is significant at the 0.01 level (1-tailed).
*. Correlation is significant at the 0.05 level (1-tailed).

However, circulating levels of leptin, both pre and post diet, showed a significant association to weight loss (%) achieved (pre-diet P=0.006, post-diet P<0.001 Spearman's Rho correlation). Higher leptin was associated to lower weight loss. (Figure 21)

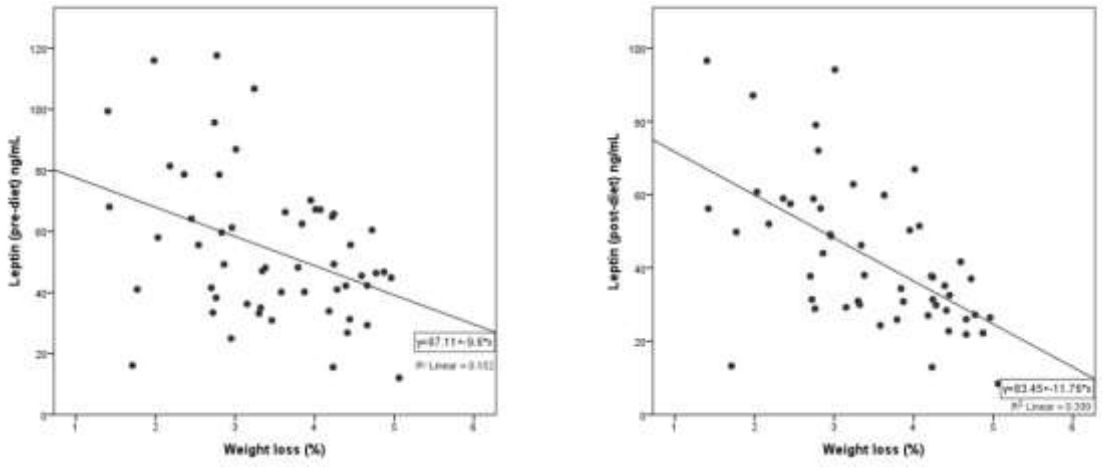


Figure 21 dot plots of weight loss (%) achieved against circulating leptin, showing significant associations pre-diet (P=0.012) and post-diet (P<0.001).*

Diabetes specific blood analyses

Pooled blood results (both diets) were separated by Diabetes status into two subgroups for euglycaemia or Diabetes/impaired fasting glucose (DM/IFG). Euglycaemia FD n=13, MRP n=16, IFG/DM FD n=13, MRP n=12. For all the biomarkers tested there was no significant difference between diabetes groups. (Table 21)

Table 21 circulating biomarkers pre and post diet, separated by Diabetes status, grouped as Euglycaemic or IFG/DM. Data are median and range, Wilcoxon signed rank test with P values for the change pre to post diet. Bonferroni correction α error probability threshold 0.0026. N represents number of samples analysed per test.

		Adi Pre	Adi Post	Pre to post diet change	Lep Pre	Lep Post	Pre to post diet change	IL6 Pre	IL6 Post	Pre to post diet change	TNF Pre	TNF Post	Pre to post diet change
		(ug/mL)		(P value)	(ng/mL)		(P value)	(pg/mL)		(P value)	(pg/mL)		(P value)
Euglycaemic	N	29	28		29	28		29	28		29	28	
	Median	8.4	8.1	0.524	55.6	40.8	<0.001	3.9	3.7	0.078	2.0	2.2	0.736
	Range	11.2	10.5		90.7	83.4		11.8	16.2		3.6	2.5	
IFG/DM	N	25	24		25	24		25	24		25	24	
	Median	6.3	6.4	0.568	45.5	34.6	<0.001	4.7	3.8	0.094	2.1	2.0	0.737
	Range	9.1	12.9		105.6	78.7		42.5	25.4		34.3	21.2	
		Glu Pre	Glu Post	Pre to post diet change	Ins pre	Ins post	Pre to post diet change	CRP Pre	CRP Post	Pre to post diet change	Fet Pre	Fet Post	Pre to post diet change
		(mmol/L)		(P value)	(mU/L)		(P value)	(mg/L)		(P value)	(ug/mL)		(P value)
Euglycaemic	N	29	29		29	28		29	28		29	28	
	Median	5.6	5.1	0.036	16.5	13.4	0.362	9.4	5.9	0.002	525.5	518.6	0.013
	Range	1.9	2.2		112.5	53.6		29.1	21.7		375.5	259.6	
IFG/DM	N	25	24		24	24		25	24		25	24	
	Median	6.3	6.3	0.032	21.9	19.5	0.378	10.0	6.6	0.009	559.1	551.9	0.018
	Range	10.1	5.6		52.6	56.0		43.2	21.8		387.7	360.5	

Table 21 (continued)

		HDL Pre	HDL Post	Pre to post diet change	LDL Pre	LDL Post	Pre to post diet change	NEFA Pre	NEFA Post	Pre to post diet change	TAG Pre	TAG Post	Pre to post diet change
		(mmol/L)		(P value)	(mmol/L)		(P value)	(mmol/L)		(P value)	(mmol/L)		(P value)
Euglycaemic	N	29	28	<0.001	29	28	<0.001	29	29	<0.001	29	28	0.113
	Median	1.2	1.0		3.7	3.2		0.5	1.0		1.3	1.3	
	Range	0.7	0.7		3.9	2.9		0.9	0.9		2.5	2.4	
IFG/DM	N	25	24	0.001	25	24	<0.001	25	24	<0.001	25	24	0.097
	Median	1.1	1.0		3.3	2.7		0.5	1.0		1.4	1.5	
	Range	0.8	1.1		3.2	3.5		0.7	1.5		2.9	3.0	
		Non-HDL Pre	Non-HDL Post	Pre to post diet change	BHB pre	BHB post	Pre to post diet change	ELF Pre	ELF Post	Pre to post diet change			
		(mmol/L)		(P value)	(mmol/L)		(P value)			(P value)			
glycaemic	N	29	28	<0.001	29	28	<0.001	28	28	<0.001			
	Median	4.4	3.9		0.2	1.2		9.2	8.2				
	Range	4.3	2.9		1.2	4.0		2.8	3.4				
IFG/DM	N	25	24	<0.001	25	24	<0.001	24	23	0.001			
	Median	4.2	3.4		0.2	0.9		9.2	8.4				
	Range	4.0	4.2		0.5	2.7		3.0	3.1				
		AST Pre	AST Post	Pre to post diet change	GGT Pre	GGT Post	Pre to post diet change	CK18M30 Pre	CK18M30 Post	Pre to post diet change	CK18M65 Pre	CK18 M65 Post	Pre to post diet change
		(U/L)		(P value)	(U/L)		(P value)	(U/L)		(P value)	(U/L)		(P value)
Euglycaemic	N	29	28	<0.001	29	28	0.750	28	28	0.001	28	28	0.027
	Median	19.5	26.9		22.3	24.0		130.0	154.7		43.5	62.6	
	Range	16.2	46.4		85.1	92.7		210.6	333.5		315.8	473.0	
IFG/DM	N	25	24	<0.001	25	24	0.059	25	25	0.061	25	25	0.476
	Median	23.1	28.8		33.2	26.9		160.9	220.3		83.1	68.1	
	Range	23.7	33.6		65.8	104.6		416.9	313.0		456.7	362.4	

Fetuin-A and NEFA

The association between NEFA and Fetuin-A was investigated. NEFA increase is shown as a shift to the right without any noticeable change in Fetuin-A level or distribution, and this was the same for both groups of euglycaemia and DM/IFG (Figure 22).

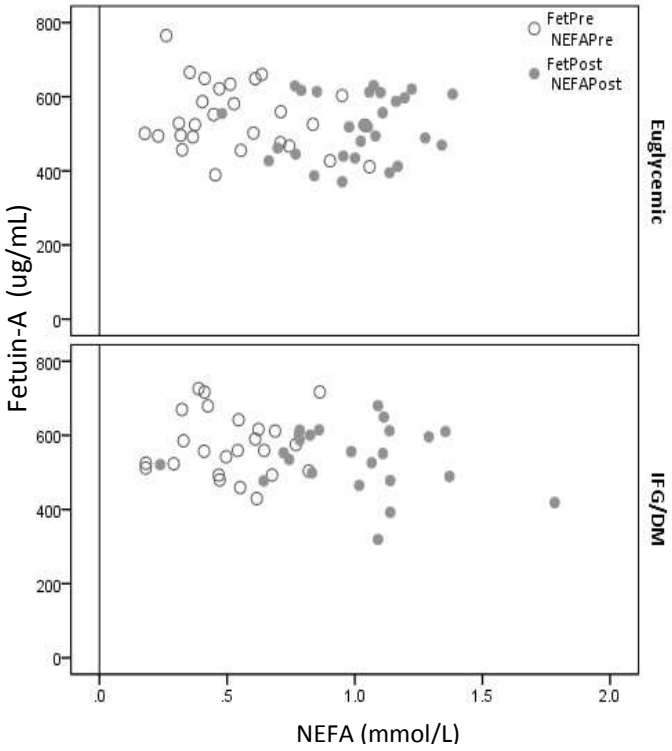


Figure 22 scatter plot of NEFA change against Fetuin-A change, by Diabetes status.

An increase in NEFA is shown as a shift to the right, but there were no discernible changes to Fetuin-A level or distribution. This behaviour was mirrored in the groups of euglycaemia and DM/IFG.

Discussion

The objectives for this work were to examine if the pre-operative diet had effects outside those of its original aim of enabling the laparoscopic approach to surgery, including modification of circulating metabolic markers; and how the diets' and their effects were perceived by patients.

TAG, NEFA, BHB, HDL, LDL, and non-HDL change pre to post diet all correlated significantly to body weight loss or excess BMI loss. As the liver is the site of lipoprotein production, they could indicate there has been a reduction in output post-diet compared to pre-diet. The majority of these results were not affected by lipid lowering medication, however both TAG and non-HDL results were altered enough by statin use to reduce pre to post diet change significance. For the majority of circulating lipids the changes observed were likely to be related to the loss of excess body weight.

Betahydroxybutyrate (BHB) was seen to increase significantly pre to post diet, in what should have been similar fasting conditions on both occasions; the median increasing by 5.7 times the original level. When combined with rising levels of BHB that are indicative of increased levels of lipid oxidation, they indicate energy intake restriction affecting NEFA release and recycling for systemic energy production, with adipose tissue reducing in mass and with both mass loss and negative energy balance affecting leptin production.

Leptin change taken together with the observation that secretory capacity of adipose tissue is not altered by levels of autophagy (Jansen et al., 2012), and that there was an increase in circulating NEFA, it suggests adipose tissue mass reduction. It has been noted that during dynamic weight loss circulating concentrations of leptin significantly reduce relative to fat mass, both due to decreased leptin gene expression in adipose tissue and increased leptin clearance rates therefore, the change in leptin reflects not only energy stores (adipose tissue mass) but also the status of energy balance (Rosenbaum and Leibel, 2014).

Following on from the discussion in the previous section regarding hunger and weight loss achieved, results could support the theory that leptin reduces as a result of the intervention, which affects a decrease in energy expenditure and potentially this combined with an increase in hunger contributes to the variability in weight loss achieved. However no significant association was found between levels of hunger and circulating leptin.

Results pre to post diet do not show a significant change in circulating levels of adiponectin. Klempel and Varady (2011) in their review determined the reliability of leptin, but not adiponectin, as a biomarker of diet-induced weight loss. They concluded that at weight loss levels equivalent to those observed in this study leptin may show a significant change. However adiponectin would only be seen to change significantly to a visceral adipose tissue loss which was greater than that observed in this study (Klempel and Varady, 2011).

Plasma glucose fell significantly pre to post diet, when the data were pooled, and this remained significant for both groups when separated into groups for Diabetes status. No significant association was found between change in glucose and weight loss. This suggests that the significant change observed must be affected by a number of factors, not limited to diet type or diabetes status.

Results for HOMA-IR show a reduction pre to post diet in the Meal plan group and those who reported being overweight/obese as a child. Considering the expandability hypothesis, these results could be interpreted to suggest that at higher starting BMI with the same reduction in BMI, those who reported being overweight/obese as a child had a greater change in insulin sensitivity than those who reported being lean as a child. This suggests that if adipose tissue dysfunction was playing a role in this insulin sensitivity, that it was more readily adaptable in severe obesity where there is greater expandability of adipose tissue.

Around 60% of this study cohort reported that they had been overweight or obese as a child. A relationship between body weight in childhood and the likelihood of developing metabolic disease has been suggested, due to limitations in adipose tissue expansion in adulthood (Spalding et al., 2008). In this study cohort there was no evidence to link those who were diagnosed with Type 2 diabetes or had impaired fasting glucose, with those who had been lean or overweight as children. However, examination of HOMA-IR suggests that those who were overweight as a child have a greater response to the dietary restriction resulting in a significant reduction in hepatic insulin resistance.

C-reactive protein (CRP) is a circulating marker of inflammation, synthesized by the liver under the regulation of the pro-inflammatory cytokines such as IL6 and TNFa. A significant drop in CRP was observed pre to post diet, alongside a reduction in IL6. CRP levels have

also been found to be associated positively to levels of leptin and negatively to levels of adiponectin (Nakamura et al., 2013). Therefore concurrent to a significant reduction in leptin, and combined with the reduction in IL6, it was unsurprising to note this reduction in CRP, and suggests a reduction in circulating inflammatory mediators during the diet.
Choi *et al* (2013) measured the effects of a 12-week energy restrictive diet (30% reduction in intake), and found Fetuin-A levels significantly dropped, associated independently with energy restriction and CRP (Choi et al., 2013). In the present cohort levels of Fetuin-A were also found to reduce significantly pre to post diet, and there was a significant correlation to weight loss (%) and excess BMI loss (%) (P=0.034, P=0.011 respectively). There was also a significant positive correlation for CRP and weight loss (%) P=0.013, and excess BMI loss (%) P=0.028. Also, circulating levels of CRP were significantly correlated to Fetuin-A, pre diet. (

Circulating levels of Fetuin-A have been found to be negatively associated with insulin sensitivity, and particularly, Fetuin-A and NEFA interacting with each other in determining insulin sensitivity (Stefan and Haring, 2013). However in the present trial, which was in a negative energy balance, NEFA increase pre to post diet was not associated with a change in Fetuin-A; as can be observed in

Figure 22. Altogether this suggests a drop in Fetuin-A pre to post diet was not associated with NEFA levels that are increased through energy restriction. However with both Fetuin-A and CRP responsive to the modifying body weight, as both were associated to weight loss, they may both be responding to another influence that was also affected by this.

IL6 reduced significantly pre to post diet, but did not correlate to weight loss or any other biomarkers; however results did correlate to levels of hepatic lobular and portal inflammation. Also circulating levels of Fetuin-A pre-diet were correlated to levels of liver cell injury post-diet. These results would indicate that circulating factors were associated with hepatic inflammatory status.

Cytokeratin-18 fragments are one of the most extensively evaluated biomarkers for diagnosis of NASH. Unfortunately their use is complicated by the ambiguity of a range in clinically significant thresholds, for example high sensitivity cut-offs range from 112-380U/L (CK18 M30[◈]) (Kwok et al., 2014). If the lower threshold was used for the present study cohort, the median value for both dietary groups would distinguish them as having NASH,

[◈] marker of apoptosis

whereas the upper threshold would exclude them. The pre to post diet rise in CK18 M30 would also indicate that NASH had increased. However, histological assessment indicates that two-thirds of the cohort had no liver cell injury and three-quarters have low levels of lobular inflammation (Stage 1 or below). The rise in CK18 M30 which was in effect a rise in apoptosis must therefore be related to other factors than liver cell injury and inflammation Liver derived biomarkers AST and CK18 increased significantly pre to post diet, and ELF fell significantly pre to post diet. AST and CK18 are used to identify clinical progression of disease; however this study's results do not correlate CK18 to indicators of disease progression, but to level of steatosis, with greater levels of CK18 in those with the highest levels of steatosis. This combined with AST increase, which is released when hepatocytes are degraded, suggests a link to cellular release of CK18 and AST as a result of cellular remodelling.

With the reduction in ELF, there was an implication of increased apoptosis as discussed above. There were also lower levels of steatosis present post-diet than might be anticipated in this high BMI cohort, and therefore changes in NAFLD severity are implied concurrent with weight loss/energy restriction. The liver derived biomarkers may therefore be an indicator to the change in cellular environment with lipid depletion.

Overall the clinical trial found that two dietary approaches of very-low-energy-diets, of Food-based and Meal replacement plan, achieved similar weight loss results for the two-week period. Also dietary evaluation revealed no significant difference in patients rating of satisfaction between the two diets, with the majority of patients satisfied or very satisfied with the diet at its completion. This was despite both mood swings and constipation being reported with higher frequency in the meal replacement plan diet.

Pre to post diet observations included favourable changes in lipids and inflammatory markers. The change in glucose and insulin was not significant and conclusions were limited as there was no measurement of duration of diabetes which will affect responses to energy restriction and weight loss. Overall there was no significant difference between dietary interventions in any of the biomarkers examined post-diet. This could indicate that the diet type did not impact on outcomes, or that the sample size was insufficient to identify any difference.

When considering clinical application the meal replacement plan diet was designed to be nutritionally complete and met all the micronutrient Reference Nutrient Intake (RNI) levels, whereas the Food-based diet showed a shortfall in a number of minerals. As previously mentioned, supplementation of a multivitamin/mineral was suggested to all patients, and therefore this was only a theoretical deficit. However bariatric patients as a population are readily deficient in some micronutrients (Schweiger et al., 2010, Ernst et al., 2009), and as a part of the care pathway of bariatric patients supplementation has now been included in new recommendations drafted by BOMSS, for a standardised protocol of micronutrient supplementation pre and post bariatric surgery (O'Kane, 2014).

Chapter 5

Outcomes of pre-operative diets for NAFLD histological assessment

Introduction

The aims of this work were to examine levels and features of NAFLD after pre-operative diets to see if they were different to the severely obese population norm.

Biopsy remains the gold-standard for diagnosis and the three recognised forms of NAFLD are separated based on their distinct histological features (Yerian, 2011, Dowman et al., 2011b), a post-diet liver biopsy was taken at the time of surgery in order to assess levels post-diet. As a pre-diet biopsy was not possible, findings were compared against comparator samples from different BMI groups and observations from other comparative severely obese populations undergoing bariatric surgery that had not followed a pre-operative diet.

Methods

A single Histopathologist (PK) assessed all the histologically stained slides of liver biopsies against the NASH Clinical Research Network scoring system (Methods Section page 84). Samples were also visually assessed for percentage of hepatocyte involvement in steatosis or fibrosis. This study did not have pre-diet tissue samples to compare the levels of NAFLD pre and post diet; however, a comparison can be made between the two dietary intervention groups', to examine if there was any difference post-diet between the two groups.

The NAFLD activity score (NAS) was developed as a tool for use in clinical trials to enable semi-quantitative measurement and monitoring of pathological features of NAFLD. It uses histological assessment, scoring the level of steatosis, lobular inflammation, and ballooning (*Table 22*).

NAS is an unweighted sum (range 0-8) of features deemed to indicate active liver injury including, steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2). NAS does not include markers of fibrosis, as it aims to identify potentially reversible factors. Although it

is used to identify active injury markers, particularly in the context of comparative research work, it cannot be used to diagnose NASH (Brunt et al., 2011).

Recent findings have suggested that NAS is not appropriate to use the activity score for diagnosis of NASH, and therefore it is suggested that this is not used alone to distinguish the disease state, but can be used in conjunction with alternative assessments, and within research studies as a relative measure of liver injury (Younossi et al., 2011, Brunt et al., 2011).

Table 22 non-alcoholic fatty liver disease (NAFLD) activity score (Brunt and Tiniakos, 2010, Kleiner et al., 2005). Sum of steatosis, lobular inflammation, and ballooning score. Maximum score is 8.

Steatosis (%)
0: <5
1: 5–33
2: >33–<66
3: >66
Lobular inflammation (foci per 20× field):
0: none
1: <2
2: 2–4
3: >4 foci per 20× field
Ballooning hepatocyte degeneration:
0: None
1: Few ballooned hepatocytes
2: Many or prominent hepatocyte ballooning

Comparisons can also be made between the clinical trial population and comparator tissue samples, from lower BMI categories. The donors' demographic data for the comparator samples is below in Table 23.

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(Appendices page 268, Figure 40 liver anatomy and structure and Figure 41 liver structure - zones, for description of zonal areas in liver structure.)

Table 23 liver comparator tissue donor demographics.

Number	BMI classification	BMI Median (kg/m^2)	BMI range (kg/m^2)	Age Median (years)	Age range (years)	Female (%)
8	Healthy	23.5	19.6-25	57	18-66	100
12	Overweight/ Obese	29.5	27-39.5	49	33-56	50

Statistics and data handling

Results are separated into the two dietary interventions, and the third column is pooled comparator tissue (details above). Where score grades are absent it indicates that no samples were given this score. Two participants on the Food-based diet did not have liver biopsies taken.

Results

Steatosis

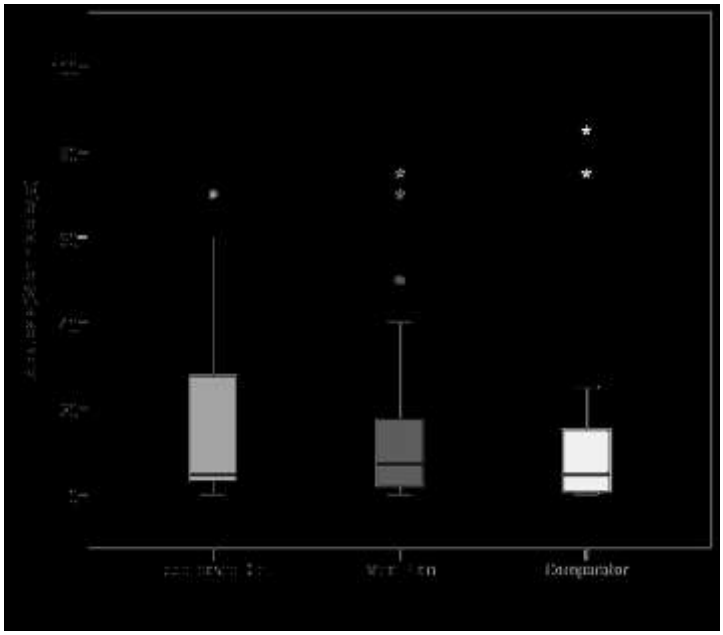
No significant association was found between intervention and steatosis grade or distribution (Kendall's Tau-C Test P=0.955, P=0.159 respectively) (Table 24). No statistically significant difference was found for steatosis percentage between interventions, or interventions and comparator (P=0.678 Kruskal-Wallis Independent Samples Test. (Figure 23 overpage).

Table 24 steatosis grade and location on histological assessment.

		Intervention		
		Food-based Diet	Meal Plan	Comparator
Steatosis grade 0-3	0	12	10	10
	% within Intervention	50.0%	35.7%	50.0%
	1	9	14	8
	% within Intervention	37.5%	50.0%	40.0%
	2	2	2	0
	% within Intervention	8.3%	7.1%	0.0%
	3	1	2	2
	% within Intervention	4.2%	7.1%	10.0%
Steatosis location 0-3	0_b	22	27	20
	% within Intervention	91.7%	96.4%	100.0%
	2	2	1	0
	% within Intervention	8.3%	3.6%	0.0%

Figure 23 percentage steatosis (hepatocyte involvement on histology) boxplots for each dietary intervention and comparator tissue.

No statistically significant difference was found for steatosis percentage between interventions, or interventions and comparator (P=0.678 Kruskal-Wallis Independent Samples Test).



Steatosis by Diabetes status and gender subgroups

When pooled dietary intervention group results were separated into subgroups for euglycaemia or Type 2 Diabetes/impaired fasting glucose (DM/IFG), a significant relationship was found between the steatosis grade and Diabetes status P=0.025 (Kendall's Tau-C Test). Results show a higher degree of steatosis in participants with DM/IFG. (Table 25)

Table 25 steatosis grade by subgroup of Diabetes status.

Steatosis Grade 0-3		Euglycaemic	DM/IFG
0	Count	15	7
	% within group	55.6%	28%
1	Count	10	13
	% within group	37%	52%
2	Count	1	3
	% within group	3.7%	12%
3	Count	1	2
	% within group	3.7%	8%

No significant relationship was found between steatosis location and Diabetes status P=0.511 (Kendall's Tau-C Test). Both groups had over 90% of steatosis in zone 0.

Gender subgroups did not show any significant relationship to steatosis grade or location (P=0.068, P=0.086 respectively).

Liver cell injury and inflammation

Liver cell injury and lobular inflammation did not show a statistically significant association to either diet group or comparator (P=0.409, P=0.173 respectively Kendall's Tau-C Test). However a significant association was found between portal inflammation and group, with lower levels of portal inflammation in the comparator group P=0.003 (Kendall's Tau-C Test) (Table 26). No samples were found to contain Mallory bodies or mega-mitochondria.

Table 26 inflammation and liver cell injury scores on histological assessment.

Liver cell injury and inflammation			**Intervention**		
			Food-based Diet	Meal Plan	Comparator
Liver cell injury (ballooning/ apoptosis) 0-3	0	Count	18_a	19_a	13_a
	1	Count	6_a	9_a	6_a
	2	Count	0_a	0_a	1_a
Lobular inflammation 0-3	0	Count	12_a	19_a	15_a
	1	Count	10_a	5_a	3_a
	2	Count	0_a	2_a	2_a
	3	Count	1_a	2_a	0_a
Portal inflammation 0-3	0	Count	$4_{a, b}$	2_b	8_a
	1	Count	11_a	15_a	12_a
	2	Count	9_a	11_a	0_b

$_{a/b}$ Z-test - denotes a subset of Intervention categories whose column proportions do not differ significantly from each other at the 0.05 level.

None of the comparator group has grade two portal inflammation, and a much higher level have no portal inflammation in comparison to the intervention groups. The association between portal inflammation and group remained significant when intervention groups were pooled and the degree of association to portal inflammation increased P=<0.001 (Kendall's Tau C Test).

Liver cell injury and inflammation by Diabetes status and gender subgroups

Liver cell injury did not show any significant relationship to subgroups of Diabetes status or gender (P=0.270, P=0.542 respectively Kendall's Tau-C Test).

Lobular inflammation score however did show a significant association with Diabetes status P=0.009 (Kendall's Tau-C Test). Results indicate a greater proportion of higher grades of lobular inflammation in the participants with DM/IFG. (Table 27)

Table 27 lobular inflammation grades by Diabetes status.

Lobular Inflammation 0-3		Euglycaemic	DM/IFG
0	Count	20	11
	% within group	76.9%	44%
1	Count	5	10
	% within group	19.2%	40%
2	Count	0	2
	% within group	0%	8%
3	Count	1	2
	% within group	3.8%	8%

Portal inflammation did not show any significant association with Diabetes status or gender P=0.067, P=0.481 respectively (Kendall's Tau-C Test).

Fibrosis

Fibrosis stage (CRN) did not show a significant association with either dietary intervention group or comparator (P=0.529 Kendall's Tau-C Test). (Table 28)

Table 28 fibrosis stage/score defined by CRN scoring definitions.

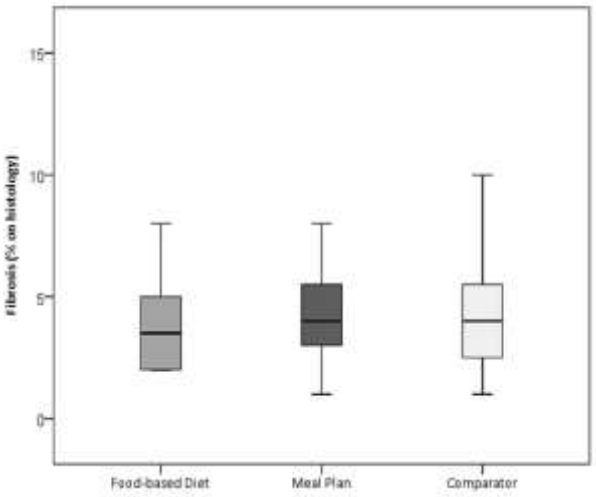
Fibrosis Stage (detailed CRN definitions)		Intervention		
		Food-based Diet	Meal Plan	Comparator
None	Count	8_a	6_a	6_a
Mild zone 3 perisinusoidal (1a)	Count	0_a	2_a	0_a
Portal/periportal (1c)	Count	12_a	14_a	8_a
Perisinusoidal and portal/periportal (2)	Count	2_a	6_a	5_a
Bridging fibrosis (3)	Count	0_a	0_a	1_a
Cirrhosis (4)	Count	2_{ab}	0_a	0_a

$_a$ Z-test - denotes a subset of Intervention categories whose column proportions do not differ significantly from each other at the 0.05 level.

$_b$ assessment suggests that these 2 samples show fibrosis levels as an artefact of the position of the biopsy location not definitive cirrhosis.

There were no statistically significant differences between distribution of fibrosis across categories of intervention or comparator (P=0.819 Independent samples Kruskal-Wallis Test), or for percentage fibrosis (hepatocyte involvement on histology) (P=0.819 Independent samples Kruskal-Wallis Test). (Figure 24))

Figure 24 percentage fibrosis (hepatocyte involvement on histology) boxplots for each dietary intervention and comparator tissue. P=0.819 between groups.



Fibrosis by Diabetes status and gender subgroups

Fibrosis stage (CRN) did not show any significant association to either Diabetes status or gender, P=0.214 P=0.237 respectively (Kendall's Tau-C Test).

NAFLD activity score (NAS)

No participant scored over 6 and the majority of participants (82.6% Food-based diet and 82.1% Meal plan) scored 3 or less. The distribution of NAS was not statistically significantly different across categories of intervention and comparator (P=0.825 Pearson Chi-Square Test) (Table 29).

Table 29 outcomes for NAS scores combined into three score categories, 0, 1-2, 3+.

NAS		Intervention		
		Food-based Diet	Meal Plan	Comparator
<= 0	Count	7_a	9_a	9_a
1 - 2	Count	10_a	13_a	8_a
3+	Count	6_a	6_a	3_a

$_a$ Z-test - denotes a subset of Intervention categories whose column proportions do not differ significantly from each other at the 0.05 level.

NAS by Diabetes status and gender subgroups

NAS was found to have a significant association with Diabetes status, P=0.017 (Kendall's Tau-C). Results indicate a higher level of NAS scores in the DM/IFG subgroup compared to those with euglycaemia. These results were not surprising considering that both steatosis stage and lobular inflammation grade were associated with DM/IFG, and these scores are used within NAS (Table 30). NAS did not show a significant association with gender P=0.158 (Kendall's Tau-C Test).

Table 30 NAS by Diabetes status.

NAS		Euglycaemic	DM/IFG
0	Count	11	5
	% within group	42.3%	20%
1	Count	7	6
	% within group	26.9%	24%
2	Count	5	5
	% within group	19.2%	20%
3	Count	0	3
	% within group	0%	12%
4	Count	3	3
	% within group	11.5%	12%
5	Count	0	1
	% within group	0%	4%
6	Count	0	2
	% within group	0%	8%

NAFLD histological assessment: relationships with circulating biomarkers of liver status, and inflammatory markers

The relationships between circulating biomarkers and levels of steatosis (%) or fibrosis (%) were examined. Pooled results (both diets) were used as biomarker results were not statistically different between diets.

Liver status

AST was examined as it increased significantly pre to post diet. A significant positive correlation (Spearman's Rho 2-tailed) was found between levels of AST pre diet and levels of steatosis P=0.010. No significant correlations were found to fibrosis levels.

Following on from this, the relationship between the circulating biomarker for cell death, CK-18, was examined. There was a significant positive correlation between levels of CK-18, both M30 and M65 (markers for apoptosis and necrosis), with percentage steatosis (Table

31). No significant correlation was found between CK-18 M30 and fibrosis, however a significant association was found between CK-18 M65 pre-diet and fibrosis P=0.038.

Table 31 Spearman's Rho correlation between circulating biomarkers of cell death (CK-18) and histological steatosis and fibrosis percentage.

		CK18 M30 pre	CK18 M30 post	CK18 M65 pre	CK18 M65 post
Steatosis (% on histology)	Correlation Coefficient	0.415**	0.436**	0.462**	0.435**
	Sig. (2-tailed)	0.002	0.001	0.001	0.001

The distribution of CK18 M30 (post-diet) was significantly different across categories of steatosis P=0.019 (Kruskal-Wallis Independent Samples Test), and there was a trend for increasing levels of CK18 M30 with increasing stage of steatosis. (Figure 25).

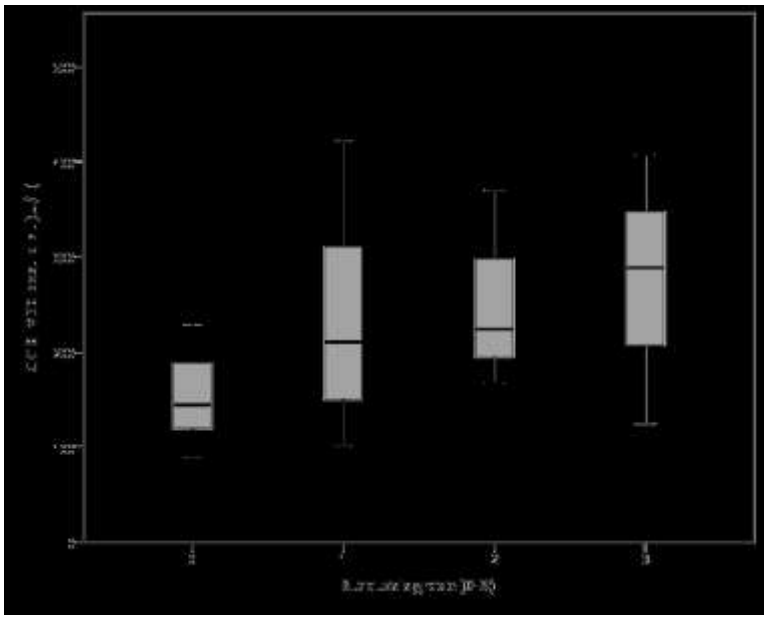


Figure 25 boxplots of steatosis grade by CK-18 M30 (post-diet).

ELF pre and post diet were assessed for association with percentage steatosis or percentage fibrosis. No significant correlation was found between ELF pre or post diet and percentage fibrosis, however a significant result was found between ELF post diet and steatosis percentage P=0.023.

NAS was also examined in a cross-tab with ELF grouped above or below median, and a significant association was found between post-diet ELF group and NAS P<0.001◘ (pre-diet P=0.411). NAS score of 1 or greater was associated with levels of ELF above median. (Table 32)

Table 32 cross-tab of NAS against ELF score group.

NAS		Post-diet ELF group	
		<= Median	Median +
0	Count	13	3
	% within group	54.2%	12.5%
1	Count	5	6
	% within group	20.8%	25%
2	Count	3	6
	% within group	12.5%	25%
3	Count	1	2
	% within group	4.2%	8.2%
4	Count	1	5
	% within group	4.2%	20.8%
5	Count	0	1
	% within group	0%	4.2%
6	Count	1	1
	% within group	4.2%	4.2%

Inflammatory markers

IL6 reduced significantly pre to post diet, but IL6 change did not correlate to weight loss or any other circulating biomarkers. When IL6 results were divided into two groups, above and below the median, and examined for connections to markers of hepatic inflammation, a significant association was found for pre and post diet circulating IL6 to both post-diet lobular inflammation and portal inflammation. Lobular inflammation to IL6 pre-diet P=0.021, post-diet P=0.015, and portal inflammation to IL6 pre-diet P=0.019, post-diet P=0.050◘. Circulating IL6 levels above the median were associated to higher grades of portal and lobular inflammation. (Table 33)

◘ Kendall's Tau-C Test

Table 33 crosstabs for lobular and portal inflammation stage against circulating levels of IL6 grouped (+/- median)

			Lobular Inflammation Grade (0-3)			
			0	1	2	3
IL6 Pre	<= Median	Count	19	7	0	0
	Median+	Count	12	8	2	3
IL6 Post	<= Median	Count	18	6	0	0
	Median+	Count	12	8	2	3
			Portal Inflammation Grade (0-3)			
			0	1	2	3
IL6 Pre	<= Median	Count	4	16	6	0
	Median+	Count	2	10	14	0
IL6 Post	<= Median	Count	4	14	6	0
	Median+	Count	2	11	13	0

IL6 pre or post diet did not show any significant associations to liver cell injury or steatosis grade (pre-diet P=0.355 and P=0.235, post-diet P=0.902 and P=0.466, respectively[✦]). Grouped (+/- median) circulating levels of CRP pre and post diet did not show any significant association to steatosis grade, liver cell injury, lobular or portal inflammation stage [✦].

Fetuin-A however shows a significant association, this time with liver cell injury grade. Results above the median were associated with higher grade liver cell injury. This association was only significant for pre-diet Fet 0.247 P=0.039, post-diet 0.176 P=0.163[✦]. (Table 34)

Table 34 crosstab of liver cell injury grade against grouped Fet (+/- median).

			Liver Cell Injury 0-3	
			0	1
Fet Pre	<= Median	Count	21	4
	Median+	Count	16	11
Fet Post	<= Median	Count	19	5
	Median+	Count	16	10

[✦] Kendall's Tau-C Test

Fetuin-A did not show any significant relationship with steatosis grade, lobular or portal inflammation stage (Pre-diet P=0.381, P=0.417, P=0.067, post-diet P=0.355, P=0.192, P=0.811, respectively[+]).

Metabolic biomarkers

Finally metabolic biomarkers, Ins, Glu and BHB, were examined. Spearman's Rho correlation found a significant positive association between Ins and Glu pre and post diet and percentage steatosis. There was also a negative association between BHB and percentage steatosis. No significant relationships were found between these biomarkers and percentage fibrosis. (Table 35)

Table 35 Spearman's Rho correlation between circulating metabolic biomarkers and histological percentage steatosis and fibrosis.

		BHB Pre	BHB Post	Ins Pre	Ins Post	Glu Pre	Glu Post
Steatosis (% on histology)	Correlation Coefficient	-0.102	-0.283*	0.300*	0.283*	0.304*	0.268*
	Sig. (2-tailed)	0.472	0.047	0.032	0.047	0.028	0.057

*Correlation is significant at the 0.05 level.

HOMA-IR

As might be expected given the correlation to glucose and insulin, HOMA-IR results also correlate positively to percentage steatosis. Fibrosis percentage did not correlate significantly to HOMA-IR. (Table 36)

Table 36 Spearman's Rho correlation for steatosis (%) and fibrosis (%) with HOMA-IR.

		HOMA-IR Pre	HOMA-IR Post
steatosis percentage	Correlation Coefficient	0.376**	0.354*
	Sig. (2-tailed)	0.007	0.012

**Correlation is significant at the 0.01 level, * Correlation is significant at the 0.05 level

NAFLD and biomarker summary

When taken together the results indicate that levels of steatosis post-diet were associated with circulating levels of insulin, and glucose pre and post diet, and higher steatosis grades were associated with Diabetes status. Diabetes status was also associated with the level of lobular inflammation, and therefore also with NAFLD activity score, whose derivatives are lobular inflammation and steatosis grade. The biomarkers for cell apoptosis and necrosis, CK-18, were also associated with levels of steatosis. Despite being a surrogate biomarker for fibrosis there was no correlation between level of fibrosis and ELF score, however ELF level was associated with NAFLD activity score. Portal inflammation grade was associated to IL6 pre and post diet.

NAFLD histological assessment: comparison between intervention and comparator groups

Results indicate the majority of NAFLD assessments produced results which were not significantly different when the diet groups (severely obese) were compared with the comparator group, separated into healthy BMI and overweight/obese. Portal inflammation was the only exception, where higher levels were found in the dietary intervention groups.

When NAFLD histological assessments were compared by BMI groupings (healthy, overweight/obese, severely obese), neither steatosis (%) or fibrosis (%) distribution or medians were significantly different between groups (Steatosis distribution P=0.076, median P=0.749; Fibrosis distribution P=0.502, median P=0.725; Mann-Whitney U Test and Kruskal-Wallis Test respectively).

There was no statistically significant association between BMI group and steatosis grade P=0.449 (Kendall's Tau-C Test). This was also true for liver cell injury grade (P=0.461), lobular inflammation grade (P=0.146), fibrosis stage (P=0.584) and NAS (P=0.398) (all Kendall's Tau-C Test). However portal inflammation did show a significant association P=<0.001, with higher levels of portal inflammation observed in the severely obese (pooled intervention) group. (Table 37) The distribution of portal inflammation grade between the three groups shows an increase in the proportion of the population at higher grades as BMI increases.

Table 37 crosstab of portal inflammation grade against BMI category, for comparator and intervention groups.

Portal inflammation (grade 0-3)		Weight status (BMI category)		
		Healthy BMI	Overweight/Obese	Severe Obese
0	Count	4	4	6
	% within weight status (BMI category)	50.0%	33.3%	11.5%
1	Count	4	8	26
	% within weight status (BMI category)	50.0%	66.7%	50.0%
2	Count	0	0	20
	% within weight status (BMI category)	0.0%	0.0%	38.5%

NAFLD histological assessment: relationships to anthropometric and dietary measures

There were no significant correlations between excess BMI loss or percentage weight loss, and percentage steatosis or fibrosis. No correlations were found between steatosis or fibrosis percentage and excess BMI loss for subgroups of Diabetes status or gender.

When the relationship between steatosis percentage and weight loss was displayed graphically with BMI (post-diet) (

Figure 26), a trend appears indicating that BMI has an effect on level of steatosis. Visually this suggests that weight loss may be related to the level of steatosis, with lower weight loss there were higher levels of steatosis. However as BMI increases, and particularly over 55kg/m^2 steatosis levels peak, suggesting that BMI level offsets the weight loss effect on steatosis.

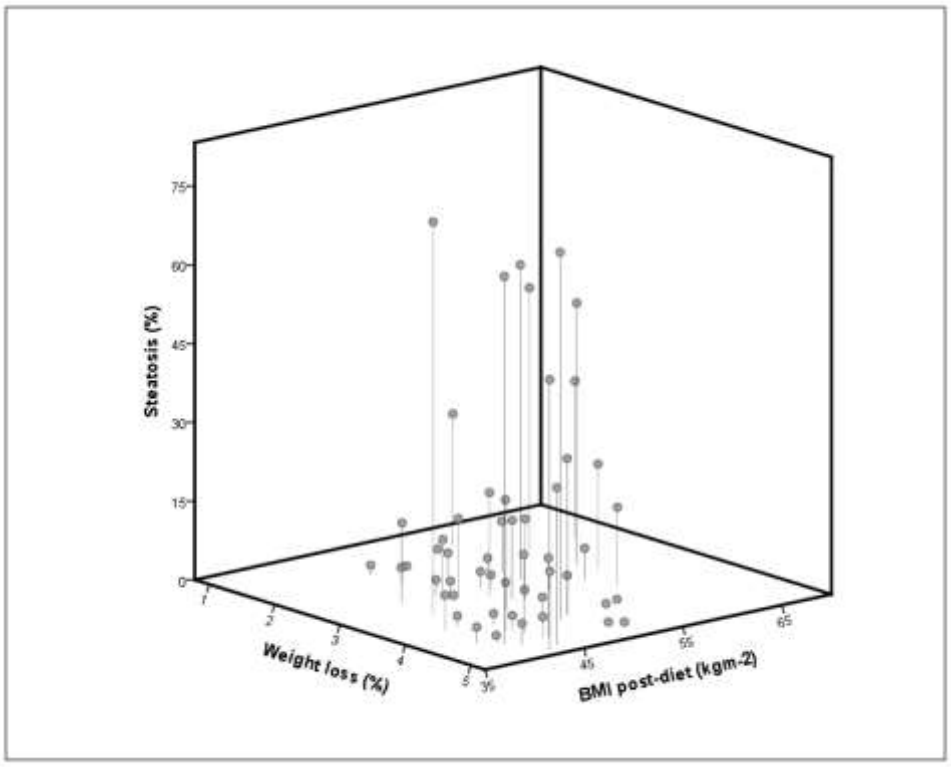


Figure 26 3D scatter plot of weight loss (%) against steatosis (% on histology) and BMI (post-diet).

A trend was indicated, with lower weight loss there were higher levels of steatosis. However as BMI increases, and particularly over 55kg/m^2 steatosis levels peak, suggesting that BMI level offsets the weight loss effect on steatosis.

In order to examine relationships with the other NAFLD category scores, results for percentage excess BMI loss were divided into two categories, above and below the median, so that crosstab analyses could be made. No statistically significant results were found for associations with steatosis stage (P=0.856), fibrosis stage (P=0.373) or lobular inflammation (P=0.365)*****.

Portal inflammation

Portal inflammation was found to rise as excess BMI loss reduces. Kendall's Tau-C Test shows a significant association -0.435 P=0.001 (Table 38).

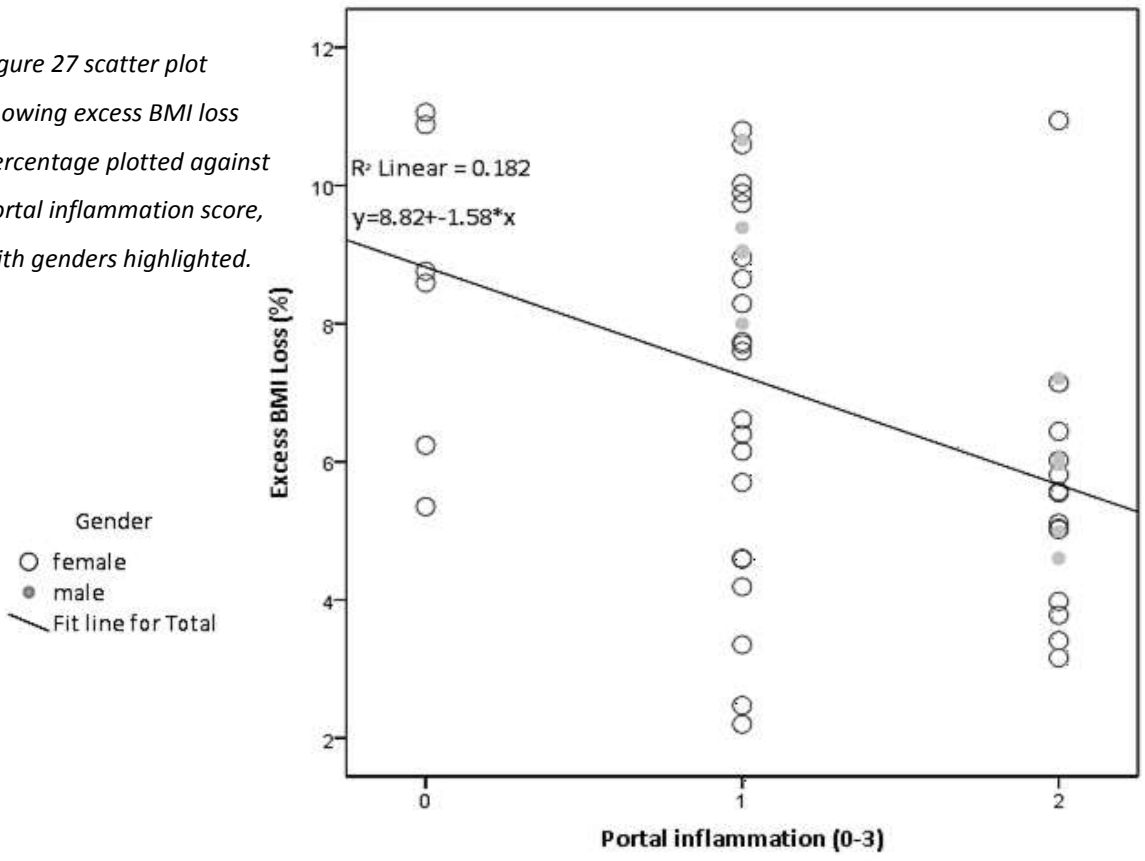
***** Kendall's Tau-c correlation

Table 38 excess BMI loss percentage (+/- median) crosstab with portal inflammation stage.

Excess BMI Loss (%)		Portal inflammation 0-3		
		0	1	2
<= 6.42	Count	2	9	16
	% within portal inflammation 0-3	33.3%	34.6%	80.0%
6.43+	Count	4	17	4
	% within portal inflammation 0-3	66.7%	65.4%	20.0%

This relationship remains significant in both subgroups of euglycaemia and DM/IFG (P=0.022, P=0.012 respectively), and both subgroups of gender, female P=0.007, male P<0.001 (Kendall's Tau-C Test). (Figure 17)

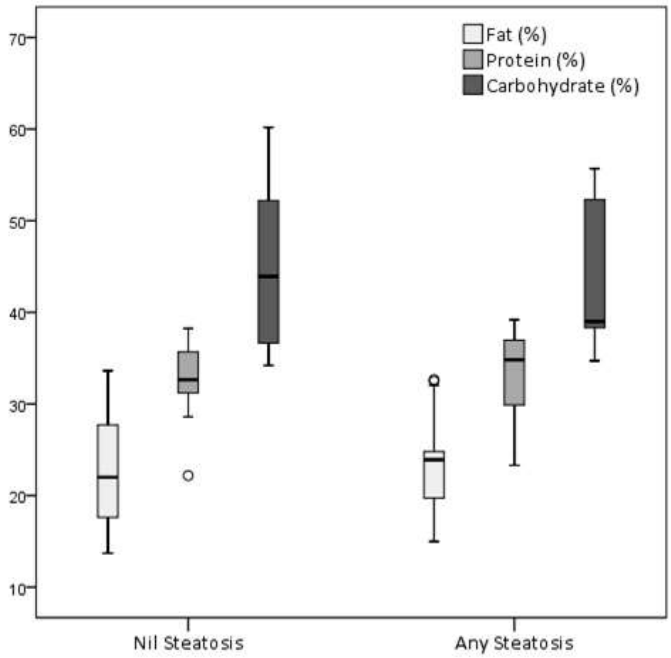
Figure 27 scatter plot showing excess BMI loss percentage plotted against portal inflammation score, with genders highlighted.



NAFLD and dietary intake

This study's pooled results separated by individual macronutrients (percentage of total energy intake) showed no statistical significant correlation to steatosis (%) or fibrosis (%) (Steatosis: carbohydrate P=0.136, fat P=0.369, protein P=0.315; fibrosis: carbohydrate P=0.311, fat P=0.388, protein P=0.225). This lack of association to steatosis was seen particularly clearly when macronutrient intake was plotted against groups for absence or presence of steatosis (Mann-Whitney U Test Carbohydrate P=0.754, fat P=0.732, protein P=0.403). (Figure 28)

Figure 28 boxplot of steatosis absence or presence against macronutrients (as % of total energy intake)



Macronutrient intake (% of total energy intake) for each macronutrient, was divided into two groups, with 50% of results in each group (+/- median intake) and a cross-tab analysis Kendall's Tau-C completed. There was also no association found between any of the macronutrient intakes, and fibrosis stage, steatosis grade, lobular inflammation grade or portal inflammation grade (Table 39).

Table 39 percentage macronutrient energy intake cross-tab with NAFLD histology assessments (statistical significance).

Percentage Energy Intake	Kendall's Tau-C Test Significance (P values)			
	Steatosis Grade (0-3)	Lobular Inflammation Grade (0-3)	Portal Inflammation Grade (0-3)	Fibrosis Stage (0-3)
Fat (+/-22.5%)	0.884	0.085	0.497	0.560
Protein (+/-32.7%)	0.843	0.865	0.585	0.535
Carbohydrate (+/-40%)	0.448	0.314	0.941	0.992

Steatosis and alcohol intake

Participants were asked to avoid alcohol for the period of the diet, and were excluded from the study if they reported having an intake over that of the current recommendations (Appendices page 227). Participants also reported if they usually took any level of alcohol, and to check if this was influencing the levels of steatosis observed a crosstab was completed. No significant association was found between those that habitually did or did not take alcohol and steatosis grade P=0.558 (Kendall's Tau-C test). (Table 40)

Table 40 reported usual alcohol intake (yes/no) crosstab against steatosis grade.

Any alcohol intake		Steatosis (grade 0-3)			
		0	1	2	3
No	Count	9	13	2	1
	% within any alcohol intake	36.0%	52.0%	8.0%	4.0%
Yes	Count	13	10	2	2
	% within any alcohol intake	48.1%	37.0%	7.4%	7.4%

NAFLD and anthropometry summary

No correlation was found between level of weight loss achieved, or BMI category, and the level of steatosis or fibrosis observed. This was also found to be the case when steatosis grade, fibrosis stage, or lobular inflammation grade were assessed against weight loss

grouped above or below median or BMI category. Also, habitual reported alcohol intake did not show any significant association to steatosis grade. However a relationship was indicated between weight loss and steatosis, with lower weight loss there were higher levels of steatosis. An association was also indicated between excess BMI loss and portal inflammation where portal inflammation was found to rise as excess BMI loss fell, which remained significant when results were subdivided by gender or Diabetes status. Portal inflammation was also shown to be associated with BMI category, with higher levels of portal inflammation in higher BMI categories.

Discussion

The objectives of this work were to examine levels and features of NAFLD after the pre-operative diet to see if they were different to the severely obese population norm.

Solga et al (2004) completed a retrospective review in a cohort of bariatric surgery patients who did not have a pre-operative dietary regime. They evaluated the NAFLD characteristics from liver biopsies taken at the time of surgery, and examined reported dietary intake (from a 24-hour food recall) taken some weeks prior to surgery, to estimate the patients' typical intake. Their baseline demographics were not dissimilar to the current clinical trial. However, their results (at time of surgery) revealed 90% of patients had some degree of steatosis, whereas 22 of this clinical trial's population (40%) had no steatosis at this point. Levels of steatosis observed in this study (at time of surgery) were also below those seen in other similar cohorts (Machado et al., 2006). Comparison with outcomes from healthy and overweight/obese groups, also indicate that the levels of steatosis observed in this trial were comparable to a lower BMI category. Levels of liver cell injury such as those defined by NAFLD activity score, were also lower than might be expected, with levels comparable to lower BMI categories.

Further to this Solga et al (2004) examined the relationship of macronutrient intake of their cohort to inflammatory status, and concluded that higher carbohydrate intake was associated with higher odds of inflammation, but had no association with steatosis (Solga et al., 2004). Browning *et al* (2011) showed that two weeks of dietary intervention reduced hepatic steatosis (TAG) by around 42% in subjects with NAFLD; and given a similar degree of weight loss, they reported a low-carbohydrate diet (less than 20g/day) resulted in significantly greater intrahepatic TAG reduction than a LED (Browning et al., 2011).

However this clinical trial did not find any association between any of the macronutrient types and levels of steatosis post-diet. However, conclusions cannot be drawn about the influence of typical dietary intake (prior to intervention), as no data were collected on this intake or NAFLD assessment made at this time.

Weight loss per se did not show a direct relationship to NAFLD findings, but a relationship was visualised between steatosis percentage and weight loss influenced by BMI (post-diet). Data can be interpreted that weight loss may influence the level of steatosis, as evidenced by lower weight loss associated with higher levels of steatosis; however as BMI increases, and particularly over 55kg/m^2 steatosis levels peak, as BMI level offsets the weight loss effect on steatosis.

Circulating levels of glucose and insulin were both observed to have an association with levels of steatosis, as was HOMA-IR, and as previous evidence suggests that insulin resistance and impaired hepatic energy metabolism influence the development of steatosis (Szendroedi et al., 2009), it was unsurprising to find this relationship.

Cusi et al (2014) in a recent clinical trial measured CK18 M30 in obese patients with and without NAFLD or NASH. They found CK18 M30 was significantly different when comparing those without to those with these conditions, and levels increased with stages of steatosis, inflammation and fibrosis, but not liver cell injury. Distribution of CK18 M30 (post-diet) in this trial was also significantly different across categories of steatosis and there was a trend for increasing levels of CK18 M30 with increasing stage of steatosis. However these findings do not support Cusi et al in that an increase in CK18 M30 aligns with increasing grade of fibrosis or inflammation, as the present results do not indicate a difference in distribution of CK18 M30 across categories for fibrosis, lobular or portal inflammation (P=0.498, P=0.063, P=0.667 respectively). Also unlike longer-term weight loss studies CK18 M30 levels pre-diet, post-diet or change, do not significantly correlate to body weight loss (pre-diet P=0.099, post-diet P=0.317, change P=0.147) or excess BMI loss (pre-diet P=0.160, post-diet P=0.386, change P=0.159) (Miyasato et al., 2014). These findings would indicate that the change observed in the present clinical trial's CK18 M30 may not have a direct relationship to weight loss, but was related to steatosis grade post-diet. This therefore was related to loss of lipid from the liver in those with higher grades of steatosis and was indicative of transient remodelling rather than apoptosis/necrosis.

Portal inflammation which is greater than mild in untreated NAFLD may be considered a marker of advanced disease (Brunt et al., 2009), and expression of TLR4 by hepatic and inflammatory cells have been found to correlate with portal inflammation, and the activity of fibrogenic cells and liver fibrosis (Vespasiani-Gentilucci et al., 2014). Also Yilmaz *et al* (2014) established associations between higher grades of portal inflammation in individuals with NAFLD when this was accompanying Type 2 Diabetes (DM) or pre-diabetes (including impaired fasting glucose (IFG)) (Yilmaz et al., 2014). This study's results do show levels of portal inflammation above those of lower BMI categories, and higher levels show a significant association with the severely obese category, with 38% of the participants had moderate levels of portal inflammation. However this study's data does not show a significant association between DM/IFG with fibrosis or portal inflammation. This study did find a significant relationship between portal inflammation and excess BMI loss, with the highest levels of inflammation in those with the least losses; this association remaining significant in groups for gender and Diabetes status.

Given that 38% of this study's participants could be considered to have advanced NAFLD, it was surprising that fibrosis levels were comparable with lower BMI categories. There was an indication that there may have been higher levels of portal inflammation, as there was a discrepancy between levels of portal/periportal fibrosis which were present in 50% of the cohort. Considering the relationship found between portal inflammation and excess BMI loss, this would indicate that portal inflammation was transitory and sensitive to energy restriction/weight loss, with fibrosis levels slower to respond.

Chapter 6

Outcomes of pre-operative diets for surgeon assessment

Introduction

Laparoscopic bariatric surgery requires retraction of the liver to enable access to the stomach and gastro-oesophageal junction. The "liver reducing" diet was introduced to reduce the volume of the liver and improve liver flexibility to enable this approach. However there is limited published evidence to support this approach (van Wissen et al., 2016). The objectives for this work were for bariatric surgeons' to visually assess levels of liver steatosis after pre-operative diets had been followed, and assess if their visual assessment relates to the ease of access or difficulty associated with the laparoscopic approach to bariatric surgery.

Methods

It was decided by the lead clinician that only the three most experienced surgeons should complete the assessment, to limit inconsistency in the results, therefore for only 22 cases out of the total of 54 participants, surgeons' completed a visual assessment of access to the gastro-oesophageal junction, liver volume and rigidity.

Study participants were asked not to reveal the type of diet they had followed to the surgeon prior to surgery in order to enable blinding prior to them completing the assessment.

Perceived difficulty of the procedure for three categories related to surgical access and procedural efficacy, were noted on a 100mm visual analogue scale for which 100 and 0mm represented the highest and lowest degrees of difficulty, respectively.

The three categories were:

1. Rigidity of liver
2. Liver volume/size
3. Obstruction of gastro-oesophageal junction

Results

Correlation analysis (Spearman's Rho) showed that increasing perceived difficulty was associated with higher percentages of steatosis assessed histologically. The correlation between histological assessed steatosis (%) and the visual assessment of the surgeons produced R^2 values for liver rigidity 0.37, liver volume 0.40, and gastric access 0.20. (Table 41)

Table 41 summary of Spearman's correlation findings for visual analogue scale of surgical difficulty against steatosis percentage.

		Steatosis (% on histology)	Gastric access	Liver volume	Liver rigidity
Steatosis (% on histology)	Correlation Coefficient	1	0.446*	0.639**	0.609**
	Sig. (2-tailed)		0.042	0.002	0.003
Gastric access	Correlation Coefficient	0.446*	1	0.719**	0.661**
	Sig. (2-tailed)	0.042		<0.001	0.001
Liver volume	Correlation Coefficient	0.639**	0.719**	1	0.796**
	Sig. (2-tailed)	0.002	<0.001		<0.001
Liver rigidity	Correlation Coefficient	0.609**	0.661**	0.796**	1
	Sig. (2-tailed)	0.003	0.001	<0.001	

*. Correlation is significant at the 0.05 level.

**. Correlation is significant at the 0.01 level.

There were no statistically significant differences found between diets (Food-based diet N=8, Meal plan N=14) for the three visual analogue assessments; gastric access P=0.513, liver volume P=0.891, liver rigidity P=0.702.

Discussion

The objectives for this work were for bariatric surgeons' to visually assess levels of liver steatosis post-diet and examine how this relates to the ease of access or difficulty associated with the laparoscopic approach to bariatric surgery. One of the aims was to investigate the suggested benefits of the pre-operative diet on operative difficulty.

Correlation was found between the three visually assessed categories of operative difficulty indicating that scoring of all three was related. Surgeons' were not aware of the diet undertaken by the participant at the time of surgery, and diet type was not differentiated in the results. Surgeons' perception of difficulty of the operation was associated to the level of steatosis.

There were limitations to this analysis as it was decided by the lead clinician that only the three most experienced surgeons should complete the assessment. This meant that only 22 cases out of the total of 54 were assessed and may have introduced a selection bias. This bias relates to bariatric case assignment, as cases were assigned to surgeons' partly based on complexity, and therefore less complex cases may not have been adequately represented. There may also be bias related to limiting surgeons' participation affecting the range of difficulty perception and breadth of responses. This limitation was unfortunate given the weighting of importance of these findings in relation to the study's outcome measures, but was unavoidable as surgeon cooperation was embedded into the wider aims of the study.

Chapter 7

Outcomes of pre-operative diets for underlying mechanisms

Introduction

This chapter examines underlying mechanisms that contribute to observed biomarker changes and tissue environment following the pre-operative diets.

As discussed previously pre-operative diets were instigated to deliver a "liver reducing" effect and observations from this clinical trial include less steatosis at the time of surgery than would be observed in the general population of severely obese people. Alongside this VLEDs have been observed to have other outcomes some of which were also observed after the two-week pre-operative diets in this clinical trial. Observations included reduction in body weight and circulating inflammatory markers, and changes in lipid profile and markers of cell death.

In order to explore the underlying mechanisms for observations post-diet, a targeted approach was used, with gene targets identified through examination of pathways related to observations, including metabolism, lipid movement, cell development/death, and inflammation. Specific transcripts for investigation were then identified using these pathways alongside any available evidence.

Methods

This work examines gene expression levels from biopsies that were taken from three tissues, omental adipose tissue (OAT), subcutaneous adipose tissue (SAT) and liver, post-diet at the time of surgery. No pre-diet tissue samples were collected, but it was decided that results could be interpreted to indicate a post-diet tissue-specific environment, and these could be elucidated in conjunction with other outcomes e.g. biomarker changes. Time and resource constraints meant that this was the only viable option for this study. The analyses use mRNA transcript expression as a surrogate for active gene expression.

Ingenuity Systems pathway analysis software (IPA) was used to complete the network analysis in October 2014.

Results

Adipose tissue

Individual TaqMan assays

CHREBP-b

After running a plate for each set of primers and probes, it was clear that the CHREBP-b primers/probe did not achieve the anticipated amplification, and the exponential algorithm failed on the software.

CHREBP-b primers were designed based on the reported findings of Herman *et al* (2012). Their reverse primer was designed to sit within an exon, and the reverse primer designed for this study was designed to sit over an exon-exon boundary to ensure that it was messenger RNA which was being amplified. The failure of these primers may indicate that the target amplified in Herman's work was not mRNA (Herman et al., 2012).

CHREBP α & α-d

Comparing results for the two dietary interventions, indicates very little difference between expression levels for either CHREBP-a or CHREBP-a-d, in either adipose tissue depot (Subcutaneous CHREBPa P=0.931, CHREBPa-d P=0.931, Omental CHREBPa P=0.822, CHREBPa-d P=0.556 Wilcoxon Signed Rank Test). (Figure 29)

However results indicate a 1.6-1.8-fold difference between OAT and SAT expression of CHREBP-a, which was significant when diets were pooled P<0.001, and in separate diets, Food-based diet P=0.016, Meal plan P=0.001. (Figure 29) For CHREBP-a-d there was no significant difference in expression for individual or pooled diets (Food-based diet P=0.889, Meal plan P=0.165, Pooled diet P=0.236) between adipose tissue depots. (All ΔCT Wilcoxon Signed Rank Test)

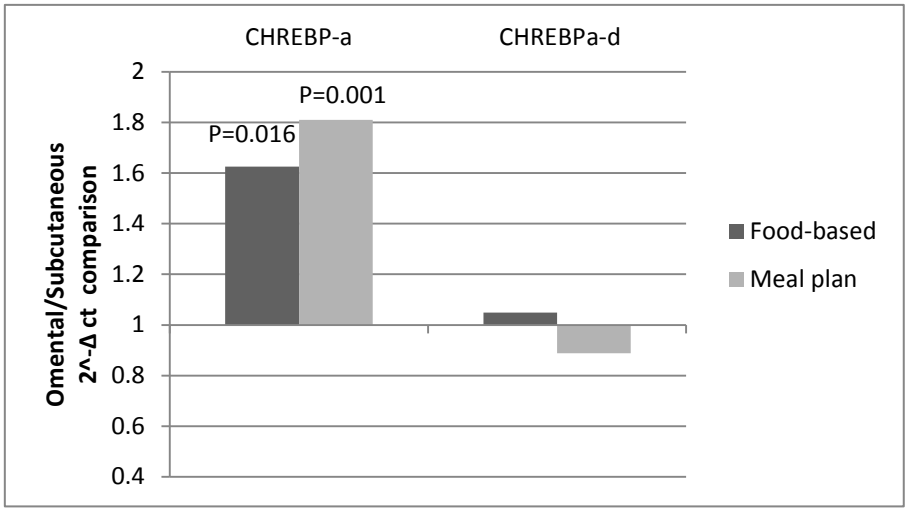


Figure 29 comparison of fold-difference between omental and subcutaneous adipose tissue depots for CHREBP variants, and both dietary interventions (medians plotted). P values indicate significant difference in fold-difference for expression between adipose depots for that transcript.

Fetuin-A

The expression levels of Fetuin-A were so low that they did not amplify within 40 cycles using the universal thermal cycling parameters, and therefore cannot be reported.

Gene array data

74 transcripts were examined in adipose tissue (Gene array card layout for adipose tissue Table 44 Page 253). No significant differences in expression levels were found for transcript expression between dietary approaches (Figure 37 page 259, Table 48 page 262).

Adipose tissue depots comparison

Data were pooled for both diets and transcripts compared between adipose tissue depots, omental (OAT) vs subcutaneous (SAT). 55 transcripts were found to have lower expression levels in OAT, compared to 19 higher. (Summary table of ΔCT median and ranges Appendices page 260)

A Wilcoxon Signed Ranks test was carried out, with a Bonferroni correction for multiple tests giving a significance level of 0.00065. The majority of transcripts (n=44) were found to have a statistically significant difference in expression levels between adipose tissue depots. These results are shown in

Figure 30 below.

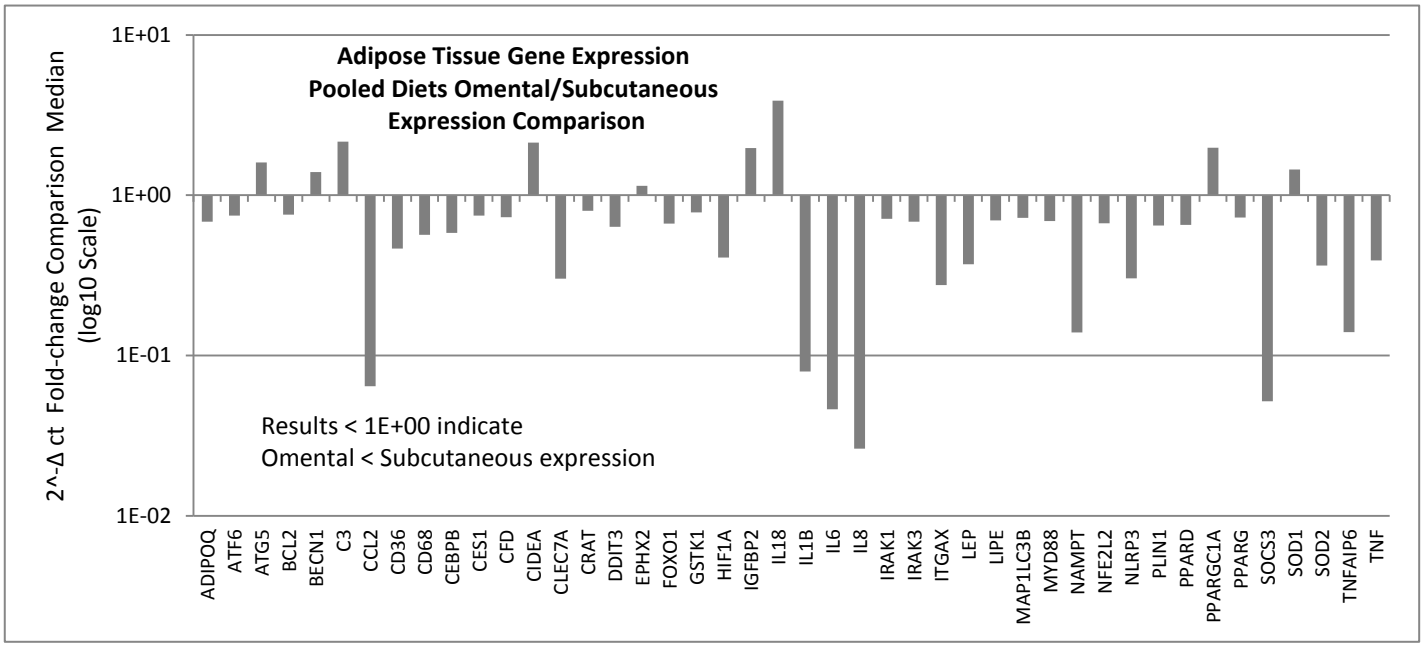


Figure 30 adipose tissue transcripts (fold-difference) for pooled diets, comparison between adipose depots. Results for significantly different transcripts shown only. 74 transcripts were examined, and 44 had a significantly different expression between tissues.

Key findings from this analysis include the differential expression of transcripts between adipose tissue depots indicating a tissue-specific environment. Also significant higher levels of pro-inflammatory transcripts such as IL6, IL1B and SOCS3 in SAT; alongside CCL2 (known as monocyte chemoattractant protein MCP-1),whose production and signalling are essential for the development of inflammation (Arner et al., 2012). This suggests a significantly different inflammatory profile between adipose tissue depots. Levels of IL8 expression were also significantly higher in SAT than OAT. This directly opposes findings elsewhere that suggest IL8 expression, from non-adipocytes within adipose tissue, is higher in obesity in OAT, and related to a more pro-inflammatory state in this depot than in SAT (Bruun et al., 2004).

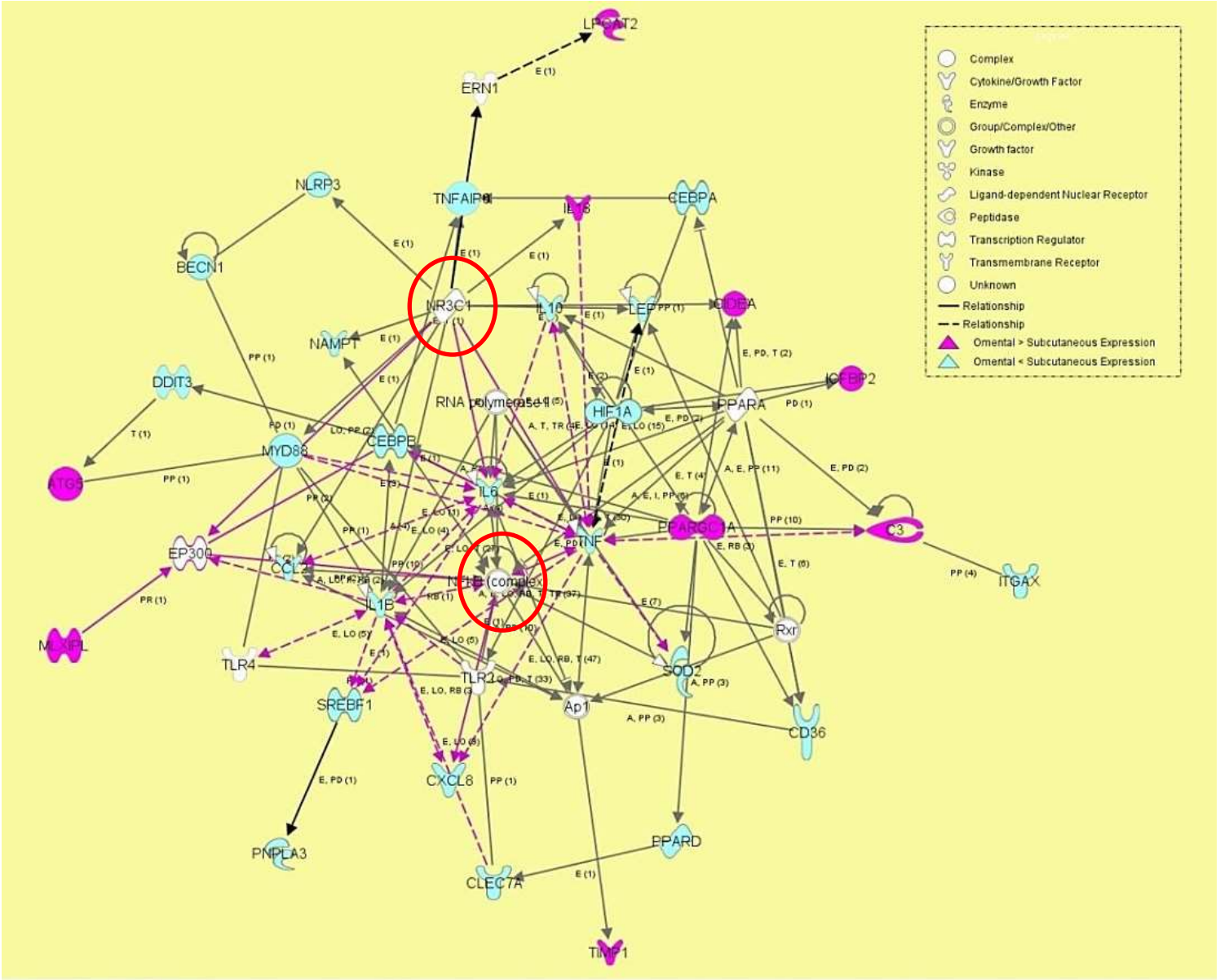
Network analysis

A group of transcript targets were selected that have the largest fold-difference between the two depots (those with greatest difference in expression levels); most equal to or greater than one-half fold-difference between adipose depots (<0.5 or >1.5 fold-difference). ATG5, C3, CIDEA, IGFBP2, IL18, LPCAT2, PPARGC1a and TIMP1 all have higher expression in OAT than SAT. CCL2, CD36, CLEC7A, HIF1a, IL1b, IL10, IL8, IL6, ITGAX, LEP, NAMPT, NLRP3, PNPLA3, SOCS3, SOD2, TNFAIP6 and TNF all have higher expression in the SAT depot.

These selected transcripts were incorporated into a network with connections indicating known links between transcripts. Figure 31 below shows this network (larger image page 264). The targets in blue were found to be more highly expressed in SAT. The targets in pink were found to be more highly expressed in OAT. Examining the number of interactions in this network, it has highlighted the glucocorticoid receptor NR3C1 (nuclear receptor subfamily 3, group C, member 1) as a major effector (highlighted), with relationships also mediated predominantly through the NFκB complex (highlighted), and multiple influences on IL6, IL1b and TNF.

Figure 31 IPA network with targets from adipose tissue whose expression levels showed the greatest expression level fold-difference between omental and subcutaneous depots.

The targets in blue were found to be more highly expressed in subcutaneous adipose tissue. The targets in pink were found to be more highly expressed in omental adipose tissue. Examining the number of interactions in this network, it has highlighted the glucocorticoid receptor NR3C1 (nuclear receptor subfamily 3, group C, member 1) as a major effector (circled), with relationships also mediated predominantly through the NFκB complex (circled), and multiple influences on IL6, IL1b and TNF.



Liver tissue

There was no pre-diet biopsy and there was no comparison of pre and post diet expression levels. Therefore the gene expression results for the liver gene array card, described in Table 43 page 251, Figure 34 page 248, and results in Table 49 page 265, were examined in below in the integrative analysis (page 166 onwards) in conjunction with expression levels from the adipose tissue depots, and within the context of other clinical trial outcomes.

Individual TaqMan assays

CHREBP a & a-d

Comparing results for the two dietary interventions, indicates no significant difference between expression levels for either CHREBP-a or CHREBP-a-d (P=0.796 and P=1.000 respectively).

Fetuin-A

Fetuin-A was expressed and fold-difference comparison between the dietary interventions revealed no significant difference (P=0.318 Mann Whitney U-test).

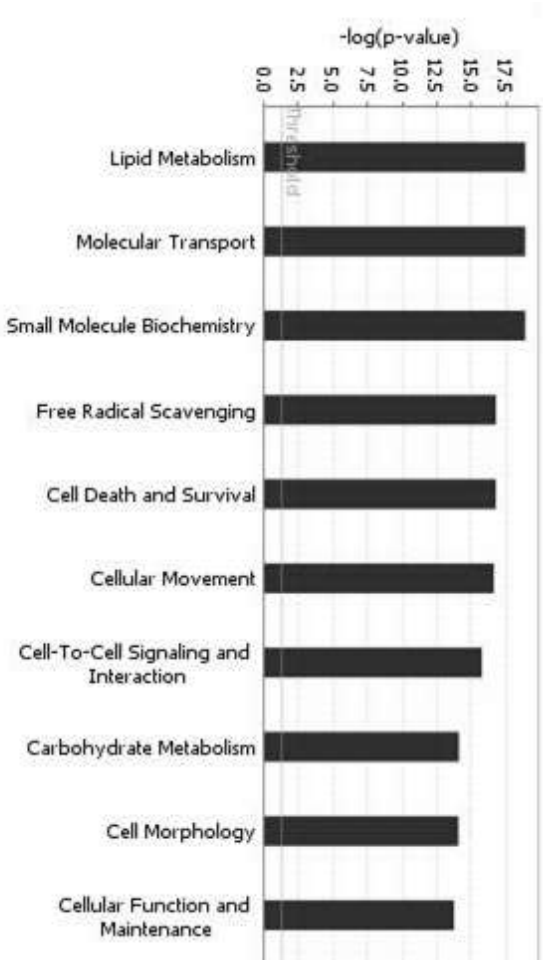
Integrative analysis of gene expression levels

Normalised transcript expression levels (ΔCT) were examined in all three tissues to identify the most actively expressed transcripts at the time of surgery (post intervention), by selecting the most highly expressed transcripts within each tissue. This group of selected transcripts, along with biomarkers that were found to change significantly pre to post dietary intervention, were analysed using IPA to identify their associations to downstream biological functions, i.e. the physiological functions that are most likely to be affected by this combination of transcripts. Results were summarised in the following Figure 32. The results indicate that lipid metabolism and molecular transport were the most active pathways post-diet at the time of surgery.

Figure 32 bar graph for pathway analysis of most highly differentially expressed transcripts between tissues and significantly responsive biomarkers to dietary intervention, showing top ten significantly associated biological systems (-log scale). Lipid metabolism and molecular transport were the most active pathways at the time of surgery, post dietary intervention.

The larger -log means a smaller p-value, and the less likely that the association was random and the more significant the association. In general, p-values < 0.05 (-log=1.3) indicate a statistically significant, non-random association.

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Upstream analysis was also completed on IPA for the same group of transcripts and biomarkers. This was to identify common regulators that were influencing expression and function. Five factors were found that associated significantly to this selection; Leptin, NFκB, TNF, PPARG and IL1b. Table 42 shows IPA upstream analysis results for the top five upstream regulators with target molecules, ordered by significance. Leptin is the factor that was most likely to be driving changes in the transcripts and biomarkers examined as it has the most significant number of associations to those examined.

Table 42 IPA upstream analysis results for the top five upstream regulators with target molecules, ordered by significance.

The overlap p-value measures whether there was a statistically significant overlap between the dataset molecules and the molecules that are regulated by a transcription regulator. The p-value of overlap was calculated by the Fisher's Exact Test.

Upstream Regulator	Molecule Type	P-value of overlap	Target molecules in dataset
Lep	Growth factor	3.20E-19	CD36,CRP,EPHX2,IGFBP2,IL10,IL1B,IL6, LEP,LIPE,NAMPT, PPARGC1A,SOCS3,SOD1,SOD2,TIMP1,TNF
NFκB (complex)	Complex	1.10E-16	C3,CD36,CRP,CXCL8,HIF1A,IGFBP2,IL10, IL18,IL1B,IL6, NAMPT,SOCS3,SOD2,TIMP1,TNF,TNFAIP6
TNF	Cytokine	4.79E-16	C3,CD36,CRP,CXCL8,HIF1A,IGFBP2,IL10, IL18,IL1B,IL6,LEP, LIPE,NAMPT,NLRP3, PPARGC1A,SOCS3,SOD1, SOD2,TIMP1, TNF,TNFAIP6
PPARG	Ligand-dependent nuclear receptor	5.67E-16	C3,CD36,CES1,CIDEA,CXCL8,IL1B,IL6, KRT18,LEP,LIPE,PPARGC1A,SOCS3,SOD1,TNF
IL1B	Cytokine	6.18E-16	C3,CRP,CXCL8,GOT,HIF1A,IL10,IL18,IL1B, IL6,LEP,NAMPT, PPARGC1A,SOCS3,SOD2,TIMP1,TNF,TNFAIP6

A key finding from this analysis was the significant role of leptin as an effector of transcript expression and, as previously identified in the adipose-specific network analysis, NFκB complex.

A detailed discussion and interpretation of these findings follows in the next section.

Discussion

This work examined the mechanisms that contribute to observed biomarker changes and tissue environment following the pre-operative diet. The following interpretation was based on gene expression transcripts and their relationships to other outcomes, and endeavours to set the anthropometric and blood results into context. These relationships were examined in detail because these transcripts showed the greatest expression levels or the greatest differential expression between tissues at the time of tissue extraction (post-diet), and therefore were the most actively expressed, of those examined, following the negative energy balance induced by the intervention.

This section includes liver gene expression results not discussed elsewhere, which have been placed into the context of the other findings, and concludes with a proposed timeline for effects following the pre-operative diet.

Inflammatory and immune response

Suppressor of cytokine signalling (SOCS3) is induced by pro-inflammatory cytokines and regulates cytokine signalling. SOCS3 action not only limits the duration of cytokine signalling to prevent over activity but it is also important in maintaining the specificity of cytokine signalling. SOCS3 expression in obesity may be increased due to elevated pro-inflammatory cytokines such as IL6, and interferes with insulin signalling through degradation of IRS1 and 2, resulting in insulin resistance (Sachithanandan et al., 2010, Moschen et al., 2010). SOCS3 can be used as a surrogate for bioactivity levels of IL6, and evidence suggests that after excess weight loss in severely obese adults SOCS3 expression is down-regulated alongside circulating levels of IL6 (Moschen et al., 2010). In this clinical trial circulating levels of IL6 were observed to reduce significantly pre to post diet, however the expression levels of IL6 in all of the three tissues did not show any association to weight loss. SOCS3 expression levels however were higher in SAT than OAT, and SOCS3 expression in liver did show a significant association to weight loss. This implies that the response of circulating IL6 to the intervention may be mediated through SOCS3 expression change in the liver. If this supposition is taken further, as circulating levels of IL6 were significantly associated with portal and lobular inflammation and weight loss, SOCS3 may also play a role in hepatic inflammation.

NLRP3 inflammasomes are implicated in identifying non-microbial originated 'danger signals', leading to production of active Caspase-1 enzyme, which in turn results in the processing of inactive precursors of IL1B and IL18 into active forms. NLRP3 inflammasome activation has been observed in obesity, promoting macrophage-mediated immune cell activation in adipose tissue and impairing insulin sensitivity. NLRP3 inflammasome activity also amplifies the immune response and exacerbates liver damage (Goossens et al., 2012, Davis et al., 2011). An association between liver-derived NLRP3 and SOCS3 was indicated, with an intra-liver correlation of 0.705 $P<0.001$. This was suggestive of a corresponding association between these inflammatory transcripts.

Nicotinamide phosphoribosyl transferase (NAMPT)[◇] is an adipokine that promotes the production of IL1b, TNF and IL6, with expression levels believed to correlate to levels of visceral adiposity in obesity (Ouchi et al., 2011). Work by Goktas *et al* (2013) found differential levels of NAMPT expression and protein amongst tissues, and concluded that in OAT they were positively correlated with HOMA-IR and insulin resistance (Goktas et al., 2013). In this clinical trial expression levels were significantly higher in SAT than OAT. NAMPT expression levels within adipose depots were also significantly correlated with each other and to pro-inflammatory related transcripts, with higher correlations in the more pro-inflammatory SAT than OAT.

Taken together with the other evidence from this clinical trial that other pro-inflammatory related transcripts such as TNF, IL6 and IL8, IL1b, NLRP3 and HIF1a were higher in SAT than OAT, this supports differential inflammatory status between OAT and SAT. It should also be taken into account that these values were taken from subjects in negative energy balance which the data from most other studies do not.

Macrophages are divided into two phenotypes based on their pro-inflammatory or homeostatic effects. M1 classically activated pro-inflammatory macrophages express CD11c (ITGAX) integrin alpha X chain protein (Wentworth et al., 2010) whereas alternatively active phagocytic macrophages can be identified by Dectin-1 (CLEC7A) (Chawla et al., 2011). Klöting *et al* (2010) investigated two groups of bariatric patients which they separated based on insulin sensitivity. They found higher levels of macrophage

[◇] also known as Visfatin

infiltration in OAT compared to SAT, and significantly higher expression levels of IL6, IL8 and HIF1a in the OAT of insulin resistant obese (Klöting et al., 2010).

In this clinical trial ITGAX and CLEC7A levels were significantly lower in OAT than SAT, which would suggest macrophage number and phenotype proportions differ between depots post-diet. It also suggests a reduced demand for phagocytic macrophages and therefore less adipocyte necrosis, as well a less inflammatory environment and higher insulin sensitivity in OAT than SAT. CLECL7A expression levels in liver also correlate to weight loss (Spearman's Rho P=0.003), indicating that there may be an intervention-related drop in macrophage density, but this would require further examination of histological samples to confirm.

Hypoxia inducible factor (HIF1a) is a transcription factor that induces a stress response to low cellular oxygen, which can occur in pathophysiological conditions such as inflammation (Rius et al., 2008). HIF1a increased expression has been found in insulin resistance, and is suggested to play a role as a chemoattractant for macrophages (Cancello et al., 2005). Monocyte chemoattractant protein-1 (MCP-1) is also known as chemokine ligand-2 (CCL2). Evidence suggests that macrophage infiltration into adipose tissue and into liver is coordinated through the C–C chemokine receptor 2 (CCR2) and its main ligand CCL2. Haukeland *et al* (2006) suggested a link between macrophage infiltration in liver to progression of NAFLD to NASH, and Baeck *et al* (2012) found inhibiting MCP-1 reduced macrophage infiltration into liver (Haukeland et al., 2006, Baeck et al., 2012).

HIF1a transcript expression levels in this clinical trial were found to be lower in OAT than SAT which is in line with observations of a lower density of macrophages (Cancello et al., 2005). CCL2 expression levels were also significantly lower in OAT than SAT. CCL2 expression in liver was not routinely measured in this clinical trial, however a small subset (n=7) show that liver expression of CCL2 was also significantly lower than SAT P=0.018, but not significantly different to OAT P=0.398. Taken together this supports the supposition that SAT has greater macrophage infiltration and a more inflammatory environment than either OAT or liver, but again this would require further examination of histological samples to confirm.

Metabolism

The network analysis carried out on the adipose tissue transcripts, found to differ most between depots, shows a marked relationship to NF-κB and NR3C1. NR3C1 is a receptor for glucocorticoids, which has a dual mode of action; as a transcription factor that binds to glucocorticoid response elements (GRE), both for nuclear and mitochondrial DNA, and as a modulator of other transcription factors. It affects inflammatory responses, cellular proliferation and differentiation in target tissues and may play a role in adipogenesis through the regulation of lipolytic and lipogenic gene expression. The major signalling pathway used by glucocorticoid receptors is via direct DNA binding and transcriptional regulation of target genes, however they can also signal by binding to other proteins, for example with transcription factors NF-κB (GeneCards, 2014, UniProt, 2014). An anti-inflammatory and anti-apoptotic role has been suggested for glucocorticoids in obesity via inhibition of TNF and IL1b release (Zhang et al., 2001). Both of these transcripts were significantly lower in OAT than SAT supporting this theory.

Circulating levels of glucose and insulin were both observed to have an association with levels of steatosis, as was HOMA-IR, and as previous evidence suggests that insulin resistance and impaired hepatic energy metabolism influence the development of steatosis (Szendroedi et al., 2009), it was unsurprising to find this relationship. Also considering the differential expression of pro-inflammatory transcripts that can affect insulin sensitivity, it is reasonable to conclude that insulin levels dropping (albeit not significantly) may be a surrogate marker for reduced insulin resistance affecting insulin production.

Insulin is the main regulator of glucose and lipid metabolism, however, evidence suggests that insulin-like growth factor (IGFI) plays an important role in coordinating energy uptake, achieved by the regulation of IGFI through IGF-binding proteins (IGFBP) such as IGFBP2 (Arafat et al., 2009, Nam et al., 1997). Fasting IGFBP2 has been positively correlated to insulin sensitivity, and acts by reducing the IGFI bioavailability, such as observed during energy restriction when IGFBP2 levels increase. Additionally, fasting IGFBP-2 levels have also been observed to be downregulated in insulin resistance, indicating that resistance to insulin actions may be contributing to reduction in IGFBP-2 (Arafat et al., 2009). In this clinical trial IGFBP2 was more highly expressed in OAT than SAT. This is concordant with differential expression of inflammatory-related transcripts, and it is also likely to be representative of tissue specific insulin sensitivity. In the liver this supposition was

supported, as the liver-derived IGFBP2 transcript expression also has significant analogous correlations to HOMA-IR pre and post diet (P=0.005 and P=0.010 respectively), but an inverse association to HOMA-IR change, which was negative pre to post diet. Also, in confirmation of the associations to inflammation and insulin resistance, in liver and OAT, transcript expressions have an inverse association to levels of lobular inflammation, with higher levels of IGFBP2 associated with lower grades of lobular inflammation (liver P=0.035, OAT P=0.014), and all three tissues transcript expression have an inverse association to steatosis (OAT P=0.015, SAT P=0.001, liver P<0.001).

Superoxide dismutases (SOD) are a group of antioxidant enzymes that degrade superoxide into hydrogen peroxide protecting cells from damage by reactive oxygen species (ROS). *In vitro* and *in vivo* work has suggested that SOD1 over-expression partially attenuates ROS mitochondrial damage in the liver which would normally initiate apoptosis, and suggests a link to hepatic insulin sensitivity (Liang et al., 2009, Kumashiro et al., 2008). In the Derby clinical trial liver-derived expression level of SOD was significantly inversely associated to circulating levels of AST post-diet, perhaps supporting the suggestion that the increase in AST pre to post diet was due to cellular recycling. All lipoprotein levels dropped pre to post diet, indicative of a reduction in endogenous lipoprotein production in line with in-liver utilisation of lipid for oxidation. Taken with the evidence for lipid mobilisation (below) and oxidation in the liver post-diet, and hepatic cellular death, this all indicates an increase in SOD1 expression as a protection mechanism to offset an increase in ROS production.

Cell cycle and lipid movement

Autophagy is a homeostatic mechanism operating a disposal system to degrade large intracellular organelles and protein groups to change cellular structure during differentiation, or to generate essential nutrients in times of energy deprivation (Jansen et al., 2012). In adipose tissue autophagy also regulates intracellular lipid stores through macrolipophagy (Singh et al., 2009). Autophagy inhibition in obesity enhances pro-inflammatory gene expression (Jansen et al., 2012, Kovsan et al., 2011). Also, visceral adiposity and visceral adipocyte hypertrophy have been found to be independently associated with higher levels of autophagy related transcript ATG5 (Kovsan et al., 2011). In liver, impaired autophagy has been cited as contributing to both steatosis development and progression of inflammation in NASH (Gonzalez-Rodriguez et al., 2014).

Autophagy-related transcript ATG5 was significantly higher in liver than OAT or SAT in this clinical trial, alongside a lower level of transcript BCL-2 whose product can both inhibit autophagy and apoptosis. As already mentioned autophagy transcript ATG5 was independently associated to hypertrophy in adipocytes, therefore higher levels in the OAT of this clinical trial may be indicative of hypertrophy, however concurrently the OAT has lower densities of macrophages, as discussed above, which would normally accompany hypertrophy-related adipocyte death. Altogether it is suggestive of higher levels of autophagy and/or macrolipophagy in the liver, and in OAT than SAT, indicative of tissue specific cellular recycling and lipid turnover. An association between higher levels of autophagy and reduced inflammation was also suggested, as alongside higher expression of autophagy transcripts there was less expression of pro-inflammatory transcripts in OAT.

As already discussed a degree of chronic inflammation has been observed in obesity. This is thought to be responsible for activation of the complement system, which is involved in the biochemical pathways for immune responses. A central component of the complement system is the C3 fraction, and both adipocytes and liver are an important source of C3 production in response to cytokines and activated macrophages. Circulating C3 has been found to increase in association with body mass index in the obese and in association to insulin resistance, and weight loss has also been shown to significantly reduce circulating levels of C3 (Hernandez-Mijares et al., 2007).

Notably the complement system is also involved in the recognition and clearance of apoptotic cells, and the number of apoptotic hepatocytes has been found to be significantly higher where C3 deposition is observed. However in the absence of rapid clearance, apoptotic cells undergo secondary necrosis releasing their pro-inflammatory contents, and therefore activation of the complement system and C3 deposition can be positive response (Rensen et al., 2009).

In this clinical trial C3 was indicated to have higher expression levels in OAT compared to SAT, also C3 expression in adipose depots was associated to BMI, inversely in OAT and analogously in SAT. This again suggests a differential environment post-diet in the two adipose depots. If it is accepted that OAT has a more dynamic environment following a short-term negative energy balance, then C3 expression in OAT is more likely to be related to adipocyte clearance following lipid depletion.

In the liver C3 levels may be indicative of a pro-apoptotic state of remodelling, in line with pre to post diet rises in circulating markers for apoptosis (CK18), and would account for increasing circulating levels of AST, which would also be released during apoptosis. Taken together with liver-derived levels of autophagy-related gene expression, and the association of CK18 to levels of steatosis, plus the mobilisation of hepatic lipid which was evidenced by lower levels of steatosis in this clinical trial than might be expected in this population, this is all indicative of transient remodelling related to loss of lipid from liver in those with higher grades of steatosis.

Lipolysis is mediated by multiple enzymes including hormone sensitive lipase (LIPE) that catalyses the hydrolysis of diacylglycerol, triacylglycerol and cholesterol-ester, adipose triglyceride lipase (PNPLA2) and carboxylesterase 1 (CES1) (Shuichi Nagashima, 2011). All three enzyme transcripts have tissue-specific expression levels in this clinical trial, with more CES1 expression in liver, more LIPE expression in SAT, and more PNPLA2 expression in OAT. Adipose tissue derived CES1 was analogously associated to weight loss (Spearman's Rho OAT P=0.004, SAT P=0.009) as might be expected as a result of a period of energy restriction. However, CES1 transcript expression in liver was contradictory increasing with levels of steatosis (Spearman's Rho P=0.038). From these results it can be posited that CES1 expression was altered with its tissue specific role, and within the liver this was associated to lipolysis relative to levels of steatosis. Concomitant to this liver-derived LIPE and PNPLA2 were associated to circulating BHB, which is suggestive of lipolysis in the liver directly feeding into lipid oxidation.

Gene expression results also reveal a differential association between adipose-derived Leptin transcripts with steatosis, with OAT having an analogous association to increased steatosis and SAT having an inverse association (Kendall's Tau-C OAT P=0.009, SAT P=0.003). This discrepancy can be rationalised as OAT has depleted as a result of the period of energy restriction (negative energy balance), whereas SAT was a much larger depot and evidence suggests it is less likely to be effected in the short-term, as rapid weight loss demonstrated with very low energy diet studies show a very early but unsustained exceptional preferential loss of visceral fat (Chaston and Dixon, 2008). The OAT leptin transcript association to steatosis was also indicative that both were affected by the intervention, but although SAT-derived Leptin expression associates with weight loss

(Spearman's Rho P=0.037), any OAT-derived Leptin association was already modified by depletion of this depot.

Patatin-like phospholipase domain containing-3 (PNPLA3) is a multifunctional enzyme which has both triacylglycerol lipase and acylglycerol-acyltransferase activities, and can therefore affect both lipid catabolism and anabolism. PNPLA3 expression is higher in the liver than adipose tissue (Huang et al., 2010), and a PNPLA3 genetic polymorphism has been found to contribute to inter-individual differences in hepatic fat content and susceptibility to NAFLD (Romeo et al., 2008). In this clinical trial PNPLA3 expression was higher in liver, than SAT or OAT, as expected. When taken alongside other indicators of liver-derived lipolysis, PNPLA3 levels in the liver were likely to be associated with lipid catabolism.

Tissue Inhibitor of Metalloproteinase (TIMP1) is implicated in the fibrogenic response to Leptin in liver by regulation of extra cellular matrix homeostasis (Handy et al., 2011). Liver TIMP1 expression in this clinical trial was significantly inversely associated to weight loss and excess BMI loss (P=0.011, P=0.010 respectively), suggesting its fibrogenic activity is inversely associated to the negative energy balance induced by the intervention.

Efficiency and capacity of adipocytes to esterify NEFA into TAG is controlled within large lipid droplets surrounded by a phospholipid layer and lipid droplet proteins such as CIDEA (Puri et al., 2008). CIDEA expression has been noted to increase in obesity during an energy restrictive diet (Dahlman et al., 2005) and independently of weight loss, when insulin sensitivity improves (Montastier et al., 2014). LPCAT is an enzyme that plays a role in phospholipid formation and is located on the surface of lipid droplets (Moessinger et al., 2011). The CIDEA transcript was higher in the OAT depot than SAT in this clinical trial, which would indicate that this may be as a result of weight loss, or better insulin sensitivity in this depot above that of the subcutaneous depot, supported by the evidence from other transcripts. Together with higher levels of LPCAT2 this is suggestive there was lipid droplet mobilisation within the OAT.

Summary

To summarise, transcripts for inflammatory mediators and macrophage infiltration were observed to be lower in the OAT than SAT following negative energy balance and dynamic

weight loss post-diet, alongside concomitant lipid droplet mobilisation. Increasing oxidation of lipid in the liver was also evident pre to post diet, potentially associated to reduction in steatosis, and it can be hypothesised that there was a reducing level of inflammation related to cellular recycling as well as reduction in inflammatory mediators in the tissue environment. Post-diet in OAT there were suggestions of lipid droplet mobilisation, with better responsiveness to insulin than in SAT. Improved insulin sensitivity was also implied within liver pre to post diet, alongside a suggested reduction in transcripts that could modify insulin sensitivity. SAT post-diet was a contradictory complex of inflammation and lipid mobilisation, which was indicative of a less dynamic tissue response to the diet in SAT than OAT.

The following chronology was implied; energy restriction promoting mobilisation of lipid in liver and OAT via macrolipophagy alongside reduction in inflammatory status of these tissues, facilitating a reduction in IR. In the liver, portal and lobular inflammation were all improved pre to post diet. SAT was last to respond, by the end of the two weeks it still has a pro-inflammatory and potentially insulin resistant environment, but with indications of some lipid mobilisation.

The most notable observation from results was the significant differential expression of transcripts between adipose tissue depots. Due to the low number of significant results when comparing transcript expression levels between dietary interventions in all tissues, alongside lack of association to NAFLD and insubstantial and contradictory biomarker outcomes, it was unlikely that the type of diet undertaken and therefore the macronutrient composition, affected outcomes observed elsewhere. This would suggest that any deviation from observations in comparative populations, equivalent to this clinical trial population's condition pre-diet, may be related to weight loss or energy restriction, but not macronutrient intake.

Chapter 8

Conclusions

Considerable work has already been undertaken elsewhere examining the potential roles and mechanisms for hepatic lipid deposition, inflammation and IR in metabolic dysfunction in obesity. However, there was a lack of consolidation of concepts and examination of reversibility in relation to progressive NAFLD and adipose tissue dysfunction in severe obesity.

Specific objectives for this thesis were to examine the application and effects of a very low energy pre-operative diet prior to bariatric surgery, used with the severely obese population. Within this examination it was also intended to explore the post-diet prevalence and features of progressive NAFLD, and the contribution of adipose tissue dysfunction. Also, to apply a novel approach in this setting, by comparing two distinct formats of diet, to compare efficacy and outcomes.

This thesis examined the application and format of a pre-operative "liver reducing" diet in clinical practice in bariatric surgery services in the UK. The findings of the survey revealed dietary choices varied and appeared to be defined by clinicians based on observed efficacy at the time of surgery, in respect of liver volume and flexibility, and ease of access to the gastro-oesophageal junction. However the Bariatric Services that responded to the request for information provided very little evidence to justify their chosen format and duration. Background research highlighted the lack of evidence for approaches used, such as milk only, and that there was conflicting outcome data not wholly supportive of the necessity or benefits of applying a pre-operative diet. Also, given data elsewhere of wider physiological effects of VLEDs in the obese population, the survey findings could be interpreted to suggest that diets were being applied naively without consideration for how the approach could affect other outcomes, for example inflammatory changes.

The current clinical trial applied a comparative approach, comparing two formats of very low energy diet (VLED) in the pre-operative phase prior to bariatric surgery, one using normal foods, the other using meal replacement products. The decision was made to use the most frequently used format of diet from clinical practice in the clinical trial. Inclusion

of the meal replacement plan was based on its frequency of use in other related research. However, to inform best practice it might have been useful to include a third format using the milk-only diet. This dietary format lacks evidence for its application or effects, and its examination would have been particularly valuable considering it is currently in use. The comparative approach used in the study within this clinical context had not previously been carried out; subsequently in Brazil, a bariatric cohort study has been carried out in a similar setting comparing two dietary approaches with a similar energy intake, but with different dietary formulations for food-based and liquid-based approaches (Faria et al., 2015).

In the clinical trial reported in this thesis, after two weeks of VLED there was a median weight loss of 3.4%, range 1.4-5.1%, which was similar to the Brazilian cohort, whose minimum body weight loss achieved was 1.3%, and maximum 4.9%. Weight loss achieved is therefore variable in this population and setting. The range in weight loss observed could be related to adherence to the dietary approach or may be influenced by appetite or adaptive thermogenesis (page 117). By pooling data in the current clinical trial there was a significant negative relationship between weight loss percentage achieved and hunger score, with higher scores for hunger frequency in the lower weight loss group. This is contrary to the findings in the Brazilian cohort who did not find any association between weight loss and degree of hunger.

The macronutrient intake (energy source) in this clinical trial also differed between the two diets. The food-based diet had higher intake of carbohydrate (median 52.3% compared to 38.1%), whereas the meal plan had higher intakes of protein (median 36.5% compared to 29.9%) and fat (median 25% compared to 18.9%). All three macronutrients intakes were statistically different between groups. But there were no significant associations between macronutrient type and weight loss achieved or reported hunger. An association was found between levels of reported constipation and hunger, which was not found to be related to fluid intake. However with differing fibre content in the diets, it would have been advantageous to examine any associations between fibre intake, constipation and hunger.

Energy expenditure was not measured during this clinical trial due to practical limitations of equipment availability on site, participant access and schedule. But, given the evidence that in the first weeks of a hypoenergetic diet there will be some level of adaptive

thermogenesis, measurement of resting energy expenditure (REE) would have been informative. Particularly as the absence of these data mean that it cannot be discounted as playing a role in weight loss variability. Use of predictive equations in the severely obese population is problematic even with the development of an equation specifically for this population (Horie et al., 2011), therefore indirect calorimetry would have been the ideal method for measurement of REE.

Weight loss results were not significantly different between diet groups at the end of two weeks for this clinical trial. However, Faria et al (2015) report significantly less weight loss in a food-based diet compared to a liquid diet over two weeks. Their results could however be related to the significant difference in baseline body mass index between the groups. One of the strengths of the clinical trial reported in this thesis was no significant difference in demographic or anthropometric profile between diet groups at baseline. Also, contrary to the postulation that a liquid format diet is easier to follow and may encourage compliance in the short term compared to a food-based approach (Faria et al., 2015); the evaluation of the dietary approaches in this clinical trial revealed no significant difference in acceptability or patient satisfaction.

The size of the current clinical trial sample was dependent on organisational compliance to a pre-operative schedule. This schedule should have allowed time and access for potential participants to undertake a pre-operative assessment, and two weeks of diet, prior to their surgery. Unfortunately as patients were brought in for surgery at short notice, or transferred offsite, it was not always possible for verbally consented patients to participate. Thus, the study sample became smaller than expected, with 105 verbally consented but only 60 being randomised. Improving recruitment could have been achieved by ensuring the study intervention fitted into the real life pre-operative schedule rather than the ideal.

The majority (81.5%) of participants in the current clinical trial were female, and although this is in line with the national average for patients undergoing bariatric surgery (BOMSS, 2011, BOMSS, 2014c), it does create a gender bias. Even though results did not suggest significant differences between genders, the validity of these results for men is in question with so few male participants. In the results of a recent systematic review, gender-specific effectiveness for different weight loss interventions was not found to be significant. However, as described in the review the interventions were heterogeneous, for duration,

type and gender (Williams et al., 2015). If the Derby clinical trial had achieved greater recruitment the gender differences could have been explored fully. Further work could include examination of the gender-specific differences in the relative distribution of adipose tissue to see if this plays any role.

The current clinical trial provides further supporting evidence that following a short-term pre-operative energy restrictive diet does not inhibit severely obese patients from having laparoscopic surgery, confirming what has been observed elsewhere (Colles et al., 2006, Collins et al., 2011, Edholm et al., 2011, Van Nieuwenhove et al., 2011, Reeves et al., 2013). There was proportionally less steatosis observed in current clinical trial at the time of surgery than would be observed in the general population of severely obese people (Machado et al., 2006, Cordeiro et al., 2015). Also there was a strong correlation found between visual assessment of the liver by the surgeons at the time of surgery, for liver rigidity and volume, and gastric access, and levels of histologically assessed steatosis. The pre-operative diet could therefore be reasoned to have enabled surgery, from the perspective of reducing difficulty of the surgery, due to limiting steatosis. However this clinical trial did not record or report perioperative or postoperative outcomes that are used as surrogate measures for operative efficiency and safety, such as operating time, blood loss, length of time before discharge, or re-operative rate. There were also limitations in the collection of the visual assessment data, due to only some of the surgeons being allowed to take part. Therefore although in one subjective measure the diet was seen to enable the laparoscopic surgical approach, the work was not conclusive.

Concordant with the Brazilian study, there was very little difference in the changes observed in fasting blood results pre and post diet between dietary approaches in the current clinical trial. Both studies observed positive changes in circulating cholesterol, glucose and CRP over the duration of the diet. However contrary to the Brazilian study one diet, the meal replacement plan, was observed to have a greater effect and enable a significant reduction in HOMA-IR. HOMA-IR was also found to have a significant association to levels of steatosis, and again when separated by diet format this was only significant in the meal replacement plan group. In examining the possible associations between diet type and these results, no association was found in the current clinical trial to macronutrient intake, which was significantly different between diet groups. However this

could be explained by the significant association in the Meal Plan group to self-reported body weight status as a child. Those who reported being overweight/obese as a child had a significant reduction in HOMA-IR when following the Meal plan that was not seen on the Food-based diet. This significant change in HOMA-IR was also not observed in the lean morphology group on either diet. These findings suggest a link to the adipose tissue expandability hypothesis, and that the status of adipose tissue may impact on reversibility of insulin resistance during periods of energy restriction.

In the current clinical trial post-diet NAFLD findings were not associated with any of the macronutrient levels during the diet, but conclusions cannot be drawn about the influence of typical dietary intake (prior to intervention), as no data were collected on this intake and no NAFLD assessment was made pre-diet. Although steatosis levels in the Derby clinical trial were not measured pre and post diet, surrogates for lipid depletion in the liver, and lower levels of NAFLD than might be anticipated in this population, suggest that both VLEDs may be effective in reducing steatosis over two-weeks. This could have been confirmed by use of non-invasive imaging, but practical limitations of equipment excluded this option at the time of the study.

When results for the current clinical trial were separated into those with euglycaemia or Type 2 Diabetes/impaired fasting glucose (DM/IFG) (as defined at baseline), a significant relationship was found between steatosis grade and DM status. Results show a higher degree of steatosis in participants with DM/IFG post-diet. NAS was also found to have a significant association with DM status. Results indicate a higher level of NAS scores post-diet in the DM/IFG subgroup compared to those with euglycaemia. This association suggests a residual steatotic outcome post-diet, irrespective of weight loss. Further work would be required to elucidate if this residual steatosis was related to duration of NAFLD or IFG/DM and irreversible, or to establish if there is an optimum dietary format or duration that might enable more equitable results in this group.

Notably, concern over what could be interpreted as detrimental increases in markers of progressive liver disease (AST and CK18) that were observed in the current clinical trial pre to post diet, can be moderated by findings that depletion of excess lipid in the liver is likely to result in cellular recycling. Underlying gene expression suggests a dynamic metabolic environment in the liver, supporting the likelihood that lipid was being mobilised and there

was cellular remodelling. Changes in these markers of liver status were therefore likely to be a response to energy deficit rather than disease progression.

Also, importantly in respect of progressive NAFLD, levels of portal inflammation in the current clinical trial were found to be significantly associated to severe obesity, such that greater BMI loss was associated to lower levels of portal inflammation. A link between inflammation and circulating levels of IL6 was also observed, with IL6 decreasing significantly pre to post diet, and a correlation to levels of hepatic lobular and portal inflammation. It would therefore be reasonable to suggest that a VLED is favourable for severely obese patients with NAFLD, and could support reversal of disease progression.

The adipose tissue gene expression results support the observations elsewhere that adipose tissue inflammatory status, which impacts upon circulating inflammatory drivers, is affected by energy restriction and weight loss (Cancello et al., 2005, Clément et al., 2004, Mardinoglu et al., 2015). However these observed changes were in both subcutaneous and omental adipose tissue and were after longer periods of energy restriction, and post bariatric surgery. In contrast in the current clinical trial it was the omental but not subcutaneous adipose tissue which was observed to have a comparatively lower inflammatory gene expression profile after two weeks of energy restriction. This suggests a short-term energy restriction elicits a response which is different to that expected if the intervention had been longer duration. The gene expression data can also be interpreted to suggest differential inflammatory environments between adipose depots, and that the subcutaneous adipose tissue was a more pro-inflammatory environment in comparison to the omental adipose tissue. If this outcome was a result of the acute energy restriction, this would support evidence elsewhere that the subcutaneous adipose tissue is not as adaptable in these circumstances (Cancello et al., 2013), and the omental depot has responded to the acute energy restriction to become more representative of a metabolically healthy phenotype (Esser et al., 2013). Also, as omental adipose tissue has previously been found to have a greater association with circulating inflammatory markers, than subcutaneous (Park et al., 2005), this finding recommends a short-term energy restriction to support reduction in circulating inflammatory biomarkers. These suggestions would have to be confirmed, ideally by testing of both depots pre and post a similar intervention, alongside circulating biomarkers. However ethically and practically this was

very difficult to achieve, as demonstrated by the paucity of similar research studies undertaken elsewhere, and the predomination of subcutaneous only sampling.

Implications for practice

In the current clinical trial both dietary approaches achieved a similar weight loss and excess BMI loss, and other outcomes such as NAFLD assessment and gene expression were also comparable between diets. Patients on the meal replacement plan reported a higher frequency of constipation and mood swings; however patient evaluation of overall satisfaction could not be separated between diets. No observed results can be reasonably ascribed to macronutrient content or format of the dietary approaches, and in so much as results were not significantly different for the majority of outcomes between food-based and meal replacement approaches, it does suggest that either approach could be used in practice. It was also notable however, that the food-based diet would not be micronutrient complete without supplementation, and this would be a recommendation for all restrictive diets where intake variety and quantity could limit intake of micronutrients, particularly as this population group have a high prevalence of micronutrient deficiencies and with the long-term post-operative nutritional restrictions. The requirement for a micronutrient supplement has a cost implication for practice, but not as substantial as the cost implication of applying a complete meal replacement plan.

Steatosis levels were observed to be related to DM status, and post-diet levels of steatosis were higher in those with DM or impaired fasting glucose. Although these findings require confirmation with assessment of liver volume pre and post diet, it is reasonable to interpret these as relevant to application of the pre-operative diet. In the absence of other evidence it would suggest that those with DM or impaired fasting glucose would benefit from additional intervention to further improve their steatosis prior to surgery.

Despite the shortcomings of this thesis, the findings can inform the format and application of a short-term pre-operative diet in severe obesity. It has established that pre-bariatric surgery patients were satisfied with either format of VLED when applied in clinical practice. It has also extended the existing evidence base on the effects of a short-term VLED, specifically the potential beneficial effects on progressive features of NAFLD, and adipose tissue inflammatory status, which could be of value in the management of the disease.

Future research

Adipose tissue inflammatory status: confirming effects of energy restriction

The Derby clinical trial observed differences between omental and subcutaneous adipose tissue through gene expression levels. This was not unexpected in some respects as a differential expression has been observed elsewhere (Linder et al., 2004, Vohl et al., 2004). However, the differential expression of pro-inflammatory genes, with lower expression levels in omental rather than subcutaneous adipose tissue was novel and warrants confirmation. Specifically work is required to confirm if expression levels change pre to post diet, as pre-diet samples were not available, and further work is needed regarding the comparison of adipose tissue depots. To enable this comparison in the severely obese population, omental and subcutaneous adipose tissue biopsies could be taken during abdominal surgery where a pre-operative diet was not applied, for example unplanned or open surgery, for patients who are not suffering with disease that is likely to affect adipose tissue inflammation. These data could then be compared with the current clinical trial.

NAFLD legacy: maintenance of improvements after VLED

Further work is required to assess if there is a legacy effect from the short-term energy restriction in NAFLD activity and progression. Do changes observed in the short-term have any longer lasting effects? Legacy effects have been described from intensive glucose control in patients with DM during follow-up studies of the United Kingdom Prospective Diabetes Study (UKPDS) and the Epidemiology of Diabetes Interventions and Complications (EDIC) study (Holman et al., 2008). Given the dramatic reversal of hepatic IR that was observed after just a short duration of VLED, further work is required to estimate if changes observed in liver lipid and inflammation, continue post-intervention, and if there is any impact on rates of disease progression observed with weight increase. Steven and Taylor (2015) suggest that improvement could last for years, but is dependent on the maintenance of weight loss and limiting intrahepatic lipid levels , but there could also be a quick switch back to metabolically unhealthy obesity if visceral adipose tissue accumulates (Hwang et al., 2015). Legacy could be assessed as a follow-up to the Derby clinical trial, examining liver lipid non-invasively alongside circulating biomarkers measured previously, however results would be confounded by the dramatic weight loss and associated metabolic effects resulting from the bariatric surgery. An alternative would be to examine a cohort of severely obese patients who are awaiting surgery, where a short-term energy

restrictive diet could be used as a motivator for weight loss during the preparatory stages (Tier 3) in the 24 months before referral for surgery. Non-invasive imaging would be used to minimise risk, and final assessments completed just prior to surgery.

Severity and reversibility of NAFLD: dependence on duration of steatosis

Is steatosis duration important in the severity and reversibility of NAFLD? Steatosis is described as a benign asymptomatic stage in NAFLD. However steatosis development is necessary before active inflammation and progression to NASH and fibrosis, and development of NAFLD will affect life expectancy (Adams et al., 2005). It is also consistently present before the onset of classical Type 2 diabetes (Taylor, 2008). The majority (58%) of the current clinical trial participants did have steatosis post-diet. This may be in part related to the severity of the disease pre-diet and was more severe in those with impaired fasting glucose/diabetes. Given the asymptomatic nature of steatosis, and the earlier onset of obesity, in children and adolescents, it is important to assess if duration of steatosis prior to diagnosis affects management. The first step would be to complete a screening study to assess levels of steatosis at different BMIs, ages and genders, before prospectively following the cohort to assess NAFLD incidence and time to progression. Non-invasive imaging techniques such as dual energy x-ray absorptiometry (DXA) could be used to visualise body composition alongside circulating markers for liver function and inflammation.

Adipose tissue expandability hypothesis: impact on insulin sensitivity during energy restriction

Further work is required, following on from the findings in this clinical trial, to confirm the association between child weight status and reversibility/flexibility of insulin sensitivity in adulthood during periods of energy restriction. Ideally a cohort of children would be prospectively followed, recording anthropometric and medical data. Periodic imaging, such as DXA, would provide data on adipose tissue volume and location, and liver lipid content. Adipose tissue biopsies, as well as measures of insulin resistance, would be taken during childhood and pre and post a dietary restrictive regime in adulthood. Histological assessment of adipose tissue would provide data on adipocyte morphology, volume and number, and inflammatory status. However, ethically and practically biopsies of omental adipose tissue from children would only be available sporadically from medically essential

surgical procedures, but subcutaneous adipose tissue biopsies could be collected routinely. If prospective collection of data was not possible, then a cohort of children and adolescents, who had their child weight status collected during the National Child Measurement Programme would be followed up in adulthood. Periodic anthropometric data from routine medical records would provide a weight status and medical history.

This work would complement that of Roy Taylor who has been examining pathophysiological effects of a very low energy diet on the pancreas in DM responders and non-responders (Steven et al., 2016)

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Appendices

Pre-op Diet for Bariatric Surgery

You need to follow this diet for the two weeks immediately before your operation. It will reduce the fat and glycogen stored in your liver and make the operation easier for the surgeon. If the surgeon finds your liver is too big your operation will not be able to go ahead.

This diet is ONLY recommended for 14 days before surgery and should NOT be followed at any other time as it is too restrictive.

Have three meals a day and use the 'extra' fruit or yoghurts as desserts or as snacks.

Try to vary the foods as much as possible during the fortnight to help make sure you are getting a good range of nutrients. It may be a good idea to start having the multi-vitamin and iron supplement (that you are recommended to take for the first six months after your band) while you are on this restricted diet.

Do not guess weights – use scales to measure portions accurately.

No sugar, cooking oils, margarine, butter, low fat spread, mayonnaise or sauces are allowed.

The following drinks and flavourings are allowed freely:

Water, tea and coffee (using milk from allowance), low calorie squash.

A small amount of the following can be added to food if needed: salt, lemon juice, vinegar, balsamic vinegar, garlic, herbs and spices, pepper, artificial sweeteners

Include at least 2 litres (4-5 pints) of fluid a day.

Breakfast

30g non sugar-coated and nut-free cereal e.g. porridge oats, ready brek, branflakes, rice crispies, special K, cornflakes, shreddies, sultana bran

or

1½ weetabix or shredded wheat

or

1 portion of fruit

or

1 diet yoghurt

or

1 small slice bread or roll

Lunch and Dinner

1 small slice bread or small roll

or

2 crispbreads

or

100g potato (uncooked weight) – jacket, boiled or mashed with milk from allowance.

or

30g (uncooked weight) of rice, couscous, pasta or noodles

With

100g of vegetables

or

cereal bowl of salad with 1 teaspoon of low calorie dressing (no salad cream or mayonnaise)

With

Choose one of the following - all are cooked weights:

150g steamed or poached fish. No batter or breadcrumb coatings

or

50g lean meat. Trim off all fat before cooking

or

1 large egg e.g. poached, boiled or scrambled with milk

or

110g Quorn or Tofu

or

25g cheese e.g. cheddar, brie, stilton etc.

or

50g low fat soft cheese e.g. Philadelphia light, Laughing cow light etc.

or

75g chicken or turkey

or

100g tinned tuna/pilchards (not in oil)

or

50g tinned salmon/sardines (not in oil)

Each day also have:

260ml (½ pint) of skimmed or semi-skimmed milk

2 portions of fruit (one of which could be swapped for a small (150ml) glass of fruit juice)

1 diet/light/virtually fat-free yoghurt or fromage frais

A portion of fruit is:

1 large fruit e.g. banana, apple, pear, orange, grapefruit

or

2 small fruits e.g. kiwi, plum, satsuma

or

200g strawberries, raspberries, blueberries, blackberries, rhubarb or gooseberries

or

10 grapes or cherries

or

100g tinned fruit in juice

If you have any queries about this diet, please contact the Dietitian on 01332 785573 (Mon-Fri 8.30am – 4.00pm)

Appendix 2 ***Meal plan***

Meal Replacement Plan

Meal replacement products were all supplied free of charge by LighterLife. All products were either dry powder which could be reconstituted with water or milk, or cereal-type bars.

Participants were given a limited choice of products, and a regime to follow (over page), which included three meal packs per day, one cereal bar and an allowance of milk.

Products included milkshake style packs (m), soup mixes (s), meal mixes (mm) and cereal bars:

- Crispy Cranberry bar
- Crispy Peanut bar
- Strawberry flavour Foodpack (m)
- Vanilla flavour Foodpack (m)
- Banana flavour Foodpack (m)
- Chocolate flavour Foodpack (m)
- Carbonara flavour Foodpack (mm)
- Chicken flavour Foodpack (s)
- Chilli Con Carne flavour Foodpack (mm)
- Vegetable flavour Foodpack (s)

Participant preferences were recorded and they were asked to inform us of any issues with tolerance. Participants were offered replacements as or when necessary in order to encourage compliance.

Participant: No:

Initials: DOB:

Meal Replacement Plan – 2 weeks

Daily - 3 foodpacks, 1 bar, 350ml whole milk

You have been allocated to follow the Meal Replacement Plan for the 2-week diet before surgery.

This means that you have a choice of foodpacks and bars, from a list which is provided. Please follow the instructions below. You will be provided with all the foodpacks and bars. During the 2-week diet do **not** eat or drink anything that is not listed below. If you have any questions, please contact the researcher Emma Baldry on 07527427818.

Daily - 3 foodpacks, 1 bar, 350ml whole milk

- Bars - Each day you should take 1 bar.
- Foodpacks - Each day you should take 3 foodpacks.

Foodpacks should be made up following the instructions on the packet. They can be made up using water or milk (from your allowance).

- Milk – Each day you should take 350ml whole milk (not provided)

This can be used to make up the foodpacks or can be used as separate drinks.

In addition to the packs/drinks above, you can also take plain water, or black coffee/leaf tea with unlimited tablet sweeteners. Take at least 2 litres (4-5 pints) of drinks a day (including any milk or water).

Example meal plan:

Daily - 3 foodpacks, 1 bar, 350ml whole milk

In addition to the packs/drinks above, you can also take plain water, or black coffee/leaf tea with unlimited tablet sweeteners. Take at least 2 litres (4-5 pints) of drinks a day (including any milk or water).

Time of Day	Description of food/drink	Amount consumed
Breakfast 8am	Banana Shake Foodpack Tea no sugar and whole milk	1 sachet made up with 100ml whole milk 100ml water Large mug – 200ml (25ml milk)
Morning 10-12noon	Flavoured water (no added sugar) Toffee bar	2 x Glass – 400ml ½ bar
Lunch 1.30pm	Vegetable Foodpack Water	1 sachet made up with water Glass – 200ml
Afternoon 2-4pm	Water Milky coffee with sweetener (whole milk) Toffee bar	2 x Glass – 400ml Cup – 150ml ½ bar
Evening 6.30pm	Pasta carbonara Foodpack Coffee with sweetener and whole milk	1 sachet made up with water Large mug – 200ml (25ml milk)
Evening 8-10pm	Tea no sugar and whole milk	2 large mugs - 400ml (50ml milk)

The majority of patients are referred into the service via their general practitioner (GP) according to the East Midlands Specialist Commissioning Group (EMSCG) guidance. Bariatric surgery is recommended as a treatment option for adults with obesity only if all of the following criteria are fulfilled (Parry, 2011):

- BMI of 50kg/m^2 or more, or 45-50kg/m^2 with other significant disease (for example type 2 diabetes, hypertension) that could improve with weight loss
- Have been receiving and complied with weight management support, both medical and psychological as required, in a specialised obesity hospital or a community based equivalent
- Aged 18 years or over
- There is evidence that all appropriate and available non-surgical measures, which may include commercially provided weight loss support programmes, have been adequately tried for a period of a least 6 months but ideally 12 to 18 months but has failed to maintain significant weight loss (i.e. ≥10%)
- There are no specific clinical or psychological contraindications to this type of surgery and the individual is generally fit for anaesthesia and surgery
- Patients must be committed to the need for follow-up by a doctor and long-term compliance with an altered lifestyle and dietary habit post-operatively
- Patients should not have smoked for at least 6 weeks before surgery

Referrals are also accepted from other agreed contracts:

- BMI equal to or greater that 50kg/m^2.
- BMI between 45–50kg/m^2 the presence of significant co-morbid conditions that could be improved by weight loss and the co-morbidity is hypertension or diabetes.
- All patients referred for surgery should have exhausted all other non-surgical ways to reduce weight including dietary support, exercise and drug treatment.

Appendix 4 ***Alcohol intake recommendations***

In the England the Department of Health is responsible for Government health policy on the harmful use of alcohol.

The UK Chief Medical Officers recommend:

- women should not regularly drink more than 2-3 units a day; and
- men should not regularly drink more than 3-4 units a day.

'Regularly' means drinking every day or most days of the week

What is a unit?

The idea of counting alcohol units was first introduced in the UK in 1987 to help people keep track of their drinking.

Units are a simple way of expressing the quantity of pure alcohol in a drink. One unit equals 10ml or 8g of pure alcohol, which is around the amount of alcohol the average adult can process in an hour. This means that within an hour there should be, in theory, little or no alcohol left in the blood of an adult, although this will vary from person to person.



The number of units in a drink is based on the size of the drink as well as its alcohol strength. For example, a pint of strong lager contains 3 units of alcohol, whereas the same volume of standard lager has just over 2 units.

Using units is a simpler way of representing a drink's alcohol content, which is usually expressed by the standard measure ABV, which stands for alcohol by volume. ABV is a measure of the amount of pure alcohol as a percentage of the total volume of liquid in a drink.

You can find the ABV on the labels of cans and bottles, sometimes written as "vol" or "alcohol volume" or you can ask bar staff about particular drinks. For example, wine that says "12% ABV" or "alcohol volume 12%" means that 12% of the volume of that drink is pure alcohol.

Taken from http://www.nhs.uk/Livewell/alcohol/Pages/alcohol-units.aspx accessed 20th October 2011

Appendix 5 LighterLife meal replacement – exclusion criteria

If a patient has any of the conditions below or is taking any of the medications listed below, they will be unsuitable for LighterLife's weight-loss programmes.

- Some cardiac conditions (including heart failure, arrhythmias, valve disease, requiring treatment).
- Some cerebrovascular conditions. Patients who have suffered cerebrovascular disease may participate if the following conditions are met:
- More than six months has passed.
- Patient's condition is stable.
- Schizophrenia, delusional and bipolar disorder, psychosis.
- Epilepsy or history of seizure.
- Kidney / liver disease (severe).
- Total lactose intolerance.
- Type 1 diabetes.
- Anorexia or bulimia or other eating disorder undergoing or awaiting treatment.
- Porphyria.
- Taking prescribed anticoagulants, digoxin, lithium or monoamine oxidase inhibitors (MAOIs).
- Unstable angina.
- Major depressive disorder.

The above list is not exhaustive and some aspects of a patient's medical history may need further clarification before suitability can be fully assessed.

Taken from http://www.lighterlife.com/hp/ - accessed 13th July 2011

Appendix 6 ***Adverse event definitions (AE)***

Any untoward medical occurrence in a clinical study subject.

An AE did include a / an:

- Exacerbation of a pre-existing illness.
- Increase in frequency or intensity of a pre-existing episodic event or condition.
- Condition detected or diagnosed after intervention even though it may have been present prior to the start of the study.
- Continuous persistent disease or symptoms present at baseline that worsen following the start of the study.

An AE did not include a / an:

- Pre-existing disease or conditions present or detected at the start of the study that did not worsen.
- Situations where an untoward medical occurrence has not occurred (e.g. hospitalisations for cosmetic elective surgery, social and / or convenience admissions).
- Disease or disorder being studied or sign or symptom associated with the disease or disorder unless more severe than expected for the participant's condition.
- Overdose of concurrent medication without any signs or symptoms.
- Change in body weight/shape due to weight loss, unless outside expected parameters

Possible effects, as listed in the Participant Information Leaflet, which may have occurred during application of the pre-surgery/meal replacement diet; including, changes in bowel habits such as constipation and flatulence, headaches, dizziness or fatigue, dry skin, sensitivity to cold or bad breath. (Although all effects reported were scrutinized by the research team.)

Any adverse events that occur as part of the routine NHS procedures (venepuncture and tissue biopsies during the operation) will be dealt with in accordance with the NHS Trust policy.

Serious adverse event (SAE)

Was defined as any untoward and unexpected medical occurrence or affect that:

- Results in death.
- Is life-threatening – refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.
- Requires hospitalisation, or prolongation of existing in-patients' hospitalisation.
- Results in persistent or significant disability or incapacity.

Causality

Defined as:

- Not related or improbable: a clinical event including laboratory test abnormality with temporal relationship to intervention administration which makes a causal relationship incompatible or for which other treatments, chemicals or disease provide a plausible explanation. This will be counted as "unrelated" for notification purposes.
- Possible: a clinical event, including laboratory test abnormality, with temporal relationship to intervention administration which makes a causal relationship a reasonable possibility, but which could also be explained by other interventions, chemicals or concurrent disease. This will be counted as "related" for notification purposes.
- Probable: a clinical event, including laboratory test abnormality, with temporal relationship to intervention administration which makes a causal relationship a reasonable possibility, and is unlikely to be due to other interventions, chemicals or concurrent disease. This will be counted as "related" for notification purposes.
- Definite: a clinical event, including laboratory test abnormality, with temporal relationship to intervention administration which makes a causal relationship a reasonable possibility, and which can definitely not be attributed to other causes. This will be counted as "related" for notification purposes.

Appendix 7 ***Participant information leaflet***

Participant Information Sheet

Study title:

Effects of short-term energy restriction on liver lipid content, metabolism and inflammatory status in severely obese adults.

Draft Final version 1.2 **Date**: 8th June 2012

We would like to invite you to take part in our research study.

Before you decide, we would like you to understand why the research is being done and what it would involve for you.

This leaflet will tell you about the purpose of this study and what will happen to you if you take part. It includes detailed information about the conduct of the study.

Please contact the researcher, Emma Baldry, on 07527 427818 if anything is not clear or you have any questions. She will be happy to call you back to discuss the project.

Study outline:
The study is supported by Derby Hospitals, and is being run by the University of Nottingham.

It is working with patients, like you, who are having bariatric (weight loss) surgery. It will be looking at the effects of the low energy diet that is followed for two weeks before surgery.

While you are on the diet, we want to find out what happens to your liver and body fat. We also want to find out how your body responds in other ways.

At the moment this di*et al*lows you to eat normal meals, but restricts certain foods and limits portions. It is designed to be lower in energy than the body needs. It should help reduce the size of your liver, to make it easier for the surgeon to carry out the surgery.

We want to compare this diet, to one which replaces normal meals with ready-prepared products. Patients on this alternative diet will take meal replacement products such as soups, milkshakes and bars. We will then look at the differences in how the body responds to the two diets.

What is the purpose of the study?
For two weeks before bariatric surgery you will be following a low energy diet. This helps to reduce the size of your liver, to give the surgeon better access to complete the surgery. During this diet you will lose fat, which is stored in the liver. We want to know if losing this liver fat has any effects, particularly on how well the liver works.

Unfortunately, the pre-surgery diet is not always followed. Occasionally patients are not allowed to have surgery because their liver is too large. We want to find out if a meal replacement plan would help to improve the number of patients that stick to the diet. On this plan patients are provided with meal replacement products instead of taking normal foods. We will look at how many patients stick to each diet. We will also look to which diet is more effective at shrinking the liver and if they have any other effects.

Why have I been invited?
Any patient who has been accepted for bariatric surgery at Derby Hospitals could be eligible to take part in the study. You have been invited because you are on the waiting list for surgery. We are inviting 100 participants like you to take part.

Do I have to take part?
No. Taking part in the research is entirely voluntary.

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. *You are free to withdraw at any time, without giving a reason. This will not affect the standard of care you receive or your legal rights.*

What happens next?
When you have had a chance to read this leaflet about the study, you will be contacted by telephone. The researcher will ask if you have any questions or if there is anything you would like to discuss. They will ask if you are interested in taking part and explain the process of consent. Let them know if you do not want to take part and you will not be contacted again about this research.

What will happen to me if I take part?
You will continue to be cared for by the bariatric team at Derby Hospitals. You will have the same opportunities for appointments and follow-up as any other patient.

If you consent to take part, you will have closer monitoring than other patients. However, you will not have to attend any extra appointments. You will be seen by the bariatric team and follow the normal pre-operative process.

If you take part you will be told whether you will be following the normal diet or the meal replacement plan. This decision is random (**Randomised trial** below), and patients will not know who will be assigned to each diet.

At your pre-surgery appointment (about 3 weeks before the operation), the researcher will speak to you about consent. You will be asked to sign a consent form. If you have changed your mind and you do not want to take part, just let the researcher know. Your decision will not affect the care you receive.

If you consent to take part, an extra a fasting blood sample will be taken (**Fasting sample** below) when you have your normal blood tests taken.

Then before you start the pre-surgery diet, the researcher will contact you again to arrange a home visit at the best time for you. At this visit your weight and height will be checked, and any meal replacement products will be provided. *You do not have to buy any products (**Expenses** below).*

When you are on the diet (normal or meal replacement) you will be asked to fill out a food diary. This will help us to assess how much you have had to eat and drink, and we will analyse the results. *You will be provided with a food diary and given instructions on how to fill it out.*

On the day of your operation we will check your weight. We will also collect your food diary.

Then when you are ready for the operation the surgical team will take another fasting blood sample. This sample will be compared to the previous one.

The only other extra process will happen during the operation. The surgeon will take very small samples of your liver and fat (biopsies). It will be their decision to do this.

After the operation you will be followed-up in clinic, and will have the same post-operative care as other bariatric surgery patients.

Randomised trial *Sometimes we don't know which way of treating patients is best. To find out, we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to if one is better. To try to make sure the groups are the same to start with, each patient is put into a group by chance (randomly).*

Expenses *You will not be asked to attend extra clinics or appointments. All meal replacement products will be provided for you if they are needed. You should not incur any extra costs or expenses if you take part, and no payments will be made.*

Fasting sample *This means you will have to fast, and avoid eating or drinking for at least 10 hours before it's taken. But, you can drink water in this time.*

Summary of extra things that will happen if you take part:

What	When and where
Consent	Before you take part. After you have read the information and we have answered any questions. At the pre-surgery appointment.
Assigned to diet (normal or meal replacement)	After you consent. (A choice of products will be provided if you are on the meal replacement plan.)
Pre-diet – weight, height and blood sample	Fasting blood sample – at the pre-surgery appointment at hospital, when other blood samples are taken. Weight and height measured - at home. (Products will be supplied at this visit, if you are on the meal replacement plan.)
Food diary	To be filled out with food/drinks or meal replacement products as you take them. At home during the 2-week diet.
Post-diet – weight, tissue and blood samples	Weight – on the day of surgery at hospital. Fasting blood sample – just before the operation at hospital. Tissue samples - fat and liver biopsies taken at the time of the operation.

What are the alternative diets (treatments)?
You will be allocated to one of two groups.

- One group will follow the normal pre-surgery diet. If you are following this diet, you will only be allowed a certain range of foods/drinks. You will also need to weigh the foods to make sure you have the right portion sizes. The dietitians will have provided you with a copy of this diet and talked you through how to follow it.
- Group two will follow a meal replacement plan. If you are following this diet the researcher will contact you to ask you which products you would like. You will be given all the products you need and a plan of when to have them.

Could there be any side effects while on the diets?
When you are following the low energy diet or meal replacement plan you may experience some effects. These include:

- Changes in bowel habits such as constipation and flatulence
- Headaches, dizziness or fatigue
- Dry skin
- Sensitivity to cold
- Bad breath

If you suffer any symptoms and are concerned, please contact the researcher: Emma Baldry Tel: 07527 427818

What will happen to any samples I give?
The samples of blood and tissue that you provide will be labelled with your date of birth, initials and a unique number to identify the study. They will not be labelled with your name and will remain anonymous to anyone outside the study. They will be securely stored by the University of Nottingham at the Queens Medical Centre at Nottingham.

We would also like to k your consent for any remaining samples (after the tests for this study has been completed) to be stored and used in possible future research. *This is optional (please indicate you agree to this on the consent form).* The samples will be stored securely with a code unique to you at the University of Nottingham under the universities Human Tissue Research Licence (no 12265).

Some of these future studies may be carried out by researchers other than the team who run this study, including researchers working for commercial companies. Any samples or data used will be anonymised, and you will not be identified in anyway.

If you do not agree to this any remaining samples will be disposed of in accordance with the Human Tissue Authorities codes of practice.

What are the possible disadvantages and risks of taking part?
We will try to limit any inconvenience or disturbances. But, it is important that we keep in contact with you and arrange monitoring.

Fasting blood samples are part of the monitoring process, and we will try to minimise any discomfort or risk related to taking these. We have arranged them to be taken at the same time as your usual blood tests, so that you do not have to have any extra procedures. When you have your operation the surgeon will take small samples (biopsies) of liver and fat. Taking these samples is an extra procedure to the normal surgical process. There is a chance that the areas where the samples are taken may be sore, or bleed. The surgeon is aware of this and will try to minimise any discomfort and manage any risks. It will be the

surgeon's decision to take samples. If they do not feel it is essential they will not take them.

What are the possible benefits of taking part?
Overall, the study should help to identify which type of diet is of most benefit to patients undergoing weight loss surgery.

The results of the study may also help to show if there are benefits of following this type of short-term diet for a wider range of people, for example those trying to lose weight without surgery.

Also, as part of the monitoring process, we will inform the bariatric team of any of your results which may help your care. *However*, w*e cannot promise the study will help you personally.*

What will happen if I don't want to carry on with the study?
You can withdraw from the study at any time, without giving a reason. As your results will be important, we will still use the data collected up to the point where you withdraw.

If you consented to samples being held for further research within the research unit, any blood or tissue samples that can still be identified as yours will be stored. However we will destroy all your identifiable samples, if you wish.

Will any genetic tests be done?
No.

However we will be looking to see how your body responds to the low energy diet. We will look for changes in the amount of enzymes and other chemicals that regulate the body. As part of this we will check to see if the genes responsible for these chemicals have increased or decreased.

Will my taking part in the study be kept confidential?
Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected about you during the course of the research will be kept strictly confidential. It will be stored in a secure and locked office, and on a password

protected database. Any information about you which leaves the hospital will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it.

Your personal data (address, telephone number) will be kept for 1 year after the end of the study so that we are able to contact you about the findings of the study and possible follow-up studies (unless you advise us that you do not wish to be contacted). All other data (research data) will be kept securely for 7 years. After this time your data will be disposed of securely. During this time all precautions will be taken by all those involved to maintain your confidentiality. Only members of the research team will have access to your personal data.

Involvement of the General Practitioner/Family doctor (GP)

If you consent to take part in the study, you are allowing us to view your medical notes. We will also let your doctor know that you have agreed to take part, and will provide them with information about the study.

What if there is a problem? Or something goes wrong?

If you have a concern about any aspect of this study, you should ask to speak to the researcher who will do their best to answer your questions. The researcher's contact details are given at the start of this information sheet. If you remain unhappy please contact the Co-Investigator, Mr Paul Leeder. His contact details are at the end of this leaflet.

The local patient advice and liaison service (PALS) offers help, support and advice to patients, relatives or carers, about any issues relating to Derby Hospitals. You can contact them if you have any concerns that you do not wish to discuss with the research team, or for independent advice or support. They also provide information and support should you wish to complain formally.

PALS at Derby can be contacted by telephone or email:

Freephone: 0800 783 7691
pals@derbyhospitals.nhs.uk

What happens when the research study stops?

You will continue being cared for by the Bariatric surgery team. You will be offered appointments and will be followed-up the same as any other patient.

What will happen to the results of the research study?

Once all the samples have been analysed the results will be collated by the researcher. It is expected that the study will be completed by October 2014.

Full results will be made available to Derby Hospital NHS Foundation Trust. It is hoped that results will also be published in academic journals. The study detail and results will also be published as part of the evidence towards a postgraduate degree, for the researcher.

As a participant you can ask to receive a copy of the full results. *You will not be identified in any report/publication.*

Who is organising and funding the research?
The University of Nottingham is paying for the research. It is being partly funded by the Schools of Biomedical Sciences and Graduate Entry Medicine and Health. They are also supporting the study by providing laboratory space, training, and access to library and computer resources.

The meal replacement products, such as soups and milkshake mixes, have been provided free of charge by the company that distributes them.

None of the health professionals in the bariatric team that are looking after your care have been paid to recruit you. They will not receive any additional payments for providing your care. None of the research team will receive any additional payments for recruiting you.

Who has reviewed the study?
All research in the NHS is looked at by independent group of people to protect your interests, called a Research Ethics Committee. This study has been reviewed and given favourable opinion by the National Research Ethics Service - West Midlands.

Further information and contact details:
If you would like to speak to someone involved in organising this research project, you would like advice as to whether you should participate, or if you are unhappy with the study; please contact:

Mr Paul Leeder
Consultant General Surgeon
Royal Derby Hospital, Uttoxeter Road
Derby, DE22 3DT
Tel: 01332 789430

Appendix 8 ***Informed consent form***

CONSENT FORM

Title of Study: EnR-Lin REC ref:

Name of Researcher: _____

Name of Participant: _____ Please **initial** box:

1. I confirm that I have read and understand the information sheet dated..... for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐
3. I understand that relevant sections of my medical notes and data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐
4. I understand and agree to complete a food and drink diary for two weeks during the study, which will then be analysed. ☐
5. I understand and agree that blood samples will be taken on two occasions for analysis. ☐
6. I understand and agree to samples of liver and body fat being removed during the bariatric operation for analysis. ☐
7. I agree to my GP being informed of my participation in this study. ☐
8. I agree to take part in the above study. ☐

Optional:

I agree that samples can be held for future research at the Research Tissue Bank at the University of Nottingham. ☐

(Where participants do not agree to the future use of the samples they will be destroyed in accordance with the Human Tissue Act, 2006.)

Name of Participant	Date	Signature
Name of Chief Investigator	Date	Signature

Appendix 9 ***GP letter***

School of Biomedical Sciences
University of Nottingham
Medical School
Queens Medical Centre
Nottingham NG7 2UH

23 April 2017

Dear Dr

Re: EnR-Lin Research Study Participation

Patient Name:	**Address:**
NHS Number:	**Date of Birth:**

The above patient is under the care of the Bariatric Team at the Royal Derby Hospital.

They have consented to take part in a study which the University of Nottingham is running in conjunction with the hospital.

The study is recruiting from patients accepted for Bariatric surgery. It will examine the effects of the pre-operative low energy diet, on liver lipid, and associated effects on metabolism and inflammatory status.

The patient will continue to follow the same care pathway as any other Bariatric surgery patient.

Prior to their operation they will be randomised into one of two groups;
1. Following the standard pre-operative low energy diet,
2. Following a meal replacement plan.

They will have additional monitoring, which includes:

	Pre-intervention	Dietary Intervention 2-weeks	Post-intervention
Fasting blood samples and body weight	X		X
Food and fluid diary		X	
Liver biopsy			X
Subcutaneous and omental adipose biopsy			X

I have enclosed an information sheet which provides further details, but please do not hesitate to contact me if you would like further information.

Yours sincerely

Emma Baldry
Postgraduate Student

Enc Patient Information Leaflet

Appendix 10 ***Case Report Form***

Case Report Form

Informed consent	☐	Withdrawal	☐
Date	/ /	Date	/ /
		Reason:	

Inclusion criteria checked	☐	
	Patient accepted for Bariatric Surgery	
Exclusion criteria checked	☐	
	Secondary NAFLD (contributors to)	LighterLife exclusions:
	Anti-inflammatory medications	Cardiac conditions
	Insulin	Cerebrovascular disease
	GLP-1 agonist	Impaired Mental health
	Pioglitazone	Epilepsy
	Pregnancy/lactation	Kidney disease
	Excess alcohol (Protocol Appendix 5)	Liver disease
		Total lactose intolerance
		Type 1 Diabetes
		Eating disorder
		Porphyria
		Anticoagulant therapy
Participant demographics	Age at initial consent	
	Gender	Male/Female
Ethnicity	Unknown/prefer not to answer	White
	Other	Mixed
		Asian or Asian British
		Black or Black British
Diet history	Any alcohol intake? Yes/No	Details:
Mobility/Physical Activity	Mobile - With/without assistance	Details:
	PA levels – regular activity?	Time/frequency
	PAL estimate	

Weight history	Overweight as child?	Yes/No	Number of years overweight		
	Weight change - last 18 months	Date		Weight	
		Date		Weight	
		Date		Weight	

Medical history	DM	☐
	IGT	☐
	No record of DM or IGT	☐

	DM Medication	☐ List below	
	Other Medication	☐ List below	
Allergy/ Intolerance	Any allergies or intolerances?	Yes/No	
	Milk ☐	Other ☐ List below	
Intervention	Meal Replacement ☐	Standard Diet ☐	
	Food & fluid diary received ☐		
Anthropometrics	Pre-intervention weight	Date / /	Weight
	Post-intervention weight	Date / /	Weight
	Height	Date / /	Height
	Pre-intervention blood test	Date / /	
Laboratory data	Pre-surgical routine blood test	Date / /	Copy enclosed ☐
	Post-intervention blood test	Date / /	
Tissue sampling		Date / /	
	Subcutaneous adipose biopsy ☐ Omental adipose biopsy ☐	Liver biopsy ☐ If not reason:	
Adverse events	Reported by participant	Date / /	
	Report to Chief Investigator	Date / /	
	Report to REC/sponsor (if applicable)	Date / /	

Appendix 11 ***Diet Evaluation Questionnaire***

Please draw a circle to answer each question. Use the number scale to show how you feel about each topic by grading each answer. Please circle only one number per question.

The diet was easy to follow:	Complicated	1	2	3	4	Easy
I enjoyed the foods/drinks taken:	Not at all	1	2	3	4	Very much
I had regular food and/or drink:	Never	1	2	3	4	Always
I felt hungry:	Never	1	2	3	4	Always
The diet was a positive experience:	Not at all	1	2	3	4	Very much
While on the diet I noticed changes in my weight or shape:	Not at all	1	2	3	4	Very much
While on the diet I noticed changes in my health/wellbeing:						
Constipation	None	1	2	3	4	Many times
Bad breath	None	1	2	3	4	Many times
Feeling cold	None	1	2	3	4	Many times
Feeling energised	None	1	2	3	4	Many times
Feeling tired	None	1	2	3	4	Many times
Mood swings	None	1	2	3	4	Many times
Overall satisfaction of the diet:	Very Dis-Satisfied	1	2	3	4	Very Satisfied

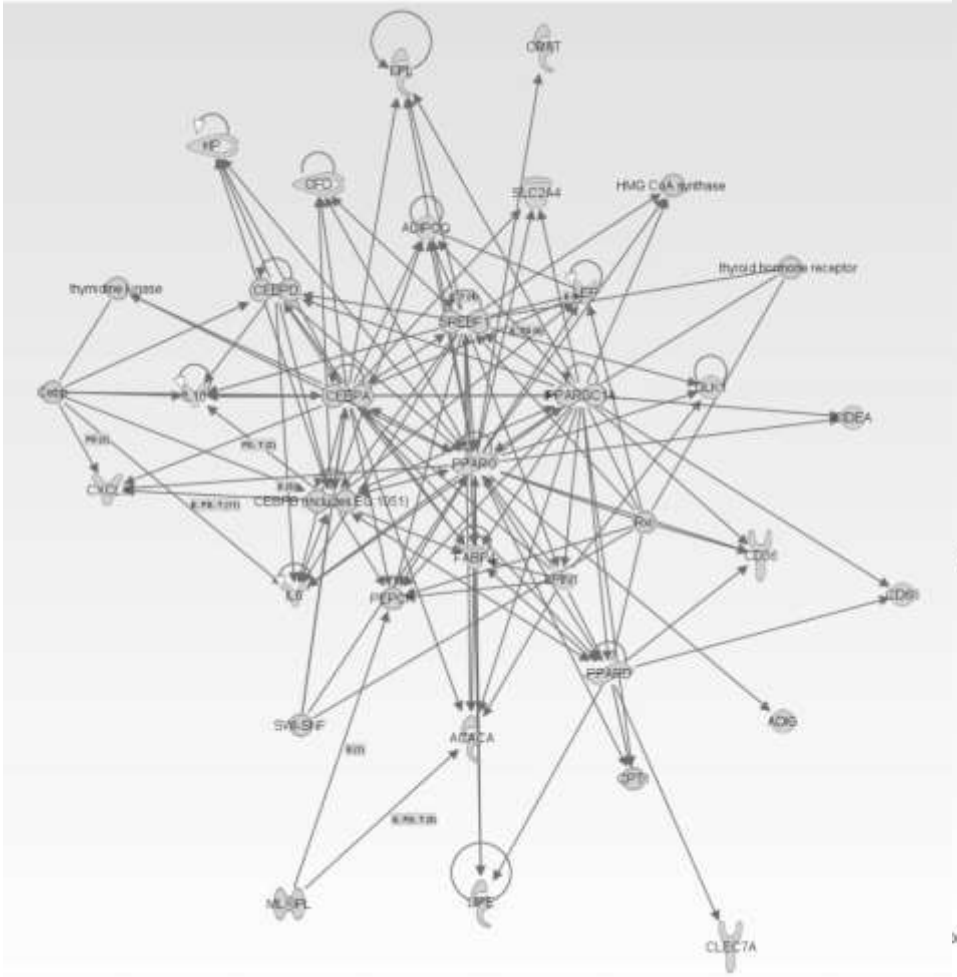
Appendix 12 ***Gene expression targets***

Figure 33 adipose tissue targets for investigation within wider networks

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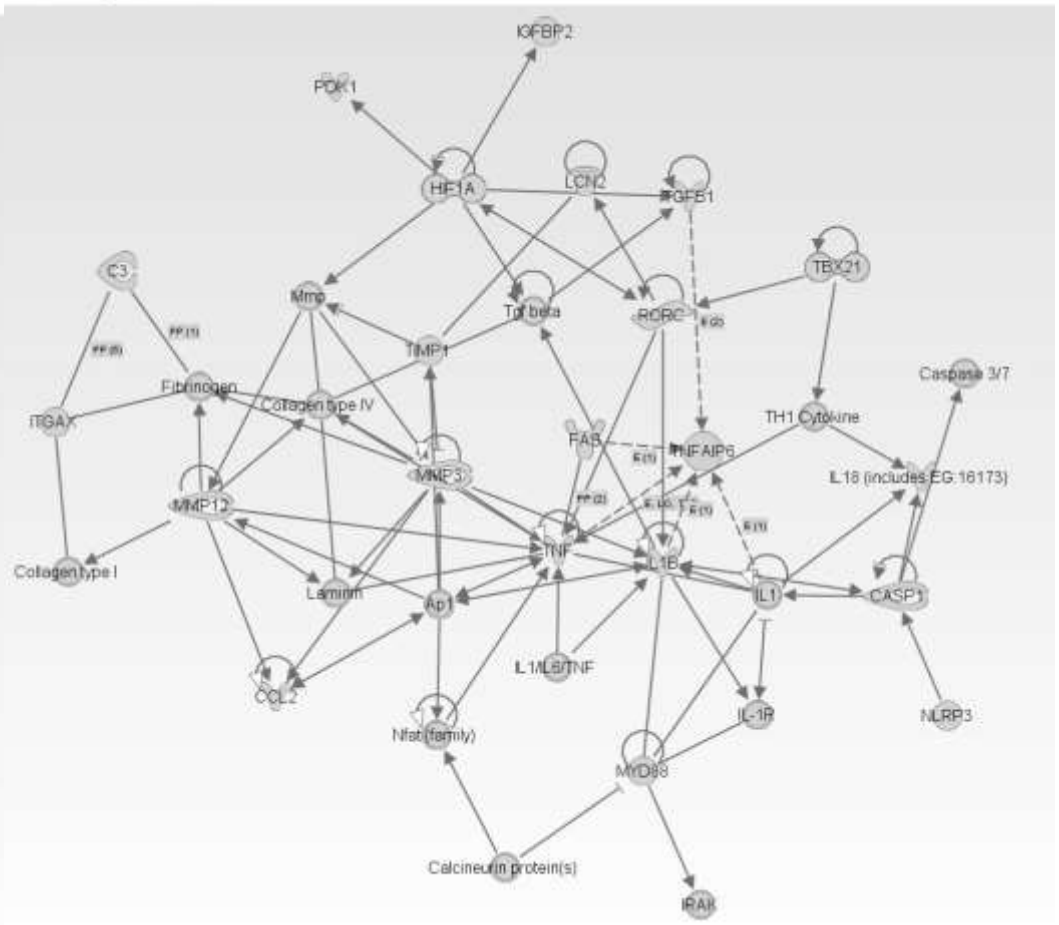
Network ID	Focus Molecules	Molecules in Network	Top Functions
1	27	ACACA,ADIG,ADIPOQ,CD36,CD68,Cebp,CEBPA,CEBPB (includes EG:1051),CEBPD,CFD,CIDEA,CLEC7A,CPT1,CRAT,DLK1,FABP4,HMG CoA synthase,HP,IL6,IL8, IL10,LEP,LIPE,LPIN1,LPL,MLXIPL, PEPCK,PPARD,PPARG,PPARGC1A,Rxr,SREBF1,SWI-SNF,TH2 Cytokine,thymidine kinase,thyroid hormone receptor	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
2	20	Ap1,Calcineurin protein(s),CASP1,Caspase 3/7,CCL2,Collagen type I,Collagen type IV, C3, FAS, Fibrinogen,HIF1A,IGFBP2,IL1,IL-1R,IL1/IL6/TNF,IL18 (includes EG:16173),IL1B,IRAK,ITGAX,Laminin,LCN2,MMP3,MMP12,Mmp,MYD88,Nfat (family),NLRP3,PDK1,Pro-inflammatory Cytokine,RORC,SAA,TBX21,Tgf beta,TGFB1,TH1 Cytokine,TIMP1,TNF,TNFAIP6	Infectious Disease, Inflammatory Disease, Neurological Disease
3	20	Akt,ATF6, ATG5,BCL2,BECN1,CASP3,CASP7,caspase,Cbp/p300,CD3E,Creb,DDIT3,estrogen receptor,Focal adhesion kinase,FOXO1,GATA3,GST,HERPUD1,IRS1,IRS2,IRS,IRS4 (includes EG:16370),JAK,NCK,NFE2L2,P38 MAPK,p85 (pik3r),PI3K (complex),PLIN1, PNPLA2,PP2A,RNA polymerase II,SLC2A4, SOCS3,SOD1,SOD2,Sod,STAT5a/b	Cell Death and Survival, Cell Morphology, Cellular Function and Maintenance
4	14	15-LOX,AAMP,AGPAT6,ALOX15,ALOXE3,ATG7,CEP120,CES1, CLN6 (includes EG:315746),DERL2,EDEM2,FAM65A,FUNDC1,GPX8,GSTK1,GSTO2,HSD17B11,IRAK1,IRAK3,LPCAT1,LPCAT2,MAP1LC3A,MAP1LC3B,MX2,NAMPT,NFkB (complex),NSA2,OGDHL,PNPLA3,PNPLA8,RAB3GAP2,SIRT1,SLFN5,STAT3,TRIM14,UBC	Lipid Metabolism, Small Molecule Biochemistry, Cell Morphology

Adipose Path Designer Network 1



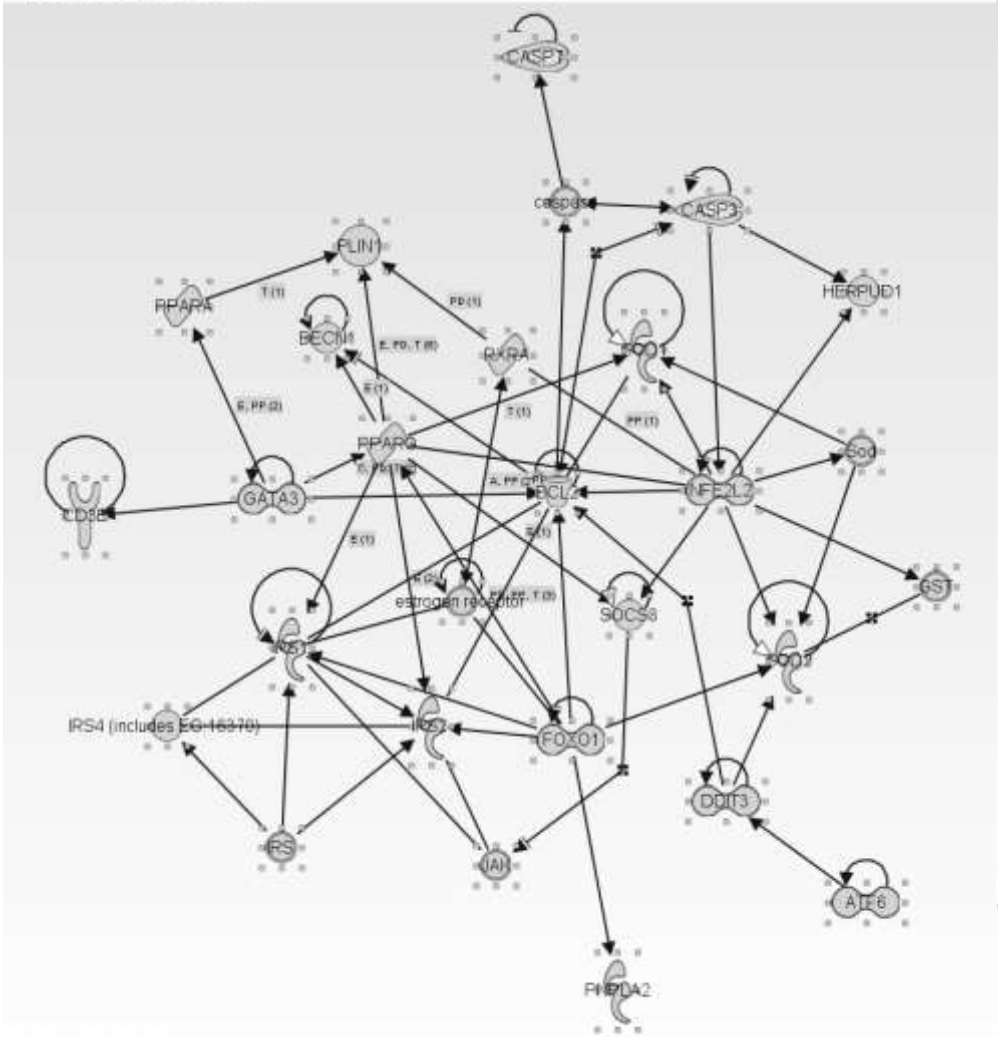
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Adipose Path Designer Network 2



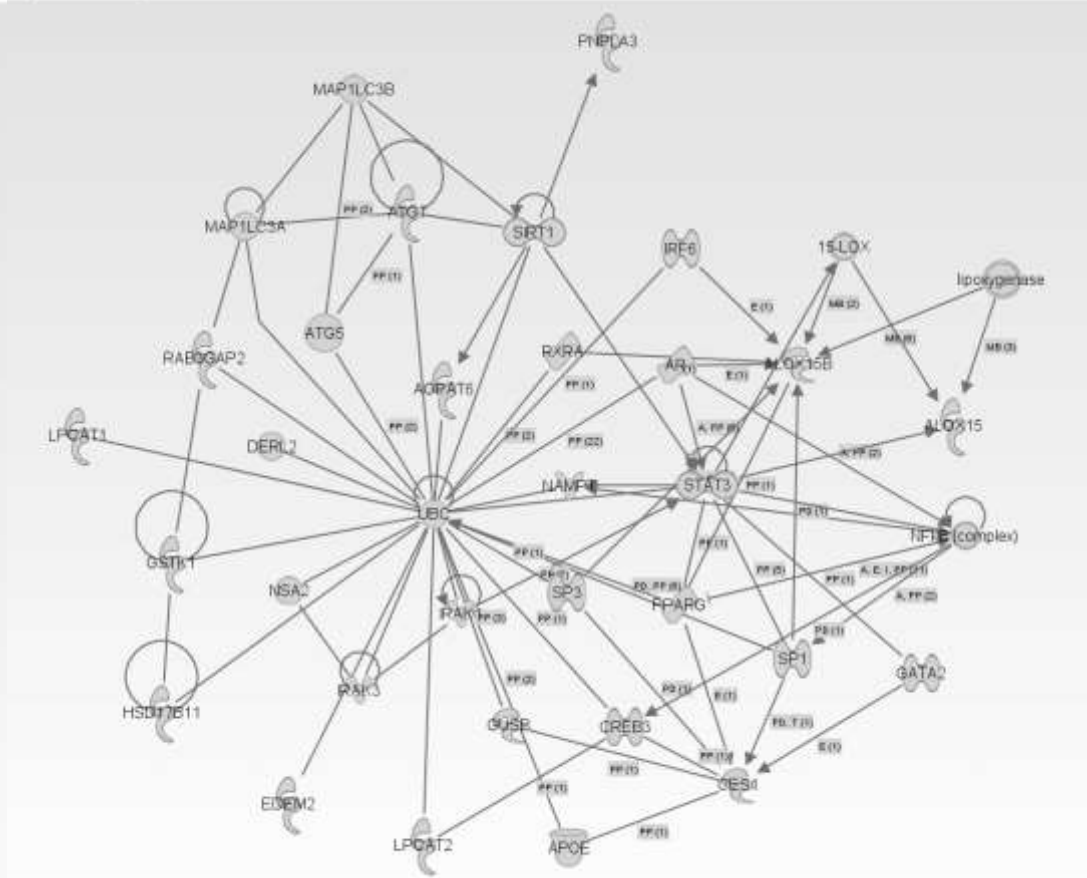
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Adipose Path Designer Network 3



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Adipose Path Designer Network 4



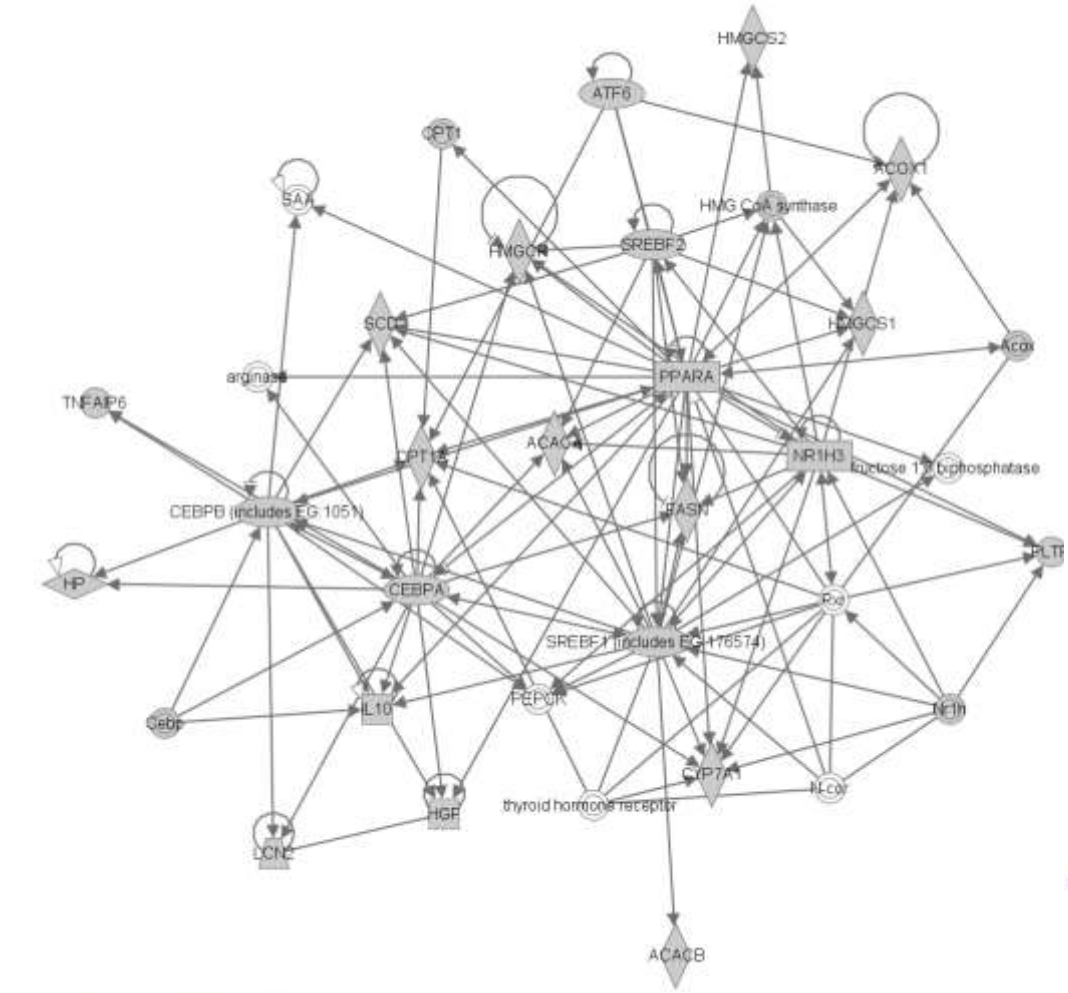
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Figure 34 liver tissue targets for investigation within wider networks.

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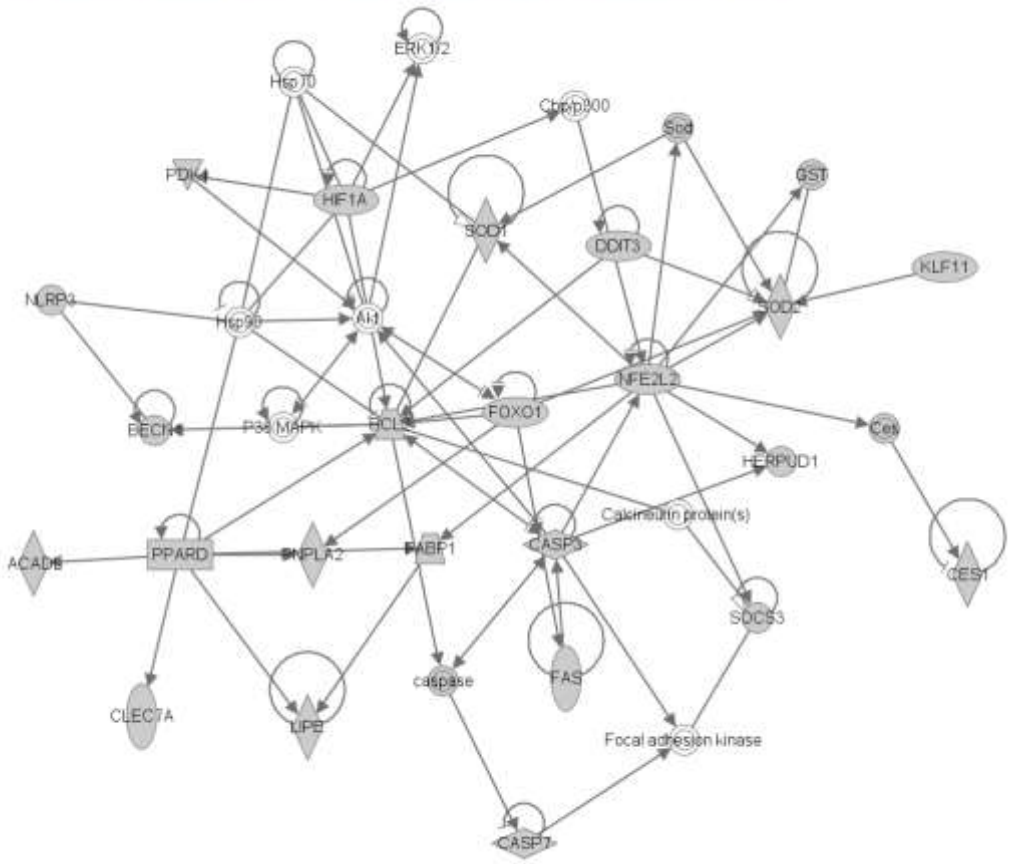
Network ID	Focus Molecules	Molecules in Network	Top Functions
1	23	ACACA,ACACB,ACOX1,Acox,arginase,ATF6,Cebp,CEBPA,CEBPB (includes EG:1051),CPT1,CPT1A,CYP7A1,FASN,fructose 1,6 biphosphatase,HGF,HMG CoA synthase,HMGCR,HMGCS1,HMGCS2,HP,IL10,LCN2,N-cor,NR1H3,Nr1h,PEPCK,PLTP,PPARA,Rxr,SAA,SCD,SREBF2,SREBF1 (includes EG:176574),thyroid hormone receptor,TNFAIP6	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
2	23	ACADL,Akt,BCL2,BECN1,Calcineurin protein(s),CASP3,CASP7,caspase,Cbp/p300,CES1,Ces,CLEC7A,DDIT3,ERK1/2,FABP1,FAS,Focal adhesion	
3	20	kinase,FOXO1,GST,HERPUD1,HIF1A,Hsp70,Hsp90,KLF11,LIPE,NFE2L2,NLRP3,P38 MAPK,PDK1,PNPLA2,PPARD,SOCS3,SOD1,SOD2,Sod	Cell Death, Tumour Morphology, Cell Morphology
4	18	Actin,ALOX15,ATG7,BDH1 (includes EG:100037356),C11orf73,CD36,CHIC1,CRAT,Creb,CYP2C8,CYP2C9,CYP2J2,DERL2,EDEM2,FLT1,GNPAT,GSTK1,Histone h3,Histone h4,HNF4A,LDLR,LPCAT1,LPCAT2,LPCAT3,MAP1B,MAP1LC3A,MAP1LC3B,MTRF1L,PEMT,PI3K (complex),PNPLA3,RPS19BP1,SIRT1,TP53 (includes EG:22059),UBCAp1,Collagen type IV,DLK1,Fibrin,Fibrinogen,IGF1,Igf,IGFBP2,IGFBP3,Igfbp,IGFBP1 (includes EG:16006),IL1,IL6,IL-1R,IL1/IL6/TNF,IL18 (includes EG:16173),IL1B,IRAK1,IRAK3,IRAK,Laminin,MMP3,MMP12,Mmp,MYD88,NAMPT,NFkB (complex),Pro-inflammatory Cytokine,PTGS2,RNA polymerase II,STAT5a/b,Tgf beta,TGFB1 (includes EG:21803),TIMP1,TNF	Lipid Metabolism, Small Molecule Biochemistry, Cardiovascular System Development and Function Cellular Development, Cellular Growth and Proliferation, Skeletal and Muscular System Development and Function

Network 1 : EnR-Lin - Liver tissue direct - 2012-07-23 : EnR-Lin - Liver tissue : EnR-Lin - Liver tissue direct - 2012-07-23

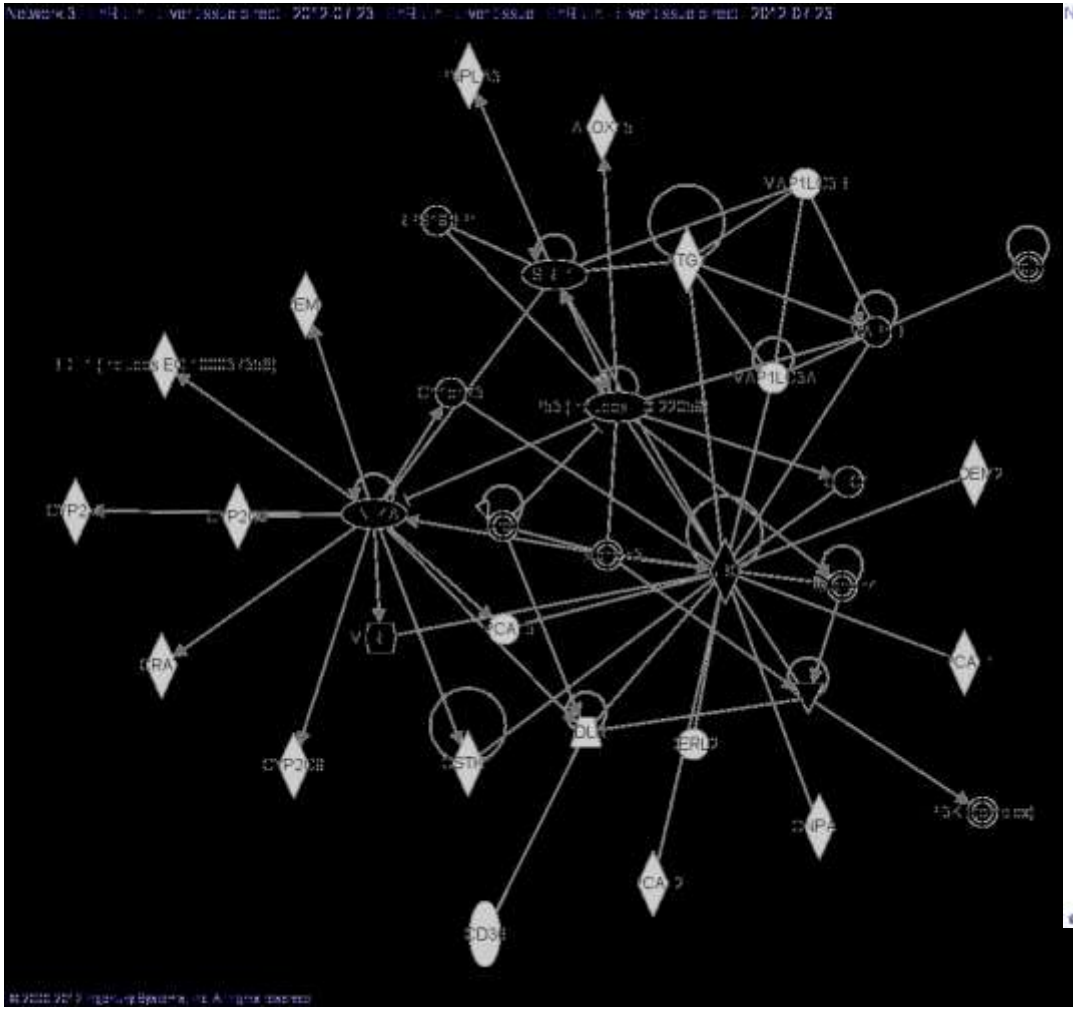


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Network 2 : EnR-Lin - Liver tissue direct - 2012-07-23 : EnR-Lin - Liver tissue : EnR-Lin - Liver tissue direct - 2012-07-23



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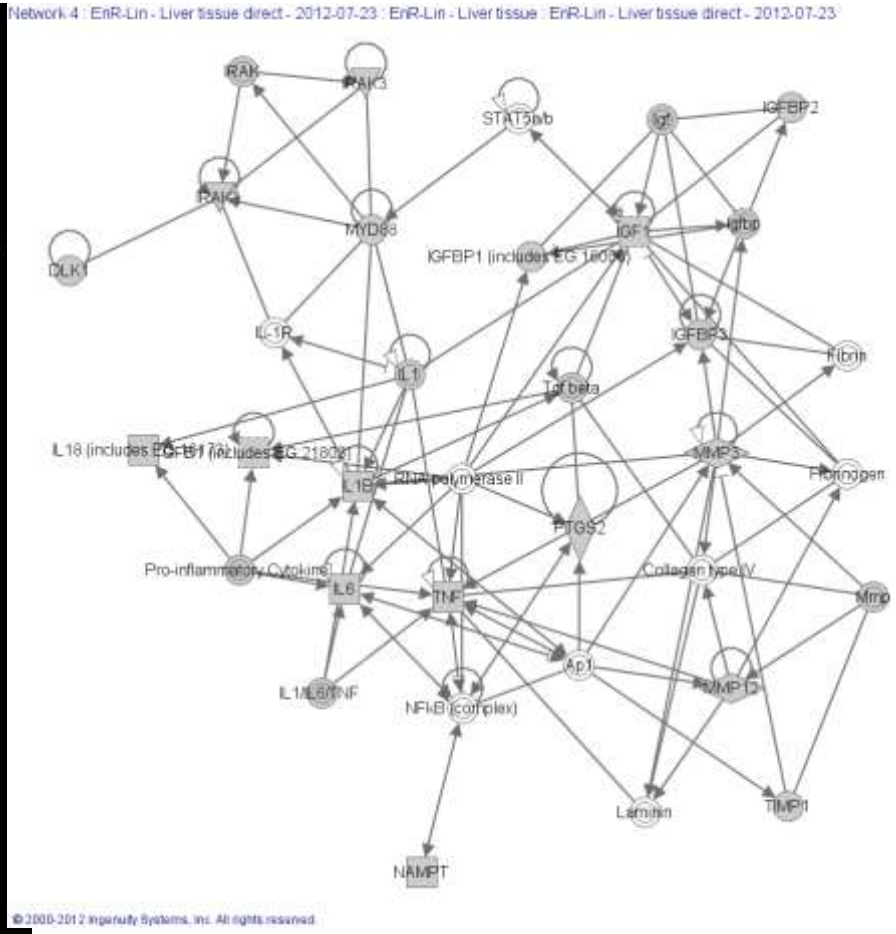

Network 4 : EnR-Lin - Liver tissue direct - 2012-07-23 : EnR-Lin - Liver tissue : EnR-Lin - Liver tissue direct - 2012-07-23
RAK
PAK3
STAT5a/b
Igf
IGFBP2
PAK2
MYD88
IGF1
Igfbp
DLK1
IGFBP1 (includes EG:1608)
IL-1R
IGFBP3
Fibrin
IL1
Tgfbeta
IL18 (includes EG:3606)
MMP3
Fibrinogen
IL1B
RNA polymerase II
PTGS2
Pro-inflammatory Cytokine
IL6
TNF
Collagen type IV
Mmp
Ap1
IL1/IL6/TNF
NFkB (complex)
TIMP3
Laminin
TIMP1
NAMPT
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Table 43 Gene array card layout for liver tissue

	Applied Biosystems, Inc. (http://www.appliedbiosystems.com)					
	Taqman Low Density Array configuration: 7				4342259	
	TLDA Production Number:				1719881	
	Card Name:				EnR-Lin Liver(1)(1)	
	Assay guide:					
	1	2	3	4	5	6
a	Hs01046047_m1	Hs00153988_m1	Hs00609608_m1	Hs00232586_m1	Hs00169468_m1	Hs00197348_m1
b	Hs00236330_m1	Hs01054576_m1	Hs00153153_m1	Hs00167151_m1	Hs00961620_g1	Hs00174097_m1
c	Hs00228747_m1	Hs00987011_m1	Hs02330328_s1	Hs00916176_m1	Hs00167309_m1	Hs01088691_m1
d	Hs00918082_m1	Hs00172885_m1	Hs00187067_m1	Hs01561850_m1	Hs00540979_m1	Hs01067287_m1
	7	8	9	10	11	12
a	Hs00608023_m1	Hs00186838_m1	Hs01100879_m1	Hs00354836_m1	Hs99999905_m1	Hs00234387_m1
b	Hs01038788_m1	Hs00174131_m1	Hs01567912_g1	Hs00155570_m1	Hs00200502_m1	Hs00194353_m1
c	Hs00998130_m1	Hs99999139_m1	Hs00174128_m1	Hs01113602_m1	Hs00380882_m1	Hs00605917_m1
d	Hs00386101_m1	Hs00228747_m1	Hs00276880_s1	Hs00231882_m1	Hs00987011_m1	Hs00211693_m1
	13	14	15	16	17	18
a	Hs00169152_m1	Hs00354519_m1	Hs00269972_s1	Hs00270923_s1	Hs00275607_m1	Hs00224028_m1
b	Hs00227357_m1	Hs00215225_m1	Hs00738808_m1	Hs00797944_s1	Hs00899662_m1	Hs00968305_m1
c	Hs00234140_m1	Hs00154355_m1	Hs00270931_s1	Hs00157263_m1	Hs00154455_m1	Hs01086177_m1
d	Hs01573471_m1	Hs01682761_m1	Hs02330328_s1	Hs00916176_m1	Hs00167309_m1	Hs01088691_m1
	15	16	17	18	19	20
a	Hs00912963_m1	Hs01090850_m1	Hs00211351_m1	Hs00171584_m1	Hs00217168_m1	Hs00157403_m1
b	Hs00182082_m1	Hs00237184_m1	Hs00232352_m1	Hs00918082_m1	Hs01561850_m1	Hs00386101_m1
c	Hs00699125_m1	Hs00178563_m1	Hs00275843_s1	Hs00269220_s1	Hs00174217_m1	Hs00174877_m1
d	Hs01081784_m1	Hs00998130_m1	Hs99999139_m1	Hs01113602_m1	Hs00174128_m1	

Liver card

	Gene symbols:					
	1	2	3	4	5	6
a	ACACA	ALOX15B	ALOX15	ATF6	ATG5	ATG7
b	FAS	FOXO1	HIF1A	IGFBP2	IL10	IL1B
c	PNPLA3	PPARD	SOCS3	SOD1	SOD2	SREBF1
d	NLRP3	NR1H3	NR5A2	PDK1	PEMT	PLTP
	7	8	9	10	11	12
a	BCL2	BECN1	C3	CASP1	GAPDH	CASP3
b	IL18	IL6	IL8	IRAK1	IRAK3	LCN2
c	TGFB1	TIMP1	TNF	TNFAIP6	ADIG	ADIPOQ
d	PNPLA2	PNPLA3	POLDIP3	PPARA	PPARD	PPME1
	13	14	15	16	17	18
a	CASP7	CD36	CEBPA	CEBPB	CES1	CLEC7A
b	LPCAT1	LPCAT2	MAP1LC3A	MAP1LC3B	MMP12	MMP3
c	CCL2	CD68	CEBPD	CFD	CIDEA	FABP4
d	PTGS2	SCD	SOCS3	SOD1	SOD2	SREBF1
	15	16	17	18	19	20
a	CRAT	DDIT3	DERL2	DLK1	EDEM2	EPHX2
b	MYD88	NAMPT	NFE2L2	NLRP3	PDK1	PNPLA2
c	FAM101A;ZNF664-FAM101A	IRS1	IRS2	IRS4	ITGAX	LEP
d	SREBF2	TGFB1	TIMP1	TNFAIP6	TNF	

Table 44 Gene array card layout for adipose tissue

	Applied Biosystems, Inc. (http://www.appliedbiosystems.com)					
	Taqman Low Density Array configuration: 7				4342259	
	TLDA Production Number:				1719882	
	Card Name:				EnR-Lin Adipose(1)(1)	
	Assay guide:					
	1	2	3	4	5	6
a	Hs01046047_m1	Hs00153988_m1	Hs00609608_m1	Hs00232586_m1	Hs00169468_m1	Hs00197348_m1
b	Hs00236330_m1	Hs01054576_m1	Hs00153153_m1	Hs00167151_m1	Hs00961620_g1	Hs00174097_m1
c	Hs00228747_m1	Hs00987011_m1	Hs02330328_s1	Hs00916176_m1	Hs00167309_m1	Hs01088691_m1
d	Hs00299515_m1	Hs00173425_m1	Hs00159345_m1	Hs00160173_m1	Hs01115513_m1	Hs01016724_m1
	7	8	9	10	11	12
a	Hs00608023_m1	Hs00186838_m1	Hs01100879_m1	Hs00354836_m1	Hs99999905_m1	Hs00234387_m1
b	Hs01038788_m1	Hs00174131_m1	Hs01567912_g1	Hs00155570_m1	Hs00200502_m1	Hs00194353_m1
c	Hs00998130_m1	Hs99999139_m1	Hs00174128_m1	Hs01113602_m1	Hs00380882_m1	Hs00605917_m1
d	Hs00220767_m1	Hs00381858_m1	Hs00168966_m1	Hs99999153_m1	Hs00922328_m1	Hs00210861_m1
	13	14	15	16	17	18
a	Hs00169152_m1	Hs00354519_m1	Hs00269972_s1	Hs00270923_s1	Hs00275607_m1	Hs00224028_m1
b	Hs00227357_m1	Hs00215225_m1	Hs00738808_m1	Hs00797944_s1	Hs00899662_m1	Hs00968305_m1
c	Hs00234140_m1	Hs00154355_m1	Hs00270931_s1	Hs00157263_m1	Hs00154455_m1	Hs01086177_m1
d	Hs00206652_m1	Hs00978377_m1	Hs00943410_m1	Hs01076122_m1	Hs00894392_m1	
	15	16	17	18	19	20
a	Hs00912963_m1	Hs01090850_m1	Hs00211351_m1	Hs00171584_m1	Hs00217168_m1	Hs00157403_m1
b	Hs00182082_m1	Hs00237184_m1	Hs00232352_m1	Hs00918082_m1	Hs01561850_m1	Hs00386101_m1
c	Hs00699125_m1	Hs00178563_m1	Hs00275843_s1	Hs00269220_s1	Hs00174217_m1	Hs00174877_m1
d						

Adipose card

	Gene symbols:					
a	1	2	3	4	5	6
b	ACACA	ACACB	ACADL	ACOX1	ALOX15B	ALOX15
c	CYP2C9	CYP2J2	CYP7A1	DDIT3	DERL2	DLK1
d	IGFBP2	IGFBP3	IL1B	IL10	IL18	IL6
	LPIN1	LPL	MTX1	PLIN1	PPARG	PPARGC1A
	7	8	9	10	11	12
a	ATF6	ATG5	ATG7	BAAT	GAPDH	BCL2
b	DNAJB12	EDEM2	EPHX2	FABP1	FASN	FAS
c	IL8	IRAK1	IRAK3	KLF11	LCN2	LDLR
d	RETN	RPUSD3	SLC2A4	CD3E	GATA3	GSTK1
	13	14	15	16	17	18
a	BDH1	BECN1	CASP3	CASP7	CD36	CEBPA
b	FOXO1	GNPAT	GSTK1	HGF	HIF1A	HMGCR
c	LIPC	LIPE	LPCAT1	LPCAT2	LPCAT3	MAP1LC3A
d	HERPUD1	HP	LIPE	RORC	TBX21	
	15	16	17	18	19	20
a	CEBPB	CES1	CRAT	CPT1A	CLEC7A	CYP2C8;CYP2C19
b	HMGCS1	HMGCS2	HERPUD1	HP	IGF1	IGFBP1
c	MAP1LC3B	MMP12	MMP3	MYD88	NAMPT	NFE2L2
d						

Figure 35 gene expression levels for Fetuin-A (AHSG) in different tissues

Image taken from Genevestigator for data search of anatomical parts with relative gene expression levels (https://www.genevestigator.com). (accessed 4/4/14)

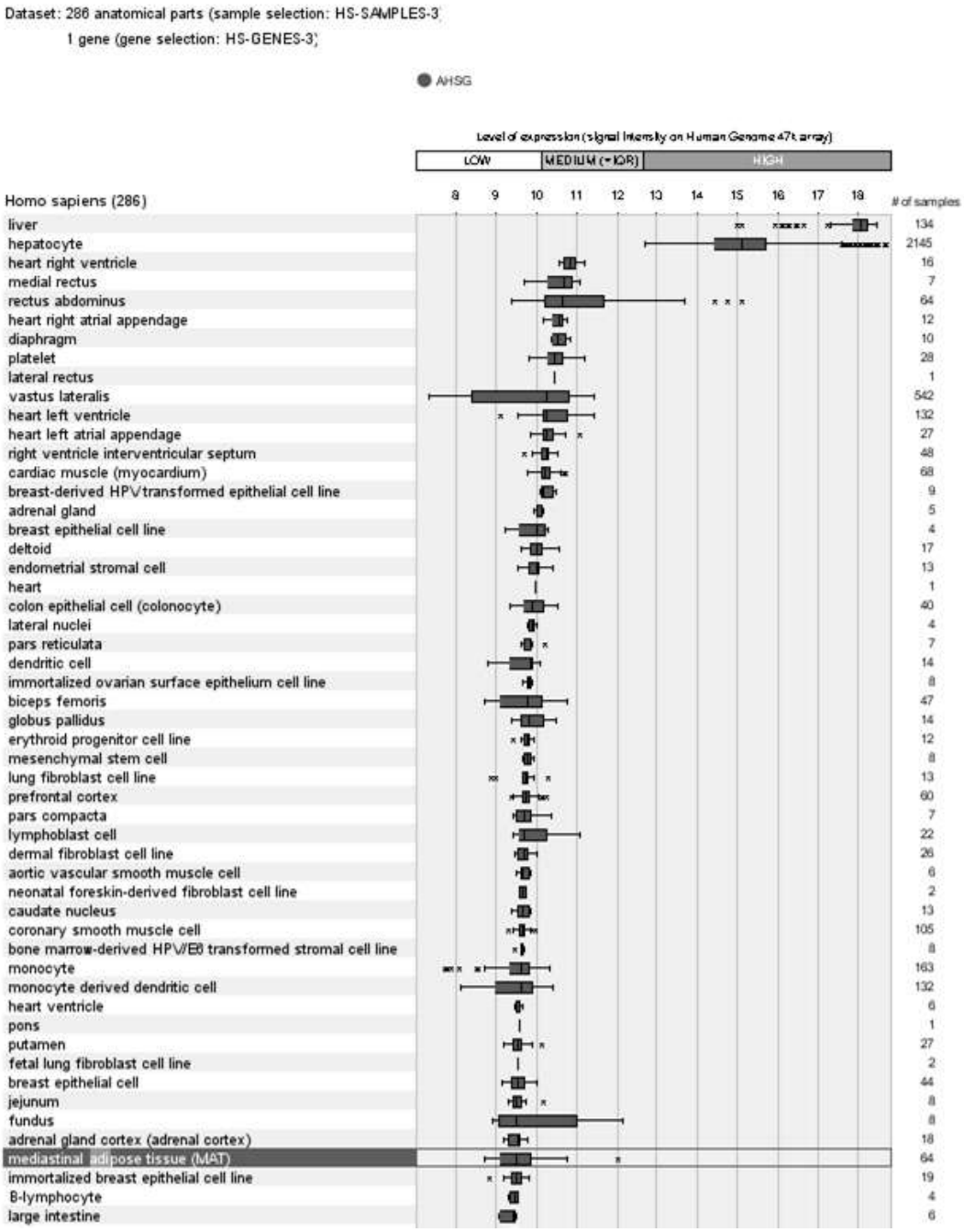


Figure 36 gene expression levels for GAPDH in different tissues

Image taken from Genevestigator for data search of anatomical parts with relative gene expression levels (https://www.genevestigator.com). (accessed 4/4/14)

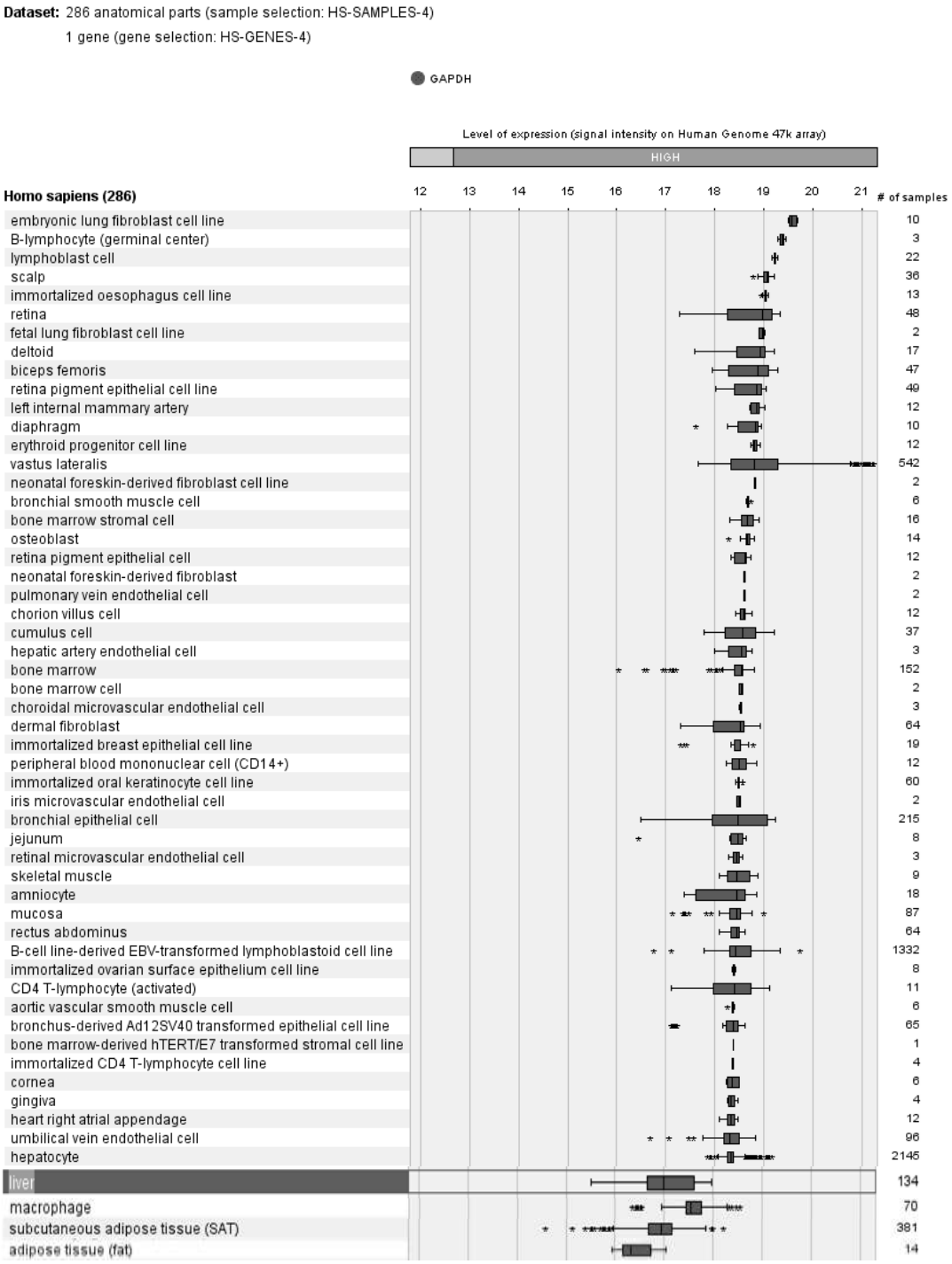


Table 45 housekeeper gene correlations in liver tissue (pooled diets).

Spearman Correlation (1-tailed)		GAPDH	DNAJB12/ MTX	PPME/ RPUSD3
GAPDH	cc	1.000	.957**	.814**
	p		<0.001	<0.001
DNAJB12/ MTX	cc	.957**	1.000	.826**
	p	<0.001		<0.001
PPME/ RPUSD3	cc	.814**	.826**	1.000
	p	<0.001	<0.001	

cc – correlation coefficient, p – significance 1-tailed

**. Correlation is significant at the 0.01 level

Correlation matrix for liver tissue housekeepers.

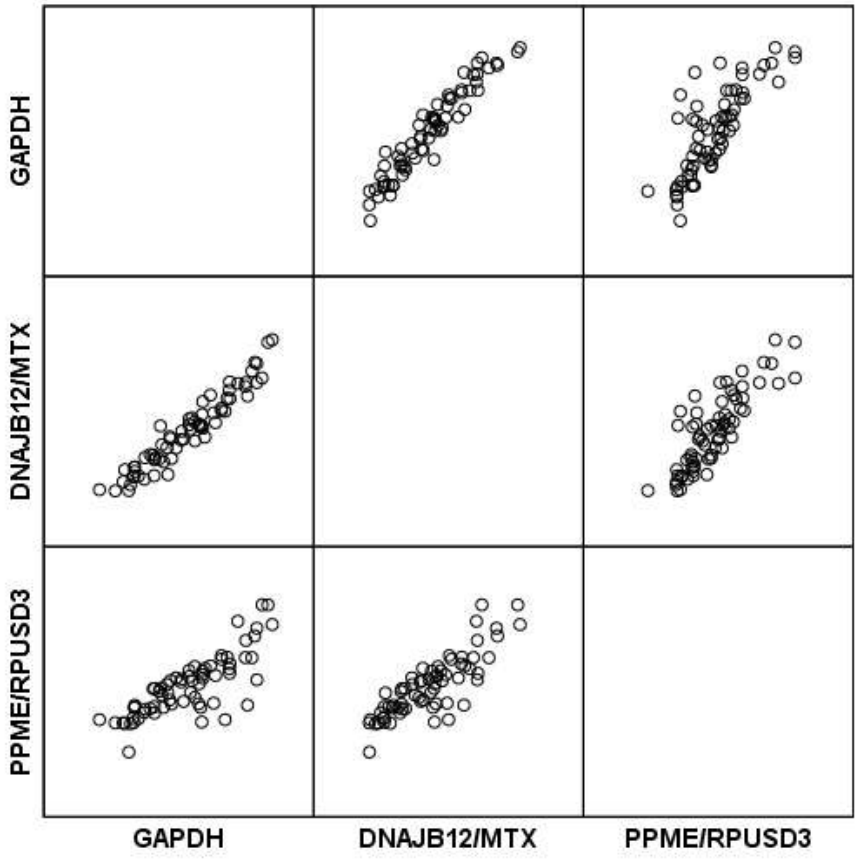


Table 46 housekeeper gene correlations for adipose tissue

Spearman Correlation (1-tailed)		MTX oa	GAPDH oa	RPUSD oa	MTX sa	GAPDH sa	RPUSD sa
MTX oa	cc	1	0.833**	0.833**	-0.040	-0.056	0.011
	p		<0.001	<0.001	0.387	0.343	0.469
GAPDH oa	cc	0.833**	1	0.721**	0.093	0.033	0.162
	p	<0.001		<0.001	0.252	0.405	0.121
RPUSD oa	cc	0.833**	0.721**	1	-0.128	-0.103	-0.053
	p	<0.001	<0.001		0.178	0.230	0.351
MTX sa	cc	-0.040	0.093	-0.128	1	0.919**	0.920**
	p	0.387	0.252	0.178		<0.001	<0.001
GAPDH sa	cc	-0.056	0.033	-0.103	0.919**	1	0.856**
	p	0.343	0.4.5	0.230	<0.001		<0.001
RPUSD sa	cc	0.011	0.162	-0.053	0.920**	0.856**	1
	p	0.469	0.121	0.351	<0.001	<0.001	

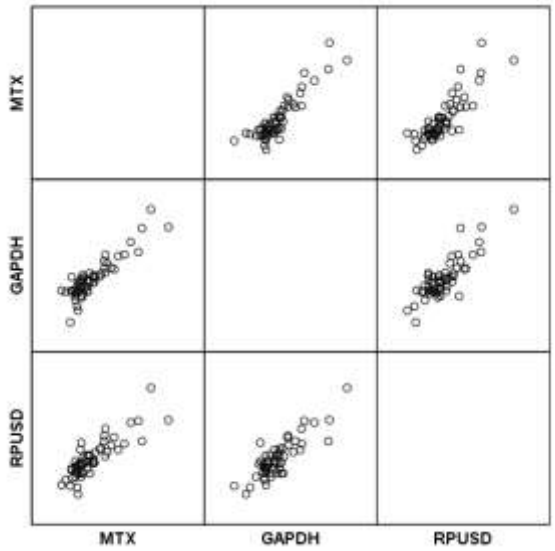
cc – correlation coefficient, P – significance 1-tailed

**. Correlation is significant at the 0.01 level

sa = subcutaneous, oa = omental

Correlation matrix for subcutaneous and omental adipose tissue housekeepers.

Omental



Subcutaneous

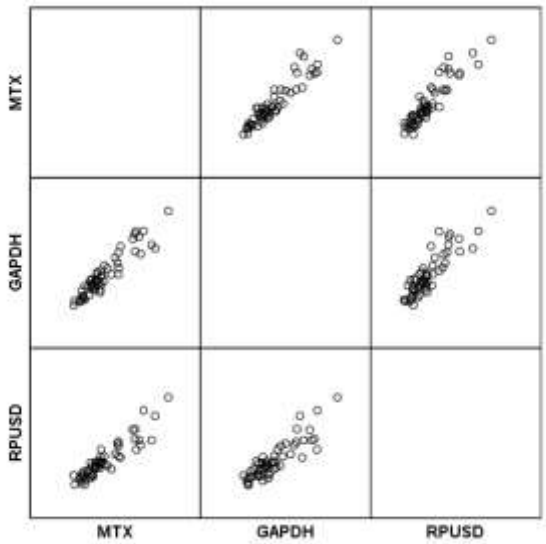


Figure 37 summary of expression fold-difference comparisons between dietary interventions, for both adipose tissue depots.

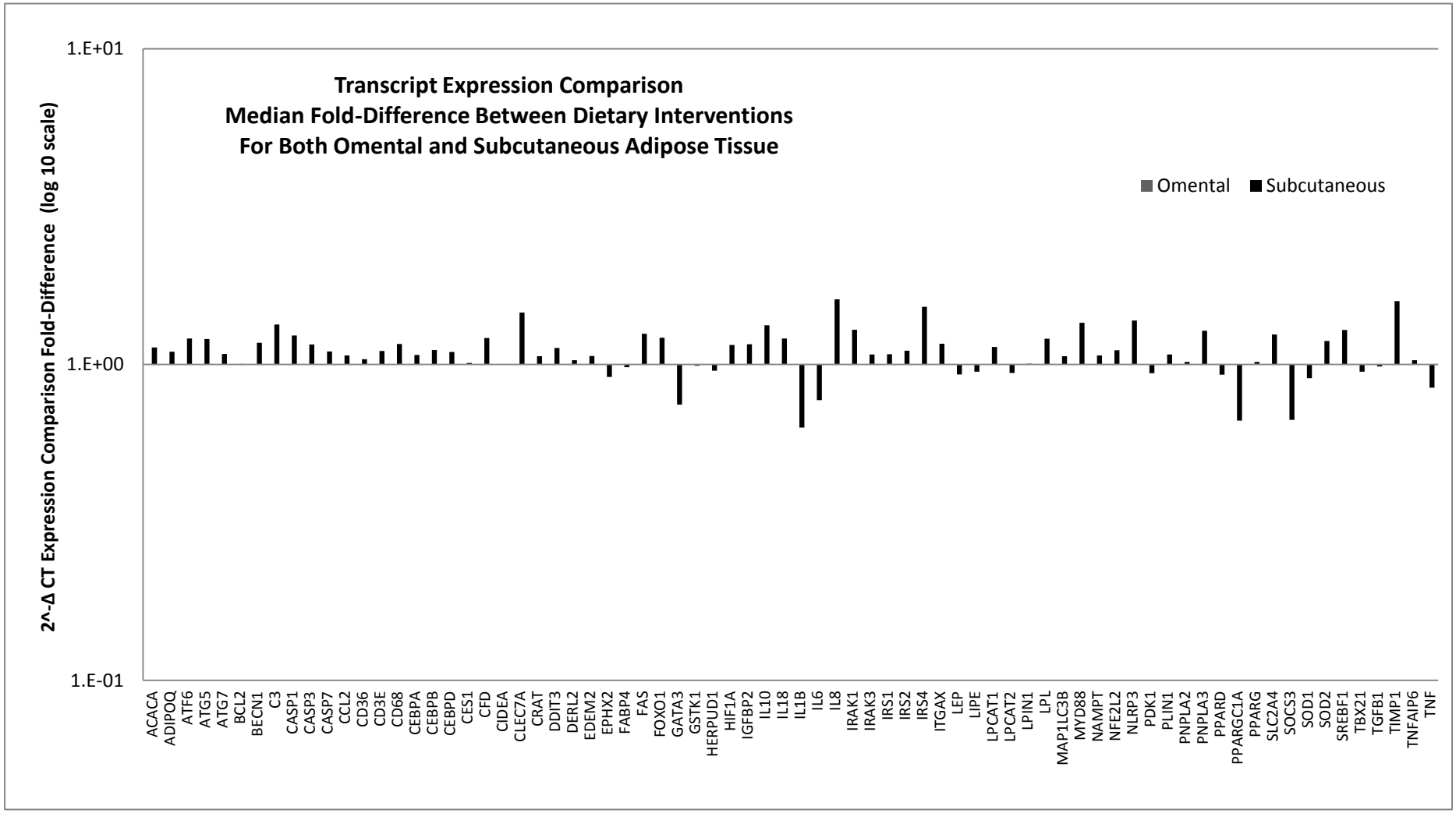


Table 47 expression levels ΔCT median and range for adipose tissue transcripts

	ACACAo	ACACAs	ADIPOQo	ADIPOQs	ALOX15Bs	ALOX15o	ATF6o	ATF6s	ATG5o	ATG5s
Median	0.77	0.74	-5.57	-6.07	4.48	0.46	-0.20	-0.57	3.36	4.06
Range	2.94	4.14	4.74	2.97	7.65	15.31	2.50	6.09	5.01	4.56
	ATG7o	ATG7s	BCL2o	BCL2s	BECN1o	BECN1s	C3o	C3s	CASP1o	CASP1s
Median	1.62	1.31	0.21	-0.17	2.60	3.12	-4.72	-3.79	2.42	2.62
Range	2.49	5.09	3.33	6.56	4.84	5.96	3.64	4.47	3.31	4.04
	CASP3o	CASP3s	CASP7o	CASP7s	CCL2o	CCL2s	CD36o	CD36s	CD3Eo	CD3Es
Median	2.68	2.57	1.66	1.46	2.30	-1.69	-4.92	-6.04	2.33	2.51
Range	5.91	3.86	2.55	4.13	8.20	7.67	4.67	2.46	6.95	6.43
	CD68o	CD68s	CEBPAo	CEBPAs	CEBPBo	CEBPBs	CEBPDo	CEBPDs	CES1o	CES1s
Median	-1.93	-2.69	-2.04	-2.17	-2.42	-3.24	-0.35	-1.10	-1.96	-2.32
Range	2.82	4.55	3.27	3.27	4.61	5.81	5.30	6.24	4.79	4.05
	CFDo	CFDs	CIDEAo	CIDEAs	CLEC7Ao	CLEC7As	CRATo	CRATs	DDIT3o	DDIT3s
Median	-4.84	-5.33	-0.25	0.86	3.42	1.55	-0.42	-0.76	1.98	1.35
Range	4.12	3.35	5.69	4.76	6.96	5.40	2.56	2.33	2.73	4.44
	DERL2o	DERL2s	EDEM2o	EDEM2s	EPHX2o	EPHX2s	FABP4o	FABP4s	FASo	FASs
Median	1.02	0.83	2.72	2.55	1.41	1.69	-6.53	-6.72	1.39	1.46
Range	1.19	6.03	2.68	6.88	2.21	3.51	4.01	4.34	2.41	4.02
	FOXO1o	FOXO1s	GATA3o	GATA3s	GSTK1o	GSTK1s	HERPUD1o	HERPUD1s	HIF1Ao	HIF1As
Median	-0.86	-1.37	2.82	2.57	-1.34	-1.67	-0.82	-0.67	-0.71	-2.03
Range	1.99	3.41	4.43	5.60	2.04	3.27	2.12	2.00	3.52	4.49
	IGFBP2o	IGFBP2s	IL10o	IL10s	IL18o	IL18s	IL1Bo	IL1Bs	IL6o	IL6s
Median	1.62	2.48	6.03	4.51	3.81	5.77	5.05	1.81	7.26	2.85
Range	4.54	3.89	10.73	7.94	7.80	6.37	10.81	8.57	8.71	11.35
	IL8o	IL8s	IRAK1o	IRAK1s	IRAK3o	IRAK3s	IRS1o	IRS1s	IRS2o	IRS2s
Median	5.44	0.30	0.79	0.31	0.48	-0.08	1.31	0.95	-1.03	-1.35
Range	11.73	12.12	5.70	3.67	4.25	3.14	3.28	4.56	3.25	6.70
	IRS4o	IRS4s	ITGAXo	ITGAXs	LEPo	LEPs	LIPEo	LIPEs	LPCAT1o	LPCAT1s
Median	5.72	5.35	3.53	1.69	-1.82	-3.27	-3.26	-3.80	0.84	0.63
Range	9.79	15.63	6.98	5.59	6.02	3.48	3.57	3.67	3.05	3.88

	LPCAT2o	LPCAT2s	LPIN1o	LPIN1s	LPLo	LPLs	MAP1LC3Bo	MAP1LC3Bs	MYD88o	MYD88s
Median	3.63	4.60	1.45	1.75	-3.67	-3.95	-1.85	-2.31	1.19	0.65
Range	3.68	6.88	3.00	3.62	4.88	2.92	3.04	6.39	3.10	3.91
	NAMPTo	NAMPTs	NFE2L2o	NFE2L2s	NLRP3o	NLRP3s	PDK1o	PDK1s	PLIN1o	PLIN1s
Median	-0.04	-2.91	-2.38	-2.93	3.76	1.96	2.33	1.99	-5.55	-6.15
Range	7.09	6.30	3.13	2.82	6.35	6.52	2.33	2.55	3.86	4.17
	PNPLA2o	PNPLA2s	PNPLA3o	PNPLA3s	PPARDo	PPARDs	PPARGC1Ao	PPARGC1As	PPARGo	PPARGs
Median	-3.18	-3.14	6.81	5.78	-0.09	-0.57	3.64	4.81	-1.68	-2.12
Range	3.96	3.24	5.46	9.10	2.46	6.80	3.96	4.38	4.25	2.72
	SLC2A4o	SLC2A4s	SOCS3o	SOCS3s	SOD1o	SOD1s	SOD2o	SOD2s	SREBF1o	SREBF1s
Median	1.06	0.90	0.58	-3.66	-0.85	-0.33	-2.07	-3.44	0.40	0.31
Range	3.88	4.97	8.70	10.55	4.13	5.54	4.83	4.66	3.74	3.10
	TBX21o	TBX21s	TGFB1o	TGFB1s	TIMP1o	TIMP1s	TNFAIP6o	TNFAIP6s	TNFo	TNFs
Median	6.79	7.21	-1.27	-1.60	-2.83	-2.01	4.79	2.04	5.67	4.36
Range	4.82	6.90	2.80	4.90	4.14	4.93	8.53	8.81	10.71	10.11

Table 48 Mann-Whitney U-test for adipose tissue transcript expression (ΔCT) comparison between dietary interventions

	ACACAo	ACACAs	ADIPOQo	ADIPOQs	ALOX15Bs	ALOX15o	ATF6o	ATF6s	ATG5o	ATG5s
Asymp. Sig. (2-tailed)	0.206	0.917	0.742	0.246	0.756	0.373	0.019	0.291	0.137	0.835
	ATG7o	ATG7s	BCL2o	BCL2s	BECN1o	BECN1s	C3o	C3s	CASP1o	CASP1s
Asymp. Sig. (2-tailed)	0.591	0.959	0.341	0.691	1.000	0.835	0.097	0.533	0.080	0.556
	CASP3o	CASP3s	CASP7o	CASP7s	CCL2o	CCL2s	CD36o	CD36s	CD3Eo	CD3Es
Asymp. Sig. (2-tailed)	0.146	0.510	0.146	0.260	0.568	0.835	0.446	0.039	0.580	0.898
	CD68o	CD68s	CEBPAo	CEBPAs	CEBPBo	CEBPBs	CEBPDo	CEBPDs	CES1o	CES1s
Asymp. Sig. (2-tailed)	0.137	0.396	0.972	0.901	0.291	0.556	0.387	0.653	0.545	0.010
	CFDo	CFDs	CIDEAo	CIDEAs	CLEC7Ao	CLEC7As	CRATo	CRATs	DDIT3o	DDIT3s
Asymp. Sig. (2-tailed)	0.206	0.769	0.387	0.703	0.050	0.822	0.876	0.556	0.128	0.200
	DERL2o	DERL2s	EDEM2o	EDEM2s	EPHX2o	EPHX2s	FABP4o	FABP4s	FASo	FASs
Asymp. Sig. (2-tailed)	0.890	0.324	0.835	0.591	0.350	0.522	0.972	0.115	0.239	0.729
	FOXO1o	FOXO1s	GATA3o	GATA3s	GSTK1o	GSTK1s	HERPUD1o	HERPUD1s	HIF1Ao	HIF1As
Asymp. Sig. (2-tailed)	0.299	0.478	0.119	0.835	0.545	0.782	0.945	0.769	0.146	0.835

(continued) Mann-Whitney U-test for adipose tissue transcript expression (ΔCT) comparison between dietary interventions

	IGFBP2o	**IGFBP2s**	**IL10o**	**IL10s**	**IL18o**	**IL18s**	**IL1Bo**	**IL1Bs**	**IL6o**	**IL6s**
Asymp. Sig. (2-tailed)	0.478	0.457	0.351	0.748	0.945	0.920	0.978	0.701	0.915	0.572
	IL8o	IL8s	IRAK1o	IRAK1s	IRAK3o	IRAK3s	IRS1o	IRS1s	IRS2o	IRS2s
Asymp. Sig. (2-tailed)	0.118	0.621	0.111	0.406	0.151	0.831	0.616	0.364	0.141	0.945
	IRS4o	IRS4s	ITGAXo	ITGAXs	LEPo	LEPs	LIPEo	LIPEs	LPCAT1o	LPCAT1s
Asymp. Sig. (2-tailed)	0.240	0.822	0.350	0.653	0.876	0.406	0.467	0.931	0.315	0.769
	LPCAT2o	LPCAT2s	LPIN1o	LPIN1s	LPLo	LPLs	MAP1LC3Bo	MAP1LC3Bs	MYD88o	MYD88s
Asymp. Sig. (2-tailed)	0.776	0.826	0.795	0.500	0.533	0.033	0.324	0.931	0.024	0.808
	NAMPTo	NAMPTs	NFE2L2o	NFE2L2s	NLRP3o	NLRP3s	PDK1o	PDK1s	PLIN1o	PLIN1s
Asymp. Sig. (2-tailed)	0.457	1.000	0.268	0.890	0.226	0.716	0.187	0.963	0.467	0.959
	PNPLA2o	PNPLA2s	PNPLA3o	PNPLA3s	PPARDo	PPARDs	PPARGC1Ao	PPARGC1As	PPARGo	PPARGs
Asymp. Sig. (2-tailed)	0.426	0.945	0.660	0.716	0.324	0.631	0.107	0.318	0.678	0.467
	SLC2A4o	SLC2A4s	SOCS3o	SOCS3s	SOD1o	SOD1s	SOD2o	SOD2s	SREBF1o	SREBF1s
Asymp. Sig. (2-tailed)	0.945	0.755	0.146	0.959	0.945	0.769	0.206	0.876	0.604	0.929
	TBX21o	TBX21s	TGFB1o	TGFB1s	TIMP1o	TIMP1s	TNFAIP6o	TNFAIP6s	TNFo	TNFs
Asymp. Sig. (2-tailed)	0.667	0.782	0.755	0.522	0.097	0.568	0.557	0.200	0.985	0.462
	CHREBPa_s	CHREBPa_o	CHREBPad_s	CHREBPad_o						
Asymp. Sig. (2-tailed)	0.931	0.822	0.931	0.556						

Appendix 13

Figure 29 IPA network with targets from adipose tissue whose expression levels showed the greatest expression level fold-difference between omental and subcutaneous depots.

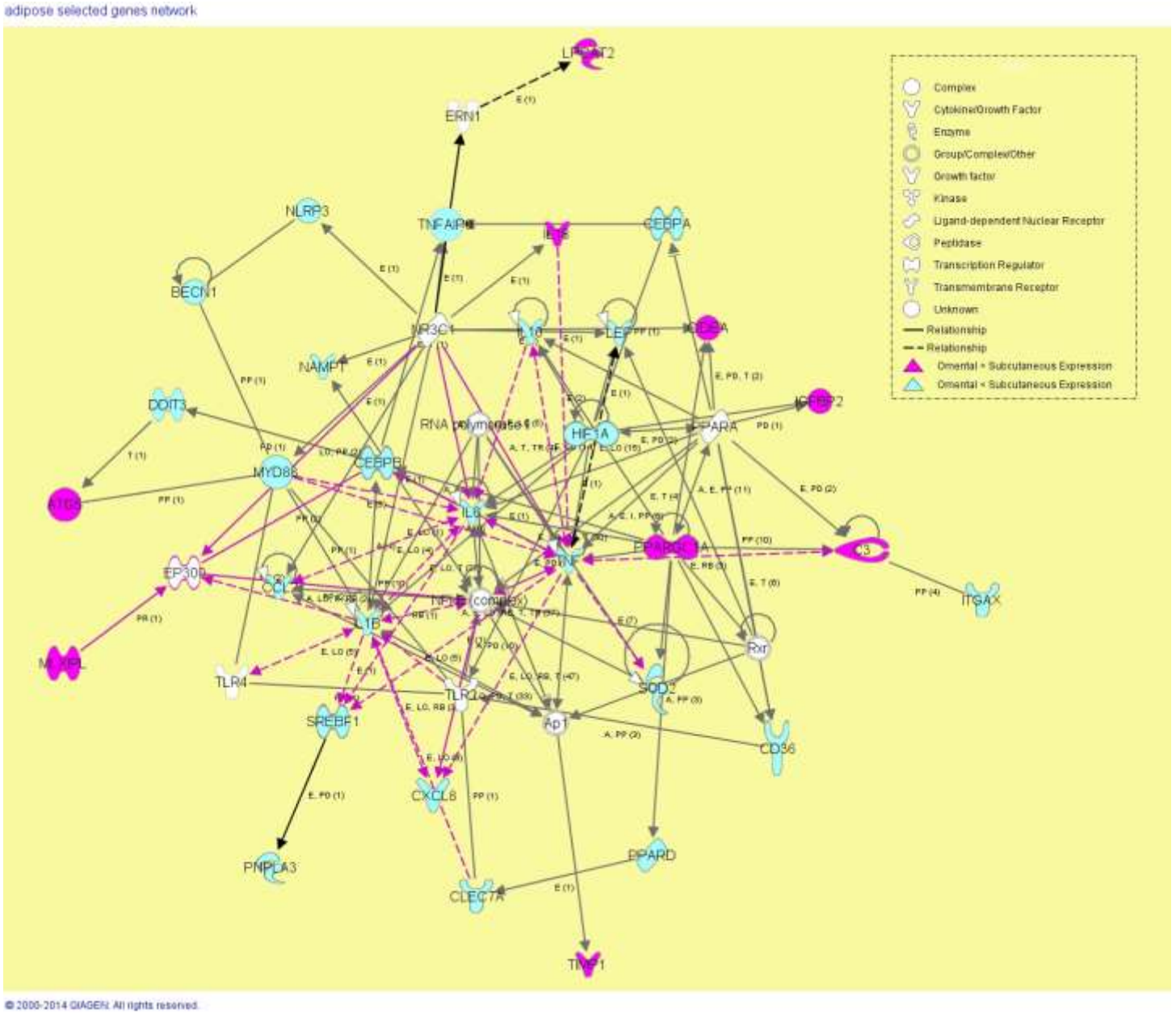


Table 49 liver transcript expression ΔCT median and range.

	ACACA	ATF6	ATG7	ATG5	BAAT	BCL2	BECN1	CASP3
Median	0.66	-0.34	2.94	2.39	-2.30	3.15	2.44	1.90
Range	8.09	2.94	3.38	3.48	4.50	4.10	3.77	5.94
	CASP7	CD36	CEBPA	CEBPB	CES1	CLEC7A	CRAT	DDIT3
Median	1.83	-0.42	-2.14	-2.93	-5.31	2.62	-2.37	2.45
Range	2.72	3.15	5.30	3.58	3.67	4.40	1.88	4.52
	DERL2	EDEM2	EPHX2	FAS	FOXO1	GSTK1	HERPUD1	HIF1A
Median	0.95	1.72	-2.29	1.07	-0.15	-2.58	-1.78	-0.74
Range	2.63	6.20	2.44	4.01	5.27	4.09	2.65	3.13
	IGF1	IGFBP2	IL10	IL18	IL1B	IL6	IL8	IRAK1
Median	-0.34	-0.27	5.52	4.56	4.06	9.28	3.73	1.18
Range	5.22	6.75	7.49	3.71	6.77	9.50	10.14	6.00
	IRAK3	LIPE	LPCAT1	LPCAT2	MAP1LC3B	MYD88	NAMPT	NFE2L2
Median	2.71	4.93	1.59	4.49	0.00	0.41	-1.70	-1.79
Range	4.74	39.66	4.30	3.95	5.39	37.57	5.13	2.64
	NLRP3	PDK1	PNPLA2	PNPLA3	PPARD	SOCS3	SOD1	SOD2
Median	3.95	2.18	0.33	0.35	0.67	0.57	-3.62	-2.35
Range	5.09	3.90	4.43	5.61	3.77	13.23	3.24	3.75
	SREBF1	TGFB1	TIMP1	TNFAIP6	TNF			
Median	-1.13	-0.59	-1.22	6.54	5.94			
Range	3.04	3.49	3.63	7.26	5.74			

Please indicate perceived difficulty of the procedure on the 100-mm visual analogue scale - 0mm lowest and 100mm highest degree of difficulty.

1. Rigidity of liver



2. Liver volume/size



3. Obstruction of gastro-oesophageal junction



Appendix 15 ***Micronutrient reference nutrient intake levels***

Table 50 reference nutrient intake (RNI) levels and recommended daily allowance (RDA) for micronutrients.

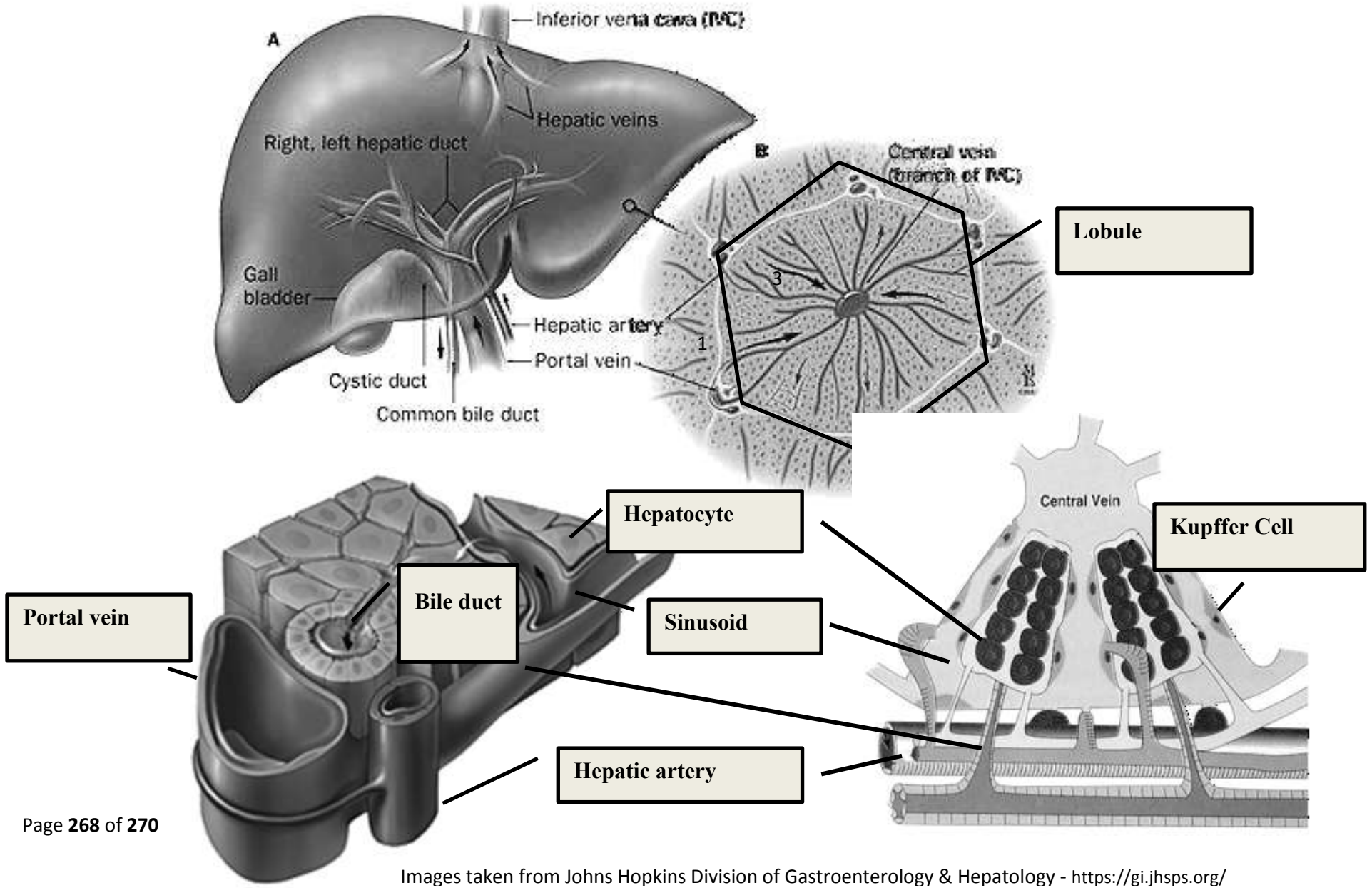
	RNI[▸]	RDA[✱]
Sodium (mg)	1600	
Vitamin C (mg)	40	80
Thiamin (mg)	0.8-1	1,1
Riboflavin (mg)	1.1-1.3	1.4
Niacin (mg)	12-18	16
Vitamin B6 (mg)	1.2-1.5	1.4
Folic acid (µg)	200	200
Vitamin B12 (µg)	1.5	2.5
Calcium (mg)	700-1000	800
Phosphorus (mg)	550-775	700
Iron (mg)	8.7-14.8	14
Magnesium (mg)	270-300	375
Zinc (mg)	7-9.5	10
Iodide (µg)	140	150
Potassium (mg)	3500	2000
Copper (mg)	1-1.2	1
Manganese (mg)	1.4	2
Selenium (µg)	60-75	55

[▸]Department of Health 1991 Dietary Reference Values for food and energy nutrients for the United Kingdom. The highest RNI values were used where values differed for genders, and for those that do not RNI levels, the safe limits or expected adequate levels were used.

[✱] Commission Directive 2008/100/EC of 28 October 2008, amending Council Directive 90/496/EEC, on nutrition labelling for foodstuffs as regards recommended daily allowances, energy conversion factors and definitions.

Appendix 16 ***Liver anatomy***

Figure 38 liver anatomy and structure



Images taken from Johns Hopkins Division of Gastroenterology & Hepatology - https://gi.jhsps.org/

As seen in the image above the liver is divided anatomically into hexagonal lobules, with a central vein, and a triad of portal vein, bile duct and hepatic artery at each corner. "…three zones are distinguished, which contain blood that is increasingly depleted in oxygen and exogenous substances." (Dancygier and Allescher, 2010) Zone 1 is the nearest to the triad of vessels (numbered 1 in the image above and histological slide below) and is described as periportal. It contains the highest oxygen concentration and the highest levels of substances originating from the intestinal circulation. Zone 3 is described as perivenular, and is the closest zone to the central vein (numbered 3 in the image above and histological slide below) (Dancygier and Allescher, 2010).

Figure 39 liver structure - zones

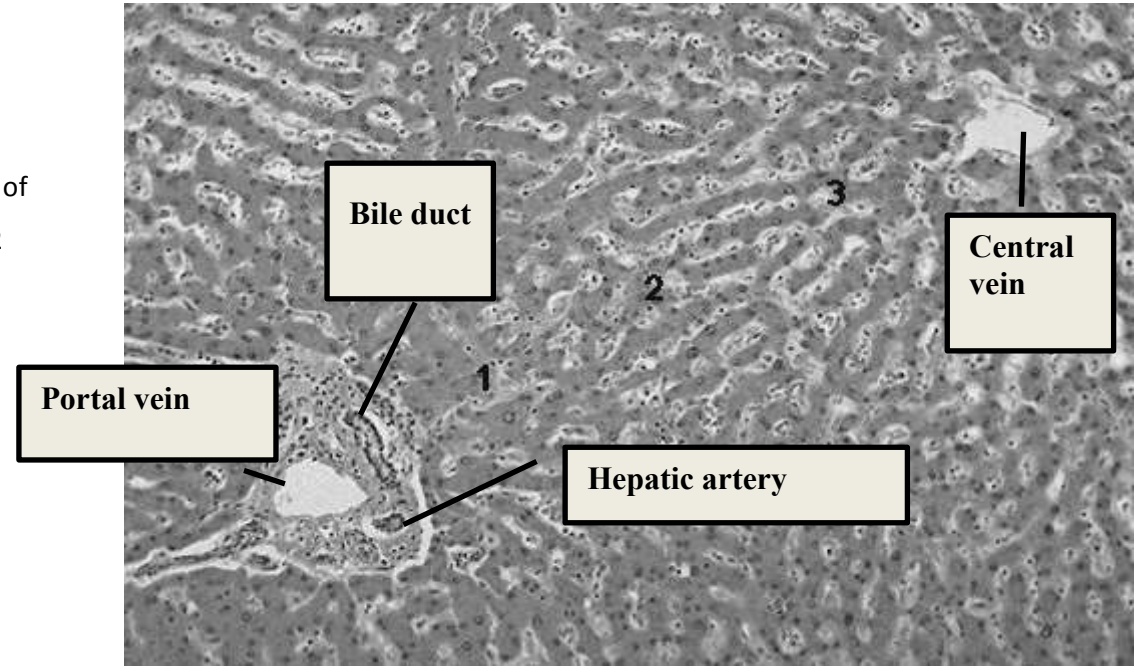


Image from Medical Histology and Virtual Microscopy, University of Michigan - http://library.med.utah.edu/WebPath/ORGAN.html#2 (29/7/14)

OBES SURG (2014) 24:416–421
DOI 10.1007/s11695-013-1125-6

ORIGINAL CONTRIBUTIONS

Pre-operative Dietary Restriction for Patients Undergoing Bariatric Surgery in the UK: Observational Study of Current Practice and Dietary Effects

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Abstract
Background Bariatric surgery is effective at achieving weight loss in the severely obese, with the majority of procedures performed laparoscopically. A short-term pre-operative energy restrictive diet is widely adopted to enable surgery by reducing liver size and improving liver flexibility. However, the dietary approach is not standardised. This observational study reports on pre-operative restrictive diets in use across bariatric services in the UK.
Methods Between September and November 2012, information was collected from bariatric services on current or past pre-operative diets, and any research providing evidence for the use or modification of their diets.
Results Around one third of bariatric services (28) in the UK responded, with a total of 49 diets in current use. Types of diet include low energy, low carbohydrate and liquid, with 59 % offering low energy/low carbohydrate food-based, 21 % milk/yoghurt, 18 % meal replacement (liquid) and 2 % clear liquid. Diet duration varies between 7 and 42 days. Limited anecdotal evidence was provided by services evaluating the pre-operative diet, and its alternative approaches, with dietary choice primarily clinician-led.
Conclusions This study has highlighted variability and lack of consensus in the form of pre-bariatric surgery diet used across different centres. Further research comparing outcomes for alternative diets would support best practice in the future.

Keywords Bariatric · Obesity · Surgery · Pre-operative · Short term · Diet

Abbreviations

BOMSS	British Obesity and Metabolic Surgery Society
BMI	Body mass index
NBSR	National Bariatric Surgery Registry

Introduction/Purpose

Bariatric surgery is the most effective means of achieving long-term weight loss in the severely obese, improving health-related quality of life alongside reduction in co-morbidities such as hypertension and type 2 diabetes [1–3].

Between 2009 and 2010, there were over 7,000 bariatric surgery procedures carried out in the UK [4]. The majority of current procedures are performed laparoscopically, which enables more rapid patient recovery [1, 4]. This surgical approach, however, may be problematic or impossible in patients who have a very high body mass index (BMI) and central obesity or a large liver [3].

Rapid pre-operative weight loss facilitates the laparoscopic approach, by decreasing liver size and improving liver flexibility by reducing stores of glycogen and lipid, and reducing visceral adipose tissue depots, enabling access to the upper stomach and oesophagus [5–8]. This is applied in practice with the use of a short-term pre-operative energy restrictive diet, or "liver reducing" diet [9].

In 2008, a consensus document was produced by dietitians for the British Obesity and Metabolic Surgery Society

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