Nutritional modulation of hepatic lipid metabolism in health and disease

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Abstract

The objective of this thesis was to assess the impact of altering macronutrient intakes on hepatic lipid metabolism. Two separate studies were performed, with liver triglyceride content being the principal outcome of both. In the first study 32 healthy and centrally overweight males were randomised to 2 periods, each of 2 weeks, of either a high fructose or glucose intake in a non-crossover fashion. Isoenergetic status was maintained by providing foodstuffs during the first period, followed by a 6 week washout and then a second period of *ad libitum* overfeeding. In the second study 55 patients with biopsy proven non-alcoholic fatty liver disease were randomised to 3 months 5g a day of capsules containing either n-3 polyunsaturated fatty acid or oleic enriched sunflower oil.

The main findings are summarised. High intakes of fructose and glucose in the isoenergetic period resulted in a stable weight, and no change in hepatic, serum and ectopic triglyceride content. There was a raised serum uric acid with fructose. During the hyperenergetic period there was a tendency for greater uric acid with fructose, whilst both groups had a matched weight gain, elevation of liver biochemistry and an increase in hepatic, serum and muscle triglycerides. Changes in liver biochemistry and triglycerides were associated with changes in weight. During both periods there was calorimetric evidence for a shift in whole body metabolism towards that reflective of a high carbohydrate intake. There was no alteration in renal function or cardiovascular haemodynamic parameters or consistent change in insulin resistance.

The n-3 polyunsaturated versus oleic acid study resulted in significant alterations of serum fatty acid profiles between the groups, which were in line with the capsules' contents. These changes however failed to translate, in the whole group, to any detected metabolic or hepatic changes beyond a reduction in serum triglyceride with n-3 polyunsaturated fatty acids. Only 43 of the 55 patients had elevated liver triglycerides on baseline MRI. Amongst this 43 there was a reduction in liver triglyceride with n-3 polyunsaturated fatty acids, but no other associated metabolic changes.

The uric acid findings support the notion of fructose and glucose differing in their pre triose metabolism. There was however no differing outcomes in terms

of lipid synthesis or storage. There was a suggestion of reduced liver triglycerides with n-3 polyunsaturated fatty acids though this was an isolated result only found amongst those with a steatotic liver at baseline. Ultimately the exquisite sensitivity of the liver to nutrient intakes was highlighted by the 0.8% gain in weight in the fructose / glucose study resulting in a 24% increase in liver lipid. This affirms the notion that dietary energy intakes have a profound influence on hepatic metabolism, but there is no evidence from this thesis that this influence is macronutrient specific. In the future macronutrient comparisons need to be made.

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Abbreviations

^{1}H MRS	Proton magnetic resonance spectroscopy
^{31}P MRS	Phosphorus magnetic resonance spectroscopy
Adipo-IR	Adipose tissue insulin resistance
ALT	Alanine aminotransferase
ARA	Arachidonic acid
ATP	Adenosine triphosphate
BMI	Body mass index
CHREBP	Carbohydrate responsive element binding protein
CRP	C-reactive protein
DEXA	Dual energy X-ray absorptiometry
DHA	Docosahexaenoic acid
DNL	De novo lipogenesis
EMCL	Extra-myocellular lipid
EPA	Eicosapentaenoic acid
F6P	Fructose-6-phosphate
FAS	Fatty acid synthase
FFQ	Food frequency questionnaire
G6P	Glucose-6-phosphate
GLUT	Glucose transporter
HFCS	High fructose corn syrup
HFrD	High fructose diet
HOMA	Homeostasis model assessment of insulin resistance
HTGC	Hepatic triglyceride concentration
IMCL	Intra-myocellular lipid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid
NHANES	National Health and Nutrition Examination Survey
PDE	Phosphodiester
Pi	Inorganic phosphate
PME	Phosphomonoester
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid

REE	Resting energy expenditure
RQ	Respiratory quotient
SREBP	Sterol regulatory element binding protein
TEE	Total energy expenditure
TLR	Toll-Like receptor
TNF	Tumour necrosis factor
TZD	Thiazolidinedione
UCP	Uncoupling protein
VAS	Visual analogue scale
VLDL	Very low density lipoprotein

Chapter 1. Introduction

1.1.   The function of adipose tissue

Triglycerides comprise around 95% of dietary lipids and can be stored within the specialised storage organ adipose tissue, or within other organs, principally liver and muscle. Lipids play essential roles in energy storage, vitamin absorption, cellular membrane maintenance and cellular signalling. The fatty acid content of the body originates either from exogenous dietary sources or from endogenous synthesis. Circulating lipoprotein and chylomicron-bound lipids increase following an intake of lipid containing foodstuffs. Lipoprotein lipase breaks down the triacylglycerol (triglyceride) from these circulating complexes, releasing non-esterified fatty acids (NEFA) which are taken up into adipocytes, where they are esterified into triglyceride and stored. The high energy density of triglyceride (9kcal/g) means that it is a more efficient energy store than protein (4kcal/kg) or carbohydrates (3.75kcal/kg). During a period of energy demand which is greater than exogenous supply, the stored triglyceride is hydrolysed by hormone-sensitive lipase and re-mobilised back into the circulation as NEFA. These opposing actions of lipid storage and lipolysis are under strict hormonal control. The hormones adrenaline, adrenocorticotropic hormone (ACTH) and glucagon promote lipolysis, whereas insulin promotes lipid storage and esterification. This hormonal profile is ultimately influenced by metabolic need, metabolic reserves and stress.

Traditionally adipose tissue was considered as being merely the major storage organ for triglyceride, and little significance was attributed to ectopic lipid storage in liver or muscle. This has radically changed over the past decade or so. Adipose tissue has emerged as a major endocrine organ, muscle lipids have been implicated in insulin resistance and hepatic lipid in altering local and whole-body metabolic and inflammatory status.

Adipose tissue secretes over 100 differing factors including adipokines, steroid hormones, fatty acids, and prostaglandins. With the exception of adiponectin, these factors are all released in greater amounts with increasing adipose tissue volume. Adiponectin, a key regulatory factor, promotes insulin sensitivity and β-oxidation. The reasons for the change in hormone and cytokine production rates by adipocytes with increasing adipose volume are uncertain,

though suggested roles include adipocyte hypertrophy and hyperplasia, chronic hypoxia due to relative vascular insufficiency, and mitochondrial dysfunction and oxidative stress (Kraemer and Shen 2006). There is emerging evidence for the existence of a biological limit to which a fat depot can expand, above which metabolic decompensation occurs (Christodoulides, Lagathu et al. 2009).

Adipose tissue and ectopic lipid storage has been shown to be closely linked with inflammatory and immune function. Metabolic and immune systems are among the most fundamental requirements for survival. Energy excess or deficiency results in an impaired immune response (Hotamisligil and Erbay 2008). Recent evidence suggests that the processes of metabolic and inflammatory responses may have co-evolved, as both processes are controlled by a single functional unit or organ termed the 'fat body' in primitive organisms such as the *Drosophila melanogaster* (Leclerc and Reichhart 2004). Indeed there remains a close association between metabolism and inflammation in the tissues of higher organisms. The principal metabolic cells (hepatocytes and adipocytes) are in close proximity to immune cells (macrophages, Kupffer cells, lymphocytes and dendritic cells). Indeed lymph nodes are embedded in adipose tissue. The adipocyte derived hormones, termed adipokines, influence not only adipocyte function in an auto- and paracrine fashion, but also are released systemically via the bloodstream to influence whole body metabolic and inflammatory pathways. As a result of this systemic release the anatomical location, and hence venous drainage paths, appears to critically determine the functional importance of adipose tissue. This has led to the portal/visceral hypothesis (Ronti, Lupattelli et al. 2006). There is much to support this theory and it will be frequently discussed in this thesis. In an elegant and simple study, Klein et al. assessed the metabolic outcomes following liposuction removal of over 30% of abdominal subcutaneous tissue (Klein, Fontana et al. 2004). Around a 6% body weight loss was achieved, though as a result of this selective weight loss visceral and muscle lipid contents remained unchanged 12 weeks later. There was a significant reduction in leptin (reflecting a reduction in adipocyte mass, though no alteration of systemic, hepatic or adipocyte insulin resistance or circulating mediators of inflammation. The long-term (7-17 years) follow up of this original study has been subsequently published and showed that long term weights were identical to those at 10 weeks and that long-term insulin resistance and lipid profiles remained unchanged from before liposuction (Mohammed, Cohen et al. 2008).

A criticism of these studies is that there is no control arm. Similar amounts of weight loss through lifestyle changes have however been repeatedly shown to improve all of these parameters (Blackburn 1995). The explanation for these findings is that lifestyle induced weight loss reduces the metabolically critical stores in visceral and ectopic stores as well as the relatively benign subcutaneous stores. The difference between these is their anatomical distribution, and hence venous drainage. The venous drainage of intra-abdominal, or visceral, adipocytes means that factors released are transported straight to the liver via the portal system. In comparison, the venous drainage from subcutaneous fat enters the wider systemic circulation. This theory is further reinforced by the observation that central obesity (apple shaped) results in greater cardiovascular risk than subcutaneous (pear shaped) obesity (Kannel, Cupples et al. 1991). To conclude, it is not so much how much adipose tissue you have, as where you have it.

1.2.   Overview of hepatic fatty acid metabolism in health

The liver is the key organ in determining the metabolism and distribution of fatty acids. It is the source of endogenous synthesis and degrades or interconverts exogenous fatty acids. The resultant fatty acids may either be stored in the liver itself, or exported to adipose tissue and muscle. Hepatic fatty acid metabolism is dynamic with triglyceride turnover occurring every 2 days (Boberg, Carlson et al. 1972). As depicted in figure 1.1, hepatic fatty acids are derived from two sources: circulating fatty acids or endogenous *de novo* lipogenesis (DNL) which uses monosaccharides as its principal substrates.
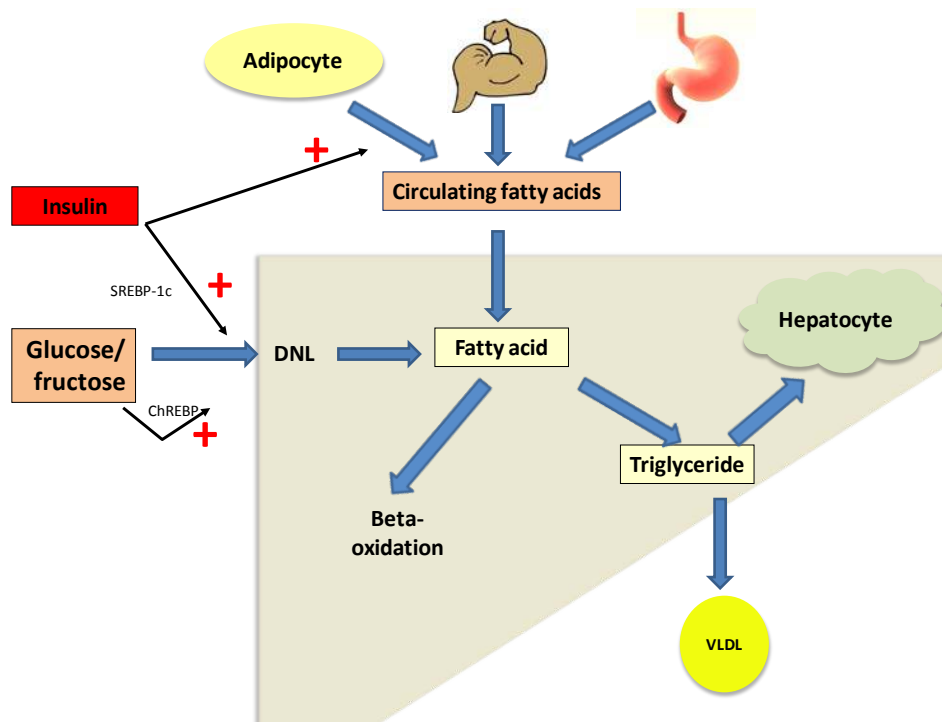
Figure 1.1: Overview of hepatic fatty acid metabolism depicting intra and extra-hepatic influences including the roles of adipocytes, muscle and diet. Abbreviations used: DNL- *de novo* lipogenesis, SREBP-1C - sterol regulatory element binding protein-1c, VLDL - very low-density lipoprotein. Adapted from (Tamura and Shimomura 2005).

Circulating fatty acids are either bound to lipoproteins or albumin. Lipoprotein-bound lipids, which include chylomicron complexes, are internalised into cells following the formation of a specific apolipoprotein-receptor complex. Dietary medium-chain triglycerides enter the portal circulation directly, whereas longer chain fatty acids enter the vascular circulation complexed with chylomicrons via the lymphatic system and the thoracic duct.

Historically it was believed that in contrast to lipid-lipoprotein complexes, the albumin bound NEFAs are internalised into cells via simple and direct penetration of the plasma membrane. The recently discovered cluster differentiation protein 36 (CD 36) forms a pathway for hepatic fatty acid uptake which is upregulated by insulin and experimental models of NAFLD (Ge, Zhou et al. 2010; Larter, Chitturi et al. 2010)As a result NEFAs are the key fatty acid source for the liver with an uptake that is directly proportional to its delivery rate (Havel, Kane et al. 1970) and potentially increased by insulin resistance and NAFLD. The concentration of circulating NEFAs is dependent on their release from adipocytes and myocytes. This is regulated by hormone sensitive lipase,

which is stimulated by adrenaline, and inhibited by insulin (Qureshi and Abrams 2007).

Insulin also acts to reduce circulating glucose concentrations by promoting its tissue uptake. Insulin resistance however results in increased circulating concentrations of insulin, and the principal hepatic fatty acid substrates, namely glucose and NEFA. As a result insulin resistance promotes hepatic lipogenesis as will be further discussed later.

Intra-hepatic fatty acids are cytotoxic (Gibbons, Wiggins et al. 2004) and so are further metabolised by three potential and separate processes: either beta-oxidation, VLDL synthesis or intra-hepatic storage as triglycerides. In the presence of high energy demand, intra-hepatic fatty acids undergo oxidation to generate energy in the form of ATP. If there is a low energy demand then intra-hepatic fatty acids are esterified into triglycerides and either stored in the hepatocyte or exported as VLDL.

1.   Oxidation

Within the mitochondria fatty acids in the form of acyl-CoA molecules are progressively cleaved by β-oxidation to generate ATP. The process is initiated at the carboxyl end and involves the successive disruption of the link between the α-2 and β-3 carbon atoms (Lavoie and Gauthier 2006). At the end of each cycle the chain is reduced by two carbon atoms, and one molecule of $FADH_2$, NADH and acetyl CoA is produced. The acetyl-CoA is then further oxidised within the mitochondria via the citric acid cycle, while the $FADH_2$ and NADH enter the electron-transport chain. The process is repeated until the whole chain is oxidised.

Microsomal (α and ω) oxidation occurs within the endoplasmic reticulum by members of the cytochrome P450 family. They catalyse the oxidation of a variety of exogenous and endogenous compounds and play a relatively minor role in fatty acid oxidation.

2.   VLDL synthesis

The addition of a single glycerol molecule to three fatty acids forms a triglyceride. Triglycerides cannot freely cross hepatocyte membranes, and so are either stored within the hepatocyte itself, or are coated in lipoproteins, incorporated within VLDL, and exported into the systemic circulation. VLDLs are formed within the liver and are a complex fusion of lipoproteins (predominantly

Apolipoprotein B-100), lipids and phospholipids. VLDL secretion facilitates the transfer of intra-hepatic fatty acids to peripheral adipose stores (Gibbons, Wiggins et al. 2004).

3.   Storage within the hepatocytes

The final option for fatty acids is of conversion to triglycerides and storage within the hepatic cytosol. Factors that directly promote hepatic triglyceride storage are poorly understood. Intra-hepatic storage appears to occur when fatty acid production exceeds the liver's oxidation or exportation abilities (Gibbons, Islam et al. 2000; Lavoie and Gauthier 2006; Serviddio, Sastre et al. 2008).

1.3.   Introduction to Non-Alcoholic Fatty Liver Disease (NAFLD)

Pathologically excessive intra-hepatic triglyceride storage, termed steatosis, may result from a variety of liver insults including alcohol, medication and hepatitis C. The histological criteria for steatosis is the presence of triglyceride stored in more than 5% of hepatocytes (Hubscher 2006). Non-alcoholic fatty liver disease (NAFLD) is diagnosed in the absence of clinical and histological evidence of such insults. Furthermore, NAFLD has characteristic histological patterns of lipid storage and injury, if present.

The term NAFLD encompasses a spectrum of disorders. The majority of NAFLD patients are asymptomatic at presentation and hence present incidentally following a routine health check-up, or as part of an evaluation for another health process (Cortez-Pinto, Chatham et al. 1999). In these situations NAFLD may be suspected by the presence of abnormal blood liver function tests or liver appearance on radiology. The minority of NAFLD patients present with complications of advanced liver disease, such as a variceal bleed or ascites.

1.4. NAFLD epidemiology
1.4.1. Prevalence of NAFLD

Accurate assessment of the prevalence of NAFLD is hampered by the lack of a cheap, non-invasive, accurate and widely available tool for disease screening and staging. Use of 'liver function' blood tests is extremely limited as they are normal in 80% of those with radiological evidence of steatosis (Browning, Szczepaniak et al. 2004), and they correlate poorly with the severity of injury and fibrosis (de Ledinghen, Combes et al. 2004). Radiology can establish the presence

of steatosis with reasonable accuracy, but it cannot reliably differentiate between the differing stages in the disease spectrum. As a result, routine clinical practice relies on a liver biopsy for disease staging, though its invasive nature means that it cannot be used in population based studies.

The prevalence of any condition depends upon the cohort studied. Globally massive differences exist between population body habitus and lifestyle. As a consequence estimates of NAFLD prevalence vary from 9% to 37% (Ong and Younossi 2007). NAFLD is the most prevalent liver disease in the Western world (Clark 2006). The US-based third National Health and Nutrition Examination Survey (NHANES III) analysed liver function tests in 15,676 adults. A presumptive diagnosis of NAFLD was made following abnormal liver function test findings in the absence of an alternative explanation, although this method clearly underestimates true prevalence. This approach found that prevalence of presumed NAFLD was greatest during the fourth and sixth decades (Ruhl and Everhart 2003), and present in more men than women (9.3% vs. 6.6%, p=.002) (Clark, Brancati et al. 2003). These observed associations may be explained by the increasing obesity and insulin resistance with age, and that men are more prone to visceral than peripheral adiposity (Frith, Day et al. 2009).

The largest population-based survey reported a prevalence of fatty liver disease at 34% in Dallas, USA (Szczepaniak, Nurenberg et al. 2005). 2,349 adults underwent hepatic lipid content assessment by proton magnetic resonance spectroscopy (^{1}H MRS). Their cohort had a mean age of 45 years, 43% were obese, and there was a 6% rate of alcohol abuse.

1.4.2. Natural history of NAFLD

NAFLD appears to be a spectrum of related disorders as shown in figure 1.2. The natural history and rate of progression through the various stages is contentious. There is evidence to support both progression and regression along the various stages, up until the development of hepatocellular carcinoma. Understanding to date is critically hampered by the absence of a study that has prospectively and systematically followed up a large cohort of NAFLD patients with a repeat liver biopsy after a pre-determined time interval. Several groups have presented ad-hoc case series, though the resultant data is difficult to interpret.

60-90% — Normal liver / Simple steatosis

10-30% — Steatohepatitis

2-5% — Cirrhosis

Hepatocellular carcinoma
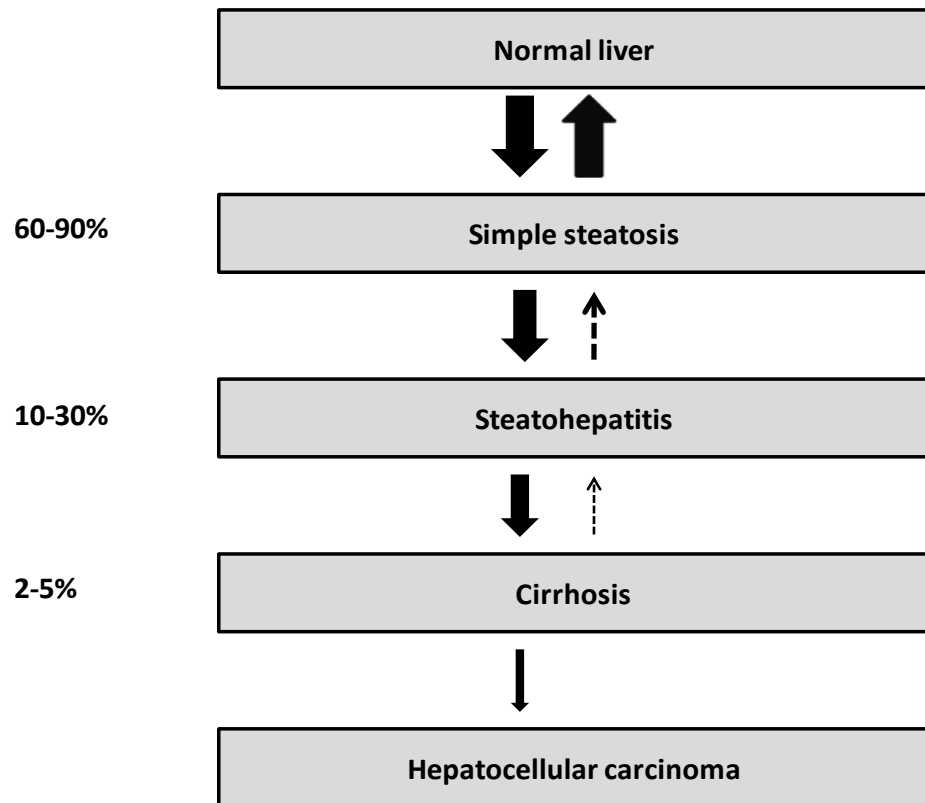
Figure 1.2.: The differing stages of NAFLD and estimates of their relative prevalence. Adapted from (Hubscher 2006).

The population prevalence of NAFLD in the US is estimated to be 30% though the majority have simple fatty change, termed steatosis, i.e. the presence of increased fat without significant inflammation or fibrosis, whereas only 3-5% have NASH (Vernon, Baranova et al. 2011). Simple steatosis appears to have a low rate of progression to NASH. Consequently it is believed that the majority of NASH patients, i.e. those with fibrosis or liver injury, progress rapidly through the stage of simple steatosis due to a pro-inflammatory state (Ong and Younossi 2007). A recent analysis of 545 NAFLD liver biopsies however has questioned this assumption, as steatosis severity was positively associated with the degree of lobular inflammation, fibrosis and NASH (Chalasani, Wilson et al. 2008). So a severe steatosis may drive an inflammatory state, an observation which is in direct conflict with the two separate and independent hit hypothesis. Multivariate analyses have demonstrated that independent clinical factors predictive of fibrosis on biopsy are age, body mass index (BMI), hypertension and insulin resistance (Argo and Caldwell 2009).

The elucidation of formal factors involved in the aetiology and prediction of progressive fibrosis development is an ongoing area of considerable scientific scrutiny. Argo et al. performed a recent systematic review of 10 studies comprising 221 patients that were assessed for histological progression in untreated NASH (Argo, Northup et al. 2009). 38% of patients with fibrosis develop progressive fibrosis, 41% have no progression, and 21% have fibrotic regression. Factors that were predictive for progressive fibrosis were age and the presence of fibrosis on the initial biopsy. Although some groups have previously shown metabolic features to be predictive for fibrosis (Ekstedt, Franzen et al. 2006), this was not borne out in this systematic review. As a result of progressive fibrosis, between 9 and 20% of patients with fibrosis or liver injury develop cirrhosis (Ong and Younossi 2007). The 5 year rates of hepatocellular carcinoma or liver-related deaths in a Japanese study of NAFLD cirrhotic patients were 20% and 7% respectively (Hashimoto, Yatsuji et al. 2005) .

Most NAFLD patients do not have a liver-related mortality. As compared the general population, NAFLD patients do have an increased mortality with a standardised mortality rate of 1.57 as compared to the general population (Musso, Gambino et al. 2010). This primarily originates from cardiovascular disease, followed by non-liver malignancy, with the third cause of death being liver-related in 6% (Adams, Lymp et al. 2005; Ong, Pitts et al. 2008; Rafiq, Bai et al. 2009). Indeed only 22% of NASH cirrhotic patients die from liver complications or receive a liver transplant (Ong and Younossi 2007). Ultimately NAFLD can be viewed as being primarily a metabolic as opposed to a hepatic disorder.

Until recently the largest prospectively followed cohort of NAFLD patients came from Esktedt *et al.*'s work in Sweden (Ekstedt, Franzen et al. 2006). This cohort of 129 patients was consecutively followed up with a repeat liver biopsy after a mean of 13.7 years. Amongst the 58 patients with simple steatosis there was no increased mortality over that predicted. Furthermore, of these 58 patients, only 8% developed bridging fibrosis, and none developed cirrhosis. Progression of liver fibrosis occurred in 41% and regression in 16%. Nine percent of the cohort developed cirrhosis over the study period. This led people to argue that simple steatosis had a very benign liver prognosis.

A recent review compiled all the studies assessing mortality with a greater than 5 years follow-up (Angulo 2010). This entailed 2 recently published large cohorts (Rafiq, Bai et al. 2009; Soderberg, Stal et al. 2010). The average follow

up period for the 798 patients with simple steatosis and 342 patients with NASH was 15 years. The overall all cause mortality was 32.5% for simple steatosis and 40.5% for NASH, which was not significantly different. There was however a significantly greater cirrhosis development rate (0.7% and 10.8%) and liver related death rate (0.9% vs. 7.3%) amongst those with NASH.

From the above it appears that NAFLD patients as a whole are at an increased risk of cardiovascular disease, and those with NASH are at the greatest risk of liver morbidity and mortality.

1.5. NAFLD Aetiology

The pathophysiology of NAFLD is complex and not completely understood. Classically NAFLD was believed to result from 'two hits' (Day and James 1998). The first hit is the development of steatosis, whist the second hit is the development of inflammation and fibrosis in the steatotically primed liver. This has since been shown to be an overly simplistic approach as these two pathological processes are not distinct from each other and instead the concept of multiple parallel hits has been generated (Tilg and Moschen 2010). The complexity of the current model means that even the paper proposing multiple parallel hits is predominantly structured along the lines of the two hit hypothesis (Tilg and Moschen 2010), and so will this thesis for simplicity's sake.

1.5.1. 1st hit – Hepatic steatosis development

It remains unclear as to what the origin is of the increased fatty acid stores. Donnelly *et al*. have performed the most in-depth lipid kinetic assessments in NAFLD to date, with liver biopsy fatty acid profiles performed after oral lipid isotope labelling (Donnelly, Smith et al. 2005). The subjects were viscerally obese and systemically insulin resistant, and they were fed oral lipid isotopes in conjunction with a four day low-fat (30%), high carbohydrate (55%, of which 72% was mono or di-saccharides) diet. Fifty nine percent of hepatic triglycerides arose from NEFA, 26% from *de novo* lipogenesis (DNL), and 15% directly from recent diet intakes of fat. It is notable that most of the hepatic triglycerides appeared to have originated from peripheral or visceral adipocyte stores via NEFA, with a relatively small recent dietary fat contribution. This does not

minimise the importance of diet intakes as ultimately the source of all of the lipogenic pathways is dietary.

Interestingly the observed rate of DNL in NAFLD by Donnelly *et al.* was greater than previously described in healthy subjects (Schwarz, Linfoot et al. 2003). Strikingly this increase in DNL rates was mainly in the fasted state, with a relative lack of post-prandial DNL stimulation. There are two potential interpretations of these observations. Firstly it may have purely resulted from the high carbohydrate diet consumed in the Donnelly protocol. Secondly it may be that the hepatic lipogenic capacity in NAFLD is persistently and maximally upregulated. If the latter is true then reducing DNL substrate availability, mainly glucose and fructose, may be a key step in NAFLD prevention or therapy. It was not possible to assess the specific influence of insulin in this study, though a blunted diurnal pattern in DNL has previously been noted in insulin-resistant subjects (Schwarz, Linfoot et al. 2003).

There are however some limitations in interpreting the findings of this excellent study. Firstly, only 38% of hepatic triglycerides on biopsy had their source accounted for. Furthermore, there was no healthy control and so the findings may not be NAFLD-specific. Finally, as acknowledged by the authors, the DNL contribution to hepatic triglyceride was augmented by the high carbohydrate intake, and the low direct dietary contribution may in part reflect the low fat intake.

Comparisons between NAFLD and healthy volunteer lipid kinetics have been made though in a less rigorous manner. Obese NAFLD patients have been shown to have rates of hepatic DNL 3-fold greater than slim healthy subjects, with a blunted post-prandial response also noted (Diraison, Moulin et al. 2003). Fabbrini et al. compared the rates of NEFA release from adipocytes into the circulation, and the rates of hepatic VLDL-triglyceride and VLDL-apoB100 secretion, in 14 NAFLD patients and 14 age, sex, BMI and body fat percentage matched non-steatotic controls (Fabbrini, Mohammed et al. 2008). There was a greater NEFA release in NAFLD, but unfortunately no assessments of DNL were made. Interestingly there was a greater hepatic VLDL secretion rate with NAFLD which reached a maximal plateau. Indeed the rate of VLDL-apoB100, reflecting the number of VLDL particles secreted, was not different between the groups. So it appears that the capacity for the liver to export triglyceride had been exceeded in NAFLD.

Taken together these data suggest that NAFLD results from persistently elevated rates of DNL both in the fed and fasted state, greater NEFA delivery to the liver and impaired VLDL exportation. The influence of oxidation remains uncertain.

The two key clinical associations with steatosis are insulin resistance and visceral obesity, and they underpin many of the above observations. Genetic status has also recently been implicated in NAFLD development. These factors will now be discussed.

1.5.1.1.   Hepatic steatosis and insulin resistance

Insulin drives an anabolic state by promoting lipid and carbohydrate storage and protein synthesis (Choudhury and Sanyal 2004). These actions are balanced by the actions of the catabolic hormones glucagon, adrenaline and growth hormone. The term 'insulin resistance' is generally used to describe an impaired ability to clear glucose from the systemic circulation into tissues. Clinically significant insulin resistance results in type 2 diabetes. However due to its plethora of actions, the term insulin resistance can be classified as being both tissue and action specific. It is standard to define insulin resistance by its carbohydrate as opposed to lipid or amino acid actions, and it is these actions that will be principally referred to in this thesis.

The term 'systemic insulin resistance' refers to disruption of normal insulin mediated peripheral glucose uptake and utilisation such that normoglycaemia can only be achieved by an increase in insulin secretion to overcome the peripheral tissues resistance to the action of insulin. This can be assessed by measuring fasting glucose concentrations alone or in combination with insulin. It can also be assessed by determining the plasma glucose response to a glucose or insulin load. This is further discussed in the methods section.

The term 'hepatic insulin resistance' refers to an impairment in suppressing fasting hepatic glucose production (Seppala-Lindroos, Vehkavaara et al. 2002). The rate of this is more difficult to assess, with no test available in the routine clinical arena. The most common assessment involves tracer labelled glucose infusions, as will be described in the methods chapter.

Finally 'adipocyte insulin resistance' refers to the sensitivity of adipose tissue to insulin-mediated suppression of lipolysis. Lipolysis is the process

whereby stored trigylcerides are hydrolysed into diacylglycerols (DAGs), then monoacylglcerols and finally into NEFAs and glycerol. Insulin potently inhibits the lipolytic actions of hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and monoacylglycerol lipase (MGL) (Lafontan 2008). Adipocyte insulin resistance is therefore assessed by the glycerol and NEFA response to insulin administration.

The differentiation between these terms is a fundamental metabolic concept. Hepatic insulin resistance results in increased hepatic glucose production by reducing rates of glycogen synthesis in association with increased rates of glycogenolysis and gluconeogenesis (Choukem and Gautier 2008). In an attempt to maintain normoglycaemia there is a matched rise in pancreatic insulin secretion. So combined hepatic and systemic insulin resistance results in elevated circulating concentrations of both insulin and glucose. The hepatic consequences of insulin resistance are:

1. Increased lipogenesis via insulin: Insulin directly up-regulates the transcription and proteolytic maturation of the transcription factor SREBP-1c. This action is independent of the insulin receptor (IRS-1) that mediates insulin's glucose influence (Shimomura, Bashmakov et al. 1999; Sajan, Standaert et al. 2004). SREBP-1c induces a family of lipogenic genes within the liver including fatty acid synthase (FAS) (Dentin, Benhamed et al. 2006).

2. Increased lipogenesis via hyperglycaemia: The elevated glycaemia directly up-regulates nuclear translocation of carbohydrate responsive element-binding protein (ChREBP) (Ishii, Iizuka et al. 2004). CHREBP acts in synergy with SREBP-1c to further induce lipogenic gene expression and provide the precursors for lipogenesis by also inducing glycolytic gene expression (Denechaud, Dentin et al. 2008).

3. Increased NEFA delivery to the liver: Adipocytes are normally exquisitely insulin sensitive (Reaven 1997). Adipocyte insulin resistance results in a reduced suppression of lipolysis, and thus an increased NEFA release into the circulation and hepatic uptake. Of note there appears to be no regulation of the rate of NEFA uptake by the liver.

4. Decreased VLDL excretion: Insulin attenuates apolipoprotein B, and hence VLDL, formation (Lewis, Uffelman et al. 1993). This impairs hepatic triglyceride exportation ability. The mechanism behind this frequently observed phenomenon has not been fully determined, though may it be occur via microsomal triglyceride transfer protein (MTP) down regulation (Lin, Gordon et al. 1995; Olofsson and Boren 2005). MTP is a critical determinant of triglyceride and apolipoprotein B incorporation into VLDL and its subsequent plasma excretion (Hussain, Shi et al. 2003). MTP-specific inhibition reduces plasma triglycerides and VLDL, whilst increasing hepatic triglycerides (Chandler, Wilder et al. 2003).

From the above actions it is clear that insulin resistance is a key factor in hepatic lipid balance and steatosis development. In response to a high carbohydrate intake, insulin resistant subjects have a much greater rate of hepatic DNL and reduced rate of muscle glycogen synthesis than insulin-sensitive controls (Petersen, Dufour et al. 2007).

When rigorously assessed most non-diabetic NAFLD patients have sub-clinical insulin resistance with evidence of impaired glucose peripheral uptake. Sub-clinical systemic and hepatic insulin resistance correlates strongly with intra-hepatic steatosis (Seppala-Lindroos, Vehkavaara et al. 2002; Sakurai, Takamura et al. 2007; Korenblat, Fabbrini et al. 2008). Indeed hepatic insulin resistance can pre-date the development of systemic insulin resistance, but this is rarely assessed as it is both laborious and invasive (Kraegen, Clark et al. 1991).

The above accepted, there is clearly more to NAFLD than purely being the hepatic consequence of insulin resistance. Most NAFLD patients do not have overt systemic insulin resistance i.e. type 2 diabetes, and only around 50% of type 2 diabetics have NAFLD (Choudhury and Sanyal 2004; Targher, Bertolini et al. 2007).

1.5.1.2. Hepatic steatosis and obesity

Obesity is the most frequently observed clinical association with NAFLD, with studies reporting its presence in 25% to 93% of patients (Ong and Younossi 2007). As previously discussed the anatomical distribution of adipose tissue determines its venous drainage and hence its metabolic effects. Using tracer methods, Nielsen *et al.* demonstrated a direct correlation between the degree of

visceral fat and the post-absorptive NEFA delivery to the liver (Nielsen, Guo et al. 2004). Furthermore, a review of clinical and epidemiological studies demonstrated a direct association between visceral and hepatic fat content (Jakobsen, Berentzen et al. 2007).

The association between visceral obesity and steatosis appears however to be more than just increased NEFA delivery. The key inflammatory regulators of hepatic metabolic homeostasis are primarily adipocyte derived, and are the pro-inflammatory cytokine TNFα and the anti-inflammatory adipokine adiponectin (Tilg and Hotamisligil 2006). Visceral adiposity and NAFLD are independently characterised by an increased TNFα and reduced adiponectin production. Of note this pattern not only induces a pro-inflammatory, but also an insulin-resistant, state (Schaffler, Scholmerich et al. 2005). To summarise, visceral adiposity results in an increased portal concentration of NEFA, a pro-inflammatory cytokine profile and insulin resistance. The influences of adiposity and insulin resistance on hepatic metabolism are therefore closely entwined.

1.5.1.3.   Hepatic steatosis and genetics

Recently a non-synonymous sequence variation I148M (rs738409) in the PNPLA3 gene has been shown to have a homozygous population prevalence of 7.5%, and to confer NAFLD susceptibility (Romeo, Kozlitina et al. 2008). PNPLA3 is a 481-residue protein with *in vitro* lipase activity which is highly expressed in the liver (Lake, Sun et al. 2005). This association has been reproduced by several other groups. PNPLA3 status does not appear to influence systemic insulin resistance, but may exert its effect via limiting hepatic hydrolysis of triglycerides (He, McPhaul et al. 2009; Kantartzis, Peter et al. 2009; Romeo, Sentinelli et al. 2009).

Recent data however shows that the presence of a mutated PNPLA3 genotype adds little to clinical markers for predicting hepatic steatosis (Kotronen, Peltonen et al. 2009). Whereas there is evidence for greater liver injury with a mutated genotype, possibly via increased lipotoxicity (Sookoian, Castano et al. 2009). The precise role and relevance of this mutation thus awaits full determination.

This whole field has been widened following the recent publication of a genome wide association study in NAFLD (Chalasani, Guo et al. 2010). An

inheritability of NAFLD was placed at around 27% with three associations made with steatosis: variants near PNPLA3, NCAN and PPP1R3B and with inflammation or fibrosis, and in or near PNPLA3, NCAN, GCKR, LYPLAL1 but not PPP1R3B. As with all GWAS studies, more questions than answers were generated but it does provide a fascinating insight into the critical genetic and molecular pathways that may become future therapeutic targets. PPP1R3B region encodes for a protein that regulates glycogen breakdown. The role of LYPLAL1 is unclear though it may have a role in triglyceride breakdown. NCAN encodes for an adhesion molecule.

Interestingly GCKR encodes glucokinase regulatory protein (GKRP), which regulates glucokinase activity. The role of glycolysis in NAFLD will be discussed in full detail later. Glucokinase initiates glycolysis and hence glucose storage or disposal in the liver. A common variant in GKRP is the P466L mutant. This mutant has recently been shown to result in reduced regulation by physiological concentrations of F6P (Beer, Tribble et al. 2009). So there was increased GCK activity. This mutant therefore could result in increased rates of de novo lipogenesis, though there are no data to support or refute this at present.

1.5.2. 2nd hit – Steatohepatitis development

The presence of steatosis plus inflammation, cellular ballooning and/or fibrosis is termed non-alcoholic steatohepatitis (NASH). The processes implicated in NASH development include a pro-inflammatory cytokine profile, generation of reactive oxygen species, and decreased hepatic ATP production (Perez-Carreras, Del Hoyo et al. 2003).

Mitochondrial dysfunction is centrally implicated in the development of NASH (Pessayre 2007). Re-entry of protons back into the mitochondria via ATPase is the final step in ATP generation by the electron transport chain. Uncoupling proteins (UCPs) bypass ATPase and hence the protons re-enter the mitochondria with energy generated as heat as opposed to ATP. Despite the discovery of UCP2 in 1997, its precise physiological role remains highly contentious (Yonezawa, Kurata et al. 2009).

Animal models of NASH are characterised by a progressive reduction in hepatic ATPase activity and ATP concentrations, in association with an increase in UCP2 expression and markers of oxidative stress (Chavin, Yang et al. 1999;

Serviddio, Bellanti et al. 2008). Progressive histological clinical studies have been rarely done, though patients with NASH have greater hepatic oxidative stress and UCP2 expression than healthy controls (Serviddio, Bellanti et al. 2008).

The aetiology and pathways behind these changes in NASH is uncertain, though increased UCP2 expression has been speculated as being an attempt to remove excess energy stores, or an attempt to minimise oxidative stress by limiting mitochondrial reactive oxygen species generation (Pessayre 2007; Serviddio, Bellanti et al. 2008). This uncoupled and ATP depleted state however leaves the liver vulnerable to external injuries (Caraceni, Bianchi et al. 2004).

As previously mentioned, the balance of circulating pro and anti-inflammatory cytokines plays a key role in hepatic metabolism and inflammatory status. These cytokines are mainly visceral adipocyte derived though may also be hepatically sourced. Hepatic production of pro-inflammatory cytokines is stimulated by oxidative stress and circulating pro-inflammatory cytokines (Day 2006).

As a result of this systemic inflammatory status oxidative stress can be detected in the plasma of NASH patients (Yesilova, Yaman et al. 2005; Madan, Bhardwaj et al. 2006). These observations have led to the search for systemic biomarkers of NASH and fibrosis as an alternative approach to the invasive liver biopsy (Guha 2009).

1.6. Medical and weight loss therapy for NAFLD

Several therapeutic strategies exist for this condition. To summarise, no therapy has been shown to fulfil the following criteria: effective, safe, and improves long-term prognosis. As of yet there is no approved medical therapy. Given the high prevalence and relatively benign prognosis for many, the potential for any toxicity is a major concern in what will need to be a long-term therapy. The experiences from the differing approaches are presented below:

1.6.1. Lifestyle and weight loss therapy

Diet and lifestyle influence NAFLD development primarily via their effects on visceral adiposity and insulin resistance and so are logically central to a NAFLD therapeutic strategy (Harrison and Day 2007). The American Gastroenterology Association recommends the first line strategy for overweight

NAFLD patients to be a target weight loss of 10% (AGA 2002). There is however scant clinical evidence to support this recommendation outside the bariatric surgery experience and in practice such weight loss is rarely achieved or maintained. To date there is liver biopsy data assessing the efficacy of weight loss from only 5 studies containing less than 150 patients (Bellentani, Dalle Grave et al. 2008). As a result there is inadequate data to define what the ideal macro- and micro-nutrient structure should be of such weight loss diets, or how much weight should be lost, and at what rate.

A recent small trial of 21 NASH patients who prospectively underwent either direct dietetic review on 39 occasions in 12 months to promote a low energy and fat diet, behavioural strategies and exercise or no advice in the controls (Promrat, Kleiner et al. 2010). The intervention arm lost 9.8% of their original weight, and none in the control arm. Insulin sensitivity was unchanged, but there were greater reductions in the NAFLD activity score (NAS) in the repeat biopsies of the intervention as opposed to the control arm. Weight loss of more than 7% appeared to be the most beneficial. A previous uncontrolled study involved similarly highly intensive support but only managed to result in 3.3% weight loss (Huang, Greenson et al. 2005). There was some reduction in inflammation, but nil in steatosis or fibrosis. These data clearly describes the potential benefits and also the logistical difficulties in generating meaningful long-term weight loss in NAFLD patients.

The experience of combined diet and exercise regimens is a little more encouraging, though again the studies frequently lack a control arm. Ueno *et al*. introduced energy restriction and increased activity by jogging in 25 patients for three months (Ueno, Sugawara et al. 1997). A mean reduction of BMI by 3kg/m^2 resulted in reduced steatosis on repeat biopsy. There have been several other lifestyle studies, though they have used indirect markers of liver content as opposed histological or ^{1}H MRS evaluation, and so only limited conclusions can be deduced from them (Harrison and Day 2007).

NAFLD has been frequently assessed in bariatric surgery patients. A liver biopsy is simple to perform at the time of surgery, and the vast majority have steatosis. The largest initial cohort was performed by Mathurin *et al*. who repeated liver biopsies in 121 patients 1 year following bariatric surgery (Mathurin, Gonzalez et al. 2006). There was a mean weight loss of 27kg over this year. Steatosis was significantly reduced, but alarmingly fibrosis increased. This same

group then presented similar data from a larger cohort of 381 patients followed up for 5 years (Mathurin, Hollebecque et al. 2009). This time there was evidence of improved steatosis and a non significant reduction in mean fibrotic score and it was shown that a lack of long-term histological improvement could be predicted by a failure in early improvement in systemic insulin resistance.

Orlistat induces fat malabsorption. It results in no greater changes in NAFLD histological outcomes than weight loss from an energy restricted diet (Harrison, Fecht et al. 2009)..

1.6.2. Promoting insulin sensitivity and antioxidants

After addressing lifestyle and adiposity, the next logical approach is to promote insulin sensitivity. Thiazolidinediones (TZDs) act via peroxisome proliferator-activated gamma (PPARγ) agonism to directly promote insulin sensitivity. TZDs induce fat store transfer from liver and muscles to peripheral adipocytes. Patients therefore gain peripheral weight, but become more systemically insulin sensitive. All three published randomised placebo controlled studies describe histological improvement with TZD therapy, and these findings are irrespective of whether the patient is diabetic or not (Belfort, Harrison et al. 2006; Aithal, Thomas et al. 2008; Ratziu, Giral et al. 2008). Unfortunately the favourable effects on liver biochemistry and histology are not continuous with rosiglitazone as there are no additional improvements after the first year of treatment (Ratziu, Charlotte et al. 2010), it is not known if this is the same with pioglitazone which does appear to have importantly different biological activity. Furthermore, any improvements revert on the discontinuation of TZDs, and so in order to maintain the initial benefits TZDs would have to be long-term therapies (Lutchman, Modi et al. 2007). This is an issue as there are considerable concerns over the side effects of TZD therapy including congestive cardiac failure, fluid retention and osteoporosis (Rizos, Elisaf et al. 2009).

The story has recently become even more interesting. Until very recently there was no histological data to support the use of antioxidants (Duvnjak, Lerotic et al. 2007). The latest TZD trial randomised 247 biopsy proven NASH patients to either 30mg per day of pioglitazone (a TZD), vitamin E (800IU per day), or placebo (Sanyal, Chalasani et al. 2010). Repeat biopsy was performed in 90% at 96 weeks with the aim to re-assess histological injury. Pioglitazone did not fulfil the pre-determined histological primary end-point and so this can be interpreted as

a negative study. The interpretation of this data however is fraught as many patients in the pioglitazone arm were recruited on the basis of having NASH, though on subsequent central pathologist review did not have adequate injury on their initial biopsy to justify their initial entry into the study. As a result it was impossible to demonstrate injury regression in those without injury at baseline. Fascinatingly vitamin E resulted in no side effects and 43% showed a reduction in liver injury to meet the primary end-point. This was opposed to 34% for pioglitazone and 19% for placebo. These vitamin E findings are currently being reproduced by other centres. Indeed there is some concerns re vitamin E safety with some highly controversial data suggesting that vitamin E intakes greater than 400IU/day may be associated with an increased mortality (Miller, Pastor-Barriuso et al. 2005).

1.6.3. Dyslipidaemia treatments

Clofibrate, a PPARα agonist, does not improve liver histology after one year (Laurin, Lindor et al. 1996). The HMG CoA reductase inhibitor atorvastatin has been used in three small studies with a combined total of 56 patients (Duvnjak, Lerotic et al. 2007). There is evidence of histological improvement, but this needs to be reproduced in a placebo controlled large-scale setting before it becomes an accepted therapy.

1.7. Interactions between diet and NAFLD

Observations detailed below have lead many authors to speculate on the role of specific dietary macronutrient intakes as opposed to simply energy excess in the aetiology and treatment of NAFLD (Cave, Deaciuc et al. 2007; Sullivan 2009). Ultimately all hepatic lipids originate from dietary intakes of carbohydrates or lipids. Furthermore, dietary contents have been shown to be powerful inducers and suppressors of the pathways central to hepatic fatty acid metabolism.

1.7.1. Dietary history assessments in NAFLD

Dietary assessments are frequently performed at the time of bariatric surgery but are poor indicators of long-term intake patterns. Such patients are keen to report and perform weight-loss promoting behaviour which commonly includes energy or macronutrient restriction. As a result only the non-bariatric diet history experience will be discussed here. There have been seven main studies comparing the diets of non-bariatric surgery NAFLD patients with that of healthy

controls (Musso, Gambino et al. 2003; Cortez-Pinto, Jesus et al. 2006; Zelber-Sagi, Nitzan-Kaluski et al. 2007; Allard, Aghdassi et al. 2008; Thuy, Ladurner et al. 2008; Abid, Taha et al. 2009; Oya, Nakagami et al. 2010). The findings are summarised in table 1. Only two of these studies however have used body mass index (BMI) matched controls. The reliability of this data in terms of underreporting of nutrient intakes is not presented by any paper. It would appear to be an issue as the mean predicted energy intakes (Schofield formula (Schofield 1985)) needed for a 30-59 year old weighing 85kg and a standard activity factor of 1.6 is 2960kcal/day in men and 2482kcal/day in women. This is not adjusted or accounted for in any of the assessments.

	Musso	Cortez-pinto	Thuy	Allard	Abid	Oya	Zelber-Sagi
Weight (kg)	80	83	-	92	-	-	-
BMI	26	31	28	32	30	25.0	30
Reported energy intake (kcal/day)	2638	2253	2242	2151	2186	1952	2493
Energy	↔	↔	↔	↔	↔	↔	↔
Total Protein	↔	↓	↔	↔	↔	↔	↔
Total Fat	↔	↑	↔	↔	↔	↔	↔
Total Carbohydrate	↔	↓	↔	↔	↔	↔	↔
SFA	↑	↓	.	↔	.	↔	↔
MUFA	↔	↑	.	↔	.	-	↔
PUFA	↓	↔	.	↔	.	-	↔
n-3 PUFA	.	↔	.	↔	.	↓	↔
n-6 PUFA	.	↑	.	.	.	-	
Simple carbohydrates	↔	↔	.	↔	↑	-	↑
Fibre	↓	↔	.	↔	.	-	↔
Fructose	.	.	↑	.	.	-	.

Table 1: Macronutrient intake patterns in NAFLD as compared to healthy controls. (Abbreviations used: SFA = Saturated fatty acid; MUFA = Monounsaturated fatty acid; PUFA = Polyunsaturated fatty acid. Significance determined at p<0.05, the symbol . means that there was no data supplied).

Musso *et al*. analysed seven-day food records from 25 Italian patients with NAFLD (Musso, Gambino et al. 2003). These were compared with records from 25 sex, age and BMI-matched healthy controls who had no evidence of NAFLD on liver enzymes and abdominal ultrasound. The individuals studied were trained to use dietary records, and their responses were analysed by published Italian food composition databases. The reported dietary intakes by the two groups were matched in terms of overall energy, total fat and carbohydrate intakes. The NAFLD patients however reported significantly greater intakes of daily saturated fatty acids (40g vs. 29g, p=.001), and reduced intakes of polyunsaturated fatty acids (10.3g vs. 13.4g, p=.019), and fibre (12.9g vs. 23.2g, p<.001).

Cortez-Pinto *et al*. used a semi-quantitative food frequency questionnaire (FFQ) to analyse food intakes over the preceding year in 45 NAFLD patients and 856 controls in Portugal (Cortez-Pinto, Jesus et al. 2006). The controls were age and sex balanced to the patients, though were neither BMI-matched nor screened for the absence of NAFLD. A further issue was that the 82-item FFQ has not been adequately validated and was unpublished. The data was BMI-adjusted, although, as the unadjusted data was not presented, it is unclear as to what effect this had. The findings in part conflict with those of Musso *et al*. Again there was no difference in terms of energy intakes, but the NAFLD patients consumed less carbohydrates (243g vs. 262 g, p=0.003), a similar amount of fibre (24.1g vs. 22.6, p=NS), a tendency to a greater amount of simple sugars (109g vs. 101g, p=NS), and a greater amount of total fat (79.7g vs. 73.0g, p<0.001). The fatty acid profile was assessed in greater detail than in the work by Musso *et al*., and identified a greater intake of n-6 polyunsaturated fatty acids (PUFAs) by the NAFLD patients (9.3g vs. 8.3g, p=0.003) and an identical combined long and short chain n-3 PUFA intake of 1.4g per day between the two groups.

12 biopsy proven NAFLD patients and 6 opportunistically obtained and neither sex nor BMI matched healthy controls had a dietary history analysed by an undisclosed method (Thuy, Ladurner et al. 2008). Energy adjusted data was not presented. The groups had identical macronutrient intakes including glucose, though fructose intakes were significantly greater in the NAFLD patients.

Allard et al. analysed 7 day food diaries and liver biopsy fatty acid profiles in 73 patients with elevated liver enzymes with a normal viral, genetic, autoimmune, medication and alcohol screen and hence assumed to represent NAFLD (Allard, Aghdassi et al. 2008). Seventeen had a normal biopsy, 18 simple steatosis and 38 had NASH. The NASH patients had a significantly greater BMI. There was no difference in macronutrient intakes between the three groups.

Two Israeli studies have assessed general intakes but focused on the intake of simple sugars in NAFLD patients. Zelber-Sagi *et al*. identified 349 people randomly selected from the Israeli National Health and Nutrition Survey, who were all negative on screening for alcohol abuse, viral or drug induced liver disease (Zelber-Sagi, Nitzan-Kaluski et al. 2007). They then divided their cohort into 108 individuals who had evidence of NAFLD on ultrasound, and 241 who did not. The benefits of this approach were that it was community based, and that it excluded the principal non NAFLD liver diseases. The limitation was that the

NAFLD arm contained more men and a greater BMI. The investigators interviewed all of the subjects face-to-face using a semi-quantitative 111-itemed FFQ. Multivariate analyses adjusting for age, sex, BMI and total energy intake demonstrated greater intakes amongst the NAFLD cohort of sugars from soft drinks (odds ratio 1.45 (1.13-1.85), p=0.005) and a tendency for reduced intakes of n-3 PUFA containing fish (odds ratio 0.73 (0.52-1.04), p=0.08).

Abid *et al*. recruited 60 patients with abnormal liver biochemistry and a clinical and ultrasound diagnosis of NAFLD (Abid, Taha et al. 2009). Seven day food records and activity levels were compared to those of 30 age, sex and BMI matched controls. There was no difference between the groups in terms of overall energy intake, macronutrient contribution to energy intakes, or physical activity. The mean daily intake of added sugars was greater in the NAFLD patients at 75g vs. 30g (p<0.001) and the majority (43%) of this originated from sugar-sweetened soft drinks. Unfortunately although the controls had normal liver biochemistry, they were not further assessed for the absence of NAFLD.

A Japanese group assessed for dietary factors predictive of NASH as opposed to simple steatosis. They compared 3 day food records of 18 patients with biopsy proven simple steatosis and 28 with NASH (Toshimitsu, Matsuura et al. 2007). Both arms had similar BMI profiles. Diets associated with NASH had a greater energy contribution from carbohydrates and sugars, and reduced protein, zinc and PUFA intakes.

Finally another Japanese group compared intake patterns of nearly 800 apparently healthy alcohol abstinent adults, with a normal 'liver screen' including viral studies, who they subdivided by gender and the presence or absence of a fatty liver on ultrasound scan (Oya, Nakagami et al. 2010). The subjects were recruited from a hospitals health check-up programme and dietary data collated by a Japanese foodstuff food frequency questionnaire. The percentage dietary energy contribution of all n-3 PUFAs, and EPA and DHA were lower in men with steatosis on ultrasound scan than those with a normal scan. There was no such difference amongst the women. These associations persisted after adjustments for age, waist and physical activity levels. There was however some energy underreporting in the patients with steatosis as despite having a greater BMI the men reported similar, if not lower, energy intakes and similar physical activity levels.

Using an entirely different approach to the previous studies, Kechagias *et al*. performed a fascinating study by analysing the hepatic effects of short-term fast-food overfeeding with intakes twice that of normal and exercise restriction to less than 5,000 steps per day (Kechagias, Ernersson et al. 2008). Over 4 weeks 18 healthy volunteers had a mean weight gain of 9.5%. Serum alanine transferase (ALT), a marker of hepatic injury, increased by 2.1-fold, and intra-hepatic lipid stores by 1.5-fold. Nutrient intakes prior to and during the intervention period were assessed by 3 day food diaries. The increases in ALT correlated with monosaccharide intakes, with no correlation with protein or fatty acids. There was no further sub analysis of the influence of individual macronutrients presented. As highlighted by my recent communication, this study however had several flaws (Johnston, Aithal et al. 2009). Firstly, pre-existing liver disease was not excluded from the cohort, and secondly the intervention lacked a control. Therefore the authors' conclusions that the findings are directly attributable to fast-food are not tenable.

In summary there is no dietary intake pattern that has been repeatedly shown to be characteristic for NAFLD. There are flaws in the data collection processes and degree of nutrient analyses in all of these studies, so a large scale and robustly performed study is urgently needed. The only macronutrients implicated in two or more studies are n-3 and n-6 PUFAs and simple carbohydrates and fructose. These limited observations form a key part of the background to this thesis.

1.7.2. Dietary assessment in NAFLD employing liver biopsy lipid analyses

An alternative method to assess hepatic exposure to dietary fatty acids is to quantify liver biopsy fatty acid profiles. This process assumes that hepatic fatty acid profiles directly correlate with dietary intakes, and that the liver handles the different fatty acid subtypes in the same manner during health as in disease. Inflammation and oxidative stress could alter synthesis from precursors, or metabolism of complex lipids including peroxidation. Therefore any observations may be either cause or effect of the disease. The above assumptions accepted lipidomic analyses remain an interesting nutritional biomarker assessment technique in assessing the interactions of nutrients and hepatic fatty acid metabolism. The published data however has failed to produce a clear picture.

Araya *et al*. analysed liver fatty acid profiles by gas liquid chromatography in 19 NAFLD patients and 11 non-BMI matched controls (Araya, Rodrigo et al. 2004). The relative contribution of saturated and monounsaturated fatty acids did not significantly differ, whereas there was a significant reduction in total polyunsaturated fatty acids (PUFAs) in the NAFLD patients (18.7% vs. 33.0%, $p<0.05$). Within the polyunsaturated fatty acids, there was reduced n-3 PUFAs (1.6% vs. 7.2%, $p<0.05$), and reduced n-6 PUFAs (16.8% vs. 25.5%, $p<0.05$). The reduction in n-3 PUFAs was more marked than in the n-6, such that the n-6 to n-3 PUFA ratio was greater in the NAFLD livers as opposed to the controls (11.5 to 1 vs. 3.6 to 1, $p<0.05$). The difference in n-3 PUFA content was more marked in the longer chain fatty acids with a 5.2 fold reduction in DHA in the NAFLD livers.

Puri et al. performed a similar analysis from 18 NAFLD patients and 9 age, sex and BMI-matched controls (Puri, Baillie et al. 2007). An identical data pattern to that described by Araya *et al*. was generated in terms of the saturated, monounsaturated and polyunsaturated fatty acid profiles including n-3 and n-6 PUFA. This study validated Araya's prior work with a more rigorously selected control group.

As previously described, Allard et al. analysed liver biopsy fatty acid profiles in 73 patients with elevated liver enzymes (Allard, Aghdassi et al. 2008). Patients with simple steatosis had greater n-6 PUFA content than those with normal biopsies, whilst NASH was characterised by a mild increase in monounsaturated fatty acid composition, and a marked long-chain n-3 PUFA

depletion. The combined EPA and DHA composition was 0.8% in normal biopsies, 0.4% in simple steatosis, and 0.2% in NASH.

From the above studies the dietary trends in NAFLD patients appear to involve increased intakes of simple sugars, and sugar-sweetened drinks, as well as reduced intakes of PUFAs in particular n-3 PUFAs. These dietary observations have not been universally shown, though are supported by other observations. As a result this thesis will focus on fructose and glucose, the principal components of added sugars in drinks, and on n-3 PUFAs.

1.8. Polyunsaturated fatty acids

1.8.1. Classification of polyunsaturated fatty acids (PUFAs)

Thirty-five percent of total energy intake in the UK adult diet originates from fatty acids (Swan 2004). Fatty acids are classified by their number of double bonds, with saturated fatty acids possessing none, monounsaturated possessing one, and polyunsaturated fatty acids (PUFAs) possessing two or more *cis* double bonds, see 3.1.

PUFAs are further sub-classified by the location of their first double bond starting from the methyl end of the chain. In n-3 PUFAs this double bond is at the third carbon-carbon bond from the methyl end, and is at the sixth bond for n-6 PUFAs, see figure 1.3. These two types of PUFAs have distinct and commonly opposing physiological actions. The majority of dietary PUFA intake is in the forms of the 18-carbon linoleic acid (n-6) (LA), and the 18-carbon α-linolenic acid (n-3) (ALA).
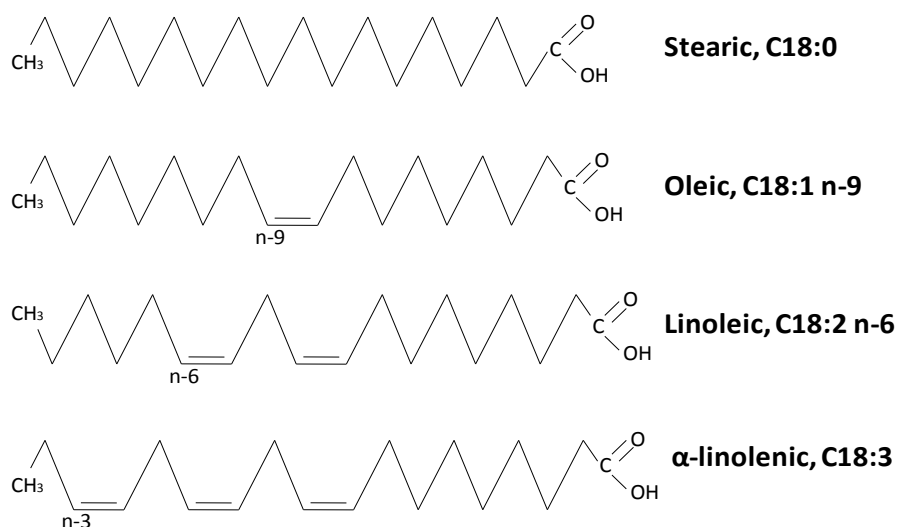
Figure 1.3.: Names and structures of common 18-carbon chained saturated (stearic), monounsaturated (oleic), n-6 polyunsaturated (linoleic) and n-3 polyunsaturated (α- linolenic) fatty acids. Adapted from (Mann 2007).

1.8.2. Dietary sources of n-3 PUFAs

PUFAs are generated within the chloroplasts of the primary producers at the bottom of both the marine and terrestrial food chains. The primary producer in the marine world is phytoplankton. The PUFAs are absorbed, assimilated and passed directly up the food chain. The so-called 'oily' or pelagic fish, such as

mackerel, predominantly store oils in their fillets, whereas 'white' or demersal fish, such as cod, store oils in their liver.

The handling of n-3 PUFAs differs in terrestrial food chains. Green-leaf vegetables generate a small but significant amount of short-chain PUFAs. This PUFA content is however not passed on and concentrated up the food chain, as microorganisms within the rumen of herbivorous ruminants hydrogenate PUFAs to saturated fatty acids. The herbivorous animal therefore may eat a relatively PUFA rich meal, but absorbs predominantly saturated fatty acids from its intestinal tract. Vegetable oils do contain a relatively high PUFA concentration, though this is principally only the short chain n-3 PUFA α- linolenic (ALA) as opposed to the more biologically important long chain n-3 PUFAs such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) found in fish.

Intakes of PUFAs contribute only around 6% of adult dietary energy in the UK, with intakes of both long and short-chain n-3 PUFA ranging from 0.7g/day to greater than 4.5g/day (Swan 2004). It is however the long-chain PUFAs that are of principal interest. Givens and Gibbs recently analysed UK dietary intakes and sources for the long-chain EPA and DHA from published food disappearance databases (Givens and Gibbs 2008). They estimated that the average intake was 244mg/day of EPA and DHA, and that 75% originates from fish, 6% from shellfish, 15% from meat, and 4% from eggs. There are no UK data assessing self-reported dietary intakes of long chain n-3 PUFAs. French self-reported data describes reported EPA and DHA intakes of 384mg per day (Astorg, Arnault et al. 2004).

1.8.2.1. Intake trends for PUFAs and the n-6 to n-3 ratio

Given the frequently opposing n-6 and n-3 PUFA activities, the concept of an n-6 to n-3 PUFA ratio has emerged. Anthropological, nutritional and genetic data indicate that the human diet has changed significantly over the past 10,000 years (Simopoulos 2006). The Paleolithic diet (400,000 to 45,000 years ago) contained mainly wild plant leaves, seeds, fish and animals with an estimated n-6 to n-3 PUFA ratio of 1:1 (Eaton and Konner 1985). Intakes have gradually changed since then, though in the 150 years since the industrial revolution the onset of food technology and agribusiness has rapidly changed global fat intake patterns. Vegetable oil is a major modern industry, and domestic livestock now

have a very high cereal diet. United States foodstuff disappearance data describes an increase in the n-6 to n-3 PUFA ratio from 8.4 to 1 in 1935 to 10.6 to 1 in 1994 (Kris-Etherton, Taylor et al. 2000).

1.8.2.2. Determining adequacy of long-chain n-3 PUFA intakes

The short-chain n-3 PUFA alpha linolenic acid (ALA) is critical in maintaining the skin's barrier function. Symptomatic deficiency with dermatitis, growth retardation and infertility is rare, occurring only in those with severe unresolved fat malabsorption and starvation (Bjerve, Fischer et al. 1987; Lauritzen, Hansen et al. 2001). No clinical features have been specifically ascribed to long chain n-3 PUFA deficiency (Benatti, Peluso et al. 2004), though the role of DHA in retinal and neuronal function is an area of intense current study (Hoffman, Boettcher et al. 2009). So in contrast to most other micronutrients, it is the benefits of an increased intake of long chain n-3 PUFAs as opposed to the prevention of deficiency that poses the primary interest. As a result of this unusual nutrient health status, the determination of adequacy of intakes is a highly contentious area and recommendations vary dependent upon the committees' review dates, health priorities, and data interpretation, see table 2. Of importance, there are no toxicity or maximal intake recommendations. The recently estimated UK average intake of 244mg/day is below all bar one of the recommendations (Givens and Gibbs 2008).

Country	Year	Recommended EPA + DHA intake	Advisory body
UK	1994	200	Department of Health (1994)
Global	2003	500	WHO/ Food and Agriculture Organisation (WHO 2003)
UK	2004	450	Scientific Advisory Committee on Nutrition/ Committee on Toxicity (Nutrition 2004)
Global	2004	500	International Society for the Study of Fatty Acids and Lipids (Lipids 2004)
USA	2005	270	Institute of Medicine (IOM 2005)

Table 2: Recommended adult daily EPA and DHA intakes (mg/day)

1.8.3. Bioavailability of long chain PUFAs

Long and short chain PUFAs are considered to be essential dietary components as they cannot be endogenously synthesised by the human body and so must be obtained from the diet. Absorbed PUFAs can however be inter-converted. The conversion of the prevalent short chain ALA to the longer chain EPA and DHA occurs primarily within the endoplasmic reticulum of the liver. It involves a series of alternate desaturation and elongation reactions that respectively insert double bonds and 2-carbon units, see figure 1.4. From the base chain length of 18 carbons, chain lengths of 20, 22 or 24 carbons are subsequently formed. Identical enzymes catalyse these reactions for n-3 and n-6 PUFAs. As a result direct competition exists between these two metabolic pathways, dependent on enzyme and substrate availability. Tracer studies using ^{13}C or ^{2}H-labelled ALA demonstrate that 15-35% of dietary ALA is oxidised to form energy, and the fractional conversion of plasma ALA to DHA is less than 4% in males and around 9% in female (Arterburn, Hall et al. 2006). This low conversion rate may be further reduced by EPA and DHA supplementation (Burdge, Finnegan et al. 2003).

Interestingly, retroconversion of DHA to EPA has been demonstrated, and occurs at a rate of 1.4% with a standard diet (Brossard, Croset et al. 1996), and up to 9.4% following a solely DHA supplemented diet of 1.6g per day for 6 weeks (Conquer and Holub 1997).

The clear implications of this is that extremely high dietary intakes of ALA are required in order to replenish or maintain long chain n-3 PUFA intakes. This is better achieved by a diet rich in long-chain n-3 PUFAs or their direct supplementation.

```
                    n-6                n-3

              18:2 (LA)           18:3 (ALA)
                 ↓      Δ6-desaturase   ↓
              18:3                18:4
                 ↓       Elongase      ↓
              20:3                20:4
                 ↓     Δ 5-desaturase   ↓
              20:4 (ARA)          20:5 (EPA)
                       Elongase        ↓
                                  22:5 (DPA)
                       Elongase        ↓
                                  24:5
                    Δ 6-desaturase     ↓
                                  24:6
              Peroxisomal oxidation    ↓
                                  22:6 (DHA)
```
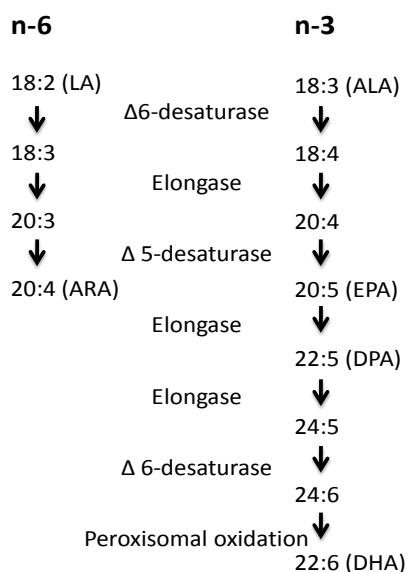
Figure 1.4.: Desaturation and elongation of n-3 and n-6 polyunsaturated fatty acids. Abbreviations: LA: Linoleic acid, ALA: α- Linolenic acid, ARA: Arachidonic acid, EPA: Eicosapentaenoic acid, DPA: Docosapentaenoic acid, DHA: Docosahexaenoic acid**.** Adapted from (Arterburn, Hall et al. 2006).

1.8.4.   Biological activity of PUFAs in relation to hepatic fatty acid metabolism

PUFAs exert their physiological effects by modifying cellular wall function, eicosanoid production and balance, and gene transcription rates. The expression of cellular proteins is genetically determined, whereas the fatty acid content of cellular membranes is largely dependent on diet. Increasing EPA and DHA intakes have been shown to replace arachidonic acid (ARA) from membrane phospholipids of almost all cells in the body including platelets, neutrophils and hepatocytes (Benatti, Peluso et al. 2004).

1.8.4.1.   PUFAs and insulin resistance

There remains considerable controversy as to whether PUFA status influences insulin resistance. No definitive mechanism behind any potential insulin sensitising effects has been widely shown as of yet. In rodents, fish oil has been shown to reduce skeletal muscle triglyceride content and hence improve whole-body insulin sensitivity (Klimes, Sebokova et al. 1993). Cultured human skeletal muscle cell line work has shown that EPA exposure increases cellular glucose uptake, via increased glucose transporter GLUT-1 expression, and

intracellular glucose oxidation (Aas, Rokling-Andersen et al. 2006). *Ob/ob* mice received a standard diet that was either enriched with 6% n-3 PUFA or not for 5 weeks (Gonzalez-Periz, Horrillo et al. 2009). There was no difference in terms of chow intake or weight gain in the two groups. The PUFA enriched group down-regulated hepatic FAS (a promoter of DNL) and up-regulated PPARα (a promoter of β-oxidation) and hence developed markedly less hepatic steatosis. Furthermore, n-3 PUFA supplementation up-regulated peripheral adipocyte expression of the insulin sensitising adipokine adiponectin. This was associated with an up-regulation of the insulin sensitising PPARγ, insulin-receptors (IRS-1 and IRS-2) and glucose transporters (GLUT-4 and GLUT-2) in adipocytes and hepatocytes. The functional outcome of these changes was confirmed by improved systemic insulin sensitivity.

Some epidemiological studies have shown a negative association between oily fish consumption and incident diabetes development. A Chinese group recently analysed fasted glucose, insulin and plasma phospholipid profiles in 180 healthy individuals and 186 type 2 diabetics (Huang, Wahlqvist et al. 2009). Moderate associations were made between n-3 PUFA status and the homeostasis model assessment insulin resistance (HOMA-IR) with a correlation coefficient of -0.42. This suggests that greater intakes improve sensitivity, though such data needs to be interpreted with caution as oily fish consumption may merely be a marker for a healthier lifestyle.

There have been very few large-scale interventional studies in either the healthy volunteer or clinical setting. A review evaluated 22 papers that assessed whether n-3 PUFAs influence glycaemic control in type 2 diabetes (De Caterina, Madonna et al. 2007). Four of these papers supplied more than 3g/day for more than a month to a combined total of only 78 patients. All showed significant plasma triglyceride reduction, whilst only one study showed an improvement in fasted insulin sensitivity.

There have been a few published studies in the non-diabetic setting. Griffin et al. demonstrated no alteration in HOMA-IR values in 61 healthy adults after a 6 month diet containing around 1g of long chain n-3 PUFAs per day (Griffin, Sanders et al. 2006). 29 overweight women received 4.2g of EPA and DHA for 12 weeks with reductions in plasma triglycerides but no alteration in insulin sensitivity (Browning, Krebs et al. 2007).

Overall there is no convincing data that altering PUFA status influences systemic insulin sensitivity.

1.8.4.2.   PUFAs and immunomodulation

Plasma membrane phospholipid PUFAs are substrates for cyclo-oxygenases (COX-1 and 2), lipoxygenase (LOX) and cytochrome p450 monoxygenases (Jump 2002). These enzymes produce the hormone-like eicosanoids including prostanoids, thromboxanes and leukotrienes. The most abundant and active precursor for these enzymes is the n-6 PUFA ARA, with EPA being the principally active n-3 PUFA. Eicosanoids influence platelet aggregation, inflammation, chemotaxis, vascular permeability and vasoconstriction. n-3 PUFAs induce less COX and LOX eicosanoid production than n-6 PUFAs through three activities (Jump 2002): Firstly, they are less effective COX and LOX substrates than n-6 PUFAs. Secondly, their resultant eicosanoid products tend to have reduced biological activity. Finally, n-3 PUFAs enhance eicosanoid catabolism via peroxisomal degradation (von Schacky, Kiefl et al. 1993). The cytochrome p450 enzymes are a large and diverse group that oxidise fatty acids to produce eicosanoids, but also oxidise hormones and medication (Capdevila, Falck et al. 2000).

The previously described systemic, and predominantly vascular, anti-inflammatory effects of n-3 PUFAs are well described. There is abundant data describing n-3 PUFA modulation of hepatic vascular insults, such as ischaemia/reperfusion injury, an area critical to liver transplant practice (El-Badry, Graf et al. 2007). Furthermore there is some evidence that it modulates response to a chemical induced hepatitis (Schmocker, Weylandt et al. 2007). However their influence on hepatic lipid inflammatory status has been much less assessed. There is evidence that n-3 PUFAs improve the adiponectin-TNFα balance (Gonzalez-Periz, Horrillo et al. 2009) and that this reduces hepatic steatotic necro-inflammation (Svegliati-Baroni, Candelaresi et al. 2006). This paper is discussed in greater detail in section 1.9.5. Malinska *et al.* have recently developed a transgenic spontaneously hypertensive rat line that is characterised by NASH and n-3 PUFA depletion and an elevated n-6 to n-3 PUFA ratio (Malinska, Oliyarnyk et al. 2010). As to what is cause and what is effect is difficult to determine as there was no n-3 PUFA replacement.

1.8.4.3. PUFAs and gene expression

n-3 PUFAs regulate intracellular, including intra-hepatic, gene expression rates and activity. Via intracellular receptors, they can either promote or inhibit gene transcription, or modify mRNA maturation or stability (Pegorier, Le May et al. 2004). The two main targets of n-3 PUFA hepatic gene modifications are the peroxisome proliferator-activated receptors (PPARs), and sterol regulatory element binding proteins (SREBPs).

1.8.4.3.1. n-3 PUFAs and peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors. They function as transcription factors regulating the expression of the peroxisomal genes which directly determine whole-body lipid and glucose metabolism. Four PPAR isoforms have been identified- α, β, δ and γ. n-3 PUFAs are natural ligands for the PPARα and PPARγ isoforms (Forman, Chen et al. 1997). n-6 PUFAs function as very weak ligands, whereas saturated fatty acids exert no effect (Price, Nelson et al. 2000).

PPARα is mainly expressed in the liver, kidney and heart. It activates fatty acid β-oxidation and lipoprotein synthesis. It therefore promotes hepatic fatty acid oxidation and exportation into the plasma (Semple, Chatterjee et al. 2006). The clinical outcome of PPARα agonism is well known as fibrate drugs are pure PPARα agonists. Fibrates are widely used to treat dyslipidaemia due to their triglyceride lowering and high-density lipoprotein cholesterol elevating effects. Their clinical benefit is modest, though they have been shown to reduce cardiovascular morbidity in diabetic patients and those with the metabolic syndrome (Staels, Maes et al. 2008). Fenofibrate has been shown to reduce steatosis in Zucker diabetic rats (Forcheron, Abdallah et al. 2009). PPARα also appears to modulate inflammation as knock-out mice have increased expression of inflammatory cytokines which are reduced with treatment of PPARα ligand replacement (Stienstra, Mandard et al. 2007). The clinical experience of hepatic outcomes from PPARα agonism is less impressive than the rodent. Twelve months therapy with clofibrate, a PPARα agonist, resulted in no biochemical or histological improvement in 16 NASH patients (Laurin, Lindor et al. 1996).

PPARγ is mainly expressed in white adipose tissue (Chui, Guan et al. 2005). It promotes differentiation and proliferation of adipocytes via targeting fatty acid binding protein 4, lipoprotein lipase and adiponectin. PPARγ

stimulation results in reduced free fatty acid release from adipose tissue, and hence improves peripheral and hepatic insulin-sensitisation (Semple, Chatterjee et al. 2006). Thiazolidinediones (TZDs) are a class of drugs that are powerful PPARγ agonists and widely used as insulin-sensitising agents in diabetic patients (Rohatgi and McGuire 2008). As previously discussed, TZDs have been shown to be beneficial therapies in NAFLD, though concerns remain over their safety.

1.8.4.3.2. n-3 PUFAs and sterol regulatory element binding proteins (SREBPs)

SREBPs are a group of transcription factors that directly enhance the transcription of more than 30 genes by binding to specific sterol regulatory DNA sequences (Horton, Shah et al. 2003). There are three main isoforms: SREBP-1a, SREBP-1c and SREBP-2.

SREBP-1c is the most pathologically implicated isoform, and has been linked to increased rates of *de novo* lipogenesis with the development of dyslipidaemia, type 2 diabetes and hepatic steatosis (Ferre and Foufelle 2007). SREBP-1c knockout mice have reduced expression rates of lipogenic genes with resultant reduced plasma triglyceride concentrations (Liang, Yang et al. 2002). Whereas, SREBP-1c overexpression in transgenic mice have a 4-fold increase in FAS levels, and a 4-fold increase in hepatic triglyceride content (Horton, Goldstein et al. 2002).

The formation of mature SREBPs is controlled by feedback inhibition. Factors central to this include intra-cellular levels of cholesterol, insulin, glucose and long-chain n-3 PUFAs (Loewen and Levine 2002). Short-chain n-3 PUFAs, such as ALA, exert only mild inhibition, whereas long chain n-3 PUFAs lower mRNA SREBP-1c levels by activating transcript delay (Worgall, Sturley et al. 1998; Clarke 2001; Xu, Cho et al. 2002). Sekiya *et al.* demonstrated that EPA decreased SREBP-1c expression in the livers of leptin-deficient ob/ob mice (Sekiya, Yahagi et al. 2003). There was a consequential reduction in the expression of lipogenic genes including FAS, and a reduction in hepatic triglyceride content and insulin resistance. There is great interest in the therapeutic potential of SREBP based therapies for NAFLD (Ahmed and Byrne 2007).

1.8.5. Summary of the observed biological activity of n-3 PUFAs

The proportionate balance of PPARα and SREBP-1c activities determines the relative rates of fatty acid oxidation and *de novo* lipogenesis (DNL). Pettinelli

et al. recently described strong correlations between hepatic long chain n-3 PUFA content and the PPARα / SREBP-1c ratio (r=0.48, p<0.02) in obese humans (Pettinelli, Del Pozo et al. 2009). The potential for n-3 PUFAs to activate PPARα and inhibit SREBP-1c results in a cascade of events up-regulating lipid oxidation, and down-regulating DNL. Furthermore PPARγ stimulation may promote insulin sensitivity, and this is further improved by correcting the adiponectin/TNFα balance. As a result n-3 PUFAs can be viewed as master switches to intracellular metabolism and promoters of pathways that would be predicted to reduce hepatic inflammation and steatosis.

1.8.6. Animal models of NAFLD and n-3 PUFA supplementation

The numerous rodent models of NAFLD and differing n-3 PUFA preparations have resulted in much study in this area assessing steatosis, gene expression, metabolic profile, fatty acid profiles and inflammatory status. A comprehensive review is therefore beyond the scope of this introduction. Three studies are worthy of in-depth discussion as they describe many of the prior discussed features:

The leptin-deficient ob/ob mice are a well validated metabolic model of obesity, insulin resistance, and hepatic steatosis. Gonzalez-Periz *et al.* recently used this model in an elegant study (Gonzalez-Periz, Horrillo et al. 2009). They randomised mice to receive either a standard chow or chow supplemented with a modest dose of n-3 PUFAs for 5 weeks. There were no differences in terms of weight gain between the two groups. In the n-3 PUFA fed mice PPARα and PPARγ were up-regulated and the hepatic expression of FAS was reduced. Adipocyte expression of adiponectin was up-regulated, whilst there was no change in TNFα. As a result hepatic steatosis was alleviated, and insulin sensitivity improved.

In a contrasting and fascinating protocol, Pachikian *et al.* have performed the only study, to the author's knowledge, to date that assessed the hepatic effects of n-3 depletion (Pachikian, Neyrinck et al. 2008). Second-generation n-3 PUFA depleted mice were fed for 34 weeks following birth a standard chow with n-3 PUFAs contributing either 2% or 9% of fatty acids. The n-3 PUFAs were predominantly short chain. The 2% fed mice had reduced weight gain, and identical amounts of visceral adipose tissue as compared to the repleted 9% fed mice. However the 2% mice developed a marked systemic insulin resistance and a

1.5-fold increase in hepatic triglyceride content. The degree of hepatic steatosis correlated very strongly with the hepatic n-6 to n-3 triglyceride ratio (r^2=0.87), though the authors were not able to identify the mechanisms behind these observations.

In standard rats a high fat/high energy diet induced visceral obesity, increased NEFAs, and TNFα, suppressed adiponectin, and induced hepatic insulin resistance (Svegliati-Baroni, Candelaresi et al. 2006). These modifications resulted in a model of NAFLD with hepatic steatosis, oxidative stress, necroinflammation, apoptosis and ultimately fibrosis. This model was then used with or without n-3 PUFA supplementation. Supplementation stimulated hepatic PPARα expression and restored adiponectin and TNFα mRNA levels. As a result the n-3 PUFA supplementation halved the amount of hepatic steatosis and necroinflammation induced.

1.8.7. Hepatic incorporation of supplemented n-3 PUFAs

Much of the prior data has originated from rodent studies. A key clinical question is whether supplemented PUFAs are incorporated within human livers, and what the time course is? There have been numerous serum and plasma incorporation studies which show maximal rate of changes of plasma phospholipid and erythrocyte n-3 PUFA content occur within 1 and 2 months of supplementation respectively (Arterburn, Hall et al. 2006).

To the author's knowledge, there is only one study that has quantified hepatic PUFA incorporation response to supplements. Senkal *et al*. randomised 40 patients undergoing elective gastrointestinal surgery into two age, sex and weight matched groups. Each group received five days of an isoenergetic diet, with one group receiving 3.7g of EPA and DHA (Senkal, Haaker et al. 2005) and the an isoenergetic control diet. Intestinal and liver biopsies were taken at the time of surgery and thus there was no pre-randomisation histology. The EPA content in the supplemented patients' liver and intestine was more than 3-fold greater than in the control group. It therefore is clear that hepatocyte phospholipid fatty acid patterns are likely to respond promptly and dramatically to supplementation of EPA and DHA. Therefore it is reasonable to expect that prolonged supplementation may result in altered hepatic metabolism.

1.8.8. Clinical trials of NAFLD and n-3 PUFA supplementation

There have been six clinical studies assessing the effects of n-3 PUFA supplementation in patients with NAFLD or related disorders:

1. 42 patients with presumed NAFLD were supplemented with 1g of EPA and DHA for 12 months (Capanni, Calella et al. 2006). The validity of the comparator has to be questioned as the 14 patients used as controls were those who had refused to take supplements. Steatosis was assessed by ultrasound scans using a 4-point graded scale. The control group had no changes on the scale, whereas 64% of the intervention group improved. The biochemistry worsened in their control group, whereas there were modest but significant reductions in the intervention group's liver enzymes, fasting glucose and triglycerides. This study had several flaws. It used a very low dose of n-3 PUFA supplements in patients with limited evidence of NAFLD (no biopsy), and it lacked a true control. Diabetics were not excluded and so alteration in their glycaemic control could have compounded the results. The biochemical changes detected were small, though the majority had normal baseline liver biochemistry. Ultrasound scans were used despite being a poorly validated tool for quantifying liver steatosis.

2. A Japanese group supplemented 23 biopsy-proven NAFLD patients to receive 2.7g of EPA per day for 12 months (Tanaka, Sano et al. 2008). Liver function tests, serum inflammatory and oxidative stress markers were improved and weight remained stable. There was no control group, and so the natural history effects cannot be fully accounted for. Seven patients agreed to a post treatment repeat liver biopsy, which showed evidence of improved steatosis, injury, inflammation or fibrosis in 6 out of the 7. However the small numbers, and lack of placebo control limit interpretation of the findings. It is noteworthy that such positive findings occurred from a Japanese cohort, as their community's n-3 PUFA intake is substantially greater than that in Western Europe and North America.

3. An Italian group randomised 40 patients with a clinical diagnosis of NAFLD (no liver biopsy) to either an energy restricted diet or an energy restricted diet plus 2g PUFA per day for 6 months (Spadaro, Magliocco et al. 2008). In those taking the PUFAs there was evidence of increased systemic insulin sensitivity and reduced inflammatory profile, plasma triglycerides, and liver steatosis as assessed by liver ultrasound. There were no such changes in the controls. Interpretation of this

study again is limited. The EPA and DHA content of the capsules was not presented, and the diet plus PUFA arm appeared to lose more weight which is a confounding factor that was not adjusted for.

4. A group from the United States have published the only negative study to date (Vega, Chandalia et al. 2008). 16 healthy volunteers with known hepatic steatosis noted on MR spectroscopy and normal liver function tests received 9g of fish oil per day for 8 weeks. The capsules contained 4.6g of EPA and 2.2g DHA. There were significant increases in plasma EPA and DHA concentrations and reductions in serum triglycerides. There was however no change in hepatic triglyceride content as assessed by proton MR spectroscopy. The authors had no explanation for their findings, though it is not certain that all the volunteers had NAFLD as they lacked a full exclusion of other liver pathology with no liver biopsy or presented alcohol intake.

5. Twenty five patients with polycystic ovarian syndrome, 13 with steatosis on their baseline 1H MRS were randomised to either 3.3g/day of EPA and DHA or oleic enriched olive oil for 8 weeks in a double blind crossover design (Cussons, Watts et al. 2009). The treatment arm resulted in reductions in HTGC, plasma triglycerides, and blood pressure. The effect on HTGC was most noted in those with steatosis at baseline and non-significant in those without steatosis at baseline. There were no changes in liver biochemistry or C-reactive protein.

6. A group from China assessed the largest cohort to date (Zhu, Liu et al. 2008). 134 NAFLD patients with associated dyslipidaemia were randomised to 6g of seal oil for 24 weeks or placebo. There were no changes in weight, whereas there were significant improvements in liver biochemistry, serum triglycerides and ultrasound assessment of liver fat stores. Again this study is limited as there was no liver biopsy performed to confirm NAFLD, we are given no data on the fatty acid profile of the active or placebo capsules, the steatosis assessment is by ultrasound only, and the biochemical differences between the two groups were at best modest.

These studies were recently entered into a meta-analysis (Parker, Johnson et al. 2011). Also include was a small Chinese study that it is not on pubmed nor

an abstract identifiable on the internet, and an ultrasound comparison of 6 versus 5 patients which I view as being too small to worthy comment on (Sofi, Giangrandi et al. 2010). The negative paper by Vega and colleagues was not included in the meta-analysis. The meta-analysis demonstrated evidence for a significant reduction in liver fat but not transaminases with n-3 polyunsaturated fatty acids. The authors however acknowledged the small sample size, assessment heterogeneity, and lack of histological confirmation in these studies. Publication bias needs also to be remembered in such small studies.

1.8.9. Safety of n-3 PUFA supplementation

Up until recently there have been no clinical concerns regarding n-3 PUFA supplementation (Schmidt, Arnesen et al. 2005), although some recent epidemiological data has suggested a link between serum PUFA status and prostate cancer risk. Over 1,500 cases of prostate cancer were matched with over 1,500 age, treatment and prostate cancer family history controls. Serum phospholipid analyses demonstrated that the highest quartile of serum DHA was associated with a 2.5 odds ratio of high grade prostate cancer as compared to the lowest quartile (Brasky, Till et al. 2011). There were no associations with EPA or n-6 fatty acids. The European Prospective Investigation into Cancer and Nutrition study (EPIC) is the second largest study to investigate this area. Prostate cancer risk was non-significantly increased in those with a high serum DHA, and high-grade cancer risk was significantly increased with increased EPA (Crowe, Allen et al. 2008). These data seem to be alarming and the authors of both papers have no explanation for their findings. It bears remembering that no biological pathway for n-3 PUFA carcinogenesis has been identified (Brasky, Till et al. 2011), and that long-term n-3 PUFA supplementation has been undertaken in many interventional studies without an increased tumour rate noted. As a result it seems premature to issue warnings.

1.8.10. Conclusion on n-3 PUFAs and NAFLD

To conclude, there is emerging evidence to support the principles and practice of n-3 PUFA supplementation. NAFLD appears to be characterised by a relative deficiency of the long chain n-3 PUFAs when assessed in the diets, plasma and livers of patients, though these are not universal observations. Hepatic lipid metabolism appears to be potentially improved by n-3 PUFAs via the promotion

of insulin sensitivity, and beta-oxidation, combined with the reduction of lipogenic gene expression, and *de novo* lipogenesis rates and anti-inflammatory immunomodulation,. It bears remembering that not all of these effects have been conclusively demonstrated outside the hepatocyte cell line or animal model experience. Clinical studies in NAFLD have been performed, though the data regarding the precise role of n-3 PUFAs remains unclear.
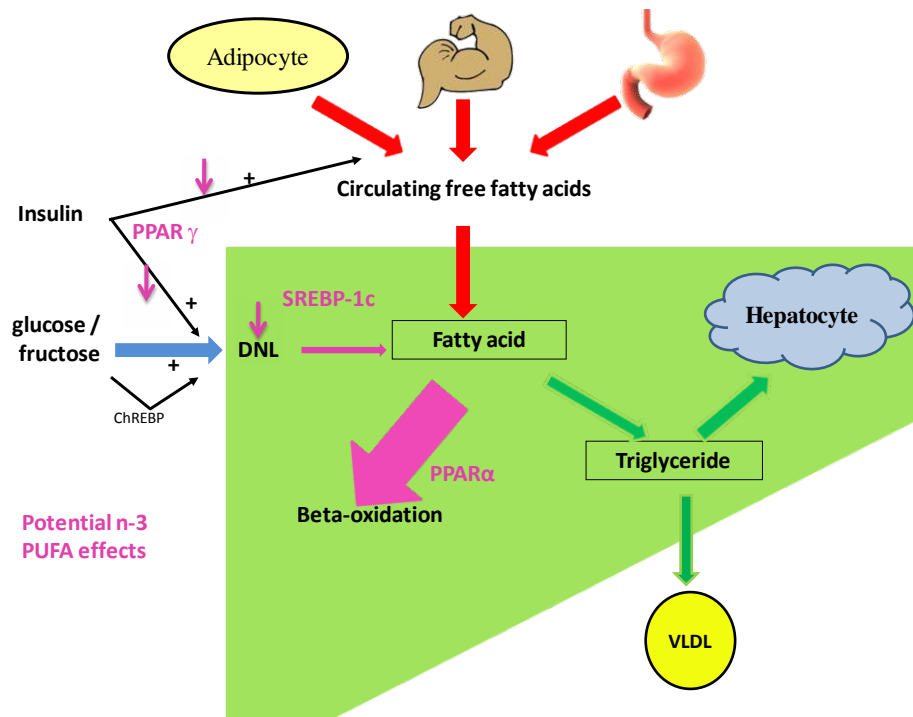
Figure 1.5. Summary of the potential actions of n-3 PUFAs on hepatic fatty acid metabolism

1.9. Fructose

1.9.1. Classification of dietary carbohydrates

Carbohydrates are structurally classified according to their size, constituent monomers and the linkages between them. The number of monomeric units is termed the degree of polymerisation. Sugars have 1-2 monomers, oligosaccharides 3-9, and polysaccharides 10 or more.

Carbohydrates can also be classified nutritionally into those which are absorbed in the small intestine, termed glycaemic carbohydrates, and carbohydrates that pass unabsorbed into the colon, termed dietary fibre. As a result it is the glycaemic carbohydrates that influence metabolism. The principal glycaemic carbohydrates are the monosaccharides glucose, fructose and galactose, the disaccharides sucrose and lactose, and starch. All of these glycaemic carbohydrates bar resistant starch are normally digested in the small intestine to form monosaccharides prior to their absorption and transfer to the liver via the portal vein.

This thesis will only review the monosaccharides, as all carbohydrates are degraded to them prior to absorption. It is important to remember that prior to digestion monosaccharides may have originated from more complex carbohydrates.

Galactose is an infrequent dietary component which undergoes the same glycolytic pathway as glucose, and hence has no intrinsic metabolic differences from glucose, see figure 1.6. Fructose and glucose do however differ in their metabolic handling. This will be the main focus for this section of the thesis.

Glucose and fructose have the same molecular content of $C_6H_{12}O_6$, though their ring structures differ, see figure 1.6. Sucrose or table sugar, a disaccharide of fructose and glucose, is the main dietary source for both of these monosaccharides.
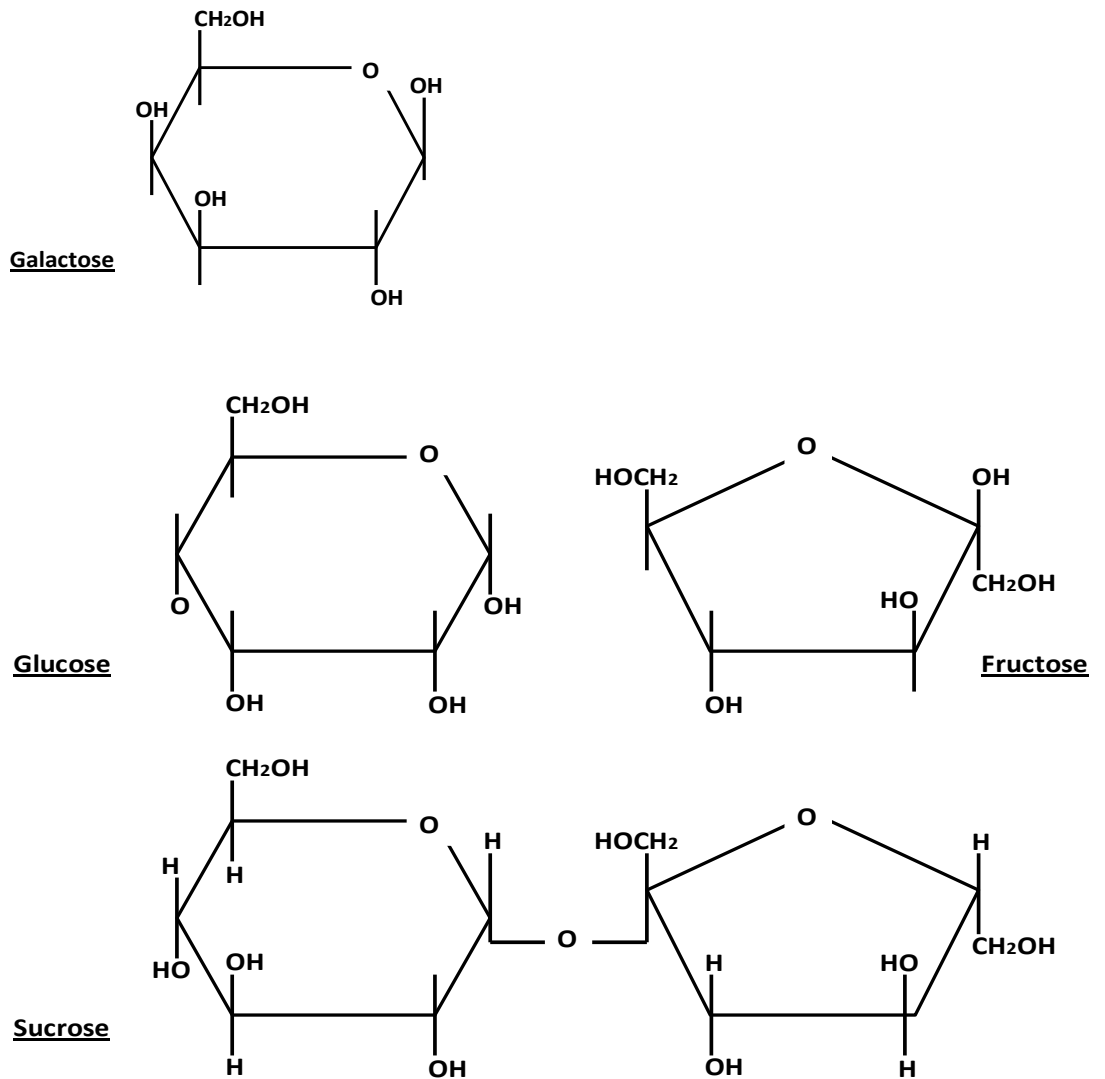
Figure 1.6.: The ring structures of galactose, glucose, fructose and sucrose

1.9.2. Dietary origins of fructose

Fructose is popularly regarded as being the 'fruit sugar'. Although this is true, modern agricultural practices means that the current principal source of fructose is from table sugar or sucrose, and that fruit and fruit juices only

contribute just over 10%, see table 3. The majority of data on fructose intakes originate from the USA, with no reliable UK data.

In order to identify the dietary sources of fructose in the USA, Marriott *et al.* analysed the NHANES data collected from 1999 to 2004 (Marriott, Cole et al. 2009). The NHANES survey analysed 24-hour dietary recall data collected by telephone from 25,165 children and adults. There are two limitations of this data for extrapolation to the UK. Firstly, it has been shown that the 24-hour dietary recall method underreports intakes by 15%, and that socially embarrassing (often fructose-rich) foodstuffs are the most underreported (Poslusna, Ruprich et al. 2009). Secondly, the dietary records are at least 6 years old and of a USA source. As a result the findings may have underestimated the actual intakes at the time of data collection, and the intake patterns may not be reflective of current UK dietary patterns. The above accepted, the paper by Marriott *et al* is the most accurate source we currently have to identify the dietary origins of fructose.

Food group	Male (%)	Female (%)
Dairy	5.1	7.2
Grain products	15.0	17.0
Fruit and fruit products	9.4	12.1
Vegetables and veg. products	2.5	2.8
Sugars and sweets	8.5	10.8
Beverages	55.1	46.1

Table 3. Percentage contribution of differing food groups to total fructose dietary intakes in US adults aged 23-50 years (Marriott, Cole et al. 2009)

Analysing table 3, it is clear that the main source of dietary fructose is from beverages, with fruit only providing just over 10% of intakes. The overall contribution of natural sources to fructose intakes was only 16%, the rest originating from added sources. The 95th centile for intakes of naturally sourced fructose was 19g per day. As the vast majority of fructose originates from added sources, the concern that vegetarianism may substantially increase fructose intakes does not seem valid. Indeed the fruit and vegetable intakes of vegetarians is only 44% greater than of non-vegetarians (Haddad and Tanzman 2003).

The mean fructose intake for males aged 19-30 years was 71g per day, with a 95th centile of 120g per day. The values for women of the same age were 55g and 104g per day respectively. Fructose intakes on average contributed 9.1% to the total daily energy intake, and for males aged 19-30 years the mean contribution was 10.0%, with a 95th centile of 16.6%.

There is no intake data for for individual sugars in the UK, but fructose intakes seem to be less than that reported in the US. The UK Department of Health presents data on the population's intakes of NMES (non-milk extrinsic sugar) intakes, due to its greater cariogenic potential than lactose, as opposed to individual sugars. Sucrose is the principal dietary NMES, though not exclusively. According to the 2009 National Diet and Nutrition Survey (NDNS), the average UK 25-34 year old male consumes 73g per day of NMES (NDNS 2009). This provides 13.0% of total energy intake. It is unclear as to what the relative contribution of each sugar is to this total NMES, though it is notable that 55% originates from beverages, and 35% originates from sugar, preserves and confectionary. It is therefore highly likely that the majority of this is sucrose and its constituent components of fructose and glucose.

There are two issues with the above data:

a.   Firstly, it bears remembering that the history of dietary sweetener consumption is slightly different in the USA than the UK. In the USA the contribution of high fructose corn syrup (HFCS) to the national dietary sweeteners increased from around 0% to around 35% in between 1960 and 1985 (Park and Yetley 1993). HFCS contains around 55% fructose to 45% glucose. The high fructose content of HFCS is a source of much concern in the USA, though there is no evidence that justifies this. HFCS acts metabolically the same as sucrose, which is 50% fructose and 50% glucose (Melanson, Zukley et al. 2007; Stanhope, Griffen et al. 2008). It also bears remembering that HFCS is only used as a sucrose replacement, and so it has no substantial impact on the overall intakes of glucose or fructose. HFCS is not added to foodstuffs in the UK where sucrose is the predominant sweetener.

b.   There is no such data presented on intakes of other simple sugars, presumably because there is less interest or concern over glucose than fructose. Though it begs the question as to whether a high fructose but low glucose intake is possible or whether their intakes tend to be equivalent. The majority of fructose and glucose originates from sucrose containing drinks and sweets with an even

glucose and fructose content. The foodstuffs with an uneven balance of fructose and glucose are fruit and vegetables. So intake patterns of fruit and vegetables could theoretically result in a difference in overall glucose and fructose intakes.

The five main fruits and vegetables purchased in the UK and their fructose and glucose content are presented in table 4. Combining average weekly intakes with their composition results in a 30% greater fructose than glucose intake from fruit and vegetables. This absolute value is not reliable as it does not contain all fruits and vegetables, is purchase as opposed to consumption data, and the intake data source is old. Since 1998 the survey has been switched to the Expenditure and Food survey which collects data on money spent as opposed to amount of foods bought.

Limitations withstanding, it does reinforce the notion that it is difficult to have an intake of fructose or glucose which markedly differs from the other. It bears remembering that fruit and vegetables only contribute 13% of total fructose intakes. So studies presenting an association between fructose intakes and an outcome may well have noted the same associations with glucose had they analysed / presented this data. Unfortunately in the absence of data this argument can neither be proven nor refuted.

	Purchase per head per week (g)	Fructose g (%)	Glucose g (%)
Banana	197	20.9 (10.6)	20.5 (10.4)
Apple	173	14.2 (8.2)	6.4 (3.7)
Tomato	85	1.4 (1.6)	1.3 (1.5)
Orange	63	2.8 (4.4)	2.6 (4.2)
Pear	49	3.7 (7.5)	1.3 (2.7)
Potato	889	6.2 (0.7)	7.1 (0.8)
Carrot	109	2.9 (2.7)	3.2 (2.9)
Onion	103	2.7 (2.6)	3.2 (3.1)
Cauliflower	93	0.8 (0.9)	0.9 (1.0)
Cabbage	52	0.5 (1.0)	0.6 (1.1)
Average		56.1	41.0

Table 4. Weekly purchases in the UK of the five main fruit and the five main vegetables and their fructose and glucose content (purchase data from the National Food Survey (MAFF 1998), and composition data from McCance and Widdowson 6th edition (FSA 2002)).

1.9.2.1. Trends in fructose and glucose intakes

Mintz's fascinating account of the global history of sugar estimates that in the past 150 years there has been a 2,500% increase in UK sugar intakes (Mintz 1986). Pre the 1850's honey was the main dietary sweetener, though it was never mass produced and so access to any sweetener was very limited. At that time the main carbohydrates consumed were barley, wheat, oats and rye (Johnson, Segal et al. 2007). Historical food disappearance records describe that the average consumption of sugar per capita per year in England was 1.8kg in 1700, rising to 8.1kg in 1800, and is currently 67.6kg (Johnson, Segal et al. 2007). This massive increase in consumption was largely due to the discovery of the New World. Sugar plantations were set up in the New World and West African slaves imported to work them. The subsequent industrialisation of agriculture, global transportation and ultimately food production has meant that foodstuffs such as cakes, biscuits and sugar-sweetened drinks are now widely and cheaply available. More recently the percentage of total energy intakes from added sugar rose from 15% in 1965 to 18% in 1999-2000 (Duffey and Popkin 2008). This remained stable at 17% in 2003-2204.

As already described, the use of HFCS in the USA has recently dramatically increased. Trend data for fructose itself (as opposed to sucrose or HFCS) is extremely limited. Studies analysing fructose intakes reported in the 1988 to 1994 and 1999 to 2004 NHANES surveys describe gradual increases in intakes in fructose over time though these changes are dwarfed by greater increases in total daily carbohydrate and energy intakes (Vos, Kimmons et al. 2008; Marriott, Cole et al. 2009).

1.9.2.2. Recommended intakes of fructose and glucose

There is no UK dietary reference value (DRV) for fructose, glucose or sucrose. The Department of Health advised upper limit for NMES is 10% of all dietary energy intake (DOH 1991). The WHO advises that less than 10% of all energy should originate from mono and disaccharides (WHO 2003).

The lack of DRV for sucrose – a whole food with no micronutrient content and hence no nutritional value beyond energy provision has been repeatedly questioned (Ruxton 2003). The European Food Safety Authority (EFSA) drafted a

proposal for the DRV for carbohydrate and dietary fibre in late 2009, though it felt there was insufficient data to set an upper limit for sucrose intake.

1.9.3. Metabolism of fructose and glucose

All polysaccharides and disaccharides are hydrolysed to their constituent monosaccharides (principally glucose and fructose) by intestinal brush border enzymes prior to gastrointestinal absorption. Glucose is actively transported along with two sodium ions across the intestinal mucosa by the sodium-glucose co-transporter 1 (SGLT1), whereas fructose undergoes passive facilitated transport by glucose transporter type 5 (GLUT5) (Wright, Martin et al. 2003). The lack of active transportation means that fructose malabsorption may occur either as a result of a GLUT5 deficiency (Born 2007) or following a dietary intake greater than can be passively absorbed (Rumessen and Gudmand-Hoyer 1986). Fructose malabsorption symptoms include abdominal distension, flatulence or diarrhoea. For reasons that are unclear, fructose absorption seems to be improved by the co-consumption of glucose (Rumessen and Gudmand-Hoyer 1986). As a result complete fructose absorption has been shown to occur when 50g of fructose is taken with 50g of glucose, whereas only 68% is absorbed when 50g of fructose is consumed alone. In standard dietary situations fructose is not consumed in isolation, and at smaller doses than 50g. So the majority of intakes appear to be absorbed and the effects of fructose malabsorption, if any, on systemic metabolism in standard dietary conditions remain undetermined (Bizeau and Pagliassotti 2005). Absorbed glucose and fructose enter the portal circulation and hence are transported first to the liver before entering the systemic circulation. Their initial transference straight to the liver is critical to their outcome.

The maintenance of normoglycaemia is one of the key components of homeostasis. The nervous system is not able to oxidise fatty acids and so relies predominantly on glucose as its energy source, whereas erythrocytes are entirely glucose energy dependent. Glycaemia depends upon the rate of glucose intake (absorption from the gut), tissue consumption (glycogen, amino acid and fatty acid synthesis, and glycolysis, citric acid cycle and the pentose phosphate pathway) and endogenous glucose production (glycogenolysis and gluconeogenesis). The liver is the central organ to these metabolic processes. Normoglycaemia is achieved via hormonal control of the above processes, with a

balance between the actions of insulin and insulin-like growth factors and the opposing glucagon, catecholamines, cortisol and growth hormone. As will now be discussed, hepatic metabolism of glucose is principally influenced by the degree of insulin sensitivity, and whether it is during the fasted (post-absorptive) state or during the fed (post-prandial) state.

1. Normal glucose physiology:

<u>a. The fasted state</u>

In the fasted state the availability of glucose for intestinal absorption falls, and so endogenous production increases in order to prevent hypoglycaemia. Insulin levels drop and glucagon and catecholamines are produced. This initially results in glycogenolysis, however prolonged fasting (greater than 8 to 12 hours) depletes hepatic and muscle glycogen stores such that gluconeogenesis is initiated. The liver is the main organ involved in gluconeogenesis (some renal contribution occurs during prolonged fasting) and produces glucose from amino acids, glycerol and lactate. Ketone bodies are used in prolonged fasting. Of note, during fasting there remains a continuous basal secretion rate of insulin so as to prevent excessive hepatic glucose production. The term hepatic insulin sensitivity refers to the degree of reduction in hepatic glucose production in response to insulin.

<u>b. The fed state</u>

In the fed state the majority of serum glucose arises from dietary rather than endogenous source and the catabolic state of fasting reverts to an anabolic one. In response to luminal carbohydrates, the intestinal wall produces the incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Baggio and Drucker 2007). These incretin hormones stimulate the pancreatic beta cells to release insulin before the glucose reaches the systemic circulation. A systemic glucose concentration greater than 3.3mmol/L further stimulates pancreatic insulin and amylin secretion (Cryer 2008).

Insulin acts to reduce postprandial glucose via activating translocation of GLUT4 glucose transporters to the cell surface of skeletal muscle and adipose tissue. As a result, glucose enters the cells and due to an effect of insulin within the cells is stored intracellularly either as glycogen or triglycerides for subsequent release during a further post-prandial period.

2. Glucose physiology and systemic insulin resistance

Systemic insulin resistance is characterised by fasting and postprandial hyperglycaemia due to the failure of glucose to enter insulin-dependent tissues and persistent endogenous production. This results from a reduction in cell surface GLUT4 transporter number and function (Karnieli and Armoni 2008). Consequently there is a reduction in glucose uptake by skeletal muscles and increased hepatic glucose output. Insulin also has indirect effects on glucose metabolism by suppressing adipose tissue lipolysis and modulating adipokine release (Bizeau and Pagliassotti 2005). As a result there is an initial rise in insulin secretion in an attempt to improve insulin-mediated responses followed by a subsequent fall in insulin secretion with pancreatic functional decline. These processes result in the development of a dysregulated hyperglycaemia, and ultimately type 2 diabetes which is characterised by systemic insulin resistance and loss of pancreatic β-cell function.

1.9.4. The effects of fructose and glucose on insulin profiles and sensitivity

In *ex vivo* human studies fructose directly induces less pancreatic β-cell stimulation than glucose (Curry 1989). Furthermore a fructose load has a very low glycaemic index of 23 and this further reduces the stimulus for insulin release compared to an identical glucose load (Teff, Elliott et al. 2004). The reduced glycaemic index and insulin response to fructose, as opposed to glucose, in humans is unquestioned. However the long-term effects on systemic and hepatic insulin resistance in humans are much less clear.

In rodents a high fructose or sucrose diet induces systemic and hepatic insulin resistance as well as hepatic steatosis (Thresher, Podolin et al. 2000). Unfortunately this study lacked a glucose comparator and so this cannot be viewed as a fructose or sucrose specific effect. Fructose appears to induce hepatic insulin resistance in both a direct and indirect manner. The binding of insulin to its receptor results in the phosphorylation of the intracellular portion of the receptor. Intracellular insulin receptor signalling is attenuated by c-Jun N-terminal kinase (JNK) (Tuncman, Hirosumi et al. 2006; Singh, Wang et al. 2009). High fructose diets in rodents increase hepatic JNK activity (Wei, Wang et al. 2005), which reduce post insulin-receptor signalling and hence result in hepatic insulin

resistance which is characterised by and greater hepatic glucose and fatty acid synthesis (Wei, Wang et al. 2007). These effects may be further compounded by fructose induced hepatic steatosis impairing hepatic insulin sensitivity. The association between these two states is well established, though whether hepatic steatosis is the result of insulin resistance, or whether hepatic steatosis causes insulin resistance remains unclear (Garg and Misra 2002).

Clinical studies have attempted to reproduce these rodent findings. A key limitation in assessing many of these studies is that the control failed to match the intervention in terms of energy and carbohydrate content. The studies have also varied in terms of fructose doses, supplied diets, single dose versus long-term dietary changes, and subject factors such as obesity, pre-existing insulin resistance, age, and sex. Schaefer et al. performed a comprehensive literature review and found that the majority of these studies have failed to show any significant difference at all between fructose and glucose intakes on systemic insulin resistance (Schaefer, Gleason et al. 2009). There have been numerous short-term studies involving oral or intravenous carbohydrate loading. Astonishingly there are only three published long-term healthy volunteer (i.e. non-diabetic) studies with an energy matched control that have assessed the influence of fructose or sucrose on fasted insulin sensitivity using a dynamic test. The findings of these papers will be discussed in chapter 4, but to summarise, both a high glucose and fructose intake impair insulin sensitivity. Fructose appears to result in greater insulin resistance though this has only been shown to be significantly different in a single study.

Using a longitudinal observational approach, several studies have assessed the influence of sugar-sweetened beverage consumption on incident diabetic development. Most have shown positive associations (Meyer, Kushi et al. 2000; Schulze, Manson et al. 2004; Montonen, Jarvinen et al. 2007; Palmer, Boggs et al. 2008) whereas others have found none (Liu, Serdula et al. 2004). These papers are difficult to interpret however as the consumption of sugar sweetened beverages are accompanied by an unhealthy dietary and physical lifestyle. Also it is impossible to determine which sugar is responsible for these observations.

1.9.5. The differing hepatic metabolism of fructose and glucose

Both fructose and glucose enter hepatic cells via the GLUT2 transporter (Leturque, Brot-Laroche et al. 2005). The metabolic outcome of hepatic fructose and glucose is dependent on the need for energy generation or storage as glycogen or triglycerides. In addition, fructose can undergo gluconeogenesis, the reverse does not occur.

The key difference between fructose and glucose metabolism is the proportion which is metabolised within the liver or peripheral tissues (Schaefer, Gleason et al. 2009). 70-80% of intestinally absorbed glucose passes through the liver and circulates to peripheral tissues (Cherrington 1999). The reverse is true for fructose (DeFronzo, Ferrannini et al. 1978). This effect can be observed in clinical studies. Systemic plasma glucose rises by around 3mmol/ following a high glucose meal. Following an identical high fructose meal systemic plasma fructose rises by only 0.5mmol/l (Chong, Fielding et al. 2007; Teff, Grudziak et al. 2009). Indeed within 3 hours circulating fructose concentrations return to zero (Teff, Grudziak et al. 2009).

The very high clearance rate from the circulation by the liver results from the high fructose affinity of fructokinase. This initiates fructose catabolism and the subsequent pathway lacks any feedback inhibition (Bizeau and Pagliassotti 2005). Lower rates of hepatic glucose extraction occur as the metabolic pathways are strictly regulated and rate limited. Most of the glucose extracted is used for glycogen replenishment and catabolism via glycolysis. The "extra" carbohydrate extraction with fructose leads to increased glycogen formation as well as entering alternative hepatic pathways such as aerobic and anaerobic fructose catabolism and *de novo* lipogenesis (Stanhope, Schwarz et al. 2009).
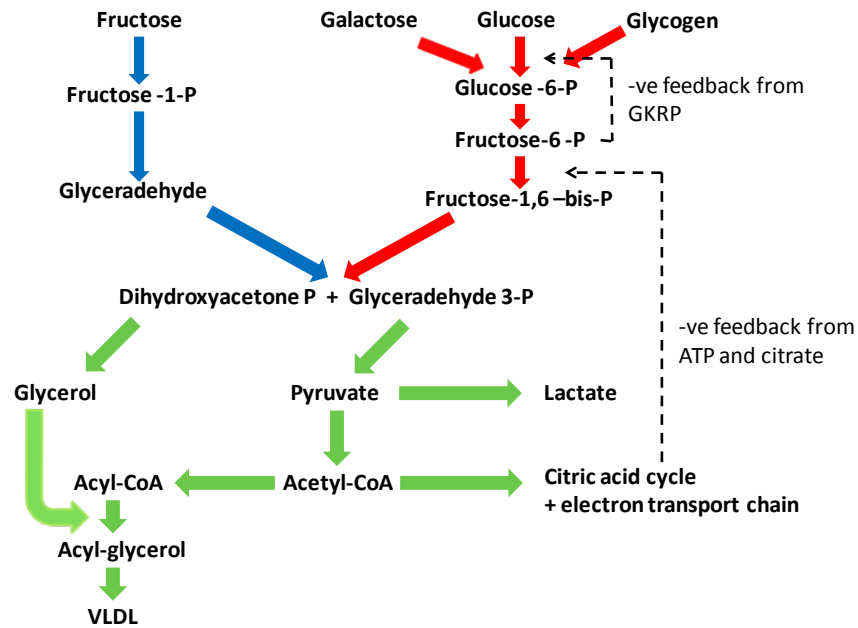
Figure 1.7. Pathways of hepatic carbohydrate catabolism. Triose formation is highlighted in blue from fructose, and red from glucose. Subsequent triose catabolism resulting in the citric acid cycle, or the formation of lactate or VLDL, is highlighted in green.

The first phase of glucose and fructose catabolism converts these 6 carbon structures to two 3-carbon trioses: glyceraldehyde-3-P and dihydroxyacetone-P. This process requires 2 ATP molecules. These trioses are interconvertible. Dihydroxyacetone-P is either catabolised to form glycerol or converted to glyceraldehyde-3-P. The second phase of catabolism involves the conversion of a triose to pyruvate, with 4 ATP molecules generated. Both of these phases occur in the cytosol.

The overall outcome of both initial phases converts one 6 carbon glucose or fructose molecule to 2 pyruvate molecules (3 carbon) with the net production of 2 ATP molecules.

$$C_6H_{12}O_6 + 2\ NAD^+ + 2\ ADP + 2\ P \longrightarrow 2\ CH_3(C{=}O)COOH + 2\ ATP + 2\ NADH + 2\ H^+$$

The subsequent potential catabolic outcomes for pyruvate are either decarboxylation to acetyl CoA or anaerobic reduction to lactate. Any acetyl CoA formed either enters the citric acid cycle or forms triacylglycerol (triglyceride) via Acyl-CoA and glycerol. Following acetyl CoA entrance into the citric acid cycle hydrogen molecules are released and enter the electron-transport chain via NADH and FADH$_2$. The transference of 2 pyruvate molecules into the citric acid cycle

and electron transport chain via acetyl CoA generates a further 34 ATP molecules. So the full catabolism of a single glucose or fructose molecule via the aerobic pathway has a net production of 36 ATP molecules.

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + 36 \text{ ATP}$$
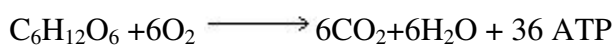
Anaerobic catabolism occurs either in the presence of inadequate tissue oxygen, or if the functional capacity of the mitochondria is exceeded. In anaerobic catabolism pyruvate is reduced to lactate. This occurs within the cytosol by lactate dehydrogenase and generates no further ATP. So the catabolism of a single glucose or fructose molecule via the anaerobic pathway has a net gain of only 2 ATP molecules. Clearly aerobic catabolism is a much more energy efficient process.

$$C_6H_{12}O_6 \longrightarrow 2 \text{ CH}_3\text{CHOHCOOH} + 2 \text{ ATP}$$
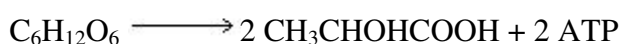
The catabolic pathways for glucose and fructose are identical after the first phase – the formation of trioses. The key difference is that the enzymatic control of this initial critical phase differs between the two. Fructose enzymes lack the strict feedback inhibition present for glucose and so they differ in their rates of triose formation. This is central to their differing metabolic outcomes. In glucose catabolism (glycolysis) the second step forms glucose-6-phosphate (G6P) which has 3 possible outcomes:

1.   continue glycolysis via the formation of fructose 1,6-bis phosphate

2.   entrance into the pentose phosphate pathway

3.   glycogen formation

These first two potential outcomes are energy generating, whereas the latter is energy storing. Strict feedback control exists to ensure that there is a balance between these potential differing outcomes. This is achieved by the end products of the citric acid cycle (citrate and ATP) providing feedback inhibition on glucokinase and phosphofructokinase action.

Glucokinase phosphorylates glucose to form glucose-6-phosphate. Fructose-6-phosphate (F6P) is then formed by phosphoglucose isomerise. F6P provides negative feedback inhibition on glucokinase by enhancing glucokinase regulatory protein (GKRP) mediated inhibition of glucokinase activity. So synthesis and accumulation of initial glycolytic intermediaries causes feedback inhibition on glycolysis. This is important physiologically as it prevents the

otherwise unregulated and excessive production of distal metabolites including triacylglycerol and lactate from glucose.

Distal aerobic glycolytic metabolites, citrate and ATP, also provide feedback via phosphofructokinase. Phosphofructokinase forms fructose 1,6-bis phosphate from fructose-6-phosphate. The rate of this step is strictly regulated as, once formed, fructose 1,6-bis phosphate is irreversibly committed to undergoing glycolysis. Inhibition of phosphofructokinase results in an accumulation of F6P which switches off glucokinase via GKRP. As a result the presence of adequate cellular energy, in the form of ATP, prevents an excess of hepatic glucose catabolism. Glucose is either not metabolised at all and passes into the systemic circulation or is transferred to form glycogen.

G6P and hence F6P is also formed by galactose or glycogen catabolism, and so feedback on phosphofructokinase affects glucose, galactose and glycogen identically. Fructose does not form F6P and fructose catabolism (fructolysis) differs in that it lacks any end-product feedback control. This lack of feedback control means that more fructose can enter the pathway than is needed for energy yielding metabolism. So a high concentration of fructose results in an abundance of triose and pyruvate formation greater than a comparative glucose concentration (Mayes 1993). This high pyruvate concentration exceeds the mitochondrial ability for it to undergo citric acid cycle and electron transport chain. The excess pyruvate therefore is shunted into alternative pathways either lactate, or glycogen or VLDL formation.

The cut-off value for oral fructose intake which exceeds the liver's mitochondrial ability to handle pyruvate is undetermined. It is likely to vary and be dependent on metabolic needs (Mayes 1993). Clinical studies however do provide some evidence that support these notions of dysregulated fructose as opposed to glucose catabolism. Following a high fructose, as opposed glucose, load there is clear evidence of hepatic ATP depletion and increased hepatic pyruvate formation with resultant increased lactate, and triglyceride production:

1.   ATP depletion.

The initial phase of fructose and glucose catabolism involves the formation of trioses at the expense of 2 ATP molecules. This initial 'investment' phase is normally subsequently 'rewarded' by further aerobic or anaerobic catabolism. ATP depletion occurs if this reward is not generated by further

catabolism, and may be compounded by further ATP utilisation for lactate to undergo gluconeogenesis or glycogenesis.

^{31}Phosphorus magnetic resonance spectroscopy (^{31}P MRS) allows for frequent and accurate quantification of hepatic *in vivo* ATP, fructose-1-P and inorganic phosphate (Pi) concentrations (Solga, Horska et al. 2005). The intra-observer correlation coefficient for interpreting the spectra is high at between 0.83 and 0.92 (Solga, Horska et al. 2008). Several groups have given healthy volunteers a single large dose of fructose and assessed their hepatic response using ^{31}P MRS (Terrier, Vock et al. 1989; Segebarth, Grivegnee et al. 1991; Boesch, Elsing et al. 1997; Cortez-Pinto, Chatham et al. 1999). A consistent, rapid and dramatic picture emerges from these studies. The first change to occur is a rise in the phosphomonoester (PME) peak, which represents a rise in fructose-1-P. This returns to normal within 20 minutes of the fructose administration. The next change to occur is approximately a 50% reduction in ATP levels from the baseline values. That Pi levels rise following the fall in ATP further reinforces the assumption that the ATP depletion is due to a net catabolism. These changes normalise within an hour in healthy volunteers, though are more prolonged in those with NAFLD (Cortez-Pinto, Chatham et al. 1999; Nair, V et al. 2003).

ATP consumption within the liver results in an increased formation of adenine nucleotides. These are degraded to form uric acid (Tran, Yuen et al. 2009). There is moderately convincing evidence that a high fructose intake results in hyperuricaemia and indeed gout as is discussed in chapter 5.

2.   Increased lactate production

Brundin and Wahren performed an extraordinary and highly informative set of experiments (Brundin and Wahren 1993). In healthy volunteers they analysed blood samples taken from hepatic venous catheters before and after oral intakes of 75g of fructose or glucose. The findings are summarised in table 5. It is clear that hepatic release of monosaccharides was much smaller following fructose than glucose, indicating that the majority of the fructose was metabolised within the liver. This was reinforced by the greater hepatic release of pyruvate and lactate following oral fructose than glucose, suggesting increased rates of triose catabolism and anaerobic glycolysis.

	15 min post fructose	15 min post glucose	60 min post fructose	60 min post glucose
Fructose	638	.	659	.
Glucose	788	5180	503	5653
Pyruvate	249	3	317	107
Lactate	1653	13	2316	501

Table 5: Increases in hepatic venous concentrations of fructose, glucose, lactate and pyruvate as compared to baseline following consumption of either 75g of fructose or glucose (units expressed are µmol/min) (Brundin and Wahren 1993)

Similar acute systemic elevations in lactate have also been described by other authors, though none have employed such invasive catheterisation techniques (Delarue, Normand et al. 1993; Chong, Fielding et al. 2007). Post-prandial elevations in lactate synthesis have also been shown in long-term high fructose diet studies (Le, Faeh et al. 2006).

3.   Increased hepatic de novo lipogenesis and plasma triglyceride concentration

The evidence that fructose results in greater rates of hepatic de novo lipogenesis (DNL) than glucose is limited. Two pathways have been suggested. Firstly, by greater rates of fructolysis than glycolysis resulting in increased triglyceride formation. Secondly, by up-regulating hepatic lipogenic transcription factors and enzymes such as carbohydrate responsive element binding protein-1c (CHREBP-1c) and fatty acid synthase (FAS) (Roglans, Vila et al. 2007; Koo, Wallig et al. 2008). These findings are widely reported, but the findings by Roglans et al. demonstrated an identical response to glucose.

Faeh et al. demonstrated increased rates of DNL following fructose and energy overfeeding in healthy male volunteers, though the lack of adequate control failed to show that this was a fructose specific effect (Faeh, Minehira et al. 2005). A fructose specific has been shown however in a single study, with an increase in DNL rates of 27% following a 10 week high glucose diet and by 75% following a matched 10 week high fructose diet (Stanhope, Schwarz et al. 2009).

The key hepatic question from these observations that remains to be fully resolved is what is the consequence of the (possibly) increased DNL rate? This will now be discussed.

1.9.6. Potential mechanisms for differing hepatic triglyceride synthesis rates between fructose and glucose

1.9.6.1. A greater rate of *de novo* lipogenesis with fructose than glucose

Potential outcomes of an increased DNL rate are either increased intra-hepatic storage as triglyceride, catabolism via beta oxidation or exportation into the plasma as VLDL incorporated triglyceride, see figure 1.1. Rodent data describes increases in all three processes with a high fructose diet (Nagai, Nishio et al. 2002; Roglans, Vila et al. 2007). These three processes have been assessed to a limited extent in only a single clinical trial with an adequate control.

A high fructose diet has previously been shown to elevate fasting plasma triglycerides, but only once in a study with an adequate and energy balanced control (Silbernagel, Machann et al. 2011). This did show an increased fasted triglyceride concentration following 4 weeks of fructose versus glucose, this however was the only significant difference between these two groups and therefore the significance of this isolated finding is uncertain.

As opposed to fasted assessments, post-prandial assessments in the acute or long-term setting, have repeatedly demonstrated an elevated plasma triglyceride profile in comparison to a high glucose intake (Bantle, Raatz et al. 2000; Teff, Elliott et al. 2004; Swarbrick, Stanhope et al. 2008; Stanhope, Schwarz et al. 2009).

The aetiology of the post-prandial fructose induced hypertriglyceridaemia appears to be both the result of both increased production (DNL) and reduced clearance by lipoprotein lipase (LPL) (Chong, Fielding et al. 2007; Stanhope, Schwarz et al. 2009). The reduced post-prandial insulin release following fructose results in reduced LPL activation, and hence reduced lipolysis of chylomicrons and VLDLs and their removal from the circulation into adipocytes. To further substantiate the reduced LPL activation, circulating NEFAs, a by-product of LPL lipolysis, are lower following a high fructose than glucose diet (Le, Ith et al. 2009; Stanhope, Schwarz et al. 2009).

The final critical difference between glucose and fructose metabolism is the ultimate storage location of the synthesised circulating VLDL. In their excellent and balanced study, Stanhope et al. demonstrated that a 10 week high fructose or high glucose diet resulted in identical amounts of weight gain at around 1.5kg (Stanhope, Schwarz et al. 2009). The increased adiposity was mainly subcutaneous following glucose and visceral following fructose. This adipose deposition pattern had never been previously assessed. A recent study that failed to show any differences between 4 weeks of 23% fructose or glucose in any parameter, bar fasted triglycerides, showed no such changes in subcutaneous versus visceral lipid deposition (Silbernagel, Machann et al. 2011). Ngo Sock et al. published the only other energy matched study comparing the lipid deposition patterns in the two key alternative organs to adipocytes – liver and muscle (Ngo Sock, Le et al. 2010). No difference was shown between glucose and fructose though there was greater IMCL deposition with glucose.

The reasons behind these disparate findings are unclear as will be further discussed in chapter 4. *Ex vivo* human adipose tissue studies have demonstrated a greater LPL insulin sensitivity in subcutaneous adipose tissue than visceral (Fried, Russell et al. 1993). No mechanistic evidence was generated by Stanhope et al. and hence their explanation for the findings, that the greater circulating insulin with a high glucose diet resulted in preferential subcutaneous as opposed to visceral fatty acid deposition, can only be viewed as conjecture.

1.9.6.2. The prebiotic nature of fructose acting on Toll-like receptor 4

If the ability of the small bowel to passively absorb dietary fructose is exceeded then it enters the colon. This colonic entry has a prebiotic effect on the intestinal microflora. Dietary glucose has no prebiotic effects as it is actively absorbed and so does not enter the colon. In rodents a high fructose diet has been shown to increase portal endotoxin concentrations, with a high glucose diet having no such effect (Bergheim, Weber et al. 2008). This same paper demonstrated that the use of non-absorbable antibiotics (polymyxin B and neomycin) markedly reduced the steatosis resulting from a high fructose diet. No data was shown as to whether the antibiotics did reduce portal endotoxin levels, though the authors felt that they had discovered a further and novel pathway for fructose induced hepatic steatosis – via portal endotoxin release.

Toll-Like receptor 4 (TLR4) is a complex expressed on the surface of the hepatic macrophage kupffer cells. TLR4 complexes are activated by portal endotoxin and result in a pro-inflammatory reaction that results in hepatic injury and fibrosis (Rivera, Adegboyega et al. 2007). Following this initial paper the same group compared dietary carbohydrate intakes with plasma endotoxin and hepatic TLR4 mRNA in 12 biopsy proven NAFLD patients and 6 healthy controls (Thuy, Ladurner et al. 2008). As expected there was a close correlation between the concentration of circulating endotoxin and the expression of TLR4 mRNA, whereas there was no correlation between fructose intakes and either plasma endotoxin concentrations or hepatic TLR4 mRNA.

Following this negative paper, the same group further explored the potential links between fructose, endotoxin, TLR4 and hepatic steatosis. TLR4 wild-type and knock-out mice were randomised to water or fructose in their drinks (Spruss, Kanuri et al. 2009). The authors claim that they demonstrated a link between hepatic steatosis, TLR4 status and a fructose-specific effect. Unfortunately there are many limitations in this paper as highlighted in my letter to the authors (Johnston, Macdonald et al. 2010). TLR4 status has a clear influence on overall hepatic metabolism and thus limits the utility of the assessment model. Furthermore, the two diets of chow with either water or water plus 30% fructose were not matched in terms of carbohydrate, energy or prebiotic intake. As a result it is not possible to determine whether the observed changes are fructose, energy or prebiotic specific. Additionally, there is a weight gain disparity between the fructose fed TLR4 wild-type and knock-out groups and consumption data are not presented. Finally, there appeared to be no difference in the response to a high fructose diet dependent on TLR4 status when the paper's findings are analysed relative to each groups' control. This intriguing and attractive area of work therefore needs further study before any definitive conclusions can be drawn.

1.9.7. High fructose diet as an animal model of NAFLD

A high fructose diet is an established rodent model for NAFLD inducing insulin resistance, obesity, hepatic steatosis, lobular inflammation, fibrosis and necrosis with acidophil bodies (Ackerman, Oron-Herman et al. 2005; Anstee and Goldin 2006). It has been shown to be a better model for inducing steatosis and lobular inflammation than a high fat diet (Kawasaki, Igarashi et al. 2009), though

the inflammatory changes are not entirely predictable. The key limitations in translating rodent dietary observations to clinical studies are that rodent and human hepatic metabolism differs, and the doses used in rodent studies are typically much greater than that observed or tolerated in human diets.

Fructose contributes 10% of energy to an average male diet, with a 95th centile contribution of 17% (Marriott, Cole et al. 2009), whereas rodent model studies can supply as much as 70% of energy originating from fructose (Kawasaki, Igarashi et al. 2009). When such a high proportion of dietary intakes originate from a single macronutrient there is a risk of deficiencies of other macro or micro-nutrients. Some elegant and relatively low dose fructose studies have however shown that it can be a model for NAFLD. Roglans et al. supplied rats with drinks containing either water, or 10% fructose or 10% glucose on top of standard chow for 2 weeks (Roglans, Vila et al. 2007). There were identical intakes of sugars between the fructose and glucose arms. Similar weight changes were observed in all three arms as the glucose and fructose fed rats compensated for the energy containing drinks by eating less chow. There was no difference in terms of systemic insulin resistance, whereas there was a 1.8-fold increase in hepatic triglyceride content in the fructose group with a non-significant increase in the glucose group. Histological assessment of liver injury was not performed in this study, though it is clear that a relatively low intake of fructose as opposed to glucose did result in greater hepatic steatosis.

1.9.8. High fructose diet and prior clinical experience of hepatic fatty acid metabolism

As presented in section 1.8.1., NAFLD diets have been frequently characterised by a high fructose intake. Ouyang et al. further explored these fructose observations (Ouyang, Cirillo et al. 2008). They took dietary histories in 6 biopsy proven NAFLD patients and 6 healthy volunteers with a normal liver biopsy. Both the cases and controls were matched in terms of age, sex and BMI. The daily fructose beverage intake was 91g in the NAFLD patients and 43g in the control arm (p<0.05). A murine hepatocyte cell line was next exposed to differing concentrations of fructose. Fructokinase was shown to be upregulated in terms of its activity and expression by the addition of fructose in a dose-dependent manner. This demonstrated that hepatic fructokinase can be viewed as a biomarker of hepatic exposure to fructose. The group then quantified fructokinase mRNA in

their two human liver biopsies. In comparison to the normal livers, hepatic fructokinase mRNA expression was increased by more than 2-fold, and fatty acid synthase (FAS) was increased by more than 3-fold in the NAFLD livers. This confirms the dietary histories and shows that the NAFLD livers had been exposed to and metabolised more fructose than the healthy BMI matched control livers. This however does not prove a causal nor fructose-specific relationship as glucose intakes were not assessed or presented.

There are relatively few clinical studies that have compared fructose versus glucose outcomes on hepatic metabolism and so they will be repeatedly referred to. In order to minimise repetition they will be individually discussed below:

Professor Luc Tappy's group (Lausanne, Switzerland) have performed five of the prior six studies that have assessed the hepatic metabolic consequences of a high fructose diet (HFrD) in healthy volunteers. The studies are all very similar in design, though difficult to interpret. Unfortunately five out of the six lack either an energy or carbohydrate matched control. All of the studies provide fructose in drinks consumed in addition to food intakes. They involve a random crossover between the arms with a washout period of 2-4 weeks. Only one (negative) study presents baseline data including weights (Le, Faeh et al. 2006). This is critical as it is impossible to determine the adequacy of the washouts, or the absolute changes with the interventions. Instead the post intervention findings in the other four papers are compared with the findings post a control period where food is supplied. This 'control', 'weight maintenance', or 'isoenergetic' arm also has no weight maintenance or metabolic data presented and so it is impossible to be certain that there is not some underfeeding and hence metabolic alteration occurring in this comparator arm. Furthermore the fructose fed group consumes the same control diet plus additional fructose. The resultant energy difference between the fructose-fed and control group is around 800-1,000kcal per day. It is therefore theoretically possible that the results merely reflect energy overfeeding (with fructose) versus energy underfeeding. As a consequence it is impossible to state that the effects observed are fructose specific.

A further major issue is that the supplied food results in carbohydrate overfeeding providing 50-55% of the energy. The mean carbohydrate contribution to total energy intake in UK men is 43.0±7.3% (SD) with the 97.5% centile at 56.1% (NDNS 2009). So the control group are not consuming a standard

carbohydrate intake. Finally, there is no mention of investigator blinding in any of the protocols. The six papers by this Lausanne group led by Luc Tappy are presented below:

1. The first study involved 7 healthy and slim male volunteers with a mean body fat of 16.5% (Faeh, Minehira et al. 2005). Both groups consumed an identical isoenergetic diet for 6 days with or without 3g/kg of fructose per day. At the end of the 6 days body weights were greater with the HFrD than the energy reduced control, though with no differences in systemic insulin resistance. Interestingly, hepatic insulin resistance was induced, and hepatic de-novo lipogenesis and fasting triglycerides increased by 588% and 79% respectively.

2. A similar protocol was observed where 8 healthy men and 8 healthy women received an isoenergetic diet for 6 days with or without 3.5g/kg per day of fructose (Couchepin, Le et al. 2008). Very similar post intervention period differences were shown in terms of systemic and hepatic insulin resistance in the men, though no differences were observed in the women despite greater fasted plasma triglycerides. In the men there was greater plasma ALT (a marker of hepatic injury) following the high fructose and energy period. The authors were uncertain as to the reason behind the differing responses between the sexes. They speculated as to whether this may be a result of differing hormonal profiles, body composition or hepatic metabolism. No further study has compared the influence of sex on the hepatic insulin sensitivity response to a HFrD.

3. Two further studies have assessed hepatic lipid content using proton MR spectroscopy. The first took 7 healthy slim males with a low fructose intake, who consumed 18% fructose in addition to their baseline intakes for 4 weeks (Le, Faeh et al. 2006). There was no control group though baseline data were presented. There was an increase in fasting plasma triglycerides, lactate and reduced ketogenesis as evidenced by reduced beta-hydroxybutyrate. Insulin sensitivity and hepatic and muscle steatosis were assessed at baseline and weeks 1 and 4. There were no significant changes noted in these parameters. This could be explained by compliance or sample size issues. Weight gain was minimal at 0.3%, which is surprising as the fructose supplied 18% extra energy for 4 weeks. Carbohydrate and lipid oxidation rates were unchanged at week 4. As foods were not supplied and dietary intakes not presented, it would appear that there was either considerable dietary adaptation or non-compliance. The number of volunteers was very small at 7.

4.   The group clearly also felt that their prior study had limitations as they essentially repeated the study, this time with a larger cohort, greater fructose dose and shorter intervention period. 16 healthy slim men with a first degree relative with type 2 diabetes, and 8 men with no such family history, took 7 days of a high fructose diet providing 35% of their energy needs followed by a standard diet (Le, Ith et al. 2009). The energy difference between the two groups was again around 810kcal per day, and as a result there was a 1.3% weight difference at the end of the study between the groups. In comparison to the low energy 'controls', the high fructose diet resulted in a 78% increase in hepatic lipids, and 5% increase in fasting hepatic glucose output. These changes were more marked in those with a family history of type 2 diabetes than those without. The reason for this is unclear. There are no baseline data to allow us to compare these two groups. Comparing the post-intervention data for the 'control', the 16 volunteers with a family history of type 2 diabetes had greater body weight and fat, greater hepatic lipid content, and were more systemically insulin resistant than the 8 healthy volunteers. There was no difference in terms of hepatic insulin resistance. It is therefore impossible to speculate as to which of these factors resulted in an increased susceptibility to the HFrD.

5.   Fructose overfeeding was compared with fat or fructose and fat overfeeding in healthy slim men (Sobrecases, Le et al. 2010). An isoenergetic diet, with no baseline data, was compared with 35% energy overfeeding with fructose for 7 days, and 30% energy overfeeding with fat for 4 days, and 35% energy overfeeding with fructose and 30% energy overfeeding with fat combined for 4 days. This extraordinary design meant that the groups all differed in macronutrients, energy and duration too. As a result little can be reliably inferred from it. The combined fat and fructose resulted in a greater increase in hepatic triglyceride content (HTGC) than fat or fructose alone, but the influence of the greater energy supply cannot be discounted for.

6.   The above studies lacked any true control and so cannot be viewed as being fructose specific. The final study performed by the same group addressed this issue and was published after the initiation of my study (Chapter 2). Ngo Sock et al. performed a 7day 35% fructose versus 35% glucose study versus a control diet in 11 healthy males (Ngo Sock, Le et al. 2010). Unfortunately there are the same issues with the data presentation of this study. There was only a 2-3 week washout period employed between three arms, and no baseline data.

Furthermore the absolute HTGC values are not presented, despite being the primary outcome measure. Instead the mean log HTGC (no units) is presented graphically post intervention, so it is very unclear as to what the units of HTGC were.

The cohort recruited had a BMI between 19 and 25 and were insulin sensitive (HOMA-IR of 1.0). Significant increases in the fructose and glucose treatments were seen for HTGC (52% vs. 58%), IMCL (49% vs. 84%), and VLDL (59% vs. 31%). As expected, carbohydrate oxidation increased in both. Curiously there was an identical increase in lactate and uric acid in both treatments and a similar reduction in NEFAs and beta-hydroxybutyrate. There is no comment on the outcome of systemic insulin resistance whilst hepatic insulin resistance reduced in both. The authors state that the HTGC changes in this study are smaller (52% versus 79%) than they have previously found with similar (uncontrolled) fructose overfeeding (Le, Ith et al. 2009), and that the inter-individual variability may have been a factor. As a result the authors do not conclude that glucose and fructose have the same hepatic lipid outcomes.

Since the onset of my current work a group separate from Luc Tappy's published another related paper. This paper was in many ways well designed and presented, with the investigators being blinded to the randomisation process (Silbernagel, Machann et al. 2011). The cohort however was small at 10 in each arm and also of mixed sex that may have added another factor. 150g of fructose or glucose was consumed three times a day. Food was not supplied, but they were instructed to consume a diet containing 50% carbohydrates, 35% fat and 15% protein and the physical activity factor was fixed at 1.6. The cohort was evenly balanced at study entry. They were relatively slim with a mean BMI of 25.9kg/m^2 and low body fat of around 23%. As a result they were insulin sensitive (mean HOMA-IR of around 1.7) and had a low initial HTGC (around 1.5%).

There were no interim visits and unfortunately the two arms seemed to differ markedly in their energy intakes during the intervention period. The fructose group's weight change was only 0.2±0.6kg (SEM) (p=0.40), indeed several seemed to lose weight. Weight gain seemed to be universal in the glucose arm as the changes were +1.7±0.4kg (p=0.001). So as a result the fructose arm appears to be isoenergetic and the glucose hyperenergetic. It is an interesting study, though the marked difference in weight gain between the two groups significantly hampers any real interpretation. Ultimately the only changes that

differed from baseline in either arm (including uric acid or NEFA) were an elevated fasting triglyceride with fructose, and an increased HOMA-IR with glucose. There were no changes that were different between the two arms.

There was a non-significant increase in HTGC of 34% as compared to the baseline value in the fructose arm and 33% in the glucose arm, though the wide variation in responses meant that neither of these changes was significantly different from the baseline values. The changes in IMCL were again non-significant at +24% and -6% respectively.

There is one final paper that is worthy of a mention though it did not assess hepatic lipid content (HTGC). Stanhope et al. randomised 32 (obese men and post-menopausal women who were older than prior studies (mean age 54), to 10 weeks of drinks providing 25% of predicted energy needs as either fructose or glucose. Compliance and weight gain were matched equally between the arms. The study protocol was metabolically exhaustive and involved 2 weeks of baseline inpatient stay within the unit, 8 weeks as an outpatient and a final 2 weeks as an inpatient. There was no crossover. During the initial pre-intervention 2 weeks, weight was maintained by provided food that had a very low fructose (1.5%) and glucose (2.9%) content. The next 8 weeks were then as an outpatient with *ad libitum* food intakes but with no sugar-containing drink bar the fructose or glucose which was mixed with water. The same inpatient food was then finally consumed during the last 2 weeks with the fructose or glucose. These details are critical to interpreting the study. Pre-study habitual food intakes are not presented, though there was clearly a washout of all monosaccharides before the intervention started and of the non supplemented monosaccharide for two weeks pre repeat assessment. This approach meant that very real changes in intakes could be assured, though it compared the outcomes of dramatic changes in monosaccharide intakes from around 2% to 30% during the outpatient phase. Beyond these observations there are little other critical comments that can be directed towards this excellent and blinded paper.

Glucose had no influence on systemic insulin sensitivity, whereas fructose impaired sensitivity by 17% as assessed by a 3 hour oral glucose tolerance test and by deuterated glucose disposal. 24-hour circulating triglycerides increased by 18% with fructose and by 3% with glucose. These changes were in association with increased postprandial DNL and reduced postprandial lipoprotein lipase (LPL) activity (non-significant). The authors attribute the LPL changes to lower

postprandial insulin concentrations. Ectopic lipid storage was only assessed by CT scans of the umbilical region to determine subcutaneous and visceral fat content. Weight changes were the same in both groups though fructose resulted in greater increases in visceral adiposity (14.0% vs. 3.2%) and abdominal subcutaneous adiposity too (7.3% vs. 4.6%). It therefore is unclear as to where the weight was deposited following glucose, though one assumes that it was in peripheral adipose stores. This is supported by the findings in the gluteal subcutaneous fat biopsies. Indeed lipogenic and lipid desaturation gene expression increased following glucose and was unchanged or reduced following fructose. Interestingly the adipose tissue adiponectin: TNFα balance was worsened following glucose and improved with fructose. Unfortunately there was no systemic TNFα or adiponectin data presented.

In conclusion there is a conundrum. The single best and most long-term paper has demonstrated changes in all the factors associated with hepatic steatosis namely insulin resistance, visceral adiposity and increased DNL. However it failed to assess hepatic lipid content. No other controlled paper has shown all of these findings or that hepatic lipid content is increased following a high fructose intake. The aim was therefore to reproduce many of the key features of the paper by Stanhope et al. and to avoid some of the others' pitfalls.

1.9.9. Conclusions on fructose and glucose on hepatic fatty acid metabolism

The hepatic metabolism of fructose and glucose primarily differ as a result of their hepatic extraction rates. This is summarised in figure 1.8. Fructose results in a greater hepatic production of lactate and lipids, and increased hepatic insulin resistance and visceral adiposity. Compiling all the mechanisms previously described, fructose may result in greater hepatic steatosis than glucose by:

1. increasing *de novo* lipogenesis, both through substrate provision and by upregulation of lipogenic factors.
2. increasing visceral adiposity resulting in increased portal NEFA delivery to the liver
3. inducing hepatic insulin resistance either by stimulating hepatic JNK activity and thus impairing post insulin-receptor signalling, or as a potential consequence of hepatic steatosis itself.

4. acting as an intestinal prebiotic resulting in greater portal endotoxin induced TLR4 activation. There is as yet however very limited evidence for this pathway.
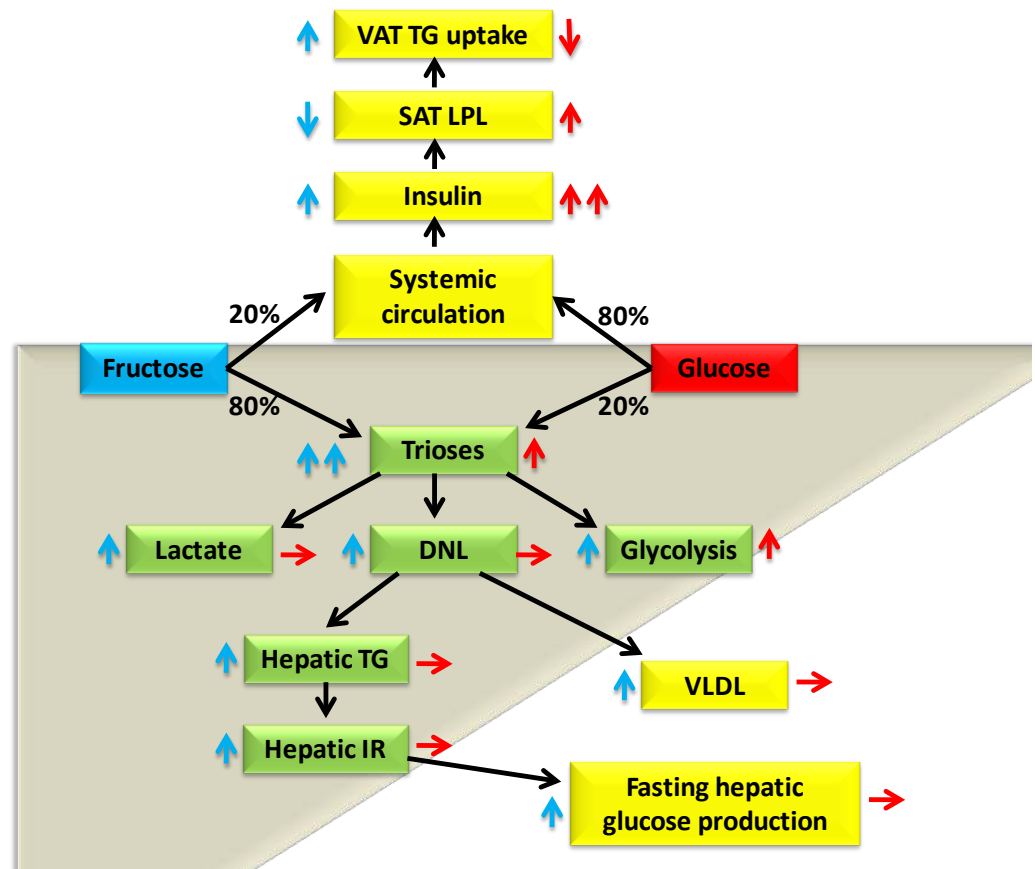
Figure 1.8: Comparative overview of proposed fructose (blue) and glucose (red) metabolism. Hepatic effects are shown in green and systemic effects in yellow. (DNL = de novo lipogenesis; TG = triglyceride; IR = insulin resistance; VLDL = Very Low Density Lipoprotein; SAT LPL = Subcutaneous adipose tissue lipoprotein lipase; VAT TG = visceral adipose tissue triglyceride.)

This hypothesis that a high fructose diet (HFrD) may induce steatosis is further supported by the following observations:

a.   Biomarker and self-reported food intake patterns describe greater fructose intake patterns in NAFLD patients than matched controls.

b.   That a HFrD (albeit often a very HFrD) is a reliable rodent model for NAFLD

c.   That a HFrD has been shown to increase hepatic triglyceride content, though this has not been shown to be a fructose-specific effect.

d.   A single study demonstrating that a HFrD as opposed to glucose results in greater insulin resistance, visceral adiposity and DNL.

This thesis aims to explore the impact of intakes of differing macronutrients on hepatic lipid metabolism. Given the background data described I chose to assess outcomes following altered intakes of fructose and n-3 PUFAs. Two studies were performed, both with the primary outcome measure of hepatic triglyceride content, whilst additional explanatory measures were also assessed.

A high fructose intake was compared with a high glucose intake in healthy overweight male volunteers, whilst the use of n-3 PUFA supplements were compared with oleic enriched sunflower oil supplements in patients with biopsy proven NAFLD. The fructose versus glucose study is detailed in chapters 3 to 5 and the n-3 PUFA study is detailed in chapter 6.

Chapter 2. Methods

2.1. Energy predictions

The determination of resting energy expenditure (REE) by formulae is controversial and fraught with challenges. This is particularly an issue in the obese. The Schofield equation is widely employed, and uses the criteria of gender, age and weight to determine resting energy expenditure (Schofield 1985). The formula originates from a database of a combination of prior published studies. The result is both a strength and a weakness, with over 60% of the data on men aged between 10 to 60 years originating from Italian studies published between 1936 and 1942. There are therefore concerns that the Schofield database does not reflect that observed in modern populations, and there have been observations that the energy requirements of Italians appear to differ from that of Northern Europeans.(Shetty, Henry et al. 1996)

The metabolic rate of adipose tissue is lower than that of lean tissue. As a result, it has been shown that REE increases in a non-linear fashion with weight, with a slower increase in men weighing more than 75kg.(Horgan and Stubbs 2003) As a result linear formulae tend to over predict energy needs in the overweight or obese. This is a particular problem when using the Schofield equation, and there is little data to support its use in the obese setting.(Frankenfield, Roth-Yousey et al. 2005)

As a result Henry re-appraised the prior published energy requirements data in 2005.(Henry 2005) New predictive formulae were generated using height as an additional criterion to those of Schofield. The combination of weight and height results in an improved, but still imperfect, proxy for adiposity. The basal metabolic rate predicted by the Henry formula results in a 1-4% lower value in overweight young men than the Schofield equation. These equations have been assessed in the overweight male setting by 2 further groups, both of which have shown the formulae to be more accurate than the Schofield formula. (Ramirez-Zea 2005; Weijs 2008)

The Henry formulae for men are

18 - 29 years: **REE = 14.4 x W + 313 x H + 113**

30 - 59 years: **REE = 11.4 x W + 541 x H - 137**

Where W is weight in kilograms, and H is height in meters, and REE is kcal/day

The determination of total energy expenditure (TEE) requires adjustment for the activity of the subject. Using doubly labeled water, Tooze et al. described a mean physical activity factor of 1.7 in overweight and obese middle-aged men.(Tooze, Schoeller et al. 2007) Subjects' physical activity was assessed using the self-completed short version of the International Physical Activity Questionnaire (IPAQ) at the initial screening assessment (Hagstromer, Oja et al. 2006). The IPAQ was developed in the late 1990s by a multi-national group, supported by the WHO. The short version of the IPAQ is quick and simple to administer and has been shown to correlate acceptably with objective evidence of physical exercise.(Mader, Martin et al. 2006) It assesses physical activity levels during the preceding seven days and generates a low, moderate or high activity score.

2.2. Indirect calorimetry

Direct calorimetry determines total energy expenditure (TEE) by measuring heat loss in a thermally sealed chamber and thus estimating heat production. Indirect calorimetry quantifies resting energy expenditure (REE) by measuring respiratory oxygen consumption and carbon dioxide production. REE is converted to TEE by:

TEE = (REE + diet induced thermogenesis) x activity factor

Diet induced thermogenesis varies little in most physiological circumstances and impacts little on the determination of TEE.

Resting energy release arises from the oxidation of stored macronutrients:

Substrate + O_2 $\longrightarrow$ CO_2 + H_2O + Heat
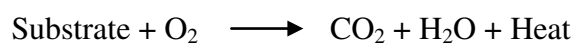
The amount of respiratory oxygen consumed (VO_2), and carbon dioxide exhaled (VCO_2) are measured by the calorimeter. The ratio between VCO_2 and VO_2 is termed the respiratory quotient (RQ), and reflects substrate oxidation. Oxidation of each of the main macronutrients results in a predictable amount of oxygen consumption and carbon dioxide production. The complete oxidation of glucose consumes 6mol of O_2 and produces 6mol of CO_2. As a result the RQ is 1.0. The RQs for lipid and protein oxidation are 0.69 and 0.81 respectively (Haugen, Chan et al. 2007).

Using indirect calorimetry substrate oxidation rates can be calculated by the non-protein equations of Peronnet and Massicotte (Peronnet and Massicotte 1991):

Carbohydrate oxidation (g/min): $4.585 * VCO_2 – 3.226 * VO_2$

Fat oxidation (g/min): $1.695 * VO_2 – 1.701 * VCO_2$

These calculations assume that there is no accumulation or excretion from the body of a metabolic intermediary or end-product other than those in the equation, namely CO_2, water or heat. Such states of accumulation or excretion can exist however, and include lipogenesis, gluconeogenesis, ketogenesis and lactate formation (Frayn 1983). As a result, net carbohydrate oxidation rates generated by the above equations during periods of *de novo* lipogenesis also include the rate of conversion of glucose to fatty acids. This elevates the observed RQ, as the RQ of lipogenesis is 5.6. Furthermore, during lipogenesis calculated lipid oxidation values can become negative, the apparent negative rates of fat oxidation quantitatively represents net rates of fat synthesis.

The gluconeogenic oxidation of lactate and pyruvate do not influence the calculations as the Cori cycle does not involve gaseous exchange. Protein oxidation resulting in gluconeogenesis consumes CO_2 whilst producing no O_2, though this process is rare in the absence of a very high protein diet or diabetes (Livesey and Elia 1988). This has no influence on lipid oxidation rates and only a minimal influence on carbohydrate oxidation rates (Frayn 1983). Ketogenesis may need to be taken into account when studying subjects undergoing prolonged fasting. The formation of lactate may influence acid-base balance and hence displace CO_2 from bicarbonate stores. This effect is most important in anaerobic exercise studies. Any influence however is very small in the absence of rapid changes in blood lactate concentrations (Frayn 1983).

During an insulin clamp a steady state of plasma glucose is achieved. As a result, the glucose infusion rate is assumed to equal the whole body glucose disposal rate (G_{total}). During the clamp procedure the rate of glucose oxidation (G_{ox}) can be determined by indirect calorimetry. As a result the rate of non-oxidative glucose disposal (predominantly glycogen storage) can be calculated as:

$G_{non-ox} = \quad G_{total} – G_{ox}$

2.3. Satiety assessment

There is a very large inter-subject variability in reported satiety and energy during periods of routine intake or following a dietary intervention. Visual

analogue scales (VAS) are widely used in to monitor satiety and its changes. Subjects are asked to mark along a line as to where their response fits between two opposing statements. The main advantage of this is its ease of design, but the interpretation is difficult as they cannot be assumed to measure the absolute intensity of a sensation. For example, a mark at 30mm along a line does not necessarily infer that the sensation is half as intense as a mark at 60mm.

2.4. Whole body dual energy X-ray absorptiometry (DEXA)

This technique was originally developed to quantify bone mineral density, though it has been extended to assess a whole body's bone, fat and lean tissue mass. X-rays produced by tungsten tubes result in photons of differing energies passing through the body which are quantified by filters in a sequential and planar projection. The molecular compositions of fat, lean tissue and bone result in differing absorption and scattering patterns for low and high energy photons. From these characteristic patterns the relative proportions of these tissues can be determined (Brownbill and Ilich 2005). Regional, as opposed to compartmental, data can be generated as only a single plane is assessed. As a result no differentiation is possible between fat content within the abdominal visceral and subcutaneous spaces. Cross-sectional CT or MRI images are needed to reliably assess compartmental compositions.

There is no true gold standard *in vivo* method for analysing body composition. The correlation between whole body MRI taken at 10mm slices and DEXA was excellent, with an r^2 of 0.95 (Vogt, Ruehm et al. 2007). The analysis of MRI data is extremely laborious and the investigation expensive. DEXA scans are the most commonly performed body composition assessment modality due to their speed of assessment and analysis, cost and low radiation exposure. There are however four potential limitations to the use of DEXA: the use of ionising radiation, the influence of hydration status, size of the absorption field, and its failure to distinguish overlying tissues types.

The dose of ionising radiation supplied varies slightly according to the size of the individual, though it is small at around 0.2μSv (Bezakova, Collins et al. 1997). The annual background exposure dose is greater than 1000μSv and so the procedure results in a minimal increase. The failure of DEXA to measure body water does however limit its accuracy. A five percent change in the water content of the lean body mass alters DEXA derived estimates of whole body fatness by 1-

2.5% (Lohman, Harris et al. 2000). Such variations are rare in healthy and stable conditions, and so in this setting the influence of hydration is negligible (Pietrobelli, Wang et al. 1998). The limbs of severely overweight individuals can fall outside the scanning area of the DEXA scanner, and overlapping of body parts increases thickness in those regions (Brownbill and Ilich 2005). The filters cannot distinguish between three tissue types in the same area. So when fat and lean tissue overlies bone the values cannot be directly resolved and have to be interpolated. The above accepted it is an acceptably reliable, safe and reproducible assessment.

2.5. Quantifying insulin resistance

2.5.1. Homeostasis model assessment of insulin resistance (HOMA)

This is a simple assessment of insulin resistance in the overnight fasted state from a single blood test. It assumes a dynamic and functional feedback between the liver and pancreatic Beta cells. Insulin concentrations reflect Beta cell response to plasma glucose concentrations, whereas hepatic glucose production is regulated by the insulin concentration. As a result a high glucose concentration and hence HOMA value primarily reflects fasted hepatic resistance to insulin-mediated reduction in gluconeogenesis.

The formula is:

HOMA = {[fasting insulin (μIU/ml)] * [fasting glucose (mmol/l)]} / 22.5. (Matthews, Hosker et al. 1985)

The denominator of 22.5 is employed as a normalising factor; as the product of normal fasting plasma insulin (5 U/ml) and normal fasting plasma glucose (4.5mmol/l) is 22.5. Hence a healthy HOMA is approximately 1.0, and the higher the value the more resistant the individual. Though due to lack of standardised insulin assay techniques the inter-assay values means that it is not possible to define cut off points for insulin resistance. The other main issue with the use of HOMA is its low reproducibility of around 23% (Sarafidis, Lasaridis et al. 2007).

2.5.2. Adipose tissue insulin resistance (Adipo-IR)

Recently the quantification of a fasted adipose insulin resistance index has been described, based on feedback between insulin and adipose tissue lipolysis via hormone sensitive lipase (Gastaldelli, Cusi et al. 2007). Adipo-IR correlates with hepatic fat (r=0.54) (Gastaldelli, Harrison et al. 2009). The formula is:

Adipo-IR = Free fatty acids (mmol/L) x fasted plasma insulin (pmol/L).

2.5.3. Hyperinsulinaemic euglycaemic clamp

This technique is recognised as being the 'gold standard' for determining whole body glucose sensitivity to insulin in vivo (Muniyappa, Lee et al. 2008). It differs from the HOMA assessment in that it mimics the postprandial, as opposed to fasted, state and also it predominantly assesses tissue sensitivity to insulin-mediated glucose uptake as opposed to hepatic sensitivity to insulin-mediated regulation of gluconeogenesis.

The delivery of exogenous insulin, as opposed to relying on endogenous insulin production, increases its reproducibility over homeostasis models and glucose tolerance tests. The clamp procedure can be used to quantify sensitivity to several of insulin's actions beyond that of whole body glucose disposal. Adipose tissue sensitivity to insulin-mediated lipolysis can be assessed by free fatty acid concentrations during the procedure. Hepatic insulin sensitivity and glucose output can be determined with the addition of a glucose tracer, as will be discussed further. Finally, the combination with indirect calorimetry facilitates the quantification of glycogen synthesis (i.e. non-oxidative glucose disposal) as previously described.

Determining systemic insulin sensitivity by use of a hyperinsulinaemic euglycaemic clamp

Using the protocol described by DeFronzo *et al.* (DeFronzo, Tobin et al. 1979), exogenous insulin is delivered as a loading bolus over 10 minutes followed by a constant infusion at a pre-determined dose. A stable plasma glucose concentration is maintained by the simultaneous infusion of glucose. The delivery of a high insulin concentration mimics the postprandial state and is assumed to fully suppress endogenous glucose production. As a result of the suppression of endogenous production, the rate of exogenous glucose supplied equals the rate of whole body glucose disposal at steady state plasma glucose (M). M is typically

normalised to fat free mass as glucose uptake can only occur in these tissues (Ferrannini and Mari 1998).

From the above it is clear that the determination of the timing of 'steady state' is critical to the analysis of the data generated. Two approaches can be taken, either defining an arbitrary time point towards the end of the clamp study, or by taking data from a period of objective stability more than an hour into the clamp (Muniyappa, Lee et al. 2008). The objective criteria proposed include a period greater than 30 minutes during which the coefficient of variation of plasma glucose, plasma insulin, and glucose infusion rate (GIR) is less than 5%. The latter approach is more rigorous, though may not be achieved during every clamp.

Determining adipocyte insulin sensitivity

Insulin suppresses lipolysis in adipocytes via inhibition of hormone-sensitive lipase and thus reduces circulating plasma NEFA concentrations. The relative reduction in plasma NEFA concentrations following insulin delivery during a clamp can thus be used to assess adipocyte insulin sensitivity.

Determining hepatic insulin sensitivity

This was performed by using a stable glucose isotope dilution technique during basal and insulin stimulated periods. The addition of a label to exogenously delivered glucose enables differentiation of the origin of circulating glucose from exogenous and endogenous sources.

Deuterated glucose is a stable isotope of glucose containing ^2H as opposed to the naturally occurring ^1H. The differing atomic masses of ^2H and ^1H glucose facilitate their individual quantification by mass spectrometry. The location of the isotopic label in the glucose molecule determines which metabolic pathways the tracer enters post glucose catabolism. Dideuterated glucose with deuterium in the 6th position ($[6,6-^2H_2]$) was selected as it is less susceptible to intermediary step analyses than the 2nd or 3rd position, and hence estimates total glucose turnover with greater accuracy (Choukem and Gautier 2008). The rate of appearance (R_a) of $[6,6-^2H_2]$ is its rate of infusion, and hence at steady glucose state the proportion of $[6,6-^2H_2]$ dideuterated glucose amongst total glucose facilitates estimations of endogenous glucose production.

There are 3 main assumptions made about the behaviour of tracer glucose:

1. The tracer is treated and distributed the same as unlabelled glucose.
2. At steady state the rate of tracer infusion is proportional to its disposal.
3. The tracer is not recycled

The first two assumptions have been shown to be essentially valid (Wolfe RR 2004). However, recycling has been shown to occur. Labeled glucose can be taken up peripherally and metabolised to pyruvate and then lactate. The labeled lactate can then return back to the liver and re-form glucose. The resultant endogenous, hepatically synthesised, glucose will be labeled and circulate in the plasma as either ($[6,6-^2H_2]$) or ($[3,3-^2H_2]$) glucose. As a result some of the products of this recycling and endogenous re-synthesis are indistinguishable from exogenous labeled glucose. The magnitude of this effect is not believed to be great (Finegood, Bergman et al. 1987), though it results in an underestimate of the rate of hepatic glucose production.

Equations of Steele:

The most commonly used equations to determine hepatic insulin sensitivity are the equations of Steele (Steele 1959). These equations rely on the assumptions that there is only a single compartment of distribution and that it has a constant volume. Several authors have questioned these assumptions (Cobelli, Mari et al. 1987; Finegood, Bergman et al. 1987), though these original equations remain the most widely used (Choukem and Gautier 2008).

Ra*

Ra   **C*** + **C**   **Rd**

Rd*
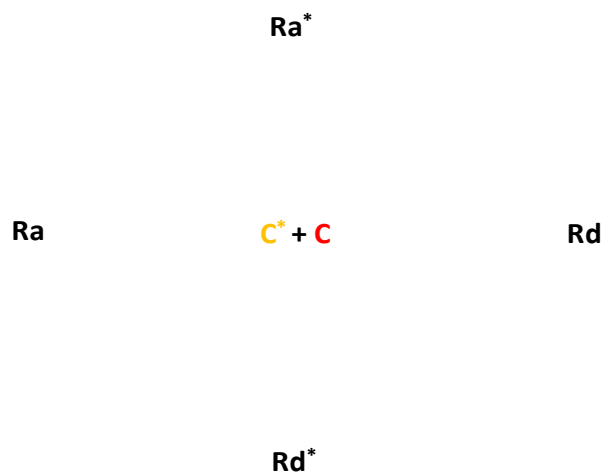
Figure 2.1. Depiction of a one compartment model (adapted from (Choukem and Gautier 2008)). R_a: rate of appearance of unlabelled (hepatic + exogenous sources) glucose. R_a^*: rate of appearance of tracer glucose. C: unlabelled plasma glucose concentration. C^*: labeled plasma glucose concentration. R_d: rate of disappearance of unlabelled glucose. R_d^*: rate of disappearance of tracer glucose.

As depicted above in figure 2.1., during steady-state conditions, the combined rate of glucose appearance ($R_a + R_a^*$) equals its combined disappearance rate ($R_d + R_d^*$). Furthermore, the ratio of labeled to unlabeled plasma glucose (C^* / C) also reflects the relative rate of the labeled tracer infusion to the rate of endogenous glucose production. So the equations of Steele for hepatic glucose production (HGP) are:

HGP = TIR / (C^* / C)

Where TIR is tracer infusion rate. The greater the HGP the more resistant the liver is to the effects of insulin. HGP can be further related to plasma insulin to form a hepatic insulin resistance index (Matsuda and DeFronzo 1999).

Hepatic insulin resistance index = 1 / (HGP x plasma insulin) x 100

It bears remembering that the liver is not the only glucose-producing organ during fasting conditions. The renal contribution is at least 5% (Choukem and Gautier 2008).

Modifications to the equations of Steele:

In the postabsorptive (fasted) state there is essentially no glycolysis of glucose within the liver, and therefore all hepatically produced glucose enters the circulation. A one-compartmental model can therefore be used in this setting. However as previously discussed, in the presence of glucose and insulin administration glucose is disposed of in muscle and liver either by oxidation or by non-oxidation (glycogen formation). This results in a non-static volume of distribution during a clamp.

If 'A' is the amount of tracer (C^* in figure 2.1.) and 'B' is the amount of tracee (C in figure 2.1.), then the enrichment 'E' is:

E = A/B

If we determine values at a specific time $_{(t)}$ and changes as δ then:

$\delta A_t / \delta_t = E_t (\delta B_t / \delta_t) + B_t (\delta E_t / \delta_t)$ (equation 1)

Changes in the total amount of tracee within a pool (δB_t) over a time period (δ_t) are equal to the differences between its rate of entry (Ra) and disappearance (Rd):

$\delta B_t / \delta_t = Ra - Rd$ (equation 2)

Changes in the total amount of tracer within a pool (δA_t) over a time period (δ_t) are equal to the rate that it is infused (F) minus the rate that it leaves the pool (RdE):

$\delta A_t / \delta_t = F - RdE$ (equation 3)

combining equations 3 and 1:

$E_t (\delta B_t / \delta_t) + B_t (\delta E_t / \delta_t) = F - RdE$

Combining this with equation 2:

$E_t (Ra - Rd) + B_t (\delta E_t / \delta_t) = F - RdE$

Hence

$Ra = [F - B_t (\delta E_t / \delta_t)] / E_t$

As glucose tracee and tracer do not mix instantaneously additional factors need to be added, where 'p' is the fraction of the total pool that does not rapidly mix, and the total extracellular glucose space is 'V'. As a result the amended formula is:

$$Ra = [F - pV ((C_2 + C_1)/ 2) * ((E_2 - E_1) / (t_2 - t_1))] / ((E_2 + E_1) / 2)$$

Where C is concentration, E enrichment and t is time at two time points ($_1$ and $_2$). The commonly used value for p is 0.65 (Cowan and Hetenyi 1971), and 25% of body weight for V (Rebrin, Steil et al. 1999).

A large volume of exogenous glucose is typically infused during a glucose clamp which results in a fall in glucose enrichment. The problem of negative values for glucose production can be avoided by the addition of tracer to the exogenous glucose infused, a so-called 'hot infusion' protocol (Finegood, Bergman et al. 1987) (Levy, Brown et al. 1989). Such an approach was used by Powrie et al. who developed a formula for Ra (hepatic glucose production) (Powrie, Smith et al. 1992):

$$Ra = (F / Ep_{(t)}) + (Evar * Ivar_{(t)} / Ep_{(t)}) - ((p*V*G_{(t)}* (\delta Ep_{(t)} / \delta t) / Ep_{(t)})$$

Where F is the glucose tracer constant infusion rate, $Ep_{(t)}$ is the plasma enrichment at time t, Evar is the enrichment of the variable glucose solution used during the

clamp, $Ivar_{(t)}$ is the infusion rate of that solution, and G is the prevailing glucose concentration.

It will be noted that at steady state the value for $\delta Ep_{(t)}$ is zero and hence Ra is the sum of infusion divided by enrichment of the constant and variable solutions.

From this, endogenous (hepatic) glucose production = R_a – exogenous glucose infusion rate

2.6.   MRI and MRS

In order for a nucleus to be observable by magnetic resonance (MR) it must have a non-zero spin, i.e. an odd number of protrons such as ^1H, ^{13}C, ^{19}F, ^{23}Na, and ^{31}P. Hydrogen is the most naturally abundant element (99.98%) and hence ^1H produces the most intense MR signals. As a result ^1H is the most frequently used element for MR imaging and spectroscopy assessment, and most systems incorporate their own ^1H receiver. Additional receivers need to be added within the magnet if other nuclei such as ^{13}C or ^{31}P are to be assessed.

The basic principles of MR involve a series of steps. An object is first placed within a powerful and homogenous external magnetic field. The nuclei within the object thus align themselves parallel or anti-parallel to the field. A brief pulse of radiofrequency (RF) energy is then applied tangential to the magnetic field polarity. This pulse decreases the longitudinal magnetisation and increases the transverse magnetisation, and so the nuclei are displaced. Once the RF pulse is stopped there is a period of relaxation when the excited and displaced nuclei re-align themselves to their original position. The signal generated by this re-alignment is detected by a coil as free induction decay (FID). The FID is assessed at multiple moments during the relaxation period and hence its exponential reduction can be plotted as intensity versus time. This FID data undergoes Fourier transformation, by computer analysis, generating either an image (MR imaging (MRI)) or a frequency spectrum (MR spectroscopy).

In MR spectroscopy the spectra are plotted on a graph with frequency in parts per million (ppm) on the x-axis and spectral amplitude on the y-axis. See figure 3. The spectroscopic peaks generated have four principle characteristics:

A. resonance frequency (ppm), i.e. their position on the x-axis.

B. height

C. width

D. peak area
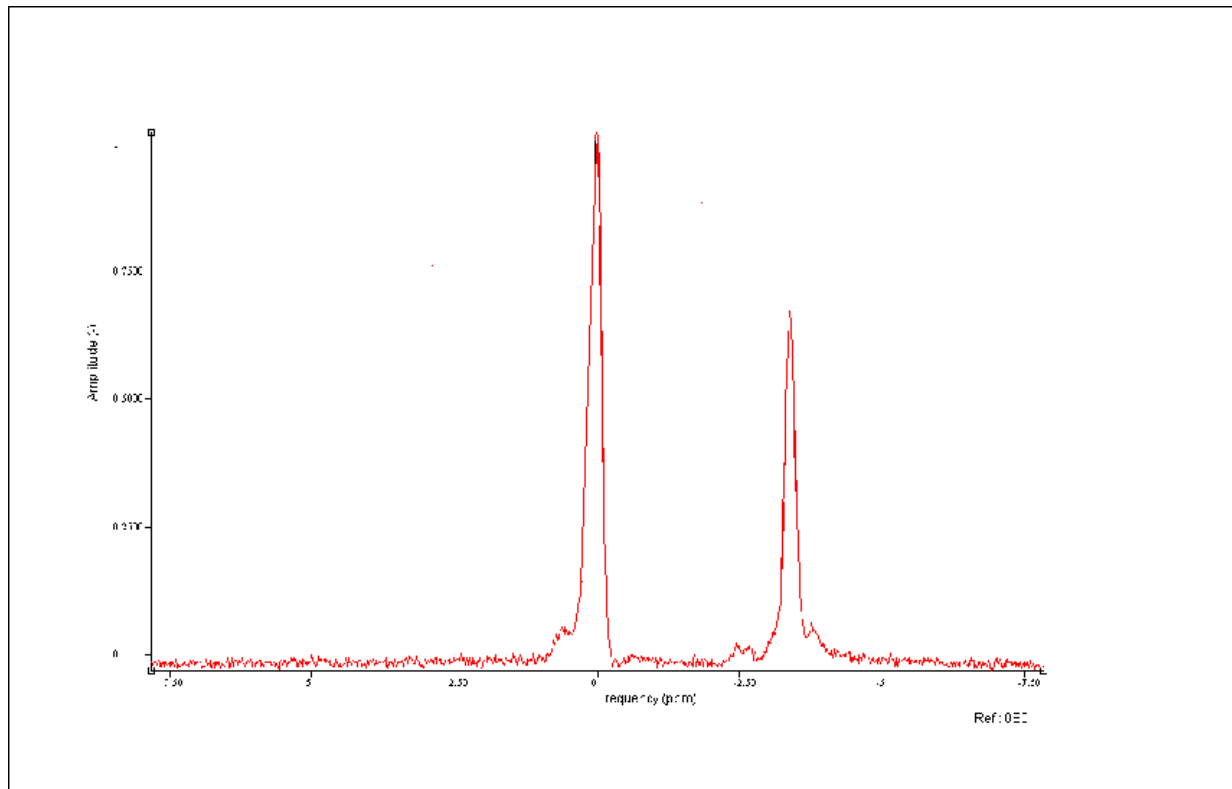
Figure 2. Typical ^{1}H MRS spectroscopic image with the main water peak and the smaller methylene lipid peak.

It is the peak area which yields relative measurements of the concentration of each nucleus. Furthermore, each nucleus can be identified by its own unique and specific resonant frequency. These frequencies however are slightly modified by the molecular structure, or environment, of the compound that the nucleus is in. This frequency modification process is termed chemical shift, and it results from neighbouring electrons forming a cloud which 'shield' the nuclei from the overall magnetic field. The hydrogen in water (H-O) bond are less shielded than the hydrogen in lipids (H-C) bond, and so the hydrogen nuclei in water rotate at a higher resonant frequency than those in lipids (Mehta, Thomas et al. 2008). This difference in resonant frequency means that hydrogen within water can be separately identified and quantified to that within lipids etc.

This separation effect can be intentionally maximised to improve the resolution of the differing spectra peaks. Improved resonance frequency separation, and reduced signal-to-noise ratio of spectra can be achieved by increasing the strength of the external magnetic field, improving the homogeneity of the magnetic field through shimming, and by repeating the excitation sequence so that multiple spectra are combined and analysed. As a result compounds with

close spectral frequencies can separated, and compounds with tissue concentrations as low as 0.1-1 mmol/L can be detected (Roden 2007).

The final issue is the spatial localisation of the origin of the signals received. This is achieved by applying static and/or pulsed gradients along the magnetic field. This change in gradient within the field allows for a change in frequency of the signal and hence localisation. In MR imaging this facilitates the generation of cross sectional or even 3D images of the human body.

2.6.1. Proton magnetic resonance spectroscopy (^{1}H MRS) of liver

Non-invasive *in vivo* hepatic fat quantification by ^{1}H MRS involves the evaluation of the two main hydrogen-containing peaks, namely water and lipid (Szczepaniak, Nurenberg et al. 2005). There is a strong correlation between the liver fat quantification of histology by a pathologist and ^{1}H MRS data, with correlation coefficients ranging from 0.88 and 0.93 (Thomsen, Becker et al. 1994; Cotler, Guzman et al. 2007). However it is important to remember that a strong correlation between assessment techniques does not mean that they quantify the same factors or generate similar absolute values. Pathologists quantify liver biopsy fat content as the percentage of hepatocytes with visible fat droplets, whereas ^{1}H MRS determines the fractional volume of fat within the liver (Schwenzer, Springer et al. 2009). As a result the values determined by ^{1}H MRS are consistently lower than histopathological values (Cotler, Guzman et al. 2007; McPherson, Jonsson et al. 2009). Confusion can arise over this as both techniques generate scores with percentages as their value. The absolute values for both techniques have been shown to differ by a factor of approximately 1.8 (Longo, Pollesello et al. 1995).

A value of 5.56% has been described as the upper limit of normal for ^{1}H MRS determined hepatic triglyceride count (Szczepaniak, Nurenberg et al. 2005). This value was derived from being the 95th percentile value from assessments in 345 subjects in Dallas, USA, with no risk factors for fatty liver disease. The subjects had a BMI less than 25kg/m^2, low self-reported alcohol consumption, no history of liver disease, normal plasma transaminase enzyme levels, and no impaired glucose tolerance or diabetes. It was assumed that none of these individuals would have increased liver fat stores and so the 95th percentile was an appropriate cut-off value. This assumption cannot be fully maintained given the absence of a confirmatory normal liver biopsy, accurate alcohol history, and that

15% of the cohort were Hispanic – an ethnicity with a high rate of steatosis. It therefore seems plausible that more than 5% of the cohort had steatosis, and that the true cut-off value for steatosis is somewhat less than 5.56%. A large scale study with comparative assessment with liver biopsy data would be helpful to clarify this, though this may never be performed.

Ignoring the interpretation of the absolute value generated by ^{1}H MRS assessment of hepatic triglyceride count, it is clear that it is a useful assessment tool as it is both non-invasive and reproducible. The inter-examination co-efficient of variation assessed by removing volunteers from the scanner and repositioning them and the voxel is excellent at 7.0% (Thomas, Hamilton et al. 2005).

Recently Johnson *et al.* furthered ^{1}H MRS assessment of liver fat by identifying and defining the individual resonant frequencies of differing fatty acid functional groups (Johnson, Walton et al. 2008). This was done by generating a highly homogenous field, and by suppressing the water peak to increase the resolution within and adjacent to the lipid peak. Formulae were generated for determining the indexes of fatty acid saturation, unsaturation and polyunsaturation within human liver *in vivo*. This work was done firstly by *in vitro* analysis of oils with a known fatty acid composition, followed by *in vivo* analysis of 27 lean and obese volunteers using a 1.5 Tesla (T) scanner. There was no correlation data presented for the *in vitro* lipid chromatography versus ^{1}H MRS work, and there was no comparative assessment with another assessment modality performed in the *in vivo* studies. As a result the paper produced no direct evidence to validate what may or may not become a useful non-invasive hepatic lipid profile assessment tool.

The formulae generated by Johnson *et al.* involve the signal amplitudes of the described fatty acid peaks:

Unsaturated index =   (Allylic + Diallylic) / (Allylic + Diallylic + Methylene + Methyl)

Saturated index =   1 - (Allylic + Diallylic) / (Allylic + Diallylic + Methylene + Methyl)

Polyunsaturated index =   Diallylic / (Allylic + Diallylic + Methylene + Methyl)

2.6.2. Proton magnetic resonance spectroscopy (^{1}H MRS) of calf muscle

Triglyceride is stored in two compartments within muscle: intra- and extra-myocellular. Interest in intra-myocellular triglyceride content (IMCL) arises as its metabolites appear to impair insulin sensitivity (Hulver and Dohm 2004).

Extra-myocellular lipid (EMCL) appears to be relatively biologically inert. The main proton signal from muscle is from water, though following water suppression the main peak is lipid, and EMCL and IMCL are closely opposed but can be separately identified (see figure 4) and modeled for. Unlike the liver, movement due to respiration is not an issue. The reproducibility of these findings for IMCL is acceptable with a coefficient of variation of 13% using 1.5 T scanner (Torriani, Thomas et al. 2005), which would be predicted to be even more reproducible with 3T.
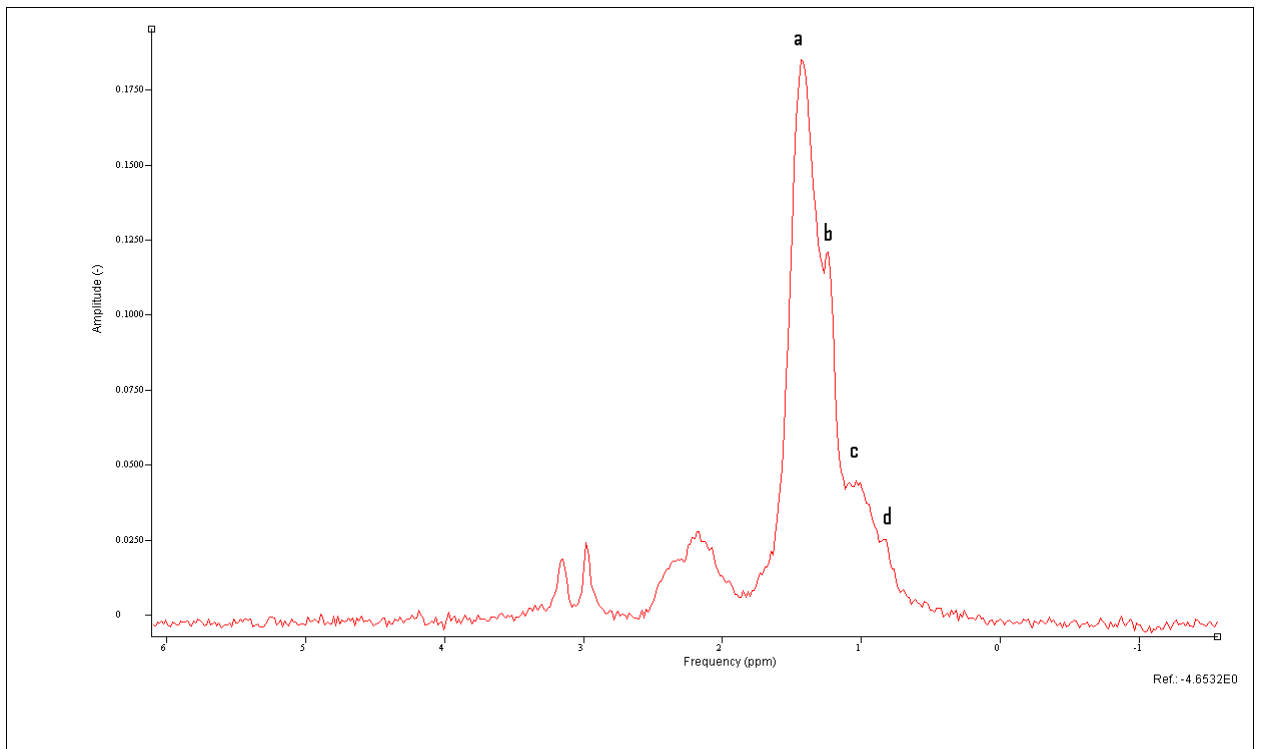
Figure 4. Proton magnetic resonance spectroscopy (^{1}H MRS) of calf soleus muscle. Peaks d and b are IMCL (methyl and methylene respectively) Peaks c and a are EMCL (methyl and methylene respectively).

2.6.3. Phosphorus magnetic resonance spectroscopy (^{31}P MRS) of liver

^{31}P has a 100% natural abundance, however it has a much lower sensitivity relative to ^{1}H at 6.6% versus 100% respectively (Roden 2007). It results in sharp peak lines over a wide chemical shift range (broad x-axis). External ^{31}P volume coils are rare i.e. coils integrally built into the scanner external magnetic system. Such external volume coils generate a voxel as per ^{1}H MRS. Commonly, as with the Sir Peter Mansfield Magnetic Resonance Centre, a ^{31}P surface coil has to be placed over the region of interest and its correct positioning confirmed by imaging prior to data collection. The limitation of this approach is that signal intensity has

been shown to reduce with a greater distance between the coil and the region of interest (Meyerhoff, Karczmar et al. 1990), i.e. subcutaneous muscle and fat overlying the liver. As a result it is the ratios between parameters, as opposed to their absolute values, which are commonly presented. The tissue detection limit of ^{31}P MRS in the liver is however reported to be excellent at around 0.1 mmol/l. The signals that are detected by ^{31}P involve 4 main spectral groups as shown in figure 5.
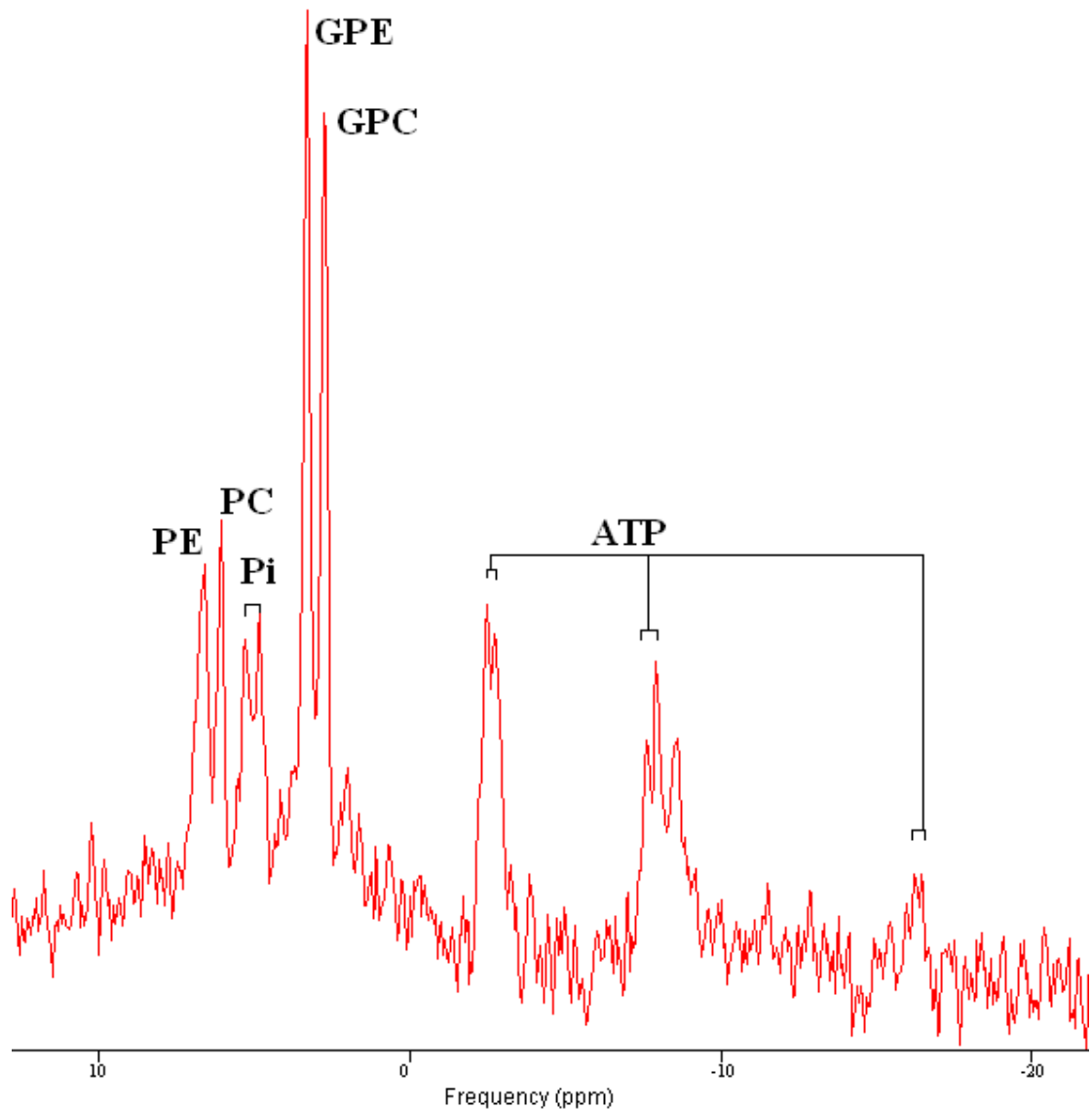
Figure 5. 31P MRS of the liver demonstrating spectral peaks for the phosphomonoesters (PME) PE and PC, the phosphodiesters (PDE) GPE and GPC, inorganic phosphate (Pi), and ATP (γ, α, and β).

A major limitation in the interpretation of ^{31}P MRS spectral data is that the PME and PDE peaks are not specific to one compound as they represent a heterogeneous mix of compounds with a phosphomonoester or phosphodiester bond, such as phospholipids and sugar phosphates (Solga, Horska et al. 2005).

PME rises following the administration of fructose, and this is attributed to the generation of fructose-1-phosphate (Boesiger, Buchli et al. 1994).

The reproducibility of ^{31}P MRS liver data has never been analysed in a large scale study. Sijens et al. repeated the analysis in six volunteers on differing days in a 2 Tesla scanner (Sijens, Dagnelie et al. 1998). The nature of the subject preparation and analysis time was not described. The intra-subject variability of the spectral peaks assessed by a single analyser ranged from 10-16%.

2.7.   Finometer

The finometer provides continuous non-invasive beat to beat monitoring of haemodynamic status. It measures systolic and diastolic blood pressure, heart rate, inter beat interval, cardiac output, stroke volume, left ventricular ejection time, total peripheral resistance, and baroreflex sensitivity. Digital artery pulse waveforms are tracked by an infrared photo-plethysmograph built into a finger cuff. It uses a volume clamp method whereby inflation of the finger cuff opposes arterial pressure on a beat-to-beat basis to maintain a constant finger volume.

The algorithmic equations used from the finger generated assessments derive intra-arterial and pressures and hence cardiac function non-invasively. As such it is clearly a highly useful assessment tool. Though being non-invasive it makes many assumptions about the cardiac and vascular compliance of the individual. As a result the true validity of using a finometer is in tracking intra-individual changes than for the absolute values it generates (Owen, Priestman et al. 2009).

Digital artery pressure tends to be lower than brachial artery pressure due to progressive arterial narrowing. The finometer remodels and reconstructs the digital pressures into a brachial pattern by using a return-to-flow measurement from an additional brachial cuff (Bogert and van Lieshout 2005). As a result the difference in mean arterial pressure is 2 mmHg between brachial artery catheter pressures and finometer derived pressures (Guelen, Westerhof et al. 2008).

The finometer also comprises Modelflow algorithms to reconstruct a central aortic waveform (Wesseling, Jansen et al. 1993). The model assumes the presence of a normal aorta and aortic valve, and the absence of pulmonary or abdominal hypertension. Modelflow model uses the parameters of gender, age, height and weight to calculate aortic pressures and cardiac function. The waveform is integrated to generate stroke volume, and cardiac output is computed as the product of stroke volume and heart rate. Total peripheral resistance is computed

as a function of mean arterial pressure and cardiac output. In a recent head-to-head comparison of cardiac thermodilution versus 3 non-invasive cardiac output algorithms, the Modelflow performed the best with an observed bias between the two assessments of 0.33 l/min (de Wilde, Geerts et al. 2009).

The above accepted there clearly are several assumptions made by a finometer on the cardiovascular health of the individual. The machine itself is also not externally calibrated pre each assessment. It is however highly reproducible with an intra-individual variation in stroke volume of 3% and less than 1% variation in blood pressure at assessments performed at least a week apart (Voogel and van Montfrans 1997).

2.8. Serum spectrophotometry

This was used to quantify serum creatinine, alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), uric acid, free fatty acids and triglycerides using enzyme based assays for the fructose versus glucose study. In brief samples are first mixed with a reagent appropriate for each clinical parameter. The combination is then homogenised by a mixing palette. The spectrometer is a tungsten-halogen white bulb which transmits light to the sample mixture. Light that passes through this is then separated by a chromator into pre-determined wavelengths which are then focused onto the photometer to quantify the amount of light at each wavelength.

Each reagent has its own pre-determined absorbing wavelength. As the amount of absorbance is proportional to the sample concentration, the comparison between the fraction of light that passes through a reference and test solution allows the spectrophotometer to be quantitative.

The use of a bichromatic analysis further strengthens the quantitative reliability. One wavelength is selected to be within the absorption range, whereas another is outside this. This minimises any errors caused by sample turbidity (Gavella 1988).

The ABX Pentra 400 system (Horiba Medical, Montpelier, France) is well validated with the company reporting co-efficient of variations (CoV) for the repeated use of the reagents of less than 3%, they also report a correlation coefficient of greater than $r^2=0.99$ with another commercial reagent.

2.9.   Statistical analyses

All raw data were initially entered onto excel spreadsheets, and the data entry re-checked. Formulae were employed within Excel, where necessary, to generate further data such as body mass index etc. The raw and transformed data was then transferred into SPSS version 17.0 and databases formed and analysed. All data are presented as the mean ± standard deviation (SD) unless stated otherwise. Comparisons between baseline values were done by the independent samples T test. The Student's T test was used for paired data. All studies involved two groups. Analysis of the significance of change between the two groups at two paired assessments was done by an independent samples T test of the absolute change between the two assessments in both groups. Associations were assessed for by 2-tailed Pearson correlations. Discrete data was analysed by Chi squared analyses. Repeated measures over time were assessed by a repeated measures one-way ANOVA with a Greenhouse-Geisser correction.

Chapter 3. Whole body effects of a high fructose versus high glucose diet

3.1. Introduction

The rate of hepatic synthesis of trioses from monosaccharides undergoes strict feedback inhibition when glucose is involved, which is absent for fructose, as previously described in 1.10.3.2. This has led to speculation that fructose may lead to greater amounts of post triose metabolic products than glucose. Such products include lactate from anaerobic glycolysis, citric acid from aerobic glycolysis and triacylglycerols. The determination of which of these potential end products predominates is dependent on whole body metabolic status and insulin resistance profile. So data on whole body status is presented in this chapter, and insulin resistance in chapter 4 and hepatic effects in chapter 5.

Healthy overweight males were randomised to a high fructose or high glucose diet for two separate periods of 2 weeks, firstly in an energy balanced (isoenergetic) setting and subsequently in an energy overfeeding (hyperenergetic) setting. The monosaccharides were consumed mixed with water four times a day and provided 25% of predicted energy requirements. The differing energy periods were achieved by firstly providing all food to be consumed and secondly by subjects consuming the monosaccharides in addition to their habitual intakes.

The assessment processes were identical pre and post each intervention period. Specific to this chapter, assessments of weight and satiety were undertaken to assess energy intakes. Cardio-renal outcomes were assessed by serum creatinine and Finometry, a non-invasive beat to beat assessor of haemodynamic status. Indirect calorimetry assessed whole body substrate oxidative status.

3.2. Method

The following is an overview of the method for the data described in chapters 3, 4 and 5.

3.2.1. Recruitment protocol

The study was recruited for by a variety of methods. Firstly, e-mail invites were sent to all overweight male subjects on the local research databases of interested future volunteers. Secondly, a purchase order list was obtained from Royal Mail, and 2,100 men living in close proximity to the research facility were sent a covering letter and study flier. Thirdly, the study was advertised on the free

classified website 'gumtree'. Finally, posters were placed around the university's medical school.

All interested subjects made contact with the investigator themselves and were sent the volunteer information sheet version 2.0 (dated 19/10/2009). Interested volunteers were then screened for their suitability by telephone and then by clinical review.

The aim was to recruit young men who were healthy, weight stable, centrally overweight with no evidence of liver or renal disease, or diabetes. The subjects also had to have a relatively 'standard' carbohydrate intake, and a low intake of fructose from drinks. As a result the study inclusion criteria were:

Inclusion criteria

1. Body mass index 25-32
2. Waist > 94 cm
3. Age 18-50 years
4. Male

Exclusion criteria

1. Active health problems requiring ongoing medical review, or chronic treatment.
2. Evidence of metabolic or viral liver disease as screened for by hepatitis B and C serology, and plasma ferritin.
3. Alcohol intake greater than 21 units per week
4. Abnormal full blood count, liver or renal function tests
5. Evidence of diabetes with a random glucose greater than 11.0mmol/L.
6. Evidence of cardiovascular disease – abnormal electrocardiogram or blood pressure greater than 140/80 mmHg.
7. Reported weight change greater than 3 kg in prior 3 months
8. Regular high intensity physical activity (total energy requirements are difficult to reliably predict in such individuals)
9. Contraindications to MRI or DEXA scanning (standard departmental screening protocols employed)
10. Intolerance to a test drink containing 50g of fructose
11. Symptoms of functional bloating or irritable bowel syndrome (as these may be exacerbated by a subsequent high fructose intake)
12. Vegetarianism (their normal and supplied food intake would differ from non-vegetarians)

13. Normal daily fructose intake from drinks greater than that in 500ml of coca cola (26g).

14. Abnormal carbohydrate contribution to energy in the baseline diet – defined as greater than 2 standard deviations from the mean. Taking the National Diet and Nutrition Survey 2002 data for men aged 25-34, this equates to carbohydrates intakes contributing less than 36% or greater than 59% (Swan 2004).

3.2.2. Recruitment phase

During an initial face to face meeting potential subjects were screened for the above criteria. The protocol was discussed and informed consent taken in those subjects who were willing and appropriate. Such subjects then underwent physical examination, anthropometric assessment, resting electrocardiography and non-fasted venesection. The subjects were randomised in a double blind fashion to receive 25% of their predicted total energy expenditure (TEE) from either fructose or glucose.

The subjects then prospectively completed three day food records (one weekend and two weekdays). These were analysed for the percentage contribution to energy from macronutrients and fructose. The results of this and screening tests were reviewed prior to a subject's formal study inclusion.

Figure 1. Study time-frame with time in weeks along the bottom and food intakes and visits (overleaf).

3.2.3. First assessment period

The study was divided into two interventional periods, both lasting 14 days, with an intervening six week washout period. During both periods the sugars were supplied in food-grade sachets and dissolved in water taken four times a day with food. During the first interventional period the supplemented sugars were taken in a carefully energy balanced setting with the explicit aim of weight maintenance. In order to achieve this all foodstuffs consumed during this period were provided. Weight and satiety were monitored after one week and energy intakes modified if necessary in order to ensure weight maintenance throughout the 2 week period. The energy from the provided foodstuffs and sugars combined comprised 55% from carbohydrate, 15% from protein and 30% from fat. The macronutrient profiles are presented in table 4. These proportions are a modest deviation from the average intakes of men aged 24-34 years in the UK of 48%, 16% and 36% respectively (Office for National Statistics and Food Standards Agency. Department of Health 2003), and are the same proportions used in prior related studies (Bantle, Raatz et al. 2000; Le, Ith et al. 2009; Stanhope, Schwarz et al. 2009). Further to this the foodstuffs were designed to be reasonably 'healthy' with an average of 3 portions of fruit or vegetables a day, a total salt content lower than the national male average of 11g per day (FSA 2008), and a saturated fatty acid content within the recommended range of 11% of total energy provision (SACN 2008).

Four food plans were devised with differing energy contents energy, which in the absence of the supplementary sugars provided 2,000, 2,200, 2,400 or 2,600 kcal per day. Subjects were allocated the food plan whose energy content most closely approximated their requirements. The food plans were designed and nutrient profile analysed with 'Microdiet' software (Downlee Systems Ltd., Salford, UK). The differing food plans had minimal foodstuff deviation between them with the differing energy values primarily originating from portion sizes. Subjects reviewed and agreed the food plans, and minor modifications were made for a handful of volunteers based on their preferences. These modifications resulted in minimal deviation from the standard plan. The supermarket delivered the foods to the volunteer's home on four occasions. All deliveries were received, though on occasion the supermarket substituted the ordered product for an alternative similar product due to stock-related issues.

Detailed written instructions as to what to consume at which meal on which day were provided. Additional sugar or sugar-sweetened drinks were forbidden, and the subjects were asked to not drink more than 2 units of alcohol per day. The subjects started to consume the provided foods on the day before the first assessment, and consumed identical foods on the day before the week 2 assessment.

3.2.4. Washout period

Previously, 3-5 weeks following a high fructose diet had been reported as adequate an adequate washout period (Faeh, Minehira et al. 2005; Abdel-Sayed, Binnert et al. 2008; Le, Ith et al. 2009). A washout period of six weeks was chosen for this study. During this period subjects were asked to maintain their habitual food intakes and lifestyles

3.2.5. Second assessment period

During the second period the same amount and type of monosaccharide was consumed by each subject, though this time in addition to their routine food intakes. The only dietary restriction during the second assessment period was to consume less than 2 units of alcohol per day. On the days prior to the main assessments similar food intakes were encouraged. The subjects were asked to maintain stable physical activity during the study period.

3.2.6. Assessment visit protocols

At each of the main study assessments the subjects first underwent early morning fasted MRI and MR spectroscopy in the Sir Peter Mansfield MR centre. On the same day they then walked the short distance to the physiology laboratory, voided urine, were weighed and then rested for 30 minutes. They then underwent 20 minutes of indirect calorimetry and Finometry measurement. Finally venesection was performed; satiety visual analogue scales self-completed and then 20 out of the 32 subjects left the physiology laboratory having been provided with breakfast. Twelve subjects remained fasted and underwent a hyperinsulinaemic euglycaemic clamp. This procedure is described later on.

The mid-study assessments involved a brief fasted visit to the laboratory for weighing, compliance and side effect discussions, self completion of satiety visual analogue scales and venesection.

3.3. Power calculation:

The primary outcome measure was a change in liver triglyceride content. In a previous study, mice randomised to consume *ad libitum* 10% fructose drinks for 2 weeks developed 80% greater liver triglyceride concentrations than those drinking 10% glucose or water (Roglans, Vila et al. 2007). This occurred in the absence of weight differences between the groups.

Stanhope *et al*. conducted a 10-week 25% fructose diet which resulted at 10 weeks in a 25% increase in abdominal visceral adiposity, and a 21% increase as compared to glucose. No assessment was made of liver fat stores. Prior MRI studies by Thomas *et al*. have shown a strong correlation between abdominal visceral fat and liver fat stores (Thomas, Hamilton et al. 2005). Using the formulae generated by Thomas' paper, the changes in visceral fat in Stanhope's paper correspond to a 145% absolute increase in liver fat, and a 124% increase relative to glucose. No pre 10 week abdominal visceral adiposity data was generated by Stanhope's study.

Prior to the present study's development only one large-scale study had assessed the hepatic effects of a high fructose diets in human volunteers. As compared to the baseline diet, 35% fructose energy overfeeding for 7 days increased liver fat stores by 79% in slim but mildly insulin resistant volunteers, and by 76% in slim insulin sensitive volunteers (Le, Ith et al. 2009).

Provision of greater energy from monosaccharides than 25% was felt to make it very difficult to achieve an isoenergetic period. A two-week intervention duration was selected as metabolic changes including insulin resistance and hypertriglyceridaemia were detected in the Stanhope paper at 2 weeks (ectopic lipids were not measured then). Indeed this same group continues to recruit for a further fructose versus glucose study providing 25% energy or less for 2 weeks (ClinicalTrials.gov NCT01103921) with a primary outcome measure of 24 hour triglycerides and secondary measure of insulin sensitivity. As a result of the above the duration of 2 weeks was chosen, with the monosaccharides providing 25% of energy.

Power was calculated with a predicted 75% increase in liver triglyceride count in the fructose group and no change in the glucose group. Using a two-sided p-value of 0.05 and power of 80%, 29.1 participants were the minimum required. A baseline steatosis of 4.4% and standard deviation of 3.18 was assumed, as

shown in prior ^{1}H MRS assessments of 375 overweight white male volunteers (Browning, Szczepaniak et al. 2004).

3.4. Study approval, registration and timelines

The study was approved by the University of Nottingham Medical School Research ethics committee; reference number D/10/2009 on 04/11/2009. It was approved by the Nottingham University Hospitals NHS trust Research and Development committee on the 11/01/2010, with the identification number of 09GA017. The study was registered on the ClinicalTrials.gov website with the identifier NCT01050140 on the 14th of January 2010. The study started on the 14th of April 2010 and finished on the 13th of December 2010.

3.5. Randomisation process and provision of randomised monosaccharide

It was a double blind study. Enrolled subjects' age, initials, weight and monosaccharide dose were entered into the Nottingham Clinical Trials Unit online randomisation package. Non-stratified simple randomisation was employed using varied block sizes and the outcome communicated via automatic e-mail with sterile services in the Nottingham University Hospital's clinical trials Pharmacy Department. There the food-grade glucose or fructose was then weighed and placed in sealed sachets with the subjects' name on. The sachets were labeled as containing either fructose or glucose (not which one) with instructions to consume four times a day mixed with water. The sachets were collected pre study initiation and two weeks' worth given to the subjects following their first main assessment of both periods.

As a result of the above, the only individuals aware of the randomisation were the team in sterile services and Clinical Trials Unit. Both glucose and fructose were fine white powders and tasted similarly sweet. As it was not a crossover study there was no potential for subjects to compare the tastes.

3.6.   The protocols for data presented within this chapter:

3.6.1.   Physical assessment of body composition

Weight was measured using a SECA medical calibrated digital weighing scale and height by an Avery beam stadiometer. Body surface area was determined using the Mosteller formula (Mosteller 1987). Waist circumference was measured halfway between the lower border of the ribs and the iliac crest in a horizontal plane. Hip circumference was measured at the widest point over the buttocks.

3.6.2. DEXA assessment of body composition

Whole body DEXA assessments were done at baseline and not repeated as significant changes in weight and composition were not predicted. Subjects were screened for contraindications for DEXA scanning such as recent radiation exposure and the presence of foreign objects. A signed consent form was taken and the subjects then rested on the base of a Lunar Prodigy DEXA (GE Medical Systems, Bedford, UK) whilst the scintillation counter swept down the body cranio-caudally. Quality assurance tests (coefficient <2%) were performed using a phantom block with known attenuation before the subjects were assessed. All subjects fitted into the screening area of the machine. Specific precautions were not taken regarding hydration, though all assessments were done in the fed state.

3.6.3. Energy predictions

The Henry modified Schofield formulae were used to predict resting energy expenditure (REE), and an activity factor determined by the short form of the International Physical Activity Questionnaire (IPAQ). An activity factor of 1.5 was ascribed to those with a low IPAQ score, an activity factor of 1.6 to those with a moderate IPAQ score, and an activity factor of 1.7 to those with a high IPAQ score. The total energy expenditure (TEE) was calculated and subjects were ascribed one of the pre-determined food plans designed in phase 1 to provide 75% of predicted TEE (see table 4 for the precise macronutrient breakdown of each plan). This was reviewed after one week and if weight or satiety changes implied an incorrect plan allocation then it was switched for the subsequent week. The amount of fructose or glucose supplied provided 25% of this predicted TEE.

3.6.4. The assessment of the energy and macronutrient content of habitual intakes and foodstuffs supplied

The subjects were taught how to complete 3 day food records. The records were of two weekdays and one weekend day before study enrolment. The completed records were analysed using 'Microdiet' software (Downlee Systems Ltd., Salford, UK). Microdiet is a nutrient analysis software package that incorporates UK based nutrient data of with the ability to edit and add additional foodstuffs as needed. Nutrient content of foodstuffs not within the database was obtained from food labels and published values. The subjects' reported intakes of energy, macronutrient contribution to energy intake and fructose contribution to energy intake were then determined.

The Microdiet software was also used to design the food plans for the two weeks when the subjects received food. The food plans primarily differed in portion size as opposed to foodstuff or macronutrient content.

3.6.5. Satiety assessment

Five 100mm horizontal lines were printed with the responses 'never' to the left and 'all the time' to the right. Above each line the following statements were written: 'Over the last week I have enjoyed my food', 'Over the last week I have felt full of food', 'Over the last week I have felt satisfied by food', 'Over the last week I have felt hungry', 'Over the last week I have lacked energy'. The subjects marked their responses on these five lines at all four main study assessments and both mid-study assessments. They did so unsupervised and any significant changes from the prior week were then discussed. Quantification was done by measuring the distance from the left of the line.

3.6.6. Indirect calorimetry

Resting pulmonary VO_2 and VCO_2 exchange was measured in rested, awake subjects lying supine on a bed. It was measured in a fasted state with 24 hour prior abstinence of stimulants such as caffeine and alcohol. Air was collected via a ventilated canopy and analysed by a Gas Exchange Monitor (GEM; Nutren Technologies Ltd, Manchester, UK). The entire GEM system was calibrated every month by an alcohol burn, and the gas analysers were calibrated pre each measurement by gases of known composition. All measurements were done for 20 minutes, with the first 5 minutes discarded from analysis.

3.6.7. Finometer assessment of rested cardiovascular status

Cardiovascular status was non-invasively assessed by Finometry, as described in section 2.7. The Finometer provides beat to beat monitoring of haemodynamic status. Subjects were fasted rested supine for 15 minutes. A Finometer PRO (Finapres Medical Systems, Netherlands) was used according to the manufacturer's standard operating procedure. Briefly, the subjects' age, sex and weight were entered and then at each subject's assessment an identical upper arm cuff, wrist box and middle finger cuff were placed.

Height correction was made between the level of the heart and the finger cuff. The machine was then started for a couple of minutes until the 'physiological measurements' were classified as 'great' or 'excellent.' Further correction for the pressure gradient between the brachial and radial pulse was based on return-to-flow calibration. Fifteen minutes of data was then generated, stored and exported

via the manufacturer's 'Beatscope Easy v01.02' software for a visual review prior to transfer to an Excel spreadsheet.

Subjects were asked to lie still, though movement did occur and it did appear to temporarily distort data. This was despite the manufacturer stating that the addition of a height correction unit facilitates the automatic correction for hydrostatic pressure changes due to movement in hand position relative to the heart. As a result the presence of temporarily distorted data was reviewed by inspection of the 'Beatscope Easy v01.02' image of pulse, blood pressure and cardiac output. In Excel the average findings of the final ten minutes were computed once any distorted data (infrequent) was removed.

3.6.8. Blood sample handling (identical in chapters 4 and 5)

Whole blood was sampled either from the antecubital fossa for overnight fasted values, or from retrogradely placed cannulae during a hyperinsulinaemic euglycaemic clamp. Blood was placed in an SST tube with no additives for quantification of liver biochemistry, insulin, creatinine, and uric acid. Blood was placed in a lithium-heparin tube with no additives for the quantification of triglycerides and deuterated glucose enrichment. For NEFA analysis 75µl of EGTA-glutathione and 5µl of tetrahydrolipstatin were added to lithium-heparin tubes in order to prevent ongoing in vitro lipolysis (Krebs, Stingl et al. 2000).

SST tubes were stored for 30 minutes to allow for sample clotting pre-centrifugation, whereas the lithium-heparin tubes were shaken so as to allow mixing with the additive and centrifuged instantaneously. These samples were centrifuged for 10 minutes at 4000 rpm and at 4°C. The resultant plasma or serum was separated from the red blood cells, placed in labeled eppendorf vials and frozen at –80°C prior to analysis.

3.6.9. Serum spectrophotometry

This protocol generated data for this chapter as well as chapters 4 and 5. The serum creatinine, alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), uric acid, C reactive protein, non-esterified fatty acids and triglycerides were quantified by bi-chromatic light absorbance using spectrophotometry with the ABX Pentra 400 as previously described. Validated calibrations were carried out with each reagent change, and following each 60 subject samples with both normal (healthy range) and pathological (unhealthy range) calibrators as provided by the company. The resultant coefficient of variations from these repeatedly assessed calibrator

samples performed at the time of the sample analyses were excellent and ranged from 0.4% to 2.0%, and the absolute values generated were very close to their target value.

3.6.10. Adrenaline and noradrenaline analysis

The protocol followed the principles as previously described (Forster and Macdonald 1999). Samples were thawed, mixed and centrifuged at 3000rpm for 5 minutes. Into a scintillation vial 100µl of internal standard, 500µl of the sample, 250µl of ammonia buffer (containing diphenyl borate-ethanolamine complex) and 1 ml of heptane solution (containing tetra-octyl ammonium bromide and 1% octanol) were added. This was vortex mixed and centrifuged at 3000rpm for 5 minutes. 750µl of the top organic layer was pipetted and added to 380µl of octan-1-ol and 40µl of 400mM acetic acid. This mixture was again vortexed and centrifuged. The acid droplet was removed from the base of the tube. The mixture underwent HPLC with a pump flow rate of 0.2 ml/min at 2000 – 3000psi and the electrochemical detector potential set at 0.65V. The column was a Phenomenex Hypersil / Hyperclone C18 100mm x 2.0mm internal diameter. The detector output was measured on a Spectra Physics SP4290 integrator. Calculations were done as per the manufacturer's standard operating procedures.

3.7. Results

3.7.1. Cohort demographics

There were 15 subjects in the fructose group and 17 in the glucose group. The study was designed to have 10 subjects in each group undergoing the assessments without a hyperinsulinaemic euglycaemic clamp, and 6 with the clamp. One subject in the fructose group was not able to tolerate the hyperinsulinaemic euglycaemic clamp though continued with the study in the non-clamp setting. This transference from the clamp to non-clamp setting was inadequately adjusted for by the randomisation package and hence the imbalance of 15 versus 17 occurred.

All the subjects were overweight with body mass indices (BMI) ranging from 25.9 to 32.2. Body fat percentage, as measured by a whole body DEXA, ranged from 26.8 to 45.0%. This indicates that the increased weight primarily originated from excess adipose tissue, as opposed to muscle. The two groups were well matched in all parameters bar waist to hip ratio which was slightly greater in the glucose group.

	Fructose (n=15)	Glucose (n=17)	Significance (p)
Age	35±11	33±9	0.60
Weight (kg)	96.8±7.4	93.9±8.7	0.32
BMI (kg/m^2)	30.0±1.4	28.9±1.7	0.07
Body fat (%)	34.5±4.6	33.9±4.2	0.70
Waist (cm)	103.8±4.9	103.3±5.2	0.77
Waist > 102cm	11/15	11/17	0.60
Waist : hip ratio	1.02±0.03	1.04±0.02	0.02 †

Table 1. Subject pre-study initiation characteristics, mean ± SD. † = p<0.05 between the groups

3.7.2. Energy requirements and monosaccharide dose

The two groups were matched in terms of their predicted energy requirements and hence energy supplied in the first period and monosaccharide amount provided during both periods.

	Fructose (n=15)	Glucose (n=17)	p
Predicted REE (kcal/day)	2003±165	1974±158	0.62
Activity factor	1.63±0.09	1.65±0.10	0.55
Supplemented monosaccharide during both periods (g/day)	217.6±16.1	215.5±13.7	0.70
Total energy provided during 1st period (kcal/day)	3276±260	3243±204	0.70

Table 2. Subject energy requirements and supplemented monosaccharide quantity, mean ± SD. (REE = resting energy expenditure)

3.7.3. Subjects' self reported food intake

Data from the three day food records of the subjects' pre study is presented in table 3, with no significant differences between the two groups. The pattern of macronutrient contribution to overall energy intakes was similar to that described amongst men aged 19-64 in the UK: fat 34.2±4.5%, carbohydrate 46.8±6.6%, protein 14.7±2.4%, alcohol 4.3%, saturated fat 13.0±2.5% (NDNS 2009). The cohort tended to have a lower alcohol intake and a higher fat intake than the national average. There are no UK data on fructose intakes. The intake of salt was similar in both groups at 9.3±2.8 g/day in the fructose group, and 8.1±4.2 g/day in the glucose group.

	Total energy (kcal/day)	Fat	Carbo hydrate	Protein	Alcohol	Saturated fat	Fructose
All	2835±375	38.4±4.1	43.9±4.4	16.1±2.4	2.0±2.1	14.3±2.0	5.4±2.1
Fructose	2724±282	37.3±3.9	44.2±4.3	17.0±2.2	1.7±1.7	14.2±1.6	6.1±2.2
Glucose	2932±427	39.3±4.3	43.7±4.6	15.3±2.4	2.4±2.4	14.5±2.4	4.8±1.9

Table 3. Total energy intakes and its percentage macronutrient origin in the subjects' ordinary diets as analysed by Microdiet software from three day food records, mean ± SD.

Assuming that weight was stable during the period of the three day records then the above reported energy intakes should equate to total energy expenditure (TEE). The TEE was calculated by combining an activity factor with the two measures of resting energy expenditure (REE), namely the Schofield formula and indirect calorimetry. The observed associations of reported energy intakes and these two measures of TEE were reasonable at r=0.50 (p<0.01), and r=0.52 (p<0.01) respectively. However the absolute values for the reported energy intake

were lower than these predictions of TEE. Indeed 3 subjects were classified as low energy reporters (daily energy less than 1.2 times the Schofield predicted BMR). These subjects were split between the groups.

The energy profile of food supplied during the first period is presented in table 4. The food alone component had a very similar macronutrient profile to the three day records, although it tended to be greater in terms of fat and protein contribution and lower in terms of carbohydrate and fructose contribution. The combination of food plus drink resulted in a high overall carbohydrate intake and a 25% difference in energy contribution from fructose between the groups. The average fructose energy contribution during the first period in those randomised to additional glucose was 2.3% overall. This is a lower contribution than their normal intakes of 4.8%. The mean sodium intake was slightly greater than the subjects' ordinary diets at 4039mg/day (range 3294 to 4329).

Food plan name	No. subjects on plan	Food or food plus drink	Total energy (kcal/ day)	Fat	Carbohy drate	Prote in	Alco hol	Satura ted fat	Fructose in fructose group	Fructose in glucose group
'2000'	1	Food alone	2045	37.3	43.5	19.3	0	15.2	3.3	3.3
		Food + drink	2667	29.8	54.4	15.4	0	12.2	27.6	2.5
'2200'	7	Food alone	2248	39.5	42.1	18.5	0	15.3	3.3	3.3
		Food + drink	2933	31.6	56.3	14.8	0	12.2	27.6	2.5
'2400'	6	Food alone	2431	40.4	41.7	18.0	0	15.5	3.1	3.1
		Food + drink	3200	32.3	52.1	14.4	0	12.4	27.5	2.3
'2500'	3	Food alone	2513	40.3	42.0	17.8	0	15.5	3.0	3.0
		Food + drink	3333	32.2	52.5	14.2	0	12.4	27.4	2.3
'2600'	15	Food alone	2595	40.2	42.3	17.5	0	15.6	3.0	3.0
		Food + drink	3467	32.2	52.9	14.0	0	12.5	27.4	2.3
Average		Food alone	2444	40.0	42.2	17.9	0	15.5	3.1	3.1
		Food + drink	3259	32.0	52.8	14.3	0	12.4	27.5	2.3

Table 4. Energy intakes and their mean percentage macronutrient origin in the food supplied during the first period by food plans including the contribution of fructose in the fructose and glucose groups

3.8. Intervention outcomes

3.8.1. Tolerability/side effects

There were no subject drop outs, and all six study assessments were attended by all. There were no procedure or investigation related incidents. One subject reported headaches during the first period. These were ascribed to his self-

enforced concurrent caffeine restriction and resolved when he increased his caffeine intake back to his baseline level (glucose group). All other reported symptoms were gastrointestinal in origin. Such symptoms were more common with fructose than glucose, as shown on table 5, p=0.08. Most symptoms were mild and only two subjects were sufficiently troubled to contact the lead investigator in between study visits to discuss them. One was regarding constipation during the first period, which may well be attributed to the change in food intake during this period. The other subject was in the fructose group and was significantly troubled by bowel frequency. This was partially alleviated by modifying the monosaccharide consumption pattern though tended to persist throughout both two week periods.

	Fructose (n=15)	Glucose (n=17)
Frequency	6	1
Constipation	0	1
Wind / gurgling	3	2
Bloating / pain	2	2
Nausea	1	0
Any GI symptom	8	4

Table 5. Gastrointestinal side effects reported during the study.

3.8.2. Changes in weight and metabolism between the two baseline assessments

The absolute change at the second baseline assessment as compared to the first of all principal variables measured for chapters 3, 4, and 5 is presented in table 6. There were no significant differences, or trends, in terms of weight, ectopic lipids, serum triglycerides, insulin resistance, renal and liver function, and whole body oxidative metabolism. Indeed no subject had a second baseline weight that differed by more than 3.5% from their first baseline weight, and 27 out of the 32 had a repeat weight within 2% of the first.

Amongst all the subjects there was a significant change in only one factor, NEFA, and near significance for uric acid (p=0.052). Between the groups there was a trend for a difference in uric acid only.

	All	Fructose	Glucose	Significance between groups (p)
Weight (kg)	0.33±1.37	0.59±1.7	0.09±1.0	0.32
Resting energy expenditure (kcal/day)	-39.6±114.1	-72.0±130.8	-11.0±91.7	0.13
Respiratory quotient (RQ)	0.05±0.038	0.0003±0.31	0.010±0.04	0.50
Lipid oxidation (mg/kg/min)	-0.16±0.31	-0.20±0.35	-0.13±0.29	0.54
Carbohydrate oxidation (mg/kg/min)	0.48±0.51	0.44±0.54	0.51±0.50	0.78
Hepatic triglyceride (%)	0.15±2.09	0.25±1.88	0.07±2.30	0.81
Intra-myocellular lipid (%)	0.01±2.26	0.23±1.96	-0.18±2.55	0.62
Extra-myocellular lipid (%)	-0.54±3.13	0.42±3.37	-1.39±2.72	0.10
Triglycerides (mmol/L)	-0.14±0.56	-0.18±0.63	-0.10±0.50	0.68
HOMA-IR	0.32±1.22	0.25±1.52	0.38±0.91	0.76
Glucose disposal (mg/kg/min)	-0.14±1.11	-0.23±0.86	-0.05±1.40	0.80
Noradrenaline (nmol/L)	-0.07±0.62	-0.01±0.69	-0.13±0.57	0.60
Adrenaline (nmol/L)	-0.04±0.17	-0.04±0.19	-0.04±0.17	0.97
Non-esterified free fatty acids (mmol/L)	-59±161*	-34±149	-80±171	0.44
Creatinine (µmol/L)	-1.8±5.6	-0.9±6.8	-2.5±4.5	0.43
Uric acid (µmol/L)	-20.6±57.6	0.2±64.2	-38.9±45.4	0.054
Alkaline phosphatase (IU/L)	-1.6±6.2	-1.1±7.4	-2.1±5.0	0.66
Alanine transaminase (U/L)	-1.7±9.0	-4.3±9.3	0.7±8.3	0.12
Aspartate transaminase (U/L)	-1.4±5.2	-2.2±5.6	-0.8±4.9	0.45
Gamma glutamyl transpeptidase (U/L)	-1.9±6.5	-2.2±4.2	-1.7±8.2	0.82

Table 6. The absolute change within the entire cohort and each group at the second baseline assessment compared to the first baseline assessment, mean ± SD. (*=p<0.05)

3.8.3. Weight changes during the study

The changes in weight reflected the differing energy statuses of the two periods, with weight maintenance during the first period and gain during the second, see table 7 and figure 1. During the first period 26 volunteers maintained their weight within 1kg of their baseline weight. Four volunteers lost more than 1kg and 2 gained more than 1kg. In order to maintain stable weights, the food

plans were increased after the week 1 visit by 200kcal/day in two volunteers and reduced by 200kcal/day in two volunteers.

During the second period both groups gained significant amounts of weight at both weeks one and two. By week two weight increased by +1.03±1.37 kg in the fructose group (p=0.01) and by +0.57±1.00 kg in the glucose group (p=0.03). There was no significant difference between the groups in terms of the amounts of change.

		All (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
1st period	baseline	95.3±8.1	96.8±7.4	93.9±8.7	0.32	
	week 1	95.3±8.2	96.9±7.6	93.9±8.7		0.84
	week 2	95.1±8.1	96.6±7.2	93.8±8.9		0.62
2nd period	baseline	95.6±8.4	97.4±8.1	94.0±8.6	0.26	
	week 1	96.2±8.6**	98.1±8.3*	94.5±8.3*		0.63
	week 2	96.4±8.5**	98.4±8.3*	94.6±8.5*		0.29

Table 7. The subjects' weight (kg) during the study, mean ± SD. (*=p<0.05, **=p<0.01 compared to baseline value in that group.)
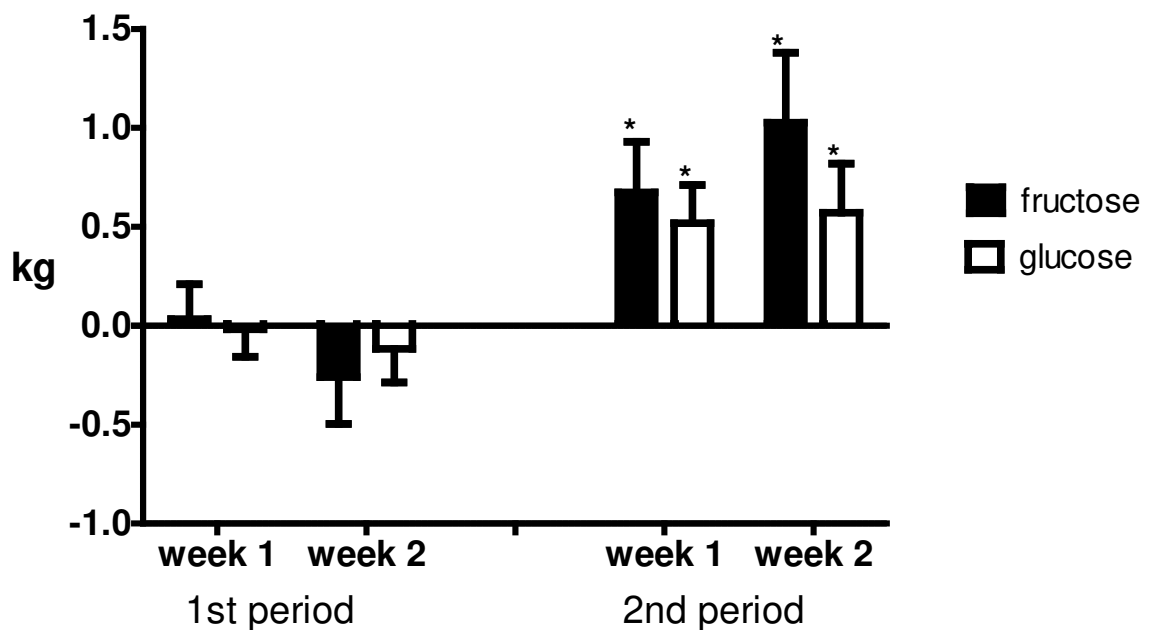
Figure 1. The change in weight from baseline during the two study periods, mean ±SEM. (*=p<0.05 from baseline in that group)

3.8.4. Satiety outcomes

a. <u>First period</u>

The self completed visual analogue scale responses during the first period for subject's satiety, energy and food enjoyment are presented in table 8. The baseline responses were essentially identical between the groups except that the glucose group reported less food satisfaction. It is however the change in response as opposed to the absolute value that is of greater interest. Overall the subjects reported increased fullness and reduced hunger during this first period despite weight maintenance. The degree of change was small and matched between the groups.

Over the last week I have…		All	Fructose	Glucose	Difference between groups at baseline (p)	Sig of absolute change between groups (p)
Enjoyed my food	baseline	70±14	72±15	69±13	0.54	
	week 2	73±16	69±20	76±9*		0.18
Felt full of food	baseline	62±19	66±17	59±20	0.30	
	week 2	69±18*	72±13	67±22		0.69
Felt satisfied by food	baseline	71±14	76±11	67±14	0.04†	
	week 2	73±11	74±11	72±12		0.13
Felt hungry	baseline	39±19	34±19	41±21	0.37	
	week 2	23±17**	21±14*	26±19		0.73
Felt I lacked energy	baseline	31±19	25±18	35±19	0.12	
	week 2	26±18	18±10	34±20		0.58

Table 8. Visual analogue scale responses during the first period to the statement: 'Over the last week I have...' (A score of 0 = never, whereas a score of 100 = all of the time), mean ± SD. (*=p<0.05, **=p<0.01 compared to baseline value in that group. † = p<0.05 difference between the groups at baseline.)

b. <u>Second period</u>

Baseline responses in the second period were not matched. The fructose group reported significantly greater food enjoyment and satisfaction, and a lower hunger and sense of lacking energy, see table 9. The reasons for this are unclear, though again what is more important is the change in values during the period

itself. Despite weight changes there were minimal changes in responses, and these were even more blunted than during the first period. During both periods there were no significant associations between change in weight and changes in visual analogue scale responses.

Of note, hunger levels reduced in the glucose group during the second period (p=0.042), whereas there was no change in the fructose group. The interpretation of this isolated finding is uncertain as there were no changes in satisfaction, fullness or energy in this group.

Over the last week I have…		All subjects	Fructose	Glucose	Difference between groups at baseline (p)	Sig of absolute change between groups (p)
Enjoyed my food	baseline	71±11	76±12	67±9	0.01†	
	week 2	74±13	79±11	69±14		0.83
Felt full of food	baseline	63±16	67±17	60±15	0.25	
	week 2	65±16	69±15	60±16		0.60
Felt satisfied by food	baseline	70±10	74±10	66±10	0.03†	
	week 2	70±11	75±12	67±9		0.92
Felt hungry	baseline	34±17	27±14	41±17	0.02†	
	week 2	33±20	31±23	34±17*		0.06
Felt I lacked energy	baseline	32±19	24±19	40±17	0.02†	
	week 2	34±19	28±20	40±16		0.45

Table 9. Visual analogue scale responses during the second period to the statement: 'Over the last week I have...'. (A score of 0 = never, whereas a score of 100 =all of the time) , mean ± SD. (*=p<0.05 compared to baseline value in that group. † = p<0.05 difference between the groups at baseline.)

3.8.5. Renal outcomes

The fasted serum creatinine data at each main visit are presented in table 10. There were no significant changes during the study or differences between the groups.

		All (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
First period creatinine	Baseline	90±11	91±14	89±8	0.52	
	Week 2	89±11	90±12	88±9		0.84
Second period creatinine	Baseline	88±12	91±14	86±9	0.33	
	Week 2	87±12	88±14	86±11		0.25

Table 10. Fasted serum creatinine (μmol/L) before and after two weeks of a high fructose or glucose intake, mean ± SD.

3.8.6. Whole body oxidative metabolism outcomes

The baseline oxidative metabolic values were matched between both groups, see table 11. The baseline respiratory quotient values were relatively low reflecting the subjects' habitual low carbohydrate and high fat intakes, and the overnight fast. The initial resting energy expenditure value was strongly associated with the Henry modified Schofield equation prediction of the basal metabolic rate, $r = 0.51$, p=0.003.

After two weeks during both periods there was an increase in the respiratory quotient value and the carbohydrate oxidation rates. This reflected the increased intakes of carbohydrate. These changes were very similar between the periods, though there was a greater rise in carbohydrate oxidation rates during the second period than the first, reflecting the greater energy overfeeding and lipogenesis.

		Whole cohort (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between baseline values (p)	Absolute change between groups (p)
VO$_2$ 1st period (ml/kg*min)	Baseline	226±31	227±35	225±27	0.87	
	Week 2	227±25	223±25	230±25		0.24
VO$_2$ 2nd period (ml/kg*min)	Baseline	220±27	217±31	223±24	0.49	
	Week2	225±26*	222±31	226±21		0.73
VCO$_2$ 1st period (ml/kg*min)	Baseline	164±24	165±27	164±21	0.90	
	Week 2	171±22*	168±20	174±23*		0.27
VCO$_2$ 2nd period (ml/kg*min)	Baseline	161±21	157±25	164±17	0.37	
	Week2	171±26*	170±27*	171±25		0.45
RQ 1st period	Baseline	0.726±0.034	0.725±0.037	0.726±0.031	0.94	
	Week2	0.756±0.058**	0.755±0.050	0.758±0.066*		0.93
RQ 2nd period	Baseline	0.731±0.032	0.725±0.032	0.736±0.033	0.38	
	Week2	0.763±0.064*	0.767±0.014*	0.759±0.073		0.45
Resting energy expenditure 1st period (kcal/day)	Baseline	1531±208	1537±240	1525±182	0.88	
	Week 2	1544±167	1517±167	1567±168		0.23
Resting energy expenditure 2nd period (kcal/day)	Baseline	1491±186	1465±213	1514±161	0.46	
	Week2	1532±179	1519±214	1543±148		0.63
Lipid oxidation 1st period (mg/kg/min)	Baseline	1.11±0.19	1.09±0.20	1.12±0.19	0.86	
	Week 2	0.98±0.26*	0.96±0.25	1.00±0.28*		0.96
Lipid oxidation 2nd period (mg/kg/min)	Baseline	1.12±0.24	1.14±0.22	1.10±0.25	0.99	
	Week2	0.94±0.29**	0.89±0.23**	1.00±0.32		0.56
Carbohydrate oxidation 1st period (mg/kg/min)	Baseline	0.355±0.239	0.353±0.274	0.356±0.212	0.97	
	Week2	0.736±0.531**	0.724±0.453	0.746±0.610*		0.65
Carbohydrate oxidation 2nd period (mg/kg/min)	Baseline	0.396±0.214	0.345±0.216	0.425±0.216	0.41	
	Week2	0.866±0.526**	0.849±0.462	0.882±0.602*		0.77

Table 11. Whole body oxidative metabolism before and after two weeks of a high fructose or glucose intake, mean ± SD.

3.8.7. Cardiovascular outcomes

3.8.7.1. Haemodynamic outcomes

The resting cardiovascular function outcomes as assessed by a Finometer are presented in table 12. No variable changed significantly from the baseline. There was a trend during both periods for systolic and diastolic pressures to remain unchanged with fructose and to reduce with glucose. This trend was non-significant however even when the absolute changes during both periods are combined, (p=0.15 for combined systolic changes).

		All (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
Heart rate 1st period (bpm)	Baseline	65.5±8.9	64.3±9.8	66.5±8.2	0.51	
	Week 2	65.6±9.7	62.2±9.9	68.5±8.8		0.08
Heart rate 2nd period (bpm)	Baseline	66.0±8.0	63.0±8.9	68.6±6.2	0.04†	
	Week2	66.3±8.4	63.9±6.7	68.5±9.3		0.65
Systolic pressure (mmHg) 1st period	Baseline	123.2±10.3	120.8±9.9	125.3±10.5	0.22	
	Week 2	122.2±9.0	120.5±8.7	123.6±9.2		0.62
Systolic pressure (mmHg) 2nd period	Baseline	126.2±10.5	123.3±10.8	128.7±9.8	0.16	
	Week2	124.5±7.8	123.7±7.0	125.3±8.6		0.22
Diastolic pressure (mmHg) 1st period	Baseline	71.7±6.7	71.4±6.8	72.0±6.8	0.82	
	Week2	70.7±6.2	71.3±7.0	70.2±5.6		0.43
Diastolic pressure (mmHg) 2nd period	Baseline	72.6±6.5	72.5±7.1	72.6±6.2	0.98	
	Week2	72.1±4.7	72.9±3.5	71.5±5.6		0.49
Cardiac output 1st period (l/min)	Baseline	7.12±1.21	6.95±1.28	7.26±1.17	0.48	
	Week 2	7.37±0.99	6.98±0.74	7.71±1.08		0.31
Cardiac output 2nd period (l/min)	Baseline	7.11±1.19	6.82±1.08	7.36±1.25	0.21	
	Week 2	7.41±1.39	7.36±0.89	7.45±1.75		0.30
Stroke volume 1st period (ml)	Baseline	110±17	110±20	110±15	0.96	
	Week2	114±14	114±14	113±15		0.88
Stroke volume 2nd period (ml)	Baseline	108±15	109±13	108±17	0.84	
	Week2	112±15	116±14	108±16		0.15
Total peripheral resistance 1st period (dyn.s/cm^5)	Baseline	769±226	762±292	775±154	0.88	
	Week 2	745±100	784±79	710±105		0.24
Total peripheral resistance 2nd period (dyn.s/cm^5)	Baseline	801±157	825±145	780±169	0.43	
	Week 2	769±167	760±89	777±216		0.21

Table 12. Resting, fasted cardiovascular haemodynamic parameters before and after two weeks of a high fructose or glucose intake, mean ± SD.

3.8.7.2. Adrenergic outcomes

During the first period, a high fructose intake resulted in reduced resting adrenaline concentrations with no change in noradrenaline. With glucose there was no change in adrenaline whereas there was a reduced noradrenaline concentration during the first period. There were no significant changes in the second period.

		All	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
Adrenaline 1st period	baseline	0.30±0.17	0.32±0.15	0.29±0.18	0.72	
	week 2	0.26±0.14*	0.25±0.11*	0.28±0.17		0.01
Adrenaline 2nd period	baseline	0.26±0.09	0.27±0.11	0.25±0.06	0.59	
	week 2	0.25±0.08	0.23±0.08	0.27±0.08		0.08
Noradrenaline 1st period	baseline	1.31±0.55	1.16±0.50	1.44±0.56	0.16	
	week 2	1.15±0.41*	1.18±0.46	1.15±0.38*		0.04
Noradrenaline 2nd period	baseline	1.25±0.48	1.17±0.57	1.31±0.39	0.42	
	week 2	1.28±0.63	1.07±0.46	1.47±0.71		0.10

Table 13. Fasted, resting plasma adrenaline and noradrenaline concentrations (nmol/L) before and after two weeks of a high fructose or glucose intake, mean ± SD.

3.9. Discussion

Summary of the findings

The intervention resulted in only one difference between the groups. During the first, isoenergetic, period plasma adrenaline and noradrenaline concentrations differed between the groups. This isolated finding failed to translate itself into any changes in haemodynamic profile and so its significance appears limited. In both groups there was an increase in carbohydrate oxidation rates, consistent with increased carbohydrate intakes; and in the second, hyperenergetic, period there was weight gain in both groups, consistent with energy overfeeding.

The two groups were well matched at baseline and for their exposure to the intervention. The waist to hip ratios differed between the two groups, though the absolute numerical difference was small. Further to this there was a tendency

for a greater body mass index in the fructose group. These surrogate markers of adiposity were not reflected in DEXA determined total body adiposity. Indeed there is no evidence that this impacted on the study as there were no differences in the metabolic and cardiovascular variables measured at baseline. The fructose group reported greater abdominal and diarrhoeal symptoms. During the first period weight was maintained whereas the sense of fullness increased and hunger decreased. There was calorimetric evidence for a shift in whole body metabolism towards a pattern reflective of a high carbohydrate intake. There was no alteration in renal function or cardiovascular haemodynamic parameters. A high fructose intake resulted in reduced resting adrenaline concentrations with no change in noradrenaline. With glucose there was no change in adrenaline whereas there was a reduced noradrenaline concentration during the first period.

The washout period resulted in a return to baseline levels in all parameters measured bar NEFAs, though the reduction in NEFAs at the start of the second assessment was matched in both groups.

During the second period weight increased equally in both groups and there was minimal change in satiety measures. Again there was evidence for a shift in whole body metabolism to that representative of a high carbohydrate intake, with increased carbohydrate and reduced lipid oxidation rates, with no differences between the groups. There was no alteration in renal, cardiovascular haemodynamic or catecholamine profiles.

3.9.1. Issues with the design of the study

Certain issues need to be addressed prior to interpreting the data as a whole. Compliance needs to be considered as a potential issue in this outpatient / community dietary intervention. The next issue is the phenotype of the cohort, and as to whether they are representative of the general population or a clinically relevant sub-group. The final issue is whether the washout period was adequate such that the two intervention periods can be viewed as being truly separate assessments.

a. Compliance with the study

The subjects appeared to be well motivated. All visits were attended and no drop outs occurred. Direct observation of monosaccharide and food consumption was only possible at study visits. There are no biomarkers specific for intakes of monosaccharides, though a few assessments can be used to infer compliance in this study. These are weight change, serum triglycerides and rates

of fasted carbohydrate oxidation. Due to the isoenergetic nature of the first period these 'compliance' assessments are only feasible during the second hyperenergetic period. In this period the energy content in the monosaccharides was greater than that normally consumed in drinks by the subjects, and so one would expect weight, carbohydrate oxidation and serum triglycerides to increase. This occurred in 25, 27 and 23 out of the 32 subjects respectively. Overall 2 failed to have either of these features present during the second period and 3 had only 1 feature present. They were evenly split between the groups. Neither of these assessment processes was designed, nor wholly reliable, as compliance monitors. The above suggests that compliance can be assumed to be acceptable overall.

b. The cohort's phenotype

The cohort was explicitly recruited on the basis of them being centrally overweight healthy men who were not vegetarian, did not drink excessive alcohol, and drank small volumes of sugar-sweetened drinks. These factors will now be discussed in turn.

The health screen identified active health problems requiring ongoing medical review, or chronic treatment, and screened for previously unidentified diabetes, liver, renal or cardiac disease as well. The primary research question was to ascertain the hepatic effects of a high monosaccharide intake and so liver biochemistry had to be normal and the presence of hepatic disease including viruses, haemochromatosis, or alcohol abuse were actively screened for.

In order to classify as being centrally overweight the target measurements were a body mass index (BMI) between 25 and 32kg/m^2 with a waist circumference greater than 94cm, a predictor of increased coronary heart disease risk (Han, Lean et al. 1996). The addition of a minimum waist circumference helped to exclude individuals whose elevated BMI originated primarily from increased muscle as opposed to adipose tissue. This simple process was chosen as it was felt to be easy to understand and reproduce. Significantly overweight individuals were excluded due to the ethics of modestly overfeeding them and the feasibility of performing MRI based assessments. Central (visceral) obesity drives systemic and hepatic insulin resistance. Le *et al.* previously demonstrated an increased metabolic response to fructose in an overweight and insulin resistant cohort compared to a healthy weight and insulin sensitive cohort. A waist greater than 94cm is an independent and more powerful predictor for metabolic risk than waist to hip

ratio or body mass index (Wang, Rimm et al. 2005) and a useful predictor of coronary artery risk (Han, Lean et al. 1996).

Gonadal hormones influence carbohydrate metabolism. As these differ between men and women and vary with the menstrual cycle, women were excluded. Furthermore, women had previously been shown to be more resistant to the metabolic changes induced by fructose (Bantle, Raatz et al. 2000; Couchepin, Le et al. 2008; Stanhope, Schwarz et al. 2009). Recently a further paper has explored this in greater detail (Tran, Jacot-Descombes et al. 2010). Given the differing body sizes and composition of men and women these are very difficult studies to do as it is unclear if the amount of monosaccharide in gender comparative studies should be uniform, or weight or lean mass adjusted. Tran *et al*. compared outcomes in age and BMI matched men and women, but not weight or body fat percentage matched. The amount of fructose consumed was dependent on fat free mass and hence was significantly smaller in the female than the male group. The influence of this on the findings is difficult to fully account for. The insulin, lactate and uric acid response was smaller in women than men. There was lower tracer-labeled fructose enrichment into VLDL with women and a failure to suppress lipid oxidation. This supports the theory that women may be less vulnerable to fructose induced lipid disorders, though of course they also received less fructose.

Vegetarians were excluded from the present study as their requirements for differing foodstuffs than omnivores would have added another potentially confounding or complicating variable. In order to minimise the potential for symptomatic monosaccharide malabsorption, subjects were screened for pre-existing gastrointestinal symptoms or an intolerance to a test drink containing 50g of fructose.

Self reported alcohol consumption greater than 21 units a week was used as an exclusion factor. The reliability of such volunteer supplied information has to be questioned. This exclusion was not mentioned in the volunteer information sheet and so potential recruits were not forewarned. Liver biochemistry was also checked pre study enrolment. Ultimately the reliable exclusion of alcohol excess is impossible, though this mirrors the clinical setting that this translational study was designed to address. During the study periods alcohol consumption was allowed at two units a day and nil for 24 hours pre a study visit. It was felt that complete

abstinence would in some subjects have resulted in the study being as much an assessment of the washout from alcohol as a 'wash-in' of monosaccharides.

Another difficult assessment issue was that of baseline fructose consumption. The enzymes that initiate hepatic fructose metabolism, fructokinase and aldolase, are induced by fructose exposure (Koo, Wallig et al. 2008; Ouyang, Cirillo et al. 2008). Clearly the metabolic response to a high fructose diet may differ between high and low-baseline consumers. The clinical evidence to support this hypothesis however only comes from a single and very small study (Stirpe, Della Corte et al. 1970). In that study the postprandial uric acid concentration following consumption of 1g/kg of fructose in the fasted state was measured for 2 hours following normal food intakes, a 2 week low fructose intake and a 3 week high fructose intake in a patient with gout and the child of a patient with gout. The increase in postprandial uricaemia reflected the preceding fructose intake. This data has never been replicated. Nonetheless the aim was to exclude high baseline fructose consumers. A complete dietary assessment was not practical prior to enrolment of each subject, and there are no data to help identify which cut-off value should be used. The total monosaccharide dose supplied in the present study was equivalent to that present in 2 litres of cola per day. A pragmatic approach taken was to exclude those with daily sugar sweetened beverage consumption greater than 500ml of cola. Again this exclusion was not mentioned in the volunteer information sheet and so they were specifically not forewarned prior to reporting their standard intake patterns.

The subjects were clearly a select group, though representative of a substantial proportion of the UK male adult population. Indeed 48% of UK adult males have both a waist greater than 94cm and a BMI between 25 and 35kg/m^2 (HSE 2006), and 72% drink less than 21 units of alcohol a week (Alcohol 2010).

c. Effects and adequacy of the washout phase.

Many prior studies of fructose lack an energy control. As a result it remained unclear as to whether much of the previously published data could be attributed to a high fructose or high energy intake. Glucose was chosen as the energy comparator as it is a similar macronutrient with the same energy density.

The study was not a crossover design. This was specifically designed so as to facilitate the comparison of a high fructose or glucose intake in an energy balanced and overfeeding setting. The reason for this was that most of the prior data generated had been hyperenergetic and no study had previously compared the

findings in both settings. It was anticipated that only limited changes may occur during the isoenergetic phase and so a crossover of two isoenergetic phases may have generated limited outcome differences. It was also felt that if the study was merely a crossover of two hyperenergetic periods then the outcomes could only be viewed as a consequence of fructose versus glucose in combination with energy overfeeding and not directly attributable to the macronutrient. The approach decided upon facilitated the exploration of both energy settings. The limitation of this approach is that inter-monosaccharide outcome comparisons are not as robust as the inter-energy setting comparisons as they were comprised of differing individuals.

The first period for each subject was the isoenergetic phase as this was predicted to induce less of a metabolic challenge. It was then followed by six weeks 'washout' and then the hyperenergetic phase. No prior study has published with repeated baseline assessments and so it was not possible to be certain that a six week duration of the 'washout' would be truly adequate. As a result, all of the analyses were repeated at the baseline of the second period. Hence the final issues that need to be addressed are whether the washout phase was adequate and whether the two groups had truly returned to their baseline 'metabolic state'.

In comparison between the two baseline assessments there were no differences in terms of weight, ectopic lipids, serum triglycerides, insulin resistance, renal and liver function, and whole body oxidative metabolism. There was a significant difference in NEFAs in both groups and a trend for lower uric acid in the glucose group, though this did not reach statistical significance. The interpretation of the biological/clinical significance of these isolated findings is not possible. As a result it seems likely that the washout was adequate and that the two baseline assessments were reflective of each other.

3.9.2. Subject specific issues

3.9.2.1. Phenotypic profile

The cohort had a mean age of 34 years. The mean body mass index was at the upper end of the recruitment range (25-32) at $29.4kg/m^2$ as potential recruits with a relatively low body mass index tended to not have a waist greater than 94cm.

The DEXA body composition scans confirmed that the subjects were correctly identified as having increased adiposity as opposed to muscle bulk. The two groups were closely matched for all anthropometric factors bar waist to hip

ratio which was mildly greater in the fructose group. The two groups did not differ in terms of their baseline metabolic profile and so this was not felt to be significant.

In light of their baseline findings the cohort cannot be classified as entirely 'healthy' and a more accurate statement would be that they were without 'obvious end-organ disease detectable in routine clinical practice'. As will be discussed in the next chapters the cohort's central adiposity was associated with relative baseline insulin resistance, dyslipidaemia and steatotic livers. They were all screened to exclude high blood pressure, and although they had a high resting cardiac output it was reflective of their weight.

3.9.2.2. Baseline nutrient intake

The groups were well matched in terms of their self reported energy consumption patterns pre study entry. The use of three day food diaries commonly results in energy underreporting. Low energy reporting has been defined as reported energy intakes less than 1.2 times the respondent's Schofield equation predicted basal metabolic rate. This was demonstrated in 33.6% of the Whitehall cohort (Brunner, Stallone et al. 2001), and was more prevalent amongst the obese and those with a lower employment status. The rate in the present study was 3 out of 32 and so clearly better than the Whitehall cohort but still remains imperfect. The source of this low energy reporting discrepancy is impossible to individually determine. During the monitoring period subjects may fail to document all of their intakes or alternatively actively, or unintentionally, restrict food intake (Friedenreich, Slimani et al. 1992). Either way it is likely that more socially embarrassing, or so called 'unhealthy', foodstuffs are either avoided or underreported during assessments. This limits the reliability of such data. However, food frequency questionnaires and 24-hour recall interviews which can be used as alternatives do not adequately overcome this issue.

The above accepted, the energy intake data reported by this cohort were strongly associated with their predicted total energy expenditure. This implies that the data were reliable. Furthermore the pattern of macronutrient contribution to energy was similar to that found in the recent UK NDNS survey which also gathered data by food diaries (NDNS 2009). One cannot expect too precise a similarity with the NDNS data as our cohort was not fully representative of the general adult male population. The lower alcohol consumption in this cohort will in part reflect that consumption greater than 21 units a week was an exclusion

criterion, though it may also reflect a subject's reluctance to report a high alcohol intake for fear of removal from the study. The cohort had a greater fat intake than the NDNS male data.

3.9.3. Nutrient and energy intakes during the first 'isoenergetic' period

In order to maintain energy intakes (hence weight), and to standardise intakes during the first period of two weeks, all food was supplied direct to the volunteers' homes. To avoid underreporting, I did not use 3 day food records. Instead standard predictive formulae were used to predict REE which were modestly associated with their subsequently measured fasted REE at baseline (r=0.51, p=0.003).

Energy provision from food and supplements combined was on average 3259kcal/day, range 2720-3485, which resulted in a non-significant weight change of - 0.18kg (range of -2.2kg and + 1.3kg) (p=0.21). The maintenance of weight during the first period means that the data for the groups can be viewed as originating from an 'isoenergetic' phase. This is in stark contrast with the marked weight gain that occurred during the second 'hyperenergetic' phase.

An issue surrounding food delivery in the isoenergetic period was that of what macronutrient ratios to supply. There were two options. First, foodstuffs with standard macronutrient ratios and content such that the addition of monosaccharide drinks resulted in an overall high carbohydrate intake. Second, supply food with an abnormally low carbohydrate (hence high protein and fat content) such that standard overall ratios were maintained once the drinks were factored in. It was felt preferable to provide an overall high monosaccharide intake, resulting from standard foods in combination with the drinks, than a standard monosaccharide intake, resulting from nonstandard foods and the drinks. The use of nonstandard foods would have been as much an intervention as the addition of the monosaccharide drinks. Thus the foods aimed to provide 40% of energy from fat, 40% from carbohydrate and 20% from protein. The resultant averages from foodstuffs alone of 40.0%, 42.2% and 17.9% mimicked the subjects' own intakes of 38.4%, 43.9% and 16.1% respectively. With the addition of the monosaccharide drinks the total intake patterns were 32.0%, 52.8% and 14.3%. So it was a high carbohydrate, low fat and protein intake.

The content of the supplied foodstuffs for sodium and saturated fatty acids were within the guidelines (FSA 2008; SACN 2008), as deviation from these may themselves have resulted in metabolic or cardiovascular changes.

3.9.4. Nutrient and energy intakes during the second 'hyperenergetic' period

During the second period the subjects were advised to continue with their normal food intakes in terms of pattern and content and to take the monosaccharide drinks four times a day. I requested subjects to avoid any alcohol binges during this period. During both periods they were instructed to continue with their normal physical activities.

As a result the subjects were energy overfed by up to 25%, depending on dietary compensation, by a monosaccharide (217g/day) for 14 days. This resulted in an average weight gain per week of 0.4%. The degree of compliance to the foodstuffs and supplements is difficult to be certain of, though it is possible to compare this rate with prior published data.

Carbohydrate overfeeding has been previously shown to induce less energy storage and weight gain than fat overfeeding. This was attributed to greater increases in rates of energy expenditure and substrate oxidation (Horton, Drougas et al. 1995). Indeed the doubling of carbohydrate oxidation rates in this current study demonstrates that a large proportion of the dietary carbohydrates supplied were not used for energy storage, whereas the reduction in lipid oxidation rates will have meant that a greater proportion of the dietary fat will have been available for storage. Frustratingly a lot of the carbohydrate overfeeding literature presents changes in oxidative and hormonal profiles as opposed to weight. As a result there are few data on the weight effects of carbohydrate overfeeding for periods of around two weeks. The outcomes in the studies involving *ad libitum* fructose or simple sugar (sweets) overfeeding are presented below in table 14. The current findings are actually of greater weight gain per week than previously published as compared with similar amounts of overfeeding.

	Carbohydrate	Energy overfeed (%)	Duration of overfeeding (weeks)	Mean weight gain per week (%)
Le et al. 2006	Fructose	18	4	0.07
Silbernagel et al. 2011	Fructose or glucose	23	4	0.3
Johnston et al. (Present study)	fructose or glucose	25	2	0.4
Stanhope et al. 2009	Fructose or glucose	25	8	0.2
Perez-Pozo et al. 2010	Fructose	30	2	0.35
Claesson et al. 2009	Sweets	~50	2	0.6

Table 14. Current and prior published rates of weight gain with simple carbohydrate overfeeding with type of carbohydrate, amount and duration of overfeeding. (Note I have estimated the degree of energy overfeeding in the paper by Claesson by using the Henry modified Schofield formula and an activity factor of 1.6)

The interpretation of weight changes is aided in the papers by Perez-Pozo and Stanhope by their presentation of dietary intakes pre and during the intervention. The absolute increase in overall energy intakes were 24% (30% supplied) and 8.4% (25% supplied) respectively. A reduction in voluntary carbohydrate intake was the main source of accommodation in the Perez-Pozo paper; no such data were available from Stanhope. This reduction in alternative carbohydrates would have been predicted given the sweetness of fructose and glucose. The weight changes in this current study are almost identical to the Perez-Pozo study and so one can speculate that similar modifications occurred.

Repeat body composition analyses were not performed in this study and so it is therefore not known what the increased weight during the hyperenergetic period represents. Horton and colleagues overfed obese subjects with 50% energy for two weeks with mixed complex and simple carbohydrate foodstuffs (Horton, Drougas et al. 1995). The resultant weight gain was approximately two thirds fat mass and one third fat free mass. This makes sense as in the presence of energy excess carbohydrates are converted to lipids by *de novo* lipogenesis as will be discussed later.

3.9.5. Changes in self reported satiety

During the first period of the present study, subjects were supplied unfamiliar foodstuffs, to be consumed at specific meals. Most important, only 75% of their energy intakes were from solid foods. So, there were concerns that satiety may change and this in turn may affect outcomes during this or the subsequent hyperenergetic period. I chose low energy-dense foodstuffs specifically, and monitored satiety with visual analogue scales. Satiety is notoriously difficult to assess. It was not a major outcome measure and I wanted to be able to detect major changes.

There were no differences between the two groups in terms of their changes in visual analogue scale (VAS) satiety parameters or weights during the study periods and so I believe that energy intakes were similar between groups. There has been a suggestion that fructose may induce a reduced satiety. Over a 24 hour period there was a reduced rise in serum insulin and leptin concentrations and blunted reduction in ghrelin with intakes of 30% fructose compared to 30% glucose (Teff, Grudziak et al. 2009). The authors concluded that this may result in differing long-term satiety levels and intake patterns. These short term differences in satiety hormone profiles have never been reproduced, and differences in long-term satiety have not been found (Monsivais, Perrigue et al. 2007), indeed the current data also refute this hypothesis.

The changes in VAS satiety responses were small, though greater in the first than second period. This small change in the first period implies that the overall food energy, content and pattern of consumption were not dramatically altered from the subject's habitual intakes. Interestingly during this period subjects reported increased fullness and reduced hunger despite no significant weight change. This discrepancy between reported changes in satiety and weights may reflect the supplied foods' low energy density, or that the food was predominantly taken in a traditional regular three times a day meal pattern. It seems unlikely that abdominal bloating due to malabsorption of the monosaccharide drinks may have been mis-interpreted as fullness, as this should only have occurred with fructose.

During the second period energy intakes were increased, with marked weight gain, though the changes in satiety and food satisfaction were minimal. This implies that the subjects adapted the additional monosaccharides into their normal intakes and did not 'sense' their overall increased energy intakes. Whether liquids fail to trigger satiety sensations as well as occurs with solid food is an area of

intense debate and scrutiny with protagonists on both sides of the fence (Malik, Schulze et al. 2006; Drewnowski and Bellisle 2007). The popular opinion however is that satiety is less well stimulated by drinks and this may explain the discrepancy between increased energy intakes, increased weight gain and the minimal effect on satiety noted in the second period.

The subjects reported an increased sense of fullness during the energy balanced period and no change during energy overfeeding. The only difference between the two assessment periods was the food consumed with the monosaccharide drinks. This implies a lack of awareness of energy overfeeding from monosaccharide drinks, and an energy-independent effect on the sensation of fullness with the supplied foods. This latter effect probably resulted from the relative low energy density of the supplied foodstuffs.

3.9.6. Side effects

The volunteers were warned about gastrointestinal side effects. During the first period the supplied food was divided into three main meals with additional snacks which could be taken whenever the subject chose. This facilitated the taking of the monosaccharide drinks four times a day in combination with food in order to help reduce the incidence of malabsorption. Studies where fructose is taken *ad libitum* continuously throughout the day have reported a high incidence of symptomatic malabsorption that resulted in 9 out of 83 subjects withdrawing from a two week study and a further 22 reporting diarrhoea, abdominal cramps or flatulence (Perez-Pozo, Schold et al. 2010). Most of the symptoms in the present study were mild in nature and resulted in no drop outs. The majority of side effects occurred during the first period when both the monosaccharides and foodstuffs were different from habitual intakes.

3.9.7. Renal outcomes

Monosaccharides are not known to have renal interactions though the protocol may have altered hydration status by two opposing mechanisms. Firstly, the monosaccharides were consumed with around 2 liters of fluid a day, and secondly any malabsorption induced diarrhoea may have resulted in dehydration. The creatinine values were therefore primarily assessed to monitor hydration and safety. No changes were either predicted or occurred.

3.9.8. Whole body oxidative metabolism

Oxidation involves the utilisation of oxygen to convert carbon based nutrients into carbon dioxide, water and heat. The data generated by indirect calorimetry represents gas exchange in the lungs and thus originates from the whole body, as opposed to a specific organ. The main oxidative organs in the fasted and rested state are the liver, brain and skeletal muscle.

The complete oxidation of glucose results in a greater respiratory quotient (RQ) than for lipid or protein. An increased RQ and carbohydrate oxidation rate and reduced lipid oxidation rate are features of carbohydrate and energy overfeeding (Horton, Drougas et al. 1995; McDevitt, Poppitt et al. 2000). Short term studies have not previously shown a differing oxidative response to 96 hours of 50% fructose or glucose and energy overfeeding (McDevitt, Poppitt et al. 2000).

In this current study there were no differences in oxidative response between glucose and fructose although there was a significant increase in the RQ during both periods and a marked increase in carbohydrate oxidation and reduction in lipid oxidation rates. During the second period plasma triglyceride concentrations increased, and there were greater changes in lipid and carbohydrate oxidation rates than in the first period.

The intervention periods resulted in a whole body oxidative metabolic status reflective of a high carbohydrate intake. There was no difference between a high fructose or glucose intake, and the greater changes noted during the second period are presumably due to the concurrent energy overfeeding.

3.9.9. Cardiovascular outcomes
3.9.9.1. Background

In rats a high fructose diet (66% of all energy), as compared to standard chow, resulted in similar weight changes though an increased blood pressure (Hwang, Ho et al. 1987). Such changes do not progress after 5 weeks, implying that in rodents there is a plateau after a relatively short period of time (Sharma, Okere et al. 2007).

Human data on the effects of fructose on BP are scant and it remains uncertain as to whether any changes occur. There are epidemiological data to support a link, with a 1.36-fold risk for blood pressure being greater than 140/90 mmHg in those with fructose intakes greater than the median 74g per day (Jalal,

Smits et al. 2010) and 1.87-fold risk for a blood pressure greater than 160/100 mmHg. These data were adjusted for multiple factors including demographics, energy intake, salt intake and activity.

However, the mechanism of such a potential hypertensive effect is unclear. It has been proposed to be due to a reduced vasodilatatory response following fructose and an increased adrenergic stimulation. Insulin induces splanchnic and skeletal muscle vasodilatation (Vollenweider, Tappy et al. 1993), and so the reduced insulin response following fructose may contribute to the reduced systemic vasodilatation noted following oral ingestion of fructose as opposed to glucose (Brundin and Wahren 1993; Brown, Dulloo et al. 2008). Such insulin-induced vasodilatation would be expected to induce hypotension. Fructose is proposed to induce hypertension through inhibition of endothelial nitric oxide production and activation of the renin-angiotensin system (Schwarz, Acheson et al. 1992; Tran, Yuen et al. 2009). The proposition that fructose may impact on blood pressure via adrenaline has to be questioned however as the magnitude of changes in adrenaline previously reported are unlikely to be clinically significant. Though the only study with a glucose control published to date has shown a greater acute noradrenaline response with glucose than fructose in young normotensive individuals (Jansen, Penterman et al. 1987).

These data have recently been summarised by Tran et al. (Tran, Yuen et al. 2009) Figure 2 is adapted from their paper, it bears remembering that these pathways originate from extremely high dose rodent studies, which may not translate into clinical experience.
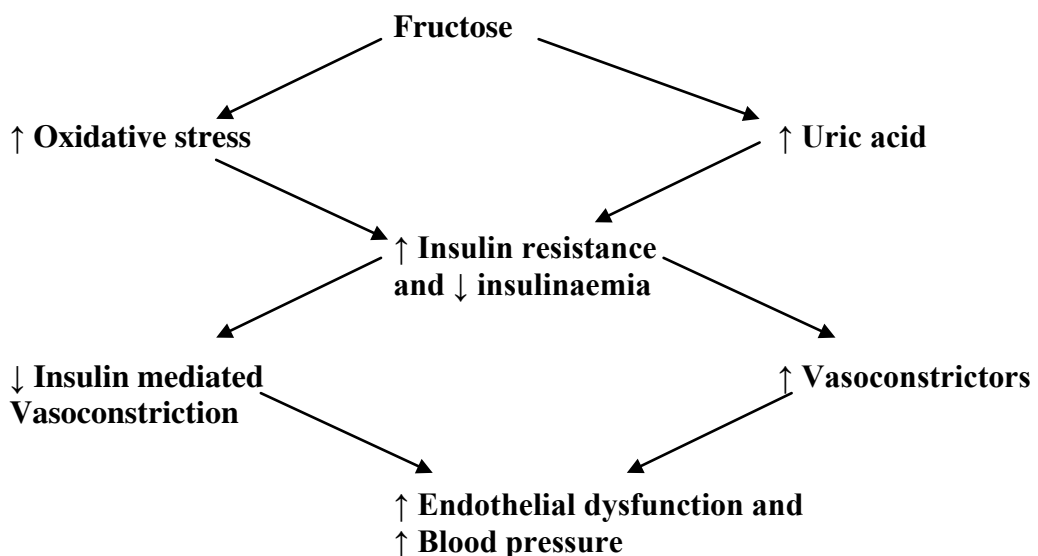
Figure 2. Proposed mechanism of fructose induced hypertension. (Adapted from (Tran, Yuen et al. 2009))

3.9.9.2. Prior short term clinical studies

One study has reported a 3mmHg greater rise in systolic blood pressure immediately post fructose consumption than following glucose or water, (Brown, Dulloo et al. 2008), whereas a subsequent paper found no such change (Bidwell, Holmstrup et al. 2010). The paper by Brown and colleagues used continuous measurements by task force monitor, which employs the same non-invasive principles as a finometer. In contrast, the Bidwell paper only measured the acute cardiovascular responses in ten volunteers at six time points, and they compared the responses following glucose and sucrose (45% glucose, 55% fructose) as opposed to pure fructose.

Brown et al. studied 15 healthy slim normotensive adults who consumed 60g of fructose or glucose or water in a single blind fashion (Brown, Dulloo et al. 2008). The mean rise in systolic pressures over the subsequent 120 minutes was around 1mmHg for water and glucose and over 4mmHg for fructose. A similar pattern was noted in the diastolic pressures. The increase in pressures with fructose was attributed to a rise in heart rate and cardiac output with no change in total peripheral resistance (TPR). Glucose also increased heart rate and cardiac output, though TPR fell such that pressures were relatively unchanged. The authors concluded that the difference in pressure was primarily due to the changes in TPR and that this was due to differing insulin and catecholamine responses, though no such confirmatory data were collected.

3.9.9.3. Prior long-term clinical studies

Three prior long-term high fructose diet studies have only crudely and discretely assessed cardiovascular status with a resting automatic cuff sphygmomanometer (Swarbrick, Stanhope et al. 2008; Stanhope, Schwarz et al. 2009; Silbernagel, Machann et al. 2011). No changes in systolic or diastolic pressures occurred in their overweight but normotensive cohorts.

The paper by Perez-Pozo et al. is the only other long-term paper to intensively assess the cardiovascular response to fructose (Perez-Pozo, Schold et al. 2010). They measured ambulatory 24 hour blood pressure in 74 middle-aged overweight normotensive men pre and post 2 weeks of a high fructose diet. There was a 7±2mmHg rise in systolic pressures and a 5±2mmHg rise in diastolic pressures during the day and a smaller rise during night time. Unfortunately this

paper lacked a nutrient control and so the changes could solely be attributed to energy (excess of 618kcal for 14 days) or carbohydrate overfeeding. In an attempt to demonstrate that this was a fructose-specific effect however the comparative group consumed fructose and allopurinol. Both groups had similar changes in weight, insulin and lipid profiles, though the fructose and allopurinol group had a significantly blunted change in systolic and diastolic blood pressures of 2.1 vs. 6.9 mmHg and 1.0 vs. 4.7 mmHg respectively. The authors offered no specific mechanism for these effects beyond reduced uric acid production (-113μmol/L vs. +65μmol/L). Heart rate was also increased in the fructose alone group, though no catecholamines were analysed to facilitate the interpretation of this.

3.9.9.4. Current cardiovascular findings

The use of finometry paired with adrenergic analyses in this study allowed for an in-depth assessment of cardiovascular function. The cohort gave no history of cardiac disease and had a normal resting blood pressure and ECG at screening.

Finometry was not performed at screening. Most of the finometry parameters are not routinely assessed and hence do not have widely accepted normal range values. The mean basal cardiac output was 7.12 L/min (normal is 4-8 L/min) with 5 subjects having elevated readings. The normal range for cardiac output is contentious given the influence of body size. The cardiac index has been developed to adjust for the effect of body size. It is the cardiac output divided by body surface area in metres^2 with a normal range of 3.0 to 4.2 L/min/m^2 (Greim, Roewer et al. 1997). Using the Mosteller formula for body surface area, only one subject had an elevated cardiac index at entry and so the cohort can be viewed as having normal cardiac function. Again there were no significant differences in the absolute values or changes between the groups in their cardiac index values.

There were no significant changes in cardiovascular function as assessed by a finometer during the study or between the groups. The non-significant trends found however did reproduce prior data. Systolic and diastolic pressures remained essentially unchanged with fructose whereas there was a tendency for a reduction with glucose. During the first period there was a non-significant trend for greater TPR with fructose and a fall in TPR with glucose as previously reported. Though cardiac output was unchanged with fructose and increased with glucose (p=0.31), and so pressures were similar between the groups. During the second period, there

again was no evidence of any difference between fructose or glucose or change with them.

In contrast to the lack of cardiovascular parameter changes there were alterations in catecholamine profiles during the first period, with reductions in adrenaline with fructose and noradrenaline with glucose. The aetiology of these changes is impossible to determine as mechanistic data were not collected. Changes could result either from alterations in rates of synthesis or clearance. Of note, the findings were in direct contrast to that previously published where there was a greater acute noradrenaline response with glucose than fructose in young normotensive individuals (Jansen, Penterman et al. 1987). As the primary stimulus for the reported noradrenaline changes is believed to be circulating insulin concentrations, the overnight fast will have resulted in a differing and blunted response than the acute postprandial state. The lack of 'end-organ' cardiovascular changes also means that interpretation of these adrenergic findings is limited.

Ultimately the lack of changes observed during this study is overwhelmingly convincing, though there remains the possibility of a type 2 error. The cardiovascular assessments were not formally part of the study power process, and they were done in the fasted, as opposed to fed, state and only whilst rested. . The number of subjects consuming fructose in this current paper (15) were however similar to that reported by Brown (15) and by Perez-Pozo (36). This current study may have involved assessments at an inappropriate time point. The hypothesis of Brown *et al*. is that differing endocrine responses to fructose and glucose drive the differing cardiovascular responses. Such endocrine differences are greater in the fed than fasted state. The greater than 10 hours fast may have resulted in a blunted or absent cardiac and/or endocrine memory of the previously ingested monosaccharide. Indeed the two papers with positive cardiac outcomes have measured parameters in the immediate postprandial or semi-continuously postprandial (sipping) state (Brown, Dulloo et al. 2008; Perez-Pozo, Schold et al. 2010). Another alternative is that resting may have blunted any adrenergic responses. The positive nocturnal findings of Perez-Pozo would argue against that, but that paper lacked a control and may merely reflect energy overfeeding. Finally the lack of long-term changes may be a true finding. Further studies need to assess this area using continuous ambulatory monitoring and comparing

fructose with another energy matched carbohydrate control to see if this is truly a fructose specific effect.

3.10. Conclusions

This study demonstrated no differences in whole body metabolism between a high fructose and a high glucose diet. The energy overfeeding period induced weight gain, as predicted, and both periods increased carbohydrate oxidation rates implying an increased intake of carbohydrates.

There are several strengths of this study:

1. The strict inclusion criteria resulted in a sharply defined cohort.

2. The balance of energy and macronutrient profiles between the two groups meant that the monosaccharides were the only factor that differed between them.

3. The food provided had a very similar macronutrient profile to habitual intakes.

4. The creation of two energy settings facilitated the comparison of the effects of energy intakes on outcomes.

5. The repeat baseline assessments ensured that a true washout had occurred.

6. The assessment of satiety and self reported 'energy' ensured that these did not compound the outcomes.

7. The comprehensive assessment process facilitated a global metabolic assessment.

 I acknowledge some limitations in this study design:

1. The sharply defined cohort means that these findings cannot be extrapolated out of the overweight healthy male population.

2. The lack of cross-over between the groups weakens analyses between the monosaccharides.

3. The relatively short-term nature of the intervention means that an effect with a longer dietary alteration cannot be excluded.

4. The haemodynamic assessments were only made in the fasted and rested state.

5. The monosaccharides were provided as their constituent powders as opposed to as a whole foodstuff. This resulted in a nutrient or metabolic comparison as opposed to that of dietary patterns. There is however no evidence that fructose differs in its metabolic outcomes when provided as a hexose from when bound to

glucose in sucrose (McDevitt, Poppitt et al. 2000; Stanhope, Griffen et al. 2008; Tappy, Le et al. 2010).

In comparison to prior studies this study is the first to provide all foodstuffs and so achieved the strictest control over total intakes. This was also the first energy-controlled long-term cardiovascular study. This study adds to the growing body of appropriately controlled work which reports that there is no difference between fructose and glucose in terms of whole body metabolism, impact on satiety or potential for weight gain. The differences in weight between the two periods clearly highlight the impact of energy overfeeding on obesity.

Chapter 4. The responses to a high fructose versus high glucose diet in terms of insulin resistance, and non-hepatic lipid content

4.1. Introduction

These data are derived from the Study fully described in chapter 3. This chapter focuses on the impact of a high fructose or high glucose diet on insulin resistance, and plasma and myocellular lipid contents.

In brief, healthy overweight males were randomised to a high fructose or glucose diet for 2 weeks firstly in an energy balanced (isoenergetic) setting and subsequently in an energy overfeeding (hyperenergetic) setting. The monosaccharides were consumed mixed with water four times a day and provided 25% of predicted energy requirements. The differing energy periods were achieved by firstly providing all food to be consumed and secondly by subjects consuming the monosaccharides in addition to their habitual intakes.

The assessment processes were identical pre and post each intervention period. Specific to this chapter, the 32 subjects underwent fasted proton magnetic resonance spectroscopy (^{1}H MRS) of their calf and fasted serum was analysed for triglyceride, non-esterified fatty acids, glucose and insulin. A subset of 12 subjects underwent a hyperinsulinaemic euglycaemic clamp with indirect calorimetry repeated during the clamp.

4.2. Protocols for the methods employed in this chapter

4.2.1. Hyperinsulinaemic euglycaemic clamp

Subjects were fasted overnight and rested supine. They were initially delivered a loading bolus dose of 4mg/kg of deuterated glucose [6, 6-^{2}H$_2$] (CK Gas Products Ltd, Hook, Hampshire, UK). This was followed by a continuous infusion at 40μg/kg/min for the rest of the study. The deuterated glucose was delivered at a concentration of 20mg/ml made up in sodium chloride 0.9% (Baxter Healthcare Ltd., Norfolk, UK). This infusion was started 2 hours pre the initiation of the insulin glucose clamp and continued at the same post-loading rate for the full 5 hours throughout the visit.

A 20 gauge catheter (Becton Dickinson, Helsingborg, Sweden) was inserted retrogradely into a dorsal hand vein, and the hand placed in a warmed air box (55^{0}C) in order to arterialise the venous samples. The difference in blood glucose concentrations between arterial and arterialised sampling is minimal at 0.1±0.3mmol/l due to shunting reducing tissue uptake (Liu, Moberg et al. 1992). A slow running infusion of 0.9% saline was used to maintain catheter patency.

Blood samples were drawn via a 3-way tap, with the first 1ml being discarded to avoid dilution with saline. The arterialised plasma samples were taken at baseline and every 30 minutes during the clamp for the measurement of tracer concentration and free fatty acids.

25 units of insulin (Human Actrapid, Novo Nordisk, Bagsværd, Denmark) was mixed with 48 ml of 0.9% saline (Baxter Healthcare Ltd., Norfolk, UK) and 2ml of the subject's venous blood. This infusion was started following the 2 hour loading of deuterated glucose following the protocol described by DeFronzo *et al.* (DeFronzo, Tobin et al. 1979). Priming and maintenance insulin infusion rates were calculated based on body surface area (DeFronzo, Tobin et al. 1979) to provide 30 mU/min/m^2 in a 3 hour single step clamp. Arterialised plasma glucose concentrations were measured every 5 minutes during the clamp (YSI 2300, Yellow Springs Incorporated, Ohio, USA), and maintained at 4.5mmol/L by a concurrent, variable rate infusion of 20% glucose (Baxter Healthcare Ltd., Norfolk, UK) with 1g (1%) of deuterated glucose spiked into the bag. The addition of deuterated glucose in the 20% clamp glucose generates what is termed a 'hot' glucose infusion protocol. This 'hot' protocol reduces inter-subject variation and improves the physiological plausibility of the endogenous glucose production data (Finegood, Bergman et al. 1987).

The insulin dose was selected to mimic the postprandial response in mildly insulin resistant subjects. The target plasma glucose concentration was chosen at 4.5mmol/l, as it was predicted to be the mean fasting concentration. Indirect calorimetry was performed for 20 minutes in every hour during the clamp by the same methods as previously described in section 3.6.5.

4.2.2. Insulin quantification by radioimmunoassay

This technique incubates a fixed concentration of labelled antigen tracer with a limited amount of antiserum such that only a limited proportion of the tracer is antibody bound. Unlabeled antigen is then added at known and increasing concentrations. The amount of bound tracer decreases as the concentration of added unlabeled antigen increases. This is due to competition between the labelled tracer and the unlabeled antigen for the limited amount of antibody binding sites. The fraction of bound to free antigen is then counted at each unlabeled antigen concentration and hence the amount of unknown antigen can be calculated from the resultant curve.

The Millipore Human Insulin assay was utilised with ^{125}I-labeled Human Insulin and a Human Insulin antiserum to determine the level of Insulin in serum. The manufacturer's protocol was followed. Briefly, six glass tubes had 1.0mL of assay buffer and serial dilutions of the standard added. Then 100μL of hydrated ^{125}I-Insulin was added to all tubes. 100μL of Human Insulin antibody was pipetted to all tubes except the total count tubes. The mixture was vortexed, covered and incubated overnight at room temperature. The next day 1.0mL of cold (4°C) precipitating reagent was added to all tubes (except total count tubes), the mixture vortexed and incubated for 20 minutes at 4°C. This was then centrifuged for 20 minutes at 2,000g. A firm pellet was formed and the supernatant decanted. The tubes were then counted in a gamma counter for 1 minute and the concentration of insulin calculated by using an automated data reduction procedure.

4.2.3. Deuterated glucose quantification by gas chromatography mass spectrometry

50μl of plasma was first mixed with 10μl of internal standard (methyl glucopyranose 50μg/ml). The addition of the internal standard means that a characteristic spectrum is subsequently formed and the peak of interest is then divided by the internal standard peak to ensure that losses are accounted for. Next 300μl of ice cold absolute ethanol was added to the mixture to deproteinise it. This mixture was spun down and the supernatant removed. A tenth of the supernatant was evaporated under N_2 at 90°C to form a dried solid residue. To this residue 50μl of pyridine and hydroxylamine (25mg/ml) was added to form an oxime group. This was then incubated at 70°C for 45 minutes and allowed to cool slightly. 50μl of BSTFA (1% TMCS) was added and it was incubated at 70°C again for 45 minutes.

This was then injected in a gas chromatography mass spectrometer at 240°C for up to 240 minutes. The glucose peak tended to come off at 10 to 12 minutes. An enrichment curve was generated by making up 0.5%-5% deuterated glucose standards. The internal standard peak was at 261. Unlabelled glucose peak was represented by a peak at 319 and deuterated glucose by a peak at 321. Two glucose peaks for each sample were obtained both with excellent enrichment curve correlation with the deuterated glucose standards of r^2 of 0.9662 and 0.9905. As the enrichment curve was slightly better using the 2nd peak, analyses just focus on this peak.

4.2.4. ^{1}H MRS calf data collection

MRI of the right calf was performed to identify the soleus muscle. A 20x20x50mm^3 voxel was positioned within the soleus muscle and an image was stored so that the voxel could be similarly placed at subsequent visits. Spectra were obtained from this voxel using a STimulated Echo Acquisition Mode (STEAM) sequence with the following parameters: echo/repetition time 13/7000 ms, bandwidth 2000 Hz, 1024 samples. 16 averages were collected with water-suppression applied and 2 without.

4.2.5. ^{1}H MRS calf data analysis

Water-suppressed spectra were individually phase corrected and realigned using jMRUI (Java-based MR user interface) (Naressi, Couturier et al. 2001) before averaging. Residual water peaks at approximately 4.75ppm were removed to simplify lipid quantisation using a HLSVD filter (Poullet, Sima et al. 2007). To determine the relative IMCL to EMCL contribution to the lipid peaks, the Advanced Magnetic Resonance Metabolite (AMARES) algorithm within jMRUI was then used to quantify individual metabolite signals. The protocol devised by Torriani et al. was then followed (Torriani, Thomas et al. 2005). Briefly, prior knowledge was fitted into the analysis algorithm as follows:

a. the IMCL -CH3 had a fixed resonance of 0.9ppm

b. the EMCL -CH3 had a fixed resonance of 1.1ppm

c. the IMCL -CH2 had a fixed resonance of 1.3ppm

d. the EMCL –CH3 had a fixed resonance of 1.5ppm

The first and zeroth order phase corrections were fixed to zero, and peaks were assumed to have a Gaussian shape. All resonances were fitted to ensure no contamination from neighbouring peaks were included, however only peak areas from the methylene groups were used for analysis. In order to calculate absolute lipid percentages (%IMCL and %EMCL), the total lipid (integrated peak areas from 1.7-0.5ppm in Matlab) to water ratio was determined from the non water suppressed spectra (as described earlier for liver). The IMCL and EMCL to water ratios are then found by relating the total lipid areas (IMCL(CH$_3$) + EMCL(CH$_3$) + IMCL(CH$_2$) + EMCL(CH$_2$) calculated from the water suppressed data in AMARES) to the total lipids to water ratio (calculated in MATLAB). To convert the methylene to water ratios to absolute values a correction was applied as described in equation 1 substituting with T$_{2H2O}$=31ms, T$_{2IMCL}$=89ms,

T_{2EMCL}=78ms, T_w =0.810g/ml and ρ_{tg} = 65.2mmol/ml (Szczepaniak, Babcock et al. 1999).

4.2.6. Statistical analyses

All data are tabulated as the mean ± standard deviation (SD). Comparisons between baseline values were done by the independent samples T test. The Student's T test was used for paired data. Analysis of the significance of change between the two groups at two paired assessments was done by an independent samples T test of the absolute change between the two assessments in both groups. Repeated measures over time were assessed by a repeated measures one-way ANOVA with a Greenhouse-Geisser correction. Associations were assessed for by 2-tailed Pearson correlations. Any negative rates of endogenous glucose production data were transformed to a rate of zero.

4.3. Results

4.3.1. Fasted insulin resistance

No subject had a fasted glucose greater than 6.0mmol/L. There was evidence of fasted insulin resistance according to HOMA, with the mean baseline HOMA value of the whole cohort at study entry being 3.28 (range 1.45-3.85).

In the whole cohort, concentrations of both insulin and glucose rose in the first period and were relatively unchanged in the second. As a result fasting insulin resistance increased in the first but not the second period.

Comparing the two groups, blood glucose concentrations rose in the fructose group during both periods and tended to remain the same or fall in the glucose group. In contrast the trends for insulin were similar between the groups. As a result there was a difference in terms of insulin resistance between the two groups, with a significant increase in HOMA noted during the first period with fructose (+23±29%, p=0.005) and not with glucose (+7±23%, p=0.45). Due to technical issues, fasted glucagon was analysed in 14 fructose and 16 glucose samples. There were no changes during the study.

		All (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	absolute change between groups (p)
Glucose 1st period (mmol/L)	Baseline	4.58±0.34	4.50±0.20	4.65±0.42	0.20	
	Week 2	4.68±0.37	4.71±0.29*	4.64±0.44		0.12
Glucose 2nd period (mmol/L)	Baseline	4.58±0.38	4.45±0.29	4.73±0.43	0.60	
	Week 2	4.59±0.35	4.60±0.29	4.60±0.41		0.10
Insulin 1st period (mIU/L)	Baseline	16.1±5.5	17.9±5.1	14.6±5.6	0.09	
	Week 2	17.9±6.9**	20.8±6.8*	15.4±6.2		0.11
Insulin 2nd period (mIU/L)	Baseline	17.7±6.6	19.5±7.4	16.2±5.6	0.16	
	Week 2	17.0±5.4	18.6±5.6	15.6±5.0		0.82
HOMA 1st period	Baseline	3.28±1.16	3.58±1.04	3.02±1.24	0.19	
	Week 2	3.71±1.40**	4.34±1.39**	3.15±1.19		0.03 "
HOMA 2nd period	Baseline	3.61±1.35	3.83±1.52	3.41±1.19	0.39	
	Week 2	3.48±1.19	3.83±1.31	3.17±1.00		0.47
Glucagon 1st period (pg/ml)	baseline	50.0±13.6	53.5±15.3	47.0±11.6	0.20	
	week 2	49.8±11.4	51.5±12.8	48.3±10.1		0.30
Glucagon 2nd period (pg/ml)	baseline	50.8±11.0	53.9±11.8	47.8±9.5	0.12	
	week 2	51.6±12.2	55.2±14.1	48.4±9.5		0.79

Table 1. Blood glucose and serum insulin and glucagon concentrations before and after the consumption of a high fructose or glucose diet for two weeks, mean ± SD. (In comparison to the baseline value, *=p<0.05, **=p<0.01, ***=p<0.001. Absolute change between groups, "=p<0.05)
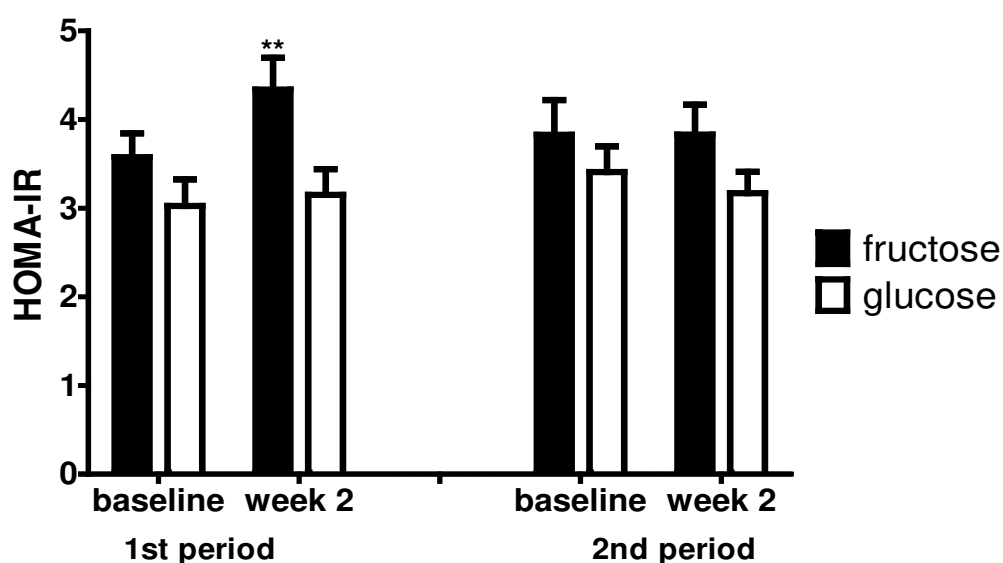
Figure 1. Changes in HOMA values (units arbitrary), mean ± SEM. (** p<0.01 in comparison to baseline value in that group)

4.3.2. Postprandial systemic insulin sensitivity

4.3.2.1. The degree that the 12 subjects undergoing the hyperinsulinaemic euglycaemic clamp were representative of the entire group

A self selected subgroup of 12 subjects underwent further assessment with a hyperinsulinaemic euglycaemic clamp to assess postprandial systemic insulin sensitivity. In terms of clinical features they were however representative of the whole cohort, and the 6 who underwent a clamp and took fructose were no different to the 6 who underwent a clamp and took glucose, see table 2.

			Difference from rest of group (p)	Difference between clamp groups (p)
Age (years)	Whole	34±10		
	Clamp fructose	36±10	0.61	
	Clamp glucose	39±11	0.19	0.64
Weight (kg)	Whole	95.3±8.1		
	Clamp fructose	95.6±10.5	0.91	
	Clamp glucose	91.7±7.5	0.24	0.48
BMI (kg/m^2)	Whole	29.4±1.7		
	Clamp fructose	29.2±1.4	0.73	
	Clamp glucose	28.0±1.9	0.07	0.23
Body fat (%)	Whole	34.2±4.4		
	Clamp fructose	35.4±3.1	0.45	
	Clamp glucose	32.2±3.6	0.23	0.13
Waist (cm)	Whole	103.6±5.0		
	Clamp fructose	103.6±4.7	0.99	
	Clamp glucose	101.8±5.9	0.33	0.63
Waist : hip ratio	Whole	1.03±0.03		
	Clamp fructose	1.04±6.7	0.47	
	Clamp glucose	1.05±0.03	0.15	0.62
Total energy provided during 1st period (kcal/day)	Whole	3259±229		
	Clamp fructose	3210±322	0.57	
	Clamp glucose	3233±190	0.77	0.88
Supplemented monosaccharide during both periods (g/day)	Whole	216.5±14.7		
	Clamp fructose	216.0±19.1	0.93	
	Clamp glucose	213.3±12.3	0.57	0.78
Weight change 1st period (%)	Whole	-0.19±0.87		
	Clamp fructose	-0.28±0.98	0.78	
	Clamp glucose	-0.41±0.62	0.49	0.79
Weight change 2nd period (%)	Whole	0.83±1.2		
	Clamp fructose	1.01±0.02	0.70	
	Clamp glucose	0.49±0.91	0.46	0.47

Table 2. Comparisons of the fructose and glucose clamp groups, mean ± SD. (n=6 in both) with the rest of the cohort in terms of demographics, anthropometry, food and monosaccharide supplied and weight changes.

The clamp assessed 12 did not differ from the full 32 subjects in their HOMA values, though there were no significant changes noted, see table 3.

			Mean ±SD	Difference from rest of group (p)	Difference between clamp groups (p)
First period	**Baseline**	Whole	3.28±1.17		
		Clamp fructose	2.91±0.96	0.40	
		Clamp glucose	2.98±1.59	0.49	0.93
	Week 2	Whole	3.71±1.40		
		Clamp fructose	3.97±1.21	0.62	
		Clamp glucose	2.80±1.09	0.08	0.11
Second period	**Baseline**	Whole	3.60±1.35		
		Clamp fructose	3.57±1.35	0.94	
		Clamp glucose	3.02±1.45	0.25	0.51
	Week 2	Whole	3.48±1.19		
		Clamp fructose	3.94±1.58	0.30	
		Clamp glucose	2.97±1.14	0.25	0.25

Table 3. Comparisons of the fructose and glucose clamp groups (n=6 in both) with the rest of the cohort in terms of fasted insulin resistance as assessed by the HOMA, mean ± SD.

4.3.2.2. The degree of metabolic stability achieved during the clamp procedures

4.3.2.2.1. Maintenance of stable glycaemia

Arterialised blood glucose concentrations during the clamps were tightly controlled at 4.5mmol/L throughout the clamp and did not differ between the groups.
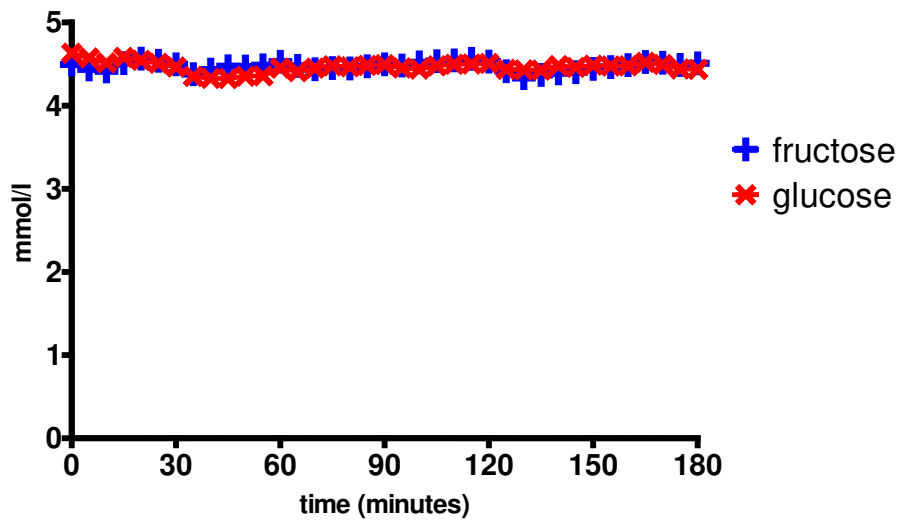
Figure 2. Arterialised blood glucose concentrations during the entire 180 minute hyperinsulinaemic euglycaemic clamp.

Whole body glucose disposal was measured during the final hour (t=120-180minutes) and the arterialised blood glucose concentrations during this assessment period are presented in table 4. There were no differences between the groups and the mean coefficient of variation of the glucose values was acceptable at less than 6.0% in all bar one of the forty eight assessments.

			Arterialised glucose (mmol/L)	Difference between groups (p)	Coefficient of variation (%)	Difference between groups (p)
First period	**Baseline**	Fructose	4.48±0.09	0.76	4.45±2.71	0.23
		Glucose	4.47±0.06		2.98±0.80	
	Week 2	Fructose	4.43±0.04	0.21	3.22±1.30	0.30
		Glucose	4.47±0.04		2.54±0.77	
Second period	**Baseline**	Fructose	4.45±0.06	0.23	3.08±0.63	0.22
		Glucose	4.48±0.02		2.60±0.62	
	Week 2	Fructose	4.45±0.03	0.61	2.88±0.80	0.77
		Glucose	4.46±0.04		2.77±0.45	

Table 4. Whole arterialised blood concentrations during the final hour of a three hour hyperinsulinaemic euglycaemic clamp, mean ± SD.

4.3.2.2.2. Maintenance of stable insulinaemia

The insulin values at each time point in all four clamp assessments are presented below. Some caution needs to be employed as unfortunately not every time period is fully represented by all 12 subjects due to sample misplacement. It

is however clear that a steady state of insulinaemia was achieved at all visits by 30 minutes and that it persisted at the same concentration throughout the 180 minute study.
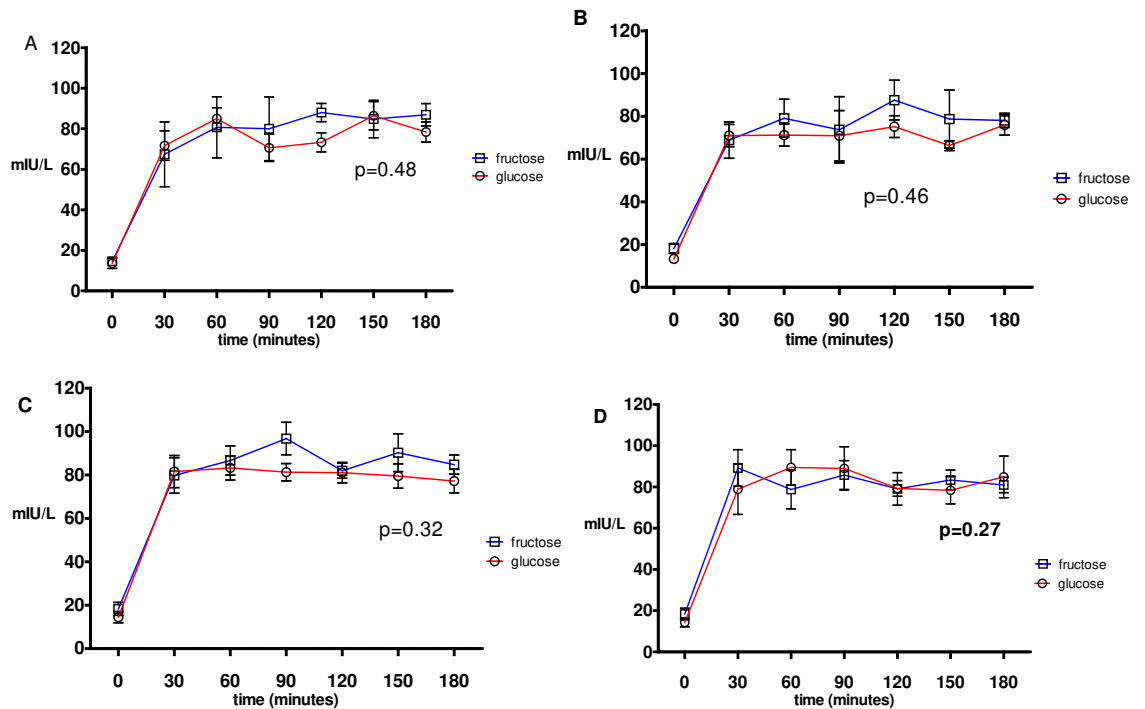
Figure 3 a-d. Serum insulin concentrations during a 180 minute hyperinsulinaemic euglycaemic clamp (units are mIU/L), mean ± SEM. A, baseline assessment during first period. B, week 2 assessment during first period. C, baseline assessment during second period. D, week 2 assessment during second period.

The insulin concentrations during the last hour are presented in table 5. Again due to sample misplacement the data is not complete and so caution needs to be exercised, there was however no differences between the groups or between the assessments.

		All (n=12)	Fructose (n=6)	Glucose (n=6)	Difference between groups (p)
First period	Baseline	86.8±12.5	89.1±11.6	84.9±14.3	0.65
	Week 2	77.9±9.3	82.2±9.2	73.6±8.0	0.15
Second period	Baseline	82.1±12.0	85.5±13.9	79.3±10.6	0.42
	Week 2	83.8±15.9	80.8±8.1	86.8±22.0	0.58

Table 5. Arterialised serum insulin concentrations during the final hour of a three hour hyperinsulinaemic euglycaemic clamp (units are mIU/L), mean ± SD.

4.3.2.3. Oxidative response to the hyperinsulinaemic euglycaemic clamp

In all 12 subjects, the clamp mimicked the postprandial oxidative state seen after a carbohydrate rich meal, with increases in respiratory quotient and carbohydrate oxidation rates and reduced lipid oxidation rates, see table 6. There was no difference between the groups in terms of the baseline or final values.

		Change in RQ	Change in carbohydrate oxidation rate	Change in lipid oxidation rate
First period	**Baseline**	0.077±0.066**	74±60**	-29±26**
	Week 2	0.051±0.063*	49±63*	-21±24*
Second period	**Baseline**	0.074±0.066**	71±63**	-26±23**
	Week 2	0.091±0.073**	86±67*	-36±27**

Table 6. Absolute changes in the respiratory quotient (RQ), carbohydrate and lipid oxidation rates (mg/min) at 150 minutes from baseline of the hyperinsulinaemic euglycaemic clamp in all 12 subjects combined, mean ± SD. (*=p<0.05, **=p<0.01)

4.3.2.4. Glucose disposal during the hyperinsulinaemic euglycaemic clamp

The rate of whole body glucose disposal during the clamp period is presented in figures 4a-d below. The groups were the same at all time points.
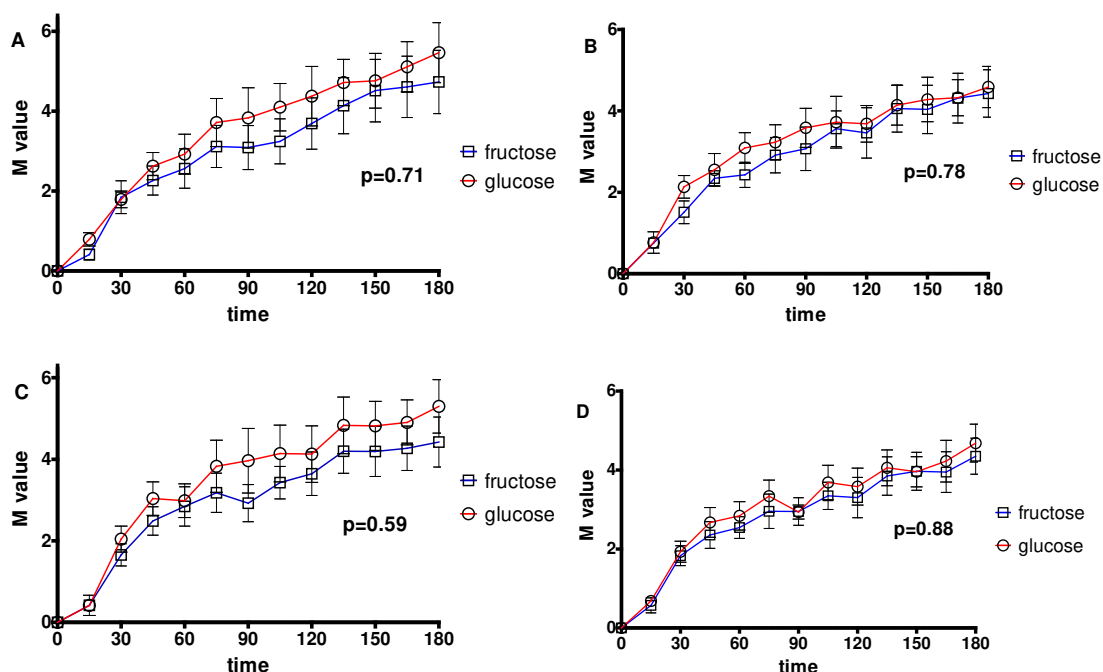
Figures 4 a-d. Whole body glucose disposal (M value) during a 180 minute hyperinsulinaemic euglycaemic clamp (mg/kg/min), mean ± SEM. A, baseline assessment during first period. B, week 2 assessment during first period. C, baseline assessment during second period. D, week 2 assessment during second period.

The average values of glucose disposal (mg/min/kg) for the last hour of the clamp (120-180 minutes) are presented in table 7. The baseline values for both periods were similar. There was a tendency for insulin-mediated whole body glucose disposal to lower during the intervention periods, though this was not significant either within the whole cohort or within the groups.

		All (n=12)	Fructose (n=6)	Glucose (n=6)	Baseline difference between groups (p)	absolute change between groups (p)
First period	**Baseline**	4.76±1.66	4.50±1.84	5.01±1.59	0.62	
	Week 2	4.27±1.25	4.21±1.42	4.33±1.20		0.58
Second period	**Baseline**	4.62±1.43	4.27±1.39	4.96±1.51	0.43	
	Week 2	4.10±1.06	3.98±1.11	4.23±1.10		0.55

Table 7. Average whole body glucose disposal (M value) during the 120 to 180 minute of a hyperinsulinaemic euglycaemic clamp (mg/kg/min), mean ± SD.

When combined together the 48 individual M values presented in table 7 were moderately associated (r=-0.45, p=0.002) with the HOMA value generated at the same assessments, as presented in figure 5.
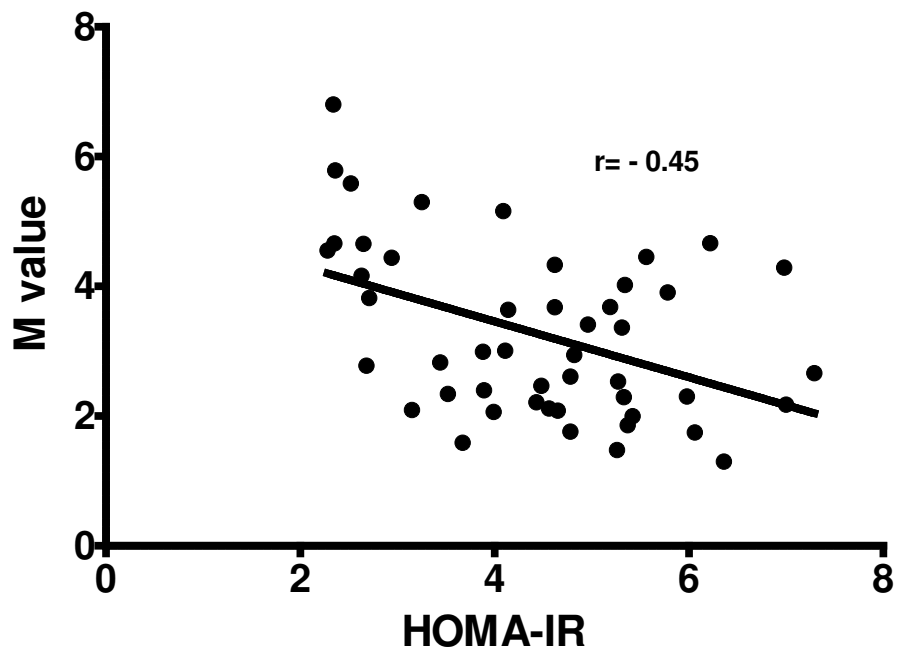
Figure 5. The association between fasted HOMA (arbitrary units) and the mean M value from t=120 to 180 minutes (mg/kg/min).

4.3.3. Fasted and postprandial hepatic glucose production

Rates of fasting hepatic glucose production were unaltered during the study. There was a tendency for it to increase with overfeeding but this was not significant, see table 8.

		Whole cohort (n=12)	Fructose (n=6)	Glucose (n=6)	Difference between groups at baseline (p)	Absolute change between groups (p)
First period	baseline	2.00±1.34	2.07±1.31	1.93±1.49	0.87	
	week 2	1.96±1.13	1.71±0.71	2.20±1.47		0.58
Second period	baseline	2.20±0.69	2.51±0.81	1.90±0.41	0.13	
	week 2	2.45±1.72	2.82±2.27	2.07±1.00		0.89

Table 8. Basal hepatic glucose output pre initiation of the hyperinsulinaemic euglycaemic clamp (mg/kg/min), mean ± SD.

By the end of the clamp hepatic glucose production was not fully suppressed, see table 9. The mean reduction of the basal rates by the end of the clamp was 48%, with no differences between the groups.

		All (n=12)	Fructose (n=6)	Glucose (n=6)	Baseline difference between groups (p)	absolute change between groups (p)
First period	**Baseline**	1.10±0.68	1.02±0.68	1.17±0.73	0.73	
	Week 2	1.29±1.52	0.91±1.08	1.66±1.90		0.47
Second period	**Baseline**	0.98±0.75	1.09±0.80	0.87±0.75	0.64	
	Week 2	1.08±1.08	0.89±0.94	1.28±1.27		0.33

Table 9. End of hyperinsulinaemic euglycaemic clamp (170-180 minutes) hepatic glucose output (mg/kg/min), mean ± SD.

As hepatic glucose production was not fully suppressed then the adjusted glucose disposal (M values) are presented in table 10.

		All (n=12)	Fructose (n=6)	Glucose (n=6)	Baseline difference between groups (p)	absolute change between groups (p)
First period	Baseline	3.7±2.0	3.5±2.2	3.8±2.0	0.76	
	Week 2	3.0±2.1	3.3±1.3	2.7±2.8		0.44
Second period	Baseline	3.7±1.6	3.2±1.2	4.2±1.9	0.32	
	Week 2	3.0±1.4	3.1±01.0	2.9±2.0		0.30

Table 10. Average whole body glucose disposal (M value) during the 120 to 180 minute of a hyperinsulinaemic euglycaemic clamp (mg/kg/min), adjusted for endogenous glucose production during 170 to 180 minutes, mean ± SD.

4.3.4. Oxidative and non-oxidative glucose disposal

At the end of the hyperinsulinaemic euglycaemic clamp the rates of whole body oxidative glucose disposal were unaltered, though there was a trend for a reduction during the first period and an increase during the second period, see table 11.

		All (n=12)	Fructose (n=6)	Glucose (n=6)	Baseline difference between groups (p)	absolute change between groups (p)
First period	Baseline	2.5±1.4	2.2±1.6	2.8±1.2	0.51	
	Week 2	1.9±0.9	1.4±0.9	2.3±0.6		0.70
Second period	Baseline	2.3±1.4	2.3±1.4	2.3±1.4	0.98	
	Week 2	2.9±1.6	3.2±1.6	2.6±1.7		0.58

Table 11. Rate of glucose oxidative disposal at the end (150 minutes) of the hyperinsulinaemic euglycaemic clamp, (mg/kg/min), mean ± SD.

There was a significant reduction in non-oxidative glucose disposal during the period of overfeeding though there was no difference between the groups.

		All (n=12)	Fructose (n=6)	Glucose (n=6)	Baseline difference between groups (p)	absolute change between groups (p)
First period	**Baseline**	2.2±1.3	2.3±1.6	2.2±1.2	0.97	
	Week 2	2.4±1.2	2.8±1.5	2.0±0.8		0.48
Second period	**Baseline**	2.3±1.0	1.9±1.2	2.6±0.8	0.26	
	Week 2	1.2±1.0*	0.8±0.5	1.6±1.2		0.87

Table 12. Rate of glucose non-oxidative disposal at the end (150 minutes) of the hyperinsulinaemic euglycaemic clamp, (mg/kg/min), mean ± SD.

4.3.5. Fasted serum triglycerides

Fasted hypertriglyceridaemia (>1.8mmol/L) at study entry was present in 9 subjects (5 fructose, 4 glucose). There was a mild increase in fasted triglycerides in the first period though this was not significant. In the second period there was a significant, though matched, increase in both groups.

		All (n=32)	Fructose (n=15)	Glucose (n=17)	Baseline difference between groups (p)	Absolute change between groups (p)
First period	Baseline	1.45±0.62	1.45±0.67	1.44±0.58	0.97	
	Week 1	1.52±0.62	1.47±0.53	1.55±0.70	0.73	0.65
	Week 2	1.48±0.79	1.38±0.60	1.57±0.94	0.51	0.35
Second period	Baseline	1.31±0.59	1.27±0.48	1.34±0.67	0.72	
	Week 1	1.63±0.70*	1.60±0.68*	1.66±0.73	0.81	0.98
	Week 2	1.65±0.72**	1.62±0.71	1.68±0.74**	0.84	0.91

Table 13. Fasted serum triglycerides (mmol/L), mean ± SD. (*=p<0.05, **=p<0.01 within that group from baseline)
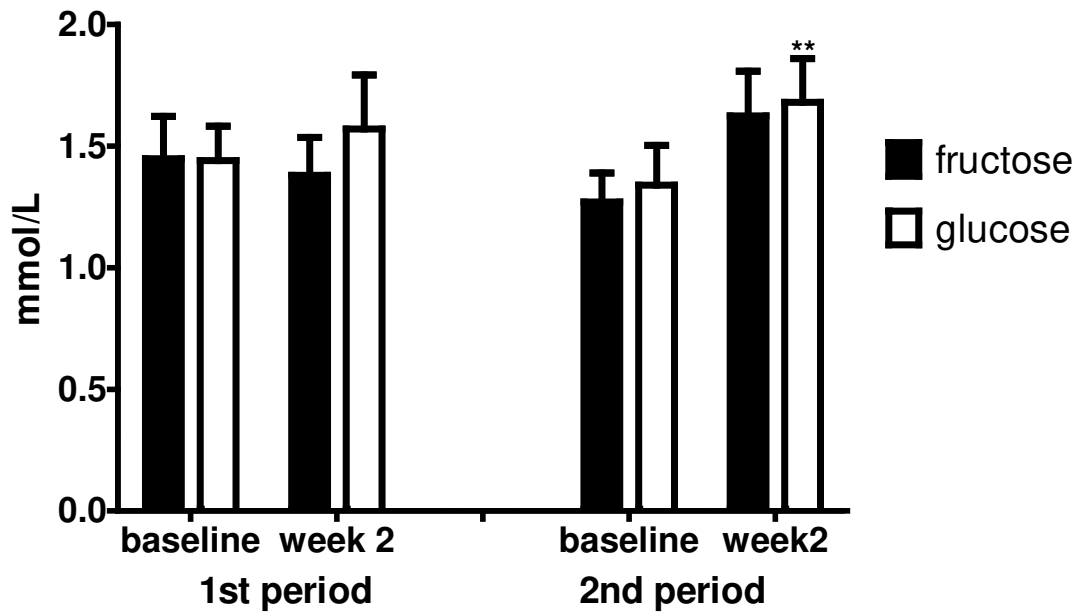
Figure 6. Fasted serum triglyceride (mmol/L) within the groups, mean±SEM.

During the first period there was neither much of a change in weight or triglycerides. The change in triglycerides during the second period was associated with change in weight (r=0.44, p=0.01).

4.3.6. Intra-myocellular lipid (IMCL)

Intra-myocellular lipid (IMCL) was relatively unchanged in the first period, and significantly increased in the second period. There were no differences between the groups.

		Whole cohort (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
1st period	baseline	8.5±3.2	8.8±4.1	8.3±2.1	0.64	
	week 2	8.8±3.2	8.4±3.0	9.2±3.4		0.34
2nd period	baseline	8.5±2.9	9.0±3.3	8.1±2.4	0.36	
	week 2	9.9±2.7*	9.9±2.6	9.8±2.9		0.51

Table 14. Intra-myocellular lipid (IMCL) percentage content of the soleus muscle, mean ± SD. (In comparison to the baseline value, *=p<0.05)
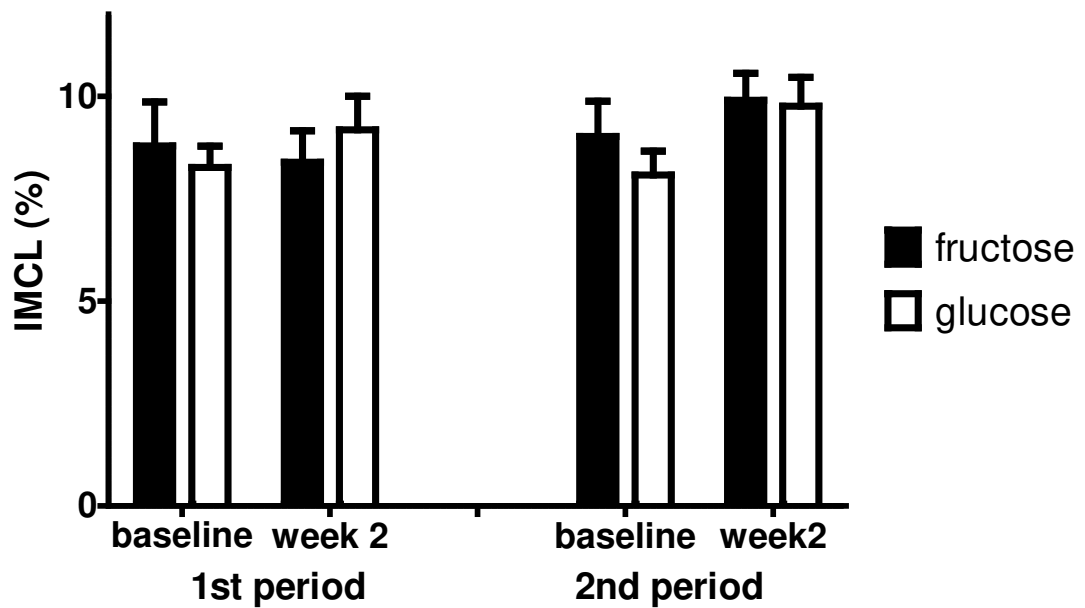
Figure 7. Intra-myocellular lipid content (IMCL) during the study, mean ± SEM.

4.3.7. Extra-myocellular lipid (EMCL)

The subjects were well matched in terms of their EMCL content, see table 15. There were no changes noted during the study.

		All (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	absolute change between groups (p)
1st period	baseline	10.8±4.4	10.6±4.4	11.0±4.5	0.78	
	week 2	10.1±3.5	10.1±3.6	10.1±3.5		0.71
2nd period	baseline	10.3±4.6	11.0±4.6	9.6±4.6	0.41	
	week 2	10.2±4.4	9.9±4.6	10.4±4.3		0.19

Table 15. The influence of two weeks of high fructose or glucose intakes on extra-myocellular lipid (EMCL) percentage content of the non-dominant soleus muscle, mean ± SD.

4.3.8. Non-esterified fatty acids

NEFA concentrations fell during the first period with fructose and were unchanged with glucose. There were identical reductions noted in both groups during the second period.

		All (n=32)	Fructose (n=15)	Glucose (n=17)	Baseline difference between groups (p)	absolute change between groups (p)
First period	Baseline	410±96	415±80	405±110	0.78	
	Week 2	376±134	334±120*	409±140		0.17
Second period	Baseline	354±126	387±139	325±110	0.17	
	Week 2	308±127	337±139	282±113		0.90

Table 16. Fasted non-esterified fatty acids (mmol/L), mean ± SD. (*=p<0.05 within that group from baseline)

4.3.9. Fasted adipose tissue insulin resistance

Fasted adipose tissue insulin resistance was unaltered during the first period, whereas sensitivity increased during the second with both fructose and glucose.

		All (n=32)	Fructose (n=15)	Glucose (n=17)	Baseline difference between groups (p)	absolute change between groups (p)
First period	Baseline	46.2±20.8	47.7±17.5	45.0±23.7	0.72	
	Week 2	46.2±24.4	46.3±23.0	46.1±26.3		0.72
Second period	Baseline	42.7±20.8	48.1±25.6	38.0±14.7	0.18	
	Week 2	36.6±20.1*	41.6±23.5	32.2±16.1		0.90

Table 17. Fasted adipose tissue insulin resistance (Adipo-IR = NEFA (mmol/L) x fasted plasma insulin (pmol/L)), mean ± SD. (*=p<0.05 within that group from baseline)

4.3.10. Serum non-esterified fatty acid concentrations during the hyperinsulinaemic euglycaemic clamp

NEFA concentrations fell markedly during the hyperinsulinaemic euglycaemic clamps, with no difference between the groups at any visit. There were no significant changes noted during the intervention periods either (p=0.47, and p=0.82 respectively).
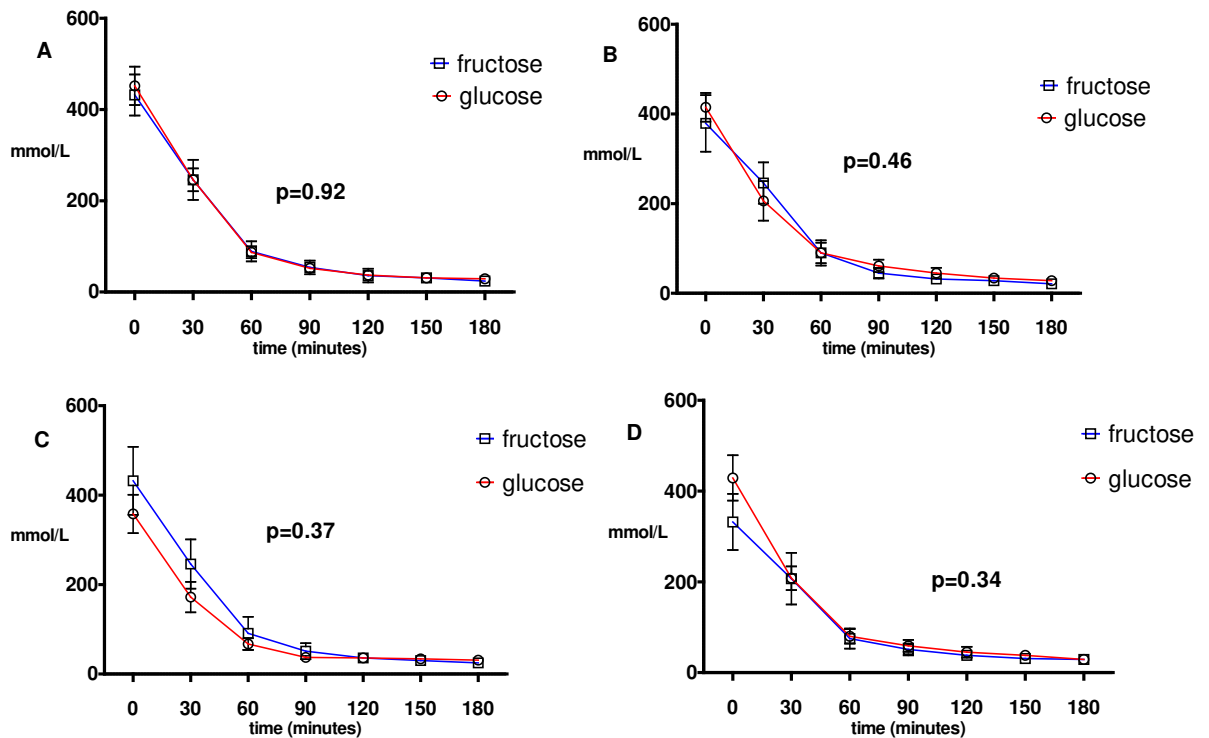
Figures 8 a-d. Systemic concentrations of non-esterified free fatty acids (NEFAs) during a 180 minute hyperinsulinaemic euglycaemic clamp (mmol/L), mean ± SEM. A, baseline assessment during first period. B, week 2 assessment during first period. C, baseline assessment during second period. D, week 2 assessment during second period.

4.4. Discussion

4.4.1. Summary of the findings

The intervention resulted in only one difference between the groups. During the isoenergetic period fructose induced a greater state of fasted insulin resistance than glucose. The interpretation of this isolated finding is therefore limited, though as will be discussed, it has been previously reported.

The subset who underwent the hyperinsulinaemic euglycaemic clamp was representative of the whole study cohort. Stable glycaemia and insulinaemia were maintained during the clamp. The postprandial state was mimicked in terms of circulating insulin concentrations (see below); reduction in hepatic glucose production, and the whole body oxidative response reflected that seen after a high carbohydrate meal.

During the isoenergetic first period, fasted insulin resistance worsened with fructose due to an increase in both glucose and insulin concentrations. There was an associated tendency for a reduction in whole body glucose disposal (M value), though this was non-significant and not different between the groups. Hepatic glucose production and non-oxidative glucose disposal did not change

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during the period or differ between the groups. Fasted serum triglycerides and ectopic lipid stores within the myocytes (IMCL) and outside them (EMCL) were unaltered. NEFA concentrations were unaltered by glucose and reduced by fructose. There was no change in fasted or postprandial adipose tissue insulin sensitivity.

During the hyperenergetic second period, there was no change in fasted insulin resistance and a tendency, as in the first period, for reduced whole body glucose disposal (M value) which was similar in both groups. Hepatic glucose production was unaltered and there was a similar trend for a reduction in non-oxidative glucose disposal in both groups. Fasted serum triglycerides rose to a matched extent in both groups as did IMCL with no change in EMCL. NEFA concentrations tended to fall in both groups to a similar extent. Fasted adipose tissue insulin resistance fell to a matched extent in both groups with no changes in postprandial adipose tissue insulin sensitivity, though the amount of insulin delivered so effectively suppressed NEFAs that reliable discrimination between the groups was not feasible.

4.4.2. Fasted steady-state insulin sensitivity

Insulin directs the major glucose homeostasis processes, namely endogenous production and tissue uptake. Maintenance of normoglycaemia is needed for the adequate function of the central nervous system, which uses glucose almost exclusively as its energy substrate. During the fasted state the level of glycaemia is primarily determined by the rate of hepatic glucose production as opposed to peripheral tissue uptake. Elevated fasting glycaemia or insulinaemia are indicative of a failure of adequate feedback between the liver and insulin and hence predominantly hepatic insulin resistance (DeFronzo and Tripathy 2009).

The background to the HOMA is discussed in the methods section. Briefly, it is a function of fasted insulin and glucose concentrations with higher values representing greater resistance. As such it is a measure of fasted insulin resistance, though its poor reproducibility means that it is a relatively crude assessment. Due to its methodological ease the HOMA assessment has however been frequently assessed following a high fructose diet. Not all studies present their HOMA values, but the calculated findings based on the insulin and glucose data are presented below in table 18. Some caution needs to be employed in interpreting this table. The data by Ngo Sock and Bantle are limited as there are

no baseline values and so the final data comparisons are made either with a 'weight maintenance' or glucose group respectively.

	Comparator to fructose	Duration (weeks)	Foodstuff provided / *ad libitum*	Hyper / iso-energetic	Baseline HOMA value	Fasted changes following fructose			Fasted changes following comparator		
						Glu	Ins	HOMA	Glu	Ins	HOMA
Swanson	starch	4	provided	iso		↑-	.	.	↓-	.	.
Ngo Sock	glucose	1	provided	hyper		↑-	↑-	↑↑	→	↑-	↑
Silbernagel	glucose	2	*ad lib*	hyper	1.8	↑*	↑-	↑*	↑-	↑*	↑↑*
Bantle	glucose	6	provided	iso		↑	→	↑	→	→	→
Stanhope	glucose	10	*ad lib*	hyper	3.1	↑***	↑**	↑↑	↓*	↑-	↑
Johnston 1st period	glucose	2	provided	iso	3.3	↑*	↑*	↑↑**	↓-	↑-	↑-
Johnston 2nd period	glucose	2	*ad lib*	hyper	3.6	↑-	↓-	→	↓-	↓-	↓-

Table 18. Fasted insulin resistance data in studies comparing fructose vs. glucose or starch. Arrows indicate changes in parameters from baseline. (glu=serum glucose, ins=serum insulin). A double arrow indicates a greater absolute change than the comparator, (* = p<0.05, ** =P<0.01, *** =p<0.001). N.B.: Johnston is the current study

Comparisons between differing groups' HOMA values are not valid or reliable due to major differences between insulin assays. Looking at the trends, the first observation is that fasted glucose increases following a high fructose diet, and falls following a high glucose diet in all prior studies bar the Silbernagel paper. Insulin concentrations rose following a high fructose or glucose diet in all prior studies with baseline data presented. As a result HOMA increased with both fructose and glucose in every prior study, and the magnitude was greater with fructose in all bar the Silbernagel paper. It bears remembering the unbalanced weight changes in the Silbernagel paper of +0.2kg and +1.7kg with fructose and glucose respectively.

This current study repeated all of these previous findings during the first period which contained provided foods in an isoenergetic environment. These changes resulted in increases in HOMA in both groups, though this was markedly greater in the fructose group (p=0.03). These changes cannot be attributed to changes in weight or calf lipid as these both remained stable during this first period. During the second hyperenergetic period of this study there was a non-significant rise in fasted glucose in the fructose group (p=0.19) and a fall in the glucose group. As a result HOMA values remained identical with fructose during this period and fell with glucose though these trends were non-significant.

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Overall, when data from both periods are combined, there was a significant rise in HOMA with fructose and no change with glucose.

It is not possible to be certain as to why these differing trends were noted between the two periods. The first period was much more strictly regulated and so the results can be viewed as being more scientifically robust, the second period was deliberately less controlled in an attempt to mimic a 'real life' less 'rat in a cage' styled experiment. This lack of control may however be responsible for the unexpected findings. Indeed the findings during the first period reflect those of Stanhope and colleagues' whereas the second period does not. During the first period identical foodstuffs were consumed during the 24 hours prior to both of these assessments, and both groups consumed identical food in the intervening period. During the hyperenergetic period the precise intakes pre each assessment and during the intervention period are unknown. Insulin sensitivity is very sensitive to variation in preceding food intakes, and as a result the insulin resistance assessments are harder to interpret in this period. Ultimately there are no significant changes in the second period and so little should be deduced from the trends.

The findings in the first period and previously of an increased fasting glucose with a high fructose diet and reduced fasting plasma glucose with a high glucose diet could either be explained by increased endogenous glucose production (hepatic insulin resistance), or by reduced glucose tissue uptake (systemic insulin resistance). Fasted endogenous glucose production, as assessed by the labelled hyperinsulinaemic euglycaemic clamp, did not alter and so it would appear that the changes evolved from tissue uptake rates.

4.4.3. Findings during the hyperinsulinaemic euglycaemic clamp

In the postprandial state the primary function of insulin is to limit endogenous glucose production and to prevent hyperglycaemia by appropriate rates of tissue uptake of glucose. The challenge with postprandial assessments is that they require a standardised exogenous perturbation of the glucose/insulin balance.

The background to the hyperinsulinaemic euglycaemic clamp was described in the methods section. Briefly, whole body insulin sensitivity is determined by the rate of exogenous glucose infusion required to maintain normoglycaemia in response to a fixed level of exogenously supplied insulin. It assumes that there is no residual hepatic glucose production and hence the rate of

exogenous supply represents the rate of whole body glucose disposal. This was not the case in our cohort. This failure to fully suppress hepatic glucose production is likely to reflect the relatively low dose of insulin administered and the pre-existing insulin resistance of the cohort. Adjusting for this did not alter the glucose disposal conclusions of no difference between fructose and glucose. The vast majority (80-90%) of this disposal is to the skeletal muscle (Thiebaud, Jacot et al. 1982) , and so what is principally assessed is the rate of insulin-mediated skeletal glucose uptake. Furthermore, the relative rates of oxidative and non-oxidative disposal can be determined by the addition of carbohydrate oxidation rates during this period as assessed by calorimetry.

Due to resource availability such assessments were performed in only 12 subjects out of the full cohort of 32. These 12 were representative of the full cohort in terms of characteristics, anthropometry and baseline HOMA values. The chosen target glucose concentration during the clamp was 4.5mmol/L which effectively maintained fasted normoglycaemia, as their mean fasted glucose concentrations were 4.66±0.47mmol/l and 4.54±0.43mmol/L at the first and second baseline assessments.

The baseline serum insulin concentration was around 14miU/L, and during the clamp there was a 6-fold increase in circulating insulin concentrations to around 80miU/L. Such changes are similar to those found postprandially in insulin resistant obese male subjects (Parra, Martinez de Morentin et al. 2005). As a result the procedure mimicked the postprandial insulinaemic response in a standardised way whilst maintaining euglycaemia and without the potential confounding of inter-subject food substrate absorption variability. To reinforce the notion that the clamp reproduced the postprandial state, the exogenous delivery of glucose and insulin resulted in an increased respiratory quotient (RQ) as compared to baseline values. This elevation in RQ reflects the rise in carbohydrate oxidation rates and a reduction in lipid oxidation rates during the three hour clamp period. There were no significant differences between the groups in the oxidation rates or their changes either within or between the assessments. There was a tendency for there to be a greater rise in carbohydrate oxidation during the second period's week 2 clamp though this was not significant (p=0.10). Such an increase could be attributed to the carbohydrate and energy overfeeding that occurred during the second period resulting in a system that was primed to cope with carbohydrate delivery.

The presented glucose disposal (M values) were calculated as the mean M value during the final hour (t=120-180). During this period the mean arterialised glucose values and insulin concentrations were at a relatively steady state and did not differ between the assessments or between the two groups. Overall the robustness of the insulin, glucose and M value balances generated facilitates a direct comparison of whole body insulin sensitivity between both groups and between visits.

4.4.3.1. Baseline findings of systemic glucose disposal

Previous studies with larger cohorts of overweight individuals have shown M values to be negatively associated with body fat (r=-0.80) (Holt, Wild et al. 2007), and with HOMA (r=-0.47) (Ruige, Mertens et al. 2006). So the M values were predicted to be low in our overweight cohort, and the current association with HOMA (r=-0.45) reflects that of prior experience.

There is no agreed cut-off M value to categorise an individual as being insulin resistant. A large European study performed euglycaemic clamps in 1,146 nondiabetic, normotensive Caucasian men and women (Ferrannini, Natali et al. 1997). The insulin dose was 1mU/min/kg body weight. No glucose disposal value suggestive of insulin resistance was generated, though the mean value amongst slim men was 7.1mg/kg/min. There were 376 overweight men with a mean BMI 28.8±3.9 (SD), and a body fat percentage of 33%. The mean M value in this cohort was 5.5±2.2mg/kg/min. The current study's baseline value is 4.8±1.7mg/kg/min. The M value for Ferrannini and colleagues, corrected for fat free mass (FFM), was 8.2mg/kgFFM/min, which is comparable to the current baseline value of 7.2mg/kgFFM/min. Such corrections for fat free mass are useful when comparing glucose disposal values between cohorts.

4.4.3.2. Changes in postprandial insulin sensitivity

Overall there was a tendency for a decreased insulin sensitivity with a reduction in the M value during both periods, though the absolute changes were small and not significant in the first (p=0.17) or second (p=0.16) period. It is worth remembering that this was not the study's primary outcome measure and hence it was not specifically powered for such assessments. Using the baseline standard deviation value of 1.66mg/kg/min, the study had 80% power to detect a difference of 2.7mg/kg/min with 12 subjects, and if all 32 had undergone the clamp then it would have had 80% power to detect a difference of 1.6mg/kg/min.

During the second period the absolute difference in change between the two groups was 0.44mg/kg/min. As such these assessments are markedly underpowered, the resultant data is non-significant, and any further interpretation needs to be done guardedly.

There are no prior published data comparing a high fructose versus high glucose diet on systemic insulin resistance using a hyperinsulinaemic euglycaemic clamp. Other groups have however assessed the non-fasted insulin resistant state. Bantle et al. measured glucose and insulin excursions at nine time-points over a 24 hour period during standardised meals after 42 days of a high fructose or glucose diet (Bantle, Raatz et al. 2000). There was no baseline analysis and so we can only compare the resultant post intervention data between the groups as opposed to the changes from baseline. There was no difference in mean hourly plasma glucose concentrations at 139 vs. 141mmol/L/hr, whereas the glucose diet had a higher insulin mean concentration at 4243 vs. 3486 pmol/L/hr.

Stanhope assessed insulin sensitivity by a 75g oral glucose tolerance test at baseline and after 9 weeks of a high fructose versus high glucose diet. As compared to the baseline there were no changes in the resultant 4 hour curves with glucose consumption but there were significantly greater insulin and glucose excursions following fructose. So either the ability of insulin to mediate tissue glucose uptake or suppress hepatic production, or both, was impaired with fructose. The addition of (15g 6,6,D_2 glucose) within the 75g of glucose aided this discrimination. The 6,6,D_2 is retained in the glucose if the glucose is not oxidised, whilst more than 90% is lost to tissue water during glycolysis. This allows the determination of the proportion of plasma glucose that undergoes glycolysis within the whole-body. This can be further transferred to an insulin sensitivity index by comparing the ^2H_2O (a marker for glycolysis) area under the curve (AUC) over the insulin AUC. Whole body glycolytic insulin sensitivity was unchanged by a high glucose intake and reduced following a high fructose intake. No direct comments can be made about hepatic insulin sensitivity, but this is implied by the associated fasted HOMA, as previously discussed.

The above assessments both differ from a clamp in that the perturbation in homeostasis originates from exogenous oral food or glucose as opposed to insulin. There is one study that used an insulin challenge. An intravenous insulin tolerance test was performed in 15 healthy volunteers who consumed a standard diet with either 1000kcal per day from fructose or glucose in drinks for a week (Beck-

Nielsen, Pedersen et al. 1980). There was identical weight gain in both groups. No changes in insulin sensitivity occurred with glucose, and there was a 25% reduction with fructose. The aetiology of these changes was not determined, though there was an observed reduction in insulin binding to monocytes, and so the authors speculated about insulin receptor modulation.

To conclude, previous data suggests that systemic postprandial insulin sensitivity worsens with fructose and is unchanged with glucose. These were not the findings in this current study, where non-significant similar reductions in systemic postprandial insulin sensitivity were found in both groups.

4.4.3.3. Hepatic insulin sensitivity

In the basal fasted state there was no change in hepatic glucose output with isoenergetic feeding and a non-significant trend for greater output following hyperenergetic feeding. As insulin was not administered in this basal state this trend may merely reflect the energy overfeeding itself. In the 'postprandial' end of clamp period hepatic glucose output was incompletely suppressed. This facilitated discrimination between the two groups in terms of the degree of suppression. Suppression (hepatic insulin sensitivity) was not altered during the interventions, though tended to be greater with fructose than glucose.

The effects of fructose versus glucose on hepatic insulin sensitivity have only once been previously assessed (Ngo Sock, Le et al. 2010). On that occasion there was a non-significant trend for a reduced sensitivity, with no differences between the two groups.

4.4.3.4. Non-oxidative glucose disposal and glycogen synthesis

The rate of non-oxidative glucose disposal approximates to the rate of glycogen synthesis. Indirect calorimetry measures whole body net carbohydrate loss, which includes the following processes: oxidation of exogenous and endogenous glucose and oxidation of glycogen without passage through extracellular glucose (Tappy and Schneiter 1997). Non-oxidative glucose disposal (NOGD) is the difference between systemic glucose disposal and carbohydrate oxidation. The potential outcomes include synthesis of glycogen, lactate or lipid (DNL). The latter two are quantitatively small and so NOGD corresponds to glycogen accumulation in either muscle or liver (Fery, Plat et al. 1999).

Insulin resistance is characterised by a low NOGD, possibly influenced by a high IMCL (Yokoyama, Mori et al. 2008). There was no change in IMCL or NOGD in the first period and an increased IMCL and a reduction in NOGD in the

second. It is unclear if the reduced NOGD is due to the increased IMCL as there was no association between the changes in these parameters (r=-0.17, p=0.6).An alternative explanation is that the overfeeding resulted in greater repletion of glycogen stores and hence glucose disposal occurred via other mechanisms.

4.4.4. Serum triglycerides

The triglycerides were measured in the overnight fasted state. This is both a strength and a weakness. A fasted sample is taken in a standardised setting and hence should be more reproducible than a non-fasted sample. The utility however of the fasted triglyceride content to predict cardio-metabolic risk has recently been questioned. As a result this area is highly contentious. Serum triglycerides areindependently predictive of cardiovascular events when assessed in the non fasted state, whereas fasted triglycerides remained dependent on traditional cardiac risk factors (Bansal, Buring et al. 2007). This study was designed to focus on the hepatic effects of a high fructose versus glucose intake, Fructose has been proposed to stimulate hepatic triglyceride synthesis and so it should also increase fasted serum triglycerides. Postprandial lipid assessments would have been interesting to have performed but they would not have addressed the primary research questions and were not feasible given the constraints of all the investigations that were undertaken.

A significant proportion of the cohort studied had evidence of dyslipidaemia at study entry with 9 subjects (5 in fructose group and 4 in glucose) having fasted serum triglycerides above the normal reference range (>1.8mmol/L). Weight was maintained during the first period and this was matched by an overall stable serum triglyceride concentration. During the second period there was a significant increase in triglycerides, though the amount of change was identical between the groups (p=0.91).

In opposition to previous findings medium-term high fructose versus glucose studies, this current study failed to demonstrate any significant changes in fasted serum triglycerides from the baseline values with fructose, see table 19. Of note the two papers which did report significant increases in serum triglycerides did not show any associated changes in hepatic triglyceride content. The findings in the second period mirror that of Stanhope and colleagues, with a significant increase with glucose and not with fructose.

	Duration (weeks)	Foodstuffs provided / *ad libitum*	Hyper / iso-energetic	Weight change (kg)	Fructose	Comparator
Bantle	6	Provided	Iso	-1.4	↓-	↓↓*
Silbernagel	2	*ad libitum*	Hyper	+0.9	↑*	→
Ngo Sock	1	Provided	Hyper	?	↑*	↑-
Stanhope	10	*ad libitum*	Hyper	+1.6	↑-	↑*
Johnston 1st period	2	Provided	Iso	-0.2	↓-	↑-
Johnston 2nd period	2	*ad libitum*	Hyper	+0.8	↑-	↑**

Table 19. Current and previously reported changes in fasted triglyceride serum concentrations following intakes a high fructose or glucose diet. (- = not significant, * = p<0.05 as compared to baseline value) (Johnston is the current study)

Interpretation of several of these studies is limited given the absence of adequate controls in terms of energy or sugar as has been discussed. The reason behind the increase in triglycerides during the second period in this current study appears to be primarily the greater energy and/or carbohydrates supplied as there was a significant association between weight change and triglycerides (r=0.44, p=0.01). Without the addition of tracers it is impossible to fully ascertain fluxes in lipid kinetics. The association with energy excess seems to have also been a factor in the prior studies as those with weight gain all had an increase in fasted triglycerides.

The paper by Silbernagel is difficult to interpret due to the significant weight discrepancy between the two groups. The rise in fasted triglycerides in the fructose group with no change in the glucose group was the only finding that differed between their groups, for which the authors offered no explanation. If I ignore this paper then the three prior medium term studies have generated opposing and conflicting fasted findings. The two studies however that have compared triglyceride data in the postprandial as opposed to fasted state (Bantle and Stanhope) have shown a greater postprandial triglyceride response following fructose than glucose.

The aetiology of the differences in the postprandial as opposed to fasted state is believed to be due to increased production by de novo lipogenesis and reduced insulin excursion with fructose resulting in a lower activation of adipose tissue lipoprotein lipase (LPL) (Stanhope and Havel 2009). In the Stanhope paper

fasted LPL and DNL rates were unchanged as compared to baseline rates in both groups. In the postprandial phase however DNL was increased 27% with glucose and 75% with fructose. In conjunction postprandial LPL excursions were increased by 20% with glucose and reduced by 5% with fructose. The resultant differences in postprandial triglyceride concentrations were relatively modest at an increase of 3% from glucose and 18% from fructose.

The prior major contribution to the understanding of DNL in the acute setting comes from Chong and colleagues (Chong, Fielding et al. 2007). They assessed the acute lipaemic responses to a single high fructose or glucose meal. The absolute difference in DNL contributions at 240 minutes from fructose or glucose was minimal with ~ 0.05% of the labelled fructose forming de novo fatty acids and ~ 0% with glucose.

To summarise, fasted serum triglycerides were unchanged during the isoenergetic period and rose in both groups during the hyperenergetic period to a matched extent. Changes in weight could partially explain the changes in the hyperenergetic period.

4.4.5. Extra-Myocellular Lipid (EMCL)

The extra-myocellular lipid (EMCL) content is quantified as part of the process of determining IMCL content. EMCL is of limited metabolic interest as it is ectopic lipid stored within adipocytes which are interspersed between muscle fibres. As it is not within the myocytes it appears to have no specific metabolic function and merely reflects the total body adiposity (Machann, Haring et al. 2004). Changes in total body adiposity were not assessed during the study, but they were unlikely to change much due to the minimal changes in total body weight (–0.19% in 1st period and +0.83% in the 2nd). As expected there were no significant changes in EMCL throughout the study.

4.4.6. Intra-Myocellular Lipid (IMCL)

The association between IMCL and insulin resistance is firmly established, though the mechanism remains yet to be fully determined. IMCL is an early marker for the development of insulin resistance (Schrauwen-Hinderling, Hesselink et al. 2006). Myocytes lack the ability to perform *de novo* lipogenesis, and so an increased IMCL content is the result of either increased circulating fatty acid delivery or reduced oxidation. It is not believed that the triglycerides

themselves mediate the insulin resistance, but that this is done through intermediary metabolites (Machann, Haring et al. 2004).

The current data appear to support these observations and theories. During the first assessment period the baseline IMCL value was not associated with HOMA from the same assessment (r=0.06), or the clamp derived M value (r=0.02), or serum triglycerides. During this period changes in HOMA, M value, weight, serum triglycerides, NEFA and lipid oxidation were also not associated with changes in IMCL.

During the second period there was an increase in IMCL in the absence of a change in systemic insulin resistance as measured by the clamp and HOMA. There was however a weak association (r=0.36, p=0.04) between changes in IMCL and changes in HOMA during this period. There was no such association with changes in weight, serum triglycerides or lipid oxidation rates. It is possible however that given time the increases in IMCL may have subsequently resulted in systemic insulin resistance, but that the time-frame was too short for this to develop.

4.4.7. Serum NEFAs

Concentrations of fasted NEFAs were relatively unchanged by glucose and reduced by fructose overfeeding. Direct assessments of lipolytic rates were not made. Previous acute overfeeding work has shown a similar reduction in NEFAs with no change in glycerol following fructose administration (Chong, Fielding et al. 2007). The authors proposed that the reduced NEFA concentrations reflect reduced spill-over of LPL derived fatty acids with fructose compared with glucose. LPL activity was not quantified in this study and so this is merely speculation, though it would explain the NEFA differences between the two groups.

DeFronzo's group has recently described adipose tissue insulin resistance (Adipo-IR) as the function of insulin and NEFA in the fasted non dynamic state (Gastaldelli, Cusi et al. 2007). Insulin mediates suppression of lipolysis via hormone sensitive lipase and this formula indirectly assesses this activity via circulating NEFA concentrations. The greater the value the less sensitive the adipocytes are to insulin. There were no changes during the first period whereas both groups demonstrated an improved sensitivity in the second period to a matched extent.

Adipose insulin sensitivity was further assessed during the clamp. Infusion of insulin resulted in a marked reduction in circulating NEFAs to a matched extent in both groups and with no change as a result of the intervention. Though the magnitude of the reduction is likely to have minimised any potential to discriminate between the two groups. It is noteworthy that despite both organs receiving the same insulin concentrations, there was a much greater response to exogenous insulin infusion from the adipocytes than from the liver. This was expected as endogenous insulin is delivered via the portal vein to the liver where there is a significant first pass effect. As a result the liver is normally exposed, and hence responds, to much greater concentrations of insulin than the rest of the body.

4.5. Conclusions

There were no convincing differences in terms of insulin resistance or non-hepatic lipid profiles resulting from a high fructose as opposed to a high glucose intake. In contrast, energy overfeeding resulted in increased serum triglycerides and intra-myocellular lipids, and reduced adipose tissue insulin resistance.

There are several strengths specific to this chapter:

1. Insulin resistance was assessed in a variety of states including systemic (fasted and postprandial), hepatic and adipose tissue.

2. This was the first study to assess the effects of fructose versus glucose on systemic insulin resistance with the gold standard assessment, hyperinsulinaemic euglycaemic clamp.

3. The clamp procedure reproduced the postprandial state in terms of circulating insulin concentrations and oxidative changes.

4. The concurrent assessment of IMCL facilitated interpretation of any changes in insulin resistance.

I acknowledge some weaknesses specific to this chapter:

1. The hyperinsulinaemic euglycaemic clamp was only performed in a representative subset of the entire group, and the study was not specifically powered for these assessments.

2. The increase in IMCL during the hyperenergetic period without any change in insulin resistance may be interpreted that the duration of the intervention was too short.

3. All assessments, bar insulin resistance, were made in the fasted state.

4. There were no assessments made of lipid kinetics, LPL activity or DNL rates. Indeed the study would have benefited from postprandial lipaemic assessments as it is in this state that differences between fructose and glucose have been most reliably reported.

This chapter refutes prior assertions that there is a significant difference between fructose and glucose on insulin resistance and non hepatic lipid stores. Further work is necessary to assess the lipaemic outcome in the postprandial state.

Chapter 5. The hepatic effects of a high fructose versus high glucose diet

5.1. Introduction

These data are derived from the method fully described in chapter 3. This final chapter of this study focuses on the primary outcome measure, namely the impact of a high fructose or high glucose diet on hepatic metabolism.

In brief, healthy overweight males were randomised to a high fructose or glucose diet for 2 weeks firstly in an energy balanced (isoenergetic) setting and subsequently in an energy overfeeding (hyperenergetic) setting. The monosaccharides were consumed mixed with water four times a day and provided 25% of predicted energy requirements. The differing energy periods were achieved by firstly providing all food to be consumed and secondly by subjects consuming the monosaccharides in addition to their habitual intakes.

The assessment processes were identical pre and post each intervention period. Specific to this chapter, the 32 subjects underwent fasted hepatic proton and phosphorus magnetic resonance spectroscopy (^{1}H MRS and ^{31}P MRS), and fasted serum was analysed for liver biochemistry, C-reactive protein, and uric acid.

5.2. Protocols for methods employed in this chapter

5.2.1. ^{1}H MRS liver data collection

This collection protocol is the same for this chapter and chapter 6. Measurements of hepatic triglyceride content (HTGC) were obtained using a Philips Achieva 3.0 Tesla, whole body system (Philips Medical Systems). A transmit/receive body coil was used. Transverse images through liver and abdomen were acquired during a single breath-hold (duration = 14.4s) using a T_1-weighted Turbo Field Echo (TFE) sequence with the following parameters: in plane resolution= 2mm^2, Field Of View (FOV) = 382x400mm^2, with 36, 7mm slices in the foot-to- head direction, repetition time (TR) = 3.1s, echo time (TE)=1.5ms.

Following image acquisition a 27 cm^3 spectroscopic volume of interest (voxel) was positioned in the right lobe of the liver. The voxel avoided major blood vessels, intrahepatic bile ducts, and the lateral margin of the liver as previously described (Szczepaniak, Nurenberg et al. 2005). A screen shot was

stored at each visit to enable repositioning of the voxel in the same location at subsequent visits.

For estimation of total fat percentages in liver, MR spectra were collected using a respiratory triggered Point RESolved Spectroscopy (PRESS) sequence (Bottomley 1987) with the following parameters: TR = 5000ms, Number of samples = 1024, bandwidth=2000Hz. In order to account for possible changes in signal relaxation times (the time over which signal dephases) with changes in fatty composition, 24 spectra were acquired with TE=40ms, 8 spectra with TE=50ms, 8 spectra with TE=60ms and 8 spectra at TE=70ms.

5.2.2. ^{1}H MRS liver data analysis

Spectra were individually phase corrected and realigned using jMRUI (Java-based MR user interface) (Naressi, Couturier et al. 2001) before averaging across each echo time. The water and methylene proton peak areas at each echo time were determined using in-house software built in Matlab (Mathworks, Matick, United States). In order to calculate the absolute HTGC percentage, a correction to account for spin-spin relaxation (T_2) and to convert to absolute %HTGC was calculated as follows:

$$\%HTGC = 100(ZRT_w) / (ZRT_w + ((\rho_{tg} / \rho_{H2O}) (D_{H2O} / D_{tg})))$$

where $Z = (\exp(-TE/T_{2H20}))/(\exp-(TE/T_{2TG}))$ is the T_2 correction factor for the measured HTGC/water ratio (R) at TE=40ms, T_w is the fractional mass of tissue water (T_w=0.711g/ml (Longo, Pollesello et al. 1995)), ρ_{tg} (=70.35mmol/ml (Szczepaniak, Babcock et al. 1999)) and ρ_{H2O} (=111.11mmol/ml (Szczepaniak, Babcock et al. 1999)) are the density of protons in 1ml of triglyceride and water respectively and D_{tg} (=0.9g/ml (Szczepaniak, Babcock et al. 1999)) and D_{H2O} (=1g/ml (Szczepaniak, Babcock et al. 1999)) are the density of triglyceride and water respectively.

T_2 values, for both water and methylene, were calculated for each individual subject using the peak areas (S) at each echo time (TE) and the following equation:

$$S = S_0 \exp(-TE/T_2)$$

where S_0 is the un-relaxed signal (a constant). T_2 is the gradient of a graph of ln (S) against (-TE).

5.2.3. Liver volume analysis

The data was collected during a single breath hold. The image sequences were weighted by the tissue's T1 relaxation via application of a pulse at 180 degrees to the field at the beginning of the sequence. A three dimensional turbo field echo (3D-TFE) acquisition mode was used to gain an entire image out of a single sequence. The planar resolution of each T1-weighted image was 2.08x2.08mm with 7mm slice thickness. Thirty six slices were acquired per analysis. Data was acquired from voxels measuring 2.08x180mm. The repetition time (TR) between sequence acquisitions was 3.11 ms with total scan time of 14.4 seconds. Images were individually analysed by drawing the hepatic outline in Analyze9 software which summated the image areas to calculate the hepatic volumes.

5.2.4. ^{31}P data collection

For measurement of ^{31}P metabolite levels, a single loop (diameter=140mm) coil tuned to 3T ^{31}P frequency, was positioned over the liver of the subject. A ^{1}H MR image (acquired on the ^{1}H body coil) was acquired to ensure the coil was positioned well over the liver (based on a ^{1}H visible marked placed in the centre of the coil) and for positioning of the voxel for ^{31}P MRS completely within the liver, as near to the coil as possible. Again, a screen shot was stored to aid positioning at the same location in subsequent visits. ^{31}P data were acquired using a respiratory triggered Image Selected *In vivo* Spectroscopy (ISIS) localization with optimized proton decoupling and Nuclear Overhauser Effect (NOE) and the following parameters: TR=5000ms, 2048 samples, Bandwidth=3000Hz, 96 averages.

5.2.5. ^{31}P data analysis

Spectra were truncated to 1024 points to reduce noise level before line broadening (12Hz Lorentzian) and phase correction in JMRUI. Spectral peaks for phosphoethanolamine (PE), phosphocholine (PC), glycerophosphorylcholine (GPC), glycerophosphoethanolamine (GPE), phosphoenolpyruvate (PEP), nicotinamide adenine dinucleotide (NAD) and uridine diphosphate (UDP) were then fitted to singlet Lorentzian line shapes in AMARES. γ-ATP and α-ATP were fitted to two double peaks (doublets) with prior knowledge constraining the line widths and amplitudes to be equal for each peak in the doublet, as well as setting the doublet splitting to 20Hz. β-ATP was fitted to three peaks with prior knowledge giving an amplitude ratio of 1:2:1 with line widths equal and

frequency shift =20Hz. The inorganic phosphate peak was fitted to two unconstrained peaks since separation can occur when there is a pH gradient between the intra- and extracellular compartments. Metabolite concentrations are routinely given as a ratio to each other since no internal reference exists for ^{31}P spectra. The GPE and GPC peaks were combined as phosphodiesters (PDE) and the PE and PC were combined as phosphomonoesters (PME).

There are no ^{31}P metabolite findings which are characteristic for NAFLD (Cortez-Pinto, Chatham et al. 1999). The largest case series published to date (85 patients) demonstrated that there are progressive changes in PME/ATP, PDE/ATP and PDE/PME ratios with worsening liver disease (Menon, Sargentoni et al. 1995). Using ^{31}P MRS acute fructose loading has been shown to temporarily deplete human liver of ATP and increase Pi for 40 minutes post an intravenous bolus (Boesch, Elsing et al. 1997). As a result the absolute values and the following ratios are presented: PME/ATP, PDE/ATP, PDE/PME and ATP/Pi.

5.2.6. Liver biochemistry and CRP

This was as previously described in 3.5.7 and 3.5.8.

5.2.7. Statistical analyses

All data are presented as the mean ± standard deviation (SD). Comparisons between baseline values were done by the independent samples T test. The Student's T test was used for paired data. Analysis of the significance of change between the two groups at two paired assessments was done by an independent samples T test of the absolute change between the two assessments in both groups. Associations were assessed for by 2-tailed Pearson correlations.

5.3. Results

5.3.1. Hepatic triglyceride content (HTGC)

In one subject the repeat baseline assessment at the start of the second period was not performed due to failure of the magnetic coil, the baseline value was carried over from the first visit. The groups were well matched for HTGC at baseline of both periods, see table 1. The mean HTGC at the first assessment was 7.6%. Indeed 17 of the 32 had steatosis using the recognised ^{1}H MRS cut-off value of 5.5% (Szczepaniak, Nurenberg et al. 2005).

The changes in hepatic triglyceride content (HTGC) in both groups combined are shown in figure 1. There was no significant change during the first period and during the second period there was a +24.3±35.1% (mean±SD) increase in absolute values from 7.77% to 9.65% (p<0.001).
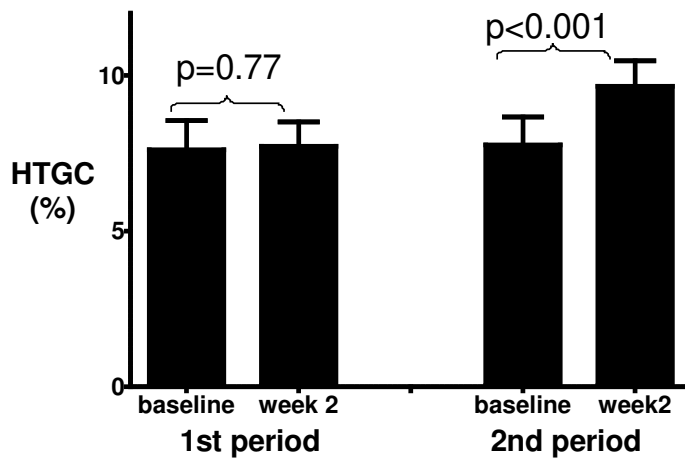
Figure 1. Hepatic triglyceride content (HTGC) in both groups combined during the study, n=32, mean ± SEM.

Analysing the HTGC outcomes by group, during the first period there were no significant changes in either group's HTGC. In the second period significant increases occurred but the changes again were matched between the groups. The changes relative to the baseline value in the second period for both groups were +22.8% (p=0.02) with fructose, and +25.5% (p=0.01) with glucose. Those with a baseline HTGC greater than 15% did not develop a greater HTGC with monosaccharide overfeeding.
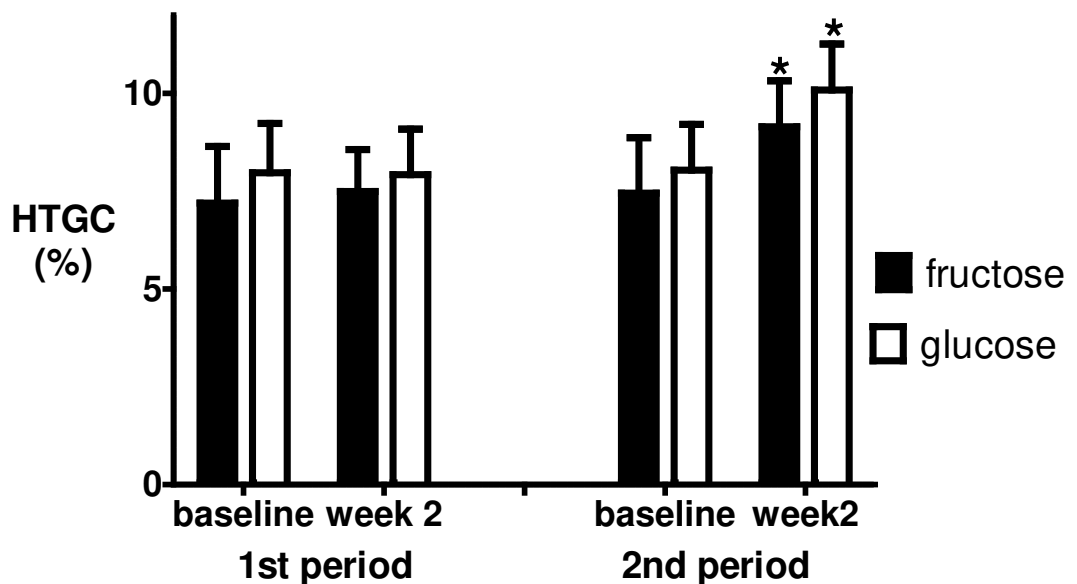
Figure 2. Hepatic triglyceride content (HTGC) before and after two weeks of a high fructose or high glucose intake. (*=p<0.05 from baseline in that group), mean ± SEM.
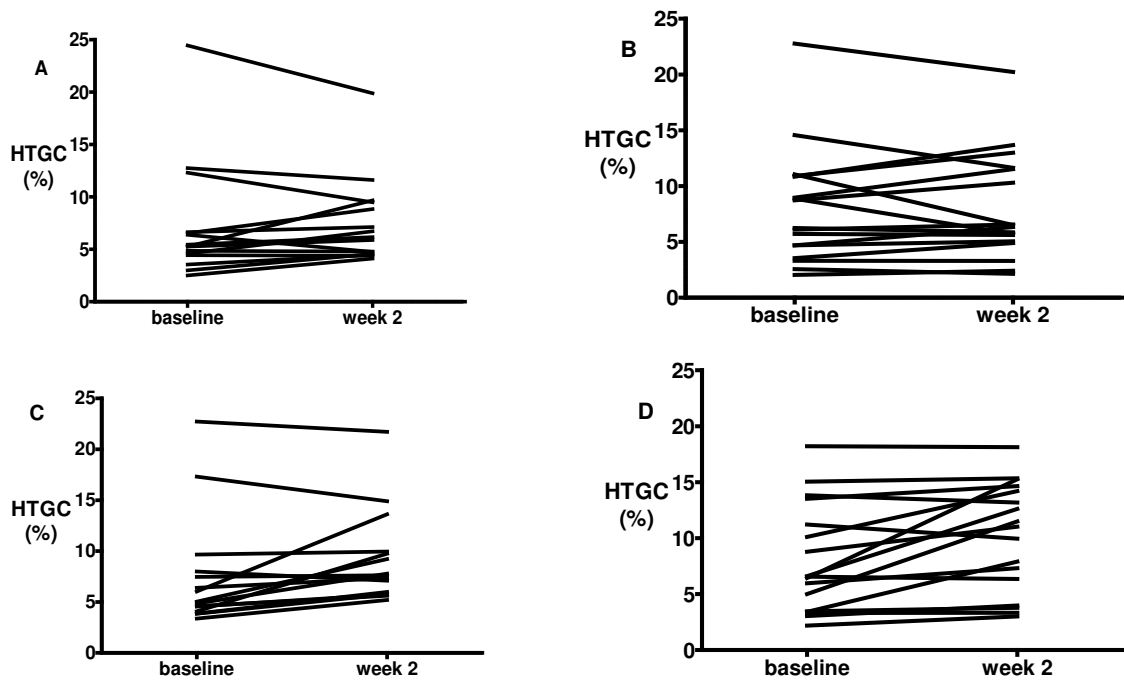
Figure 3a-d. Changes in individual subject hepatic triglyceride content (HTGC) during the study in the two groups during the two periods. (A – fructose 1st period, B – glucose 1st period, C – fructose 2nd period, D glucose 2nd period)

		Whole cohort (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
First period	Baseline	7.61±5.3	7.20±5.6	7.98±5.2	0.69	
	week 2	7.72±4.43	7.50±4.1	7.92±4.8		0.65
Second period	baseline	7.77±5.08	7.45±5.5	8.04±4.8	0.75	
	week 2	9.65±4.64***	9.15±4.5*	10.09±4.8*		0.73

Table 1. The effect of 2 weeks of a high fructose or glucose intake on percentage hepatic triglyceride content (HTGC), mean ± SD. (In comparison to the baseline value, *=p<0.05, **=p<0.01, ***=p<0.001)

The observed changes in HTGC were partially explained by changes in weight during the first period but not during the second as shown in table 2.

		R	p
Whole cohort (n=32)	1st period	0.54	0.002**
	2nd period	0.18	0.34
Fructose (n=15)	1st period	0.56	0.03*
	2nd period	0.41	0.13
Glucose (n=17)	1st period	0.54	0.03*
	2nd period	-0.03	0.90

Table 2. Association between changes in weight and changes in HTGC. (2-tailed Pearson correlation, *=p<0.05, **=p<0.01)
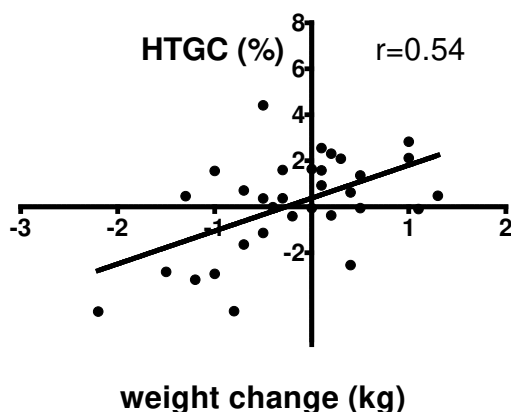
Figure 4. Association between changes in weight and HTGC in all 32 subjects during the first period.

There was an association noted between fasted serum triglyceride concentrations and HTGC which was most pronounced at the first baseline assessment, as shown in table 3.

		All (n=32)	Fructose (n=15)	Glucose (n=17)
1st period	Baseline	0.51**	0.29	0.76***
	Week 2	0.21	0.09	0.26
2nd period	Baseline	0.35*	0.33	0.38
	Week 2	0.42*	0.36	0.47

Table 3. Association between fasted serum triglycerides and HTGC at the initial baseline. (* =p<0.05, ** =P<0.01, ***p<0.001)
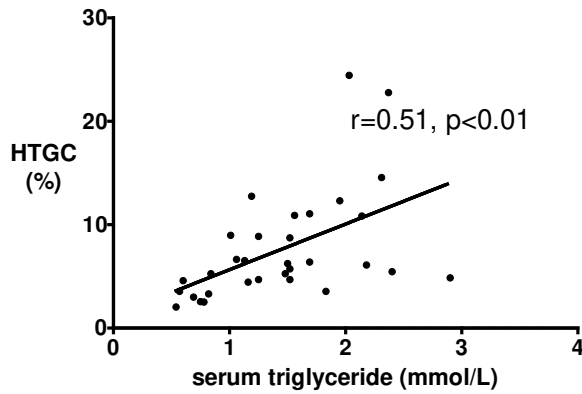
Figure 5. Association between fasted serum triglyceride concentrations and HTGC in all subjects at the initial assessment.

5.3.2. Liver volume

Liver volumes were unchanged in the first period and increased in both groups during the second.

		Whole cohort (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
First period	baseline	2.05±0.33	2.09±0.38	2.02±0.28	0.53	
	week 2	2.07±0.28	2.11±0.31	2.04±0.26		0.96
Second period	baseline	2.11±0.31	2.12±0.37	2.10±0.26	0.91	
	week 2	2.21±0.32**	2.28±0.37*	2.14±0.25		0.09

Table 4. The effect of 2 weeks a high fructose or glucose intake on liver volumes, mean ± SD. (In comparison to the baseline value, *=p<0.05, **=p<0.01)

5.3.3. Liver biochemistry

The two groups were well matched in terms of their liver biochemistry at baselines of both periods, see table 5. In the whole cohort liver biochemistry improved significantly, bar alkaline phosphatase, during the first period. In the second period they all significantly worsened. The changes were matched between the groups for all, bar alkaline phosphatase during the first period which worsened in fructose and was unchanged in glucose.

		All (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
ALT 1ˢᵗ period (U/L)	Baseline	28.7±12.3	31±15	27±10	0.31	
	Week 1	27.9±11.3	31±13	25±9		0.58
	Week 2	25.3±9.5*	27±11	24±8		0.67
ALT 2ⁿᵈ period (U/L)	Baseline	27.0±9.5	27±12	27±7	0.90	
	Week 1	32.0±13.3*	37±18**	28±6		0.009 ^^
	Week 2	32±12**	33±14*	31±10		0.62
AST 1ˢᵗ period (U/L)	Baseline	23.9±6.6	24±8	24±5	0.74	
	Week 1	23.0±4.8	23±6	23±4		0.79
	Week 2	22.1±4.9*	21±4*	23±5		0.11
AST 2ⁿᵈ period (U/L)	Baseline	22.5±5.0	22±5	23±5	0.73	
	Week 1	24.9±6.4*	26±8*	24±5		0.21
	Week 2	23.9±5.3	23±5	25±6		0.64
GGT 1ˢᵗ period (U/L)	Baseline	35.3±14.8	39±17	32±12	0.14	
	Week 1	33.8±14.4	40±17	29±9*		0.09
	Week 2	31.2±14.4**	38±18	25±8**		0.08
GGT 2ⁿᵈ period (U/L)	Baseline	33.3±13.5	37±16	30±10	0.13	
	Week 1	36.8±20.4	44±27	31±10		0.12
	Week 2	38.0±19.4*	44±24*	32±12		0.17
Alk Phos 1ˢᵗ period (IU/L)	Baseline	73.7±16.4	73±16	74±17	0.81	
	Week 1	78.6±18.5***	80±19**	77±18		0.046 ^
	Week 2	74.8±16.0	77±19**	73±15		0.003 ^^
Alk Phos 2ⁿᵈ period (IU/L)	Baseline	72.1±16.2	72±15	72±15	0.94	
	Week 1	79.8±18.1***	80±18***	80±19***		0.61
	Week 2	75.7±16.4**	75±15*	77±18*		0.49

Table 5. The effect of 2 weeks of a high glucose or fructose intake on liver biochemistry, mean ± SD. (In comparison to the baseline value, *=p<0.05, **=p<0.01, ***=p<0.001. Difference between groups in change during intervention ^ = p<0.05, ^^ =p<0.01). (ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma glutamyl transferase, Alk Phos = alkaline phosphatase).

Changes in liver biochemistry in the whole cohort (n=32) (again bar alkaline phosphatase) were positively associated with changes in weight (table 6), and only weakly associated with changes in HTGC (table 7).

		Whole cohort (r)	Fructose	Glucose
Alt	Period 1	0.64**	0.54*	0.66**
	Period 2	0.33	0.20	0.58*
Ast	Period 1	0.52**	0.45	0.62**
	Period 2	0.31	0.19	0.52*
GGT	Period 1	0.25	0.22	0.57*
	Period 2	0.40*	0.67**	0.32
Alk P	Period 1	-0.09	-0.19	0.045
	Period 2	-0.1	-0.19	-0.003

Table 6. Association between changes in liver biochemistry and changes in weight (2-tailed Pearson correlation, *=p<0.05, **=p<0.01, ***=p<0.001)

		Whole cohort (r)	Fructose	Glucose
Alt	Period 1	0.44*	0.49	0.42
	Period 2	0.09	-0.03	0.18
Ast	Period 1	0.28	0.43	0.16
	Period 2	0.17	0.08	0.21
GGT	Period 1	0.40*	0.55*	0.25
	Period 2	0.36*	0.22	0.70**
Alk P	Period 1	0.23	0.11	0.30
	Period 2	0.29	-0.12	0.51*

Table 7. Association between changes in liver biochemistry and changes in HTGC (2-tailed Pearson correlation, *=p<0.05).

5.3.4. Hepatic inflammation

There was no change in hepatic inflammation as assessed by serum high-sensitivity CRP during either period.

		Whole cohort (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
1st period	baseline	1.22±1.29	1.01±1.08	1.40±1.46	0.41	
	week 2	1.20±1.28	0.79±0.84	1.55±1.51		0.43
2nd period	baseline	1.44±1.46	1.22±0.97	1.63±1.79	0.44	
	week 2	1.69±2.81	1.13±0.95	2.19±3.75		0.37

Table 8. The effect of 2 weeks of a high fructose or glucose intake on serum HS-CRP (mg/L), mean ± SD.

5.3.5. Uric acid

At study entry fasted uric acid levels were pathologically raised (>420μmol/L) in 11 out of the 32 subjects (5 fructose and 6 glucose). In both periods, uric acid concentrations increased with fructose and were reduced with glucose, though these changes were only significant during the first period.

		Whole cohort (n=32)	Fructose (n=15)	Glucose (n=17)	Difference between groups at baseline (p)	Absolute change between groups (p)
1st period	Baseline	408±69	406±60	410±77	0.87	
	Week 1	406±72	432±76	384±62**		0.003 ^^
	Week 2	406±73	428±65	388±77**		0.004 ^^
2nd period	baseline	388±92	406±79	371±102	0.29	
	Week 1	394±71	420±59	370±74		0.45
	week 2	393±82	429±57	362±90		0.10

Table 9. The effect of 2 weeks of a high fructose or glucose intake on fasted serum uric acid (μmol/L), mean ± SD. (In comparison to the baseline value, **=p<0.01. Difference between groups in change during intervention ^^ =p<0.01).

5.3.6. Hepatic ^{31}P metabolite profiles

There was signal failure in 4 subjects. Overall there was minimal change in the metabolite profiles during the study. Pi increased during the second period to an equal extent in both arms. PDE increased during the second period with glucose and reduced with fructose.

			Whole cohort (n=28)	Fructose (n=14)	Glucose (n=14)	Difference between groups at baseline (p)	Absolute change between groups (p)
g-ATP	1st period	baseline	254±83	239±63	270±99	0.34	
		week 2	259±83	245±79	273±87		0.96
	2nd period	baseline	257±82	237±75	274±84	0.23	
		week 2	261±97	251±81	271±111		0.65
Pi	1st period	baseline	223±69	223±72	223±68	0.99	
		week 2	231±70	242±80	219±60		0.48
	2nd period	baseline	209±65	209±69	210±62	0.96	
		week 2	241±84*	236±80	246±90		0.81
PME	1st period	baseline	287±86	281±78	292±97	0.75	
		week 2	276±87	267±100	285±76		0.83
	2nd period	baseline	297±122	301±130	285±118	0.73	
		week 2	281±93	283±109	279±79		0.67
PDE	1st period	baseline	556±154	525±158	586±149	0.30	
		week 2	610±185	587±183	633±191		0.84
	2nd period	baseline	630±192	638±224	614±163	0.75	
		Week 2	649±241	533±207	756±226*		0.01^

Table 10. Fasted hepatic ^{31}P metabolite peak areas before and after 2 weeks of a high fructose or glucose intake (nil units), mean ± SD. (In comparison to the baseline value, *=p<0.05. Difference between groups in change during intervention ^ =p<0.05).

There were no consistent changes of ^{31}P metabolite ratios during the study. The changes in Pi and ATP to Pi ratio were not associated with changes in uric acid or weight.

			Whole cohort (n=28)	Fructose (n=14)	Glucose (n=14)	Difference between groups at baseline (p)	Absolute change between groups (p)
PME / ATP	**1st period**	baseline	1.41±1.21	1.22±0.36	1.58±1.66	0.43	
		week 2	1.40±1.27	1.25±0.79	1.54±1.61		0.71
	2nd period	baseline	1.45±1.28	1.30±0.46	1.54±1.72	0.62	
		week 2	1.39±1.26	1.18±0.41	1.58±1.69		0.45
PDE / ATP	**1st period**	baseline	2.45±1.06	2.22±0.44	2.66±1.41	0.28	
		week 2	2.71±1.27	2.59±1.00	2.82±1.50		0.59
	2nd period	baseline	2.71±1.14	2.63±0.41	2.73±1.52	0.82	
		week 2	3.12±1.52	2.78±1.56	3.36±1.45*		0.37
PDE / PME	**1st period**	baseline	2.01±0.47	1.92±0.53	2.09±0.40	0.35	
		week 2	2.30±0.60*	2.30±0.58	2.29±0.65		0.45
	2nd period	baseline	2.16±0.97	1.89±0.76	2.47±1.03	0.10	
		week 2	2.55±0.78	2.28±109	2.79±0.65		0.77
g-ATP / Pi	**1st period**	baseline	1.20±0.43	1.10±0.19	1.29±0.58	0.25	
		week 2	1.19±0.54	1.03±0.27	1.35±0.69		0.58
	2nd period	baseline	1.28±0.39	1.16±0.27	1.38±0.46	0.13	
		Week 2	1.13±0.36	1.12±0.36	1.13±0.38		0.30

Table 11. Fasted hepatic ^{31}P metabolite ratios before and after 2 weeks of a high fructose or glucose intake, mean ± SD. (In comparison to the baseline value, *=p<0.05.)

5.4. Discussion

5.4.1. Summary of findings

The intervention resulted in only a handful of isolated differences detected between the groups. During the isoenergetic period fructose resulted in a greater uricaemia than glucose. This observation has been widely shown previously and is attributed to their differing pre triose metabolic feedback inhibition. Interestingly there were no associated ^{31}P MRS metabolite profile differences. Two other differences occurred between the groups, with a greater alkaline phosphatase concentration with fructose in the isoenergetic period, and a greater PDE peak area with glucose in the hyperenergetic period and a smaller PDE peak area with fructose. These isolated and unrelated findings appear to be of no consequence.

During the first period there were no changes in HTGC, whereas there was an increase of 24% in HTGC relative to baseline values during the second period. This change was matched in both groups. Changes in weight were strongly associated with changes in HTGC in the first period, and less so during the second. The HTGC values were strongly associated with the serum triglyceride concentrations.

The trends for alkaline phosphatase did not reflect those of the other liver enzymes measured. The reasons for this are uncertain. The rest of the liver biochemistry improved during the first period and worsened during the second with no difference between the groups. The absolute effects were small but statistically significant. Changes in liver enzymes were associated with changes in weight and HTGC. In spite of the changes in transaminase levels there were no changes in C reactive protein concentrations.

Uric acid concentrations increased with fructose and reduced with glucose, particularly during the first period. However there were no real changes in fasted hepatic ^{31}P metabolite profiles or ratios during the study.

5.4.2. HTGC findings

The cohort had a markedly high HTGC value at study entry. Indeed it was greater than that predicted in the power calculation (7.6±5.3% vs. 4.4±3.2%). The power calculation value was derived from a previously reported large cohort of Caucasian men in Dallas, USA (Browning, Szczepaniak et al. 2004). Indeed the cohort of Browning and colleagues had almost identical BMI and waist profiles to that recruited to this current study, and so the aetiology of the current greater HTGC values is unclear. Potential factors include the younger age and lack of regular vigorous exercise in the current cohort. Alcohol abuse was an exclusion for the current study and only reported in 6% of the Dallas cohort. Ultimately the greater mean HTGC and broader standard deviation in the observed population will have weakened the study's true power.

The isoenergetic period maintained weight and HTGC, so a high monosaccharide diet in the presence of energy balance has no impact on liver fat. There was a trend for HTGC to increase in fructose and for it to decrease in glucose, though the changes were far from significant. The absolute difference in changes between these two groups was 0.36%. This does not appear to be a type 2

error as if we do a power calculation based on an 80% power and a significance of <0.05 to detect these changes then we would have had to recruit:

$(4*(1.96+0.84)^2 * 5.3^2) / (0.36^2) = 6,797$ volunteers

At 90% power the numbers needed to recruit are 9,112 volunteers.

The trend in the first period for fructose to induce a greater HTGC was reversed by glucose during the second period. As a result this study is overwhelmingly negative for there being a true difference between fructose and glucose overfeeding in HTGC changes. It bears remembering that the reproducibility of these HTGC measures using ^{1}H MRS is high, with an inter-examination co-efficient of variation of 7.0% (Thomas, Hamilton et al. 2005).

The aetiology for the increase in HTGC during the second period as opposed to the first is presumed to be its positive energy balance. Curiously there was no association between weight change and HTGC changes during the second period. As previously discussed in chapter 1, Kechagias and colleagues overfed 18 healthy male and female volunteers by twice their predicted requirements and restricted their exercise (Kechagias, Ernersson et al. 2008). Over 4 weeks the subjects had a mean weight gain of 9.5%. Serum ALT increased by 215%, and HTGC by 155%. So a total body weight gain of 1% in the Kechagias study resulted in a 16% increase in HTGC, whereas in this current study a 1% weight gain resulted in a 28% increase in HTGC. These ratios are strikingly similar. The reason behind the apparently higher ratio in this study is unclear but it may be due to its pure monosaccharide overfeeding. Indeed the contribution of sugar to overall energy intake in the Kechagias study only increased from 15.7% to 18.6%. In further support of this theory, in Kechagias' study the intake of sugars was the only nutrient change that was associated changes in liver injury according to the maximal ALT values (r=0.62, p=0.006).

The finding that there was no difference in HTGC with glucose or fructose overfeeding is not what many would have predicted. Fructose has been turned into a pariah nutrient with many believing that it turns 'everything to fat'. Indeed 6 review articles on the interactions between fructose and hepatic lipid metabolism were published in 2010. This is despite scant controlled clinical evidence that fructose and glucose metabolism differ in terms of lipid metabolic outcomes (Tappy, Le et al. 2010).

This study was started after the publication of two similar papers comparing fructose and glucose overfeeding with the primary outcome measure

being HTGC. Unfortunately for me neither study was registered on the clinical trials website and so I was unaware of their existence. Fortunately both studies have significant limitations which are discussed in-depth on section 1.10.5. In brief, the paper by Ngo Sock et al. compares outcomes from three differing intake patterns over 7 days in men: namely a weight maintenance intake; or the same intake with additional 35% fructose, or glucose. The data presentation is very unclear and there are no baseline analyses despite the short washout periods of 2-3 weeks. Silbernagel et al. report a small mixed sex study with 10 in each arm, food was not supplied and there were no interim visits. After 4 weeks the fructose group's weight change was only 0.2±0.6kg (SEM) (p=0.40), indeed several seemed to lose weight. Whereas weight gain seemed to be universal in the glucose arm as the changes were +1.7±0.4kg (p=0.001). So as a result the fructose arm appears to be isoenergetic and the glucose hyperenergetic.

Both studies enrolled slim, healthy, insulin sensitive subjects. They showed marked increases in HTGC which were matched between the groups. Neither of the papers had any real explanation for the absence of a difference between fructose and glucose and HTGC. Silbernagel and colleagues accepted it as a true outcome. Ngo Sock and colleagues stated that the HTGC changes in their study were smaller than their group had previously found (52% versus 79%) with similar amounts of fructose overfeeding (Le, Ith et al. 2009), and that the inter-individual variability may have been a factor. As a result they do not conclude that glucose and fructose have the same hepatic lipid outcomes.

My current findings compliment these prior findings and show that there is no difference in a high fructose versus high glucose intake on hepatic lipid stores and biochemical enzyme tests. My paper extends the prior studies by recruiting a larger cohort with a differing phenotype, namely centrally overweight males with a pre-existing tendency to sub-clinical insulin resistance, dyslipidaemia and hyperuricaemia. Indeed it is such a cohort who may be predicted to be most vulnerable to overfeeding. From a methodological perspective the presence of baseline, as well as post intervention, data aid the transparency of the findings' interpretation. The supply of monosaccharides in an isoenergetic as opposed to merely energy overfeeding setting means that the separate metabolic challenges in these phases can be assessed. The weight changes reflected the differing energy delivery phases and were balanced between both groups. The supply of foodstuffs provided complete intake control and energy balance during the first period, such

that the only factor that differed was the monosaccharide. Changes in HTGC were non-significant in the isoenergetic period and matched in the hyperenergetic period such that the findings can only be viewed as being the consequence of energy as opposed to nutrient overfeeding. The strong associations between the hepatic and serum triglyceride values reinforce the reliability of my assessment process.

The liver volumes also reflected the changes in HTGC, with no changes in the first period and a matched increase in both groups during the second. The aetiology of this increased liver volume is uncertain but is likely to reflect increased liver triglyceride and glycogen storage.

5.4.3. Liver biochemistry and CRP

Liver biochemistry improved in all parameters bar alkaline phosphatase in this first period. The reasons for this are unclear. They are crude and poor markers of overall liver 'health' but it would tend to suggest that the subject's livers improved during this period. This is a curious finding as HTGC and serum triglycerides were unchanged, whereas HOMA (a marker of hepatic insulin resistance) worsened in those taking fructose. This improved liver biochemistry may be explained by the nutritional quality of the provided foodstuffs being greater than habitual intakes. The supplied diet clearly changed many nutrients, it reduced intakes of fat and increased carbohydrate intakes whilst potentially changing the intakes of many micronutrients. Altered alcohol intakes may have had a role, but the reduction in Gamma GT was no greater than the reduction in ALT or AST. The absolute changes in liver biochemistry are small and so are not felt to warrant over-interpretation. There were no changes in C-reactive protein levels, which have been shown to be associated with liver enzymes, visceral adiposity and the presence of NASH (Kerner, Avizohar et al. 2005; Verrijken, Francque et al. 2010; Fierbinteanu-Braticevici, Baicus et al. 2011).

Liver biochemistry worsened during the overfeeding period and this was associated with the marked weight gain that occurred during this period. Of note the liver biochemical changes in the Kechagias study were much more dramatic than in this study (Kechagias, Ernersson et al. 2008). Kechagias and colleagues were unable to be certain as to why such high ALT values were noted in their study but they felt that it was by 'nutrient induced enzyme induction'. It may well be that their dramatic overfeeding caused overwhelming metabolic stress resulting

in the hepatic compensatory threshold being exceeded. Unfortunately this is merely speculation as the authors have not released any non-esterified fatty acid, triglyceride, uric acid or inflammatory marker data despite my personal invitation for them to do so (Johnston, Aithal et al. 2009).

5.4.4. Uric acid

A significant proportion of the cohort had hyperuricaemia at baseline. Hyperuricaemia results from either an increased production, or decreased excretion, of uric acid. In health, diet has a key role in uric acid balance. High intakes of alcohol, purine rich foods or fructose increase uric acid production, whereas a ketogenic diet reduces its excretion. As to whether hyperuricaemia is a causative, or merely an associated, factor in cardiometabolic and renal disease development is widely debated. The majority feel that it forms merely an association due to it being a marker of a high risk lifestyle (Kanbay, Afsar et al. 2011). Interestingly uric acid concentrations have been recently shown to be greater in children with NASH than without (Vos, Ryan et al. 2011).

The aetiology of fructose induced hyperuricaemia was described in section 1.10.3.2. To summarise, the initial steps of fructose catabolism involve hepatic ATP consumption which results in an increased formation of adenine nucleotides. These are degraded to form uric acid (Tran, Yuen et al. 2009). The rate of these steps is strictly limited for glucose via feedback from phosphofructokinase. As a result a high fructose intake is associated with hyperuricaemia and hence gout (Choi and Curhan 2008; Choi, Ford et al. 2008). However, as sucrose is the main dietary source of fructose a high fructose diet also involves a high glucose intake. As a result these population based studies cannot demonstrate that this is a fructose-specific effect. Indeed there are scant long-term studies comparing the uric acid outcomes following a high fructose or glucose intake.

In the acute setting hyperuricaemia has been described following overfeeding with fructose (Tran, Jacot-Descombes et al. 2010), though not as a fructose-specific effect (Teff, Grudziak et al. 2009). In long term studies hyperuricaemia with fructose has only been reported in studies lacking an energy control (Le, Ith et al. 2009), and resulting in a non-significant trend in comparison to glucose (Ngo Sock, Le et al. 2010; Silbernagel, Machann et al. 2011). As a result this is the first study to my knowledge that clearly demonstrates a difference in uricaemia following a high fructose or glucose intake. There was a trend for an

increase in uric acid with fructose and reduction with glucose in both periods. This was only significant in the first period when all intakes were controlled for. During this period however the main change was a reduction in uric acid with glucose as opposed to an increase with fructose. Following identical fructose and glucose intakes there would be predicted to be a higher rate of pre triose metabolism with fructose than glucose due to the lack of feedback inhibition. This would result in a greater uricaemia with fructose than glucose as the pre triose metabolism of both results in ATP consumption, forming adenine nucleotides which are degraded to uric acid (Tran, Yuen et al. 2009).

The reasons for the observed reduction in uric acid with glucose are unclear. During that period both food and monosaccharides were supplied. It is possible that this reflects a washout of fructose in the normal diet of the glucose arm or a glucose-specific effect. Changes in purine or alcohol intakes should have affected both groups equally. Microdiet software does not present data on purine intakes and so the purine content of the supplied food is not known. In the glucose group habitual intakes of fructose contributed 4.8% of energy. During the first period this fell to 2.3%. So there was some degree of fructose washout.

5.4.5. Hepatic ^{31}P metabolite profiles

^{31}P MRS analyses were performed in order to further assess hepatic ATP and uric acid metabolism. There were no consistent patterns of metabolite changes during the study. There was an increase in Pi during the overfeeding period presumably as a result of increased glucose and/or fructose metabolism. The absence of any other ^{31}P MRS findings conflicts with the current uric acid findings and the previously described acute responses to fructose overfeeding (Segebarth, Grivegnee et al. 1991; Boesch, Elsing et al. 1997; Cortez-Pinto, Chatham et al. 1999). The current lack of changes will most likely reflect that the assessments were made in the fasted (at least 10 hours post fructose) state as opposed to the immediately postprandial phase. It seems likely that the observed changes in plasma uric acid reflect the less transitory changes in their levels than hepatic ^{31}P metabolite profiles. One would predict tight regulation of ATP metabolism with a return to baseline as soon as the acute disturbance is removed, which would be predicted to occur within 10 hours of exposure to fructose, though this is speculation. Uric acid is not so critical to the central functioning of cells and so disturbances likely to be less rapidly reverted. It would have been

fascinating to have performed postprandial ^{31}P analyses as these may have been altered and exaggerated by induction of fructokinase with fructose.

5.5. Conclusions

The study resulted in only a handful of differences between the groups, all of which appear to be unrelated. During the isoenergetic period fructose resulted in a greater uricaemia and serum level of alkaline phosphatase than glucose, and the hepatic PDE peak areas differed during the second period. No differing hepatic metabolism pattern emerged between fructose and glucose. In contrast there were significant differences noted in both groups. The isoenergetic period merely resulted in improved liver biochemistry in both groups. In contrast the hyperenergetic period was associated with increases in liver lipid, volume, biochemistry and Pi content in both groups. So there was a clear metabolic challenge to the liver during this period which was equal with both fructose and glucose. As a result the clear messages are that over a two week period glucose or fructose overfeeding are not different, and they only impact on the liver in the presence of energy excess. The lack of an alternative nutrient comparator in this study however means it cannot be resolved as to whether the changes observed are solely the effects of energy overfeeding, or energy overfeeding specific to monosaccharides.

The strengths of this chapter lie within the numerous and in-depth assessments that were made of hepatic metabolism and function. The combination of liver lipid, volume, biochemistry and inflammatory markers provided a global assessment which is further strengthened by the whole body metabolism, insulin resistance and non-hepatic lipid data of the previous two chapters. As such it can be viewed as a comprehensive and definitive assessment.

The key limitation of this work is the lack of histological assessment of liver tissue. Such an assessment is not ethically possible within a healthy volunteer research setting. In its absence however only indirect inferences can be made on the hepatic inflammatory and fibrotic influences of the interventions. Another limitation was the timing of the ^{31}P MRS assessments. There are dramatic alterations in liver parameters in response to a fructose infusion as assessed by ^{31}P MRS. All ^{31}P MRS measurements were made in the fasted state. It would be fascinating to perform such assessments in the postprandial or post fructose infusion state following a high glucose or high fructose diet. This has

never been done before, but it would aid assessments of the degree of enzyme induction by overfeeding.

At present there are no controlled data that demonstrate a difference between fructose and glucose intakes on hepatic metabolism. As such advice on 'low fructose diets' in NAFLD are entirely unjustified. The impact of monosaccharide and energy overfeeding is however very clearly demonstrated by this study, and so further work needs to be made assessing the outcomes of low monosaccharide intakes in patients with NAFLD.

Chapter 6. The effect of n-3 PUFA supplementation on hepatic fatty acid metabolism in non-alcoholic fatty liver disease

6.1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the pathological consequence of excessive lipid storage within the liver. The aetiology of NAFLD appears to be multifactorial, though it is primarily associated with central obesity and insulin resistance. Despite all fatty acids being ultimately derived from dietary intakes, NAFLD lacks a clear dietary association. Some studies have characterised NAFLD as having a relative deficiency of the long chain n-3 PUFAs in the patients' diets, plasma and livers. Biological plausibility of these observations is reinforced as hepatic lipid metabolism is potentially improved by n-3 PUFAs. Suggested mechanisms include the promotion of insulin sensitivity, anti-inflammatory immunomodulation and beta-oxidation, combined with the reduction of *de novo* lipogenesis. Not all of these effects have been conclusively demonstrated outside the hepatocyte cell line or animal model experience. Clinical studies in NAFLD have been performed and shown divergent outcomes, though many have lacked robust assessment protocols. This study aimed to assess the impact of n-3 PUFA supplementation in patients with clear evidence of NAFLD using a robust assessment process.

6.2. Method
6.2.1. Subject identification

Adult NAFLD patients were identified from a hospital liver biopsy database in University Hospitals Nottingham, UK. I reviewed all liver biopsies over the preceding 3 years. All patients aged greater than 18 years and with a liver biopsy report suggestive of NAFLD then had a case note review to ensure that they had previously undergone detailed investigations to exclude alternative diagnosis. In addition, I excluded the following-

1. Significant hepatic architectural distortion on biopsy with bridging fibrosis or cirrhosis
2. The presence of a further hepatic diagnosis
3. Excessive alcohol intake defined as self-reported intakes greater than 21 units per week in men, and 14 units per week in women.
4. Poorly controlled diabetes (HbA1C > 7.0% (Skyler, Bergenstal et al. 2009))

5. Use of insulin replacement therapy or insulin sensitising therapies such as a thiazolidinedione, or gliclazide.

6. Initiation, or changes in the dose, of lipid altering medication within the preceding 3 months, such as a HMG CoA reductase inhibitor, fibrate or systemic steroids.

7. Fat malabsorption

8. Use of n-3 polyunsaturated capsules (including cod liver oil) in the preceding 4 months.

9. Predicted life expectancy less than 2 years.

10. Contraindications to magnetic resonance imaging.

Architectural distortion was excluded due to its influence on regional hepatic chemical composition. A four month wash-out period of polyunsaturated fatty acids has been previously shown to be adequate to return plasma erythrocyte membrane fatty acid concentrations to normal (Cao, Schwichtenberg et al. 2006). Poorly controlled diabetes was excluded as significant changes in diabetic control could influence the assessment outcomes.

6.2.2. Subject recruitment

Patients with biopsy proven NAFLD without any identified exclusion criteria were contacted by post with a covering letter signed by the hepatologist overseeing the clinical management of the patient, a study information sheet, a reply form and envelope. Patients used the reply form to indicate interest, or decline involvement in the study. The reply form was returned to me stating either an interest or not in participating in the study. Those expressing an interest were then contacted by telephone and an initial meeting arranged. A single reminder was sent to those who did not return the initial reply slip.

6.2.3. Overview of method

The initial screening assessment entailed a discussion of the study protocol and any issues or questions identified by the subjects or investigator. The subjects then underwent a semi-structured medical and physical assessment which was performed solely by me. This assessed past medical and surgical history, usage of medication, alcohol and supplements.

Subjects who were appropriate and willing to participate signed three consent forms. As per *Good Clinical Practice* one consent form was kept by the

subject, one inserted into their healthcare records (along with a patient information sheet), and the final consent form was placed in the study files. The flow chart for the study is shown below in figure 1. The assessments performed at baseline and post-intervention were identical. Eighty one were screened in order to recruit the 58 patients. 12 were unsuitable for an MRI scan (8 had internal foreign objects, 4 had a waist greater than 125cm), 4 reported current excessive alcohol intakes, 3 had recent changes in lipid altering medication, 3 were current users of n-3 PUFAs, and 1 withdrew consent due to fears of the MRI scanner.

58 NAFLD patients

Baseline assessment
MR scan, venesection, clinical

Randomise

29 patients
3 months
Daily 3.5g EPA and DHA

29 patients
3 months
Daily sunflower oil capsules

Post-intervention assessment
MR scan, venesection, clinical
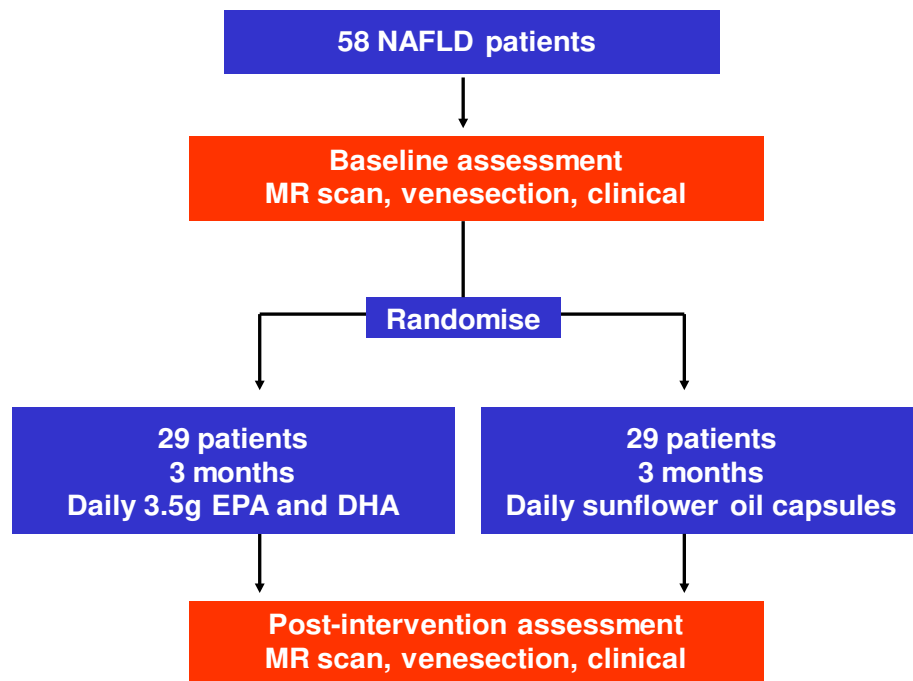
Figure 1. Overview of the n-3 PUFA study in those recruited

6.2.3.1.   Clinical assessments

Resting blood pressure was measured three times by a Dinamap Carescape V100 (General Electric Healthcare, UK) with the subjects in a relaxed quiet environment, and the mean value is presented. Weight and height were measured by the calibrated scales and telescopic column scale of a SECA 704 (SECA Hamburg, Germany). Waist, hip and mid arm muscle circumference were measured by the use of a nonextensible, flexible tape measure, and triceps skinfold thickness by a single calibrated set of callipers (Holtain Ltd., Dyfed, Wales) with a constant pressure of 10g/mm^2. I performed all of these measures myself.

6.2.3.2. Venesection

Whole blood was collected, after an overnight fast, into two BD vacutainer SST tubes with no additives. The SST tubes were stored for 30 minutes to allow for sample clotting. One tube was centrifuged for 10 minutes at 4000 rpm and at 4°C. The resultant serum was separated from the red blood cells, placed in labelled eppendorf vials and frozen at –80°C prior to analysis. The non centrifuged SST tube was submitted fresh to the University hospital's laboratory for analysis of liver biochemistry and lipids.

6.2.3.3. MR assessment

These were performed fasted using a Philips Achieva 3.0 Tesla, whole body system (Philips Medical Systems) in the Sir Peter Mansfield MR Centre, Nottingham.

6.2.3.3.1. Hepatic triglyceride quantification

The collection and analysis protocol was the same as that in chapter 5.2.1.

6.2.3.3.2. Hepatic fatty acid indexes quantification

To determine the indexes of hepatic fatty acid saturation, unsaturation and polyunsaturation a water-suppressed, respiratory triggered, PRESS sequence was used. It involved the same parameters as for hepatic triglyceride quantification, however the number of acquisitions at each echo time was doubled in order to further increase signal to noise ratio.

The resultant spectra were individually phase corrected and realigned using jMRUI (Java-based MR user interface) (Naressi, Couturier et al. 2001) before averaging. Spectra were then processed using QUEST (part of jMRUI software), as described previously by Johnson *et al*. and formulae detailed in section 2.6.1. (Johnson, Walton et al. 2008).

6.2.4. Randomisation

Following successful assessments, the subjects then received their randomly allocated oil capsules and instructions on taking them. Randomisation to active or placebo capsules was performed by a simple non-stratified internet based programme run by the University's clinical trials unit. The outcome of the random allocation for each subject was communicated to the pharmacy

department from the clinical trials unit. The capsules were labelled in an identical fashion for all the subjects, and contained information as to what the two potential options for the contents were. As a result, double blinding was attempted to be maintained throughout the study. The presence or absence of a fish oil taste was unavoidable, although it was only described by a few of the subjects. This potential loss of blinding in some volunteers did not influence the outcome analysis as all the outcomes were purely objective.

6.2.5. Profile of the capsules

The active capsules were a fish oil concentrate in a 1000mg Efamax capsule (Efamol ltd, Leatherhead, UK), and the placebo capsules were 1000mg oleic-enriched sunflower (Efamol ltd, Leatherhead, UK). Both capsules were of an identical size, shape and colour and bound in gelatine. Vitamin E is added to fish oils for stabilisation, and so an identical dose was added to the placebo. Such regular intakes of vitamin E supplements have been shown to be safe and with no known clinical outcomes at such a dose (Pocobelli, Peters et al. 2009). Certificates of analysis were provided by the manufacturing company, and the fatty acid profiles are demonstrated in table 1. There was 24 IU of vitamin E per capsule and hence 120 IU were consumed in both arms per day.

	Active	Placebo
Saturated	35	77
Monounsaturated	149	820
Polyunsaturated	797	98
n-3 polyunsaturated	746	1
n-6 polyunsaturated	48	97
n-9 polyunsaturated	3	0
Short chain n-3 PUFA	9	1
Short chain n-6 PUFA	7	97
Long chain n-3 PUFA	737	0
Long chain n-6 PUFA	41	0
Oleic acid	44	816
Vitamin E	16	16

Table 1. Content of the active and placebo capsules in mg per capsule.

6.2.6. Post intervention assessment

After three months the clinical, MRI and venesection process was repeated. In addition, capsule-related side effects were screened for in a subject-completed questionnaire, and any reported side effects were discussed between

the lead investigator and the subject. Subjects were reimbursed their travel expenses for each assessment visit, but received no other financial remuneration.

6.2.7. Serum analyses

Serum insulin and non-esterified fatty acids were quantified by the methods previously described in sections 4.2.2. and 3.6.8. respectively.

6.2.7.1. Serum fatty acid profile analysis by gas chromatography

A step-wise process measured the relative contributions of the differing fatty acids to the serum PC fatty acid content. The first step extracted the fatty acids from the serum then the extract was fractionated to collect phosphatidylchoine. The fatty acids were then converted to volatile methyl esters, which were identified using gas chromatography. This allowed determination of the proportionate contribution of each fatty acid to the total content. This is further detailed below:

1. Lipid extraction from the serum

The serum sample vials were thawed at room temperature and the lipids extracted using chloroform and methanol. This was done by pippeting 0.4ml of serum from the labelled vial into a correspondingly labelled tube, and first adding 0.4 ml of 0.9% NaCl. Next 0.1 ml of internal standard (1mg/ml in chloroform:methanol 2:1) was added to each sample, followed by 5ml chloroform: methanol (2:1+BHT 50mg/l), and finally 1ml of 1M NaCl was added. The tubes were then capped and vortexed for 15 seconds. The samples were then centrifuged (10 min, 2000 rpm, room temperature). The lower layer of the sample was aspirated and transferred into a new tube, where it was evaporated to dryness under nitrogen at 40°C.

2. Fractionation of serum total lipid extract using solid phase extraction (SPE)

The extract was dissolved in 1 ml chloroform and separated using solid phase extraction with an aminopropyl-silica column. The sample was loaded onto the column and triacylglycerol and cholesterol ester were eluted with 2ml chloroform and discarded. The phosphatidylcholine fraction was then eluted with 2 ml chloroform:methanol 60:40 And the elutant evaporated under nitrogen.

3. Transmethylation to produce fatty acid methyl esters (FAME)

0.5ml of toluene and 1 ml of methanol containing 2% sulphuric acid were added to the dried samples. The samples were capped, and mixed gently for 10

seconds. Following this they were incubated on a heat block at 50^0C for two hours. Tubes were then allowed to cool and then 1ml of neutralising agent (0.5M K_2CO_3, 0.25M $KHCO_3$) and 1ml of hexane was added. Samples were then vortex mixed for 5 seconds and centrifuged (2 minutes, 1000 rpm, room temperature). The upper layer was aspirated and transferred to a new labelled glass tube. The samples were then evaporated to dryness under nitrogen at 40^oC.

The dried sample extract was re-suspended in 75µl of hexane and vortex mixed for 5 seconds. The hexane and sample mix was transferred into a labelled Gas Chromatography vial, and a further 75µl was added to the tubes. This was vortexed for 5 seconds and transferred to a vial and stored at -80^oC.

4. <u>Gas Chromatography of FAME</u>

Fatty acid methyl esters (FAME) were analyzed using a Hewlett Packard 6890 series Gas Chromatography system (Hewlett Packard, Basingstoke, UK). The Gas Chromatography system was fitted with a BPX-70 30m x 0.22mm column, which had a coating of 0.25um (SGE Analytical Science, Milton Keynes, UK). The FAME were diluted in 150 µL of hexane, 1 µL of which was injected onto the column using an Agilent 7683 series autosampler (Agilent Technologies UK Ltd., Wokingham, UK). The injector inlet temperature was 300˚C and helium was used as the carrier gas with a pre-column split ratio of 50:1 and a head pressure of 21.89 psi. The methyl esters were separated by the following temperature program: the oven was initially heated to 115˚C and held for 2 minutes and the temperature then increased to 200˚C at 10˚C per minute. The FAMEs were identified by the comparison of retention time against known standards (in house standard mix and PUFA-3 Menhaden Oil standards, Supelco, Dorset, UK). 1,2,-dipentadecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, inc, USA) was used as the internal standard.

A flame ionisation detector within the gas chromatograph detected FAMEs. The individual FAMEs differ in their chemical and physical properties, and hence they were separated within the column. The rate at which they passed through the column's detector and their amount was noted and transferred to chromatograms. The chromatograms were plotted and analysed using Hewlett Packard Chemstation software package (Hewlett Packard, 1999). The fractional contribution of each fatty acid in relation to the total fatty acid content was calculated and converted into percentages of the total amount of fatty acid within the sample.

6.2.8. Ethical and research and development approval

Nottingham Research and Ethics Committee one approved the study on the 11th of March 2008, and Nottingham University Hospital's Research and Development department gave approval on the 27th of June 2008. No substantial amendments were required to the original protocol.

6.2.9. Power calculation

There were no prior human n-3 PUFA studies using MR spectroscopy as their primary outcome measure which could aid sample size determination. Both thiazolidenediones and n-3 PUFAs share PPARγ activity, and use of a thiazolidenedione resulted in a 39% reduction of hepatic triglyceride as quantified on ^{1}H MRS after three months (Mayerson, Hundal et al. 2002). Prior human n-3 PUFA studies had shown significant ultrasound reductions by 35% (Hatzitolios, Savopoulos et al. 2004) and 64% (Capanni, Calella et al. 2006). As a result a mean reduction of 30% in the intervention group was predicted with no mean change in the control group. 57 participants were calculated to be the minimum required using a two-sided p-value of 0.05 and power of 90%. A baseline steatosis of 11.5% and standard deviation of 4.0 was assumed, as shown in prior MR studies of Caucasians (Browning, Szczepaniak et al. 2004; Thomas, Hamilton et al. 2005).

6.2.10. Statistical analyses

All data are presented as the mean ± standard deviation (SD). Comparisons between baseline values were done by the independent samples T test. The Student's T test was used for paired data. Analysis of the significance of change between the two groups at two paired assessments was done by an independent samples T test of the absolute change between the two assessments in both groups. Associations were tested for by 2-tailed Pearson correlations. Discrete data were analysed by Chi squared analyses.

6.3. Results

6.3.1. Baseline clinical data

43 men and 15 women were recruited. During the 3 month follow up there was a single drop out, which was in the n-3 PUFA group. This was due to a change in personal circumstances as opposed to the intervention or assessment

process. Post randomisation the biopsies were reviewed by an independent liver pathologist. Two biopsies were felt to have inadequate steatosis in order to be truly labelled as having NAFLD, all the others were felt to be diagnostic for NAFLD. As a result the final analysis is of the 55 patients whose baseline demographic and clinical features are presented in table 2. Of the 55 patients, 42 were male, 20 treated for hypertension, 16 on long-term dyslipidaemia therapy, 6 diabetic, 1 diet treated and 5 on metformin.

	n-3 PUFA, n=28	Placebo, n=27	p
Age (years)	49 ± 9	52 ± 12	0.20
Male	21/28	21/27	0.81
Weight (kg)	91.2 ± 16.4	87.0 ± 15.5	0.34
BMI	30.3 ± 4.6	29.1 ± 3.8	0.29
Waist (cm)	106.0 ± 11.7	103.0 ± 10.6	0.32
Waist : hip ratio	1.03 ± 0.06	1.02 ± 0.07	0.62
Triceps skinfold thickness (cm)	2.73 ± 1.16	2.64 ± 0.92	0.75
Mid arm muscle circumference (cm)	34.5 ± 3.4	33.4 ± 3.8	0.24
Systolic BP (mmHg)	142 ± 13	142 ± 13	0.80
Diastolic BP (mmHg)	84 ± 10	80 ± 7	0.10
On hypertensive therapy	11/28	9/27	0.65
Diabetic	2/28	4/27	0.36
Alcohol intake (units per week):	7 ± 7	9 ± 8	0.28

Table 2. Baseline clinical, demographic and anthropometric data on the two groups. (mean±SD).

The cohort was typical for NAFLD being predominantly male, middle aged, and centrally overweight. The two groups were evenly balanced in terms of clinical and demographic features at baseline.

Compliance was assessed historically and by counting returned capsules. The reported compliance rates were 97% in both groups, p=0.91.

6.3.2. Side effects

Mild gastrointestinal symptoms attributed to the capsules were more common in the treatment group (6/28), as opposed to the placebo group (1/27)

(p=0.049). None reported symptoms that they classified as severe, and all reported willingness to take such capsules again in the future.

6.3.3. Serum fatty acid profile outcomes

The two groups were essentially matched for their serum fatty acid profiles at baseline, see table 3. There was a slightly greater EPA and n-3 PUFA content in the treatment group at baseline.

During the intervention there were no changes in the fatty acid profile in the placebo arm except for oleic acid. Whereas the treatment group had a significant reduction in the relative contributions of n-6 PUFAs, including arachidonic acid, and an increase in n-3 PUFAs including EPA and DHA.

	n-3 PUFA			Placebo			Difference between baseline values (p)	Absolute change between groups (p)
	Baseline	3 month	p	Baseline	3 month	p		
Saturated	44.5±1.1	44.4±1.1	0.54	44.2±1.0	44.2±1.1	0.81	0.33	0.77
Monounsaturated	13.5±2.1	13.6±2.1	0.66	13.5±2.4	13.1±1.4	0.40	0.94	0.34
Oleic acid	9.8±1.4	9.3±1.3	0.004	10.1±1.8	10.6±1.5	0.02	0.97	<0.001
Polyunsaturated	42.0±2.2	41.9±2.3	0.89	42.2±2.7	42.7±1.9	0.38	0.72	0.44
n-6 PUFA	35.1±5.9	30.6±2.8	<0.001	35.8±5.7	36.2±2.4	0.83	0.35	0.003
ARA	9.1±1.8	8.0±1.7	<0.001	9.1±2.1	9.0±2.3	0.28	0.96	<0.001
n-3 PUFA	6.9±2.1	11.3±2.4	<0.001	6.4±1.2	6.5±1.3	0.98	0.08	<0.001
n-6:n-3	5.3±1.5	2.9±1.2	<0.001	5.8±1.1	5.8±1.2	0.98	0.15	<0.001
EPA	1.6±0.9	4.2±2.0	<0.001	1.2±0.5	1.2±0.5	0.80	0.04	<0.001
DHA	3.4±1.1	4.5±0.9	<0.001	3.1±0.8	3.2±0.9	0.96	0.38	<0.001

Table 3. Percentage of serum fatty acid profiles from individual fatty acids and their classes at baseline and at three months in the active and placebo groups. (ARA = arachidonic acid). (mean±SD).

Figure 2. Percentage contribution of oleic acid, arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to total serum fatty acid content pre and post intervention in the n-3 PUFA group (A) and the placebo (B). (As compared to baseline value *=p<0.05, **=p<0.0, ***=p<0.001). (mean ±SEM).

6.3.4. Hepatic and metabolic outcomes

The groups were well matched at baseline. The biochemical findings are typical for NAFLD with an elevated ALT (normal range being 0-45 U/L in men

and 0-35 U/L in women), and a high ALT to AST ratio. There is evidence of mild systemic insulin resistance and dyslipidaemia with an elevated fasted triglyceride and total cholesterol.

At 3 months there were no significant changes between the groups. There was a trend for a greater reduction in HTGC and alkaline phosphatase with n-3 PUFA, and an increase in insulin concentrations.

	n-3 PUFA			Placebo			Difference between baseline values (p)	Absolute change between groups (p)
	Baseline	3 month	p	Baseline	3 month	p		
HTGC (%)	9.3±5.2	8.7±5.5	0.25	10.8±6.0	11.2±6.1	0.41	0.33	0.16
ALT (U/L)	54±24	49±19	0.21	55±25	51±36	0.45	0.86	0.89
AST (U/L)	37±10	36±10	0.72	40±14	40±19	0.99	0.31	0.85
GGT (U/L)	105±103	95±89	0.33	96±109	86±87	0.25	0.74	0.95
Alk P (U/L)	105±42	95±32	<0.01**	91±27	89±24	0.58	0.16	0.10
Cholesterol (mmol/L)	5.6±0.9	5.5±1.1	0.51	5.5±1.4	5.4±1.3	0.43	0.61	0.96
Triglyceride (mmol/L)	2.0±1.0	1.7±0.7	0.02*	2.2±1.7	2.1±1.2	0.77	0.69	0.25
Glucose (mmol/L)	5.5±0.5	5.4±0.7	0.59	5.9±1.6	5.8±1.5	0.48	0.18	0.89
Insulin (mIU/L)	10.3±6.0	12.4±7.9	0.04*	10.2±9.4	10.1±7.1	0.89	0.98	0.10
HOMA-IR	2.5±1.5	2.9±1.7	0.053	2.9±3.3	2.7±2.3	0.58	0.60	0.12
NEFA (mmol/L)	344±132	387±238	0.18	404±148	449±153	0.15	0.12	0.96
Adipose-IR	21±19	29±26	<0.01**	28±39	28±26	0.98	0.42	0.12

Table 4. Hepatic and metabolic measures at baseline and three months in the n-3 PUFA and placebo groups. (HTGC = hepatic triglyceride concentration, ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma glutamyl transferase, Alk Phos = alkaline phosphatase, NEFA = non-esterified fatty acids, Adipose-IR = Adipose tissue insulin resistance (NEFA (mmol/L) x fasted plasma insulin (pmol/L)). (mean±SD).
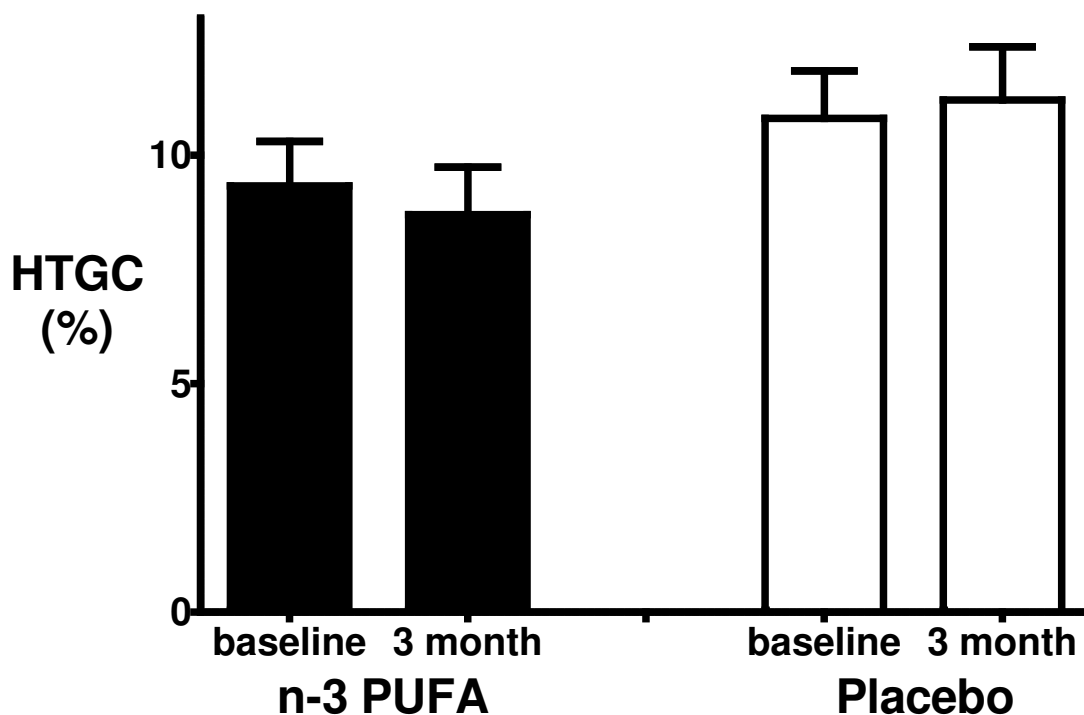
Figure 3. HTGC in all pre and post n-3 PUFA or placebo, n=55 (mean±SEM).

The baseline HTGC findings are surprisingly low given that this was a study in patients with known NAFLD. The reliability of this data however is reinforced by the association between the HTGC values and measures of obesity, insulin resistance and liver enzymes, see table 5.

	r	Significance
Weight	0.43	0.001
BMI	0.46	<0.001
Waist	0.42	0.001
ALT	0.26	0.06
HOMA-IR	0.34	0.01

Table 5. Correlations between HTGC and markers of obesity, liver enzymes and insulin resistance in the whole cohort, n=55.

6.3.5. Hepatic fatty acid profiles

Due to technical issues with the MRI fatty acid profile analyses, data are only available for 49 patients, 27 in n-3 PUFA group and 22 in the placebo group.

These values were matched at baseline. In accordance with the serum data in table 3, there were no changes in the profile of fatty acid classes during the intervention

	n-3 PUFA			Placebo			Difference between groups at baseline (p)	Absolute change between groups (p)
	Baseline	3 month	p	Baseline	3 month	p		
Saturated	95.5±1.7	95.7±1.2	0.63	95.8±1.3	95.3±2.6	0.41	0.53	0.32
Monounsaturated	4.5±1.7	4.3±1.2	0.63	4.2±1.2	4.7±2.6	0.41	0.53	0.32
Polyunsaturated	1.3±0.5	1.4±0.6	0.64	1.5±0.8	1.9±2.7	0.61	0.47	0.66

Table 6. Hepatic fatty acid percentage profiles as quantified by ^{1}H MRS (n=27 for n-3 PUFA, and n=22 for placebo). (mean±SD).

6.3.6. Effect of baseline steatosis on outcomes

The accepted ^{1}H MRS cut-off value for a normal HTGC is less than 5.5% (Szczepaniak, Nurenberg et al. 2005). It was not predicted that recruited patients would have a normal HTGC value. Post-hoc analyses comparing the 12 patients with a normal baseline HTGC and the 43 patients with an elevated HTGC found no significant differences in terms of age, anthropometry, or insulin sensitivity, liver and lipid biochemistry, see table 7. There was a tendency for those with an abnormal HTGC to have a greater body mass index.

	Normal HTGC, n=12	Abnormal HTGC, n=43	P
Age (years)	52±11	50±10	0.56
Sex (n male)	8	34	0.37 [x]
Alcohol (weekly units)	8±8	8±8	0.93
Diabetic	1	5	0.74 [x]
Weight (kg)	81.3±18.2	91.3±14.8	0.054
BMI (kg/m^2)	27.7±3.7	30.3±4.2	0.058
Waist (cm)	100.5±11.4	105.7±11.0	0.16
Waist to hip ratio	1.01±0.07	1.04±0.06	0.16
ALT (U/L)	51.1±23.8	55.5±24.3	0.58
AST (U/L)	40.1±13.0	38.4±12.3	0.68
GGT (U/L)	142.7±113.5	88.6±100.6	0.12
Alk P (U/L)	118.8±56.7	92.0±25.1	0.14
Cholesterol (mmol/L)	6.0±1.1	5.4±1.2	0.14
Triglycerides (mmol/L)	2.2±1.4	2.1±1.4	0.84
HOMA-IR	1.9±1.2	2.9±2.8	0.22
NEFA (mmol/L)	390±170	368±135	0.65

Table 7. Comparison of those patients with and without an abnormal HTGC at baseline. ([x]= chi-squared.) (mean±SD).

6.3.6.1. Findings in those without steatosis at baseline

Of the 12 patients with a normal baseline HTGC, 7 received n-3 PUFAs and 5 placebo. At baseline these two sub-groups did not differ in terms of their clinical, anthropometric or biochemical data, as shown in table 8. There were no significant changes within or between the two groups, apart from serum triglycerides which increased with placebo.

	n-3 PUFA, n=7			Placebo, n=5			Difference between groups at baseline (p)	Absolute change between groups (p)
	Baseline	3 months	p	Baseline	3 months	p		
HTGC	3.34±1.46	4.95±2.94	0.14	3.00±1.22	3.97±1.36	0.25	0.69	0.62
Weight	83.7±20.1	85.0±20.5	0.15	77.9±16.6	78.8±16.3	0.48	0.61	0.71
ALT	54.1±26.4	43.4±18.3	0.28	46.8±21.9	57.6±69.7	0.69	0.62	0.39
AST	42.3±12.2	36.6±6.5	0.22	37.0±15.0	44.2±32.2	0.65	0.52	0.36
GGT	185.6±125.1	122.7±64.3	0.11	82.6±65.2	94.2±95.7	0.58	0.13	0.11
Alk P	134.3±69.4	108.0±51.2	0.06	97.2±25.0	102.0±19.9	0.69	0.29	0.09
Cholesterol	5.7±0.8	5.7±1.4	0.90	6.4±1.5	6.1±0.94	0.47	0.26	0.46
Triglycerides	2.6±1.6	1.8±0.9	0.10	1.5±0.9	2.1±1.2	0.03	0.21	0.02*
Glucose	5.4±0.5	5.3±0.4	0.57	5.4±1.3	5.1±0.8	0.31	0.99	0.69
Insulin	7.1±4.9	8.9±3.3	0.27	8.7±5.2	9.5±2.4	0.77	0.61	0.72
HOMA-IR	1.7±1.2	2.1±0.8	0.33	2.1±1.3	2.1±0.3	0.98	0.60	0.55
NEFA	314±126	323±229	0.88	496±179	458±106	0.67	0.06	0.57

Table 8. Hepatic and metabolic measures at baseline and three months in the n-3 PUFA and placebo groups of those with normal HTGC at baseline. (mean±SD).
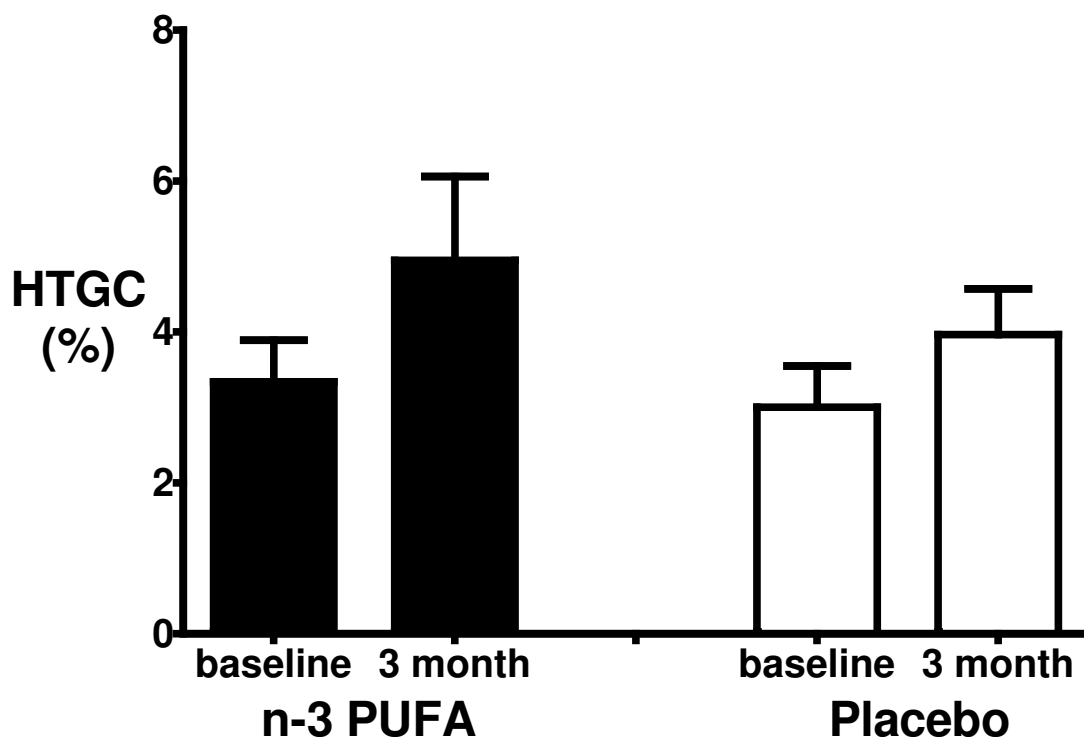
Figure 4. HTGC in those with a normal baseline HTGC assessment pre and post n-3 PUFA or placebo, n=12. (mean±SEM)

6.3.6.2. Findings in those with steatosis at baseline

Of the 43 with an abnormal baseline HTGC, there were no clinical or anthropometric baseline differences between those receiving n-3 PUFA and placebo.

	n-3 PUFA, n=21	Placebo, n=22	P
Age	47±9	53 ±11	0.07
Sex (n male)	16/21	18/22	0.72[x]
Alcohol (weekly units)	8.9±1.6	6.6±1.7	0.33
Diabetic (n)	1/21	3/22	0.32[x]
Weight (kg)	93.7±14.7	89.1±14.8	0.32
BMI (kg/m^2)	31.1±4.7	29.6±3.6	0.25
Waist (cm)	107.5±11.7	103.9±10.3	0.28
Waist to hip ratio	1.04±0.06	1.03±0.07	0.72

Table 9. Characteristics of the two groups of patients with steatosis at baseline. ([x]= chi-squared test). (mean±SD).

At baseline the two groups were also well matched in terms of liver fat content, liver biochemistry and lipids, see table 10. However, these two subgroups did respond differently to the intervention. There was a 12.2% relative reduction of HTGC in the n-3 PUFA group from the original value, and an increase of 2.2 % in the placebo group, p=0.046. Conversely there were opposing outcomes for GGT. Within the groups there were improvements in ALT with placebo and alkaline phosphatase with n-3 PUFA, though no significant decrease in serum triglycerides.

| | n-3 PUFA, n=21 | | | Placebo, n=22 | | | Difference between groups at baseline (p) | Absolute change between groups (p |
	Baseline	3 months	p	Baseline	3 months	p		
HTGC	11.3±4.3	9.9±5.6	0.02*	12.6±5.1	12.9±5.4	0.63	0.39	0.046*
Weight	93.7±14.7	93.9±14.4	0.60	89.1±14.8	89.1±14.5	0.91	0.32	0.62
ALT	53.9±23.5	51.1±19.5	0.49	57.1±25.6	49.8±26.4	0.02*	0.68	0.37
AST	35.3±9.4	36.4±11.5	0.58	41.3±14.1	39.6±15.0	0.29	0.11	0.26
GGT	78.2±80.8	85.7±95.2	0.09	98.5±117.5	84.6±87.7	0.11	0.52	0.03*
Alk P	94.6±23.1	90.3±22.1	0.03*	89.5±27.2	86.2±23.8	0.30	0.51	0.78
Cholesterol	5.6±1.0	5.5±1.1	0.40	5.2±1.4	5.2±1.3	0.78	0.31	0.53
Triglycerides	1.8±0.6	1.6±0.7	0.10	2.3±1.9	2.1±1.2	0.40	0.25	0.97
Glucose	5.5±0.5	5.4±0.8	0.78	6.0±1.7	5.9±1.6	0.75	0.17	0.98
Insulin	11.3±6.0	13.5±8.6	0.08	10.6±10.2	10.3±7.9	0.75	0.77	0.11
HOMA-IR	2.8±1.5	3.2±1.8	0.11	3.0±3.7	2.8±2.5	0.56	0.75	0.16
NEFA	354±136	409±242	0.16	383±136	447±164	0.07	0.50	0.85

Table 10. Hepatic and metabolic measures at baseline and three months in the n-3 PUFA and placebo groups of those with abnormal HTGC at baseline. (mean±SD).
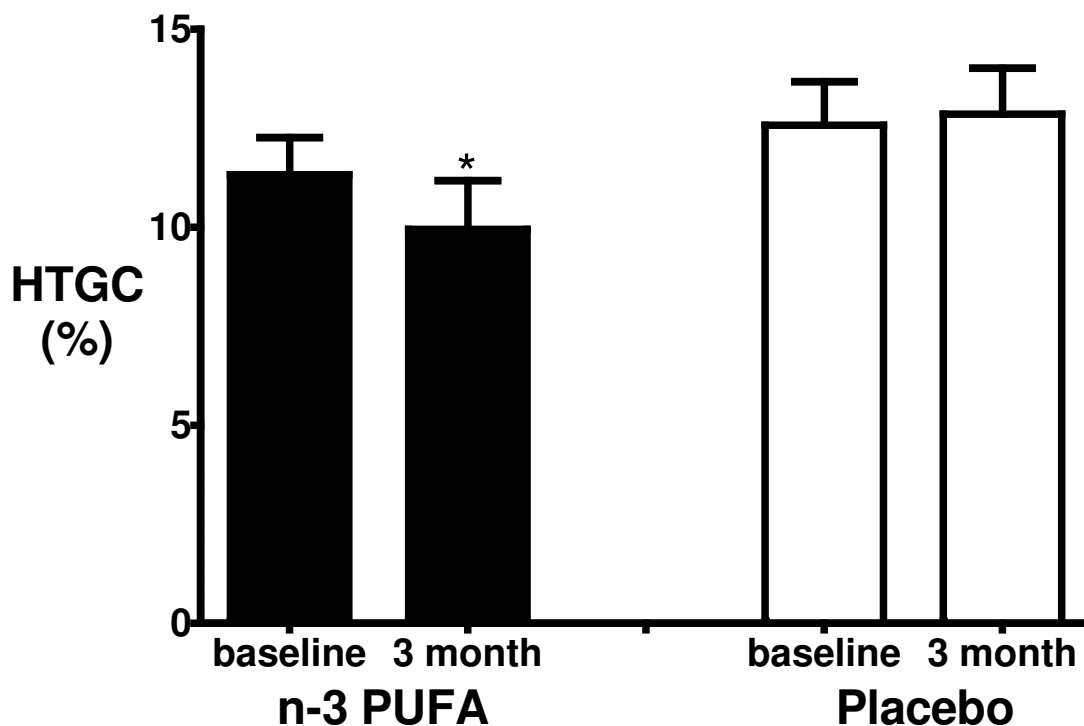
Figure 5. HTGC in those with an elevated baseline HTGC assessment pre and post n-3 PUFA or placebo, n=43. (mean±SEM)

6.4. Discussion

6.4.1. Summary of findings

The intervention resulted in significant alterations of serum fatty acid profiles between the groups, which were in line with the capsules' contents. These changes however failed to translate, in the whole group, to any detected metabolic or hepatic changes beyond a reduction in serum triglyceride. Amongst those with steatosis on biopsy and at ^{1}H MRS there was an isolated reduction in HTGC with n-3 PUFA and a reduction in GGT with placebo.

At study entry the two groups were well matched for all parameters bar a slightly greater serum EPA concentration in the treatment group. The cohort was typical for NAFLD in terms of central obesity, male predominance, abnormal liver enzymes, dyslipidaemia and insulin resistance. Not all however had steatosis according to their initial ^{1}H MRS assessment.

Both groups gained weight, though this tended to be greater in the treatment group. There was a greater incidence of mild gastrointestinal symptoms in the n-3 PUFA than placebo arm. The placebo resulted in an isolated increase in oleic acid in the serum fatty acid profile, whereas within the treatment group there was an increase in the serum n-3 PUFA proportion and a reduction in n-6 PUFA.

Within the groups of the whole cohort there were no changes in hepatic lipid or biochemical assessments with placebo. In contrast n-3 PUFA treatment was associated with a non-significant reduction in HTGC and ALT, and with a significant reduction in alkaline phosphatase and serum triglycerides. Serum insulin concentrations however increased with n-3 PUFAs and there was greater adipose tissue insulin resistance and a tendency towards greater whole body insulin resistance. Strong associations were made between these outcomes and changes in weight. As a result the greater weight gain in the treatment arm may have influenced many of the outcomes.

Those with a normal HTGC at study entry tended to have a lower weight and BMI. Amongst these there were no significant changes either within or between the groups. In those with an abnormal baseline HTGC, post hoc analyses demonstrated a significant reduction in HTGC with n-3 PUFA capsules. This was not associated however with any other significant improvement in liver enzymes or metabolic parameters over placebo.

6.4.2. Issues with the recruitment process

In routine clinical practice many patients are diagnosed as having NAFLD without a liver biopsy. This clinical diagnosis is made on the basis of abnormal liver biochemistry in the absence of an alternative explanation following clinical assessment and a 'parenchymal screen'. Such patients can however have alternative diagnoses (Skelly, James et al. 2001), and so the requirement for all patients to have had a prior liver biopsy strengthens this study. Unfortunately the histological diagnosis of NAFLD is not without its controversies. Alcoholic liver disease can be difficult to distinguish clinically or pathologically from NAFLD (Yeh and Brunt 2007). The inter-observer agreement of NAFLD histology is limited with a kappa value of between 0.23 and 0.65 depending on the parameter assessed (Gawrieh, Knoedler et al. 2011).

The patients in this study were recruited after biopsy assessments from several pathologists, some who were not specialists in hepatic pathology. The failure to have a central histological assessor of the biopsies before recruitment was a missed opportunity. It does however have prior precedent, with a recent major NAFLD histology study finding that on central review many patients already recruited lacked the histological criteria to enter the study (Sanyal,

Chalasani et al. 2010). Due to one patient dropping out and two not meeting the diagnostic criteria for NAFLD the cohort entailed 55 patients as opposed to 58.

6.4.3. Compliance

Compliance was assessed by counting returned capsules and further validated by serum fatty acid analyses. Historically very high rates of capsule consumption were reported and few capsules were returned. Objectively there was an increase in the combined serum EPA and DHA content in 25 of 28 in the active group and of oleic acid in 20 of 27 in the placebo group. This suggests high compliance rates in both the active and placebo arms, especially as the placebo will have only moderately increased total daily intakes as will now be discussed.

The only study to quantify habitual EPA and DHA intakes in NAFLD patients estimated 434mg per day (Allard, Aghdassi et al. 2008). The five n-3 PUFA capsules would have increased this by 3,455mg per day, a nearly 8-fold increase, such that an increase in serum concentrations would be universally expected. Daily intakes of oleic acid in NAFLD have not been specifically published. It is however known that NAFLD patients consume 58g per day of monounsaturated fatty acids (Musso, Gambino et al. 2003), and that oleic acid contributes to around 90% of monounsaturated fatty acid intakes in healthy adults (Hunter, Rimm et al. 1992). Hence the capsules would have only resulted in an increase of oleic acid intakes by 8%, so an increase in 20 out of 27 patients seems acceptable.

6.4.4. Disagreement between histological and ^{1}H MRS findings

Not all patients had an elevated hepatic triglyceride count as assessed by ^{1}H MRS at study entry. The reasons for this remain unclear. There was a time lag between biopsy and study recruitment, though the maximal time difference was 3 years and none reported to be actively losing weight at the time of recruitment. The time interval may however have allowed for NAFLD disease progression, regression, or indeed a new hepatic disease to develop. This is a limitation, but the primary endpoint was defined as the ^{1}H MRS findings not factors related to the original histology, and the histology was not a component of the subsequent analyses.

Although the two assessment techniques of liver biopsy and ^{1}H MRS do complement each other they also differ in what they assess. As discussed in

chapter 2, histopathologists quantify liver biopsy fat content as the percentage of hepatocytes with visible fat droplets, whereas ^{1}H MRS determines the fractional volume of fat within the liver (Schwenzer, Springer et al. 2009). Consequently the values determined by ^{1}H MRS are consistently lower than histopathological values (Cotler, Guzman et al. 2007; McPherson, Jonsson et al. 2009). Confusion can however arise as both techniques generate scores with a percentage value. The absolute difference between both techniques is a factor of approximately 1.8 (Longo, Pollesello et al. 1995). So it is possible that some may not fulfil the ^{1}H MRS criteria for steatosis whilst fulfilling the pathological criteria.

6.4.5. Duration and nature of intervention and placebo

In order for the study to be truly blinded there needed to be an oil within the placebo capsule. No fatty acids are fully biologically inert, though oleic acid is a substantial part of the standard diet and has been shown to be relatively metabolically benign. A similar preparation of oleic enriched sunflower oil was previously used as a placebo at 3.9g/day (4.08g/day in this study) for 12 weeks (Lambert, Goedecke et al. 2007). It resulted in no change in weight, body composition, insulin resistance or lipid profiles. As such it appeared to be a valid placebo. There were no changes in the placebo group serum fatty acid profiles with the exception of oleic acid itself.

Normal values for EPA or DHA serum fatty acid concentrations are not published. As a result it is difficult to interpret the cohort's baseline values. There was however a significant increase in serum EPA and DHA content in the treatment group. Indeed the proportion of patients who had at least a 50% rise in their serum EPA and DHA concentrations at 3 months was 19/28 vs. 2/27 in the placebo group, and 12/28 vs. 0/27 had more than a 100% rise. This was associated with a reduction in serum n-6 PUFA content. So it is clear that there was a marked change in serum polyunsaturated fatty acid status with the treatment capsules.

Ultimately it was the hepatic, as opposed to serum, fatty acid profile, and indeed content, that the intervention aimed to alter. As discussed in chapter 1 there are scant data on the timescale for hepatic incorporation of oral n-3 PUFAs. The only evidence available described a 3-fold increase in hepatic EPA content following 5 days of 3.7g of EPA and DHA (Senkal, Haaker et al. 2005). Due to the delivery of similar amounts of EPA and DHA (3.5g/day) for 3 months and the marked increase in serum EPA and DHA content, it seems reasonable to assume

that hepatic EPA and DHA content increased with the intervention. The ^{1}H MRS fatty acid profile analyses were designed to detect changes in total PUFA status. No changes were detected, though total serum PUFA status did not change either. As opposed to serum analyses, the ^{1}H MRS technique was not able to quantify hepatic n-3 or n-6 PUFA sub-fractional status.

The addition of vitamin E to capsules is necessary to stabilise fish oils. Identical amounts were present in both capsules. The daily dose was 15% of that used in the PIVENS study and for an eighth of the duration (Sanyal, Chalasani et al. 2010). The vitamin E arm in the PIVENS study had improved NASH features in 34% more patients than placebo. In this study it seems unlikely that the small amounts of vitamin E supplied impacted significantly on the outcomes, especially as there have been previous negative studies using the same amount as in the PIVENS study for 3 and 12 months (Kugelmas, Hill et al. 2003; Bugianesi, Gentilcore et al. 2005).

6.4.6. Potential impact of weight change on study outcomes

The reasons for the differing weight gain tendency between the capsules are unclear. Dietary and activity records were not taken. The capsule energy content of both groups was identical at 45kcal per day. Over the 12 weeks the patients received 3,780kcal from the capsules. It is estimated that dietary intake needs to increase by around 7,000kcal to gain a kilogram of fat (Katan and Ludwig 2010). As a result the weight change in the n-3 PUFA group of 0.5kg is in line with that predicted. What is interesting is that the placebo group gained less weight (0.1kg). This was not significantly different but it may itself have influenced the metabolic outcomes. One possibility for the difference between the groups is that those taking the n-3 PUFA consumed other foodstuffs around the time of taking the capsules so as to limit any gastrointestinal side effects or tastes. Such data were not collected and so this is merely speculative.

	Group	r	P
HTGC	All	0.44	0.001
	n-3 PUFA	0.59	0.001
	Placebo	0.29	0.14
ALT	All	0.27	<0.05
	n-3 PUFA	0.36	0.06
	Placebo	0.21	0.30
Triglycerides	All	0.25	0.064
	n-3 PUFA	0.20	0.31
	Placebo	0.36	0.06
HOMA-IR	All	0.21	0.13
	n-3 PUFA	0.36	0.06
	Placebo	0.07	0.73

Table 11. Associations between weight change and changes in hepatic and serum triglycerides, liver enzymes and insulin resistance.

Weight change was strongly associated with changes in HTGC and liver enzymes and tended to be associated with changes in insulin resistance and serum triglycerides, see table 11. It is therefore likely that it was a factor that negatively influenced outcomes in the intervention group. Adjusting for it would however have been a mistake as this was a clinical trial attempting to generate clinical data. If the potential beneficial effects of a therapy are negated by a side effect, then the side effect should not be ignored. That said weight gain is not a frequently reported finding in prior n-3 PUFA supplementation studies. The experience in NAFLD is limited and clearly more data is needed in this area.

6.4.7. Hepatic outcomes

The primary outcome measure for the study was HTGC. There was a trend for a reduction in the whole group with n-3 PUFA and this was significant in those with an elevated HTGC at study baseline. As this was a post-hoc subgroup analysis the study cannot be viewed as being positive. Though a greater reduction amongst those with steatosis than those without has been previously reported (Cussons, Watts et al. 2009). However the magnitude of change in HTGC was not dramatic in this study and there were scant associated metabolic changes. It is however strongly suggestive that a larger study with patients with elevated baseline HTGC may be positive.

It is noteworthy that amongst the whole cohort there was a trend, in both groups, for a general improvement in liver biochemistry and lipids. This was

never significant in the placebo group. The aetiology of this general improvement is unclear. Potential explanations include a beneficial effect of the intervention and placebo capsules, regression to the mean phenomenon, or a change in lifestyle behaviour as a result of being intensively observed in a clinical study – the Hawthorne effect (Brody and Miller 2011). Of the individual enzymes, ALT and AST are traditionally viewed as being a more significant marker of liver injury in NAFLD, and there was no change in these. Alkaline phosphatase was significantly reduced in the treatment group whereas there was little change in the placebo group. The role for alkaline phosphatase as a marker of NAFLD has been explored and suggested as a marker for NASH by a single paper, though there seems to be little other corroborative data for this story (Pantsari and Harrison 2006). Within the subgroups of those with and without an abnormal HTGC at baseline there were no additional trends for overall change in liver enzymes with the intervention or placebo.

6.4.8. Metabolic outcomes

Fasted serum triglycerides were significantly reduced in the active group as has been widely shown by prior clinical experience in the non NAFLD setting (Hartweg, Farmer et al. 2007). This was a weight independent effect.

Systemic and adipose IR tended to worsen in the n-3 PUFA group. This was due to an increase in fasted insulin with no real change in glucose or NEFA concentrations. Such a rise was an unexpected finding. Potentially n-3 PUFAs have insulin sensitising effects via PPAR gamma agonism, though this has never been shown to have a clinical effect. No mechanistic data were collected that could fully explain the aetiology of the development of insulin resistance. Weight gain was greater in the treatment than placebo group and this tended to correlate with a change in the HOMA-IR value, r=0.36 (p=0.06). As such the greater weight gain in the treatment group may in part explain this unpredicted outcome.

6.5. Conclusions

This study failed to demonstrate a difference in hepatic lipid content following n-3 PUFA treatment in patients with NAFLD. This overall finding has to be tempered however by the expectation that all patients would have a raised HTGC at study entry. Amongst those with an elevated baseline HTGC there was a reduction in HTGC, though the magnitude of reduction was small and it was an isolated finding.

The strengths of this study are:

1. Its double blind design, meticulous entry criteria, robust assessment process, and the high dose of n-3 PUFA prescribed.

2. All subjects needed histological evidence of NAFLD, as assessed by a single expert histopathologist.

3. Patients on insulin sensitising therapies were excluded.

4. The assessment of HTGC using a powerful magnetic field, coupled with serum and hepatic lipid profiling aided the interpretation of the findings.

The study does however have limitations:

1. There was no histological assessment of liver tissue pre and post intervention. This meant that assessments of hepatic inflammation and fibrosis could not be made.

2. The duration of 3 months may not have been adequate enough to fully test the hypothesis. That said, a previous 8 week study assessing liver fat with ^{1}H MRS, in a smaller cohort of people with polycystic ovarian syndrome, and using smaller n-3 PUFA doses than the current study, had positive outcomes (Cussons, Watts et al. 2009).

3. Not all patients had steatosis at study entry. This appears to have impacted on the outcome, as the findings were positive in those with steatosis.

4. Patients were not prospectively identified and so there may have been disease regression or progression in between the time of their biopsy and study entry.

The conflicting findings between the whole group and the subgroup with steatosis mean that this is not a conclusive study. There are several ongoing studies in this area registered on the clinicaltrials.gov website. So this study will add to what in the future will be a considerable body of evidence.

Chapter 7. Final summary

Non-alcoholic fatty liver disease is the hepatic consequence of obesity and insulin resistance. The vast majority of such patients are overweight or obese and as such lifestyle induced weight loss is currently the only approved therapy for NAFLD (AGA 2002). There is however only a single paper that validates this approach (Promrat, Kleiner et al. 2010), and it has been shown to be very difficult to meaningfully achieve weight loss (Huang, Greenson et al. 2005). Histological improvement in steatohepatitis has been shown with insulin sensitising medical therapies. Unfortunately these improvements appear to revert following therapy cessation and such therapies have been associated with an increased cardiovascular risk. Liver targeted anti-inflammatory therapies are currently under development though none are imminently expected.

The management of obesity and its associated complications is a major healthcare challenge. In later life the presence of obesity results in a 2 to 3-fold increased mortality due to an increased incidence of cardio-metabolic and malignant disease (Adams, Schatzkin et al. 2006). The global epidemic of obesity is therefore predicted to have an exponential impact on the future health of society and hence health care services. This has resulted in nutrient intakes and dietary patterns being politicised, with responsibilities increasingly transferred from the individual to organisations and governments. Such bodies are ultimately keen to please the public whose opinions are commonly led by lay journalism and individuals such as Jamie Oliver. The public is also vulnerable to manipulated information originating from the food and pharmaceutical industries. Whilst much of the current efforts are to be praised it does increase the pressure on the scientific community to ensure that the driving forces for nutritional change are fully informed and valid.

The message of 'eat less, do more' is rarely effective in terms of weight loss. Recently there has been an attempt to reduce energy intakes by branding whole foods as being either 'good' or 'bad' on the basis of their energy and macronutrient profile (Brand-Miller, McMillan-Price et al. 2008). This branding has been promoted by the food industry which has further developed the concept with the development of 'nutraceuticals'. So a supermarket visit is rapidly being transformed into a visit to a scientific laboratory, with the experiment being your long-lasting health (van Kleef, van Trijp et al. 2005). It is implied that the only

limits to such health benefits are your culinary skills, wallet, taste buds and penchant for 'bad foods'. The dangers of simplistic classifications of good versus bad foods are that it confuses the public and overtly vilifies certain foodstuffs. Denmark, for example, has recently imposed tax increases of 25% on ice cream, chocolate and sweets (Wilkins 2010). Whilst sweets lack any real nutritional value the same cannot be said for chocolate or ice cream. Although there are clear benefits behind the drive to highlight foods based on their individual nutrient and energy profiles, this misses the message that an overall balanced intake is more important than its individual constituents.

To date there is a clear limitation in medical advice given to NAFLD patients – 'eat less or do more'. The majority of patients are significantly overweight and hence already aware of their personal benefits of heeding such advice irrespective of the presence of liver disease. Critically the mechanisms to safely achieve this are unclear and long-term support limited within most healthcare systems. Until cirrhosis develops most are relatively asymptomatic, and so the diagnosis of NAFLD appears to result in little real motivation to change lifestyles. Recently there has been a suggestion that such an energy-focused strategy should be promoted even in compensated overweight cirrhotics (Berzigotti, Garcia-Tsao et al. 2011). This was a post-hoc analysis of a prospectively collated cohort of cirrhotic patients with a variety of aetiologies. It observed a greater rate of decompensation in patients that were overweight or obese than normal weight, as assessed by body mass index. The authors speculated that weight loss may reduce the rate of decompensation. The safety of this apparently logical and simple conclusion needs to be first assessed before it becomes routine practice, as was highlighted in my communication with the journal (Johnston, Dolman et al. 2011). I raised concerns over the potentially detrimental effects of prolonged fasting or intense aerobic exercise in cirrhosis. Plank and colleagues demonstrated that total body protein status is greatly improved by nocturnal nutritional supplements than by use of energy-matched daytime supplementation in cirrhosis (Plank, Gane et al. 2008). Furthermore, very low calorie diets and carbohydrate restriction may pose unique risks in cirrhosis by inducing hepatic stressors, namely an increased demand for endogenous gluconeogenesis and increased requirement to catabolise ketone bodies. There are limited data on a cirrhotic liver's ability to cope with such insults. Increasing exercise is ordinarily an attractive weight loss strategy but in cirrhosis there is

impaired endogenous gluconeogenesis and hence there is a potential risk of aerobic exercise induced hypoglycaemia (DeLissio, Goodyear et al. 1991). Nutrient manipulation has the potential to be a safer, more acceptable and complementary approach to weight loss in NAFLD, especially those with advanced disease. Such strategies are generally well tolerated and accepted, with individual changes having an additive effect on metabolic outcomes (Lewis, Hammett et al. 1981).

My studies were designed to identify the impacts of altering intakes of specific nutrients in hepatic lipid metabolism. The issues were whether altering intakes of certain macronutrients promotes the development of NAFLD, or can treat established disease. These studies have a clear translation into public health messages. The studies detailed in this thesis assessed the effects of fructose, one of the most notorious nutrient 'baddies', and omega 3 fatty acids, a 'goody'. Hence there has been a lot of interest and expectation in this work. The resultant messages are not as clear-cut and simple as pressure groups would like. The greater uric acid concentration with fructose supports the observation of its lack of pre triose metabolic control. It was however the post triose metabolism that was the key interest of this study, with the primary outcome being hepatic lipid content. There was no difference between fructose and glucose in this respect. It is clear that combined energy and monosaccharide overfeeding has a significant impact on hepatic metabolism, though the lack of alternative nutrient comparators (e.g. fat) means that these cannot be viewed as monosaccharide specific. Supplementing n-3 PUFAs reduced serum but not hepatic triglycerides and made no other impacts on metabolic status. So this provides no evidence for altering macronutrient, as opposed to energy, intakes.

There has been much publicity both in scientific and lay press resulting in considerable anxiety surrounding the fructose content of a modern diet. Despite there being no evidence that outcomes from high fructose corn syrup differ from sucrose there are numerous campaigns to ban its use in the United States. There is scant robust data demonstrating that fructose and glucose result in differing clinical outcomes. This has recently been acknowledged by one of the leading fructose researchers, Luc Tappy, who stated that the only convincing difference is the greater serum triglycerides with fructose (Tappy, Le et al. 2010). There is clear evidence however that overall sugar intakes are increasing and this has been associated with the development of obesity (Johnson, Segal et al. 2007). So efforts

should be addressed at reducing intakes of all sugars as opposed to an individual component. The specific demonisation of fructose appears to be largely unjustified and hence unhelpful from a public health perspective.

The background data implicating a high intake of fructose with NAFLD aetiology is derived from some circumspect observations. Firstly, NAFLD patients have occasionally been shown to have a higher fructose intake than controls (Ouyang, Cirillo et al. 2008). This may not be a fructose-specific effect however as there is a strong association between the fructose and glucose content of foodstuffs. Secondly, clinical studies have reported features associated with NAFLD such as insulin resistance and dyslipidaemia developing with a high fructose intake. There are several uncontrolled studies reporting this (Faeh, Minehira et al. 2005; Le, Ith et al. 2009), and only one controlled study that supports this (Stanhope, Schwarz et al. 2009).

Prior to the initiation of this thesis there were no controlled studies that compared the hepatic outcomes of fructose versus glucose overfeeding. Two recent papers, both with significant limitations, have reported a similar finding to this study, with no hepatic differences between glucose and fructose short term overfeeding in terms of liver lipid content or biochemistry (Ngo Sock, Le et al. 2010; Silbernagel, Machann et al. 2011). This current study adds to these similar outcomes in terms of triglyceride in the serum, liver or muscle; systemic, adipose and hepatic insulin resistance; hepatic volume and ^{31}P metabolites; whole body substrate oxidation; and cardiovascular profile.

State of the art assessment tools were used to form a full metabolic profile or picture in this thesis. It was the first to study hepatic outcomes in healthy yet overweight individuals. The cohort was precisely defined using standard clinical assessments, making it easier for others to reproduce the findings. The next key strength was that it assessed the outcomes in both an isoenergetic and hyperenergetic setting. The provision of all foodstuffs during the isoenergetic period ensured complete control over nutrient intakes and hence improves the robustness of those findings. Weight maintenance was achieved and so a high fructose or glucose intake was achieved without a concurrently high energy intake. No prior hyperinsulinaemic euglycaemic clamp data has been published in this area (the gold standard for assessing insulin resistance). This was the first long-term hepatic ^{31}P assessment study, and the first long-term study on cardiovascular health using any assessment greater than a sphygmomanometer.

The major limitations of this fructose versus glucose study are that it was only performed in men, and that there was no cross-over between the groups. This limits the generalisability of the findings and the analyses of the inter-monosaccharide outcome response. The relatively short-term nature of the intervention means that the possibility of an effect with a longer dietary alteration cannot be excluded. There was an explicit power calculation made for the primary outcome measure, but none for the numerous secondary outcomes assessed. As a result many of these potentially could be affected by a type 2 error, and over interpretation of the data as a whole could be an error. Such over interpretation is more of an issue for false positive than negative findings, and there are few positive differences between the groups in the fructose versus glucose study. This is a challenging area of study design. Hepatic lipid metabolism does not occur in isolation from the global metabolic status. Hence the assessment and presentation of the primary outcome, liver triglyceride, would be relatively meaningless without data on weight, substrate oxidation, liver biochemistry, serum triglycerides, and insulin resistance as an absolute minimum. I believe that the additional parameters paint a fuller picture, including explanatory data and assessment for potential confounders, and are not merely a 'fishing exercise' as critics may suggest. This is therefore both an interventional study and an assessment of mechanisms. Ideally each parameter should be individually powered for though this process is not feasible or indeed reliable itself.

Fructose and glucose are not normally consumed in vastly differing amounts, and so the study did not reproduce potential natural intake patterns from whole foods. The addition of a group consuming sucrose would have aided interpretation of the data from that of inter-nutrient assessments to a nutrient versus whole food assessment. Indeed there is some suggestion that fructose behaves metabolically differently when bound to glucose in sucrose, though this has been rarely shown and the explanation for this is uncertain (Bizeau and Pagliassotti 2005). Equally, the addition of an energy balanced high lipid or protein group would have facilitated macronutrient comparisons. These assessments need to be performed in future studies.

In NAFLD patients a low energy diet (~1300kcal/day), with standard macronutrient patterns, has recently been shown to reduce hepatic triglyceride to a lesser extent than a low carbohydrate diet (~25g/day) (Browning, Baker et al. 2011). The main problem with this study is that not all foods were provided and

hence energy was not explicitly controlled for between the groups. Of note the two groups lost similar amounts of weight and only differed in their HTGC reduction.

The lack of hepatic differences between fructose and glucose means that future work should focus on the hepatic effects of high or low intakes of carbohydrates versus other macronutrients in a rigorously energy controlled setting. No such work has been previously done.

Just as there are pressures to reduce fructose intakes, there are equal pressures to increase the population's n-3 PUFA intakes which are currently markedly lower than recommended guidelines. The ideal mechanism to achieve such changes in intake remains uncertain (Givens and Gibbs 2008). Oily fish and capsules could be promoted, but such advice is unlikely to be heeded by those who would most benefit, and there are limited fish stocks globally. Whole foodstuff enrichment with n-3 PUFAs is possible but fraught by losses due to fatty acid oxidation during food storage and preparation (Taneja and Singh 2011). This is of interest not only to the food industry but also the pharmaceutical industry who promote the health benefits of n-3 PUFAs even when robust evidence is still lacking. A recent article in Nature Medicine quoted a scientist as stating of n-3 PUFAs and heart disease that "there are clearly benefits. It's a matter of isolating exactly what (the benefits) are, and we haven't managed to do that yet" (Hersher 2012). This opinion contrasts with several recent meta-analyses showing no benefits with n-3 PUFAs in high risk cardiovascular patients in terms of mortality, sudden cardiac death or cardiovascular re-stenosis (Filion, El Khoury et al. 2010; Chen, Cheng et al. 2011). Both of these meta-analyses assessed randomised data on over 30,000 patients. The dwindling scientific support for n-3 PUFA supplementation is in stark contrast to their ever increasing consumption by the public (Hersher 2012).

The background data linking n-3 PUFA status and NAFLD comes from dietary assessments and analyses of lipid profiles in NAFLD liver biopsy tissue. These describe a depletion of the long chain n-3 PUFAs (Araya, Rodrigo et al. 2004; Puri, Baillie et al. 2007; Allard, Aghdassi et al. 2008). However the story is not as simple as that as these analyses have also shown a reduction in arachidonic acid. This relative depletion of both n-6 and n-3 PUFAs may come from NAFLD associated alterations in diet, fatty acid synthesis from precursor, complex lipid metabolism resulting in either less cellular incorporation or greater release, be the

result of systemic or local inflammation, or alterations in lipid peroxidation driven by oxidative stress. Whatever the mechanism the potential for hepatic benefit with n-3 PUFA supplementation is given biological plausibility by their PPARα and PPARγ agonism (Gonzalez-Periz, Horrillo et al. 2009). Rodent and non-randomised clinical studies with limited assessment techniques have suggested that supplementation with n-3 PUFAs is beneficial in NAFLD in terms of hepatic triglyceride reduction (Capanni, Calella et al. 2006; Gonzalez-Periz, Horrillo et al. 2009).

The study described in this thesis did not demonstrate such a beneficial effect of n-3 PUFA supplementation in patients with NAFLD. There was a trend for a reduction in hepatic triglycerides with n-3 PUFAs, and so the study may be criticised for being underpowered in terms of the number of patients or that the duration of supplementation was inadequate. At present one can neither prove nor refute this argument. There was a significant increase in serum n-3 PUFA fatty acid content and a reduction in serum triglycerides implying that the supplements were altering lipid metabolism. The tendency for weight to increase and insulin resistance to develop in this study may have negated any potential benefits.

The n-3 PUFA study recruited a rigorously defined cohort who all had histological evidence of NAFLD. Secondly, hepatic triglyceride content was quantified using 3-Tesla ^{1}H MR spectroscopy as opposed to ultrasound – a markedly more sensitive tool. Thirdly, double blind randomisation was performed with a valid placebo. Assessments of serum and hepatic fatty acid profiles along with fasted assessments of insulin resistance aided outcome interpretation.

There are many current studies registered that aim to assess the effects of n-3 PUFA supplementation. A full picture of the role of n-3 PUFAs in NAFLD will therefore shortly be available.

Ultimately the exquisite sensitivity of the liver to nutrient intakes was highlighted by the 0.8% gain in weight in the fructose / glucose study resulting in a 24% increase in liver lipid. This affirms the notion that dietary energy intakes have a profound influence on hepatic metabolism, but there is no evidence from this thesis that this influence is macronutrient specific.

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