The Effect of Composition of Dietary Sugars on Hepatic Fatty Acid Synthesis and Partitioning and Insulin Secretion and Sensitivity

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Authorship Declaration

This thesis and the work presented in it were completed at the University of Oxford (Radcliffe Department of Medicine, Oxford Department for Diabetes, Endocrinology and Metabolism) and Singapore (Agency for Science, Technology and Research (A*STAR), Clinical Nutrition Research Centre) between October 2014 and September 2018, under the supervision of Prof. Leanne Hodson, Prof. Jeremy Tomlinson and Dr Faidon Magkos. All contributions made by others have been stated clearly in the text. This work has not been submitted in full or partial fulfilment of any other degree.

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Abbreviations

ACC	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
ALT	Alanine aminotransferase
ATP	Adenosine triphosphate
AUC	Area under the curve
Apo	Apolipoprotein
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CHREBP	Carbohydrate responsive element binding protein
DEXA	Dual energy X-ray absorptiometry
CV	Coefficient of variance
DI	Disposition index
DNL	*De novo* lipogenesis
FA	Fatty acid
FAS	Fatty acid synthase
GC	Gas chromatography
G-6-P	Glucose-6-phosphate
GCMS	Gas chromatography mass spectrometry
GC-C-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometry
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GSIS	Glucose-stimulated insulin secretion
HDL	High-density lipoprotein
HFCS	High fructose corn syrup
HOMA-IR	Homeostasis model assessment of insulin resistance
iAUC	Incremental area under the curve
ISR	Insulin secretion rate
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
MRI	Magnetic resonance spectroscopy
MUFA	Polyunsaturated fatty acid
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid(s)
OHB	Hydroxybutyrate
$PNPLA_3$	Patatin-like phospholipase domain-containing protein 3
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
RER	Respiratory exchange ratio
S_f	Svedberg flotation value

SAT	Subcutaneous adipose tissue
SCD	Stearoyl-CoA dehydrogenase
SEM	Standard error of the mean
SFA	Saturated fatty acid
SREBP	Sterol regulatory element binding protein
STT	Sugar tolerance test
TG	Triacylglycerol
TRL	Triacylglycerol-rich lipoproteins
TTR	Tracer-tracee ratio
VAT	Visceral adipose tissue
VLDL	Very-low-density lipoprotein
Φ	Beta-cell responsivity
Φ_s	Static β-cell responsivity
Φ_d	Dynamic β-cell responsivity

Abstract: The Effect of Composition of Dietary Sugars on Hepatic Fatty Acid Synthesis and Partitioning and Insulin Secretion and Sensitivity

Increased consumption of foods containing free sugars, especially fructose, has been associated with the development of non-alcoholic fatty liver disease (NAFLD). The overall aim of this thesis is to investigate the effect of composition of fructose and glucose on hepatic fatty acid (FA) synthesis and partitioning and insulin response and to do this, three studies were undertaken.

A randomised crossover study was conducted, where subjects consumed a low fructose meal and a high fructose meal with [U^{13}C]palmitate incorporated to trace the metabolic fate of dietary fats. The two study visits were four weeks apart. Overall, it was found that consuming a high fructose meal but not a low fructose one had a marked effect on females but not males in postprandial hepatic FA synthesis and not in hepatic FA partitioning.

In the second study, subjects consumed a test meal which contained [U^{13}C]fructose to trace the metabolic fate of dietary fructose. It was demonstrated that though there was fast metabolic conversion of fructose into FAs within 2 hours, less than 1% of the ingested fructose was converted to FAs. A much larger proportion of fructose was oxidised into $^{13}CO_2$ (30.5%).

The objective of the third study was to investigate the acute and chronic effect of high fructose consumption on insulin secretion, liver fat content and hepatic *de novo* lipogenesis (DNL) in subjects. In the crossover acute arm of the study, single dose consumption of the high fructose drink resulted in significantly lower static insulin secretion rate (ISR) in females but not males. This difference in static ISR diminished following prolonged high fructose consumption. It was also found that the

daily consumption of either the low or high fructose drink did not change the liver fat content or fasting hepatic DNL in the subjects.

Overall, we found notable gender differences in FA metabolism after consumption of low and high fructose meals, the mechanistic details of which can be investigated in future studies.

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Chapter 1: Introduction

1.1 Metabolic importance of the liver

The liver is one of the largest and most important organs in the human body. It plays a key role in maintaining homeostasis of the systemic circulation in several ways, including the synthesis of bile acids, removal of toxins from the bloodstream and the regulation of metabolic pathways for dietary macronutrients (Fernández-Rojo and Ramm, 2016). Over the past two decades, there has been a worldwide increase in the contribution of chronic liver diseases to total disease-related mortality rates with the number of deaths attributed to liver diseases, such as cirrhosis, cancer and non-alcoholic fatty liver disease (NAFLD) (Leitao *et al.,* 2013). The increased prevalence of chronic liver disease is, in part, due to the changes in diet and lifestyle in many countries (Sherif *et al.*, 2016). Diets have become more energy dense, higher in free sugars and fats while lifestyles have become more sedentary in many countries. Free sugars are those added to food in any form as well as monosaccharides and disaccharides naturally present in fruits and vegetable juices, honey and syrups. It excludes lactose in milk and dairy products, and also those in cereal grains, nuts and seeds *(SACN. Carbohydrate and health. London 2015)*. Dietary and lifestyle changes have led to an imbalance between energy intake and output in many people; the primary cause of obesity (Popkin *et al.*, 2012).

1.2 Obesity and ectopic fat deposition

It is estimated 502 million people globally are obese and this number is increasing (Wang *et al.*, 2011). For instance, in Singapore, the percentage of population who were considered obese in the 1990's was 5% and this is expected to significantly increase to 15.9% by 2050 (Phan *et al.*, 2014). Obesity is tightly linked to insulin resistance and pancreatic β-cell dysfunction (Gupta *et al.*, 2012) and therefore is

associated with increased risk of type 2 diabetes and metabolic diseases such as cardiovascular disease and NAFLD.

In metabolically healthy individuals, dietary fat is principally stored in adipose tissue (Fernández-Quintela *et al.*, 2007; Ruge *et al.*, 2009). However, if adipose tissue becomes dysfunctional, dietary fatty acids (FAs) may get stored in non-adipose tissue organs (Frayn, 2002) such as the heart, pancreas and liver (Lettner and Roden, 2008); this is known as ectopic fat deposition.

Ectopic fat deposition within the liver is known as NAFLD (Unger, 2003) and there is a strong correlation between obesity, particularly abdominal obesity, and risk of NAFLD (Moore, 2010; Estadella *et al.*, 2013). It is estimated that between 57% and 74% of obese individuals have NAFLD, and this figure increases up to 90% for those who are morbidly obese (Festi *et al.*, 2004).

1.3 Non-alcoholic fatty liver disease

NAFLD is the most common form of liver disease in developed countries (Azzam and Malnick, 2015) and its prevalence is estimated at 20-40% within the general adult population (Krawczyk *et al.*, 2010). NAFLD is a spectrum of disease that begins with the accumulation of triacylglycerol (TG) within the liver (steatosis) in the absence of excessive alcohol consumption (Nascimbeni *et al.*, 2013). Hepatic steatosis is defined as a liver TG content exceeding 5% of hepatic tissue as measured by magnetic resonance imaging (Fabbrini *et al.*, 2010). The development of hepatic steatosis most likely involves an imbalance between FA input, synthesis and disposal (Fabbrini *et al.*, 2010; Hodson and Frayn, 2011; Mashek, 2013). As visceral adiposity and intrahepatic fat content are strongly associated, it is possible

that hepatic steatosis is closely linked with metabolic complications (Fabbrini *et al.*, 2009).

1.3.1 Pathogenesis of NAFLD

NAFLD is often referred to as the hepatic manifestation of the metabolic syndrome (Cave *et al.*, 2007; Petta *et al.*, 2011) and it has been suggested that the pathogenesis of NAFLD is a two-hit process (Day and James, 1998). The first hit is the initial accumulation of TG in hepatocytes which gives rise to hepatic steatosis. Following that, some individuals progress to develop inflammation (Torres and Harrison, 2015); increasing amounts of oxidative stress may lead to mitochondrial dysfunction and lipid peroxidation, which is known as the second hit. This leads to non-alcoholic steatohepatitis (NASH) which may progress to irreversible conditions such as liver cirrhosis and hepatocellular carcinoma (Chalasani *et al.*, 2012; Nascimbeni *et al.*, 2013). The occurrence of NASH is considered to be the point in the pathogenesis of NAFLD which is strongly associated with increased risk of cancer, kidney disease and cardiovascular disease (Byrne *et al.*, 2009; Lonardo *et al.*, 2013). In 2010, Dowman *et al.* proposed a 'third hit' where oxidative stress causes increased hepatocyte death that cannot be replaced by proliferation, and therefore leads to loss of tissue function (Dowman *et al.*, 2010).

A "multi-hit" mechanism was also proposed where insulin resistance and its resultant lipid imbalance is considered the first hit (Polyzos *et al.*, 2009). Liver steatosis develops and this makes it more vulnerable to other stimuli which include gut-derived bacterial toxins, cytokine or adipokine imbalance, oxidative damage, mitochondrial dysfunction and the development of inflammation. Collectively these may stimulate liver fibrosis and apoptosis of hepatocytes, leading to progressive liver disease and NASH (Tilg and Moschen, 2010).

1.4 Factors affecting the development of hepatic steatosis

There are many proposed factors that may lead to the development of hepatic steatosis including ethnicity, genetic predisposition, and dietary and lifestyle factors (Moore, 2010).

1.4.1 Effect of ethnicity on NAFLD risk

Liver disease is a major public health issue around the world, particularly in Asia; with liver cancer being the second largest contributor to cancer mortality (McGlynn *et al.*, 2015). NAFLD is often a silent liver disease which is not typically investigated unless recommendations are given by physicians to patients who are considered to be at high risk due to their increased BMI or waist to hip ratio (Grattagliano *et al.*, 2007). Asians, however, have been shown to have a higher risk of metabolic syndrome at lower BMI levels than Caucasians. Chang *et al.* (2003) found that at any given BMI, Taiwanese men and women (n=1079) had on average, a higher body fat percentage than Caucasian men and women. Wu *et al*. (2007) also found a higher percentage of body fat and increased prevalence of truncal fat in Asians at a lower BMI compared to Caucasian counterparts. At any given waist circumference, higher amounts of visceral adipose tissue and body fat have been reported in Asian compared to Caucasian populations (Kagawa *et al.*, 2007; Lear *et al.*, 2007). Therefore, it is likely that the WHO cut-off values for BMI, waist circumference and waist-hip ratio may be underestimating obesity-related risks in Asian compared to European populations (Nishida, 2004; Diaz *et al.*, 2007). As body fat percentage and BMI are associated with liver fat percentage (Ko *et al.*, 2017), these studies show that different cut-offs for body fat percentage and BMI are needed in different regions to accurately determine the risk of obesity-related metabolic diseases, such as NAFLD.

An important risk factor for NAFLD is the presence of genetic polymorphisms. In the Dallas Heart Study, the rs738409 polymorphism of Patatin-like phospholipase domain-containing protein 3 (PNPLA$_3$) (I148M) was identified as the single major genetic determinant of hepatic fat content and alanine aminotransferase (ALT) levels (Romeo *et al.*, 2008; Yuan *et al.*, 2008). This single nucleotide polymorphism involves a change of cytosine to guanine at the 148th position of the DNA sequence, encoding a change of amino acid from isoleucine to methionine (I148M). Studies conducted in different ethnicities confirmed that the I148M variant predisposes towards liver damage associated with fatty liver (Dongiovanni *et al.*, 2013). The 148M risk allele was more prevalent in Hispanics than in Europeans and less common in African-Americans explaining a consistent fraction of the inter-ethnic variability in NAFLD susceptibility (Romeo *et al.*, 2008; Hernaez *et al.*, 2013).

1.4.2 Dietary factors

1.4.2.1 Effect of energy content of diet

The energy content of diets has been shown to exert an effect in liver fat accumulation. In a review by Parry and Hodson (2017), hyperenergetic diets have been shown to increase liver fat content. For example, Kechagias *et al*. (2008) reported that a 4-week hyperenergetic diet with a higher proportion of energy from fat (43% of total energy) on 18 subjects (12 males, 6 females) aged between 16 and 26 increased liver fat content from 1.1% to 2.6%. This is supported by the findings of van der Meer *et al*. (2008) who showed that in a group of 15 healthy males fed a supplement of 280 g of fat daily for 3 days in combination with their regular diet, hepatic fat content increased from a mean of 2.0% to 4.3%.

On the contrary, hypoenergetic diets have been reported to reduce liver fat content according to a recent review by Dalla Pepa *et al.* (2017). A 4-week intervention study by Edholm *et al.* (2011) in obese females showed that severe energy restriction (800 – 1100 kcal/d) reduced hepatic TG levels by 51% whilst Colles *et al.* (2006) found that consumption of a very-low-energy diet (456–680 kcal/d) for 12 weeks resulted in a 43% reduction in liver fat in obese individuals.

The nutrient composition of the diet may be important as it has been shown that there are differential effects between diets high in carbohydrate and high in fat on hepatic TG levels. Hypoenergetic diets low in carbohydrate are suggested to be more effective than hypoenergetic diets low in fat in reducing hepatic fat content in the short term (Kirk *et al.*, 2009; Haufe *et al.*, 2011). For example, the study by Kirk *et al.* (2009) showed that in obese participants fed a hypoenergetic diet with either carbohydrate or fat restriction for 48 hours, the carbohydrate restricted group had lower intrahepatic TG content compared to the fat restricted group. However, this pattern of decrease in hepatic fat was not sustained and both groups had similar intrahepatic TG content and achieved a similar weight loss of 7% after 11 weeks. Another study by Haufe *et al.* (2011) involving 170 overweight and obese participants fed with hypoenergetic diets and split into either a low fat or low carbohydrate groups found that after 6 months that there was no difference between the 2 diets in hepatic TG levels.

In the case of isoenergetic diets, van Herpen *et al*. (2011) found that an isoenergetic diet which was high in fat (55% of total energy) increased hepatic fat content by 17% after 3 weeks while a low-fat diet (20% of total energy) decreased hepatic fat content by 13% compared to a control diet which contained 35% of total energy as

fat. The composition of fat or carbohydrate would thus seem to be more important than the absolute amount of either macronutrient.

Taken together it would appear that the development of NAFLD is influenced in part by total energy intake as well as the amount and composition of dietary fat and carbohydrate. What remains unknown is how the composition of carbohydrate in an isoenergetic meal affects postprandial hepatic FA synthesis and partitioning and this was the focus of one of my studies.

1.4.2.2 Dietary fats

Dietary fats have been proposed to play a role in the development of NAFLD, although this appears to be dependent on the type and frequency of fat consumption (Sullivan, 2010). A cross-sectional study by Kuk *et al.* (2008) found a positive correlation between habitual fat intake and liver fat content. A 10-week isoenergetic intervention study involving 61 obese participants found that saturated fatty acid (SFA) consumption was associated with increased liver TG content whilst polyunsaturated fatty acid (PUFA) consumption was associated with decreased liver TG content (Bjermo *et al.*, 2012). In line with results found in obese individuals, Rosqvist *et al.* (2014) found the same effect in healthy participants who were fed a hyperenergetic diet enriched with either SFA or PUFA for 7 weeks. A possible reason for the decrease in liver TG content on the PUFA diet was suggested to be that PUFAs preferentially undergo β-oxidation and inhibit lipogenic gene expression (Bjermo *et al.*, 2012).

SFAs have been shown to be an inflammatory mediator in hepatocytes and are associated with liver dysfunction as they are reported to increase oxidative stress in hepatocytes both *in-vitro* and *in-vivo* in rodent studies (Quiles *et al.*, 2002; Nakbi *et*

al., 2010). SFAs activate toll-like receptor 4 (TLR4) which produces tumour necrosis factor-α (TNF-α), an important inflammatory mediator, that may promote pro-inflammatory signalling pathways leading to mitochondrial dysfunction, increase in amount of reactive oxygen species, caspase release and eventually cell death (Jaeschke, 2011; Ibrahim *et al.*, 2011). SFAs have also been shown to cause a change in gut microflora with increased populations of gram-negative bacteria which may lead to an increase in the release of lipopolysaccharides (Cani *et al.*, 2007). Lipopolysaccharides are recognised by toll-like receptors on cell surfaces, which have an additive downstream effect on the direct pro-inflammatory effect of SFAs (Machado and Cortez-Pinto, 2013).

1.4.2.3 Dietary sugars

The effects of dietary sugars on the development of NAFLD have been seen in both observational and intervention studies in humans. A study by Jia *et al*. (2015) involving 4365 healthy individuals in Tianjin, China found an association between high carbohydrate consumption and risk of developing NAFLD in females but not in males. While hyperenergetic, high carbohydrate intervention diets have been reported to increase liver fat deposition, the evidence is not so clear for isoenergetic studies. Bravo *et al*. (2013) showed that a 10-week high fructose corn syrup (HFCS) or sucrose-enriched diet had no significant effect on hepatic fat content in 64 individuals when the carbohydrates were fed at 30% of total energy content compared to the control where the carbohydrates composed 8% of total energy content. In contrast, Schwarz *et al.* (2015) found that after consumption of a high fructose diet (25% of total energy content), there was significantly higher liver fat content as compared to after a diet where the fructose was isoenergetically

substituted with complex carbohydrates. Together, these studies show that the effect of high fructose diets on hepatic fat accumulation is not completely clear.

1.5 Treatment of NAFLD

There are currently no FDA approved drugs for the treatment of NAFLD, with the most effective intervention being weight loss (Kenneally *et al.*, 2017). Ghaemi *et al.* (2013) suggested that 15-20% weight loss is required to significantly improve liver function, as measured through biomarkers. Other studies found that a weight loss between 7-15% can reduce liver fat content, fibrosis and improve the condition of NASH in patients (Huang *et al.*, 2005; Romero-Gómez *et al.*, 2017).

Higher physical activity levels have been reported to be associated with lower liver fat levels (Perseghin *et al.*, 2007; Keating *et al.*, 2015). A meta-analysis of 12 studies demonstrated that regular exercise of at least 3 sessions a week for 20-60 mins can significantly lower liver fat levels irrespective of exercise intensity (Keating *et al.*, 2012). The proposed mechanism by which this occurs is due to exercise increasing levels of FA oxidation, leading to the increased utilisation of stored fat within the body, including from the liver (Horowitz and Klein, 2000). Exercise increases oxidation due to its effect on upregulating the phosphorylation of acetyl-CoA carboxylase (ACC). Phosphorylated ACC is inactive, leading to downregulation of malonyl-CoA production and therefore a decrease in FA synthesis via *de novo* lipogenesis (DNL), which is the generation of FAs from non-lipid precursors (Wakil and Abu-Elheiga, 2009; Paglialunga and Dehn, 2016). Therefore, the presence or absence of exercise in daily life may have an important role in hepatic FA metabolism and the risk of developing hepatic steatosis.

1.6 Sources of hepatic fatty acids

Hepatic FAs may come from dietary and non-dietary sources. In addition to dietary FAs, they can be synthesised via DNL and from the lipolysis of adipose tissue which results in the release of NEFA into the bloodstream. All of these FAs can be taken up by the liver and contribute to the hepatic FA pool.

Work using rodent hepatocytes demonstrated that FAs from the diet and DNL-derived enter a common pool before being partitioned into esterification or oxidation pathways (Gibbons and Wiggins, 1995). There is a storage pool of TG in the liver and both FAs and TGs are in constant turnover (Green and Hodson, 2014). TGs in the storage pool can be packaged into VLDL for export (Hodson and Frayn, 2011).

1.6.1 Dietary fatty acids

Upon fat consumption, FAs are incorporated into chylomicrons within the enterocyte of the small intestine before entering the systemic circulation (Hodson *et al.*, 2009). Lipoprotein lipase (LPL), an enzyme found on the surface of endothelium of capillaries hydrolyses TGs contained within chylomicrons, allowing FAs to be taken up into tissues for storage. With hydrolysis, chylomicron particles decreased in size, such that when they reach the liver, they are in the form of chylomicron remnants. These are taken up by the liver via LDL receptor-related protein-1 and hydrolysed in the lysosomes before the resultant FAs contribute to the FA pool (Donnelly *et al.*, 2005; Parry and Hodson, 2017).

1.6.2 Endogenous sources of hepatic fatty acids

The sources of endogenous hepatic FAs can be classified into those originating from adipose tissue lipolysis, those already stored within the liver as TG and those that are synthesised via DNL (Rui, 2014).

In the human body, TGs are stored mostly in the adipose tissue. In the fasting state, glucagon activates hormone sensitive lipase which hydrolyses subcutaneous and visceral adipose tissue TGs into NEFAs that then enter the systemic circulation and can be taken up into the liver. In the fasting state, NEFA is the primary substrate for FA oxidation and precursor for TG synthesis (Havel *et al.*, 1970).

1.6.3 Hepatic fatty acid synthesis: *de novo* lipogenesis

De novo lipogenesis is a process whereby FAs are synthesised from non-lipid precursors including amino acids such as phenylalanine and tyrosine and the monosaccharides glucose and fructose (Moore *et al.*, 2014). A pathway involved in DNL is the TCA cycle where amino acids can be converted into intermediates (such as α-ketoglutarate) or acetyl-CoA (Figure 1.1). Carbohydrates undergo glycolysis and oxidative decarboxylation to also form acetyl-CoA which can be further metabolised to form FAs through the action of fatty acid synthase (FAS) (Figure 1.1). The liver is the predominant site of DNL in the human body, although DNL can also occur in adipose tissue and intestinal enterocytes (Theytaz *et al.*, 2014).

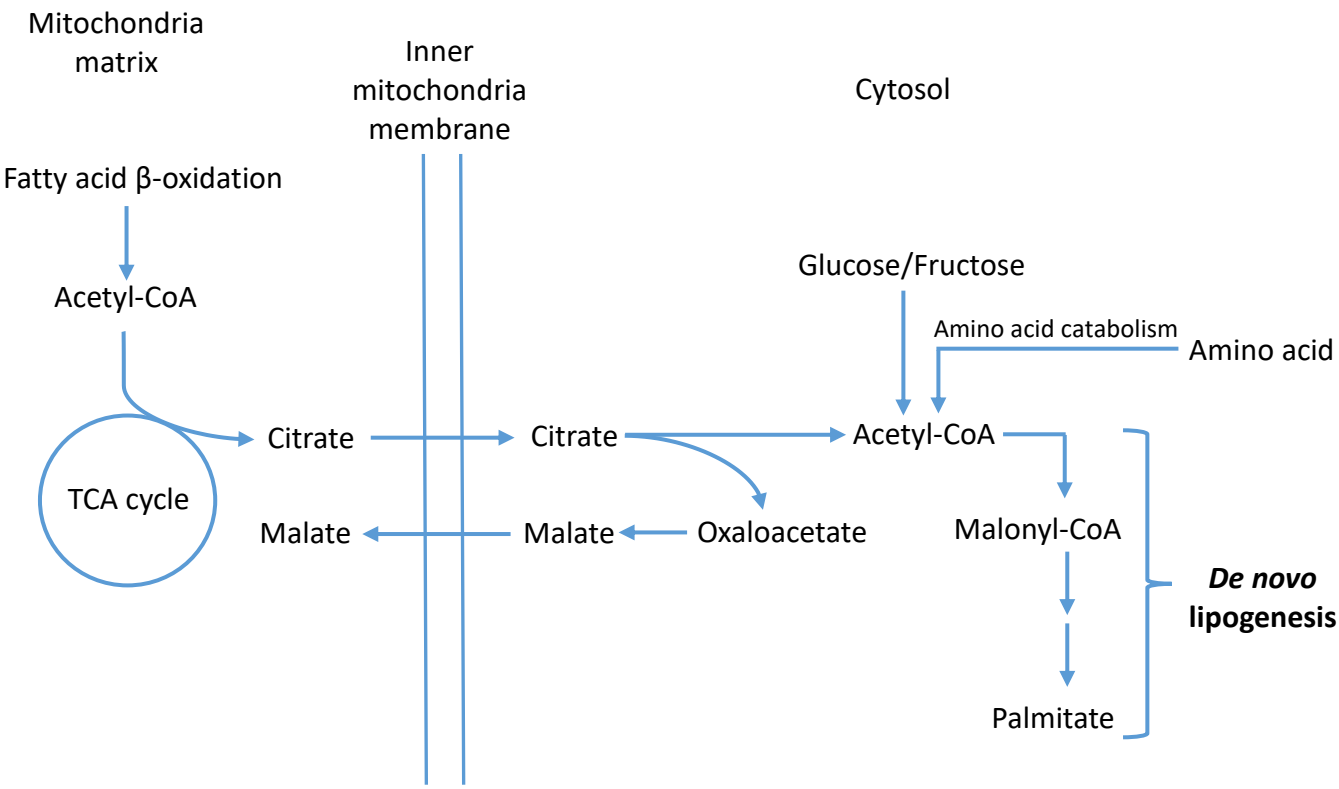

Figure 1.1: A schematic of fatty acid metabolism. Acetyl-CoA is converted to citrate via the TCA cycle. The citrate can pass through the mitochondrial membrane to enter the cytosol where it can be converted to oxaloacetate and acetyl-CoA. Monosaccharides and amino acids are converted to acetyl-CoA in the cytosol through glycolysis and amino acid catabolism respectively. The acetyl-CoA in the cytosol can be carboxylated by acetyl-CoA carboxylase to form malonyl-CoA, the precursor for *de novo* lipogenesis (adapted from Moore *et al.*, 2014).

DNL occurs when the amount of carbohydrate consumed exceeds the energy requirement of an individual and the glycogen storage capacity of the liver (Strable and Ntambi, 2010). The process of DNL starts with the conversion of acetyl-CoA to malonyl-CoA by ACC. Malonyl-CoA is an important intermediate in the process as its formation marks the point where DNL substrates are committed for conversion into FAs (Chong *et al.*, 2007) (Figure 1.1). Malonyl-CoA subsequently undergoes a series of reactions via the action of FAS, and the end product, palmitate is synthesised stepwise in 2 carbon blocks. The saturated FA (SFA) palmitate is

considered to be the end product of DNL (Hellerstein *et al.*, 1996; Aarsland and Wolfe, 1998) (Figure 1.2).

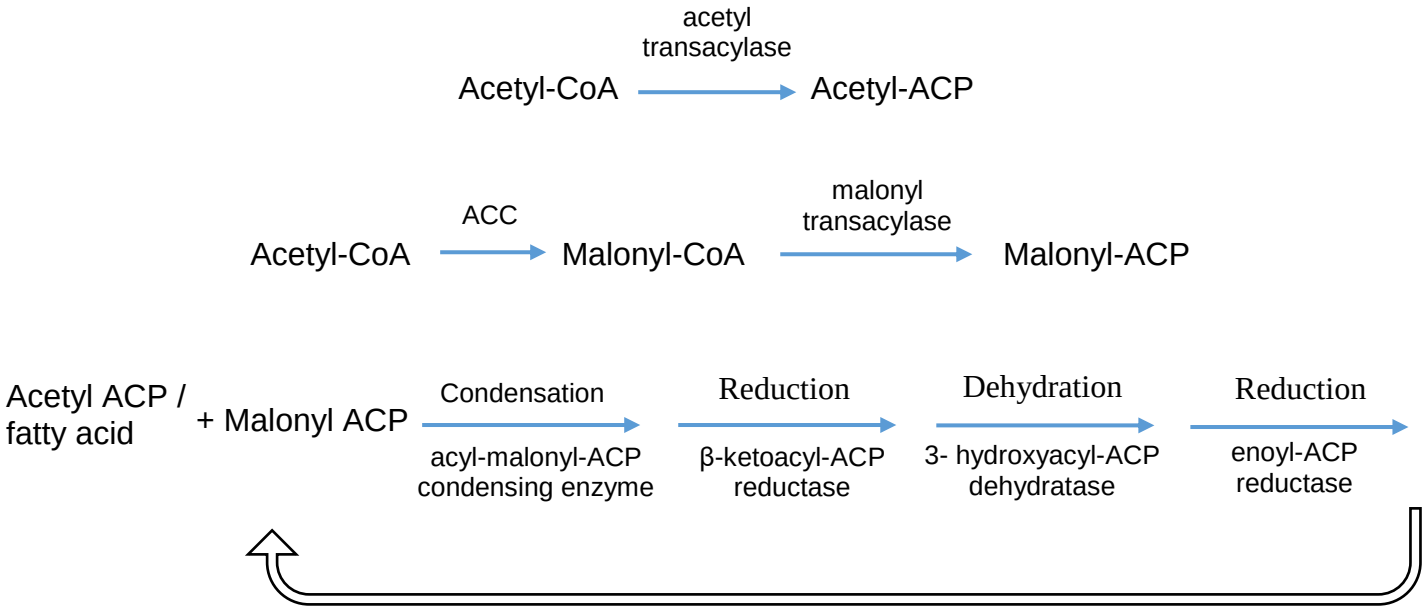

Figure 1.2: Schematic of fatty acid biosynthesis (adapted from Berg *et al.*, 2002)

Palmitate is suggested to be cytotoxic; high amounts of hepatic palmitate may disrupt cellular function and cause oxidative stress to the mitochondria and endoplasmic reticulum via lipid peroxidation (Srivastava and Chan, 2008). To counteract toxicity, it is thought that there is parallel upregulation of the enzyme stearoyl-CoA desaturase (SCD) which desaturates SFAs to form monounsaturated FAs (MUFAs) which have been shown in human studies, *in-vitro* adipocyte studies and rodent work (Chong *et al.*, 2008; Oosterveer *et al.*, 2009; Collins *et al.*, 2011).

With increased consumption of free sugars, enhanced hepatic DNL may alter hepatic FA partitioning towards esterification rather than oxidation (Chong *et al.*, 2007) as malonyl-CoA is a potent inhibitor of the enzyme carnitine

palmitoyltransferase-1 (CPT1) which transports FA into the mitochondria (Figure 1.3) (Foster, 2012), the site of FA β-oxidation.

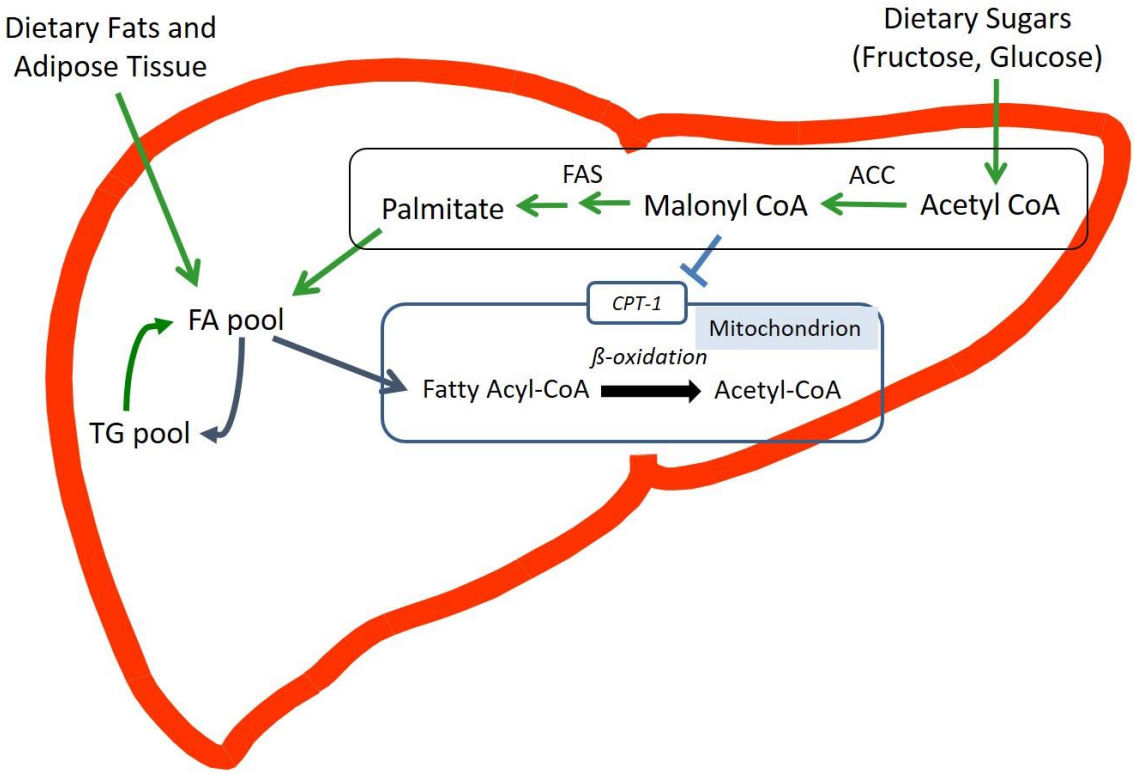

Figure 1.3: Inhibition of carnitine palmitoyltransferase-1 (CPT1) by malonyl-CoA (Foster, 2012).

1.7 Measurement of hepatic *de novo* lipogenesis

Traditionally, indirect calorimetry has been used to evaluate the amount of DNL *in-vivo* in humans. The main limitation of using this method is that it measures net DNL of the whole body, which is the difference between overall DNL and overall fat oxidation and thus is not organ specific (Schwarz *et al.*, 2003). However, whole body net DNL only happens under extreme situations e.g. overfeeding with a high-carbohydrate diet (Schwarz *et al.*, 1995) or during total parental nutrition that had glucose as the main energy source (King *et al.*, 1984).

In the 1990s, research was conducted to quantitatively measure hepatic DNL and two methods were developed: a non-isotopic and an isotopic method. Even though

these methods may produce different values, the measurements obtained are well correlated (Hudgins *et al.*, 2000).

1.7.1 Non-isotopic methods

The non-isotopic method was developed by Hudgins *et al.* in 1996. This method does not quantify the exact amount of DNL but is able to evaluate the percentage change in hepatic DNL between treatments. It is based on the dilution of the essential omega-6 FA linoleate which cannot be synthesised in the body (Hudgins *et al.*, 1996). The method compares the ratio of palmitate (16:0, the end product of DNL) and linoleate (18:2 n-6, the essential FA) in the plasma of individuals. This method has been previously used by Chong *et al.* (2008) in a crossover study to determine differences in plasma FA composition after the consumption of a 3-day isoenergetic high carbohydrate (10% fat; 75% carbohydrates) and a high fat (40% fat; 45% carbohydrates) diet. The team found that the ratio of 16:0 to 18:2 was significantly greater after the high carbohydrate diet than after the high fat diet ($p = 0.02$).

While this method is relatively easy to carry out compared to the isotopic methods, it has the clear disadvantage of requiring an adaptation period and controlled feeding over a prolonged period of time as the FA composition in the adipose tissue should remain constant throughout the study period and lipid turnover in the adipose tissue is a very slow process (Hudgins *et al.*, 1996).

1.7.2 Isotopic methods

Hellerstein *et al.* (1991) infused [^{13}C]acetate over 12 hours after an overnight fast and monitored the rate of [^{13}C]acetate incorporation into VLDL-TG palmitate. They then measured hepatic DNL using the mathematical approach of mass isotopomer

distribution analysis (MIDA) (Hellerstein *et al.*, 1991). In this study, they showed that in healthy humans, fasting DNL was quantitatively very low, contributing less than 1% of total palmitate. Using ^{13}C-acetate to measure hepatic DNL is based on equilibration in the precursor pool of acetyl-CoA as acetate is converted into acetyl-CoA for metabolism and the amount can be calculated through binomial expansion (Moore *et al.*, 2014). The main advantage of this method is that is does not require a long period of controlled diet or adaptation prior to the study. However, it requires a long infusion period of labelled acetate and this may not be practical.

Another tracer that can be used to assess hepatic DNL is deuterated water (^2H_2O). The synthesis of FAs from carbohydrates requires water; specifically during glycolysis when fructose-1,6-bisphosphate is hydrolysed into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Deuterated water incorporated in this process will label the downstream product acetyl-CoA, the final product of oxidative decarboxylation which follows glycolysis (Turner *et al.*, 2013). The labelled acetyl-CoA is then a substrate for DNL, allowing the hydrogens from the deuterated water to be incorporated into the FA molecules and the fractional synthesis of hepatic FAs can then be measured after allowing for the relative proportion of hydrogens donated by NADPH or H_2O (Moore *et al.,* 2014). The main advantage of this method is that water is not a direct substrate of DNL and this method would not artificially introduce additional DNL substrates in the bloodstream and would likely provide a more accurate measure. The disadvantage would be that deuterated water must be consumed the day before by the subject in order to be incorporated into the bloodstream.

1.8 Regulation of hepatic DNL

The hormones insulin and glucagon can regulate the hepatic DNL pathway through their effects on the expression and action of sterol regulatory element-binding proteins (SREBPs) (Horton *et al.*, 2002). SREBP-1c is primarily involved in the expression of genes which code for enzymes including ACC, FAS, and SCD (Shimomura *et al.*, 1998; Horton *et al.*, 2002). Carbohydrate responsive element binding protein (ChREBP) is another transcription factor which is partially responsible for the regulation of expression of lipogenic genes (Yamashita *et al.*, 2001).

The transcription factor liver X receptor (LXR) is also involved in the regulation of DNL (Strable and Ntambi, 2010) through the regulation of SREBP-1c (Repa *et al.*, 2000; Horton *et al.*, 2002) and ChREBP (Cha and Repa, 2007). In addition, Joseph *et al.* (2002) have shown LXRs can independently transcriptionally activate FAS.

1.8.1 Contribution of DNL in fasting and postprandial states

In healthy individuals, the contribution of DNL FAs is typically lower in the fasting state than the postprandial state. For example, Timlin and Parks (2005) found that hepatic DNL increased from 4.7% of total plasma VLDL-palmitate in the fasting state to 18.2% in the postprandial state. In individuals with insulin resistance and/or NAFLD, there is constitutive activation of the DNL pathway even in the fasting state and therefore the contribution of DNL FAs to VLDL-TG is notably higher than in healthy individuals (Tamura and Shimomura, 2005). Newly synthesised palmitate was found to contribute to 26% of palmitate in liver TG as well as 22.5% of lipoprotein-TG in the fasting state in subjects with NAFLD and the contribution to lipoprotein-TG in the postprandial state did not change markedly (Donnelly *et al.*, 2005).

1.8.2 Phenotypes that affect the contribution of postprandial DNL

1.8.2.1 Effects of metabolic syndrome on DNL

The metabolic syndrome is a group of risk factors for the development of cardiovascular diseases which includes hyperinsulinemia, obesity and NAFLD (Bremer *et al.*, 2012). Individuals with metabolic syndrome have been shown to have increased postprandial DNL (Pramfalk *et al.*, 2016).

Insulin is considered a lipogenic hormone as it stimulates lipogenesis in tissues including the liver, increases the uptake of FAs from the blood to the adipose tissue and muscle and decreases the rate of FA oxidation in the muscles and liver (Dimitriadis *et al.*, 2011). It is hypothesised that insulin resistance and its associated hyperinsulinemia would lead to higher DNL levels due to its association with hepatic steatosis (Ameer *et al.*, 2014). The mechanism for hyperinsulinemia leading to higher rates of DNL was suggested to be FoxO inactivation (Haeusler *et al.*, 2014). FoxO is a transcription factor which binds to the promoter of glucose-6-phosphatase (Nakae *et al.*, 2001) and mouse studies have shown that its inactivation would lead to increased hepatic DNL and TG content (Haeusler *et al.*, 2014).

In support of hyperinsulinemia being linked to higher DNL levels, Pramfalk *et al.* (2016) studied 37 healthy, non-obese individuals with varying levels of plasma insulin and divided them into normoinsulinemic (NI) (<11.2 mU/L) and hyperinsulinemic (HI) groups (>11.2 mU/L). The HI group had higher fasting DNL (11.5% of plasma VLDL-TG compared to 7.5% for NI group, $p < 0.05$) and lower FA oxidation ($p < 0.05$) and this difference remained in the postprandial state. The authors suggested that elevated plasma insulin, if sustained would lead to

changes in hepatic fat partitioning and metabolism which may lead to steatosis (Pramfalk *et al.*, 2016).

Insulin resistance and hyperinsulinemia are very closely linked to obesity and it is likely an individual who is obese would also have hyperinsulinemia (Ferrannini *et al.*, 1997). It is thus not surprising that obesity is independently linked and positively correlated with increased DNL levels. This is shown by Marques-Lopes *et al.* (2001) and McDevitt *et al.* (2001). Marques-Lopes *et al.* (2001) conducted a study involving 6 lean and 7 obese men who consumed a high-carbohydrate, low-fat meal and it was found that overweight men had significantly ($p < 0.01$) higher fasting and postprandial fractional hepatic DNL than lean men. In line with this, McDevitt *et al.* (2001) found that the contribution of DNL to VLDL-TG differed significantly between lean and obese subjects fed an isoenergetic diet with normal proportions of macronutrients; DNL was more than 40% greater in terms of overall percentage of hepatic VLDL contribution in the obese subjects than the lean subjects (McDevitt *et al.*, 2001).

Differences in postprandial DNL have also been reported between subjects with and without NAFLD. Fabbrini *et al*. (2008) found that in obese individuals the rate of VLDL-TG secretion in the body was more than 2-fold higher in NAFLD subjects than in those with normal liver fat content, demonstrating that the contribution of DNL was higher in individuals with NAFLD. This is supported by Lambert *et al.* (2014) who found that subjects with NAFLD had significantly greater rates of DNL compared to subjects with normal liver fat levels (2.57 ± 1.53 μmol/min in subjects with NAFLD vs 0.78 ± 0.42 μmol/min in subjects with normal liver fat levels; $p = 0.001$). Umpleby *et al.* (2017) reported that when a group of men with NAFLD

(n = 11) were fed an isoenergetic high carbohydrate diet (26% of total energy intake) or an isoenergetic low carbohydrate diet (6% of total energy intake), they had a higher rate of DNL (by 26% and 183%, respectively) than the control group of subjects without NAFLD (n = 14) fed both diets.

1.8.2.2 Effect of gender on DNL

Males and females have different body fat distribution and lipid metabolism. Males tend to accumulate more visceral fat and exhibit lower whole body FA oxidation than females (Blaak, 2001). As visceral adiposity is linked to hepatic steatosis, it is not surprising that in studies by Browning *et al*. (2004) and Graff *et al.* (2013), Caucasian males have been reported to have a higher prevalence of hepatic steatosis as compared to European American females.

However, despite the differences being seen in observational studies, limited work has been performed in elucidating the mechanism behind the differences in hepatic steatosis between the genders. Differences in DNL and hepatic FA oxidation provide some evidence as increased DNL and decreased FA oxidation are linked to increased hepatic steatosis (Chen *et al.*, 2016; Sanders *et al.*, 2018). In a 6-hour postprandial study by Pramfalk *et al.* (2015), men and women matched for age, BMI and liver fat content had similar fasting hepatic DNL and it was found that men maintained a higher contribution of DNL FAs to VLDL-TG and also had lower dietary hepatic FA oxidation when compared to women when fed the same mixed test meal. This effect was suggested to be caused in part by females having higher oestrogen levels than males. Previous findings in animal studies have also demonstrated the ability of oestrogen to decrease the activity of ACC (Diamant *et al.*, 1975).

1.8.2.3 Effect of genotype and ethnicity on hepatic DNL

$PNPLA_{3,}$ which was a gene mentioned in Section 1.4.1 that when mutated (I148M) confers susceptibility to NAFLD, also has an effect on DNL. Mancina *et al.* (2015) found in a study involving 88 healthy subjects that individuals with the $PNPLA_3$ mutation linked with NAFLD ironically had lower levels of DNL ($p = 0.04$).

In addition, Santoro *et al.* (2015) found that a variant in glucokinase regulatory protein (GCKR) rs1260326 resulted in increased glycolysis in cells and increased hepatic DNL. This finding was reported in a study which involved 14 teenagers where 7 were homozygous for the common allele (CC) and the rest homozygous for the risk allele (TT). After feeding a single meal containing 75 g glucose and 25 g fructose, subjects with TT demonstrated higher fasting fractional DNL ($p < 0.05$) and a lower increase in postprandial fractional DNL ($p < 0.05$).

In support of ethnicity affecting DNL levels, Hudgins *et al.* (2017) compared the lipogenic response to dietary sugar between Asians and Caucasians and found that Asians had higher hepatic DNL and insulin response after consumption of a single beverage made with glucose (1.5 g / kg) and fructose (1.5 g / kg).

1.8.3 Dietary factors affecting DNL

1.8.3.1 Effect of dietary fat on hepatic DNL

High fat diets not only induce hepatic insulin resistance but also impair insulin clearance *in-vitro* and *in-vivo* in animals (Svedberg *et al.*, 1991; Wiesenthal *et al.*, 1999) and humans (Carpentier *et al.*, 2000). This may lead to hyperinsulinemia observed in insulin-resistant states and NAFLD (de Piano *et al.*, 2007; Tock *et al.*, 2010). Consumption of different types of dietary fat may influence the amount of hepatic DNL.

1.8.3.2 Effect of saturated fatty acids on hepatic DNL

Rodent studies have shown that the main effect of SFA on hepatic lipid metabolism is on the levels of gene expression of enzymes required for FA synthesis such as ACC and FAS (Vallim and Salter, 2010). Higher gene expression for these genes have been seen in hamsters but not in mice (Oosterveer *et al.*, 2009; Lin *et al.*, 2012); however, these changes may not translate into increased DNL. Recently, Luukkonen *et al*. (2018) reported that overfeeding by 1000 kcal/day for 3 weeks with either SFAs, unsaturated fats or free sugars resulted in no significant change in DNL in the groups fed SFAs compared to baseline.

1.8.3.3 Effect of unsaturated fatty acids on hepatic DNL

While there is no evidence of MUFA consumption having any effect on DNL, the consumption of PUFAs has been shown, in rodent studies, to downregulate SREBP-1c expression by 63% (Xu *et al.*, 2002). This finding was reproduced in HEK-293 cells perfused with PUFAs (Hannah *et al.*, 2001). The downregulation of SREBP-1c can also be shown through the lowered expression of downstream regulated proteins including ACC and FAS (Jump and Clarke, 1999; Jump *et al.*,

2013). The mechanism by which PUFAs mediate the downregulation of SREBP-1c expression is suggested to be by preventing the activation of the LXRα response element in the SREBP-1c promoter, as shown by knockout and competition luciferase reporter assays with antagonists of the LXRα response element (Howell III *et al.*, 2009).

To further elucidate the effect of PUFAs against that of MUFAs, Hodson *et al*. (2017) conducted a study in 24 NAFLD participants fed 4 g/day of pure omega-3 FAs or 4 g/day of olive oil (mostly oleic acid, a MUFA) for 15 to 18 months. It was found that the group supplemented with omega-3 FAs had a significant decrease in VLDL-TG derived from hepatic DNL while the group supplemented with olive oil exhibited no change (Hodson *et al.*, 2017). A study on 88 healthy subjects also showed that there was no change in DNL after feeding a diet enriched with omega-6 FAs for 3 weeks (Luukkonen *et al.*, 2018).

1.8.3.4 Effect of dietary sugars on hepatic DNL

The composition and amount of dietary carbohydrate may be important in determining the contribution of hepatic DNL to VLDL-TG. For hepatic DNL to be stimulated in a high-carbohydrate diet, Parks *et al.* (1999) showed that starch has to contribute less than half of the carbohydrate component. Using both non-isotopic and isotopic methods, Hudgins *et al.* (1998) demonstrated that when a low-fat diet containing 75% of energy from short-chain glucose polymers was substituted with one that contained 75% of energy from starch, the amount of newly synthesised FAs from DNL appearing in VLDL-TG significantly decreased.

McDevitt *et al.* (2001) reported that the contribution of DNL FAs to VLDL-TG did not vary with the overconsumption of sucrose and glucose. DNL was measured using

deuterated water during 96 hours of overfeeding by 50% more energy with either sucrose or glucose and this was compared to a control energy balance intervention in 8 lean and 5 obese women. In all the subjects, DNL was 2- to 3-fold higher after overfeeding of either sugar than after the isoenergetic control diet. In a study by Lammert *et al.* (2000), subjects who consumed hyperenergetic carbohydrate-rich diets had significantly higher DNL compared to those consuming hyperenergetic fat-rich diets (20% vs 3% DNL contribution to VLDL-TG respectively).

1.8.3.5 Effect of fructose compared to glucose on hepatic DNL

Although glucose and fructose have the same empirical formula, they differ in several ways including absorption, insulin response and metabolic properties in the body. In addition, glucose and fructose also exhibit differential effects on DNL. Hudgins *et al.* (2011) found that dietary fructose was more lipogenic than glucose and increased hepatic DNL within hours of consumption.

A number of studies have involved feeding high-carbohydrate diets containing one type of monosaccharide, for example only glucose or only fructose. As diets containing just one type of monosaccharide are rarely consumed, it would be more relevant to report on diets containing a combination of glucose and fructose.

1.8.3.6 Effect of co-consumption of fructose and glucose on hepatic DNL

Fructose is rarely consumed in isolation and is typically consumed with glucose; an example of this being high fructose corn syrup (HFCS). HFCS is by composition not very different from sucrose but has sparked great interest in the scientific world in the past few decades. Evidence from epidemiology studies suggest that the severity of NAFLD is related to the increase in consumption of HFCS (Basaranoglu *et al.*, 2015).

When co-ingested with glucose, fructose absorption has been reported to be faster and more complete (Rumessen and Gudmand-Høyer, 1986; Choi *et al.*, 2003). This has been proposed to be due to glucose's ability to increase the activity of the fructose transporter GLUT5, as shown in an *in-vitro* cellular model of Caco-2 cells (Mesonero *et al.*, 1995).

Several studies have been carried out with the co-consumption of fructose and glucose to determine their combined effect on the contribution on hepatic DNL. In 2008, Parks *et al.* performed a study in 6 healthy subjects to determine if consumption of a mixture of fructose and glucose in different proportions consumed as a single bolus would stimulate hepatic DNL. They found that fructose, when consumed with glucose in a 1:1 ratio or a 3:1 ratio stimulated lipogenesis by 2-fold and elevated plasma TG levels by 76-200% when compared to glucose only. The increase in plasma TG levels was partly due to increased DNL (Parks *et al.*, 2008). In line with these findings, Hudgins *et al.* (2011) found that consumption of a glucose drink did not increase newly synthesised palmitate levels whilst consumption of fructose alone did. However, co-ingestion of the same amount of glucose and fructose in a ratio of 1:1 enhanced hepatic DNL, as evidenced by a 2-fold increase in newly synthesised palmitate in VLDL-TG when compared to the consumption of fructose alone.

1.9 Effect of hepatic DNL on insulin resistance and hepatic metabolism

Increased hepatic DNL is suggested to contribute towards the development of insulin resistance (Zammit, 2013). The cause and effect between the two is unclear but it is likely that they form a vicious cycle where both contribute to each other's severity (Farese *et al.*, 2012). In a study by Fabbrini *et al.* (2008), both plasma insulin concentrations and the HOMA2 index of insulin resistance in non-diabetic, obese subjects were significantly higher in those with NAFLD compared to individuals without NAFLD. Not everyone who has NAFLD has insulin resistance, but the reverse often holds true (Mills *et al.*, 2018). As a result, insulin resistance or sensitivity, together with the amount and rate of insulin secretion, could be good indicators of whether an individual is at increased risk of developing NAFLD (Gaggini *et al.*, 2013).

The effect of DNL on hepatic metabolism is that it may change hepatic FA partitioning but not directly via the SFA it produces. Chong *et al.* (2007) suggested that hepatic DNL may have a significant impact on the selective hepatic partitioning of FAs towards esterification rather than oxidation pathways.

1.10 Hepatic fatty acid partitioning

From the FA pool, hepatic FAs are partitioned between esterification and oxidation pathways (Hodson and Frayn, 2011). Changes in hepatic FA synthesis or partitioning may be important in the development and progression of hepatic steatosis.

1.10.1 Esterification of hepatic fatty acids

In the liver, esterification of FAs predominantly leads to the formation of TGs with the production of other lipids including cholesteryl esters and phospholipids (Hodson *et al.*, 2009). The biosynthesis of TG begins with the sequential acylation of glycerol-3-phosphate (G-3-P) by long-chain acyl-CoA thioesters to produce phosphatidic acid (PA). PA is hydrolysed by PA phosphatase (also known as lipin) to form diacylglycerol (DAG) which in turn undergoes acylation by diacylglycerol transferases (DGATs) to form TG (Hodson and Fielding, 2010; Coleman and Mashek, 2011).

VLDL is formed when the synthesised TG binds to the protein ApoB-100 through the action of microsomal triglyceride transfer protein (MTTP) (Sparks *et al.*, 2012). VLDLs are then exported out of the liver into the circulation, where the TGs within the VLDLs are transported to various organs for metabolism or storage. The remaining TGs can then accumulate in the liver, leading to hepatic steatosis (Hodson and Fielding, 2010; Fon Tacer and Rozman, 2011).

VLDL production is also highly dependent on the expression of enzymes and secretory proteins involved in this process. This is in turn controlled by hormones such as insulin, which suppresses VLDL-1 ApoB production (Hodson and Fielding, 2010). MTTP is a transfer protein essential for the assembly and export of VLDL particles in the liver (Sparks *et al.*, 2012). Human MTTP polymorphisms which lead to decreased MTTP activity are associated with greater TG accumulation in the liver and such individuals are more likely to have NAFLD (Fon Tacer and Rozman, 2011).

1.10.2 Hepatic fatty acid oxidation

During FA oxidation, fatty acyl-CoA formed through metabolism of FAs enter the mitochondrion via CPT1 in the form of fatty acyl-carnitine which is converted back to fatty acyl-CoA with the regeneration of CPT1 completing this cycle (Longo *et al.*, 2016). Fatty acyl-CoA then undergo β-oxidation, producing acetyl-CoA which can either enter the Krebs (or tricarboxylic acid) cycle or ketogenesis. In 1970, Havel *et al.* reported that in healthy individuals, after an 18-hour fast, about 2 times more FAs which entered the liver underwent ketogenesis as compared to esterification for the formation of VLDL. The amount of FAs that underwent ketogenesis was also 4 times more than the amount of FAs that went through the hepatic TCA cycle (Havel *et al.*, 1970). Mashek (2013) reported that incomplete FA oxidation to ketone bodies is the major contributor of hepatic FA catabolism, with complete oxidation to carbon dioxide being the minor component, which may explain the lower respiratory exchange ratio (RER) of fat compared to carbohydrate as a much higher percentage of carbohydrate is converted to carbon dioxide than fat is.

1.10.2.1 Ketogenesis

Ketogenesis produces the ketone bodies acetone, acetoacetate and 3-hydroxybutyrate (3OHB). They are produced from acetyl-CoA, an important intermediate from both carbohydrate and fat metabolism through the action of the enzymes β-Hydroxy β-methylglutaryl-CoA synthase and lyase (McGarry and Foster, 1976). While acetoacetate is the main ketone produced by the liver, the primary ketone circulating in the blood is 3OHB, which is often used as a proxy marker for hepatic oxidation as it is not utilised by the liver (Hodson *et al.*, 2007; Paoli *et al.*, 2015).

3OHB levels are highest in the fasted state due to the action of the hormone glucagon, which has the opposite effect of insulin. Glucagon increases lipolysis of adipose tissue leading to an increase in substrate availability for ketogenesis (Foster and McGarry, 1982). In the transition from the fasted to postprandial state, under the influence of insulin, the production of malonyl-CoA, an intermediate in the DNL pathway increases, decreasing entry of FAs into the liver's mitochondria and hence decreasing FA oxidation and ketogenesis (Alberti *et al.*, 1978; Foster and McGarry, 1982). Excess acetyl-CoA from β-oxidation of FAs can be directed towards hepatic ketogenesis (Cotter *et al.*, 2014).

1.10.2.2 Tricarboxylic acid cycle (TCA)

The amount of TCA cycle substrate flux indicates the amount of oxidation occurring in the mitochondria of a cell, with elevated TCA cycle functioning as a surrogate indicator for increased amounts of substrate oxidation (Sunny *et al.*, 2011). Individuals with NAFLD have been reported to have a 2-fold increase in TCA cycle flux. Hepatic TG content was also found to be positively correlated with hepatic TCA cycle flux (Sunny *et al.*, 2011). This increase is suggested to be linked to elevated energy requirements within the fatty liver, including the induction of gluconeogenesis (Walter *et al.*, 1966). However, Sunny *et al.* (2011) suggested that the increased levels of gluconeogenesis alone were not sufficient to account for the 2-fold increase in TCA cycle flux, and that mitochondrial damage and reduced efficiency may account for the difference (Serviddio *et al.*, 2008; Caldwell *et al.*, 2009).

1.11 Fructose metabolism

Fructose is a commonly found monosaccharide present in many types of food, both alone and in combination with glucose as sucrose. Fasting plasma concentrations of fructose are almost undetectable at around 0.01 mmol/L in adults (Bray 2007); which is notably lower than 5.5 mmol/L for glucose. Given the large difference between the plasma concentrations of the two monosaccharides, it is logical to consider the differences in their respective metabolism after consumption by humans, with a focus on fructose metabolism.

Upon ingestion, the adsorption of fructose by human body is largely via the brush border transporter GLUT5 in the jejunum of the small intestine and the liver, and by carrier-mediated facilitative diffusion (Riby *et al.*, 1993; Putkonen, 2013; Douard and Ferraris, 2008). The process of fructose absorption into the enterocytes requires energy and is therefore limited when compared to glucose which is adsorbed by multiple glucose transporters. Fructose that is not adequately absorbed by the intestinal enterocytes undergoes bacterial fermentation, producing short-chain fatty acids and gases including hydrogen, carbon dioxide and methane (Ebert and Witt, 2016). This process results in an osmotic force that raises fluid influx into the lumen. When consumed alone in large amounts, fructose malabsorption can lead to symptoms such as bloating, excessive gas, abdominal pain, irritable bowel syndrome and diarrhoea (Shepherd and Gibson, 2006; Fedewa and Rao, 2014). However, co-ingestion with glucose improves fructose absorption (Latulippe and Skoog, 2011). Figure 1.4 illustrates the differences between metabolism of fructose and glucose in the intestinal enterocyte.

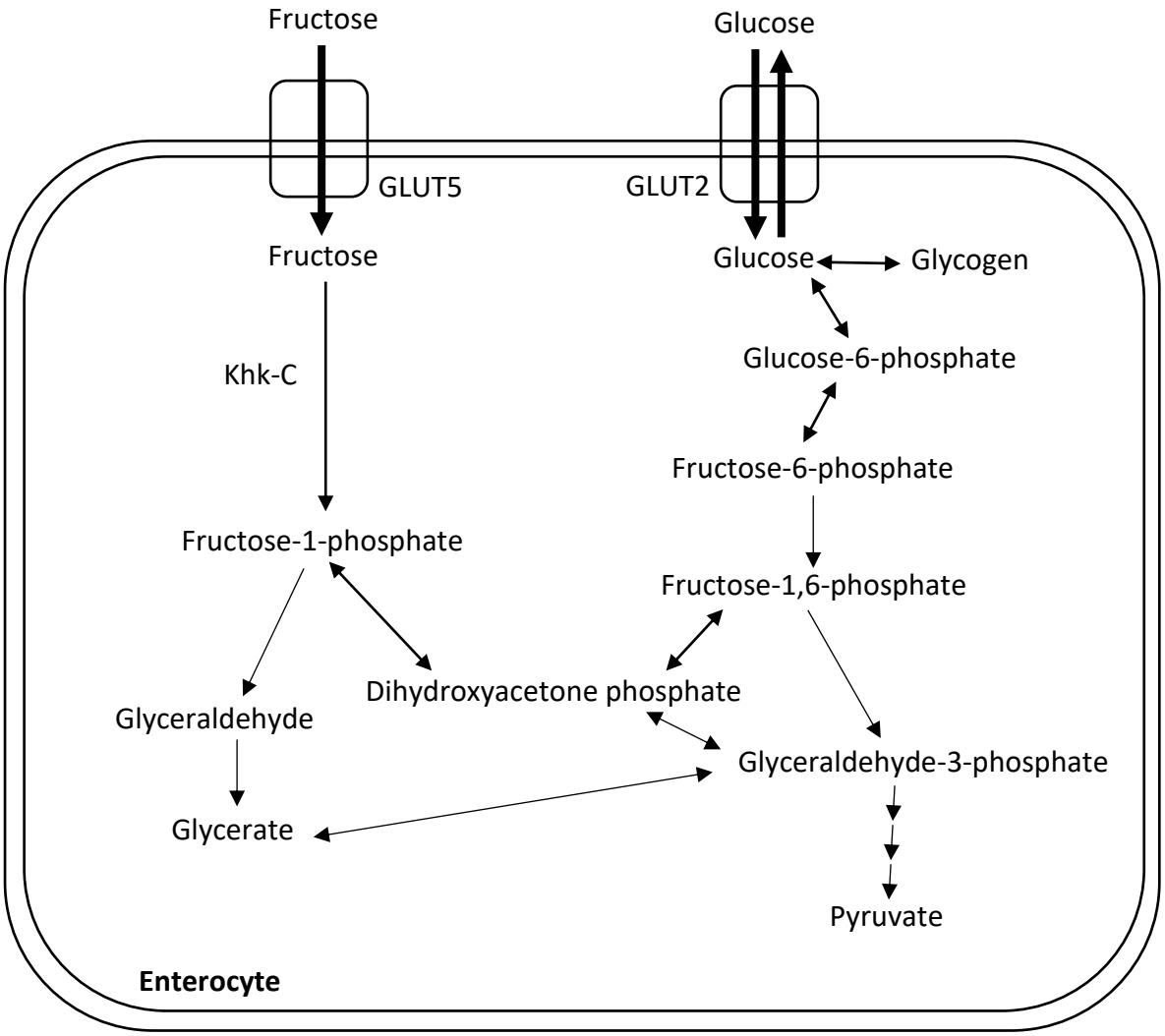

Figure 1.4: An illustration of fructose and glucose metabolism in intestinal enterocytes. Abbreviation: Khk-C, Ketohexokinase-C (adapted from Jang *et al.*, 2014).

The small intestine takes in arterial blood and releases it via the portal vein. Examination of metabolites in the portal blood suggested intestinal metabolism of fructose may also occur (Jang et al., 2014). The portal blood is enriched in metabolites from the intestinal lumen and those made via intestinal metabolism. From the small intestine, blood travels directly to the liver via the portal vein (Laughlin, 2014) (Figure 1.5).

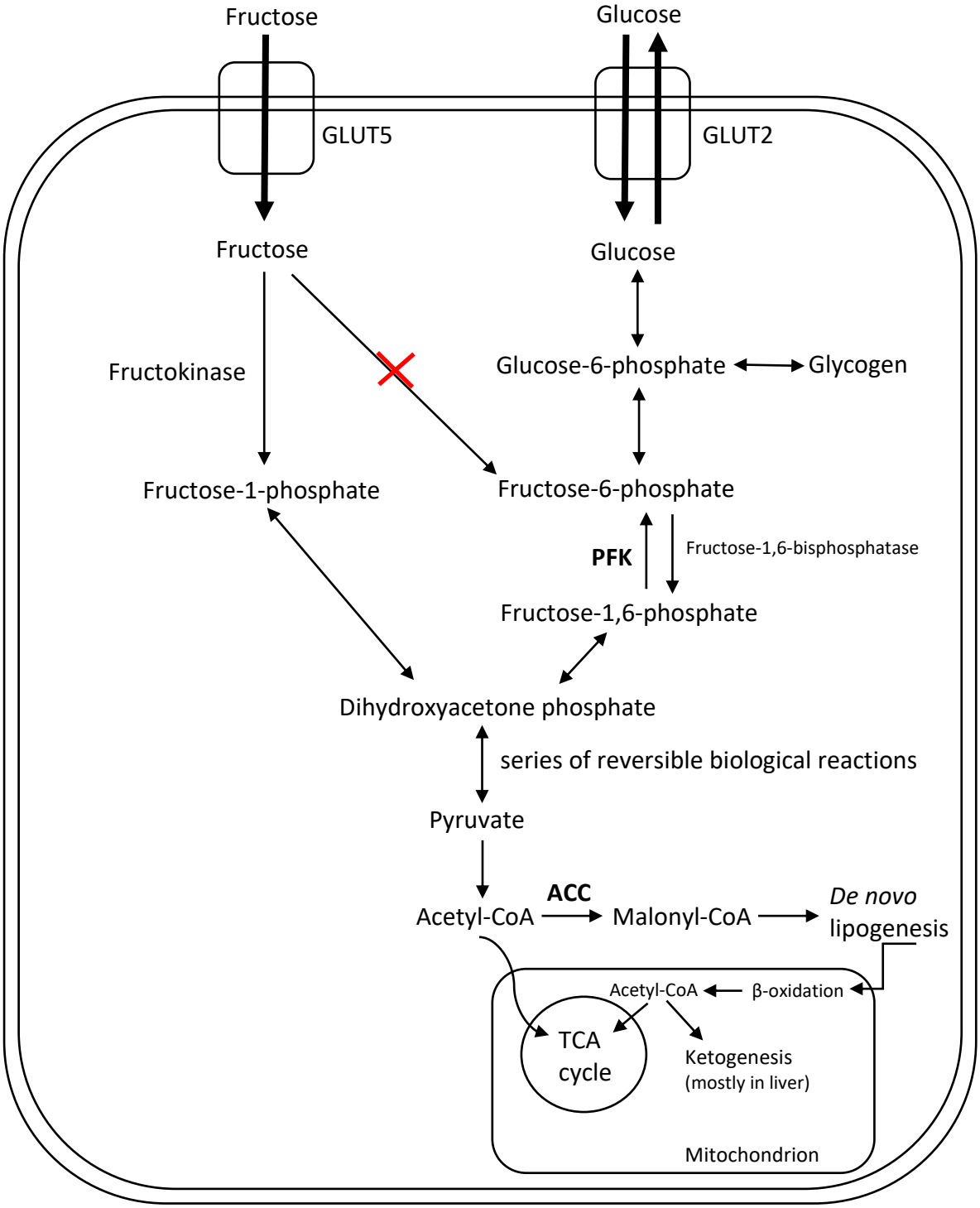

Figure 1.5: An overview of the differences in glucose and fructose metabolism in the liver. Abbreviations: GLUT, glucose transporter; PFK, phosphofructokinase; ACC, acetyl-CoA carboxylase; TCA cycle, tricarboxylic acid cycle (adapted from ter Horst and Serlie, 2017)

Both the small intestine and the liver have been suggested to be possible sites of fructose metabolism. Transcriptional data by Ishimoto *et al.* (2012) suggested that in mice, liver is the key site of fructose catabolism as it had the highest expression of ketohexokinase-C (Khk-C) which is the main isoform of fructose-phosphorylating enzyme Khk required for fructose clearance (Ishimoto *et al.*, 2012). In contrast, a recent mice study carried out by Jang *et al.* using continuous intravenous infusion of [U^{13}C]fructose showed that about 90% of dietary fructose is metabolised in the small intestine which shields the liver from being exposed to fructose by converting fructose to glucose and organic acids (Jang *et al.*, 2018). Within the intestinal enterocytes, fructose can also be converted to lactate and gluconeogenesis occurs to as much as 10-30% of all absorbed fructose (Sun and Empie, 2012; Patel *et al.*, 2015). This shielding capacity of the small intestine however, diminishes when fructose is consumed in high doses, saturating the intestinal clearance capacity. The balance between the consumption of fructose and its clearance by the small intestine determines the extent of the liver's exposure to dietary fructose (Jang *et al.*, 2018). In the liver, a large proportion of fructose is converted into glucose (about 50%). The glucose derived from fructose is either stored as hepatic glycogen or released into the plasma (Nilsson and Hultman, 1974; Tounian *et al.*, 1994).

As seen in Figure 1.5, in the liver, fructose is processed through the enzyme fructokinase while glucose is processed by glucokinase and then phosphofructokinase-1 (PFK1) to form fructose-6-phosphate (Mayes, 1993). The activity of PFK1 is tightly controlled by the hormones insulin and glucagon (Müller *et al.*, 1988; Payne *et al.*, 2005) and represents the rate limiting step for glycolysis. Fructose bypasses PFK1 and its controlling mechanisms, leading to a much larger flux through the glycolysis pathway as compared to similar amounts of glucose

(Mayes, 1993) (Figure 1.5). In addition, due to its regulation by PFK1, hepatic glucose uptake is inhibited when the energy status is high in the liver as PFK1 is inhibited by ATP and citrate. In contrast, fructose metabolism is not dependent on energy status, resulting in unregulated uptake by the liver and increased intrahepatic DNL (Stanhope *et al.*, 2009). Studies carried out by Lê *et al.* (2009) and Lecoultre *et al.* (2013) have shown that hyperenergetic diets supplemented with fructose increased hepatic fat content, as reviewed by Moore *et al.* (2014).

1.12 Insulin

Insulin, a hormone produced in and secreted by the β-cells of the pancreas, is important for normal metabolism. The precursor of insulin, proinsulin, is converted to insulin after cleavage of C-peptide. Insulin and C-peptide are stored in secretary granules awaiting release on demand (Fu *et al.*, 2013).

Insulin has numerous metabolic roles and functions – signalling and regulating the catabolism of carbohydrates and fats (Saltiel and Kahn, 2001). In addition to maintaining fasting plasma glucose levels between 4.4 to 6.1 mmol/L (Liu *et al.*, 2012), insulin is also needed for the synthesis of bile acids (Li *et al.*, 2012). In insulin sensitive individuals, with the ingestion of exogenous glucose and the subsequent secretion of insulin, glucose is rapidly taken up by muscles and hepatic glucose production is almost completely suppressed (Seino *et al.*, 2011). In the process of lipid metabolism, insulin inhibits the lipolysis of adipose tissue TG stores (Jensen *et al.*, 1989; Lafontan and Langin, 2009) and the secretion of hepatic VLDL-TG into the circulation (Lewis, 1993; Lewis, 1994).

1.12.1 Insulin secretion

Insulin secretion involves a sequence of events in β-cells that lead to fusion of secretory granules with the plasma membrane. Glucose stimulation is the primary trigger for insulin secretion, while other nutrients such as free FAs and amino acids can augment the response to glucose (Fu *et al.*, 2013). The autonomic nervous system can also stimulate insulin secretion (Grote and Wright, 2016). In addition, various hormones, such as melatonin, oestrogen, leptin, growth hormone, and glucagon-like peptide-1 (GLP-1) have been found to regulate insulin secretion (Fu *et al.*, 2013).

The study of insulin secretion is important as it is widely acknowledged that irregularities in its dynamics, especially an abnormality in the first phase of glucose-stimulated insulin secretion (GSIS), is characteristic of an early manifestation of β-cell dysfunction that precedes the development of type 2 diabetes (Seino *et al.*, 2011).

1.12.2 Mechanistic pathway of insulin secretion

Upon sensing a change in the concentration of plasma glucose, under normal circumstances, the β-cell produces the amount of insulin required to restore glucose homeostasis. This process is co-ordinated using the stimulus-secretion coupling system (Herman and Kahn, 2006).

Glucose is transported into the β-cell by the glucose transporters (mainly GLUT2) (Seino *et al.*, 2011). As glucose enters the β-cells, it is metabolised rapidly and leads to the generation of ATP. The rise in the ratio of ATP to ADP closes ATP-sensitive K^+ (KATP) channels which subsequently depolarise the β-cell membrane and open the voltage-dependent Ca^{2+} channels, allowing the influx of Ca^{2+}

(Ashcroft, 2005). The increase in concentration of cytoplasmic Ca^{2+} triggers the release of insulin granules by exocytosis (Detimary *et al.*, 1996). In addition to the KATP channel-dependent pathway, Henquin (2000) found a KATP channel-independent pathway.

Insulin secretion from the pancreatic β-cells occurs in the form of insulin secretory granules. There are two pools of granules: 1) the reserve pool, which forms the majority of the granules and 2) the readily releasable pool, which accounts for less than 5% of the total amount (Hou *et al.*, 2009). Rorsman and Renström (2003) hypothesised that the release of granules from the readily releasable pool constitutes the first phase of insulin secretion (rapid secretion of preformed insulin) and the second phase is the conversion of granules from the reserve pool to the readily releasable pool (representing newly formed insulin).

1.12.3 Dynamics of insulin secretion

Insulin is directly secreted into the portal system (Song *et al.*, 2000). The concentration of insulin in the blood oscillates and is likely to be caused by intrinsic β-cell mechanisms, with modification by exogenous signals such as membrane potential and action of kinases and pumps (Gilon *et al.*, 2002). This oscillation was also found to occur during postabsorptive periods (Lefèbvre *et al.*, 1987; Weigle, 1987) in both lean and obese individuals with a similar periodicity of 10 to 12 mins (Seino *et al.*, 2011). Oscillation in insulin secretion is suggested to be directly caused by constant changes in the concentration of Ca^{2+} (Gilon *et al.*, 2002).

When glucose is introduced into the body rapidly in the form of a bolus or by infusion, a biphasic insulin response has been observed (Curry *et al.*, 1968). The biphasic insulin response comprises of a first phase which occurs instantaneously

upon stimulation with a rapid rise and decay of a peak occurring within 3 to 4 mins, an inter-peak nadir, and a slower but increasing in-time second phase that can last for hours with constant exposure of β-cells to glucose (Caumo and Luzi, 2004). The magnitude in insulin secretion in the first phase is dose-dependent and follows a sigmoidal function of glucose concentration, with a half-maximum of about 7.5 mmol/L (Caumo and Luzi, 2004).

However, under physiological postprandial conditions, with the gradual increase in glucose concentration, instead of being biphasic, insulin secretion is pulsatile; with the absence of a well-defined first phase (Grodsky, 1972; Sorenson *et al.*, 1980). In both normal weight and obese individuals, insulin secretory pulses take place every 1.5 to 2 hours. The amplitude of the pulses is greater in obese subjects as compared to lean individuals (Caumo and Luzi, 2004).

1.12.4 Modelling of insulin secretion from C-peptide concentrations

Insulin secretion is difficult to measure as it is continually being extracted to a variable degree by the liver, kidneys, and to some extent by other organs. Therefore, its measurement is often performed in conjunction with the measurement of C-peptide, which is a by-product of insulin synthesis, and is released in equimolar amounts with insulin but is not extracted by the liver (Pacini and Cobelli, 1988). C-peptide has a much longer half-life (35 mins) than insulin (10 mins) (Ferrannini and Cobelli, 1987; van Cauter *et al.*, 1992). The gold standard kinetic model to derive C-peptide secretion from C-peptide concentrations is known as the Eaton-Polonsky approach (Eaton *et al.*, 1980; Polonsky *et al.*, 1986). It involves a deconvolution process, which is the mathematical removal of the impact of substance kinetics from the concentration measured. The kinetics of C-peptide is

determined in a separate experiment where biosynthetic C-peptide is injected in a single bolus and a decay curve is obtained and analysed (Eaton *et al.*, 1980; Polonsky *et al.*, 1986).

Toffolo *et al.* (1995) described another approach to measure insulin secretion, known as the “minimal model” that uses only C-peptide measurements. A unique feature of the minimal model is that it takes into consideration glucose control on β-cell insulin secretion and can provide information on β-cell responsiveness to glucose which is important in subjects with impaired mechanisms of insulin secretion. The minimal model was originally developed to help interpret intravenous glucose tolerance test data but has also been adapted to interpret oral glucose tolerance test data (Toffolo *et al.*, 1995; Breda *et al.*, 2001). The oral minimal model enables for the calculation of indices of insulin secretion such as Φ_s and Φ_d which reflect the inherent ability of the pancreas to secrete insulin at a fixed concentration of glucose (Φ_s) and in response to a given increase in glucose concentration (Φ_d) respectively (Breda *et al.*, 2001). The minimal model has been validated against deconvolution in experiments with slow changes in insulin secretion (Breda and Cobelli, 2001).

Despite all the above methods for the measurement of insulin secretion being based on the measurement of C-peptide concentrations, qualitatively, the profile of plasma insulin concentration is better than that of C-peptide in reproducing the shape of the profile of insulin secretion (Shapiro *et al.*, 1988). Alternative approaches which rely only on plasma insulin concentrations to estimate first and second phase insulin secretion have been developed. A crude method is to derive insulin secretion indices from calculating areas under the insulin concentration

curve during a hyperglycemic clamp (0-10 mins for 1st phase, >10 mins for 2nd phase) (Caumo and Luzi, 2004).

1.12.5 Experimental methods to estimate insulin secretory responses

There are various ways to measure insulin secretory response. However, to date, there is no consensus as to which should be considered the gold-standard; the method chosen should be based on the question being addressed. It has been proposed that intravenous glucose administration is a more appropriate way to evaluate the first phase of insulin secretion (also known as “acute response to glucose”) while the use of hyperglycemic clamp is more suitable for the measurement of the second phase (Rachman *et al.*, 1996; Fritsche *et al.*, 2000). In addition to the intravenous glucose tolerance test (IVGTT) and the hyperglycemic clamp technique, insulin secretion can be estimated reliably by using an oral glucose tolerance test (OGTT) or feeding a mixed meal and subsequent minimal modelling.

An IVGTT requires frequent blood sampling at 1 to 2 mins intervals during the first 10 mins of the test to determine the acute insulin secretory response of an individual. A bolus of glucose, often tailored according to body weight of the individual, is given into the antecubital vein and blood sampling occurs at designated intervals to assess the concentration of plasma glucose, insulin and C-peptide. Insulin sensitivity of an individual can then be obtained from the area under the curve (AUC) of insulin and C-peptide during IVGTT (Hahn *et al.*, 2011).

The hyperglycemic clamp technique allows assessment of three physiological variables: 1) the amount of insulin secretion in the early and late phases, 2) the amount of glucose metabolised during the clamp and, 3) the insulin response of

β-cells (Porte and Pupo, 1969; Sowell *et al.*, 2003). In a hyperglycemic clamp, the plasma concentration of glucose is acutely raised to beyond physiological levels by means of exogenous infusion of glucose (e.g. 7 mmol/L above baseline values). The hyperglycemic plateau is maintained for 4 hours after glucose infusion commenced with constant blood sampling for the measurement of the concentrations of insulin and C-peptide. The first phase insulin secretory response to hyperglycemia is obtained from measurements between 0 to 10 mins. The second phase insulin secretion under hyperglycemic conditions is determined from measurements between 10 mins and 4 hours (Sowell *et al.*, 2003).

With the glucose bolus given during the IVGTT and the higher than physiological levels of glucose administrated in the process of the hyperglycemic clamp, both methods do not reflect conditions under normal physiological conditions. This is because insulin responses are not linearly related to plasma glucose concentrations and results obtained are therefore not completely predictive of physiological conditions (Porte Jr. and Pupo, 1969). However, these techniques represent important tools which allow us to determine whether defects exist in the different stages of insulin secretion (Seino *et al.*, 2011).

The OGTT, like the IVGTT, is based on a single glucose bolus that is given orally. The use of a physiologic stimulus in OGTT allows the possibility to measure insulin sensitivity with a modified algorithm based on the minimal model as well as glucose effectiveness and insulin secretion. A similar approach can be used for mixed meals, where the stimulus for insulin secretion are all macronutrients and not just glucose. The main limitation of oral tests is that they are unable to differentiate between peripheral tissue insulin resistance and the reduced capacity of β-cells for producing insulin in diabetic subjects (Muniyappa *et al.*, 2008).

1.12.6 Quantifying the rate of insulin secretion

Understanding dynamic *in-vivo* systems often requires making assumptions about how the system works and describing them mathematically. In the case of insulin secretion modelling, compartmental models are often used. This includes the minimal model which describes the movement of material through the various compartments of the system by using differential equations (Mari *et al.*, 2001).

The rate of insulin secretion can be described by two components. They are related to the production of new insulin by β-cells and the secretion of insulin. At any time-point, the production of new insulin is controlled by plasma glucose concentration and this is known as the static glucose control (Φ_s). The secretion of insulin is controlled by the rate of increase in plasma glucose concentration and this is known as the dynamic glucose control (Φ_d). Dynamic glucose control can be influenced by the hormones such as oxytocin (Scerbo and Gerdes, 2017). Both Φ_s and Φ_d have been translated physiologically, with Φ_s relating to insulin granule translocation to the membrane and Φ_d relating to the exocytosis of insulin granules docked to the membrane (Pedersen *et al.*, 2010).

By measuring the kinetics of C-peptide, the insulin secretion rate can be derived from the C-peptide and glucose concentrations at different time-points and a model constructed for the rate of insulin secretion. The minimal model assumes a linear relationship between insulin secretion rate (ISR) (pmol/min) at steady state and glucose concentration. It is expressed as follows:

$$ISR_{ss} = ISR_b + \beta V_1(G_{ss}-G_b)$$

Where ISR_{ss} is insulin secretion rate at steady state, ISR_b is the basal insulin secretion rate, G_{ss} is the plasma glucose concentration at steady state, G_b is the basal plasma glucose concentration, $\beta=10^{-9}$ min^{-1} and V_1 is the C-peptide distribution volume (Cobelli *et al.*, 2014).

The minimal model describes two connected pools of insulin in the β-cells. A diagrammatic description of this model is shown in Figure 1.6.

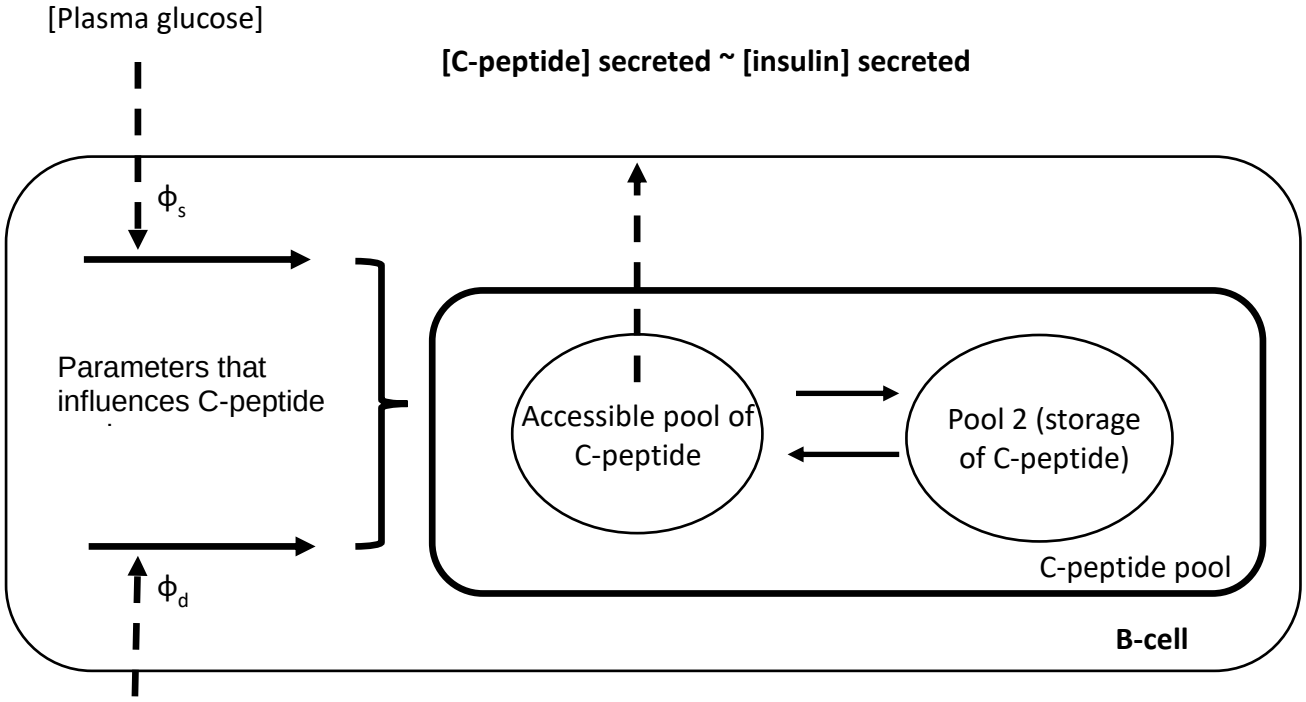

Figure 1.6: Diagrammatic description of the C-peptide oral minimal model which is used to approximate insulin secretion. Insulin secretion is modelled on two parameters which are in turn dependent on the plasma glucose concentration and the rate of increase of plasma glucose concentration (δg/δt) respectively. C-peptide concentration in the β-cell is modelled as two interconnected compartments, which are in turn influenced by the parameters that affect insulin secretion (Figure adapted from Cobelli *et al.*, 2014).

1.12.7 Factors affecting insulin secretion

1.12.7.1 Hormones and signalling molecules

Hormones such as incretins have important roles in the regulation of insulin secretion (Seino *et al.*, 2011). Oral consumption of glucose induces significantly greater insulin secretion compared to intravenous glucose injection, even when plasma glucose concentrations are matched (Nuttall and Gannon, 1991). The "incretin effect" reflects the difference between oral and intravenous glucose administration on insulin secretion and is mediated by gastrointestinal hormone

incretins, such as glucose-dependent insulinotropic polypeptide (GIP) and GLP-1 (Nauck, 2009), which potentiate GSIS by activating the cAMP signalling in pancreatic β-cells (Miki *et al.*, 2005). It has been reported that “the incretin effect” is responsible for about 60-70% of GSIS in normal healthy subjects (Holst, 2007). Studies carried out using intravenous bolus glucose administration and hyperglycemic clamps have reported that incretin hormones including GIP and GLP-1 potentiate the first and second phases of GSIS (Quddusi *et al.*, 2003).

1.12.7.2 Dietary factors

The amount of insulin secretion is versatile and can be affected and regulated by dietary intake (Fu *et al.*, 2013). β-cells respond to nutrients in the circulation which includes glucose, other monosaccharides, amino acids and FAs. About 5 times more insulin is secreted after ingestion of 75 g of glucose after 30 mins compared to the same amount of protein or fat (Chang and Goldberg, 1978).

β-cells have several means of sensing circulating glucose levels. The first is via glucose transporter 2 (GLUT2) which is one of the main glucose transporters expressed in β-cells. Glucose enters β-cells via GLUT2-mediated facilitated diffusion (Simmons, 2017). There is also the enzyme glucokinase, which phosphorylates glucose intracellularly and this is the rate-limiting step in β-cell glucose metabolism. Glucokinase is not inhibited by its substrate or its product and has a much lower affinity for glucose than other hexokinases, which allows it to play an important glucose sensing role in β-cells (Suckale and Solimena, 2008).

Co-consumption of different macronutrients can also have an effect on insulin secretion. In 1998, Gannon and team investigated insulin secretion in untreated type 2 diabetic individuals when fructose was co-ingested with protein. After an

overnight fast, 7 male subjects were fed either 25 g fructose, or 25 g protein, or 25 g fructose plus 25 g protein, or water and their plasma concentrations of glucose, insulin and C-peptide were measured over 5 hours. Results showed that insulin response to the co-consumption of fructose and protein were additive compared to either fructose or protein alone when corrected for subject's body weight and the type and amount of protein consumed (Gannon *et al.*, 1998). Gannon *et al.* (1998) suggested that fructose and protein stimulate insulin secretion by independent pathways which remain to be elucidated.

The ability of fructose to potentiate GSIS has been reported by many investigators although its occurrence under physiological concentrations of fructose requires further investigation as its mechanism remains unclear (Nuttall and Gannon, 1991). In 1968, Curry *et al.* reported that fructose could potentiate insulin secretion in combination with glucose as compared to glucose alone. A possible reason for this is that some fructose may have been enzymatically converted to glucose within the intestinal mucosa. This leads to a higher plasma glucose concentration as compared to consuming glucose alone, resulting in augmented insulin secretion (Holdsworth and Dawson, 1965; Peterson and Reaven, 1971). This finding is in agreement with that of Crapo *et al.* (1976) who found that when glucose and sucrose were given in equimolar amounts, consumption of sucrose produced greater insulin response than glucose even though they produced similar glycemic responses. In a study carried out with untreated type 2 diabetic patients, insulin responses were determined after consumption of foods that contained 50 g of fructose, sucrose or glucose with fructose. Investigators found that the response of insulin to the co-ingestion of fructose was additive or less than additive but not synergistic. No difference was found in the amount of insulin secreted with

consumption of sucrose compared to co-consumption of glucose and fructose (Gannon *et al.*, 1986).

In addition to monosaccharides and sucrose, FAs can also stimulate insulin secretion. When cultured pancreatic β-cells were perfused with SFAs and glucose compared to glucose alone, a 2-fold increase in the amount of insulin secretion was found (Cen *et al.*, 2016). However, when the process was conducted over a continuous 24-hour period, decreased secretion of insulin due to β-cell death was observed (El-Assaad *et al*., 2003). Collier *et al.* (1988) found that after a meal containing 75 g carbohydrate and 37.5 g fat, peak insulin levels obtained in response to a single 5 g intravenous dose of glucose were 60% higher than those obtained after a meal containing 75 g carbohydrate only. Xiao *et al.* (2006) found that the consumption of different types of dietary fat produce significantly different effects on insulin secretion. By feeding 7 subjects 3 different test meals containing 89% energy from SFA, MUFA or PUFA, it was found that SFA ingestion led to impaired insulin response and insulin resistance while PUFA ingestion decreased insulin secretion. A proposed mechanism for FAs to influence insulin secretion is that a free FA receptor GPR40 is expressed in β-cells. It was shown that in mouse pancreatic β-cells overexpressing GPR40, addition of SFAs was able to stimulate insulin release in the micromolar range (Itoh and Hinuma, 2005).

1.12.8 Relation of insulin secretion with insulin sensitivity

Kahn *et al*. (1993), through the minimal model, found that first phase insulin secretion ($AIR_{glucose}$) is associated to insulin sensitivity via a hyperbolic function. The hyperbolic relationship means that the product of insulin sensitivity and insulin secretion is constant for a given degree of glucose tolerance (Weyer *et al.*, 1999). This product is called the disposition index (DI), and is a metric of the appropriateness of insulin secretion for a given degree of insulin sensitivity (or resistance). Compensatory insulin secretion is usually observed when β-cells are stressed over the capacity to produce sufficient insulin to compensate for the decline in insulin sensitivity in normal individuals. The hyperbolic relationship also means that a change in one of the variables is mirrored by a reciprocal change in the other variable, provided the system functions well to maintain glucose homeostasis. A study involving 26 individuals with impaired glucose tolerance and 91 healthy individuals (31 obese and 60 lean control subjects), found that with obesity, insulin sensitivity decreased significantly and although $AIR_{glucose}$ increased, the result was a significant decline in the DI (Elbein *et al.*, 2000). The investigators found that BMI was a significant predictor of insulin sensitivity, $AIR_{glucose}$ and DI ($p = 0.002$) after correcting for age, sex and glucose tolerance status. On the basis of these observations they suggested that obesity, which is often coupled with insulin resistance, may directly lead to β-cell defects and subsequently lead to β-cell compensation (Elbein *et al.*, 2000).

1.13 Aims of thesis

The overall aims of this thesis are to investigate the effects of the consumption of fructose on FA metabolism and insulin secretion. These will be accomplished through three human physiological studies and the aims of each study are:

1) To investigate the effect that the composition of dietary sugars has on hepatic FA synthesis and partitioning (Chapter 3),
2) To trace the metabolic fate of dietary fructose in a mixed meal (Chapter 4), and
3) To explore the acute and chronic effect of consumption of low and high fructose drinks on insulin secretion and sensitivity (Chapter 5).

Chapter 2: Biochemical and Statistical Analysis

This chapter details common methodology performed on the plasma and lipoprotein samples obtained from the study days described in Chapter 3, 4 and 5. Study specific details, including ethical approval, study design, participant inclusion and exclusion criteria will be presented within the relevant chapter.

2.1 Methods for biochemical analysis

2.1.1 Blood plasma collection

Whole blood was collected via cannulation of the antecubital fossa vein into heparinised syringes (Sarstedt, Leicester, U.K.). Plasma was then separated by centrifugation at 4000 rpm for 10 mins at 4 °C. Blood samples were collected at baseline (Time 0) and regularly after consumption of the test drink.

2.1.2 Measurement of plasma metabolite concentrations

2.1.2.1 Plasma non-esterified fatty acids (NEFA)

Plasma non-esterified fatty acids (NEFA) concentrations were measured enzymatically using a Randox kit (RX MONZA, Randox, U.K.) on an ILab 600 Multianalyser (Instrumentation Laboratory, Warrington, U.K.). The assay was designed for the quantitative *in-vitro* determination of NEFA in plasma. In the reaction, NEFAs were first converted into their acyl-CoA form using the enzyme acyl-CoA synthetase, which were then oxidised to produce hydrogen peroxide. The hydrogen peroxide reacted with 4-antiaminopyridine and N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-toluidine to form a fluorescent adduct, quinoneimine, which production (colour intensity) was proportional to the concentration of NEFA in the sample. Absorbance measurements were taken at wavelength of 600 nm. The method was linear up to 2240 µmol/L of plasma NEFA concentrations. The calibrator used was the NEFA CAL provided in the kit and the QC used were Randox Assayed Multi-sera Level 2 and Level 3. The inter-assay CV was < 5 %. Within-assay quality

control was conducted with every run and the CV calculated from the differences between duplicate samples was < 1 %.

The concentration of the rest of the metabolites were measured using an ILab 650 Multianalyser (Instrumentation Laboratory, Warrington, U.K.).

2.1.2.2 Plasma triacylglycerol (TG)

The concentration of plasma TG was measured using a Randox kit (Randox, U.K.) designed for the quantitative *in-vitro* determination of TG in plasma. The assay was based on a colorimetric method. Briefly, the addition of lipoprotein lipase broke the ester bonds in triacylglycerol forming glycerol, which via glycerol kinase and later a reduction reaction which involved glycerol-3-phosphate oxidase produced dihydroxyacetone phosphate and hydrogen peroxide. The final step involved the addition of 4-chlorophenol and 4-aminoantipyrine to hydrogen peroxide to form a fluorescent adduct, which production (colour intensity) was proportional to the concentration of TG in the sample. Absorbance measurements were taken at wavelength of 510 nm. The method was linear for plasma TG concentrations up to 2260 µmol/L. The calibrator used was ReferrIL G and the QC used were Randox Assayed Multi-sera Level 1 and Level 2. The inter-assay CV was < 4 %. Within-assay QC was conducted with every run and the CV calculated from the differences between duplicate samples was < 1 %.

2.1.2.3 Plasma glucose

The concentration of plasma glucose was measured using an IL Test glucose (oxidase) kit (Instrumentation Laboratory, Warrington, U.K.) which was based on a 2-step reaction designed for the quantitative *in-vitro* determination of glucose in plasma. The first step oxidised glucose to form gluconic acid and hydrogen peroxide. Following that, the second step involved the addition of phenol and 4-aminoantipyrine forming quinoneimine which fluoresces, and the concentration of glucose was proportional to the colour intensity. The absorbance measurements were taken at wavelength of 510 nm. The method produced a linear relationship between glucose concentration and absorbance for plasma glucose concentrations up to 28.2 mmol/L. The calibrator used was ReferrIL G and the QC used were Randox Assayed Multi-sera Level 1 and Level 2. The inter-assay CV was < 2 %. Within-assay QC was conducted with every run and the CV calculated from the differences between duplicate samples was < 1 %.

2.1.2.4 Plasma 3-hydroxybutyrate (3OHB)

The RANBUT kit (Instrumentation Laboratory, Warrington, U.K.) allows the measurement of 3OHB by adding the enzyme 3-hydroxybutyrate dehydrogenase, which oxidises 3OHB to acetoacetate and reduces NAD^+ to NADH. NAD and NADH are both fluorescent but with different peak wavelengths. Therefore, 3OHB can be quantified by measuring fluorescence at 340 nm, which is directly proportional with the concentration of NADH in solution. This assay was designed for the quantitative *in-vitro* determination of 3OHB in plasma. The kinetic enzymatic method was linear up to 5750 µmol/L of plasma 3OHB concentrations. The calibrator used was the RANBUT CAL provided in the kit and the QC used were Randox Assayed Multi-sera Level 2 and Level 3. The inter-assay CV was < 3.5 %. Within-assay

quality control was conducted with every run and the CV calculated from the differences between duplicate samples was < 1 %.

2.1.2.5 HDL-cholesterol

The Randox Direct HDL-Cholesterol kit (Randox, U.K.) was used to determine high density lipoprotein (HDL) cholesterol concentrations in plasma. The assay is based on a 2-step reaction. The first step separates HDL-cholesterol from all other forms of lipids and the second step uses the hydrogen peroxide formed by the oxidation of HDL-cholesterol to react with 4-aminoantipyridine to form a quinone based dye, which fluoresces at 600 nm wavelength. The intensity of the dye produced was directly proportional to the concentration of HDL-cholesterol. This enzymatic clearance method was linear up to 4.03 mmol/L of plasma HDL-cholesterol concentrations. The calibrator used was as provided in the kit and the QC used were Randox Assayed Multi-sera Level 1 and Level 2. The inter-assay CV was < 3 %. Within-assay quality control was conducted with every run and the CV calculated from the differences between duplicate samples was < 1 %.

2.1.2.6 Total Cholesterol

Plasma total cholesterol concentrations were determined using the Cholesterol kit (Instrumentation Laboratory, Warrington, U.K.) designed for the quantitative *in-vitro* determination of cholesterol in plasma. Cholesterol was separated from lipoproteins with cholesterol esterase and then oxidised with cholesterol oxidase to form hydrogen peroxide. Peroxide then reacted with 4-aminoantipyridine which fluoresces at 510 nm, where signal measured was proportional to cholesterol concentration. This colorimetric method was linear up to 28.2 mmol/L. The calibrator used was ReferrIL G and the QC used were SeraChem Control Level 1 and Level 2. The inter-assay CV was < 4 %. Within-assay quality control was conducted with

every run and the CV calculated from the differences between duplicate samples was < 1 %.

2.1.2.7 Plasma fructose

The Randox GLUCOSE/FRUCTOSE (GLUC/FRU) kit (Randox, U.K.) allows the indirect measurement of plasma fructose. The assay was designed for the quantitative *in-vitro* determination of fructose in red and white wine, beer and fruit juices. The concentration of fructose was obtained by subtracting the concentration of glucose from the total concentration of monosaccharides present in the samples. Glucose content was measured enzymatically with the use of hexokinase and glucose-6-phosphate dehydrogenase. To determine the total monosaccharide content (glucose+fructose), fructose-6-phosphate (F-6-P) was first converted to glucose-6-phosphate (G-6-P) using the enzyme phosphoglucose isomerase (PGI). This was followed by the conversion of G-6-P to gluconate-6-phosphate. The NADH formed in this oxidative process was stoichiometric with the total amount of monosaccharides. The wavelength used to measure glucose and total monosaccharide content was 340 nm; and the assays used to measure them were linear up to 6 g/L. In the absence of glucose, the fructose assay was also linear to up 6 g/L. For conversion of units from g/L to mmol/L, values were multiplied by 5.5. The calibrator used was as provided in the kit and the QC used was a Sugar Combination standard (ThermoFisher Scientific, U.K.). The inter-assay CV was < 5 %. Within-assay quality control was conducted with every run and the CV calculated from the differences between duplicate samples was < 1 %.

2.1.2.8 Plasma insulin

Plasma insulin was measured using radioimmunoassay (Human Insulin Specific RIA kit, HI-14K, Merck Millipore, Watford, U.K.). The principle of the technique is to react insulin antibody with radioactive binding ^{125}I to form antigen-antibody complexes with the soluble antigen, plasma insulin. When more antibody is bound, less antibody is being read on gamma counter. This translates to the presence of higher plasma insulin concentrations. Antibody-bound ^{125}I-insulin was separated from the unbound fraction using the double antibody-polyethyleneglycol (PEG) precipitation technique. The limit of linearity for this radioimmunoassay was 200 µU/ml (100 µl sample size).

2.1.3 Lipoprotein isolation

Plasma lipoprotein fractions were separated from samples collected at 0, 120, 180, 240, 300 and 360 mins in the studies described in chapters 3 and 4.

2.1.3.1 Separation of lipoprotein fractions of svedberg flotation rate (S_f) > 400 and S_f 20 – 400 using density gradient ultracentrifugation

1.5 ml of sodium bromide (NaBr) (1.42 kg/l) was added to 3 ml of plasma and gently inverted to mix. 4 ml was then added into ultracentrifuge tubes (14 ml, 14 x 95 mm) (Ultraclear; Beckman Instruments, Palo Alto, CA) which had been coated with polyvinyl alcohol (BDH Chemicals Ltd., Poole, U.K.). A density gradient made up of 3 ml each of 1.063 and 1.020 kg/l sodium chloride (NaCl) solutions and 2.8 ml of 1.006 kg/l NaCl was then added, in a sequential manner to the ultracentrifuge tubes.

Ultracentrifugation was performed with an L-70 ultracentrifuge using the SW 40 Ti swinging bucket rotor (Beckman Instruments, Palo Alto, CA). To obtain the

lipoprotein fraction of S_f > 400 (chylomicrons), the parameters for the run were 40,000 rpm for 32 mins at 15°C. At the end of the run, the top 0.5 ml of the sample was collected and then NaCl solution of density 1.006 kg/l was used to top up the tube for an overnight run (16 hours) to float S_f 20 – 400 lipoproteins. This was performed at 40000 rpm at 15 °C and at the end of the spin 0.5 ml of the S_f 20 – 400 lipoprotein fraction was collected (Karpe *et al.*, 1993).

The concentration of TG in the lipoprotein fractions (S_f > 400 and S_f 20 – 400) was measured using an ILab 650 Multianalyser (Instrumentation Laboratory, Warrington, U.K.) as described previously in section 2.1.2.2.

2.1.3.2 Immunoaffinity separation by monoclonal antibodies for separation of apolipoprotein B-100 (ApoB-100)

The S_f 20 – 400 lipoprotein fraction was further separated by immunoaffinity chromatography to obtain particles containing ApoB-48 and those containing ApoB-100. This method was previously described by Heath *et al.* (2003). Briefly, the separation of the S_f 20 – 400 fraction was carried out in 1 ml columns containing antibody-coupled sepharose gel dissolved in phosphate-buffered saline (PBS, pH 7.4). The monoclonal antibodies (anti-ApoB-100) used act specifically against ApoB-100 and do not react with ApoB-48 (Heath *et al.*, 2003).

2.1.3.3 Sample loading

The 1 ml antibody-coupled columns can bind a maximum of 500 umol/L of TG. Thus, to avoid overloading of the columns, the peak concentration of TG-rich lipoproteins (TRL) from any time-point in the S_f 20 – 400 fraction was used to determine the volume of sample to be placed into all the columns as shown below:

$$\text{Volume of sample to be placed through column} = \frac{500}{\text{peak concentration of TRL}}$$

A constant volume of TRL was added into the antibody-coupled columns together with phosphate buffered saline such that the total amount of solution loaded into each column was 500 µl. To ensure optimal binding efficiency, the sample was passed through the column 8 times with the repeated loading of each flow-through of the previous step back into the column.

2.1.3.4 Elution of bound (VLDL containing ApoB-100) and unbound (VLDL and TG-rich lipoproteins containing ApoB-48) fractions

At the end of the 8th loading, the unbound fraction was collected by draining the column, and washing through with 1 ml of PBS. The columns were washed with a further 5 ml of PBS before the ApoB-100 containing VLDL that were bound to the column were eluted with 3 M sodium thiocyanate (NaSCN, pH 7.4). To maximise elution efficiency, the columns were first filled with 2x1 ml of 3 M NaSCN before a further 8x1 ml of 3 M NaSCN was added to elute the bound fraction.

The 10 ml sodium thiocyanate containing the bound fraction was transferred into polyvinyl alcohol-coated Ultra-Clear centrifuge tubes and 2.8 ml PBS was added to the top of each tube to create a small density gradient to aid the centrifugation

process. The bound fraction was then concentrated by density gradient ultracentrifugation for 17 hours at 40000 rpm and 15 °C (L-70 ultracentrifuge, SW 40 Ti swinging bucket rotor, Beckman Instruments, Palo Alto, CA). The bound samples were collected by aspiration.

Concentration of TG in the bound (VLDL containing ApoB-100) and unbound fractions (TRL containing ApoB-48) were measured using an ILab 650 Multianalyser (Instrumentation Laboratory, Warrington, U.K.)

For simplicity, the ApoB-100 containing lipoproteins will be referred to as VLDL and the ApoB-48 containing lipoproteins as the unbound fraction.

2.1.4 Lipid extraction

Lipids were extracted from the respective samples based on the method by Folch *et al.* (1957). To 0.5 ml of plasma or lipoprotein fraction, 20 µl of internal standard, 5 ml of chloroform-methanol (in a ratio of 2:1 v/v) and 1 ml of 1 M NaCl were added and the tubes were placed on the rotatory mixer to spin for 10 mins at room temperature. The internal standards were heptadecanoic acid for the NEFA fraction and triheptadecanoyl glycerol for the lipoprotein TG fractions (Heath *et al.*, 2003).

The organic and aqueous phases were then separated by centrifugation at 2000 rpm for 10 mins at 14 °C (J6-MI centrifuge using JS-4.2 rotor, Beckman Instruments, Palo Alto, CA). The aqueous layer was removed by aspiration, leaving the organic chloroform layer which was dried under N_2 at 50 °C in an evaporator (Zymark TurboVap LV Evaporator). Dried lipid extracts were later reconstituted with 1 ml chloroform to be loaded into the solid phase extraction (SPE) columns.

2.1.4.1 Solid-phase extraction of NEFA and cholesteryl esters / TG

The columns used were 1 ml solid-phase extraction (SPE) columns (Isolute NH_2 100 mg/1 ml; Biotage; Hengoed, Wales) and they separate lipids according to polarity via an anion exchange mechanism (Burdge *et al.*, 2000). Before sample loading, columns were prewashed with 2x1 ml acetone and 2x1 ml chloroform under vacuum. Samples were dissolved in chloroform and then loaded into the columns and allowed to drip through.

2 ml of chloroform was added to the columns to elute the non-polar cholesteryl esters and triacylglycerol (CE/TG) lipids. This fraction was then dried under N_2 at 50 °C in the evaporator (Zymark TurboVap LV Evaporator).

In plasma samples, after the collection of the CE/TG fraction, NEFA were eluted by the addition of 2 ml of acidified diethyl ether (2% acetic acid) under vacuum. The fraction was then dried under N_2 at 50 °C in the evaporator (Zymark TurboVap LV Evaporator).

For the extraction of TG (plasma and lipoprotein), a new set of 1 ml SPE columns (Isolute NH_2 100 mg/1 ml; Biotage; Hengoed, Wales) were used. After pre-conditioning with 4x1 ml hexane under vacuum, the dried CE/TG samples were reconstituted in 1 ml hexane and loaded into the columns and allowed to drip through. 2x1 ml of hexane was first added to the columns under vacuum to elute CE, then 2x1 ml of hexane, chloroform and ethyl acetate in a ratio of 20:1:1 was added to elute TG from the column. The TG samples were dried under N_2 at 50 °C in the evaporator (Zymark TurboVap LV Evaporator).

2.1.4.2 Preparation of fatty acid methyl esters (FAME)

To the dried NEFA and TG fractions, 400 µl of toluene (containing BHT, 10 mg/100 ml) and 800 µl of 1.5% H_2SO_4 dissolved in methanol were added, samples vortexed and placed in a water bath (JB2, Grant Instruments, Cambridge, U.K.) at 80 °C for an hour. After this, the samples were removed from the water bath. Once cooled, 2 ml of neutralising solution (0.1 mmol/L of $KHCO_3$ and K_2CO_3) and 2 ml of cyclohexane were added and the samples were mixed on the rotatory mixer for 15 mins, then centrifuged at 2000 rpm for 10 mins at 14 °C (J6-MI ultracentrifuge using TT JS 4.2 swinging bucket rotor, Beckman Instruments, Palo Alto, CA). The upper solvent phase (organic phase) was then collected and dried under N_2 at 50 °C in an evaporator (Zymark TurboVap LV Evaporator).

2.1.5 Analysis of fatty acid composition by gas chromatography (GC)

Fatty acid (FA) composition in the plasma and lipoprotein fractions were determined by gas chromatography (GC). Samples were reconstituted in chloroform are then transferred to the respective GC vials. 20 µl of external standard (methyl tricosanoate) was then added before the sample was placed on the machine. Peaks were identified by comparison of retention times with known fatty acid methyl esters (FAME) standards and the FA composition was calculated based on the area of the respective peaks. The palmitate concentration was calculated by multiplying the proportion of palmitate by the plasma concentration of NEFA or TG determined enzymatically on the ILAB (section 2.1.2.1 and 2.1.2.2).

2.1.6 Tracer analysis by gas chromatography mass spectrometry (GCMS)

2.1.6.1 Analysis of [U^{13}C]palmitate enrichments in plasma NEFA and TG FAME derivatives

[U^{13}C]palmitate enrichment in the plasma NEFA and TG FAME derivatives were determined by GC mass spectrometry (GCMS). Ions with mass-to-charge ratios of 270 (M + 0) and 286 (M + 16) were determined by selected ion monitoring. Tracer-to-tracee ratios (TTRs) for [U^{13}C]palmitate were determined by (M + 16)/(M + 0). The background measurement of natural abundance of ^{13}C in each subject was taken from their fasting (Time 0) sample on the day of their study visit. The TTR of the fasting sample was then subtracted from the TTR of samples at the different time-points to give the respective tracer enrichment which were then multiplied by the corresponding palmitate-NEFA or palmitate-TG concentrations to give plasma tracer concentrations (Hodson *et al.*, 2007).

2.1.6.2 Analysis of [^{2}H]palmitate enrichments in lipoprotein samples

TG-rich lipoproteins (TRL) and VLDL were run on the GCMS together with 6 controls for background measurement of natural abundance of ^{2}H. Each sample run was for 27 mins and ions of interest included those of molecular mass of 270 (m) and 271 (m+1), with 270 being the molecular mass of the unlabelled palmitate ion, 271 is the molecular mass of a palmitate ion with one hydrogen atom being exchanged by a deuterium atom. The ratio of 271 to 270 then gives the tracer to tracee ratio (TTR), which after accounting for the natural abundance of ^{2}H, represents the respective tracer enrichment at each time-point (Hodson *et al.*, 2007). Data collected from this analysis was used in the calculation of the percentage of fasting and postprandial *de novo* lipogenesis (DNL) in VLDL-TG,

which reflects the percentage contribution of newly synthesised palmitate in VLDL-TG.

2.1.7 Tracer analysis by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS)

GC-C-IRMS (Finnigan GC-C-III and Delta Plus XP GC combustion-isotope ratio MS) (Thermo Electron Corporation, Bremen, Germany) was used to detect the ratio of $^{13}C/^{12}C$ in the lipoprotein samples. In this methodology, samples eluted from the GC travelled along a capillary micro-reactor at 940 °C and were oxidised into carbon dioxide. Gases were then cooled to 60 °C before being inserted into the mass spectrometer. A notation δ was defined by McKinney *et al.* in 1950. This expresses the abundance of isotope A of an element relative to that in a reference material. In the case of carbon, it is Pee Dee Belemnite (PDB).

2.1.7.1 Analysis of [U^{13}C]palmitate enrichments in lipoprotein samples

The concentration of ^{13}C tracer incorporated into the TG of the respective lipoprotein fractions (chylomicrons, TRL S_f 20 – 400, VLDL) was calculated by using the equations below:

Results expressed as $\delta^{13}C\ ^{0}/_{00}$ were converted to TTR using the following formula:

TTR ($^{13}C/^{12}C$) = (($\delta^{13}C\ ^{0}/_{00}$ /1000) +1) x 0.0112372 (Chong *et al.*, 2007).

Concentration of palmitate in lipoprotein-TG at each time-point (µmol/L)

= (mol% of palmitate in lipoprotein-TG/100) x concentration of TG

Concentration of ^{13}C-palmitate in lipoprotein-TG at each time-point (µmol/L)

= TTR x concentration of palmitate in lipoprotein-TG x 3 x 16 x 17/16

where lipoprotein-TG includes chylomicron-TG, TRL-TG and VLDL-TG.

The multiplications by 3, 16, and 17/16 are used to account for the number of fatty acids per triacylglycerol, the number of carbon atoms per fatty acid, and the methyl group added during derivatization, respectively.

2.1.7.2 Analysis of $^{13}CO_2$ enrichment in breath samples

Fasting and postprandial breath samples were analysed using GC-C-IRMS. A capillary column (CPPoraPLOTQ; VarianLtd, Oxford, United Kingdom) was used to separate CO_2 from the presence of other gases. The column had dimensions of 30 m by 0.32 mm by 10 µm. Samples were injected at 100 µl using the split injection mode at 110 °C with the column flow kept at 2.0 ml/min. The sampling time was 10 mins with the oven temperature at 35 °C. (Chong *et al.*, 2007).

From each sample, the machine generates a delta value ($\delta^{13}C\ ^{0}/_{00}$) which was then converted into a tracer-to-tracee ratio (TTR) using the following equation stated in section 2.1.7.1. To account for the natural abundance of ^{13}C, the TTR of the fasting breath sample was later subtracted from the TTR of each postprandial sample.

CO_2 production was measured by indirect calorimetry using Gas Electron Multiplier (GEM) machine (GEMNutrition Ltd, Warrington, U.K.). This measurement was performed at 120 mins after meal consumption. By multiplying the TTR of each breath sample by the CO_2 production rate ($\dot{V}_{CO2}$ in mmol/min), $^{13}CO_2$ production over the course of the study could be determined.

2.1.7.3 Analysis of ^{2}H enrichment in plasma water

^{2}H enrichment in plasma water was measured using the Finnigan GasBench II (ThermoFisher Scientific, U.K.). The sensitivity of IRMS and the low natural abundance of deuterium (> 0.02%) (Crotty *et al.*, 2013) required the subjects' plasma water to be labelled to 0.3% deuterium for detection and accuracy. 10 µl of plasma was added to 500 µl of deionised water in EXETAINER® tubes (Labco, High Wycombe, Buckinghamshire, U.K.) together with a platinum catalyst for H_2 and CO_2 water equilibration to occur (Horita, 1988). GasBench II diluted and displaced the sample gas present in the tubes by flushing each tube with helium steam that contained 2% of hydrogen. After the required equilibration time, samples were analysed using repetitive loop injection (1-2 min per replicate).

2.2 Calculations for DNL

2.2.1 Calculation of deuterium (^2H) enrichment (0.3%) in plasma water

By comparing the isotopic ratios to a measured standard, an accurate determination of the isotopic make-up of the sample was obtained. In the case of hydrogen and oxygen, the reference material was Standard Mean Ocean Water (SMOW).

Equation A

Deuterium enrichment in plasma water =

$$\left[\left(\text{delta}\ \frac{^2H}{^1H}\ \text{of sample corrected to SMOW}\right) - \left(\text{delta}\ \frac{^2H}{^1H}\ \text{of blank corrected to SMOW}\right)\right] X \frac{\text{Weight of } H_2O}{\text{Weight of sample}}$$

where blank refers to tap water.

Equation B: Conversion of value obtained from Equation A to a tracer (^2H) to traceer (^1H) ratio

$$\frac{^2H}{^1H} = \frac{\text{Value achieved from Equation A}}{1000 + 1} \times 0.00015595$$

where $^2H/^1H$ is the TTR raw value obtained at each time point.

Equation C:

Plasma water enrichment at each time-point

= value from equation B – baseline enrichment

where baseline enrichment was the result from section 2.1.7.3.

2.2.2 Calculation of postprandial percentage of DNL in VLDL-TG

$$\text{Percentage of } {}^{2}H_2O \text{ in plasma} = \frac{\text{TTR}}{22 \text{ x plasma water enrichment}}$$

where 22 is the total number of substitutable hydrogen atoms in palmitate (Semple *et al.*, 2009; Santoro *et al.*, 2015)

2.3 Statistical Analysis

Data was analysed using SPSS software windows version 22 (SPSS, Chertsey, U.K.). Statistical significance was set at $p < 0.05$. Data distribution for each variable was tested for normality using the Shapiro-Wilks test. Plasma concentration of 3OHB from the 2 study days were then compared using Mann-Whitney U test which is a non-parametric related samples t-test as the data follows a non-normal distribution. For the other variables, Student's paired t-test was performed between fasting datasets to assess differences of the baseline characteristics of subjects between the study days. It was also conducted to compare the postprandial effect of various metabolites upon consumption of the two meals. For each metabolite, the baseline-subtracted values were found and incremental area under the curve (iAUC), calculated using the trapezoid method, were then compared using 2-tailed paired t-tests.

Repeated-measures ANOVA was used to investigate the change between meals over time for a series of metabolites including plasma glucose, NEFAs, 3OHB, insulin and lipoprotein fractions (chylomicrons and VLDL). 'Time' and 'meal' were used as within-subjects factors. The same analysis was also performed for [U^{13}C]palmitate concentrations of chylomicron-TG, VLDL-TG, plasma NEFA, plasma TG and $^{13}CO_2$ production.

Chapter 3: Investigation on Effect of Composition of Dietary Sugars on Hepatic Fatty Acid Synthesis and Partitioning

3.1 Introduction

Carbohydrate is a major dietary component with its consumption making up 45 to 65% of UK adults' total daily energy intake (Henderson *et al.*, 2002). Two widely consumed monosaccharides are glucose and fructose and they have the potential to play a role in hepatic fatty acid (FA) metabolism due to the human liver's ability to convert the intake of excess carbohydrates into fats; a process known as *de novo* lipogenesis (DNL). Hepatic DNL was found to increase when greater than 50% of dietary carbohydrate consumed was in the form of monosaccharides (Parks *et al.*, 1999; Schwarz *et al.*, 2017). This observation suggests that there is strong association between consumption of dietary monosaccharides and the occurrence of DNL.

Dietary fructose has been found to be more lipogenic than glucose (Hudgins *et al.* 2011) and this has been suggested to be mediated by their differing effects on insulin secretion and downstream pathways (Elliott *et al*., 2002). It has been demonstrated that compared to glucose, fructose increased markers of hepatic DNL by 3-fold within 4 to 6 hours of consumption, in both lean and obese individuals (Hudgins *et al.*, 2011). Acute fructose ingestion contributes to the synthesis of both the glycerol and fatty-acyl components of very low density lipoprotein (VLDL)-triglyceride (TG) (Chong *et al.*, 2007; Parks *et al.*, 2008). Although the contribution of FAs from the DNL pathway to circulating VLDL-TG is quantitatively minor (Chong *et al.*, 2007), fructose may play an important role in the development of fructose-induced hepatic steatosis and dyslipidaemia. Fructose is suggested to increase the expression of key lipogenic enzymes in the liver (Tappy and Lê, 2010) and inhibit hepatic FA oxidation, thus favouring FA synthesis, esterification and VLDL-TG synthesis (Topping and Mayes, 1972). While the lipogenic effect of fructose can be

seen in insulin-sensitive individuals, dyslipidaemia occurs to a greater extent in insulin-resistant individuals (Teff *et al.*, 2009). A meta-analysis postulated that consuming 50 g/day of fructose is sufficient to increase postprandial TG levels, within a 2-week period (Livesey and Taylor, 2008); it is notable that this is about consuming 3 cans of 330ml of Coca-Cola a day with each can containing 17.5 g of fructose (Coca-Cola Company Annual Review, U.S.A., 2017).

Co-ingestion of glucose and fructose has been found to be more lipogenic than the intake of the same amount of either monosaccharide (Parks *et al.*, 2008; Hudgins *et al.*, 2011). In 2008, Parks *et al.* found that fructose, when consumed with glucose in a 1:1 or 3:1 ratio stimulated lipogenesis by 100% and elevated plasma TG levels by 76-200% after a subsequent meal, when compared to glucose alone. In line with this work, Hudgins *et al.* (2011) and Theytaz *et al.* (2014) reported that consumption of fructose and glucose, in a 1:1 ratio, was more lipogenic than consuming fructose alone; an effect that increased in a dose response manner. In contrast, Stanhope and Havel (2008) found that the increase in postprandial TG was similar with the administration of equivalent amounts of fructose, sucrose, mixtures of glucose and fructose, or high fructose corn syrup (HFCS). As sucrose and HFCS contained approximately fructose and glucose in approximately 1:1 ratio, it was suggested that co-ingestion with glucose significantly potentiated the hypertriglyceridemic effect of fructose (Theytaz *et al.*, 2014). This could be due to the improved absorption of fructose with glucose co-ingestion, as discussed in Chapter 1. Another possible reason would be the increased insulin response due to the co-consumption of glucose as compared to consumption of fructose alone.

Over the past decades, with the use of HFCS in food and beverages, there has been an exponential increase in worldwide consumption of fructose due to its

classification as a natural product, low cost and favourable properties with regards to enhancing food texture and flavour (Putnam and Allshouse, 1999). The rise in consumption of HFCS has coincided with the exponential increases in obesity and metabolic disease rates worldwide (Bray *et al.*, 2004). Whether HFCS contributes directly to the obesity pandemic and its effect on hepatic FA metabolism are debatable (Moore *et al.*, 2014).

3.1.1 Aims and hypothesis

As the effect of dietary sugar composition on hepatic postprandial FA synthesis and partitioning remains unclear, the aims of this study were:

1. To investigate the effect of a single high fructose compared to a low fructose meal on hepatic DNL and postprandial FA partitioning and whole body oxidation.
2. To determine if there is sexual dimorphism in hepatic DNL and postprandial FA partitioning and whole body dietary FA oxidation after consumption of meals high and low in fructose.

The hypothesis of the study is as follows:

1. A difference would be seen in hepatic DNL and postprandial FA partitioning and whole body FA oxidation between the two test meals, with the high fructose meal resulting in higher hepatic DNL and decreased whole body oxidation.
2. Differences would be seen in postprandial hepatic DNL and whole body FA oxidation between genders upon consumption of either meal, where postprandial DNL would be higher and whole body FA oxidation would be lower in males compared to females.

3.2 Methods

3.2.1 Ethics

The study protocol was approved by the Portsmouth Research Ethics Committee (Portsmouth REC 12/SC/0267).

3.2.2 Power calculation for subject recruitment

Using data from the work of Chong *et al.* (2007) where they noted a change in the area under the curve (AUC) for VLDL-TG of 33% after feeding a fructose-rich meal compared to glucose-rich meal, I predicted a more conservative change of 25%. To detect a difference of 25% with a power of 0.80 at α of 0.05, n=14 individuals would be required. To allow for a 15% drop-out rate, I aimed to recruit 16 individuals.

3.2.3 Subject recruitment

Sixteen healthy subjects (8 males, 8 pre-menopausal females) were recruited from the Oxford Biobank (Tan *et al.*, 2006). All subjects gave written informed consent.

Prior to the commencement of the study, all volunteers attended a screening session to ensure suitability for the study. The inclusion criteria were individuals aged 30 to 50 years old, of BMI >25 but <35 kg/m^2, waist circumference > 80 cm for females and >94 cm for males. To be accepted into the study, subjects had to be healthy, free from known disease and not taking medication that could alter glucose or lipid metabolism. Participants needed to be non-smokers and consumed alcohol within recommended limits (Nascimbeni *et al.*, 2013). Exclusion criteria included those who had dyslipidaemia, intolerance to fructose or hyperglycemia. Female participants had to be not pregnant or using contraceptives (eg. oral contraceptives, hormone replacement therapy (HRT)) for the past 12 months. Prior to the study day, subjects were asked to

avoid food naturally enriched in ^{13}C (e.g. cornflakes) for 48 hours and refrain from strenuous exercise and alcohol for 24 hours.

3.2.4 Study design

The study was a randomised crossover trial involving two study days separated by a 4-week washout period (Figure 3.1).

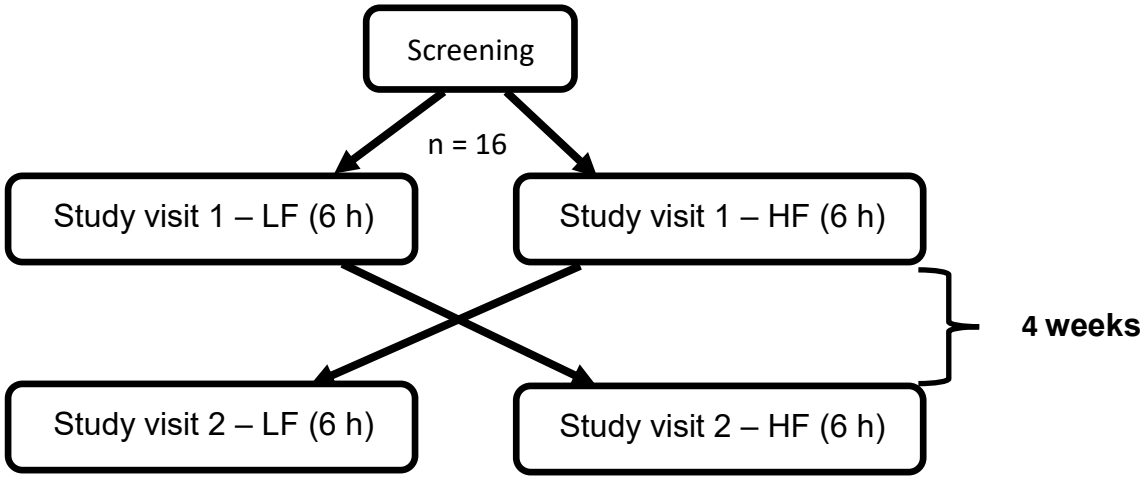

Figure 3.1: Overview of the crossover study for investigation of effect of composition of dietary sugars on hepatic fatty acid synthesis and partitioning. Abbreviations: LF, low fructose meal; HF, high fructose meal.

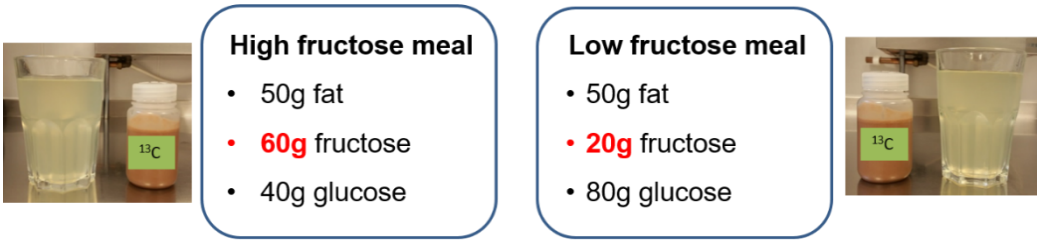

Figure 3.2: Test meals on the two study days

The ratio of fructose to glucose in the high fructose lemon-flavoured sugar drink was designed to be 60:40 to mimic the composition of fructose to glucose in HFCS which is 55:45. With the aim to have the same amount of monosaccharides in both sugary drinks for the respective study days, the low fructose drink had a third of the amount of fructose, 20 g, and contained twice as much the amount of glucose, 80g. The

total energy of the monosaccharides in the high and low fructose drinks were the same of 400 kcal, with 4 kcal / g of monosaccharide (Appendix Table 2 and 3).

Table 3.1: Composition of fatty acids in palm oil and olive oil

Type of oil	Percentage		
	SFA	MUFA	PUFA
Palm oil	50	41	9
Olive oil	18	70	12

Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

The 50 g of fat in the chocolate-flavoured fat-emulsion drink contained 30 g of palm oil and 20 g of olive oil (Figure 3.2). This was used to mimic the physiological ratio of FAs in the human blood of saturated FA (SFA) to monounsaturated FA (MUFA) to polyunsaturated FA (PUFA), which was found to be 40:50:10 (Hodson *et al.*, 2008). The total energy of the oils in the fat-emulsion added up to 450 kcal, with 9 kcal / g of fat. The drink also contained 0.4 g of [U^{13}C]palmitic acid to trace the metabolic fate of dietary fat (Appendix Table 1).

In this crossover study, for each study day, all subjects were meant to be fed with the same amount of energy to ensure that the study was isoenergetic within and between subjects for better comparison of postprandial effects on concentration of plasma metabolites upon consumption of the low and high fructose test meals. The total amount of energy per meal added up to about 920 kcal, taking into consideration the small proportion of energy from the artificial sweetener, cocoa powder and lemon juice added into the chocolate fat-emulsion and the lemon-flavoured sugary drink respectively (Appendix Table 1, 2, and 3).

3.2.4.1 DEXA and MRI scans

During one of the visits, dual-energy X-ray absorptiometry (DEXA) scan was performed to measure the whole body composition and fat distribution (Hodson *et al.*, 2014).

Intrahepatic fat was quantified on a separate day after an overnight fast and within 2 weeks of a study day, using proton magnetic resonance imaging spectroscopy (^{1}H-MRS) (Banerjee *et al.*, 2014).

3.2.5 Overview of study days

On the evening prior to the study day, subjects consumed deuterated water ($^{2}H_2O$) (3 g/kg body water) to enrich body water to 0.3% (Hodson *et al.*, 2015) and they continued to consume 0.3% enriched water throughout the study day to maintain enrichment. $^{2}H_2O$ was used to determine fasting and postprandial hepatic DNL.

On the study day, subjects arrived at the Clinical Research Unit after an overnight fast. Following cannulation of the antecubital fossa vein, fasting blood and breath samples were taken as baseline measurements. Subjects were then given a lemon-flavoured sugar drink and a fat-containing chocolate drink (Figure 3.2) to consume. The study meal on both study days contained a total of 100 g monosaccharides but varied in composition as shown below:

1) Low fructose meal: 20 g fructose and 80 g glucose
2) High fructose meal: 60 g fructose and 40 g glucose

The composition of the chocolate drink was the same for both study days and contained 0.2g [U^{13}C]palmitate (isotope purity 99%; CK Gas Products Ltd, Winchester, U.K.) to trace the fate of dietary FAs.

After consumption of the test meal, blood samples were taken regularly at 30, 60, 90, 120, 180, 240, 300 and 360 mins for the measurement of plasma glucose, insulin, fructose, TG, non-esterified FA (NEFA) and 3-hydroxybutyrate (3OHB). Samples collected at 0, 120, 180, 240, 300, and 360 mins were also used for the analysis of chylomicron-TG, triacylglycerol-rich lipoprotein (TRL)-TG and VLDL-TG. Blood was drawn into heparinised syringes and centrifuged immediately for 10 mins at 4 ^{o}C, 4000 rpm. Plasma was then aliquoted and stored in -20 ^{o}C until analysis.

Breath samples were collected at the same time-points into EXETAINER® tubes (Labco Ltd., High Wycombe, Bucks, U.K.) for measurement of $^{13}CO_2$ enrichment.

3.2.5.1 Indirect calorimetry

Indirect calorimetry was performed 120 mins after the meal consumption using a GEM calorimeter (GEM Nutrition Ltd) to examine the whole body CO_2 production, whole body respiratory exchange ratio (RER) and basal energy expenditure of the subjects. For details, please refer to Section 2.1.7.2.

3.2.6 Methods for biochemical analysis

Plasma metabolites, including insulin were analysed using biochemistry analysers and radioimmunoassay as previously described in Chapter 2. Separation of the lipoprotein fractions was carried out using density gradient ultracentrifugation and the S_f 20 – 400 fraction was further separated by immunoaffinity chromatography to obtain a pure fraction of VLDL containing ApoB-100 (detailed in Chapter 2, section 2.1.3.2).

3.2.6.1 Fatty acid composition and stable isotope analysis

To determine FA composition of plasma and lipoprotein samples, total lipids were extracted from plasma, chylomicrons, TRL and VLDL fractions before being methylated and separated by gas chromatography (GC). Isotopic enrichment of [U^{13}C]palmitate (Tracer-to-tracee ratios) in plasma NEFA and TG FAME derivatives were determined by GC mass spectrometry (GCMS) using selected ion monitoring where 270 (M + 0) is the mass of the parent ion, palmitate and 286 (M + 16) is that for ions which have all 16 carbons displaced by ^{13}C-labelled carbons in the palmitate tracer. The isotopic enrichment in lipoproteins and CO_2 were determined by GC-C-IRMS as described in Chapter 2 (section 2.1.11). The tracer concentration was determined by multiplying the concentration of plasma palmitate with the tracer-to-tracee ratio of each sample at the time-points (Hodson *et al.*, 2010).

3.2.7 Calculation

Insulin resistance was calculated using the homeostasis model assessment (HOMA2) model (Levy *et al.*, 1998).

3.2.8 Statistical Analysis

Data were analysed using SPSS 22 software (SPSS [UK] Ltd., Chertsey, U.K.) and presented as mean ± SEM unless otherwise stated. The normality of the data distribution for each variable was assessed by using the Shapiro-Wilks test. Anthropometric data and fasting plasma concentration of glucose, insulin, fructose, TG, NEFA, cholesterol and non-HDL cholesterol from the 2 study days were compared using the 2-tailed paired t-tests. Comparisons of anthropometric and biochemical data between male and female participants between study days were performed using independent sample t-tests and significance was set as $p < 0.05$. Plasma concentration of 3OHB from the 2 study days were compared using Mann-Whitney U test which is a non-parametric related samples t-test as the data follows a non-normal distribution. Postprandial AUC of concentration of metabolites from subjects on two study days were performed using 2-tailed paired t-tests. Between genders, independent t-tests or where relevant, non-parametric t-tests were used. AUC were calculated by the trapezoid method and were divided by the relevant time intervals to give time-averaged values.

Repeated measures ANOVA (RMANOVA) with factors of time and meal was used to analyse the postprandial plasma concentration of glucose, insulin, fructose, TG, NEFA, 3OHB, [U^{13}C]palmitate-TG, [U^{13}C]palmitate-NEFA, [U^{13}C]palmitate-chylomicron, [U^{13}C]palmitate TRL S_f 20 – 400, [U^{13}C]palmitate-VLDL, postprandial $^{13}CO_2$ production and percentage postprandial DNL in VLDL-TG.

With time and sex as between-subject factors, RMANOVA was performed to check if there was any significant difference between the postprandial response of males and females after consumption of the low and high fructose test meals.

Spearman rank correlations were performed to assess associations between liver fat, fasting and postprandial concentration of insulin, percentage contribution of fasting and postprandial percentage DNL in VLDL-TG, postprandial plasma concentration of 3OHB, [U^{13}C]palmitate-VLDL and $^{13}CO_2$ production per kilogram lean mass.

3.3 Results

3.3.1 Baseline characteristics

The anthropometric and biochemical data obtained from the 16 healthy individuals, 8 males and 8 females, aged between 35 and 50 are shown in Table 3.1. Between genders, age and BMI of males and females were comparable; however body fat distribution was significantly different with females having significantly more gynoid fat and lower waist-to-hip ratios compared to males (Table 3.1). Lean mass was also significantly lower in females (Table 3.1).

Table 3.2: Subject baseline characteristics

Parameter	Male (n = 8)	Female (n = 8)
Age (y)	42.8 ± 1.8	46.6 ± 0.9
Weight (kg)	93.0 ± 3.3	81.0 ± 3.3 *
BMI (kg/m^2)	29.2 ± 0.9	28.5 ± 1.2
Waist (cm)	101 ± 4	94 ± 4
Hip (cm)	106 ± 1	110 ± 2
Waist / hip ratio	0.95 ± 0.03	0.86 ± 0.03 *
Total fat (kg)	27.7 ± 2.6	33.7 ± 3.1
Total lean (kg)	61.6 ± 1.3	44.7 ± 1.7 ***
Android fat (kg)	2.8 ± 0.4	2.8 ± 0.5
Gynoid fat (kg)	4.0 ± 0.3	5.7 ± 0.4 **
Android / Gynoid fat ratio	0.69 ± 0.05	0.47 ± 0.07 *
Visceral fat (kg)	1.3 ± 0.3	0.9 ± 0.3
Liver Fat (%)	3.1 ± 1.1	3.3 ± 1.6

Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 between males (n = 8) and females (n = 8).

3.3.2 Study days

Subjects' anthropometric and metabolic variables were comparable on the both study days (Table 3.3).

Table 3.3: Fasting and postprandial values of parameters from subjects on the study days

Parameter	**High Fructose**	**Low Fructose**
Weight (kg)	87.7 ± 2.8	87.3 ± 2.7
BMI (kg/m^2)	29.2 ± 0.7	29.0 ± 0.7
Waist (cm)	97.9 ± 2.8	95.9 ± 2.6
HOMA2-IR	1.29 ± 0.12	1.32 ± 0.12
Fasting Plasma Variables		
Glucose (mmol/L)	5.2 ± 0.1	5.3 ± 0.2
Insulin (mU/L)	9.9 ± 1.0	10.1 ± 0.9
Fructose (mmol/L)	0.15 ± 0.03	0.10 ± 0.02
TG (µmol/L)	1040 ± 117	1037 ± 144
NEFA (µmol/L)	445 ± 38	459 ± 57
3OHB (µmol/L)	86 ± 20	114 ± 33
Total cholesterol (mmol/L)	4.9 ± 0.2	4.7 ± 0.2
HDL cholesterol (mmol/L)	1.4 ± 0.1	1.3 ± 0.1
Non-HDL cholesterol (mmol/L)	3.5 ± 0.2	3.4 ± 0.2
Postprandial Plasma Variables (time-averaged over 6 hours)		
Chylomicron-TG (µmol/L)	256 ± 61	230 ± 33
TRL (S_f 20 – 400)-TG (µmol/L)	784 ± 117	711 ± 102
VLDL-TG (µmol/L)	343 ± 43	345 ± 43

Data presented as mean ± SEM. Abbreviations: BMI, body mass index; HOMA, homeostatic model assessment; IR, insulin resistance; TG, triacylglycerol; NEFA, non-esterified fatty acid(s); 3OHB, 3-hydroxybutyrate; HDL, high-density lipoprotein; TRL, triacylglycerol-rich lipoprotein; VLDL, very low density lipoprotein.

On the respective study days, the concentration of fasting metabolites was comparable ($p > 0.05$) within genders. Higher fasting plasma TG and insulin levels as well as HOMA2-IR were seen in males compared to females on both study days; there were no other significant differences (Table 3.4).

Table 3.4: Fasting and postprandial values of metabolites from males and females on the low and high fructose study days

Fasting plasma variables	Low Fructose		High Fructose	
	Male	Female	Male	Female
Glucose (mmol/L)	5.5 ± 0.3	5.0 ± 0.2	5.3 ± 0.1	5.1 ± 0.3
Insulin (mU/L)	12.0 ± 1.0	8.1 ± 1.1 *	11.6 ± 1.5	8.3 ± 1.0
Fructose (mmol/L)	0.17 ± 0.06	0.17 ± 0.06	0.21 ± 0.07	0.31 ± 0.11
HOMA2-IR	1.6 ± 0.1	1.1 ± 0.1 *	1.5 ± 0.2	1.1 ± 0.1
TG (µmol/L)	1210 ± 263	864 ± 106	1082 ± 180	998 ± 159
NEFA (µmol/L)	383 ± 56	536 ± 95	416 ± 32	474 ± 69
3OHB (µmol/L)	115 ± 64	112 ± 24	98 ± 33	74 ± 23
Total cholesterol (mmol/L)	4.6 ± 0.2	4.8 ± 0.4	4.8 ± 0.3	5.1 ± 0.4
HDL cholesterol (mmol/L)	1.2 ± 0.1	1.5 ± 0.2	1.3 ± 0.1	1.6 ± 0.2
Non-HDL cholesterol (mmol/L)	3.4 ± 0.3	3.3 ± 0.2	3.5 ± 0.3	3.5 ± 0.2
Postprandial Plasma Variables (time-averaged over 6 hours)				
Chylomicron TG(µmol/L)	321 ± 41	138 ± 24 **	347 ± 110	164. ± 37
TRL S_f 20 – 400 TG (µmol/L)	860 ± 178	563 ± 82	890 ± 202	677 ± 120
VLDL TG (µmol/L)	383 ± 77	308 ± 40	347 ± 68	338 ± 57

Data presented as mean ± SEM. *p < 0.05 male vs female, **p < 0.01 male vs female. Abbreviations: HOMA, homeostatic model assessment; IR, insulin resistance; TG, triacylglycerol; NEFA, non-esterified fatty acid(s); 3OHB, 3-hydroxybutyrate; HDL, high-density lipoprotein; TRL, triacylglycerol-rich lipoprotein; VLDL, very low density lipoprotein.

On both study days, plasma glucose concentrations for the cohort peaked at 30 mins after meal consumption and then decreased back to baseline (Figure 3.3A). The same trend was seen between the genders upon consumption of either the low or high fructose meals (Figure 3.3B and C). The magnitude of increase in postprandial glucose concentration was greater after consumption of the low fructose meal as it contained two times more glucose than the high fructose meal. In line with the changes in plasma glucose concentration, there was a significant effect of the meal ($p < 0.01$) on plasma insulin concentrations (Figure 3.4A). The postprandial AUC of both plasma glucose and insulin were significantly higher after consumption of the low fructose meal as compared to the high fructose meal ($p < 0.01$). There were no significant differences in plasma insulin concentrations between genders after consumption of the low or high fructose meal (Figure 3.4B and C).

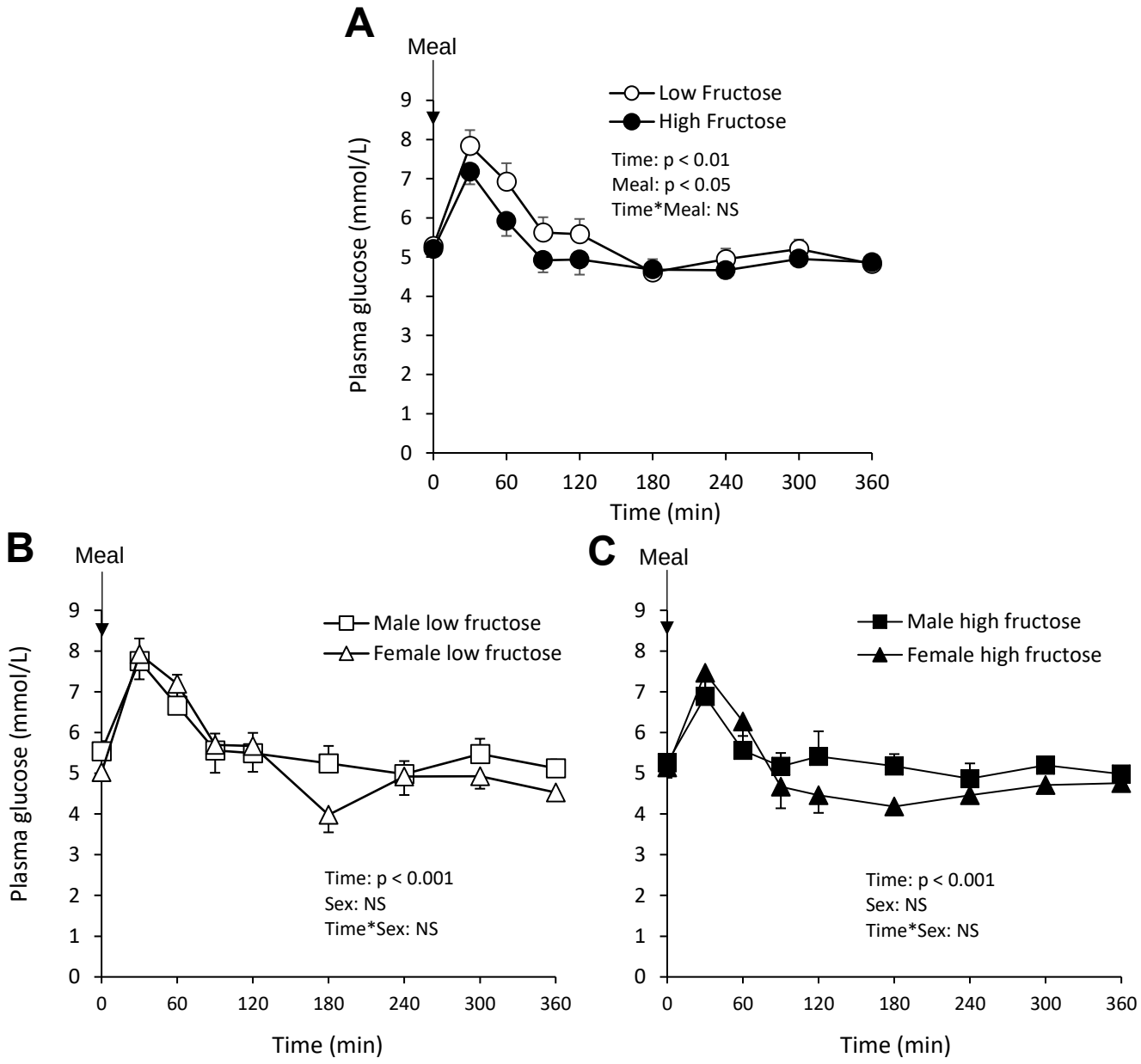

Figure 3.3: Postprandial plasma glucose concentrations (mmol/L) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviation: NS, non-significant.

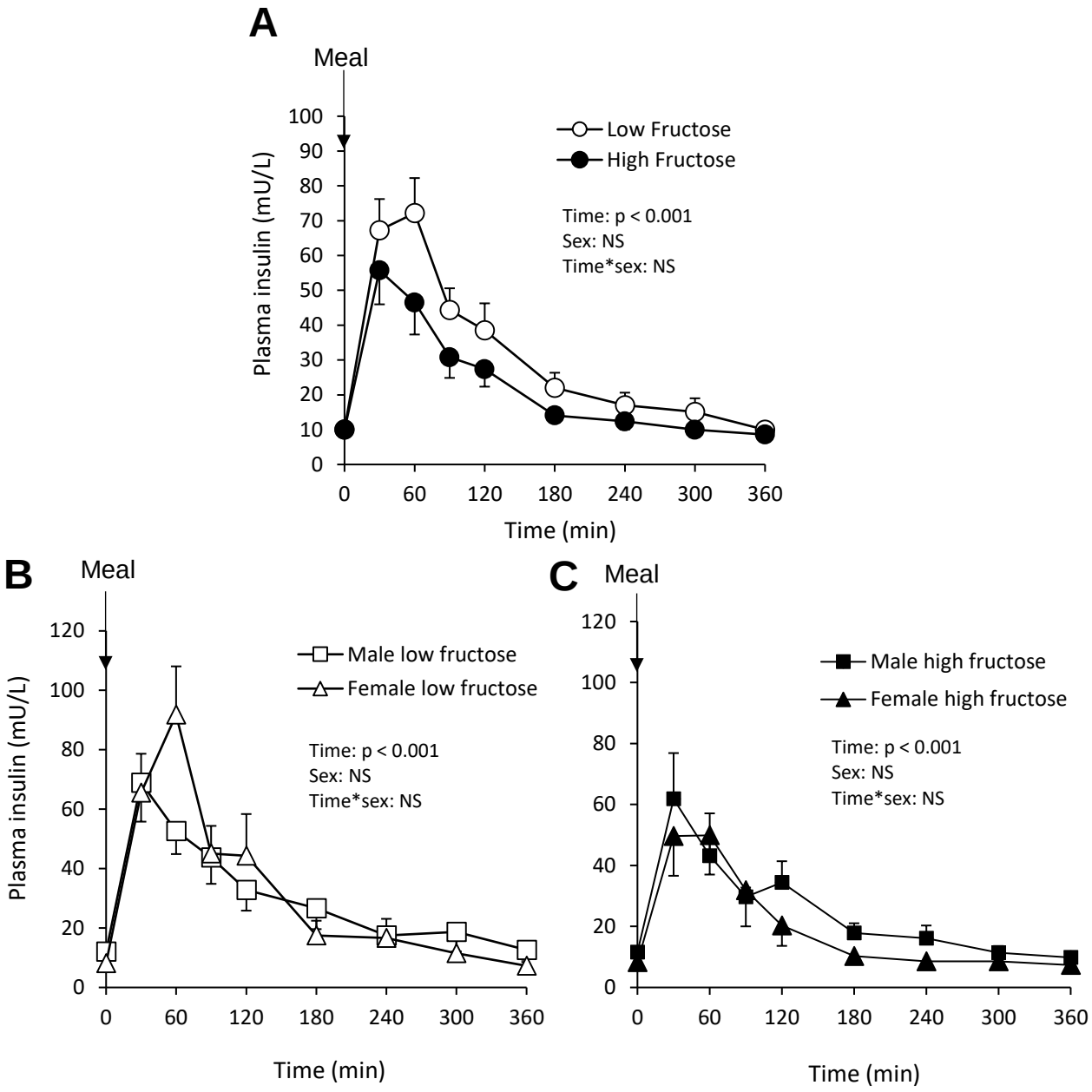

Figure 3.4: Postprandial plasma insulin concentrations (mU/L) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviation: NS, non-significant.

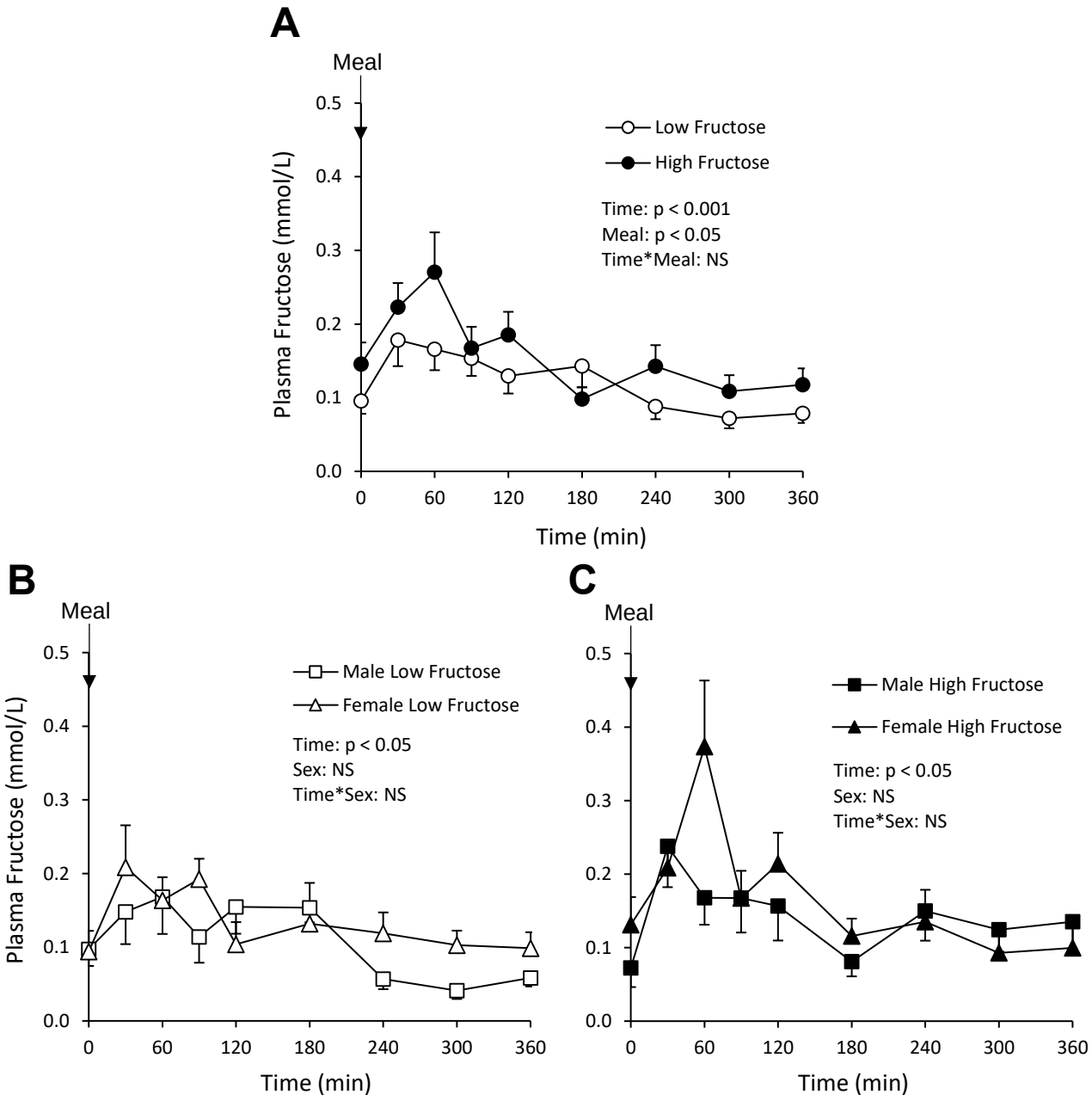

Figure 3.5: Postprandial plasma fructose concentration (mmol/L) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviation: NS, non-significant.

The pattern of change in plasma fructose concentrations over time differed between the study days. Upon consumption of the low fructose meal, after the initial increase in the first hour, plasma fructose concentrations decreased and returned to baseline after 360 mins. After consumption of the test meals, plasma fructose concentrations were significantly higher after consumption of the high fructose meal ($p < 0.05$). A significant effect on time ($p < 0.01$) was also seen as plasma fructose concentrations increased after consumption of both meals (Figure 3.5A). The high fructose meal contained 3 times more fructose than the low fructose meal and this was reflected in the postprandial AUC of plasma fructose, which was significantly higher after consumption of the high fructose compared to the low fructose meal ($p < 0.05$). There was no significant difference in the response of plasma fructose concentrations between males and females after consumption of the low or high fructose meals (Figure 3.5B and C).

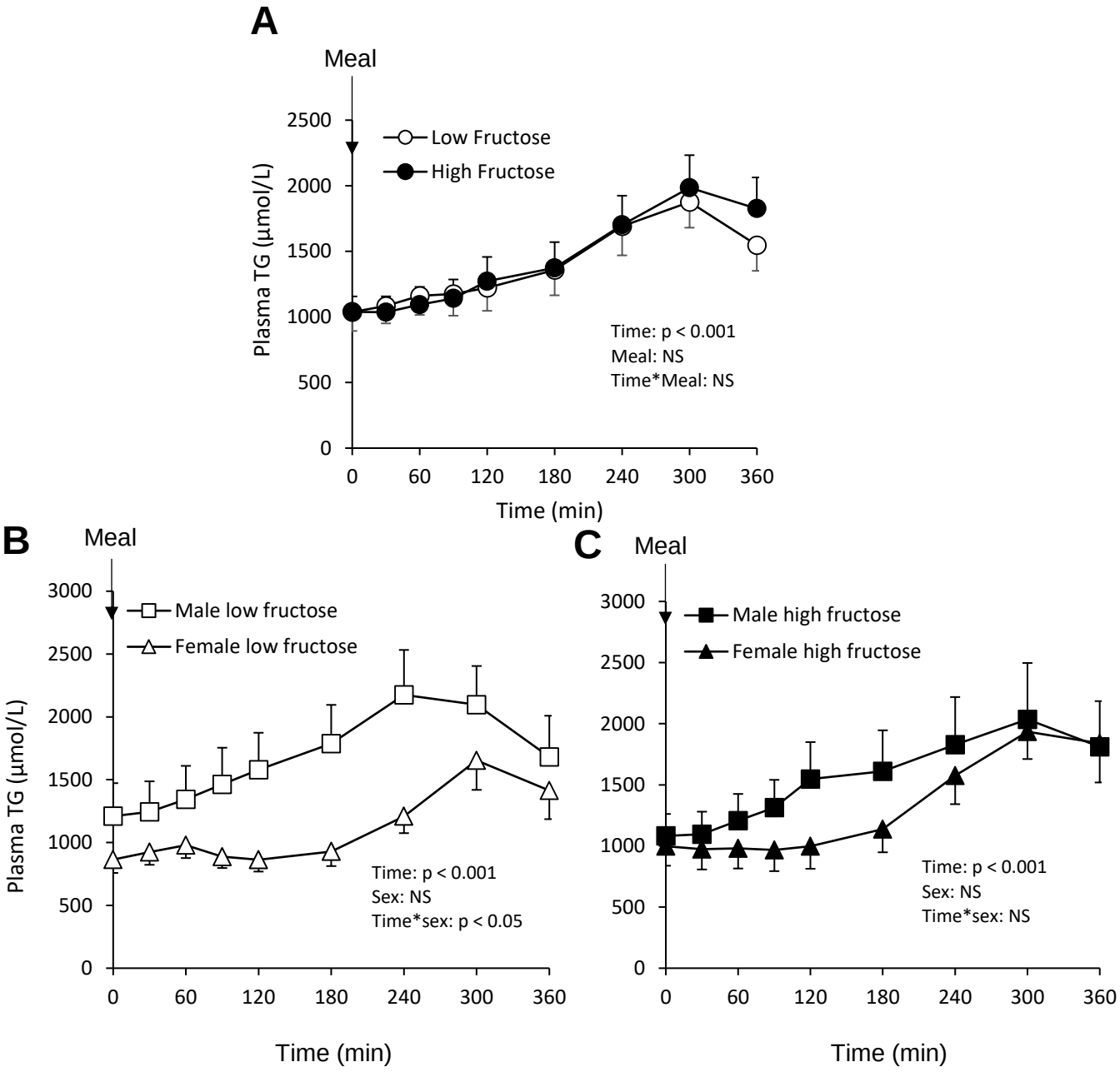

Figure 3.6: Postprandial plasma TG concentrations (µmol/L) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviations: TG, triacylglycerol; NS, non-significant.

Ingestion of the test meals did not result in significant differences in plasma TG concentrations over time between the low and high fructose meals; consumption of both meals increased plasma TG concentrations over time until around 300 mins (Figure 3.6A).

Upon consumption of the test meals, the postprandial plasma TG response differed in males and females. However, a significant postprandial time*sex interaction was only seen after ingestion of the low fructose meal (Figure 3.6B).

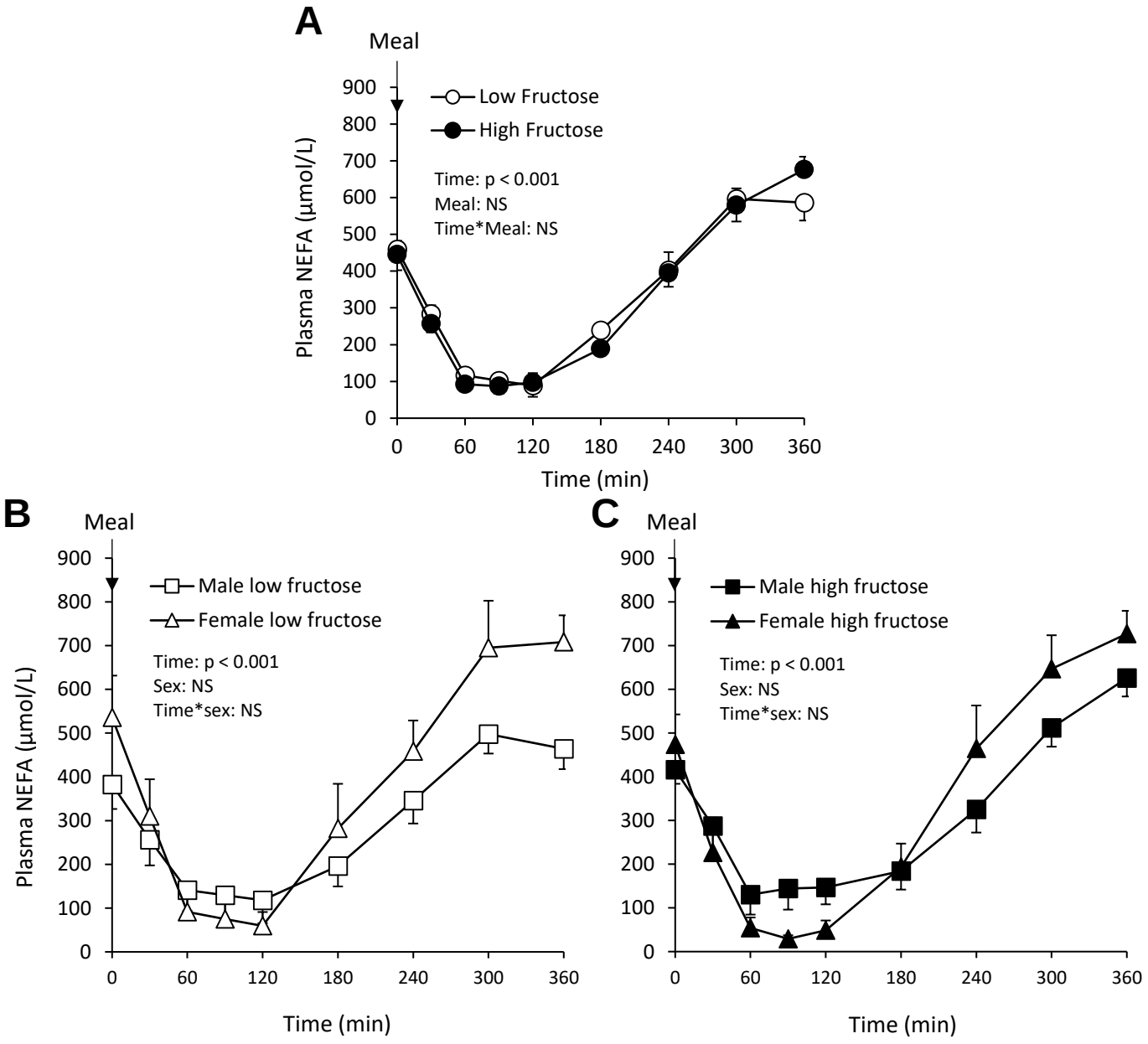

Figure 3.7: Postprandial plasma NEFA concentrations (µmol/L) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviations: NEFA, non-esterified fatty acid; NS, non-significant.

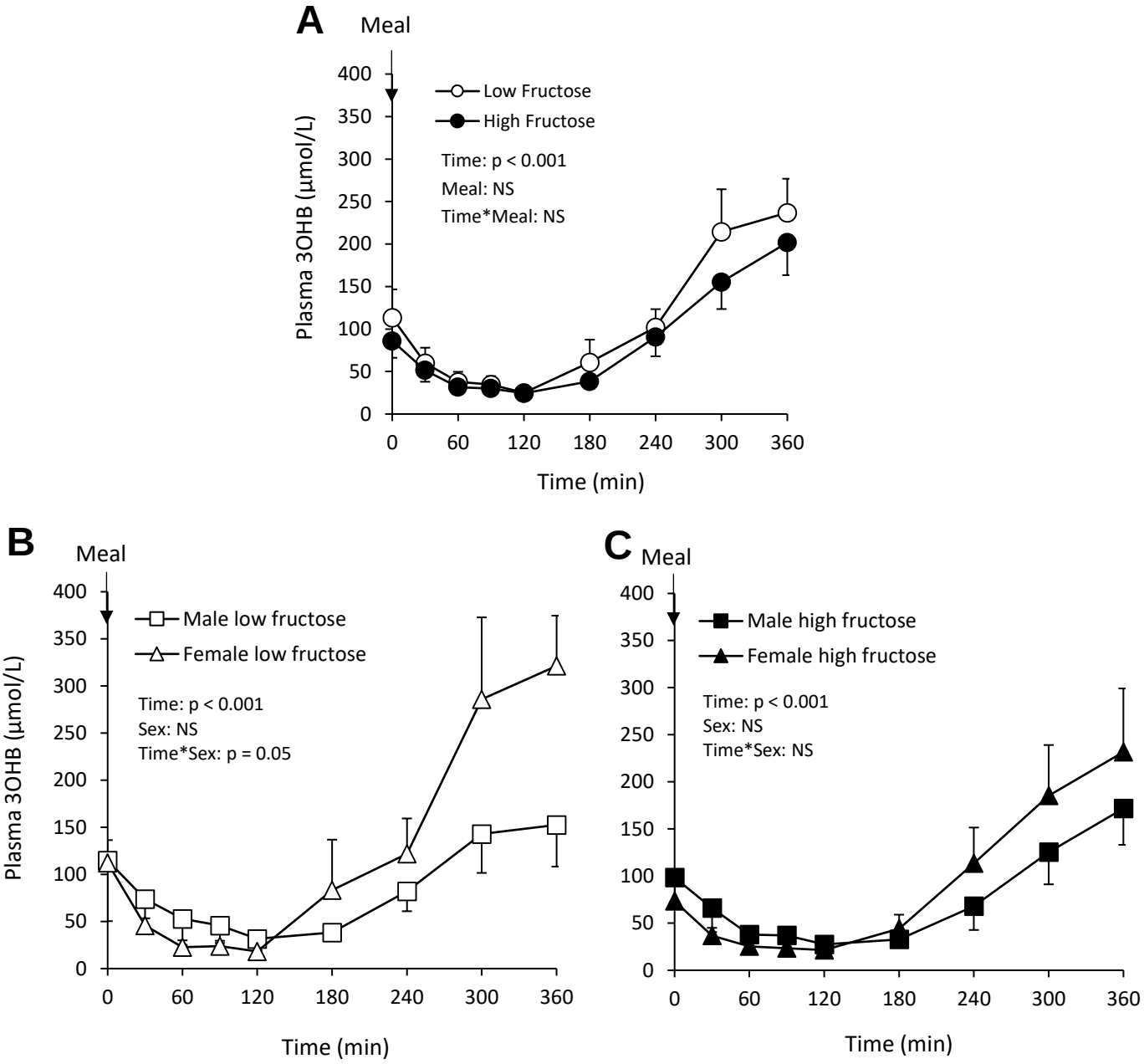

Figure 3.8: Postprandial 3OHB concentrations (µmol/L) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviations: 3OHB, 3-hydroxybutyrate; NS, non-significant.

Plasma 3OHB concentrations followed a similar response after consumption of either test meal, decreasing until approximately 120 mins after which they increased to more than double the baseline level by 360 mins (Figure 3.8A). Upon consumption of the low or high fructose meal, an initial suppression of 3OHB concentrations was seen in both males and females (Figure 3.8B and C). Plasma 3OHB concentration reached a nadir before beginning to rebound 180 mins postprandial with concentrations rebounding to a greater extent in females compared to males. This was significant after consumption of the low fructose meal where there was a significant time*sex interaction ($p < 0.05$). Although females had higher plasma concentrations of 3OHB at 360 mins compared to males after both meals, this only reached statistical significance ($p < 0.05$) after consumption of the low fructose meal (Figure 3.7B and C).

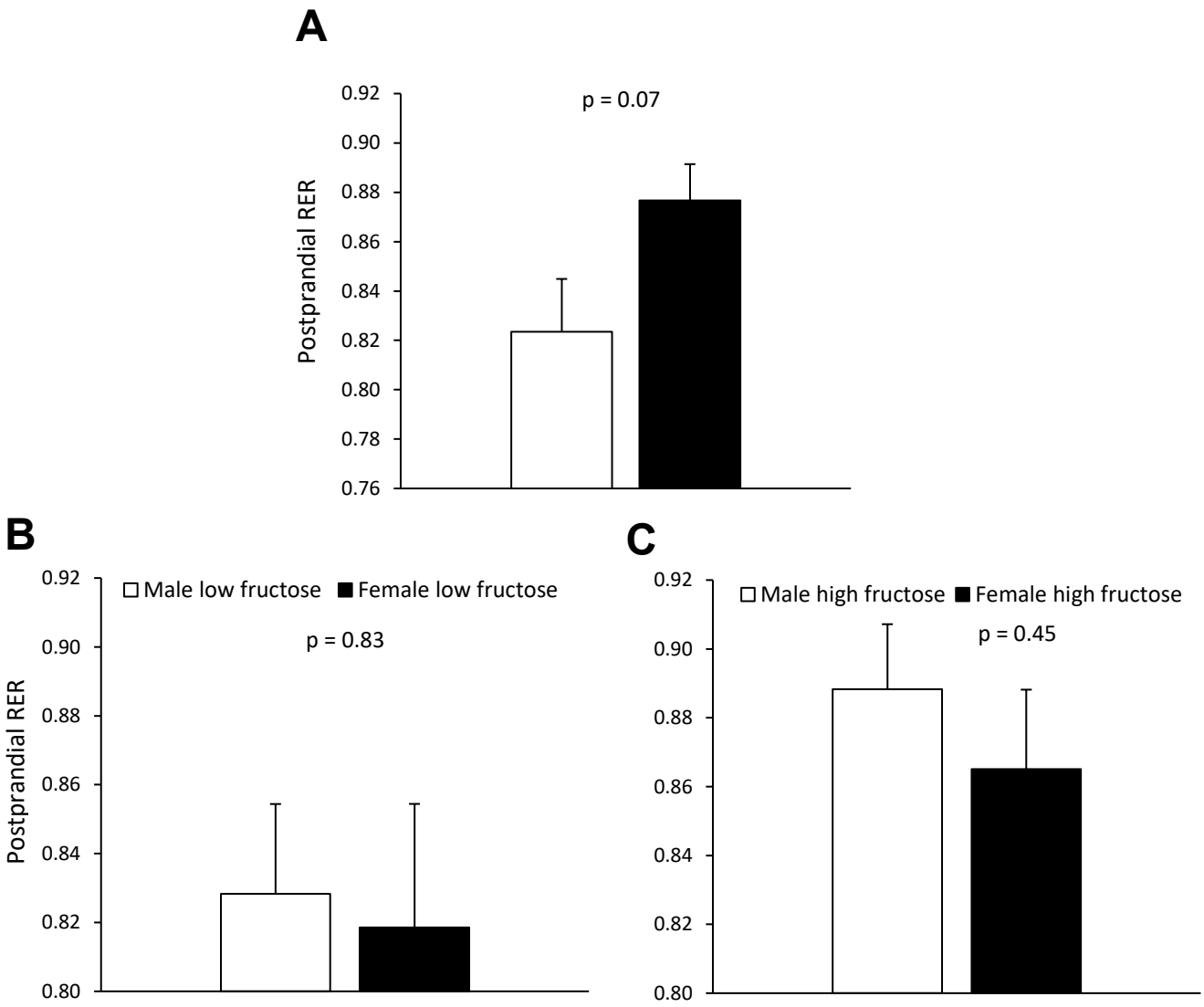

Figure 3.9: Postprandial respiratory exchange ratio (RER) at 120 mins in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, p-value calculated using 2 tailed paired t-test **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, p-value calculated using independent sample t-tests; and **(C)** males and females upon consumption of high fructose meal, p-value calculated using independent sample t-tests. Data represented as mean ± SEM.

The postprandial respiratory exchange ratio (RER) was higher, but not significantly so ($p > 0.05$), after consumption of the high fructose meal compared to the low fructose meal (Figure 3.9). The postprandial RER was not significantly different between the genders upon consumption of either meal (Figure 3.9B and C).

3.3.3 Postprandial metabolite concentrations: ^{13}C enrichment

In both the test meals, [U^{13}C]palmitate was incorporated into the chocolate drink to trace the fate of dietary FAs. After consumption of the meals, the appearance of [U^{13}C]palmitate in plasma-TG increased and peaked at 300 mins postprandial before starting to decrease; the response did not differ between test meals (Figure 3.10A). When stratified by gender, the appearance of [U^{13}C]palmitate in plasma-TG was significantly higher ($p < 0.05$) in males compared to females after consumption of the low but not high fructose meal.

Consumption of the meals resulted in the concentration of [U^{13}C]palmitate in plasma NEFA to increase until 300 mins postprandial (Figure 3.11A) and this was similar upon consumption of both the test meals in males and females (Figure 3.11B and C).

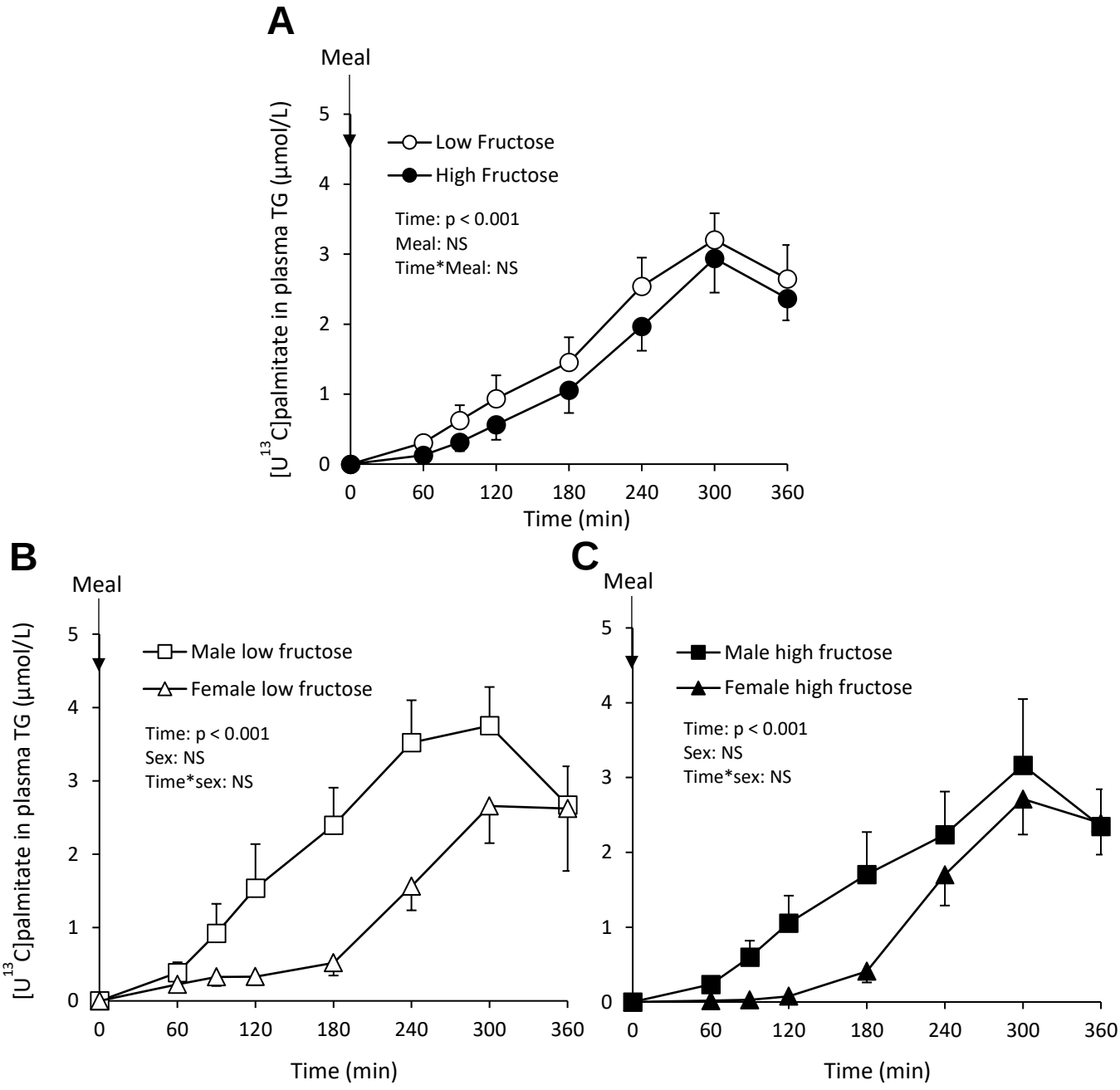

Figure 3.10: Postprandial concentration of [U^{13}C]palmitate in plasma TG (µmol/L) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviations: TG, triacylglycerol; NS, non-significant.

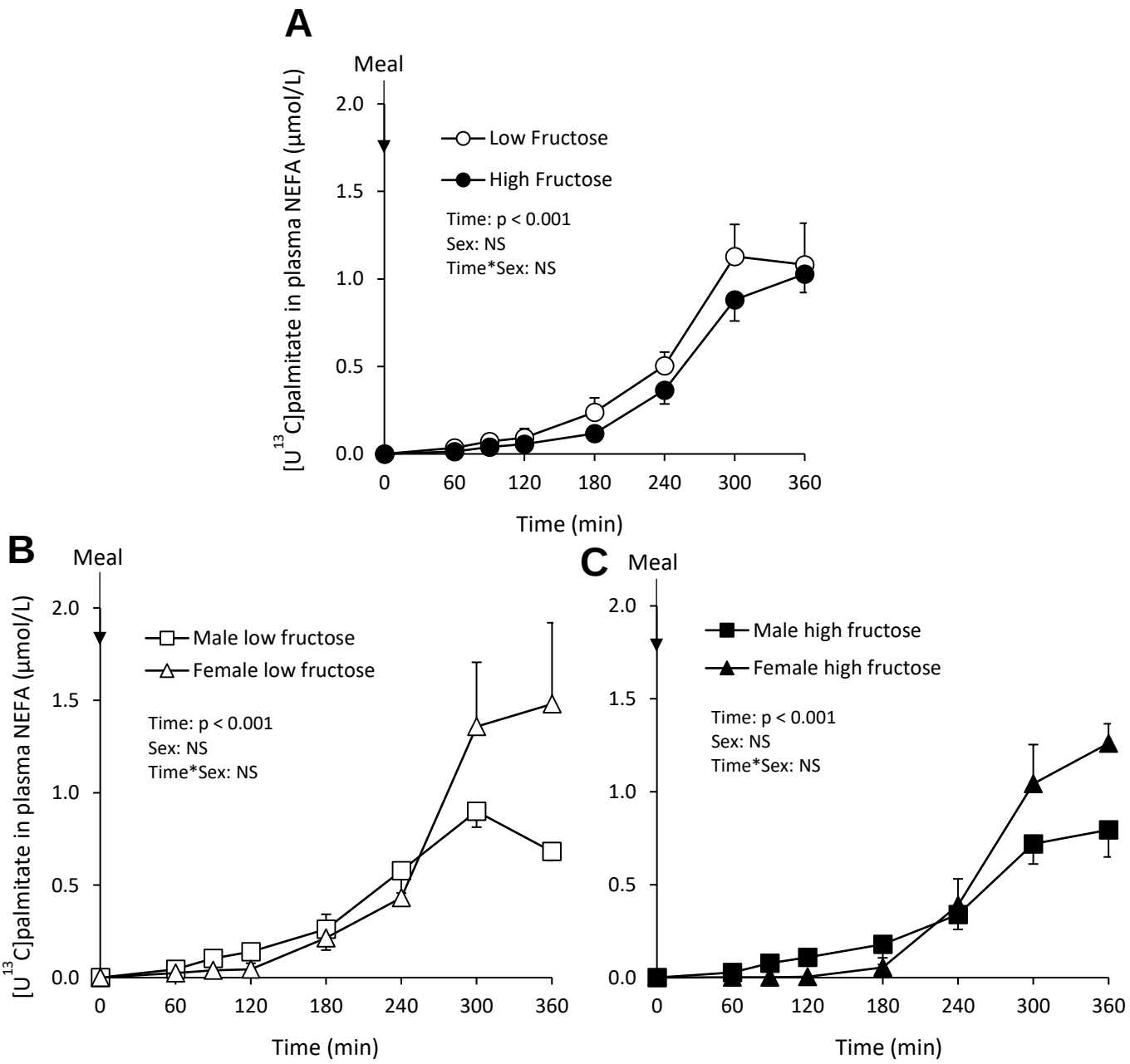

Figure 3.11: Postprandial concentration of [U^{13}C]palmitate in plasma NEFA (µmol/L) in **A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data is presented as mean ± SEM. Abbreviations: NEFA, non-esterified fatty acid; NS, non-significant.

In line with the findings for plasma TG, there was no difference in the appearance of [U^{13}C]palmitate in chylomicron-TG after ingestion of either test meal (Figure 3.12A). No significant difference was found between males and females after consumption of the high fructose meal. However, males had significantly higher [U^{13}C]palmitate in chylomicron-TG after consumption of the low fructose meal than females (Figure 3.12B and C).

For both test meals, the amount of [U^{13}C]palmitate in TRL-TG increased over the postprandial period. After consumption of the low fructose meal, the amount of [U^{13}C]palmitate in TRL-TG began to plateau after 300 mins while this trend was not seen after consumption of the high fructose meal (Figure 3.13A).

The postprandial concentration of [U^{13}C]palmitate VLDL-TG increased upon consumption of the test meals in a similar manner (Figure 3.14A). After consumption of the high fructose meal, the increase in [U^{13}C]palmitate VLDL-TG concentration was seen to slow down in both genders (Figure 3.14C). However, after consumption of the low fructose meal, the concentration of [U^{13}C]palmitate VLDL-TG continued to increase after 360 mins postprandial in females but not in males, where a plateauing was observed after 300 mins (Figure 3.14B).

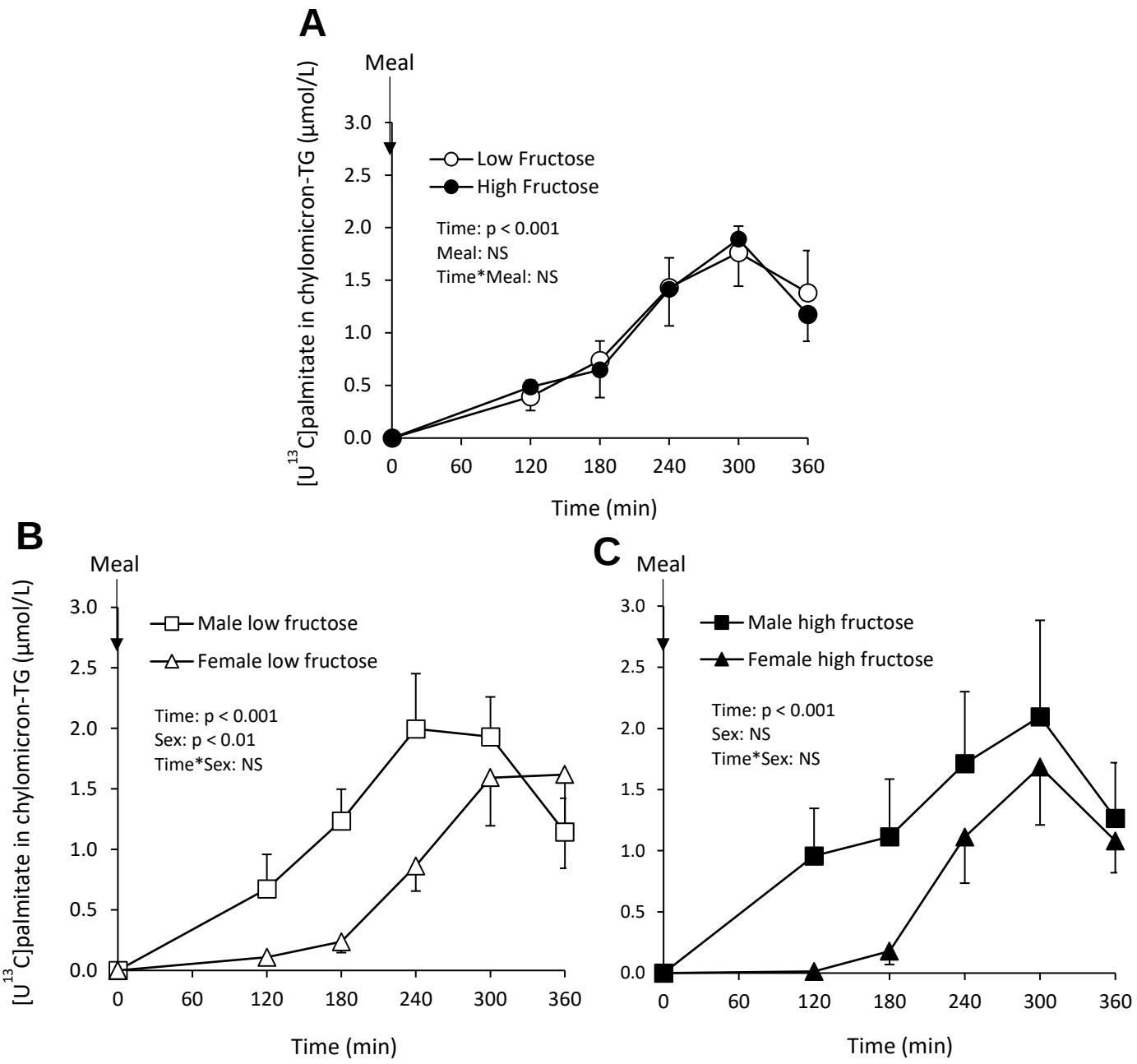

Figure 3.12: Postprandial concentration of [U^{13}C]palmitate in chylomicron-TG (µmol/L) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviations: TG, triacylglycerol; NS, non-significant.

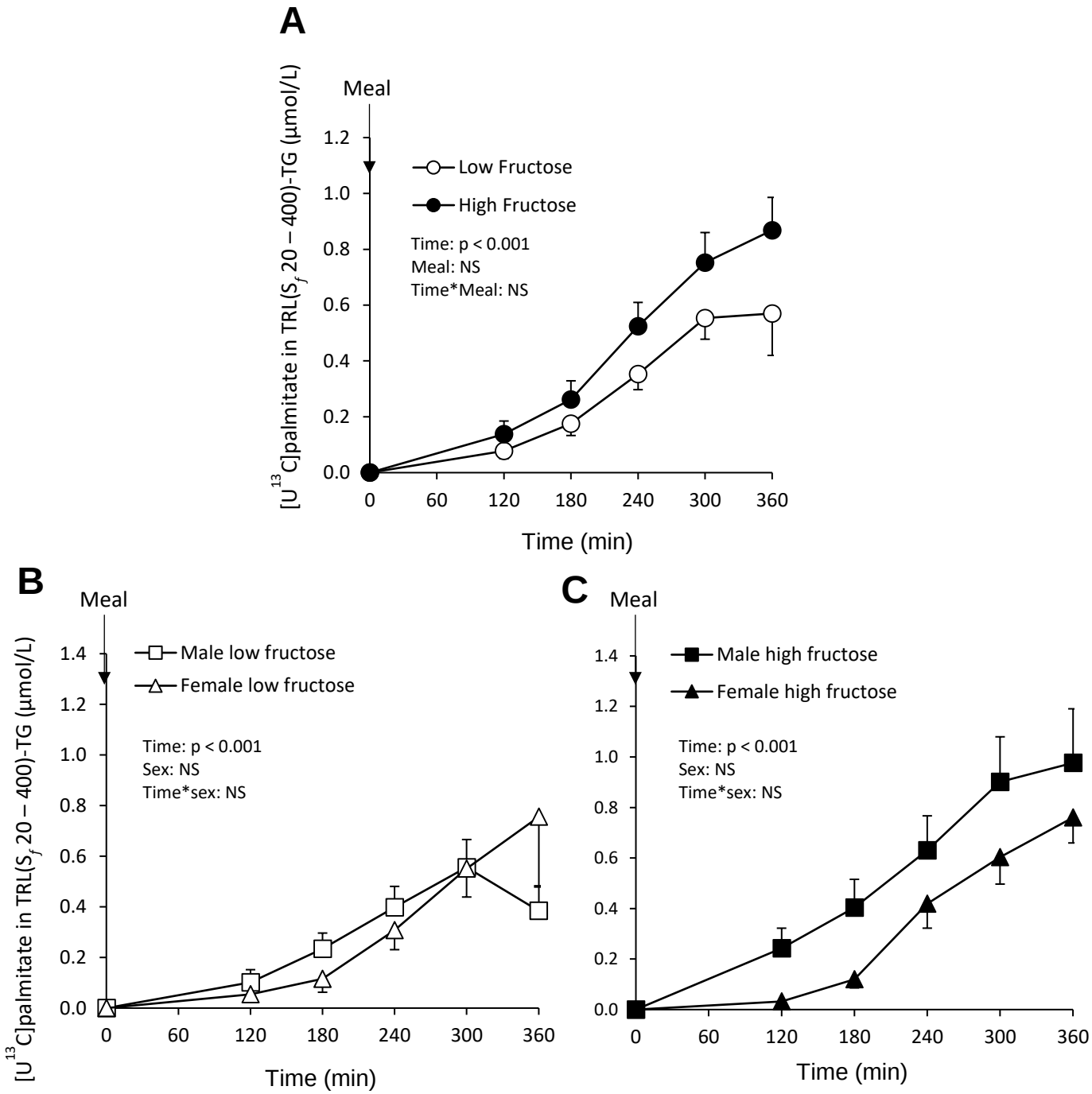

Figure 3.13: Postprandial concentration of [U^{13}C]palmitate in TRL(S_f 20 – 400)-TG (µmol/L) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviations: TRL, triacylglycerol-rich lipoprotein; TG, triacylglycerol; NS, non-significant.

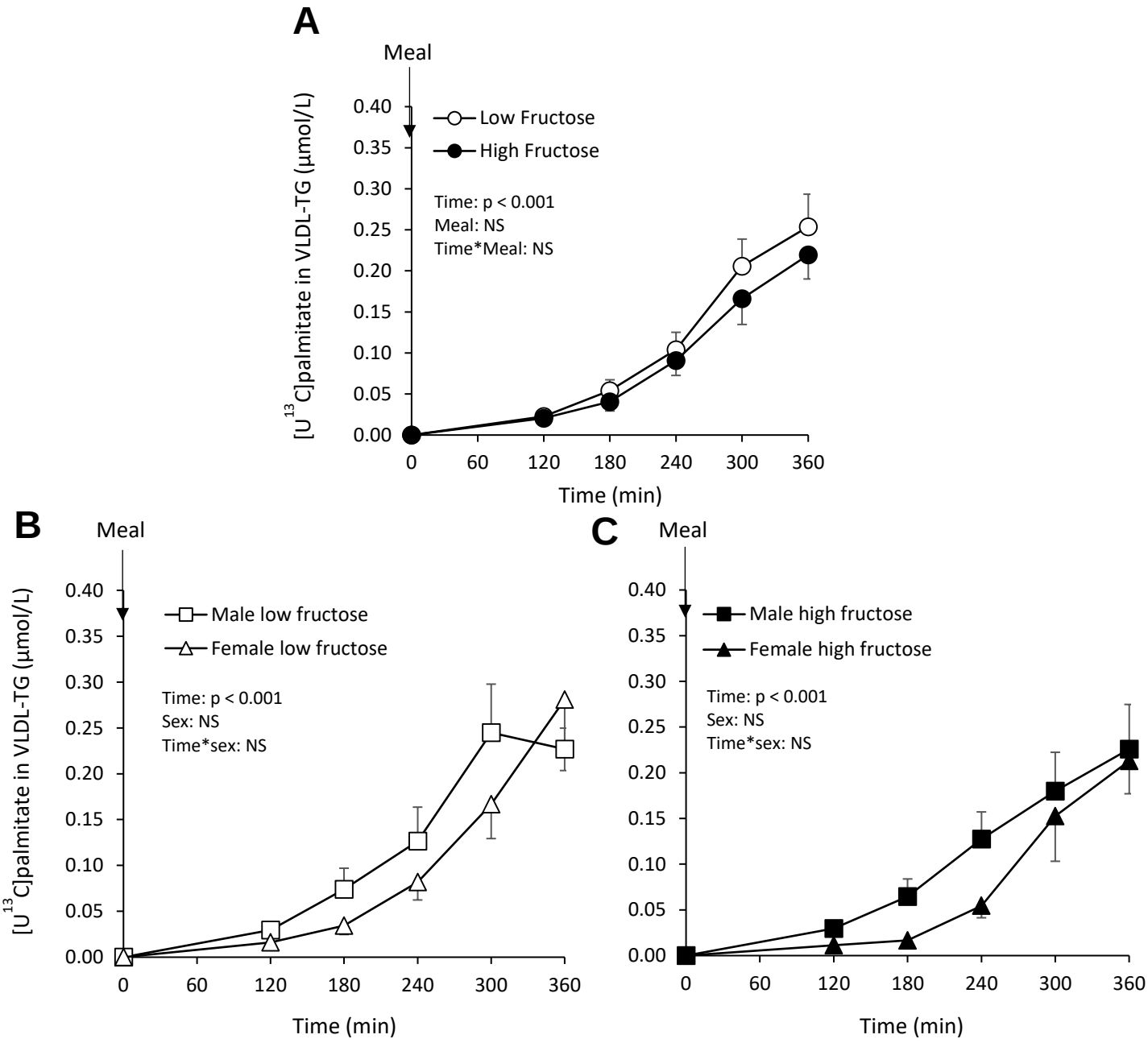

Figure 3.14: Postprandial concentration of [U^{13}C]palmitate in VLDL-TG (µmol/L) in **A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data is presented as mean ± SEM. Abbreviations: VLDL, very low density lipoprotein; TG; triacylglycerol; NS, non-significant.

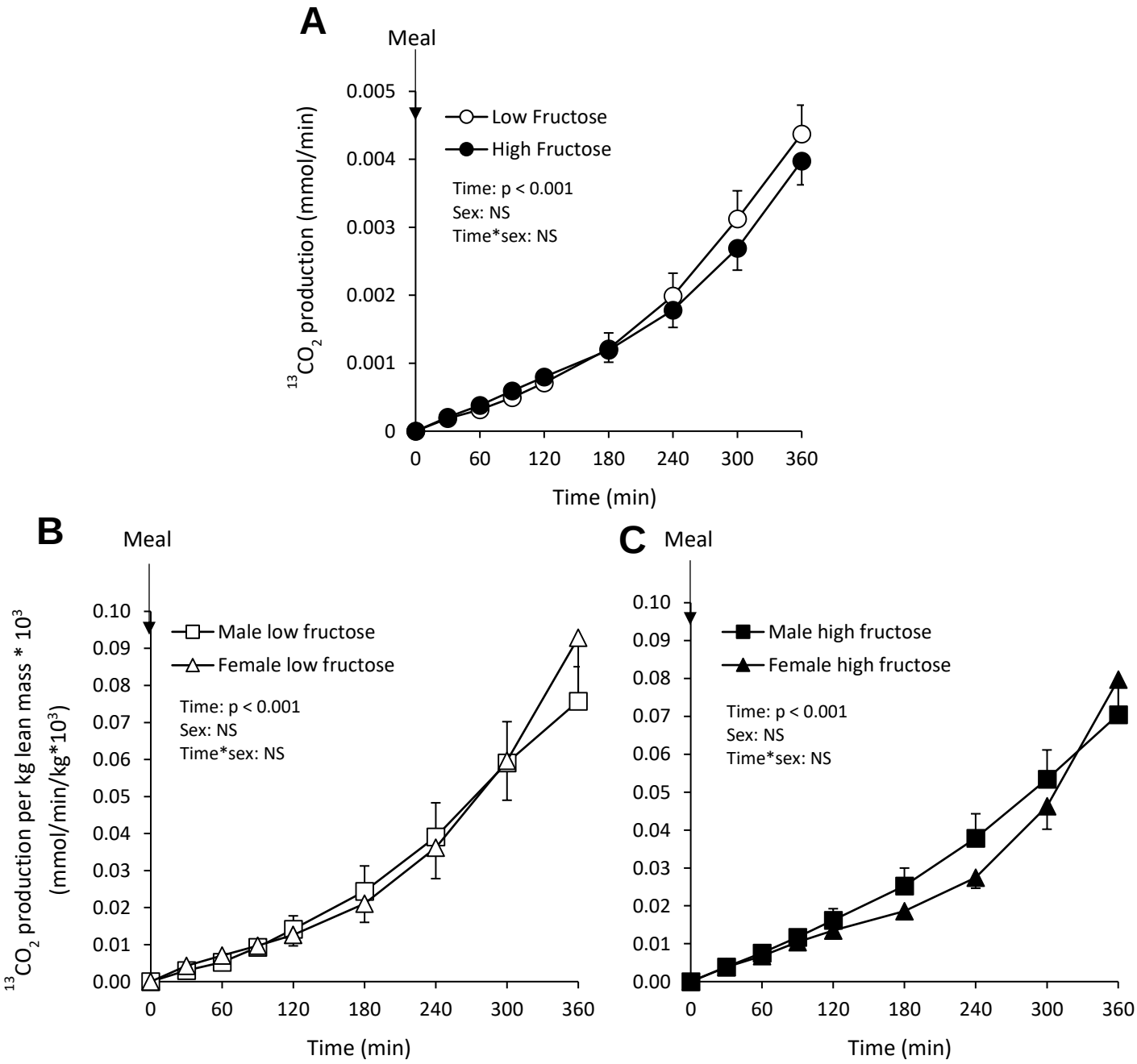

Figure 3.15: $^{13}CO_2$ production (mmol/min) in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals. $^{13}CO_2$ production per kg lean mass of **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviations: NS, non-significant.

Over time, whole body $^{13}CO_2$ production was not significantly higher after consumption of the low fructose compared to the high fructose meal (Figure 3.15A). The $^{13}CO_2$ rate of production was similar between males and females after consumption of the respective test meals (Figure 3.15B and C).

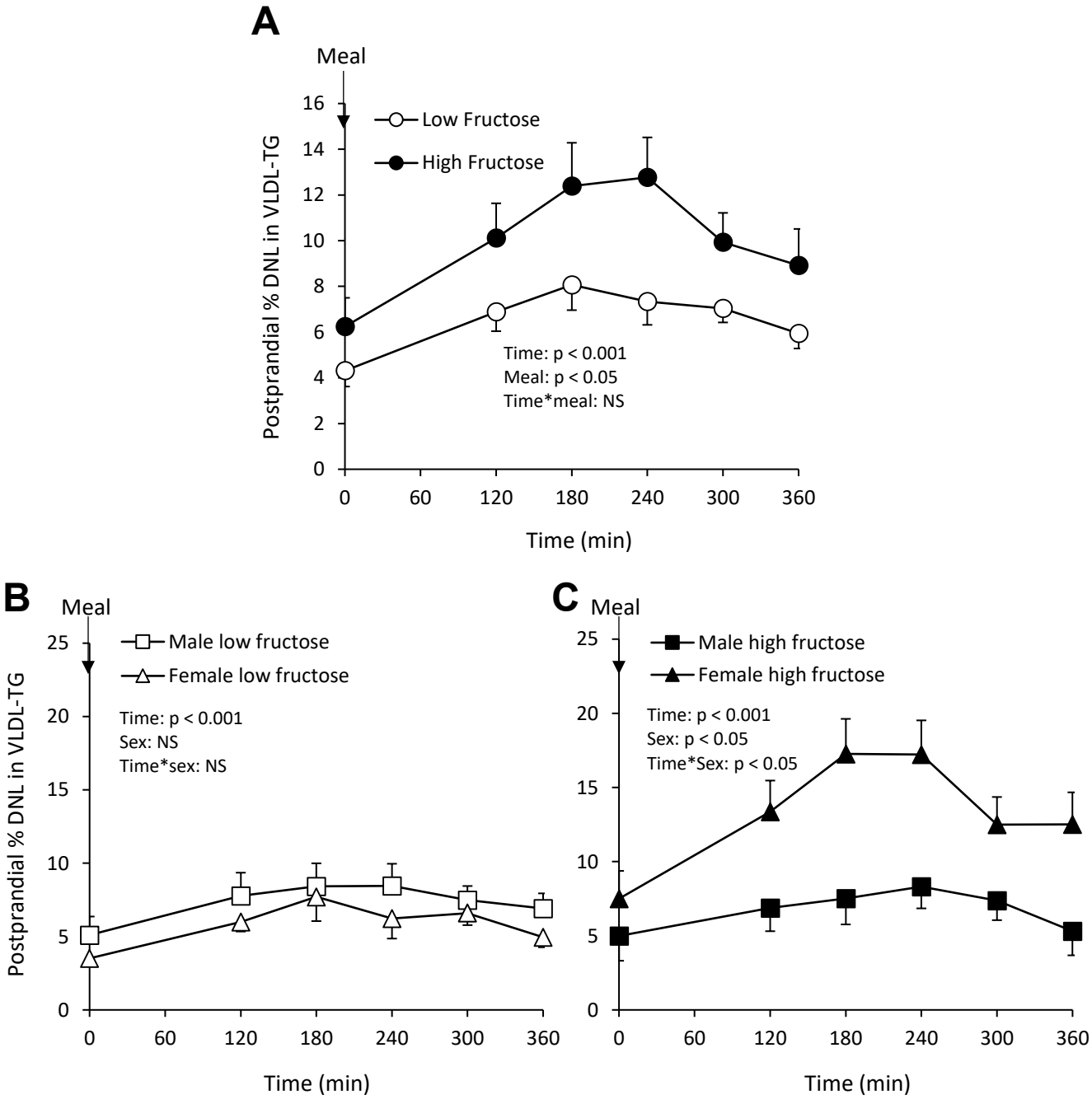

Figure 3.16: Postprandial % DNL in VLDL-TG in **(A)** all subjects (n = 16) upon consumption of low and high fructose meals, **(B)** males (n = 8) and females (n = 8) upon consumption of low fructose meal, and **(C)** males and females upon consumption of high fructose meal. Data presented as mean ± SEM. Abbreviations: DNL, *de novo* lipogenesis; VLDL, very low density lipoprotein; TG, triacylglycerol; NS, non-significant.

While fasting hepatic DNL was not significantly different between the two study days, postprandial hepatic DNL was significantly higher ($p < 0.05$) upon consumption of the high fructose compared to the low fructose meal (Figure 3.16A). When comparing between genders, the ingestion of the low fructose meal resulted in no difference in postprandial DNL between the genders (Figure 3.16B), but the consumption of the high fructose meal resulted in significantly ($p < 0.05$) higher postprandial hepatic DNL in females compared to males (Figure 3.16C).

3.3.4 Associations between hepatic fatty acid synthesis and oxidation

The association between hepatic FA synthesis and FA oxidation were investigated. Robust inverse correlations were found between postprandial hepatic DNL and postprandial plasma 3OHB concentrations after both test meals (low fructose meal: $r_s = - 0.67$, $p < 0.01$; high fructose meal: $r_s = - 0.57$, $p < 0.05$) (Figure 3.17A). There was also a significant inverse correlation between postprandial hepatic DNL and whole body oxidation, as assessed by the incorporation of ^{13}C into breath CO_2 after the high fructose ($r_s = - 0.72$, $p < 0.01$) but not the low fructose meal (Figure 3.17B).

A significant negative association was observed between postprandial plasma 3OHB concentrations and postprandial hepatic DNL in males after the low fructose meal and in females after the high fructose meal (Figure 3.18A and B). There were no associations after consumption of the low or high fructose meal between $^{13}CO_2$ production per kilogram lean mass and postprandial hepatic DNL in males or females (Figure 3.19A and B).

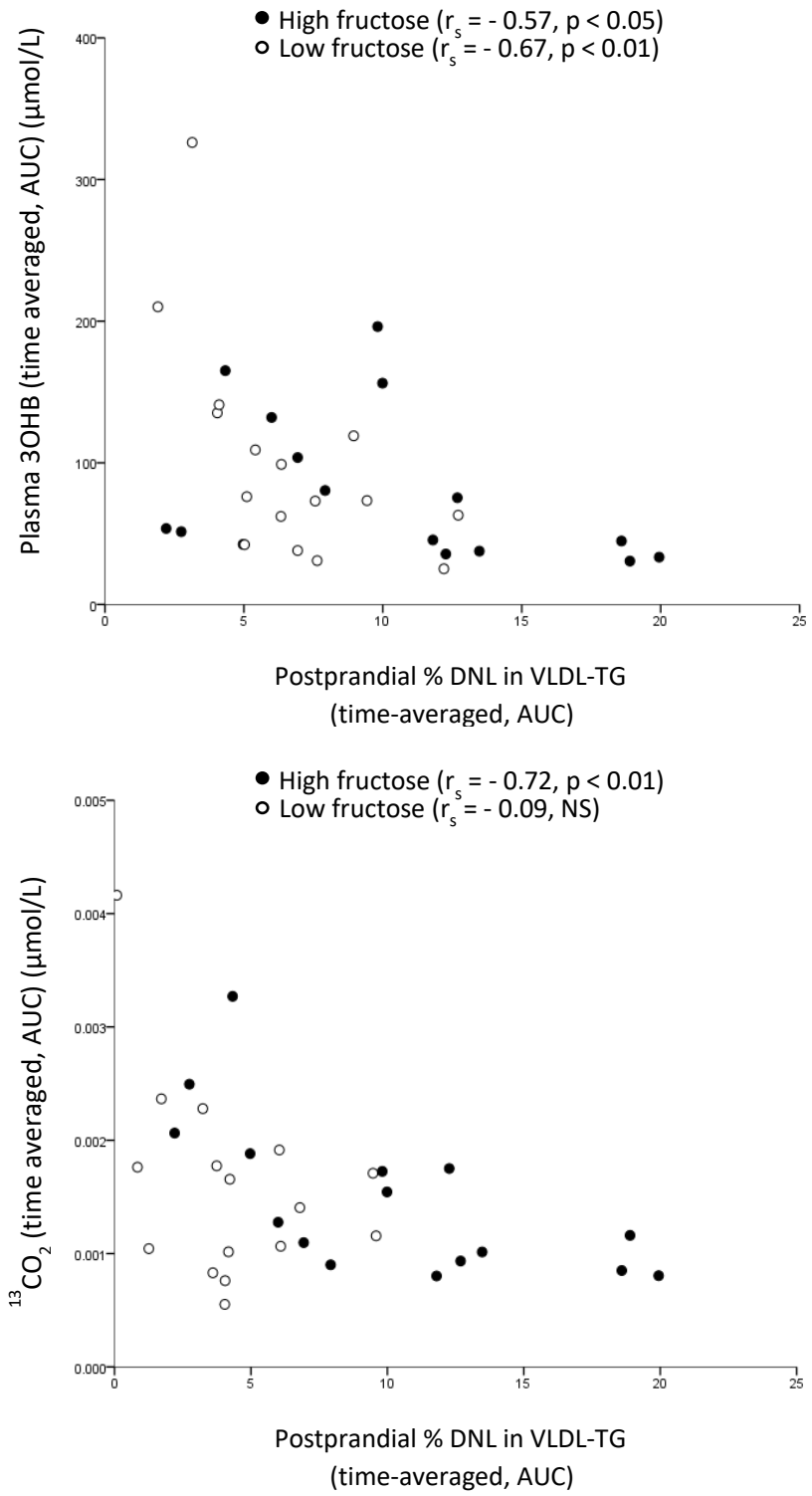

Figure 3.17: Correlations based on n = 16 between **(A)** postprandial % DNL in VLDL-TG and postprandial concentration of plasma 3OHB, and **(B)** postprandial % DNL in VLDL-TG and $^{13}CO_2$. Abbreviations: DNL, *de novo* lipogenesis; VLDL, very low density lipoprotein; TG, triacylglycerol; 3OHB, 3-hydroxybutyrate; NS, non-significant; AUC, area under curve

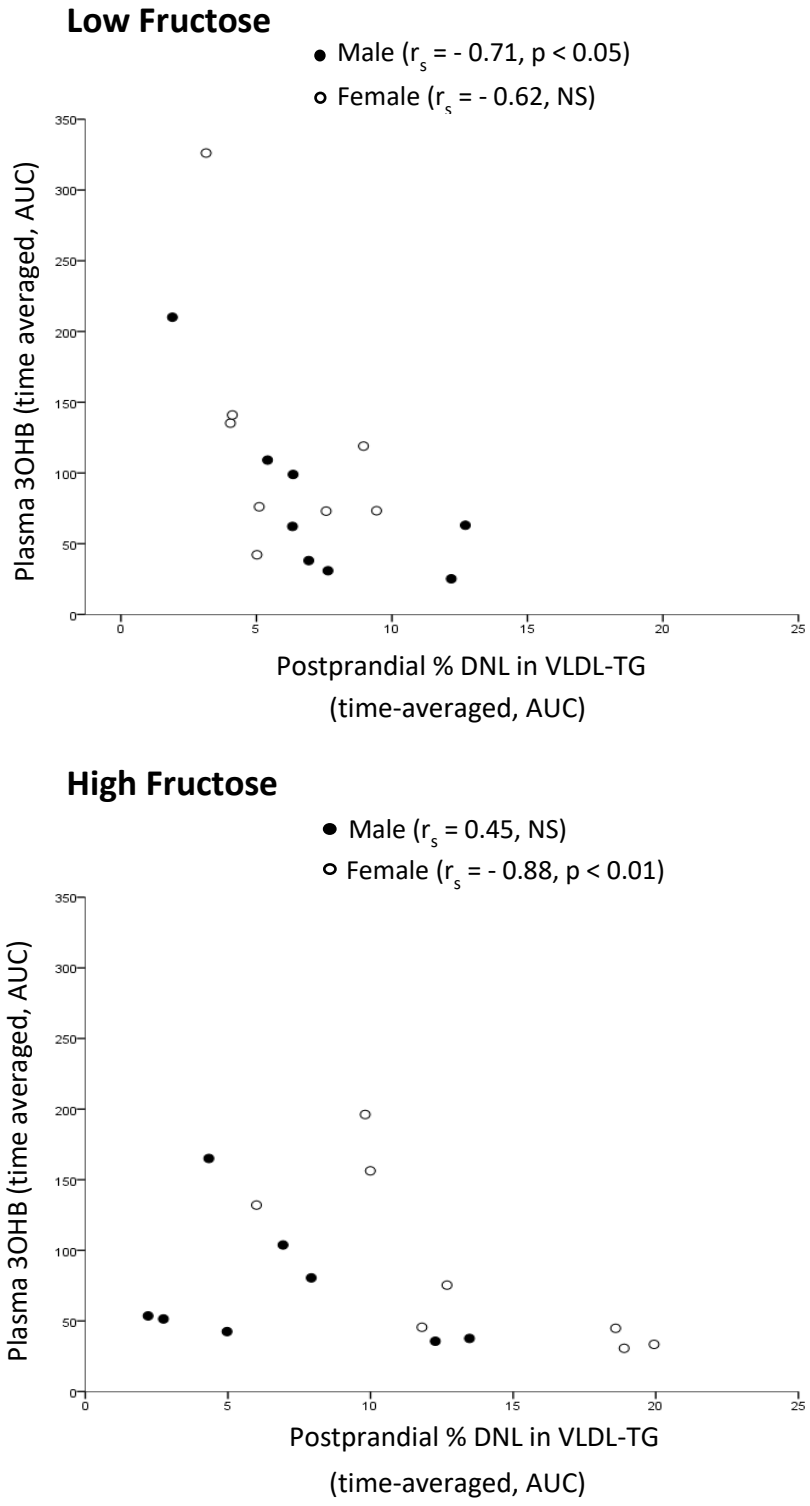

Figure 3.18: Correlation between postprandial % DNL in VLDL-TG and plasma 3OHB in males and females after consumption of **(A)** low fructose meal, and **(B)** high fructose meal. Abbreviations: DNL, *de novo* lipogenesis; VLDL, very low density lipoprotein; TG, triacylglycerol; 3OHB, 3-hydroxybutyrate; NS, non-significant, AUC, area under curve.

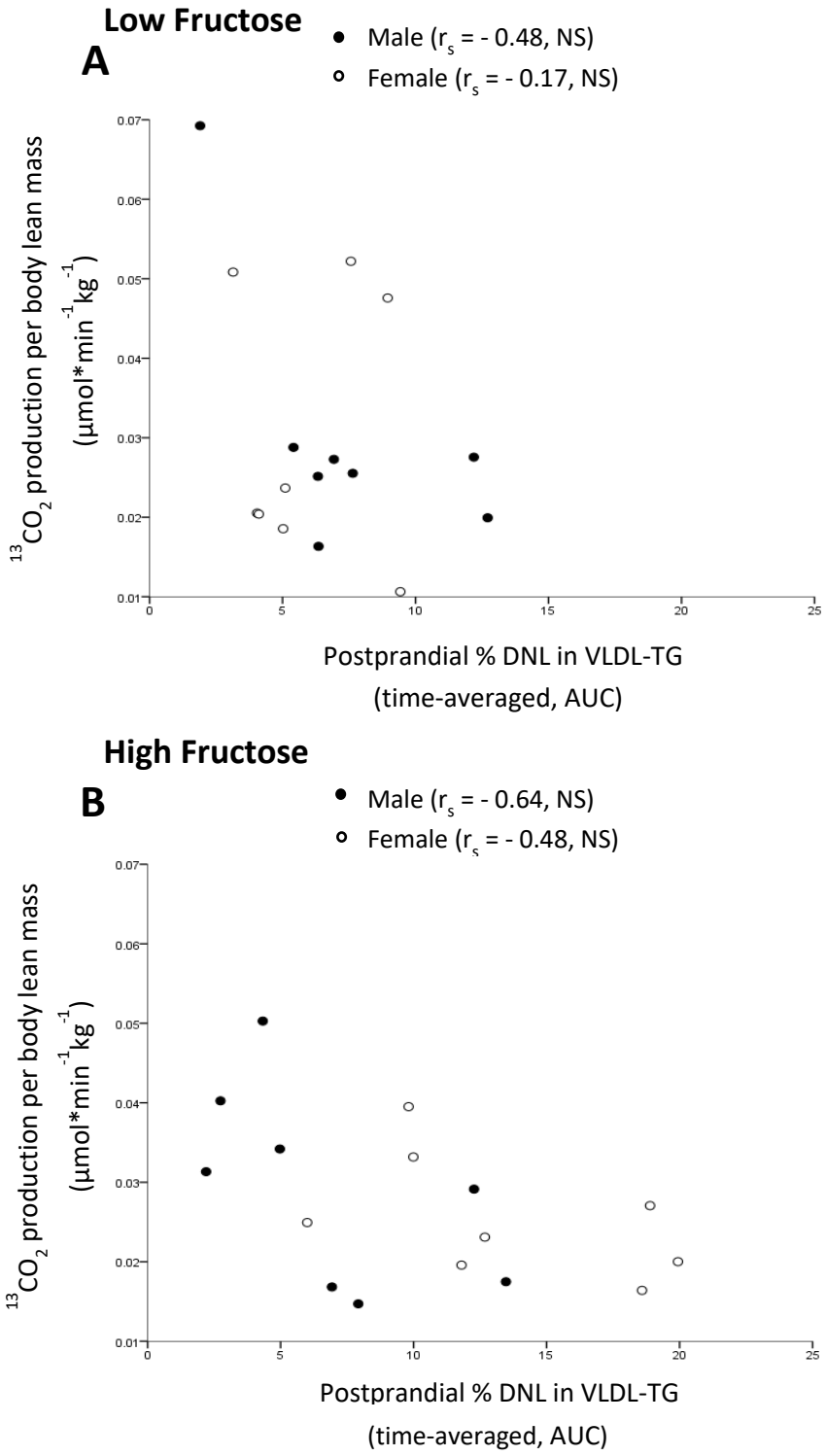

Figure 3.19: **(A)** Correlation between postprandial % DNL in VLDL-TG and $^{13}CO_2$ production per kilogram lean mass in males (n = 8) and females (n = 8) upon consumption of the low fructose meal, and **(B)** the correlation between postprandial % DNL in VLDL-TG and $^{13}CO_2$ production per kilogram lean mass in males and females after consumption of the high fructose meal. Abbreviations: DNL, *de novo* lipogenesis; VLDL, very low density lipoprotein; TG, triacylglycerol, NS, non-significant; AUC, area under curve.

3.4 Discussion

Increased consumption of fructose, mostly in the form of HFCS has often been linked to the rise in the worldwide incidence of NAFLD (ter Horst and Serlie, 2017). An increase in hepatic DNL has been suggested to contribute to the development of NAFLD and co-consumption of fructose and glucose, have been shown to be lipogenic (ter Horst and Serlie, 2017). Hence, it is of interest to investigate if meals containing both glucose and fructose, in different proportions, have a direct effect on hepatic DNL, FA partitioning and dietary FA oxidation, as this has not been previously reported. Therefore, the aim of the study was to investigate the effect of dietary sugar composition on postprandial hepatic DNL and dietary FA partitioning. Overall, it was found that compared to a low fructose meal, a meal containing a higher amount of fructose increased hepatic DNL, without changing dietary FA oxidation.

Previous studies have reported differences between males and females in postprandial DNL and hepatic FA oxidation (Blaak *et al.*, 2001; Pramfalk *et al.*, 2015); however, it remains unclear whether different meal composition would have a different impact on FA metabolism between genders. Therefore, I investigated whether there were any gender-specific effects on FA metabolism after consumption of mixed meals containing different amounts of glucose and fructose. Overall, the composition of the dietary sugars consumed had a marked effect on postprandial hepatic DNL in females but not males.

3.4.1 Effect of consumption of high and low fructose meals on plasma glucose and insulin levels

The consumption of the high fructose meal resulted in significantly lower plasma glucose and insulin levels compared to the low fructose meal. This was in line with expectations, as insulin responses increase with the amount of glucose consumed (Fu *et al.*, 2013). Fasting glucose and insulin levels were lower in females compared to males for both study visits which translated to a lower HOMA2-IR in females than males. This result was in line with previous findings (Geer and Shen, 2009) and was suggested to be linked to oestrogen production (D'Eon *et al.*, 2005).

3.4.2 Effects of consumption of high and low fructose meals on plasma fatty acid and triglyceride concentrations

Although there was no difference in postprandial NEFA concentrations after consumption of both test meals, females exhibited a larger amount of suppression and rebound in plasma NEFA concentrations than males. This difference may be explained in part, by the difference in postprandial activity of adipose tissue LPL between the genders.

With the postprandial increase in concentration of insulin, the expression of lipoprotein lipase (LPL) that hydrolyses dietary FA for cellular uptake is upregulated. Sex hormones are speculated to play a role in the regulation of adipose tissue LPL activity as its activity was found to be greater in females than males. Inverse correlation of testosterone and LPL was found in obese men (Ramirez et al., 1997) while introduction of oestrogen and progesterone has been shown to increase the activity of AT LPL in postmenstrual women (Rebuffé-Scrive *et al.*, 1987). Using [U^{13}C]palmitic acid, Piché *et*

al. traced the fate of dietary fat in a study involving 71 individuals who were fed 40 g carbohydrate and 40 g fat in a standard meal. The study found that the proportion of chylomicron-derived spillover FAs in the systemic NEFA pool was significantly higher in females compared to males ($p < 0.05$) (Piché *et al.*, 2018). It has also been previously suggested that females can clear chylomicron-TG more rapidly than males (Knuth and Horowitz, 2006). With faster clearance, less fatty acids can be taken up by adipose tissues for storage, and this results in more spillover and higher plasma NEFA concentrations in females compared to males.

Although there were no significant differences in postprandial plasma TG concentrations after the consumption of either meal, there was a difference in plasma TG concentrations after consumption of the low fructose meal between males and females. The difference in concentration of plasma TG between the genders continue to be shadowed in the concentration of [U^{13}C]palmitate in chylomicron-TG, showing the strong robustness of the analysis. In addition, as the subjects were fed a fixed amount of monosaccharides and fats, the relative dose per kilogram is generally higher in females than in males, which may lead to underestimation of the true effect of the test meals on males compared to females. The lower postprandial TG concentrations seen in females may be due to the suppressive effect of oestrogen on postprandial lipid accumulation (Couillard *et al.*, 1999). This trend is in agreement with a study by Couchepin *et al*. (2008) which showed that fructose consumption caused differential effects between the genders (n = 16) on fasting plasma triglyceride levels, with 6 days of a hyperenergetic high fructose diet causing a much larger (71%) increase in plasma TG levels in males than in females (18%).

Oestrogen has been suggested to have a favourable effect on postprandial TG levels. Using a rodent model, Diamant *et al.* (1975) demonstrated the mechanism of action of oestrogen in the reduction of postprandial accumulation of TG may be through the activity of acyl-coenzyme A-cholesterol acyltransferase 2 (ACAT2). Oestrogen has also been reported to reduce the activity of ACAT2 in primate studies (Kavanagh *et al.*, 2009). Work in mice, where ACAT2 was knocked out, have shown that global and liver-specific ACAT2 deficiency decreased the accumulation of hepatic cholesteryl esters and TG and led to higher levels of VLDL secretion and higher TG mobilisation and hydrolysis in the liver due to the lack of cholesteryl esters which exhibit a competitive inhibition effect on TG hydrolases (Alger *et al.*, 2010). In a separate rodent study, the differential role of fibroblast growth factor-21 has been shown to underpin the differences in lipid metabolism after feeding of a high fructose, high fat diet (Chukijrungroat *et al.*, 2017). Oestrogen was shown to protect against hepatic steatosis by increasing fibroblast growth factor-21 expression as mice which have no ovarian hormones were seen to have higher amounts of hepatic steatosis after feeding of a high fructose and fat diet for 12 weeks and lower expression of fibroblast growth factor-21 (Chukijrungroat *et al.*, 2017). On the other hand, androgen activity might be associated with lipid metabolism and accumulation (Nasiri *et al.*, 2015). A clinical study by Hazlehurst *et al.* (2016) showed that inhibition of 5α-reductase, the enzyme responsible for conversion of testosterone to dihydrotestosterone led to increased hepatic lipid accumulation. Other observational and rodent studies also found that lower testosterone concentrations in men were associated with increased hepatic steatosis, in line with the finding by Hazlehurst *et al*. (2016) (Völzke *et al.*, 2010; Kim *et al.*, 2012; Nasiri *et al.*,

2015). These results are in line with our finding that males had higher postprandial TG levels than females.

3.4.3 Fructose-induced hepatic DNL: the effect of consuming high and low fructose meals

The postprandial contribution of hepatic DNL FA to VLDL-TG was significantly higher after consumption of the high fructose compared to the low fructose meal. This is in line with previous findings that have reported dietary fructose to be a potent stimulant of hepatic DNL whereby hepatic DNL increased within 2 to 4 hours of consumption of a fructose-rich meal (Chong *et al.*, 2007; Parks *et al.*, 2008). A possible reason why fructose is more lipogenic than glucose is that fructose interacts directly with transcription factors carbohydrate response element binding protein (ChREBP) and sterol response element binding protein-1c (SREBP-1c) which upregulate enzymes required for FA synthesis including acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Fructose also indirectly stimulates SREBP-1c and ChREBP via PPAR-γ coactivator 1β which is a transcriptional coactivator for SREBP-1c (Nagai *et al.*, 2009). Fructose-1,6-bisphosphate, an intermediate in the metabolism of fructose can combine with glyceraldehyde to produce xylulose-5-phosphate (Bonsignore *et al.* 1962), which is found to be a strong activator of protein phosphatase 2A (Kabashima *et al.*, 2003) that has the function of activating ChREBP (Dentin *et al.*, 2006).

It has previously been suggested by Chong *et al.* (2007) that while fructose induces DNL, it only played a small direct role in DNL but potentially has a much larger effect on hepatic FA partitioning. An increase in hepatic DNL would lead to an increase in the concentration of malonyl-CoA, an intermediate in the DNL pathway, which is also a

potent inhibitor of carnitine palmitoyltransferase-1 (CPT1), the enzyme which transports FA into the mitochondria for oxidation (Sanders and Griffin, 2016). The inhibition of CPT1 would potentially lead to a downregulation in hepatic FA oxidation, increasing the flux of FAs toward the esterification pathway. I found that consumption of the different test meals resulted in a different hepatic DNL responses. Upon consumption of the high fructose meal, postprandial hepatic DNL was significantly higher in females compared to males. This was not seen after consumption of the low fructose meal though there was an overall difference between the genders. I speculate that this may be because the amount of DNL after the low fructose meal may be not high enough to see significant differences between the genders.

3.4.4 Effects of consumption of high and low fructose meals on dietary fatty acid oxidation

In the present study, FA oxidation was accessed by measuring postprandial 3OHB levels as well as tracing the appearance of ^{13}C (from dietary fat) in breath CO_2. It was found that after consumption of the meals, dietary fat oxidation was not significantly higher after the low compared to the high fructose meal as evidenced from the amount of $^{13}CO_2$ produced. There was also no significant differences found in the postprandial concentrations of plasma 3OHB between the meals. However, I did find that females had a significantly higher plasma 3OHB concentration after consumption of the low fructose meal compared to males. This result suggests that there is a gender difference in hepatic but not whole body FA oxidation. These results were in line with a study by Pramfalk *et al.* (2015) where 22 healthy subjects (11 males, 11 females) were fed 40 g fat and 40 g carbohydrate and they found a higher incorporation of ^{13}C from dietary FAs

in plasma 3OHB in females compared to males. In contrast to my study, they also found a higher appearance of ^{13}C from dietary fat in breath CO_2 in females compared to males. Tran *et al*. (2010) also investigated the effect of a single bolus of fructose fed to 18 healthy subjects (9 males, 9 females) on lipid oxidation and found that whole body lipid oxidation was suppressed in males but not in females after fructose consumption and was overall lower than in females both at baseline and after fructose consumption. This study however only fed fructose and would thus lead to a different result from my study which used a mixed fructose-glucose meal (Tran *et al.*, 2010).

3.4.5 Link between DNL and hepatic fatty acid oxidation

In the present study, a significant inverse correlation was found between postprandial hepatic DNL and the postprandial plasma concentration of 3OHB and $^{13}CO_2$ production upon consumption of the high fructose meal. This shows the strong negative association between different liver metabolites and also between liver function and whole body metabolism upon high fructose feeding. However, while consumption of the low fructose meal resulted in a significant inverse correlation between postprandial hepatic DNL and the postprandial plasma concentration of 3OHB, the negative association between liver function and whole body metabolism was not strong as the correlation between postprandial hepatic DNL and the postprandial $^{13}CO_2$ production upon consumption of the low fructose meal did not reach significance.

When the results were analysed after stratifying them by gender, there was a significant inverse correlation between postprandial hepatic DNL and postprandial plasma 3OHB upon consumption of the high fructose meal in females but not males, where a significant inverse correlation was found after ingestion of the low fructose meal. Similar

trends were seen in females after consumption of the low fructose meal and in males after consumption of the high fructose meal, though neither of these reached statistical significance. A possible reason for this is the small sample size and the possible presence of outliers which may have led to the data being seen as not statistically significant. For the association between postprandial hepatic DNL and postprandial plasma 3OHB, outliers exist within genders. For instance, in 3 males that when %DNL was below 5%, the time-averaged AUC of plasma 3OHB was below 100 µmol/L upon consumption of the high fructose meal. In females, there was also 1 case that upon consumption of the low fructose meal, while %DNL was below 5%, the time averaged AUC of plasma 3OHB was above 300 µmol/L, which doubled that observed in the rest of the females at the similar amount of percentage contribution of postprandial DNL in VLDL-TG. Significant variance existed within genders when postprandial hepatic DNL was correlated with amount of ^{13}C-labelled carbon dioxide upon consumption of either low or high fructose meals. In addition, confounding factors existed. One of which was that while increase in DNL contributed to an increase in hepatic fatty acid pool and also meant an increase in concentration of malonyl CoA, it is not a complete switch for reduction in the production of carbon dioxide as fatty acid can be partitioned into the ketogenesis pathway or into the esterification pathway for production of VLDL or formation of TG which would have then remained in the liver and contributed to the hepatic TG pool.

3.4.6 The effect of consumption of high and low fructose meals on postprandial hepatic fatty acid partitioning

The appearance of [^{13}C]palmitate in the plasma NEFA pool was lower after the high fructose compared to the low fructose meal which was in line with the findings by Chong *et al.* (2007). This may be due to decreased activation of lipoprotein lipase (LPL) (Sadur and Eckel, 1982), resulting in less hydrolysis of chylomicron-TGs and VLDL-TGs and less spillover of LPL-derived NEFAs, leading to lower concentration of plasma NEFA.

However, there was a lack of change in the amount of [^{13}C]palmitate incorporated into VLDL-TG after consumption of both meals. This data does not agree with the findings from Chong *et al*. in 2007 which found that consumption of fructose leads to a significantly higher amount of palmitate incorporated into newly synthesised VLDL-TG as compared to consumption of glucose (Chong *et al.*, 2007). My study differs from Chong *et al.* (2007) as I used mixed fructose-glucose meals rather than pure fructose or glucose and thus did not completely isolate the effects of either monosaccharide, possibly leading to a lack of difference seen.

3.4.7 Study limitations

There were some limitations to this study that should be taken into consideration. As this study was designed to give all subjects, regardless of gender, with the same amount of energy, I did not provide the test meals in accordance to their individual weight, nutritional and energy requirement. This may have resulted in the overfeeding of some individuals, especially females compared to males, leading to a potential error in the comparison of the postprandial effect of low and high fructose meals on concentration of plasma metabolites and hepatic fatty acid synthesis and partitioning. This error could also have been incorporated into the results achieved from comparisons done between genders. In this thesis, in terms of presenting the results between genders, I have reported their significant differences at each time point but not the differences between the changes in each gender. To better evaluate the results, it is important that I should have shown that the genders respond significantly differently. This can be carried out using independent sample t-tests to compare the AUC of each parameter that had results stratified by gender; for instance, the concentration of plasma metabolites and lipoprotein-TG fractions. I did not standardise the participants' diets before the first study day or over the 4-week washout period. Although advised to maintain their regular diet and lifestyle habits, it is possible that participants might have deviated and this could have affected their responses on the study days. Liver fat was only measured once for each subject in the fasting state and not before and after the test meals. Therefore, I could not comment on whether the increase in DNL after the high fructose meal altered the amount of fat within the liver. It could be speculated, given the small but significant change in hepatic DNL and lack of change in hepatic FA oxidation that liver fat content remained unchanged within this short period of time. A longer dietary intervention study

could thus be useful to determine the effect of chronic consumption of different amounts of glucose and fructose on liver FA metabolism. Schwarz *et al*. (2015) showed that consumption of a high fructose diet (25% of total energy) for 9 days was able to increase liver fat content by 137% in 8 healthy male subjects compared to a control diet where the fructose was isoenergetically replaced by complex carbohydrates. Also, my study subjects were aged between 38 to 50 years and therefore it is unclear if the results observed here could be translated to younger or older populations. It is likely that DNL increases with fructose consumption regardless of age, as Chong *et al*. (2007) showed the same trend in 14 healthy subjects (8 males, 6 females) aged 22 to 64 and a significant decrease in DNL was seen in a group of 41 children aged from 9 to 18 after 9 days of fructose restriction (Schwarz *et al.*, 2017). The assay used to measure concentration of fructose in plasma samples was important as it determined the robustness of the results. For my study, there may have been an overestimation of the concentration of fructose present in the samples as the assay was primary designed to measure the concentration of fructose in red and white wine, beer and fruit juices; and not for the quantitative *in-vitro* determination of concentration of plasma fructose. Therefore, the assay used may not have been sensitive enough to detect small concentrations of fructose in plasma. I also only investigated the effects of these meals on Caucasian individuals and it would be interesting to do the same study on other ethnicities as it has been shown that individuals of different ethnicities have different lipid metabolism. Hudgins *et al*. (2017) showed that South Asians had enhanced lipogenic sensitivity, with higher VLDL-TG levels and DNL as compared to Caucasians after consumption of a single fructose and glucose mixed beverage.

In conclusion, I observed differences between consuming low and high fructose meals and the gender specific effects on hepatic FA metabolism, with females exhibiting higher DNL after the consumption of the high fructose meal and higher 3OHB concentrations after consumption of the low fructose meal. These differences were not seen in male subjects. Although between the test meals the data suggest that consuming a single high fructose meal had little overall effect on hepatic lipid metabolism, the effect of it notably increasing hepatic DNL in females, but not males is of interest and it could be suggested that if a high fructose intake were continued for a longer period, changes in hepatic fatty acid partitioning would have become evident. In chapter 5, I investigated the effect of consuming different composition of monosaccharides over a longer period of time on hepatic liver metabolism.

Chapter 4:
Tracing the Metabolic Fate of Dietary Fructose

4.1 Introduction

4.1.1 Metabolic fate of fructose

Fructose is suggested to be primarily metabolised in the liver where it can be converted into glucose, lactate and fatty acids (FAs) via *de novo* lipogenesis (DNL) (Nilsson and Hultman, 1974; Tounian *et al.*, 1994). In the liver, fructose is phosphorylated to fructose-1-phosphate which undergoes glycolysis and oxidative decarboxylation to form acetyl-CoA. With consumption of meals high in fructose, although a large amount of acetyl-CoA is generated, only a small proportion of it is used as the substrate for hepatic DNL (Chong *et al.*, 2007).

In catabolism, the three major metabolic pathways which fructose enters involve its conversion into glucose (50%), lactate (25%) and glycogenesis (15%); after which the metabolites can be stored within the liver or oxidised into carbon dioxide via tricarboxylic acid cycle (TCA) cycle (Lustig, 2013). Studies using [U^{13}C]fructose have shown that approximately 50% of ingested fructose was expired in breath CO_2 within 4 to 6 hours of consumption of a single dose of fructose and the other 50% was stored mainly in the liver as glycogen (Delarue *et al.*, 1993; Tran *et al.*, 2010). It has also been demonstrated that when consumed with fats, dietary fructose enters the DNL pathway along with being utilised for glycerol production within 4 hours of ingestion (Chong *et al.*, 2007). As previous studies which traced the metabolic fate of fructose have mostly fed fructose to the subjects in isolation, it is unclear whether consumption of a mixed meal containing fructose, glucose and fat would result in different proportions of fructose being metabolised via oxidation or DNL.

4.1.2 Aim and Hypothesis

Aim:

To investigate the metabolic fate of dietary fructose when consumed in combination with glucose and fat.

Hypothesis:

A low percentage of dietary fructose will be utilised in the DNL pathway to make palmitate and be secreted in VLDL-TG whilst a larger proportion will undergo oxidation.

4.2 Methods

4.2.1 Ethics

The study protocol was approved by the Portsmouth Research Ethics Committee (Portsmouth REC 12/SC/0267).

4.2.2 Subject recruitment

Eight healthy subjects (4 males, 4 pre-menopausal females) were recruited and all subjects gave written informed consent. Prior to the commencement of the study, all volunteers attended a screening session to ensure they were suitable for the study. The inclusion criteria were individuals aged 20 to 50 years old, of BMI >23 but <30 kg/m^2, waist circumference > 80 cm for females and > 94 cm for males. To be accepted into the study, subjects had to be healthy, free from known disease and not taking medication that could alter glucose or lipid metabolism. Participants needed to be non-smokers and consumed alcohol within recommended limits (Nascimbeni *et al.*, 2013). Exclusion criteria included those who had dyslipidaemia, intolerance to fructose or hyperglycemia. Female participants had to be not pregnant or using contraceptives (eg. oral contraceptives, hormone replacement therapy (HRT)) for the past 12 months. Prior

to the study day, subjects were asked to avoid food naturally enriched in ^{13}C (e.g. cornflakes) for 48 hours and refrain from strenuous exercise and alcohol for 24 hours.

4.2.3 Study design

The study was a dietary trial designed to investigate the metabolic fate of dietary fructose in a meal by which the carbohydrate component mimicked the monosaccharide composition in HFCS. Therefore, I designed the trial to not have a comparator arm and only required subjects to consume a single high fructose meal which incorporated [U^{13}C]fructose to trace the metabolic fate of dietary fructose.

Subjects attended the clinical research unit after an overnight fast for a single study day, where they consumed the high fructose meal which consisted of a chocolate-flavoured fat-emulsion and a lemon-flavoured drink (Figure 4.1). The fat-emulsion had an energy content of 520 kcal and was made up of 30g of palm oil and 20g of olive oil (Appendix table 1). The fatty acid composition of palm oil and olive oil is as shown in Table 3.1. The lemon-flavoured drink was of an energy content of about 400 kcal and contained 60 g of fructose, 40 g of glucose and 500 mg of [U^{13}C]fructose (Appendix table 4). The total energy for the study meal added up to about 920 kcal.

Figure 4.1: Test meal consumed by the subjects at the start of the study day

Both males and females were given the same test meal as they were meant to be fed with the same amount of energy to ensure that the study was isoenergetic between subjects for better comparison of postprandial effects on concentration of plasma metabolites upon consumption of the high fructose test meal. The total amount of energy per meal added up to about 920 kcal, taking into consideration the small proportion of energy from the artificial sweetener, cocoa powder and lemon juice added into the chocolate fat-emulsion and the lemon-flavoured sugary drink respectively (Appendix Table 1 and 4).

4.2.4 Overview of study day

On the study day, following cannulation of the antecubital fossa vein, baseline blood and breath samples were collected from subjects. They were then served the test meal made up of both a chocolate-flavoured emulsion with 50 g of fat incorporated and a lemon-flavoured sugar drink containing 60 g fructose, 40 g glucose and 500 mg of [U^{13}C]fructose to trace the metabolic fate of fructose. Both the drinks were made using deuterated water. Subjects had also been told to consume deuterated water ($^{2}H_2O$) (3 g/kg body water) from the evening prior and this consumption continued throughout the study day to maintain the enrichment of their body water with 0.3% deuterium. Postprandial blood and breath samples were taken regularly at 30, 60, 90, 120, 180, 240, 300 and 360 mins postprandial and metabolites analysed as mentioned in Chapter 3, section 3.2.5.

4.2.5 Methods for Biochemical Analysis

The collection of whole blood, measurement of concentration of plasma metabolites, separation of lipoproteins and analysis of FA composition and the percent incorporation of stable isotope ([U^{13}C]fructose) was performed as described in Chapter 2, section 2.1.

4.2.6 Calculations

HOMA2-IR was calculated; see Chapter 3, section 3.4.

The net amount of labelled dietary fructose recovered in breath and the proportion appearing as newly synthesised palmitate in VLDL-TG were determined using the following calculations based on the work of Chong *et al.* (2007). To estimate the proportion of labelled fructose that was oxidised to produce $^{13}CO_2$ in the breath, the total amount of $^{13}CO_2$ (mmol) produced throughout the study period (6 hours) was first divided by 6 (number of ^{13}C atoms in [U^{13}C]fructose) to give an equivalent amount (mmol) of [U^{13}C]fructose. This was then divided by the amount (mmol) of [U^{13}C]fructose added to the test meal to obtain a percentage of dietary fructose recovered through $^{13}CO_2$ in the breath and thus fructose oxidation (Chong *et al.*, 2007).

To estimate the proportion of labelled fructose that was incorporated into VLDL-TG through DNL, the following formula was used:

$$\% \text{ DNL} = \frac{\text{AUC of labelled carbon in VLDL-TG x 17 x 3}}{\text{Amount of labelled carbon in sugar drink}}$$

where 17 is the number of carbons in each methylated palmitate and 3 is the number of FAs in each TG molecule (Chong *et al.*, 2007).

4.2.7 Statistical analysis

Data were analysed using SPSS 22 software (SPSS [UK] Ltd., Chertsey, U.K.) and presented as mean ± SEM in figures and median (min – max) in tables. Statistical significance was set at $p < 0.05$. Using the trapezoid method, postprandial area under the curve (AUC) of concentration of metabolites from subjects were calculated and later divided by the relevant time intervals to give time-averaged values. Repeated measures ANOVA (RMANOVA) with time was the factor was used to analyse the postprandial plasma concentration of glucose, fructose, insulin, TG, NEFA, 3OHB, ^{13}C-palmitate chylomicron-TG, ^{13}C-palmitate TRL-(S_f 20 – 400)TG, ^{13}C-palmitate VLDL-TG, postprandial percentage DNL in VLDL-TG and postprandial $^{13}CO_2$ production. Spearman rank correlations were performed to assess associations between parameters such as postprandial plasma 3OHB, ^{13}C-palmitate VLDL-TG, $^{13}CO_2$ production and postprandial percentage DNL in VLDL-TG.

4.3 Results

Subject characteristics for the 8 healthy adults (4 males and 4 females), aged 25 to 44 years are shown in Table 4.1. The BMI of participants ranged from just below 24 to 28 kg/m^2, all subjects were metabolically healthy and had fasting plasma metabolites within normal ranges (Table 4.1).

Table 4.1: Fasting and postprandial values of parameters from subjects on the study days

Anthropometric Variables	
Age (y)	32 (25 – 44)
Weight (kg)	74 (61 – 97)
BMI (kg/m^2)	24.0 (23.7 – 28.0)
Waist (cm)	84 (72 – 95)
Fasting Plasma Variables	
Glucose (mmol/L)	4.8 (4.4 – 5.6)
Insulin (mU/L)	8.9 (5.6 – 19.1)
Fructose (mmol/L)	0.14 (0.07 – 0.27)
TG (µmol/L)	863 (333 – 1512)
NEFA (µmol/L)	480 (222 – 883)
3OHB (µmol/L)	47.0 (35.1 – 212.8)
Total cholesterol (mmol/L)	4.3 (2.5 – 5.4)
HDL cholesterol (mmol/L)	1.4 (1.2 – 1.5)
Non-HDL cholesterol (mmol/L)	3.1 (1.7 – 4.0)
Postprandial Plasma Variables (time-averaged over 6 hours)	
Chylomicron-TG (µmol/L)	188 (27 – 463)
TRL (S_f 20 – 400)-TG (µmol/L)	637 (169 – 1112)
VLDL-TG (µmol/L)	245 (107 – 406)

Data presented as median (min – max). Abbreviations: BMI, body mass index; TG, triacylglycerol; NEFA, non-esterified fatty acid(s); 3OHB, 3-hydroxybutyrate; HDL, high-density lipoprotein; TRL, triacylglycerol-rich lipoprotein; VLDL, very low density lipoprotein; AUC, area under the curve.

On the study day, after meal consumption, there was a rapid initial increase in plasma glucose concentration which peaked at 30 mins and this decreased back to baseline around 90 mins after meal consumption. After 90 mins, plasma glucose concentrations decreased beyond baseline and reached a nadir at 180 mins before increasing back to the fasting levels (Figure 4.2A). Plasma fructose concentrations were more than 10 times lower than that of plasma glucose. The pattern of change in plasma fructose concentration was also different with an initial increase appearing to peak at 60 mins before decreasing. By the end of the study period, the plasma fructose concentrations were 40% lower than its baseline value (Figure 4.2B).

Plasma insulin concentrations increased after consumption of the high fructose meal, mirroring that of the plasma glucose concentrations and peaking at around 30 mins after meal consumption. However, the subsequent decline in plasma insulin levels was slower than that of plasma glucose and plasma insulin concentrations returned to baseline after 240 mins (Figure 4.2C).

Plasma TG concentrations did not significantly change over time with an increase being noted from 240 mins after meal consumption (Figure 4.2D). Plasma NEFA concentrations decreased after meal consumption, with a nadir at 90 mins before increasing beyond baseline concentrations at 360 mins (Figure 4.2E). A similar pattern was observed for plasma 3OHB where concentrations decreased until 120 mins postprandially before increasing to more than 3 times the baseline level by 360 mins (Figure 4.2F).

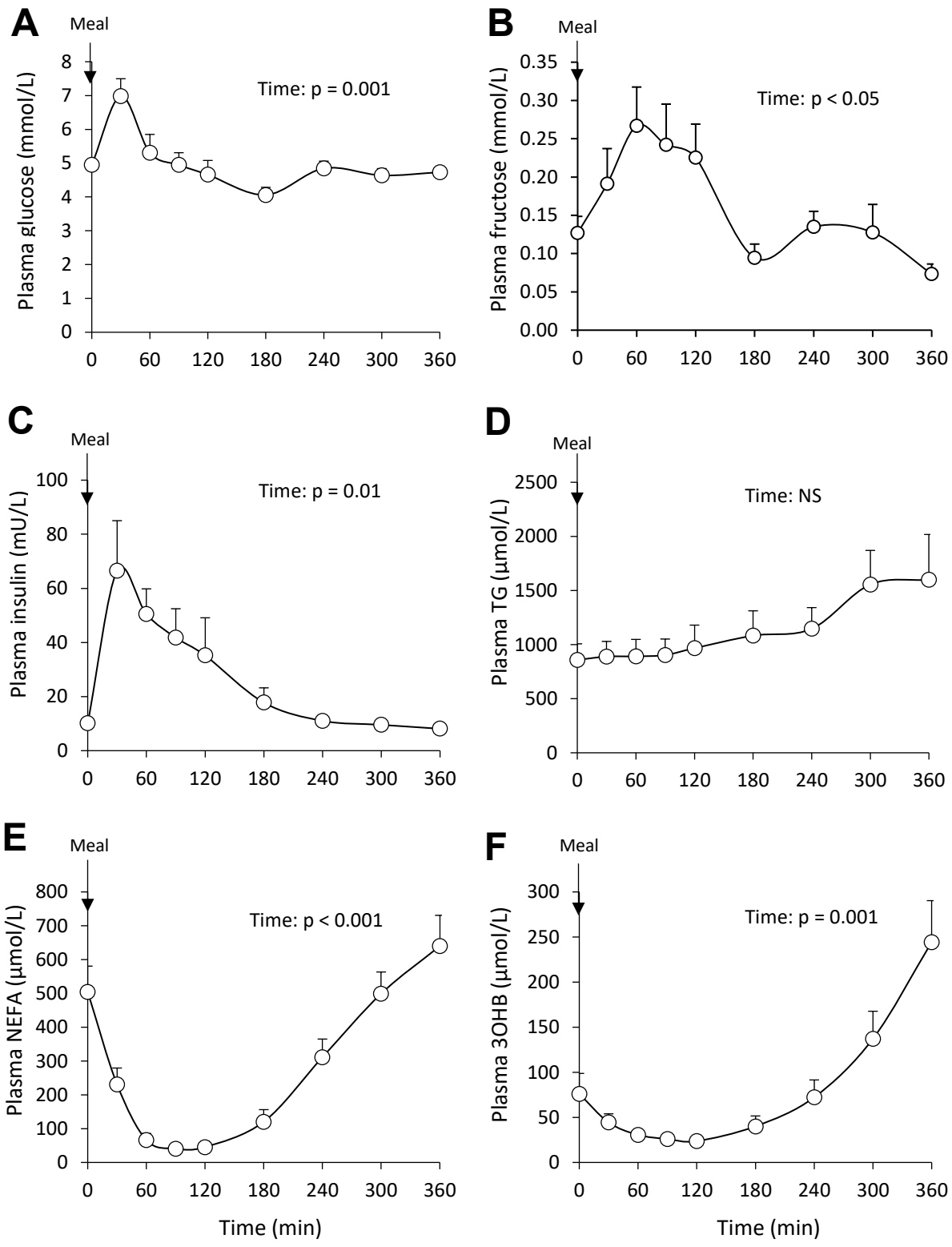

Figure 4.2: Postprandial plasma concentrations of **(A)** glucose (mmol/L) **(B)** fructose (mmol/L) **(C)** insulin (mU/L) **(D)** TG (µmol/L) **(E)** NEFA (µmol/L) **(F)**3OHB (µmol/L). Data represented as mean ± SEM (n=8). Abbreviation: TG, triacylglycerol; NEFA, non-esterified fatty acid(s); 3OHB, 3-hydroxybutyrate; NS, non-significant.

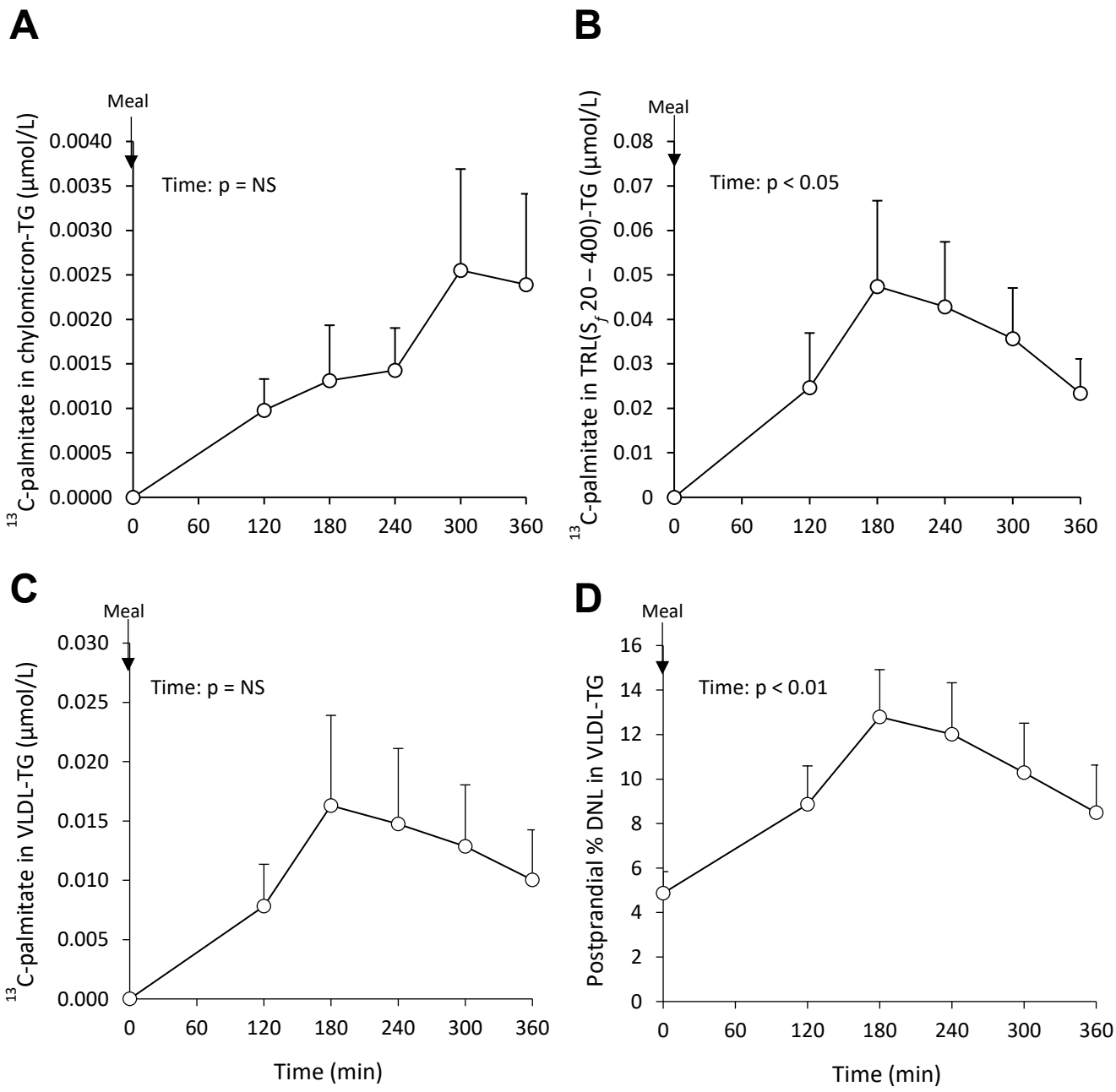

Figure 4.3: Appearance of ^{13}C from dietary [U^{13}C]fructose as in **(A)** ^{13}C-palmitate chylomicron-TG (µmol/L) **(B)** ^{13}C-palmitate TRL(S_f 20 – 400)-TG (µmol/L) and **(C)** ^{13}C-palmitate VLDL-TG (µmol/L), and **(D)** Postprandial % DNL in VLDL-TG (assessed using deuterated water). Data presented as mean ± SEM (n = 8). Abbreviations: TG, triacylglycerol; TRL, triacylglycerol-rich lipoprotein; S_f, Svedberg floatation; VLDL, very low density lipoprotein; DNL, *de novo* lipogenesis; NS, non-significant.

As fructose can be utilised to produce palmitate via the DNL pathway, I assessed the incorporation of ^{13}C from the labelled fructose contained in the test meal into lipoprotein palmitate-TG. It is of note that although uniformly labelled ^{13}C-fructose was fed, the ^{13}C-palmitate that was formed via DNL was equivalent but might not actually be uniformly labelled and thus cannot be stated as [U^{13}C]palmitate. It was found that after consumption of the test meal containing ^{13}C-labelled fructose, the appearance of ^{13}C-palmitate in chylomicron-TG was low and despite increasing over time did not achieve statistical significance compared to baseline (Figure 4.3A). In the postprandial period, the amount of ^{13}C-palmitate TRL-TG increased significantly ($p < 0.05$) over time, with a peak being achieved at 180 mins before decreasing (Figure 4.3B). The appearance of ^{13}C from dietary fructose in ^{13}C-palmitate VLDL-TG increased over time, demonstrating that dietary fructose was contributing to the synthesis of newly made palmitate, although this did not achieve statistical significance (Figure 4.3C). By measuring the amount of ^{13}C incorporated into newly synthesised palmitate, the percentage of dietary fructose recovered from DNL could be determined and this was found to be 0.5%. The postprandial percentage of DNL in VLDL-TG increased and peaked at about 180 mins before decreasing. However, the levels did not return to baseline by the end of the study period (Figure 4.3D).

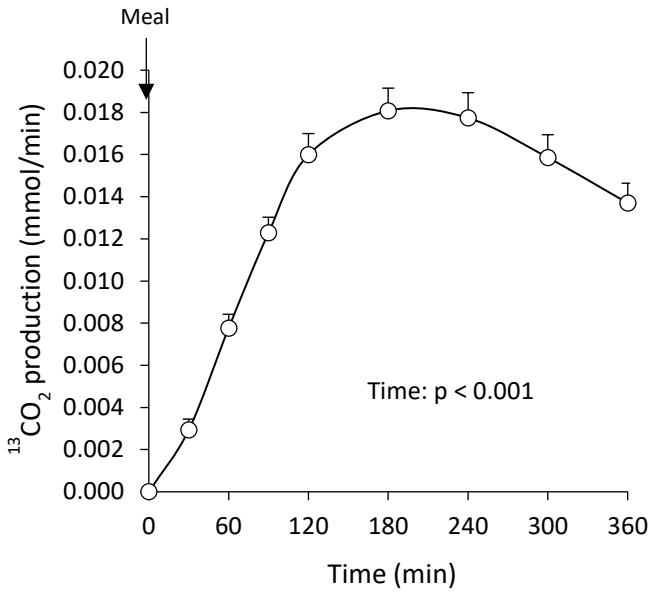

Figure 4.4: $^{13}CO_2$ production (mmol/min) (n = 8). Data presented as mean ± SEM.

I measured the appearance of ^{13}C from dietary fructose into expired carbon dioxide over time. Whole body $^{13}CO_2$ production increased steeply in the first hour and peaked between 180 and 240 mins postprandially (Figure 4.4). I calculated the recovery of the labelled fructose given in the meal via the $^{13}CO_2$ expired and found this to be 30.5%.

I assessed the associations between postprandial plasma 3OHB concentrations and hepatic DNL, ^{13}C-palmitate in VLDL-TG and hepatic DNL, and $^{13}CO_2$ and hepatic DNL and found no association between the variables (data not shown).

4.4 Discussion

Excessive consumption of fructose is often implicated as one of the causes for the development of metabolic diseases such as NAFLD and type 2 diabetes. However, fructose is rarely consumed in isolation; it is typically co-consumed with other monosaccharides and macronutrients. The aim of this study was to investigate the metabolic fate of dietary fructose when consumed with glucose and fat.

The majority of previous studies have assessed the effects of fructose when consumed in isolation. However, participants in the present study were fed a meal high in fructose content. Using [U^{13}C]fructose, I was able to trace the fate of dietary fructose into both lipogenic and oxidation pathways. It was found that 30.5% of labelled fructose consumed appeared in expired CO_2 within 6 hours of meal consumption and a very low proportion, less than 1%, appeared in VLDL-TG as labelled palmitate, demonstrating it was utilised in hepatic DNL. The results presented here are in line with previous work which has found that after ingestion of a fructose bolus, fructose preferentially enters oxidation rather than esterification pathways. By feeding ^{13}C-labelled fructose at 0.5 g / kg body weight and 1 g / kg body weight, it was demonstrated that more than half of dietary fructose ingested (56 % and 59 %, respectively) appeared in breath CO_2 over a 6-hour period (Delarue *et al.*, 1993). In the present study, I gave on average the equivalent of 0.8 g fructose/kg body weight in combination with an average of 0.5 g/kg body weight glucose and 0.7 g/kg body weight fat and found 30.5% of the labelled fructose was detected as $^{13}CO_2$. This finding is consistent with the finding reported by Chong *et al.*, (2007) who fed 0.75 g/kg body weight of fructose together with 0.5 g/kg body weight of fat and found that 6 hours after ingestion of the meal, 35 % of the

labelled fructose appeared in breath CO_2. Thus, it is likely that the difference in the appearance of ^{13}C in breath CO_2 between studies is mostly likely due to the co-ingestion of dietary lipid in the test meals which resulted in the reduction of hepatic glucose and fructose uptake (Pagliassotti and Cherrington, 1992; Moore *et al.*, 1998). The amount $^{13}CO_2$ produced did not return to baseline by the end of the study, suggesting that postprandial carbohydrate metabolism continues well beyond the 6-hour postprandial period. It is also possible that I have underestimated the recovery of fructose via oxidation as some labelled $^{13}CO_2$ may have been lost via urine (Wolfe, 1992); although it is likely this was minimal.

In my study, it was estimated that only 0.5% of the labelled fructose ingested was recovered as newly synthesised palmitate in VLDL-TG. This finding is in line with the finding of Chong *et al*. (2007) where they reported that 0.4% of circulating TG-palmitate was derived from the ingestion of dietary fructose. Patterson *et al*. (2002) also suggested that less than 1% of the ingested monosaccharide was converted to VLDL-palmitate. It should be noted that I may have underestimated hepatic DNL as a proportion of the generated FAs in the liver might have been retained in the hepatic storage pool and would not be secreted into the plasma.

The remaining 69% of dietary fructose which was unaccounted for could have undergone several other metabolic fates which I did not trace. For instance, dietary fructose could have been converted to glycerol but I did not measure for amount of ^{13}C-glycerol in plasma. By using ^{13}C-fructose and ^{13}C-glucose in experiments and analysing of results with time course metabolomics, Jang *et al.* (2018) traced the fate of dietary monosaccharides in mice and found that fructose consumption led to 11 times

the glycerate production of glucose consumption. As glycerol is an important component for triglyceride synthesis, increased consumption of dietary fructose compared to glucose can therefore lead to an increased production and storage of triglycerides.

In addition, Jang *et al.* (2018) also showed that as compared to glucose, the consumption of fructose led to a higher production of a key gluconeogenic gene *G6pc* in the small intestine of mice, which increased more than 20-fold over 2 hours postprandial. Feeding mice with a mixture of glucose and fructose in a ratio of 1:1 resulted in higher production of TCA cycle intermediates, amino acids such as glutamate, glutamine and alanine, and urea cycle metabolites ornithine and citrulline compared to mice fed only with glucose (Jang *et al.*, 2018).

Fructose, can be converted to glucose, through fructolysis, with a cascade of enzymatic reactions initiated by fructokinase and aldolase B. However, I did not measure the amount of [U^{13}C]fructose that was converted to ^{13}C-glucose. It has been shown that about 50% of ingested fructose is converted to glucose in studies which fed fructose alone (Delarue *et al.*, 1993). In my study, given the proportions of fructose found in breath CO_2 and the DNL pathway, it is plausible though unlikely, that as much as half of the fructose fed was converted to glucose as fructose was provided as part of a mixed meal to the participants in this study. The presence of glucose and by extension, glycolytic intermediates would shift the equilibrium of fructose conversion to glucose towards a lower percentage of conversion (Takasaki, 1967). A proportion of the glucose derived from dietary fructose has been reported to be converted to glycogen and stored in the liver (Nilsson and Hultman, 1974).

Measurement of the amount of fructose that was converted via glycolysis to form lactate (Mayes, 1993; Daly *et al.*, 2000) was not conducted. Lecoultre *et al*. (2010) showed that the percentage conversion from fructose to lactate was 28% in men during exercise conditions; it was suggested that the proportion of conversion was lower at rest (Lecoultre *et al.*, 2010).

In my study, ^{13}C-palmitate TG derived from dietary [U^{13}C]fructose appeared in systemic circulation 2 hours after consumption of the test meal, this suggests that fast metabolic conversion of fructose into FAs via the DNL pathway can occur. This result is in line with the finding that the turnover time of plasma VLDL-TG is around 2 hours (Patterson *et al.* 2002), showing that newly synthesised palmitate can be incorporated very quickly into plasma VLDL-TG by the liver.

Although the appearance of ^{13}C-palmitate in chylomicron-TG in systemic circulation was minimal, it was an interesting observation and it could be speculated that this was due to DNL occurring within enterocytes. In support of this, Theytaz *et al*. (2014) previously reported detectable ^{13}C in chylomicron-TG after feeding 0.5 g/kg of fructose or a combination of 0.5 g/kg of fructose and 0.5 g/kg of glucose, in addition to a mixed protein and lipid meal. The authors suggested that within the enterocyte, dietary fructose is a substrate for intestinal DNL (Theytaz *et al*., 2014). Using an animal model, Haidari *et al*. (2002) found that after feeding a high fructose diet (60% of total energy content) containing radioisotope labelled acetate to Syrian golden hamsters for 3 weeks, the radioisotope was found incorporated in fatty acids of isolated cultured hamster enterocytes, supporting the presence of enterocyte DNL. Any future

measurement of DNL from fructose should take this into consideration even though the amount seen in this study is minimal.

This study had several limitations. Firstly, in terms of absorption, I cannot exclude the possibility that not all dietary fructose was absorbed and thus the net amount of labelled fructose taken into the circulation may only be a fraction of that given. However, others have shown that co-consumption of glucose and fructose in a 1:1 ratio leads to complete absorption of fructose (Truswell *et al.*, 1988). Next, as this study was designed to give all subjects, regardless of gender, with the same amount of energy, I did not provide the test meals in accordance to their individual weight, nutritional and energy requirement. This may have resulted in the overfeeding of some individual, especially so in females compared to males, leading to a potential error in the estimation of the averaged concentration of plasma metabolites upon consumption of a high fructose meal and percentage recovery of dietary fructose via oxidation and esterification pathways. Also, as mentioned earlier, there were some metabolic fates of fructose which I did not measure, including the amount of fructose converted into lactate, glycerol and glucose. In this study, as I only fed a single meal, I cannot exclude the possibility that having multiple fructose-containing meals would lead to a change in the metabolic fate of fructose. Finally, my study has a downfall of not having a comparator and as such the results obtained from tracing dietary fructose in a high fructose meal do not have a basis for comparison. This makes it impossible to access the difference and significance of dietary fructose metabolism upon consumption of a high fructose meal compared to a low fructose meal. A possible way to make the comparison possible

would have been to design the study as a randomised crossover trial with the comparator arm being a low fructose meal which had [U^{13}C]fructose incorporated.

In conclusion, I assessed the metabolic fate of fructose after consumption of a mixed meal containing fructose, glucose and fat and found that only a small proportion underwent DNL and appeared as newly synthesized palmitate in VLDL-TG. A larger proportion was oxidized into CO_2 after 6 hours but the majority of fructose was not accounted for by these two measurements, suggesting that fructose has multiple metabolic fates. Thus, even though fructose was shown to increase DNL levels in previous studies, it is likely that this effect is indirect and likely to be mediated through changes in hepatic fat partitioning. It would be useful to investigate these changes and the mechanisms behind them.

Chapter 5: Investigation on Effect of Composition of Dietary Sugars on Insulin Secretion and Sensitivity

5.1 Introduction

5.1.1 Measuring insulin secretion

The measurement of insulin secretion is important for the diagnosis and treatment of type 2 diabetes. However, insulin secretion is difficult to measure because it is secreted into the portal vein, which is inaccessible for direct measurement (Bergman, 2000). Also, insulin passes through the liver and undergoes a large, time-varying extraction. As plasma insulin levels reflect secretion, distribution and degradation, the concentration measured in plasma is not a direct reflection of secretion (Caumo and Luzi, 2004).

Nevertheless, solutions have been found to help overcome these challenges, including measuring the concentration of plasma C-peptide. C-peptide is co-secreted with insulin from β-cells on an equimolar basis but is not extracted by the liver. Therefore, plasma concentrations of C-peptide are more representative of the amount of insulin secretion when used in combination with computational models (Caumo and Luzi, 2004). Such models are described in detail in Chapter 1, Section 1.12.4.

5.1.2 Effect of chronic consumption of fructose on carbohydrate and lipid metabolism

Although there is some information regarding the effects of chronic consumption of a combination of glucose and fructose on metabolic parameters, studies focusing on insulin secretion are limited. Stanhope and Havel (2008) reported that in energy balanced studies, the metabolic and endocrine profiles elicited by high fructose corn syrup (HFCS) consumption over 24 hours were similar to those elicited by sucrose. On the other hand, HFCS was shown to cause a larger (about 1.5 times) insulin response

after a single energy balanced meal in 6 patients with type 2 diabetes (Akgün and Ertel, 1981).

Energy balanced studies investigating the chronic consumption of either glucose or fructose in isolation have found fructose to be more lipogenic (ter Horst and Serlie, 2017). Faeh *et al.* (2005) overfed 7 healthy males for 6 days with a hyperenergetic high fructose diet which provided subjects with 25% extra energy, achieved by supplementing the 3 main meals with drinks containing 20% fructose or the equivalent of 3 g / day per kg body weight of fructose. After consumption of high fructose against the energy balanced control diet, an increase in fasting plasma glucose (by 7%), triacylglycerol (TG) (by 79%) and fractional *de novo* lipogenesis (DNL) (6-fold) were observed. Lê *et al.* (2006) reported that when 7 healthy individuals consumed a high fructose energy balanced diet (1.5 g fructose per kg body weight per day) for 4 weeks, plasma TG concentrations increased, but there was no change in liver or muscle TG deposition or insulin sensitivity. However, in another study by Lê *et al.* (2009), they found that overfeeding twice as much fructose (3 g fructose per kg body weight) over a shorter time frame (7 days) induced a significant increase in liver fat content (by 76%), which was positively correlated with fasting very low density lipoprotein (VLDL)-TG concentration. The increase in fasting plasma TG levels with chronic high fructose intake may be the result of impaired clearance of triacylglycerol-rich lipoprotein (TRL) (Swarbrick *et al.*, 2008) or increased DNL and VLDL-TG secretion (Softic *et al.*, 2016).

Apart from being lipogenic, fructose has also been shown to induce insulin resistance by contributing to defective insulin signalling in adipose tissue through compensatory hyperinsulinemia (Elliott *et al.*, 2002). Beck-Nielsen *et al.* (1980) found that 7 days of

high fructose positive energy feeding (1000 extra kcal/day) in healthy human subjects (3 males and 14 females) was associated with reduction in cellular insulin binding and insulin sensitivity, whereas high-glucose positive energy feeding did not induce significant changes in insulin sensitivity. It was reported by Faeh and team that 6 days of fructose supplementation (an extra 25% of total energy) resulted in fructose-induced insulin resistance in the liver and adipose tissue, but not in skeletal muscle (Faeh *et al.*, 2005). There is evidence that fructose is pro-inflammatory and activates cellular stress responses, and this has been suggested to be the molecular basis of the development of fructose-induced insulin resistance (Togashi *et al.*, 2000; Kawamura *et al.*, 2002; Kelley *et al.*, 2004; Cirillo *et al.*, 2009).

5.1.3 Aims and hypothesis

As high levels of insulin secretion are linked to reduced insulin sensitivity, determining the effects of high fructose drinks on insulin secretion is potentially useful to better understand the metabolic effects of consuming fructose-rich meals. Therefore, the aims of this study were:

1) To explore the acute effect of consuming drinks with different composition of fructose and glucose on postprandial insulin secretion in 20 healthy Asian subjects and to investigate if there is a gender difference in postprandial insulin secretion.

2) To investigate the effect of chronic consumption of sugar drinks with different compositions of fructose and glucose on postprandial insulin secretion.

3) To investigate the effect of chronic consumption of low or high fructose drinks on liver fat composition and parameters of FA metabolism.

The respective hypotheses for the aims were:

Hypothesis 1: Acute high fructose feeding will lead to a decreased insulin secretion when compared to acute low fructose feeding and this will be more pronounced in females than in males.

Hypothesis 2: Chronic consumption of a high fructose drink, but not low fructose drink, will lead to an adaptation of β-cell function, so that insulin secretion will be similar after intervention with either drink.

Hypothesis 3: Chronic (2 weeks) high fructose feeding will result in an increased liver fat content and a shift in hepatic fatty acid (FA) metabolism away from oxidation.

To test these hypotheses, insulin secretion and β-cell function (sugar tolerance test) and intrahepatic triacylglycerol (IHTG) content (by magnetic resonance spectroscopy) and fasting hepatic DNL (using the lipogenic index) were assessed in 20 Asian healthy males and females.

5.2 Materials and methods

5.2.1 Ethics

The study protocol was approved by the NHG Domain Specific Review Board (DSRB) of the National Healthcare Group in Singapore.

5.2.2 Subject recruitment

Twenty healthy Asian subjects were recruited via emails to students and employees at the National University of Singapore (NUS) and Nanyang Technological University (NTU), flyers and word-of-mouth. Participants included 12 males and 8 females. All subjects provided written informed consent. Of the 20 subjects recruited, one female left the study (for unrelated reasons), leaving 19 (12 males and 7 females) to complete the study.

Prior to the commencement of the study, detailed explanation of the study procedures was given to volunteers in person before they were screened to ensure suitability. The inclusion criteria were individuals aged 20 to 35 years old and of BMI >18 but <30 kg/m^2. Both genders were recruited into the study. To be accepted into the study, subjects had to be healthy, free from known disease and not taking medication that could alter carbohydrate and lipid metabolism. Participants needed to be non-smokers and consumed alcohol within recommended limits (Nascimbeni *et al.*, 2013). Exclusion criteria included those who had dyslipidaemia, fructose intolerance, hyperglycemia or had lost > 5% of their body weight over the past 6 months prior to screening. Female participants had to be not pregnant or had not been using contraceptives (eg. oral contraceptives, hormone replacement therapy (HRT)) for the past 12 months.

Prior to the study day, subjects were instructed to continue with their usual diet and to refrain from strenuous exercise and alcohol for 24 hours.

5.2.3 Study design

This study was a single-blinded trial which involved an acute randomised crossover sugar tolerance test (STT), followed by a parallel chronic (2-week) dietary intervention. The treatment arm to which each subject was assigned to for the chronic study was dependent on the drink they consumed on their 2nd visit of the acute study. The acute randomised crossover visits were scheduled one week apart (shown as visits 2 and 3, Figure 5.1). For the acute STT the test drinks contained a total of 100 g monosaccharides but varied in composition:

1) High fructose (HF) drink: 60 g fructose and 40 g glucose (Appendix Table 2)
2) Low fructose (LF) drink: 20 g fructose and 80 g glucose (Appendix Table 3)

The chronic study visits (shown as visit 3 and 4, Figure 5.1) were 2 weeks apart and when subjects came back for their last STT (visit 4), they consumed the same sugar drink they had during their third visit and over the duration of the chronic dietary intervention.

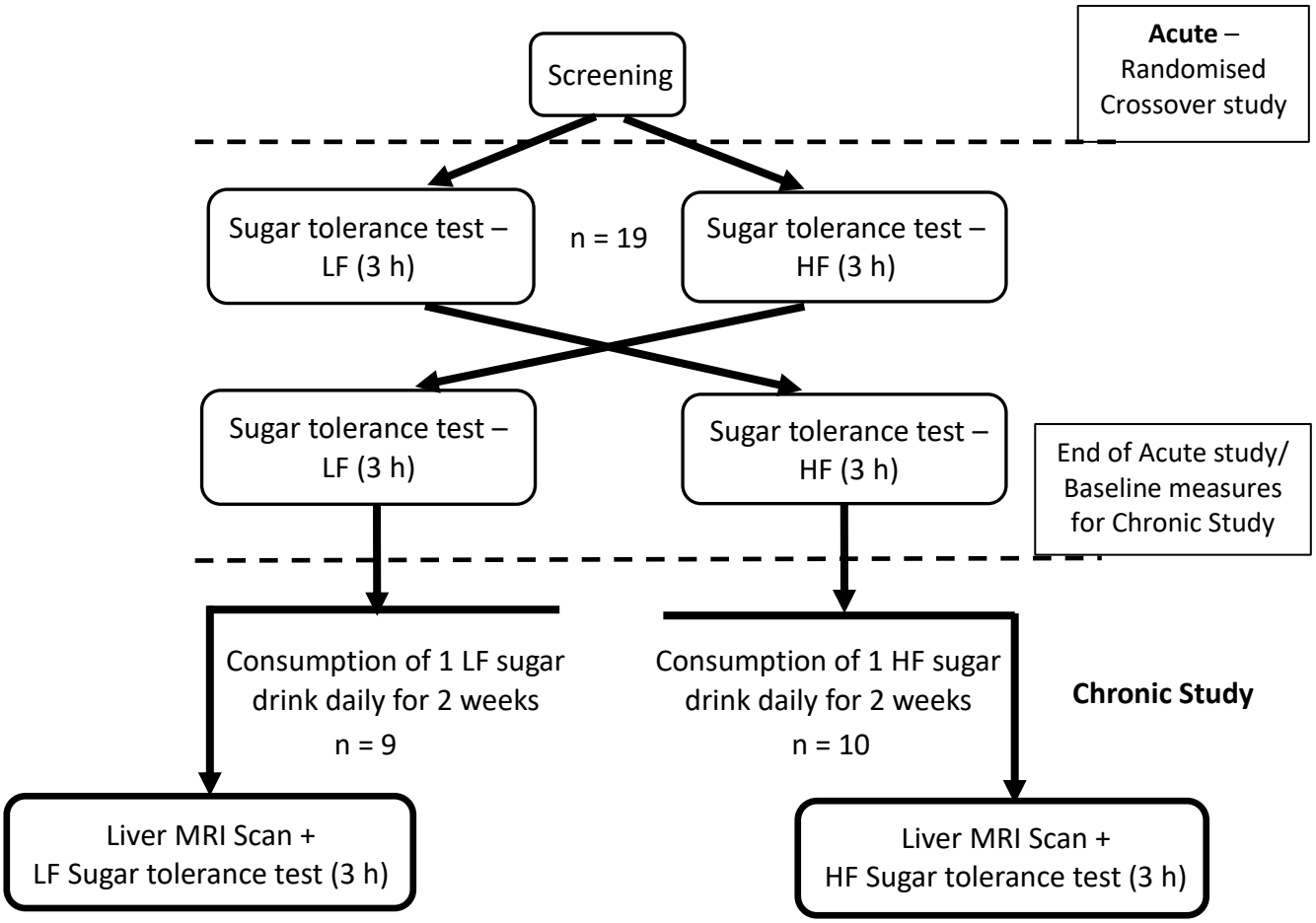

Figure 5.1: Flowchart for overview of FRU study. Abbreviations: HF, high fructose; LF, low fructose; MRI, magnetic resonance imaging

5.2.3.1 Overview of study day

On each study day, subjects arrived at the Clinical Nutrition Research Centre after an overnight fast. Before testing started, vital signs including heart rate, blood pressure and temperature were measured after 30 mins of bed rest. An indwelling catheter was then inserted into a forearm vein and fasting blood samples taken. The fore-arm was heated to 50 ◦C with the use of a thermostatically-controlled box to obtain arterialized blood samples for measurement of glucose, C-peptide and insulin concentrations over time.

A lemon-flavoured sugar drink containing 400 kcal was then consumed over 5 mins and postprandial blood samples were taken regularly over the next 3 hours; at 10, 20, 30, 45, 60, 90, 120, 150 and 180 mins. At each time-point, 10 ml of blood was collected. 6 ml of blood samples were collected into a vacutainer haematology tube containing EDTA and protease inhibitor to prevent proteolysis. The other 4 ml of blood samples were collected using silica-coated vacutainer serum separator clot-activator tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and were left to clot for 30 mins before processing to obtain serum. After obtaining blood sample at each time-point, the catheter tubing was flushed with BD PosiFlushTM saline (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) to prevent clotting. All tubes containing blood samples were placed on ice before being centrifuged, aliquoted and stored at -80 ^{o}C until further assays were performed. The whole blood samples were centrifuged at 4000 rpm for 10 mins (Hettich Rotina 420R).

5.2.4 Measurement of liver fat content and abdominal fat

Magnetic resonance imaging (MRI) scans were undertaken at the Clinical Imaging Research Centre. Each subject had 2 scans, one performed before and the other after the 2 weeks of dietary intervention. MRI scans were undertaken with the subject in a fasted state during visit 3 and visit 4, before the commencement of the STT. The liver fat content of subjects was quantified using proton MRI spectroscopy**.**

Analysis of individuals' subcutaneous abdominal and visceral adipose tissue (SAT, VAT) volumes were performed using MRI on a 3T MR scanner (Tim Trio, Siemens). MR images of abdominal fat were obtained using two-point Dixon sequence (TR = 5.28 ms, TE1 = 2.45 ms, TE2 = 3.68 ms, slice thickness = 3 mm, interslice gap = 0.6 mm).

Quantification of the abdominal fat was measured using 80 axial slices from the fat-only image between the L1 and L5 lumbar vertebrae. The IHTG, SAT and VAT depots were quantified with the use of a fully automated graph theoretic segmentation algorithm (Sadananthan *et al.*, 2015). The liver spectra were obtained from a 2×2×2 cm^3 voxel from two locations (left and right lobe) using point-resolved spectroscopy sequence (TR = 2000 ms, TE = 30 ms). The acquired spectra were processed using LCModel (Provencher, 1993) and IHTG was determined from the concentration of methyl, methylene groups of lipids and unsuppressed water signal, corrected for T2 losses and averaged. Liver fat was expressed as the ratio of fat to fat plus water (%).

5.2.5 Sample analyses

Measurement of plasma glucose concentration was performed using an automated glucose analyser (YSI 2300 Stat Plus; YSI Life Sciences, Yellow Spring, OH). Plasma insulin and C-peptide concentrations were analysed using the commercially available electrochemiluminescence assays (Roche/128 Hitachi cobas e411 immunochemistry analyzer; Roche Diagnostics, Indianapolis, USA). For measurement of concentration of non-esterified fatty acids (NEFA), triacylglycerol (TG) and 3-hydroxybutyrate (3OHB), 0.8 ml of fasting plasma was sent to Oxford Centre for Diabetes and Endocrinology, University of Oxford for analysis. Only the fasting samples from visits 3 and 4 were analysed to look at the potential effect of chronic consumption of HF and LF drinks on fasting plasma metabolites. The FA composition of plasma TG were analysed using GC (refer to section 2.1.5) to determine the lipogenic index (16:0 / 18:2n-6 ratio) as a surrogate marker of hepatic DNL before and after consuming the LF and HF drinks for 2 weeks.

5.2.6 Calculations

5.2.6.1 Insulin secretion

The β-cell responsivity (Φ) to plasma glucose and insulin secretion rate (ISR, in pmol/L/min) before and after ingestion of the sugar drink were assessed by using oral minimal model analysis of plasma C-peptide and glucose concentrations (Simulation, Analysis and Modeling Software, SAAM II version 2.3, The Epsilon Group, Charlottesville, VA). SAMM II enables for the calculation of indexes of insulin secretion such as static β-cell responsivity (Φ_s) and dynamic β-cell responsivity (Φ_d) which reflects insulin response when a precise concentration of glucose is given and in the latter case when there is a change in glucose concentration (Breda *et al.*, 2001). The minimal model also provides basal (fasting), static, dynamic and total ISR (Dalla Man *et al.*, 2005). 27 data points were obtained from the modelling process to allow for a tighter linear regression. Using the trapezoid method, cumulative postprandial ISRs (in pmol/L) were computed as the corresponding areas under the curve (AUC) for 180 mins after ingestion of each drink.

5.2.6.2 Insulin sensitivity

Insulin sensitivity of the participants was calculated using HOMA2-IR (refer to Chapter 3, section 3.2.7 for details).

5.2.6.3 Sample size

The primary endpoint, ISR was determined by using oral minimal modelling analysis of the STT data. This method was shown in past studies performed by Prigeon *et al.* (1994) and Bergman *et al.* (2002), which evaluated the reproducibility of glucose and insulin responses to repeated glucose tolerance tests to evaluate acute insulin secretory

response to intravenous glucose. Based on previous experience with STT, it was determined that 20 subjects would be required for the acute arm, and 10 subjects per group for the chronic arm, to detect differences in insulin secretion in the order of 20% using 2-tailed tests at the 5% level of significance (type I error at $\alpha = 0.05$), and with 80% power (type II error at $\beta = 0.20$).

5.2.6.4 Statistical analysis

The normality of the data distribution for each variable was assessed by using the Shapiro-Wilks test. To evaluate differences between low and high fructose consumption in the acute arm of the study (cross-sectional design), Student's paired t-test for normally distributed variables and Wilcoxon's signed rank test for non-normally distributed variables were performed. To assess the effect of the low or high fructose drink on the cohort, repeated measures analysis of variance (RMANOVA) with factors of time and meal was used to evaluate the data. To investigate if gender differences existed upon acute consumption of each drink, the data file was split according to gender before RMANOVA with factors of time and meal was used evaluate the data.

To assess the effects of fructose consumption in the chronic arm of the study (parallel design), for each study arm (low or high fructose), RMANOVA with time and meal as the two factors (baseline vs at the end of 2-week dietary intervention) was performed. All statistical analyses were conducted by using SPSS version 23 (IBM SPSS, Chicago, IL). Statistical significance was set at $p < 0.05$.

5.3 Results

5.3.1 Acute (crossover) study

5.3.1.1 Baseline characteristics

Nineteen healthy individuals, 12 males and 7 females, were recruited. The subjects were predominantly Chinese ethnicity (17 / 19; 89%), and 2 subjects were Filipino and Indian respectively. Anthropometric and metabolic data of the subjects are shown in Table 5.1.

Table 5.1: Subject anthropometric and metabolic characteristics at screening

Parameter	Mean ± SEM	Range (min – max)
Age (y)	24.0 ± 0.7	21 – 32
Weight (kg)	63.5 ± 2.2	46 – 82
BMI (kg/m^2)	22.7 ± 0.6	18.1 – 26.5
Waist circumference (cm)	76 ± 2	66 – 94
Hip circumference (cm)	83 ± 2	74 – 96
Waist-to-Hip Ratio	0.91 ± 0.01	0.79 – 0.98
Total abdominal fat (cm^3)	2350 ± 265	906 – 5275
VAT (cm^3)	601 ± 89	77 – 1650
SAT (cm^3)	1749 ± 205	643 – 4055
Liver fat (%)	2.1 ± 0.6	0.2 – 10.3
Fasting glucose (mmol/L)	4.7 ± 0.1	4.1 – 5.4
TG (µmol/L)	923 ± 154	340 – 3270
Total Cholesterol (mmol/L)	4.8 ± 0.3	3.2 – 6.7
HDL cholesterol (mmol/L)	1.5 ± 0.1	0.9 – 2.4
Non-HDL cholesterol (mmol/L)	2.9 ± 0.2	1.4 – 4.5

Data presented as mean ± SEM and range (min – max) (n = 19). Abbreviations: VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; TG, triacylglycerol; HDL, high density lipoprotein.

5.3.1.2 Study days

On the two study days, subjects' fasting plasma glucose, insulin and C-peptide concentrations were comparable, and their body weight remained constant (Table 5.2).

Table 5.2: Fasting values from subjects on the two study days

Parameter	Low fructose	High fructose	p-value
Weight (kg)	63.5 ± 2.2	63.7 ± 2.2	0.23
Glucose (mmol/L)	5.1 ± 0.1	5.0 ± 0.1	0.44
Insulin (mU/L)	6.7 ± 0.6	7.4 ± 0.8	0.65
HOMA2-IR	0.9 ± 0.1	1.0 ± 0.1	0.43
C-peptide (pmol/L)	592 ± 39	630 ± 45	0.35

Data presented as mean ± SEM (n = 19).

Upon consumption of either the high or low fructose drink, postprandial plasma glucose concentrations peaked at 45 mins and decreased back to baseline after approximately 120 mins (Figure 5.2A). Compared to consumption of the high fructose drink, ingestion of the low fructose drink increased plasma glucose concentrations to a greater extent. There was also significant effect of time ($p < 0.001$), drink ($p < 0.05$) and time*drink interaction ($p = 0.01$) between the low and high fructose drinks (Figure 5.2A). The AUC for glucose was ~8% lower ($p < 0.05$) after consumption of the high fructose compared to the low fructose drink (Figure 5.2B).

In line with the changes in plasma glucose concentration, plasma insulin concentrations rose and remained elevated to a greater extent and for a prolonged period of time after consumption of the low fructose compared to the high fructose drink (Figure 5.2C). A significant difference in plasma insulin concentrations was seen at 60 and 90 mins after consumption of the drink (both $p < 0.05$). Significant effects of time ($p < 0.001$) and drink ($p = 0.01$) were also found and the postprandial AUC for plasma insulin was

significantly lower by ~29% after consumption of the high fructose compared to the low fructose drink ($p = 0.01$) (Figure 5.2D).

Consumption of the high fructose drink resulted in significantly lower concentration of C-peptide which was maintained late into the postprandial period, from 45 to 150 mins (all $p < 0.05$) (Figure 5.2E). The AUC for C-peptide was ~24% lower ($p < 0.01$) after consumption of the high than the low fructose drink (Figure 5.2F).

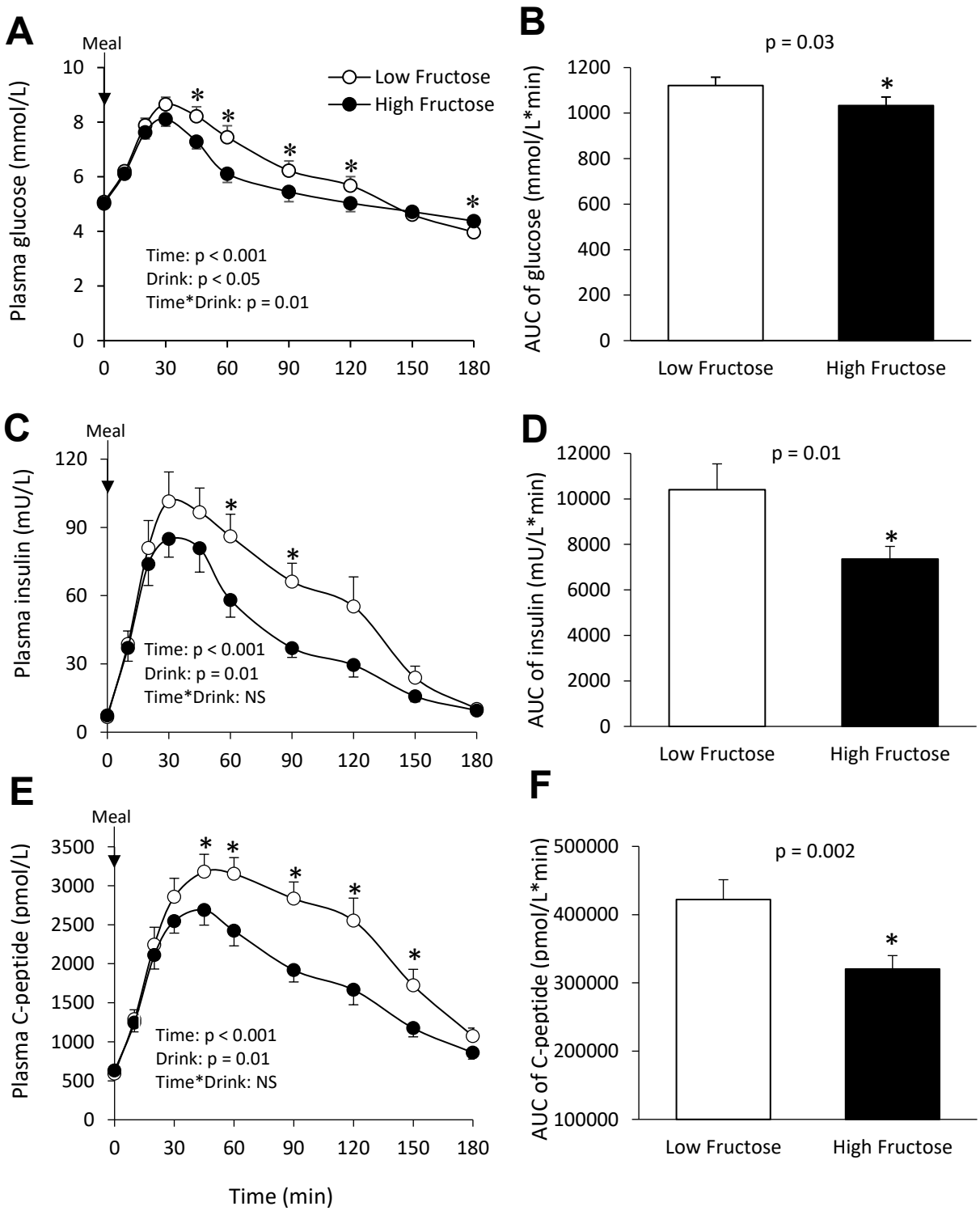

Figure 5.2: Postprandial plasma concentration of **(A)** glucose (mmol/L) **(C)** insulin (mU/L) **(E)** C-peptide (pmol/L); AUC of **(B)** glucose (mmol/L*min) **(D)** insulin (mU/L*min) **(F)** C-peptide (pmol/L*min). Data presented as mean ± SEM. Data is presented as mean ± SEM. *p < 0.05 for low vs high fructose. Abbreviations: AUC, area under curve; NS; non-significant.

The basal β-cell responsiveness and basal ISR were similar on both study days (Table 5.3). This was consistent with the similar fasting glucose, insulin and C-peptide concentrations, indicating that subjects had similar metabolic states on the 2 study days.

Table 5.3. Basal β-cell responsivity (Φ) and insulin secretion rate (ISR)

Parameter	Low Fructose	High Fructose	p-value
Basal Φ (10^9/min)	7.0 ± 0.4	7.4 ± 0.5	0.29
Basal ISR (pmol/L*min)	35.5 ± 2.4	36.8 ± 2.7	0.58

Data presented as means ± SEM (n = 19).

Upon consumption of either the low or high fructose drinks, the postprandial static ISR increased and peaked rapidly at 37.5 mins before decreasing again. Static ISR was significantly higher from 37.5 to 138 mins after ingestion of the low fructose drink than the high fructose drink. There was significant effect of time ($p < 0.001$), drink ($p < 0.01$) and time*drink interaction ($p < 0.05$) (Figure 5.3A). Overall, the AUC for static ISR was ~29% lower ($p = 0.001$) after consumption of the high fructose compared to the low fructose drink (Figure 5.3B).

Ingestion of the high and low fructose test drinks did not result in significant differences in dynamic ISR over time. A rapid increase occurred in the first 10 mins when dynamic ISR peaked (coinciding with the greater rates of increase in plasma glucose concentrations), before decreasing rapidly towards zero (in accordance with the levelling off or decreasing glucose concentrations) (Figure 5.3C). There was a significant effect of time ($p < 0.001$), but not drink ($p > 0.05$) and there was no time*drink interaction ($p > 0.05$) (Figure 5.3C). There were no significant differences between test drinks in the AUC for dynamic ISR (Figure 5.3D).

The postprandial total ISR increased and peaked rapidly at 20 mins; however, the increase with the high fructose drink was at a later time and lower amount than with the low fructose drink. By 180 mins postprandially, total ISR was below baseline after both test drinks. After ingestion of the drink low in fructose, total ISR was significantly greater from 37.5 to 138 mins postprandial ($p < 0.05$). Comparing the consumption of the two drinks, there was significant effect of time ($p < 0.001$), drink ($p < 0.01$) and time*drink interaction ($p < 0.05$) (Figure 5.3E). Overall, the AUC for total ISR was ~27% lower ($p < 0.01$) after consumption of the high fructose compared to the low fructose drink (Figure 5.3F).

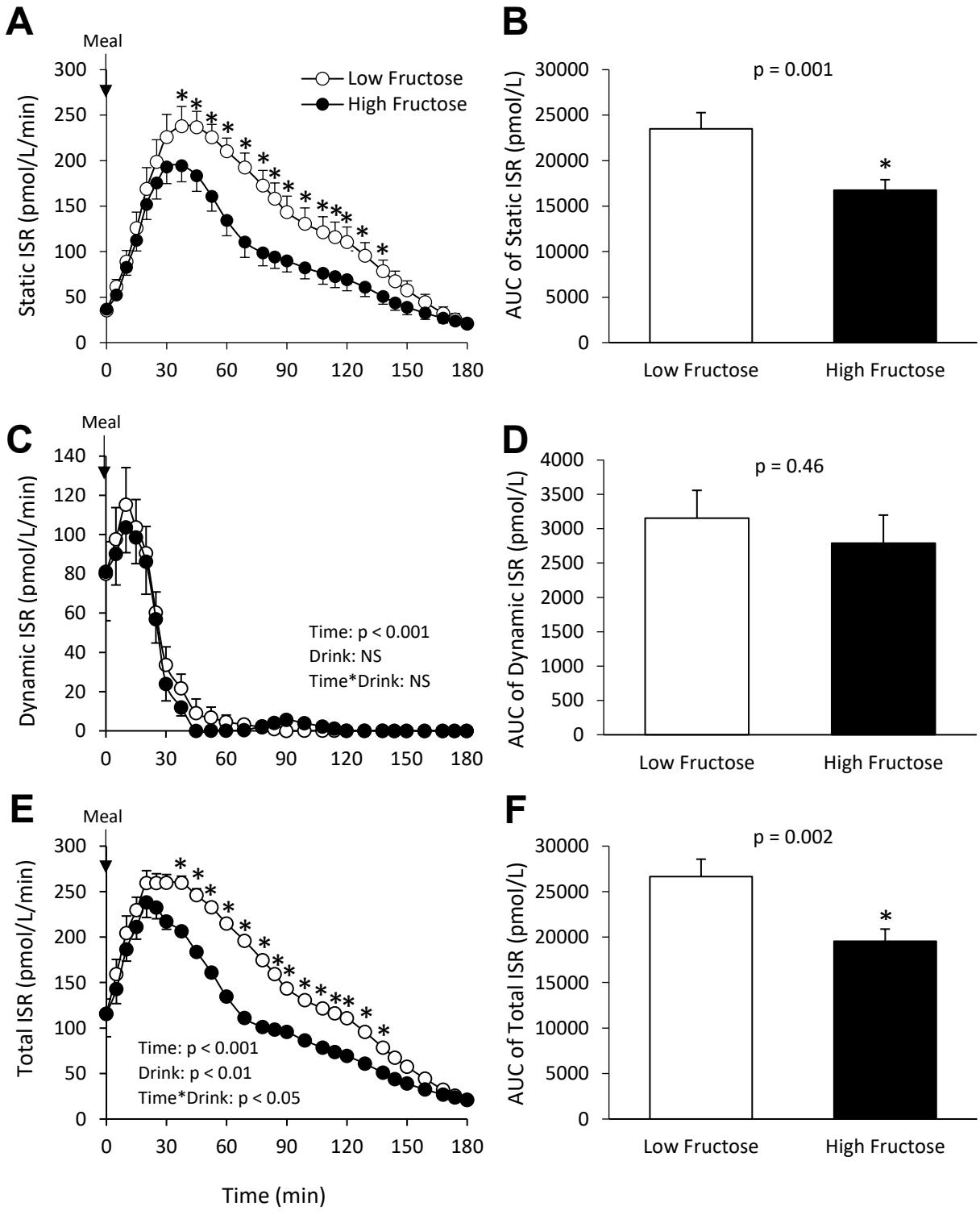

Figure 5.3: Postprandial ISR (pmol/L/min) (n=19): **(A)** Static **(C)** Dynamic **(E)** Total; AUC of ISR (pmol/L) **(B)** Static **(D)** Dynamic **(F)** Total. Data presented as mean ± SEM. *p < 0.05 for low vs high fructose. Abbreviations: AUC, area under curve; ISR, insulin secretion rate; NS, non-significant.

The insulin clearance rate was not significantly different after consumption of the high or low fructose drink (Figure 5.4), indicating that differences in circulating insulin concentrations were the result of differences in pancreatic insulin secretion.

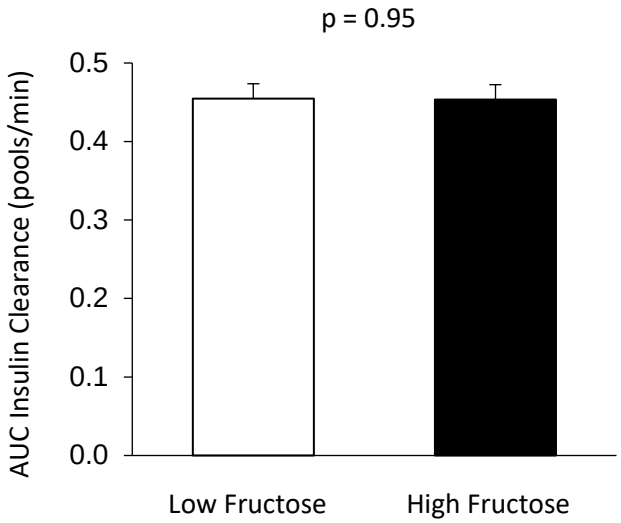

Figure 5.4: Postprandial insulin clearance rate (pools/min) upon consumption of low and high fructose drinks

5.3.2 Gender differences in the acute crossover section

The responses of males and females to acute low or high fructose consumption were examined separately. Both males (n = 12) and females (n = 7) had similar metabolic states for the 2 study days (Tables 5.4 and 5.5, respectively).

Table 5.4: Fasting anthropometric and metabolic characteristics of males on both study days

Parameter	Low Fructose	High Fructose	p-value
Weight (kg)	66.7 ± 2.7	66.7 ± 2.8	0.85
BMI (kg/m^2)	22.3 ± 0.7	22.3 ± 0.8	0.84
Glucose (mmol/L)	5.1 ± 0.1	5.1 ± 0.1	0.93
Insulin (mU/L)	6.9 ± 0.7	7.2 ± 1.1	0.79
C-peptide (pmol/L)	622 ± 51	671 ± 67	0.41

Data presented as mean ± SEM based on n = 12.

Table 5.5: Fasting anthropometric and metabolic characteristics of females on both study days

Parameter	Low Fructose	High Fructose	p-value
Weight (kg)	58.1 ± 3.1	58.5 ± 2.9	0.09
BMI (kg/m^2)	23.1 ± 1.4	23.2 ± 1.4	0.09
Glucose (mmol/L)	5.0 ± 0.2	4.8 ± 0.1	0.34
Insulin (mU/L)	6.8 ±1.3	7.3 ± 0.8	0.67
C-peptide (pmol/L)	542 ± 59	560 ± 36	0.69

Data presented as means ± SEM based on n = 7.

After the consumption of the drinks, there was a notable difference in plasma glucose and insulin concentrations in female but not male subjects (Figures 5.5A and B). Postprandial glucose concentration for both genders followed the same pattern after the consumption of the two drinks. For males, upon consumption of either the low or high fructose drink, plasma glucose concentrations peaked 30 mins postprandially; and then decreased slowly back to baseline (Figure 5.5A). However, in females, plasma glucose concentration peaked earlier after the high fructose drink (~30 mins) than after the low

fructose drink (~45 mins), and ingestion of the high fructose drink led to significantly lower plasma glucose concentrations ($p < 0.05$) between 60 and 120 mins postprandially compared to the high fructose drink (Figure 5.5B).

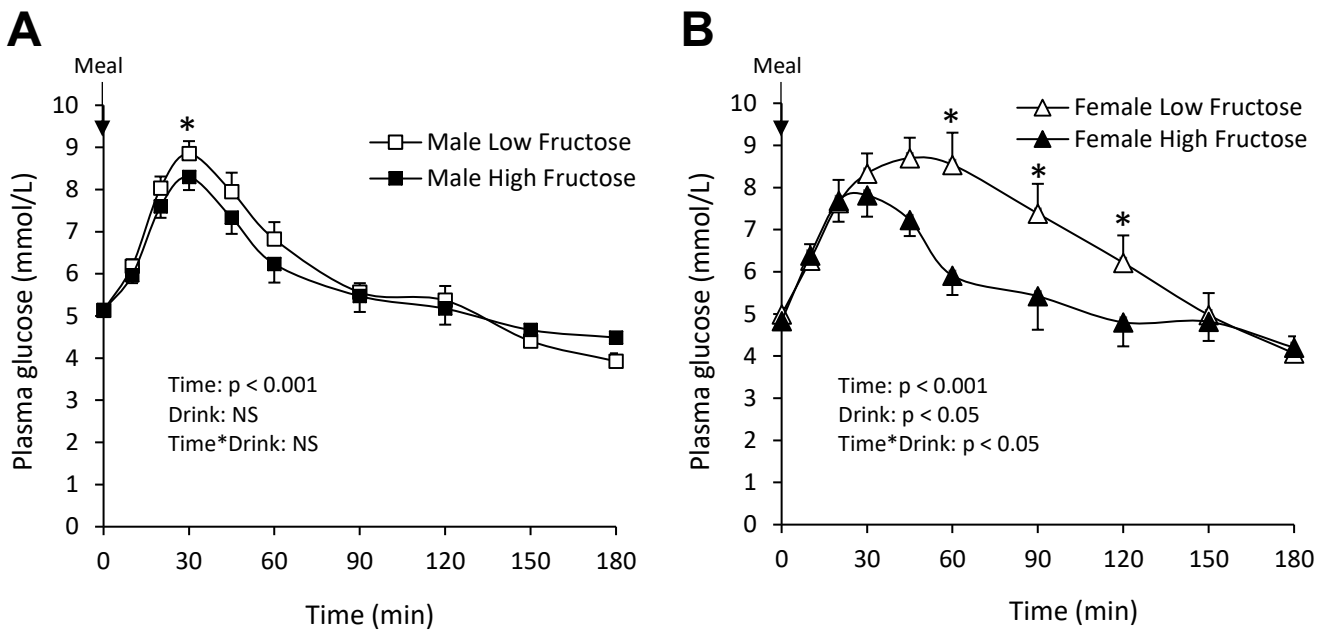

Figure 5.5: Postprandial plasma concentration of glucose (mmol/L) upon consumption of low or high fructose drink **(A)** males (n = 12) **(B)** females (n = 7). Data is presented as mean ± SEM. *p < 0.05 for low vs high fructose. Abbreviation: NS, non-significant.

Plasma insulin responses were in line with the responses for plasma glucose. In males, there were slight differences in postprandial plasma insulin concentrations after consumption of the low and high fructose drinks, but this did not reach significance at any time-point (Figure 5.6A). In contrast, the postprandial plasma insulin response in females varied upon consumption of the test drinks. Ingestion of the high fructose drink yielded a significantly lower insulin concentration at 60 and 90 mins. There was also an effect of time ($p < 0.001$) and a time*drink interaction ($p < 0.05$) (Figure 5.6B).

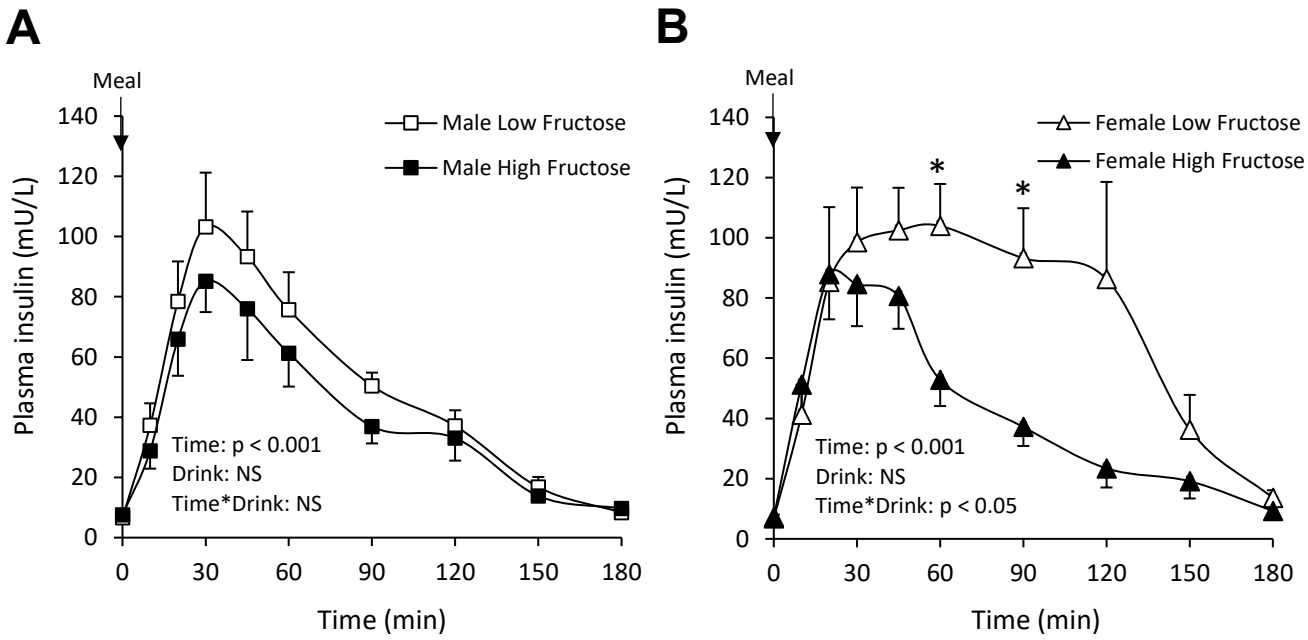

Figure 5.6: Postprandial plasma concentrations of insulin (mU/L) upon consumption of the low or high fructose drink **(A)** males (n = 12) **(B)** females (n = 7). Data is presented as mean ± SEM. *p < 0.05 for low vs high fructose. Abbreviation: NS, non-significant

The data for static, dynamic, and total ISR after ingestion of the low and high fructose drinks in males and females are shown in Figure 5.7. In females, the high fructose drink resulted in significantly lower static and total ISR than the low fructose drink compared to males.

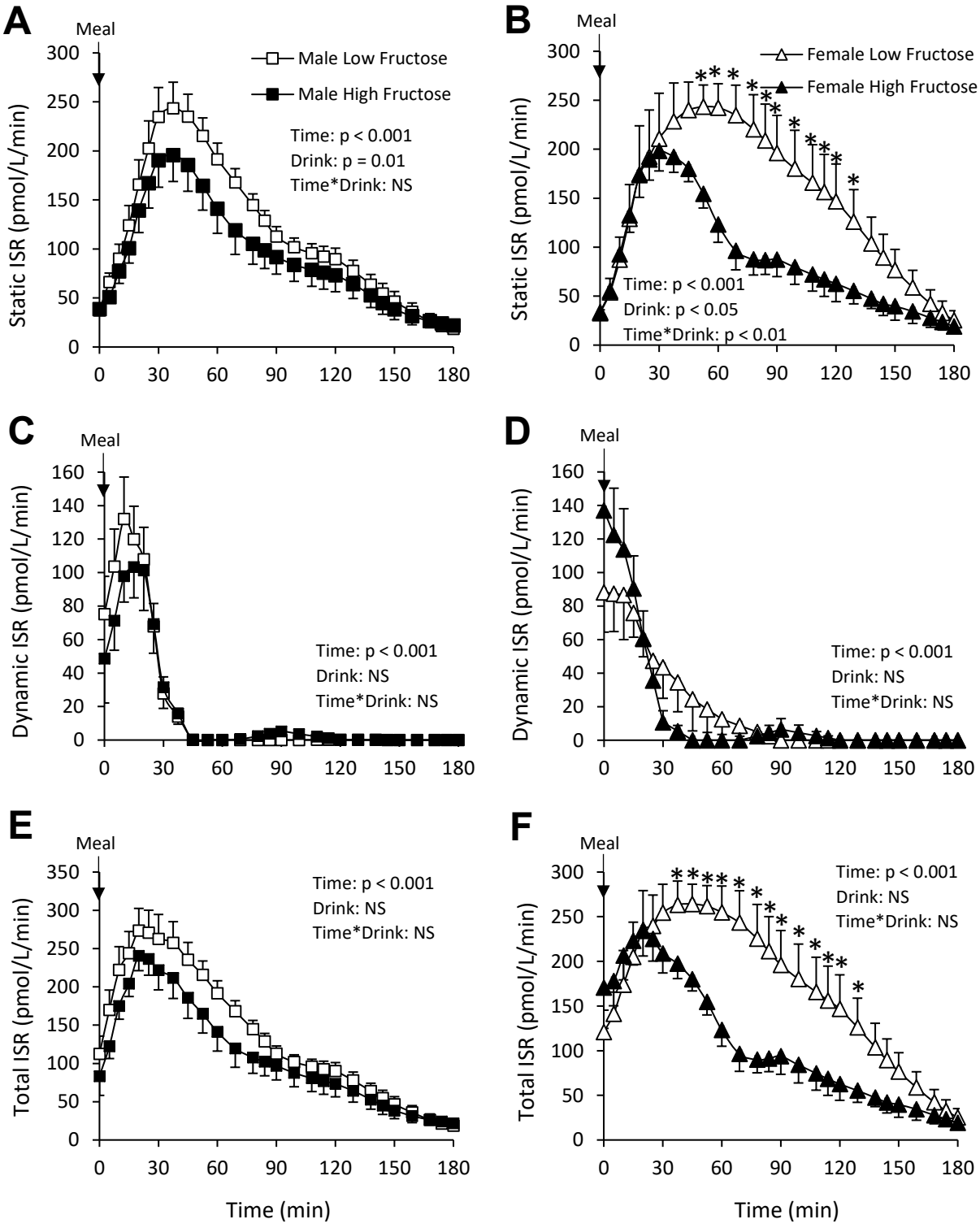

Figure 5.7: Postprandial ISR for males (n = 12) and females (n = 7) upon consumption of the low or high fructose drink **(A)** Static ISR for males **(B)** Static ISR for females **(C)** Dynamic ISR for males **(D)** Dynamic ISR for females **(E)** Total ISR for males **(F)** Total ISR for females. Data presented as mean ± SEM. *p < 0.05 for low vs high fructose drinks. Abbreviation: NS, Non-significant.

The rate of insulin clearance after the consumption of the low or high fructose drinks was not sgnificantly different in either males or females ($p > 0.05$) (Figure 5.8).

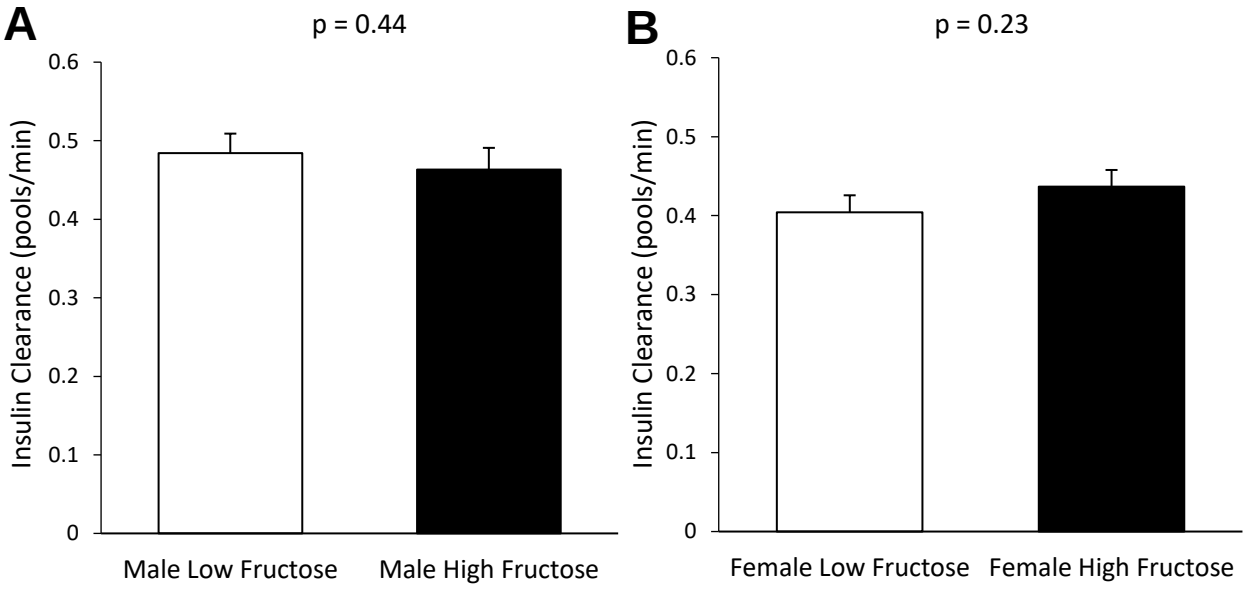

Figure 5.8: Postprandial insulin clearance rate (pools/min) upon consumption of the low or high fructose drink **(A)** males (n = 12) **(B)** females (n = 7). Data presented as mean ± SEM.

5.3.3 Results for chronic fructose consumption

5.3.3.1 Subject characteristics at baseline

A total of 10 subjects were randomly assigned to the low fructose dietary intervention (1 dropped out, leaving 9 who completed the study) and 10 subjects were randomly assigned to the high fructose dietary intervention (all 10 completed the study). Their anthropometric and metabolic characteristics at baseline are shown in Table 5.6. Although well-matched for BMI, body fat and liver fat, the high fructose group had significantly higher fasting insulin and C-peptide concentrations than the low fructose group ($p < 0.05$).

Table 5.6: Anthropometric characteristics and fasting metabolite concentrations of the subjects assigned to low or high fructose dietary intervention

Parameter	Low Fructose	High Fructose	p-value
Males / Females	6 / 3	6 / 4	-
Weight (kg)	64.2 ± 2.7	63.2 ± 3.5	0.82
BMI (kg/m^2)	22.9 ± 1.3	22.8 ± 1.6	0.97
HOMA2-IR	1.1 ± 0.1	0.8 ± 0.1	0.06
Liver Fat (%)	2.6 ± 1.1	1.8 ± 0.6	0.50
VAT (cm^3)	542 ± 83	654 ± 154	0.55
SAT (cm^3)	1786 ± 216	1715 ± 349	0.87
Glucose (mmol/L)	5.1 ± 0.1	5.1 ± 0.1	0.92
Insulin (mU/L)	8.3 ± 0.9	6.0 ± 0.5 *	0.04
C-peptide (pmol/L)	697 ± 57	551 ± 43	0.05
TG (µmol/L)	836 ± 65	683 ± 96	0.13
3OHB (µmol/L)	83.9 ± 26.3	46.2 ± 13.9	0.20
Cholesterol (mmol/L)	4.3 ± 0.2	4.6 ± 0.3	0.47
HDL cholesterol (mmol/L)	1.3 ± 0.1	1.5 ± 0.2	0.31
Non-HDL cholesterol (mmol/L)	3.0 ± 0.2	3.2 ± 0.2	0.82

Data is presented as mean ± SEM. For not normally distributed data, results presented as medians with quartiles and were compared with Wilcoxon signed rank test. *$p < 0.05$ for low vs high fructose. Abbreviations: BMI, body mass index; HOMA, homeostatic model assessment; IR, insulin resistance; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; TG, triacylglycerol; 3OHB, 3-hydroxybutyrate; HDL, high density lipoprotein.

5.3.3.2 Chronic low fructose consumption

A total of 9 subjects, 6 males and 3 females, aged between 21 and 32 years completed the low fructose dietary intervention. Body weight and BMI remained constant over the 2-week diet intervention ($p > 0.05$; Table 5.7). Chronic low fructose consumption did not affect fasting plasma glucose, insulin, and C-peptide concentrations or lipid profiles (Table 5.7).

Table 5.7: Anthropometric characteristics and fasting metabolite concentrations before and after 14 days of daily consumption of low fructose drinks (n = 9)

Parameter	Baseline	Day 14	p-value
Weight (kg)	64.2 ± 2.7	64.1 ± 2.8	0.52
BMI (kg/m^2)	22.9 ± 1.3	22.8 ± 1.4	0.50
HOMA2-IR	1.1 ± 0.1	1.0 ± 0.2	0.83
Liver Fat (%)	2.6 ± 1.1	3.5 ± 1.5	0.14
VAT (cm^3)	542 ± 83	531 ± 83	0.30
SAT(cm^3)	1786 ± 216	1775 ± 215	0.58
Glucose (mmol/L)	5.1 ± 0.1	5.1 ± 0.1	0.96
Insulin (mU/L)	8.3 ± 0.9	8.0 ± 1.2	0.85
C-peptide (pmol/L)	697 ± 57	690 ± 80	0.94
TG (µmol/L)	836 ± 65	930 ± 107	0.50
3OHB (µmol/L)	52.8 (34.1 –135.5)	24.2 (18.5 – 50.4)	0.11
Cholesterol (mmol/L)	4.3 ± 0.2	4.3 ± 0.3	0.87
HDL cholesterol (mmol/L)	1.3 ± 0.1	1.3 ± 0.1	0.52
Non-HDL cholesterol (mmol/L)	3.0 ± 0.2	3.0 ± 0.2	0.69

Data presented as mean ± SEM, except for 3OHB which is presented as median (25^{th} – 75^{th} percentile). Abbreviations: BMI, body mass index; HOMA, homeostatic model assessment; IR, insulin resistance; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; TG, triacylglycerol; 3OHB, 3-hydroxybutyrate; HDL, high density lipoprotein.

Two weeks of daily consumption of low fructose drinks did not result in a significant change in liver fat content (Figure 5.9), or postprandial plasma glucose, insulin and C-peptide concentrations (Figure 5.10A-F), or static, dynamic, and total ISRs (Figure 5.11A-F).

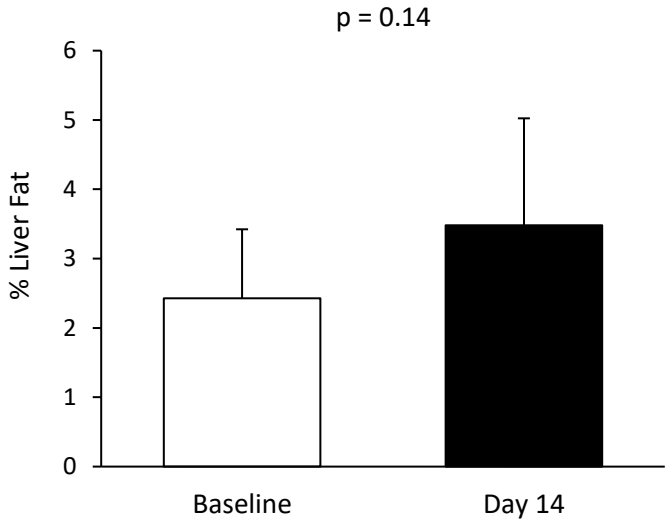

Figure 5.9: Percentage liver fat at baseline and the end of 14 days consumption of low fructose drinks (n = 9). Data presented as mean ± SEM.

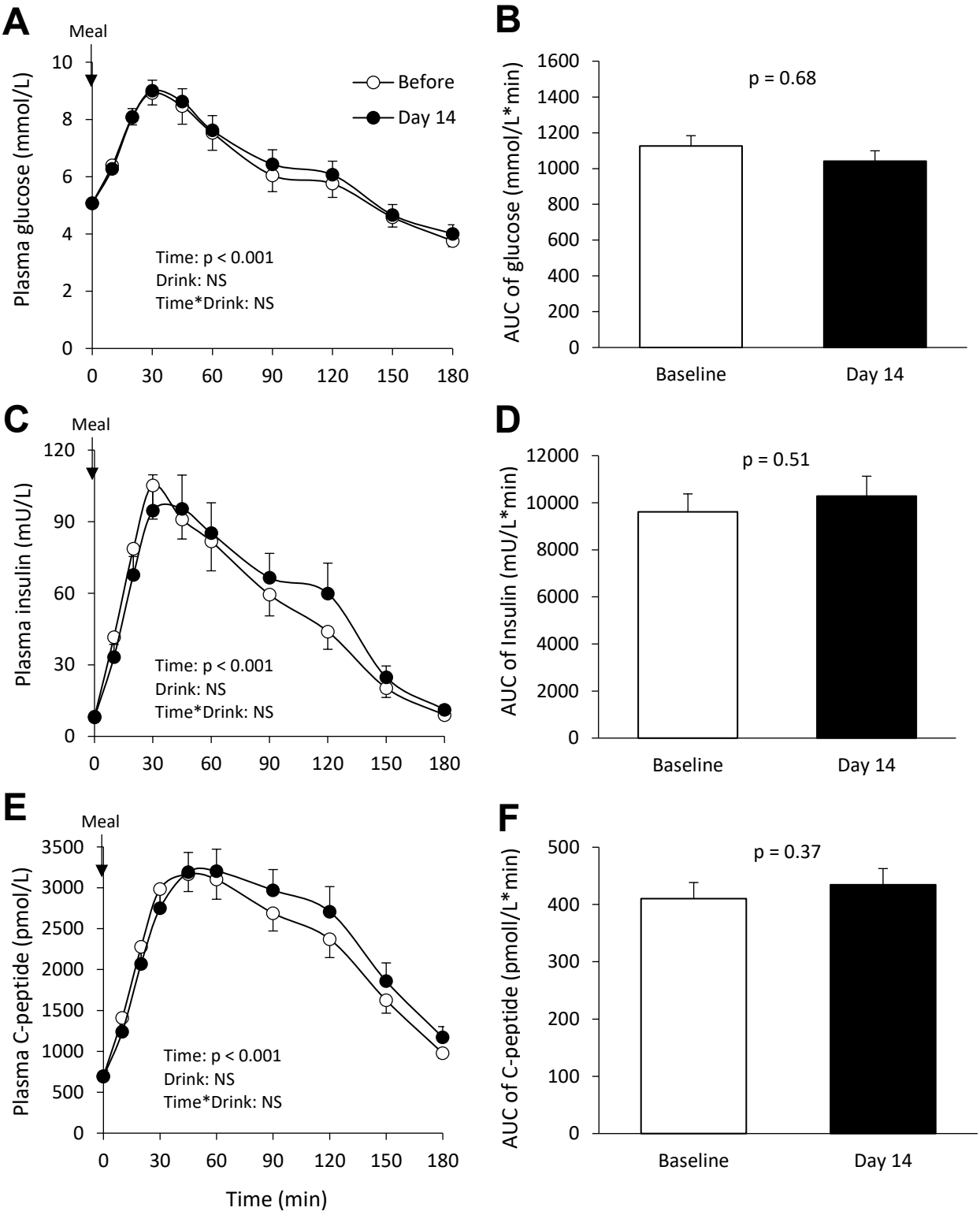

Figure 5.10: Postprandial plasma concentration at baseline and after 14 days consumption of low fructose drinks (n = 9) **(A)** glucose (mmol/L) **(C)** insulin (mU/L) **(E)** C-peptide (pmol/L); AUC of **(B)** glucose (mmol/L*min) **(D)** insulin (mU/L*min) **(F)** C-peptide (pmol/L/min). Data presented as mean ± SEM. Abbreviations: AUC, area under curve; NS, non-significant.

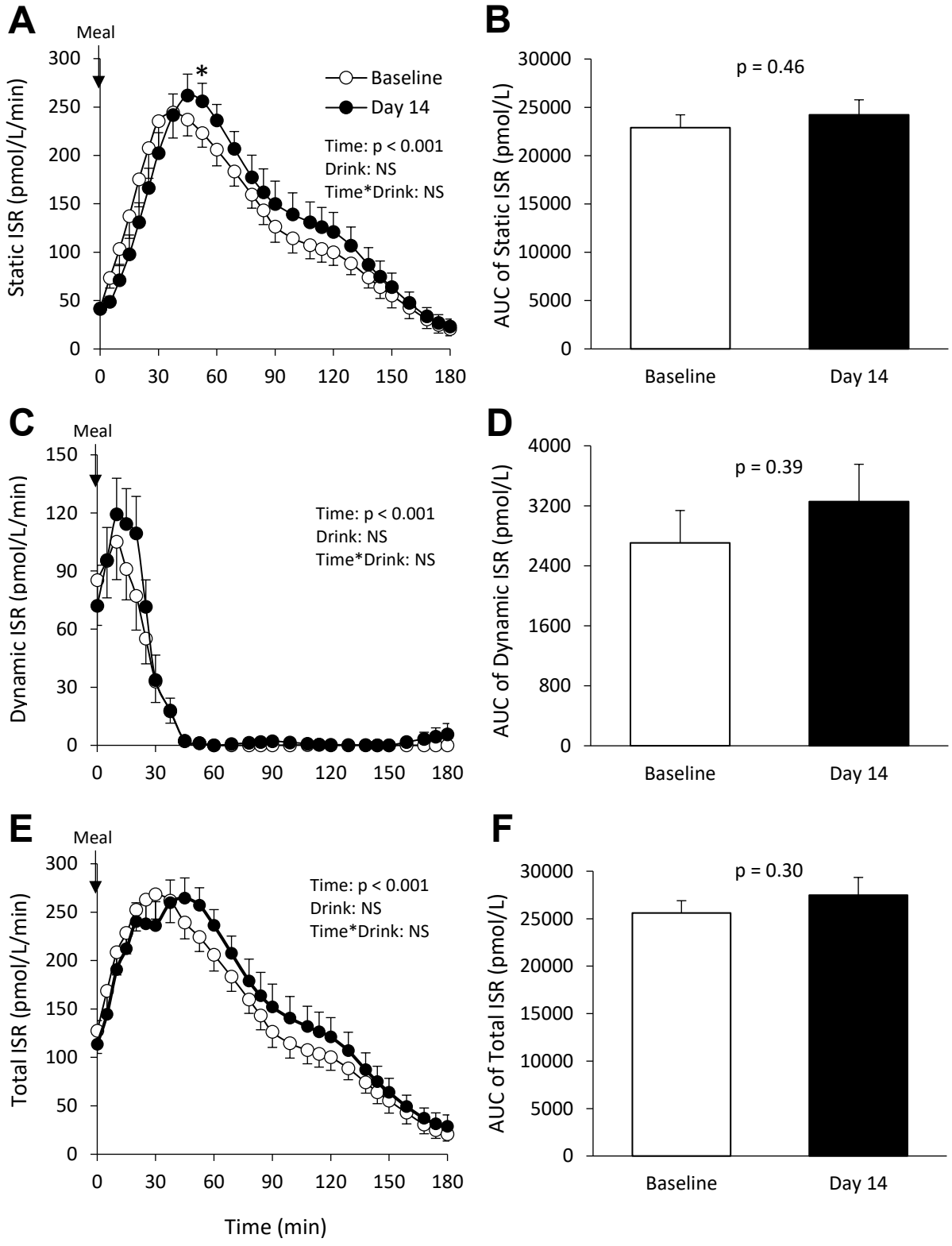

Figure 5.11: Postprandial ISR (pmol/L/min) at baseline and after 14 days consumption of low fructose drinks (n = 9): **(A)** Static **(C)** Dynamic **(E)** Total; AUC of ISR (pmol/L) at baseline and the end of 2 weeks consumption of low fructose drinks: **(B)** Static **(D)** Dynamic **(F)** Total. *p < 0.05. Abbreviations: AUC, area under curve; ISR, insulin secretion rate; NS, non-significant.

Chronic low fructose consumption for 2 weeks did not affect plasma clearance rate of insulin (Figure 5.12).

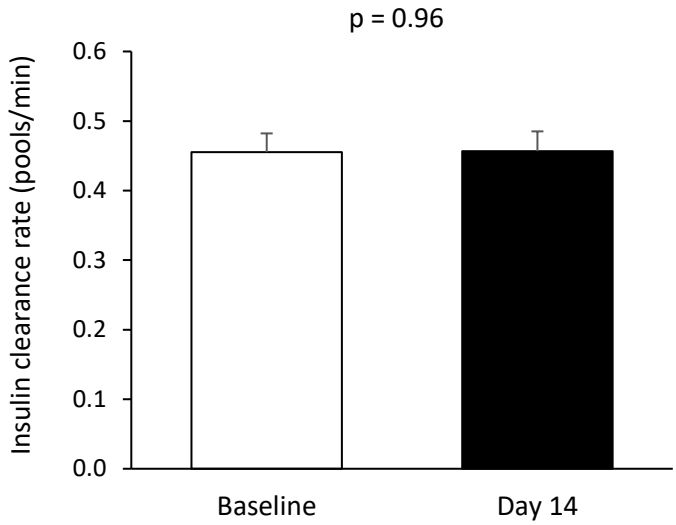

Figure 5.12: Postprandial insulin clearance rate (pools/min) at baseline and after 14 days consumption of low fructose drinks (n = 9). Data presented as mean ± SEM.

Using a surrogate marker for hepatic DNL, the lipogenic index, it was found that chronic low fructose consumption for 2 weeks did not change the lipogenic index in plasma TG, and thus presumably did not change hepatic DNL (Figure 5.13).

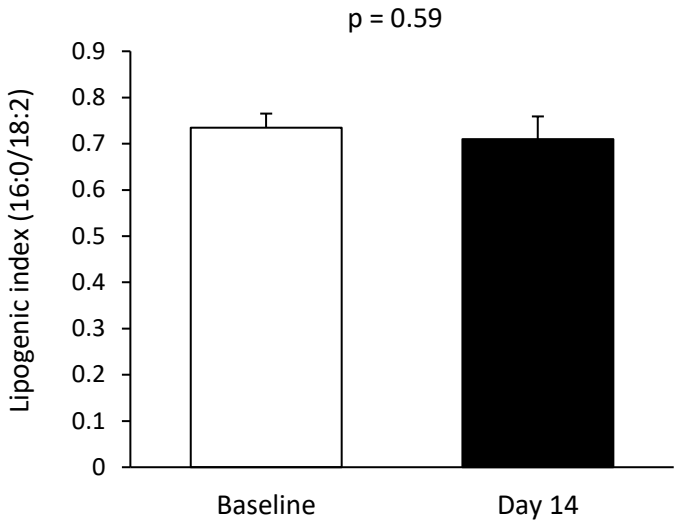

Figure 5.13: Ratio of palmitic acid (16:0) to linoleic acid (18:2) in fasting plasma VLDL-TG at baseline and after 14 days consumption of low fructose drinks (n = 9). Data presented as mean ± SEM.

Plasma 3-hydroxybutyrate (3OHB) is the proxy marker for hepatic FA oxidation. Prior to and after chronic consumption of the low fructose drink, no significant difference was noted in the fasting plasma 3OHB concentration (p = 0.12) (Figure 5.14).

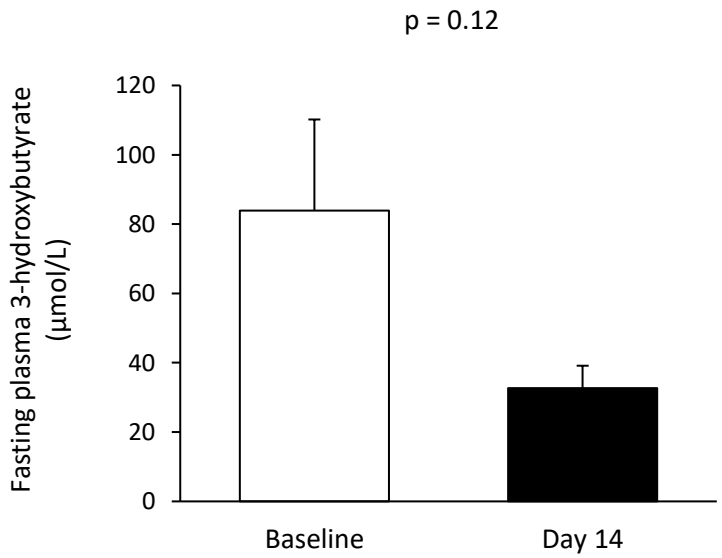

Figure 5.14: Fasting concentration of 3-hydroxybutyrate (µmol/L) at baseline and after 14 days consumption of low fructose drinks (n = 9). Data presented as mean ± SEM. Abbreviation: 3OHB, 3-hydroxybutyrate.

5.3.3.3 Chronic high fructose consumption

Chronic high fructose consumption did not affect body weight, BMI, plasma lipid profiles, or fasting plasma glucose, insulin, and C-peptide concentrations (Table 5.8).

Table 5.8: Anthropometric characteristics and fasting metabolite concentrations measured at baseline and the end of 14 days of daily consumption of high fructose drinks

Parameter	Baseline	Day 14	p-value
Weight (kg)	63.2 ± 3.5	63.6 ± 3.4	0.16
BMI (kg/m^2)	22.8 ± 1.6	22.9 ± 1.6	0.17
HOMA2-IR	0.8 ± 0.1	0.7 ± 0.1	0.43
Liver Fat (%)	1.8 ± 0.6	1.98 ± 0.87	0.77
VAT (cm^3)	654 ± 154	664 ± 166	0.59
SAT (cm^3)	1715 ± 349	1780 ± 362	0.14
Glucose (mmol/L)	5.1 ± 0.1	5.0 ± 0.1	0.49
Insulin (mU/L)	6.0 ± 0.5	5.3 ± 0.6	0.34
C-peptide (pmol/L)	551 ± 43	530 ± 56	0.52
TG (µmol/L)	683 ± 96	744 ± 139	0.46
3OHB (µmol/L)	34.5 (24.4 – 50.2)	50.2 (37.9 – 82.3)	0.20
Cholesterol (mmol/L)	4.6 ± 0.3	4.7 ± 0.3	0.85
HDL cholesterol (mmol/L)	1.5 ± 0.2	1.5 ± 0.1	0.86
Non-HDL cholesterol (mmol/L)	3.2 ± 0.2	3.2 ± 0.3	0.86

Data presented as mean ± SEM (n = 10). For non-normally distributed data, results are presented as medians with (25^{tth} – 75^{th} percentile); Wilcoxon sign rank test. Abbreviations: BMI, body mass index; HOMA, homeostatic model assessment; IR, insulin resistance; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; TG, triacylglycerol; 3OHB, 3-hydroxybutyrate; HDL, high density lipoprotein.

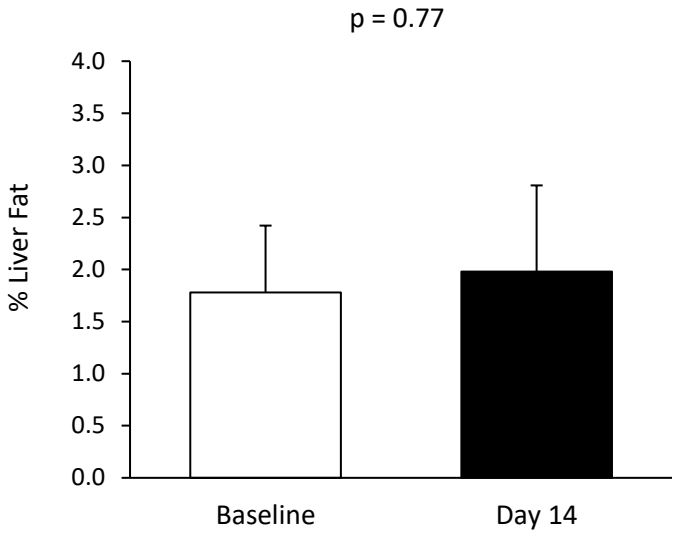

Figure 5.15: Percentage liver fat at baseline and after 14 days consumption of high fructose drinks (n = 10). Data presented as mean ± SEM.

Daily consumption of a high fructose drink for 2 weeks did not lead to any significant changes in the percentage of liver fat (Figure 5.15) or the postprandial concentrations of plasma glucose, insulin and C-peptide (Figure 5.16A – F).

The responsiveness of the β-cells and the ISR in the basal state did not change over the period of chronic high fructose intervention (Table 5.9), consistent with the absence of changes in fasting insulin concentrations.

Table 5.9: Basal Φ and ISR at baseline and after 14 days consumption of high fructose drinks

Parameter	Baseline	Day 14	p-value
Basal Φ (10^9/min)	6.3 ± 0.4	6.2 ± 0.6	0.71
Basal ISR (pmol/L x min)	31.0 ± 1.9	32.3 ± 3.4	0.63

Data presented as mean ± SEM (n = 10). Abbreviations: Φ, β-cell responsivity; ISR, insulin secretion rate.

There were also no significant differences after 2 weeks of consumption of high fructose drink in postprandial static, dynamic and total insulin secretion ISRs (Figure 5.17).

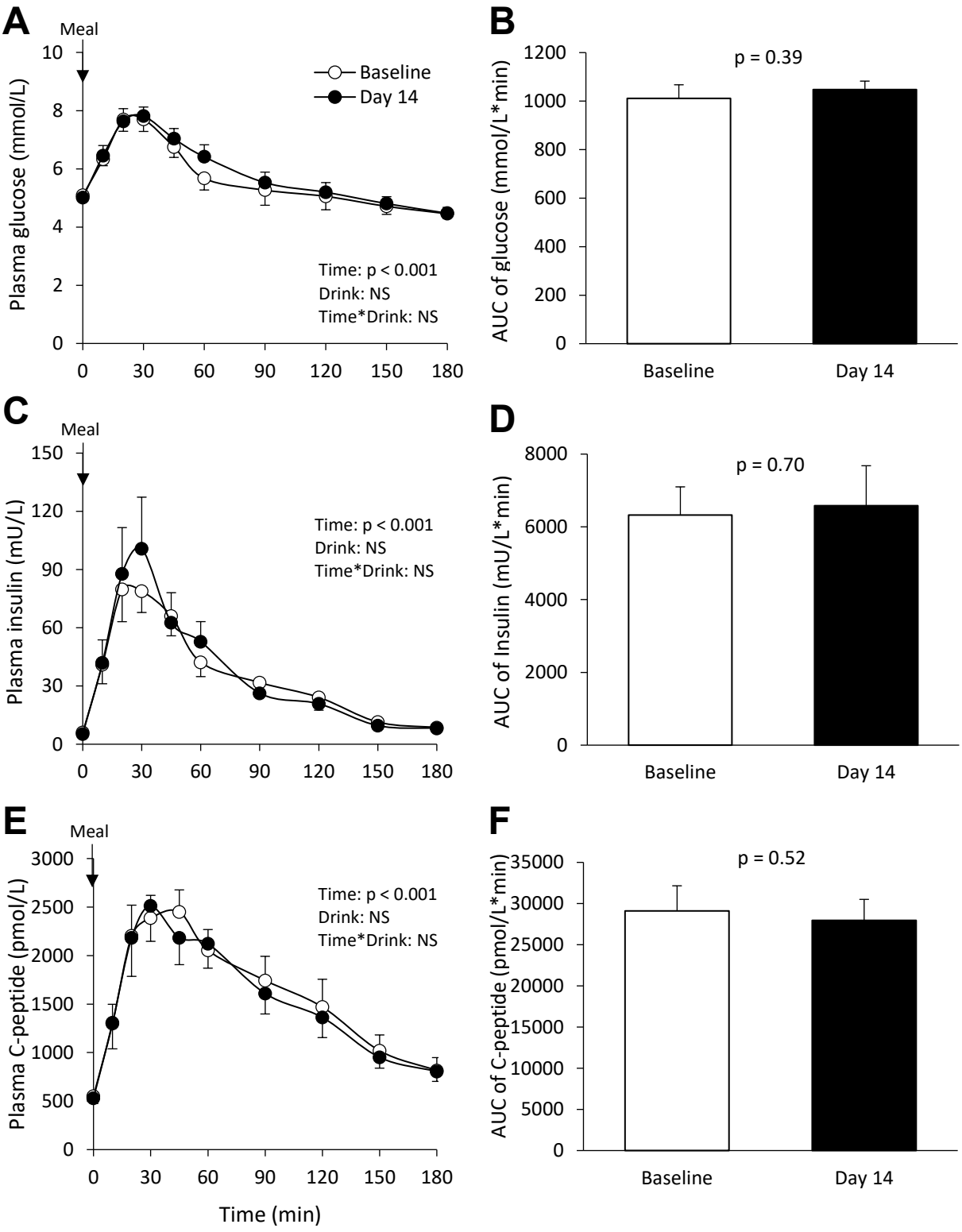

Figure 5.16: Postprandial plasma concentration at baseline and after 14 days consumption of high fructose drinks (n = 10) **(A)** glucose (mmol/L) **(C)** insulin (mU/L) **(E)** C-peptide (pmol/L); AUC of **(B)** glucose (mmol/L*min) **(D)** insulin (mU/L*min) **(F)** C-peptide (pmol/L/min). Data presented as mean ± SEM. Abbreviations: AUC, area under curve; NS, non-significant.

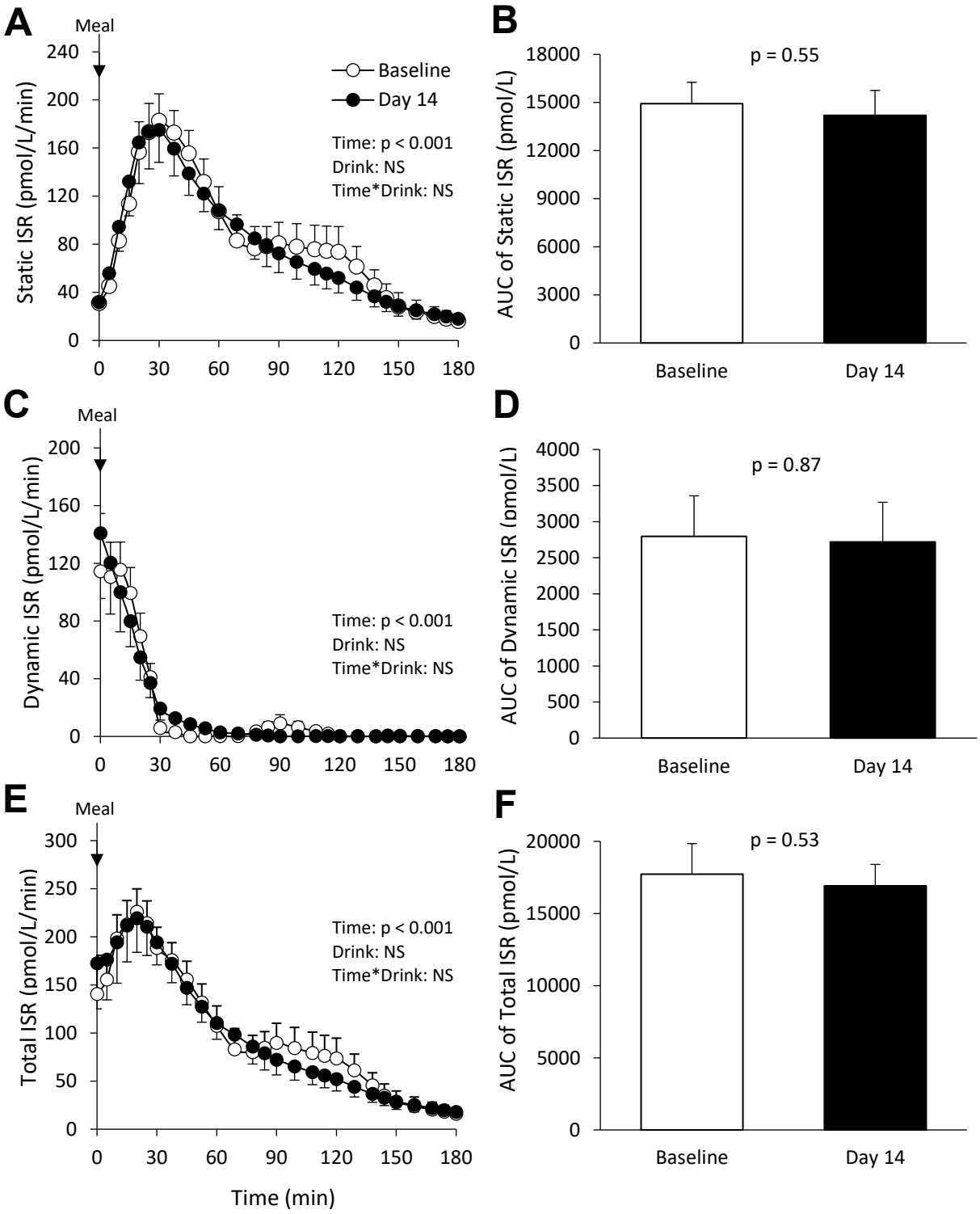

Figure 5.17: Postprandial ISR (pmol/L/min) at baseline and after 14 days consumption of high fructose drinks (n = 10): **(A)** Static **(C)** Dynamic **(E)** Total; AUC of ISR (pmol/L) at baseline and the end of 2 weeks consumption of low fructose drinks: **(B)** Static **(D)** Dynamic **(F)** Total. Data presented as mean ± SEM. Abbreviations: AUC, area under curve; ISR, insulin secretion rate.

No significant differences were found in the rate at which insulin was being cleared before and after consuming high fructose drinks for 2 weeks (Figure 5.18).

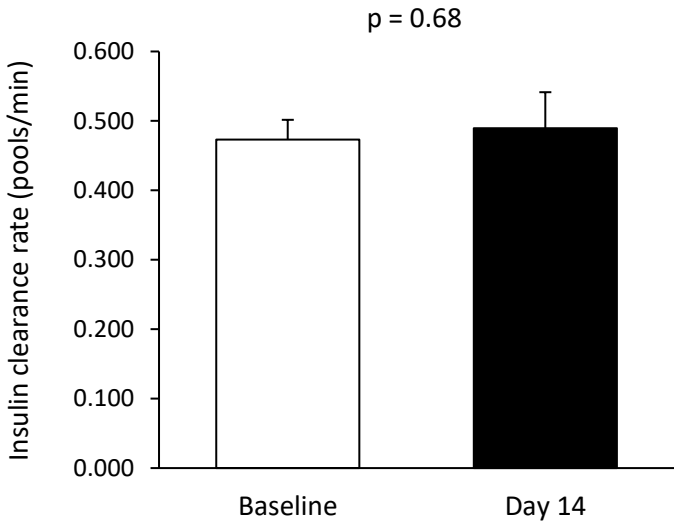

Figure 5.18: Postprandial insulin clearance rate (pools/min) at baseline and after 14 days consumption of high fructose drinks (n = 10). Data presented as mean ± SEM.

No difference was found before and after 14 days of daily consumption of the high fructose drink in the lipogenic index of the subjects' fasting plasma samples. This result suggested that there was no change in hepatic DNL over the course of the dietary intervention (Figure 5.19).

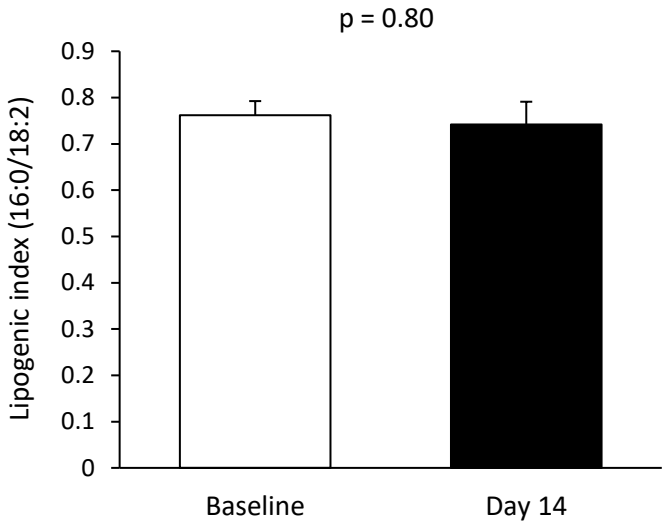

Figure 5.19: Ratio of palmitic acid (16:0) to linoleic acid (18:2) in fasting plasma at baseline and after 14 days consumption of high fructose drinks (n = 10). Data presented as mean ± SEM.

There was also no significant difference in fasting levels of plasma 3OHB before and after 14 days consumption of the HF drink, (Figure 5.20).

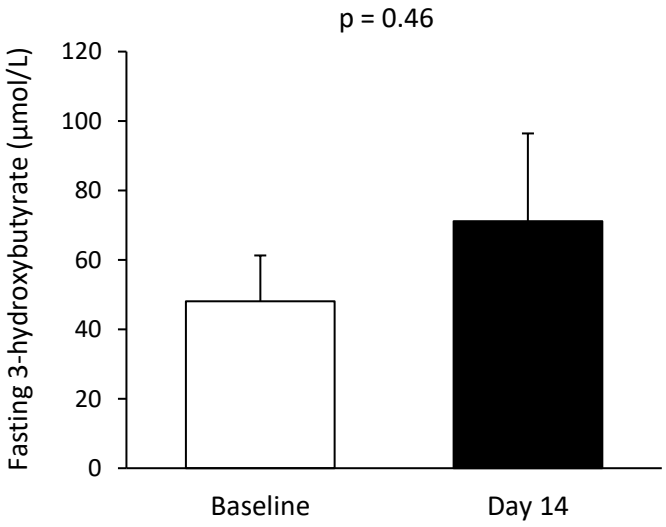

Figure 5.20: Fasting concentration of 3-hydroxybutyrate (µmol/L) at baseline and after 14 days of consumption of high fructose drinks (n = 10). Data presented as mean ± SEM. Abbreviation: 3OHB, 3-hydroxybutyrate.

5.4 Discussion

5.4.1 Acute fructose consumption

Increased consumption of free glucose and fructose in processed foods and drinks have been linked with the increased incidence of metabolic diseases such as type 2 diabetes (Rippe and Angelopoulos, 2016). In this study, investigation on the effect of the composition of dietary monosaccharides on insulin secretion was carried out by having subjects consume a lemon-flavoured drink that contained either 20 g of fructose and 80 g of glucose or 60 g of fructose and 40 g of glucose and subsequently measuring insulin secretion for up to 180 mins postprandial. The high fructose drink reflected the composition of HFCS, a common mixture of free glucose and fructose used in foodstuffs (Bray *et al.*, 2004).

In line with the higher glucose content of the low fructose drink, I found significantly higher plasma glucose, insulin and C-peptide concentrations in all subjects after consumption of the low fructose compared to the high fructose drink. Previously, insulin responses have been found to have a dose-dependent association with the glucose stimulus (Rudenski *et al.*, 1988). However, my observation that the decrease in plasma glucose concentrations were not directly proportional to the decrease in plasma insulin concentration (8% vs 29%, respectively) suggest this might be an independent effect of fructose, rather than just lower glucose content in the drink. This is consistent with previous findings which showed a 50% reduction in plasma insulin concentrations with the consumption of a drink containing 18.75 g of glucose and 75 g of fructose as compared to an isoenergetic drink containing 75 g of glucose and 18.75 g of fructose (Akhavan and Anderson, 2007). My results are also in agreement with those of Hara

and Saito (1981) who found that an isoenergetic mixture of fructose and glucose reduced insulin secretion when compared to glucose alone. The study by Hara and Saito (1981) also emphasised the importance of the oral feeding of fructose, as this inhibited glucose action on the incretin gastric inhibitory peptide (GIP), a stimulus for the secretion of insulin.

Upon consumption of the drinks, differences in insulin secretion were found in the static but not dynamic component for insulin secretion. Static ISR is directly proportional to plasma glucose concentrations and the pattern seen in the present study was expected as it mirrored the time course of plasma glucose. There was a significant difference between the high and low fructose drinks for static ISR which is in agreement with the overall trend for insulin secretion; the two drinks had different glucose content and thus were likely to stimulate insulin secretion differently. Static ISR peaked around 40 mins postprandially, which is about the time the glucose concentration peaked after consumption of both drinks, in line with expectations.

No significant difference was observed in the dynamic ISR upon consumption of the low or high fructose drink. Dynamic insulin secretion was proportional to the rate of change (increase) of glucose concentrations and this was similar for both drinks for the first 40 mins after consumption. The cut-off for insulin resistance, using HOMA2-IR, is a score above 2 in most populations (Salgado *et al.*, 2010; Gayoso-Diz *et al.*, 2013). In the present study, participants had baseline HOMA2-IR values of below 2 on both the study days, suggesting that they were insulin sensitive and therefore should have normal β-cell function. The absence of a difference in the dynamic ISR suggested that subjects were insulin sensitive as they were able to detect changes in plasma glucose

concentrations over time and maintain glucose homeostasis (40 g in high fructose drink compared to 80 g of glucose in low fructose drink). The amount of glucose given in the fructose drinks were sufficient to elicit β-cells to secrete insulin spontaneously. Dynamic ISR peaked at 20 mins postprandially, which was much earlier than static ISR. This was due to the delay required for glucose to reach the plasma and stimulate insulin secretion and is approximately in line with the increase in static ISR between 0 and 40 mins postprandially. The dynamic insulin secretion acts as a faster mechanism of control compared to the static ISR.

When the results were examined by gender, I observed that the difference in plasma glucose and insulin concentrations and the static ISR after consumption of the two experimental drinks were much greater in female than in male subjects. In all cases, it was not the peak values that were different but rather the sustained plateau after reaching peak, which was notably longer in females than males. The sustained plateau of glucose-induced insulin secretion after consumption of the low fructose may lead to higher amounts of insulin in the systemic circulation and its downstream effects such as increased FA synthesis and uptake into adipose tissue (Dimitriadis *et al.*, 2011). The high fructose drink was able to induce a much longer plateau in plasma glucose and insulin concentrations and by extension, the static ISR and total ISR. There was no significant difference in the dynamic ISR in either gender. There were differences in the peak timing of plasma glucose and insulin concentrations as well as static and total ISRs between males and females after consumption of the low fructose drink. These parameters peaked much later and at a slightly higher level, around 60 mins postprandially, in females as compared to 40 mins postprandially in males. It could be

suggested that the difference in these effects is due to the effects of in sex hormones. Oestrogen, but not testosterone has been shown to be a regulator of insulin secretion (Bruns and Kemnitz, 2004). Differences have been reported in the ISR in pre-menopausal and post-menopausal females, but were more clearly compared in post-menopausal females with and without the use of HRT. The use of HRT had been found to decrease incidence of diabetes, increase insulin sensitivity and also lead to higher ISR (Godsland, 2005).

There are limitations to the study. Firstly, the sample size was relatively small, which limited the statistical power of the study, especially when male and female subjects were compared. Moreover, the number of males was higher than that of females and this may have skewed the overall results; it would be of interest to study equal numbers of males and females. Next, I did not standardise the participants' diets before each of the study days. Although advised to maintain their regular diet and lifestyle habits, it is possible that participants might have deviated and this could have affected their responses on the study days. Subjects consumed test drinks which only contained free sugars and no other macronutrients such as fats and proteins. This does not represent the composition of a normal typical meal. Also, my study was only conducted in adults who were aged from 21 to 32 years; therefore it is possible that the findings here are not applicable to older individuals, who may be at a greater risk of metabolic syndrome and type 2 diabetes. Lastly, I only fed subjects a single meal and this does not allow long term investigation of the effect of high fructose consumption on insulin secretion. We thus proceeded to do a chronic study with a drink containing a mixture of glucose and fructose for 14 days.

5.4.2 Chronic fructose consumption

I investigated and compared the effects of chronic consumption of drinks that contained either low or high amount of fructose on insulin secretion and liver fat content. The experimental design involved consumption of one of the two drinks (low or high fructose) daily for 14 days.

No change was found in insulin secretion, fasting plasma glucose, or plasma insulin concentrations in subjects consuming either the high fructose or the low fructose drinks. No change was noted in the two components of insulin secretion (static and dynamic) investigated. From these findings, it could be speculated that there was β-cell adaptation over the period of 14 days in these normoinsulinaemic healthy adults, as the ISR response observed at baseline and after 14 days was similar. This is in line with the results of Crapo and Kolterman (1984), who demonstrated by using glucose and fructose tolerance tests that 2 weeks of fructose feeding resulted in no difference between baseline and end of study insulin secretion levels compared to sucrose feeding. Study measures were made 2 weeks after participants had consumed 63 – 99 g/day of fructose or sucrose (Crapo and Kolterman, 1984).

A recent meta-analysis, of 11 energy balanced studies investigating the chronic effect of replacing glucose or sucrose with fructose-rich foods or beverages found there was little difference in plasma glucose or insulin concentrations between the fructose and glucose groups (Evans *et al.*, 2017). However, in the studies that had subjects with impaired glucose tolerance, there was a large reduction in fasting blood glucose levels (-0.7 mmol/L) when fructose was replaced with glucose, but without a concomitant decrease in plasma insulin concentrations (Evans *et al.*, 2017). These findings

suggested that fructose replacement is potentially useful for diabetic individuals, who have lower glycemic tolerance compared to normoinsulinemic individuals. Fructose helps with glycemic control but is no longer widely used due to concerns with its lipogenic effects (American Diabetes Association, 2008). However, these findings are in contrast to those of Stanhope *et al.* (2009), who found that consumption of 25% of energy requirements from fructose for 10 weeks as part of an isoenergetic diet, increased fasting plasma glucose and insulin concentrations whilst consumption of glucose did not. Reiser *et al.* (1989) also found no difference in plasma glucose or insulin concentrations in both normoinsulinaemic and hyperinsulinaemic subjects after a 5-week isoenergetic diet where 20% of energy were given as amylose or fructose. A recent meta-analysis performed by Choo *et al.* (2018) included 155 controlled intervention studies where sources of fructose-containing carbohydrates were fed to subjects. The authors included positive, negative and energy balanced studies. Glycemic control for these subjects were assessed. It was found that isoenergetic fructose substitution for glucose or subtraction from the diet had no effect on plasma glucose or insulin levels but overfeeding of fructose or fructose substitution for non-carbohydrate macronutrients decreased baseline plasma insulin levels (Choo *et al.*, 2018). The lack of agreement amongst studies suggests that the mechanisms by which β-cells adapt to chronic fructose consumption may differ between individuals based on phenotype including, age, ethnicity and different metabolic parameters (such as glycemic control and lipid profile). Moreover, these studies differed in the energy content and likely also in macronutrient composition of the diets or meals consumed by subjects.

Previous energy balanced studies presented in this thesis have shown that acute fructose consumption can lead to increased hepatic DNL (Chong *et al.*, 2007). In this study, I compared the effects of chronic consumption of a high fructose drink and a low fructose drink over 14 days on hepatic TG content, hepatic DNL (by using the lipogenic index) and plasma 3OHB levels. No change was found in these parameters after 14 days of consumption of either drinks. These results show that consumption of a sugary drink, which contained different compositions of glucose and fructose, does not alter hepatic fat metabolism after 2 weeks in healthy individuals. The lack of change noted here may be due to short term metabolic adaptation to the addition of fructose to the diet in order to maintain homeostasis in lipid metabolism.

The results from my study do not agree with some other studies which conducted high fructose dietary intervention with the comparator arm being glucose, fructose or sucrose for 3 to 11 weeks. For instance, Hochuli *et al.* (2014) conducted a 3-week hyperenergetic intervention with a drink sweetened with fructose only compared to drinks sweetened with sucrose or glucose. They found significant differences in VLDL-TG palmitate content between the groups, with the subjects consuming the fructose sweetened drink having a higher VLDL-TG content than the other two groups of subjects. A meta-analysis performed by Chui *et al.* (2014) found that isoenergetic fructose replacement in 4 trials performed for more than 7 days had no effect on liver fat content while 5 other hyperenergetic trials showed significant differences between chronic fructose and glucose consumption, with increased liver fat content in the subjects who consumed fructose. Another meta-analysis conducted by Wang *et al*. in 2014 assessed the effect of chronic fructose consumption on postprandial triglyceride

concentrations. They found that isoenergetic fructose substitution for starch, sucrose, glucose or HFCS for more than 7 days had no effect on postprandial triglyceride levels while hyperenergetic fructose addition caused a significant increase in postprandial triglyceride levels (Wang *et al.*, 2014). Overall, it appears that isoenergetic fructose substitution results in no change in plasma lipid concentrations or liver fat content whilst the addition of fructose to make the diet hyperenergetic generally results in increased DNL and liver fat content (ter Horst and Serlie, 2017). It is worth noting that my study conducted isoenergetic and not hyperenergetic diet intervention. It is thus in agreement with the results of the meta-analysis above.

Also, similar to the trends on DNL and hepatic fat content, isoenergetic and hyperenergetic fructose substitution have different effects on plasma insulin concentration and sensitivity. A meta-analysis performed by ter Horst *et al.* (2016) found isoenergetic substitution of glucose, sucrose or starch with fructose had no effect on plasma insulin concentration or insulin resistance as measured by HOMA2-IR but the hyperenergetic addition of fructose did result in increased plasma fasting insulin concentrations and increased hepatic insulin resistance (Aeberli *et al.*, 2013). The data from the 2 studies above are consistent with those obtained from my study which involved isoenergetic substitution of glucose for fructose.

There are some limitations for the chronic fructose intervention study. First, only one drink per day was replaced with either the high or low fructose drinks and no meal diaries were kept by the subjects, so it is not known whether the subjects consumed meals of differing macronutrient composition during the study duration, though there was no change in body weight of the subjects. It was also difficult to assess other

compliance such as whether the subjects have consumed the drinks as there is currently no good biomarker for carbohydrate intake. It was assumed that the subjects complied through interviews. Also, I did not match the fatty acid composition of the test meals with those of the subjects' adipose tissue. This could be done through obtaining adipose tissue biopsy samples, which involves an invasive procedure and is likely to be less acceptable to subjects; though it will improve the accuracy of the results obtained (Hudgins *et al.*, 1996; Chong *et al.*, 2008). I did not assess the amount of physical activity undertaken by the subjects which may have influenced the results as Bidwell *et al.* in 2014 showed that high amounts of physical activity were able to counteract the deleterious effects of high fructose consumption on postprandial lipidaemia.

In conclusion, my study showed that a single high fructose test drink was able to cause changes in the insulin secretion profile in females but not males but these changes were not seen after 14 days of chronic consumption. The lack of change may suggest that whole body metabolism is able to adapt to fructose substitution over a prolonged period of time.

Chapter 6: Conclusions and Future Work

6.1 Summary and conclusions

In this thesis, I investigated the effects of consuming meals containing different compositions of glucose and fructose on hepatic fatty acid (FA) metabolism, and insulin secretion and sensitivity.

In Chapter 3, I investigated the effect of two meals, containing differing amounts of fructose, on hepatic FA synthesis and partitioning. In contrast to previous studies, I traced the fate of dietary FAs (by incorporation [U^{13}C]palmitate into the meals) to determine if consuming different amounts of fructose with glucose, together with fat altered hepatic DNL and dietary FA oxidation. I found there was little difference in whole body FA oxidation (as assessed by ^{13}C-incorporation into expired breath) after consumption of either test meal. However, when results were stratified by gender, females were found to have higher hepatic DNL than males after consumption of the high fructose meal. I also found females had higher levels of hepatic FA oxidation (as measured by plasma 3OHB concentrations) but not whole body FA oxidation than males, after consumption of the low fructose meal. The sexual dimorphism in hepatic DNL and partitioning suggests gender specific differences in metabolic regulation when fructose is included in a meal; the underlying mechanism for this remains to be elucidated.

In Chapter 4, I traced the metabolic fate of fructose after consumption of the high fructose meal only. By including [U^{13}C]fructose as part of the meal, I found 30.5% of dietary fructose in breath CO_2 at the end of the 6-hour postprandial period, whilst less than 1% of the ingested fructose was synthesised to palmitate and incorporated into VLDL-TG. This result is in line with a study by Theytaz *et al*. (2014) which found that

oxidation of fructose to CO_2 made up 36.6% of the fructose ingested and Chong *et al*. (2007) who found that the proportion of fructose converted to FAs after consumption of a fructose bolus was 0.5%. This result confirms previous findings and shows that although high fructose consumption has been shown to increase hepatic DNL, a much greater proportion of dietary fructose undergoes oxidation.

In Chapter 5, I investigated the effect of acute and chronic consumption of both high and low fructose drinks on insulin secretion and hepatic FA metabolism. In the acute study, I found that consumption of the 2 different drinks affected only the static component of insulin secretion but not the dynamic component, as modelled using the minimal model. Between genders, it was found that the change in static insulin secretion rate was only apparent in females. However, after 14 days consumption of either drink, these differences were no longer apparent, indicating the possibility of β-cell adaptation and that either a longer term study period or a higher intake of fructose consumption is required before changes in HOMA2-IR or other measures of β-cell function can occur; alternatively it may be that fructose does not have an effect on β-cell function.

In conclusion, my studies provided some insight into the effects of both acute (single meal) and longer term consumption of a combination of glucose and fructose on FA metabolism and insulin response. The findings indicated some gender-specific differences but little overall difference between a high and low fructose meal. Taken together, with previous studies, it seems that there is no change in hepatic FA metabolism from short term consumption of relatively large amounts of free monosaccharides, HFCS or sucrose despite the links between this and the metabolic syndrome.

6.2 Future work

On the basis that DNL was higher with consumption of the high but not low fructose meal it would be of interest to elucidate the cellular mechanisms behind these differences; this could be undertaken using cell-based studies with human hepatocytes (primary and cell-lines). To understand and determine the amount of fructose required to see changes in DNL, dose response studies (varying the amounts of glucose and fructose) and time course experiments could be undertaken, and changes in the expression of genes related to carbohydrate and fatty acid metabolism, along with intracellular TG could be assessed. This information would allow better understanding of cellular responses to different doses of glucose and fructose over time.

It would also be of interest to investigate if the increase in hepatic DNL, along with sex-specific differences after feeding of a single high fructose meal are sustained when a high fructose diet is consumed. This could be undertaken by assessing hepatic DNL regularly over the course of a study period, which determine when changes occur in hepatic DNL and if they are maintained. FA oxidation and substrate utilisation could also be assessed concurrently. This would provide a more complete picture of changes in lipid and carbohydrate metabolism after high fructose diet feeding.

The differences in hepatic DNL and 3OHB seen between the genders were suggested to be due to hormonal differences, specifically the effect of oestrogen. An approach to investigate hormonal differences is to perform the study described in Chapter 3 on groups of post-menopausal female subjects with and without hormone replacement therapy. Investigation on the effect of ethnicity, age and gender on the effect of high fructose consumption on hepatic FA synthesis and partitioning could also be carried out

to determine whether genetic factors or ageing play a role in how fructose affects lipid metabolism.

A difference in static ISR was also shown after consumption of the high and low fructose meals when a single meal was consumed, but no difference after continued consumption of these meals for 14 days. This could be due to β-cell adaptation and a possible future experiment would be to investigate this via a cell-based approach. I could use cultured pancreatic islet cells perfused with a mixture of glucose and fructose and measure insulin secretion in the media to check for their function over time course and dose-response experiments, varying the amounts of glucose and fructose in the media. Alternatively, as the current study's chronic treatment was conducted only over a 2-week period and only replaced a single meal of subjects with either the high or low fructose drinks, to justify the current finding of suggested β-cell adaptation, a dietary intervention study could be carried out with consumption of a high amount of fructose in a controlled isoenergetic diet over a longer period of time. Measurement of β-cell adaptation in humans can be done by modelling β-cell function after acute and chronic consumption of different ratios of glucose and fructose and determining if there is any difference across groups of subjects.

Appendix

A) Chocolate fat-emulsion test drink

The following recipe was used to make the chocolate fat-emulsion test drinks used in chapter 3 and 4.

Appendix Table 1: Ingredients needed for the chocolate fat-emulsion and their respective energy percentages in the drink

Ingredient	Amount by weight (g)	Energy content (kcal)	% contribution
Palm oil	30	270	52
Olive oil	20	180	34
Cocoa Powder	2.4	60	12
Artificial sweetener	1.2	5	1
Emulsifier	0.4	3	0.6
[U^{13}C]palmitic acid	0.2	2	0.4
Boiled heavy drinking water	40	N.A.	0
	Total energy content:	520	100%

First, the amount of palm oil and olive oil needed were weighed into a 600ml glass beaker. Myverol 18-04k emulsifier (Surfachem Group Ltd, Leeds, UK) was then added into the oil mixture and heated in microwave (high mode) for 20 sec. The beaker was taken out of the microwave to give a swirl and check if the emulsifier has dissolved. The temperature of the oil mixture was checked to be below 70 ^{o}C. In the case, where the emulsifier has not completely dissolved, the mixture was reheated in cycles of 10 sec

until all emulsifier had dissolved (usually no more than three short bursts were needed). Following that, the beaker was removed from the microwave and placed in a bowl of hot water to keep warm. Next, with swirling, [U^{13}C]palmitic acid (Merck, UK), cocoa powder (Cadbury Bournville Cocoa, UK), artificial sweetener (Tesco, UK) and boiled heavy water were sequentially added to the mixture. The contents in the beaker were then whisked for 2 mins, until a fine light-brown chocolate-coloured emulsion was produced. The emulsion was left to cool slightly before transferring into a plastic bottle and placed in a baby warmer at 65 °C until it was served to the participants.

B) Lemon-flavoured sugar drink (without isotope)

The following recipe was used to make the lemon-flavoured sugar drink used in Chapter 3 and 5.

Appendix Table 2: Ingredients needed for the lemon-flavoured high fructose drink and their respective energy percentages in the drink

Ingredient	Amount	Energy content (kcal)	% contribution
Glucose	40 g	160	40
Fructose	60 g	240	59.5
Lemon juice	10 ml	2	0.5
Boiled heavy drinking water	180 ml	N.A.	0
	Total energy content:	402	100%

Appendix Table 3: Ingredients needed for the lemon-flavoured low fructose drink and their respective energy percentages in the drink

Ingredient	Amount	Energy content (kcal)	% contribution
Glucose	20 g	80	20
Fructose	80 g	320	79.5
Lemon juice	10 ml	2	0.5
Boiled heavy drinking water	180 ml	N.A.	0
	Total energy content:	402	100%

First, the amount of glucose and fructose needed were measured into a 250 ml glass. Boiled heavy drinking water was then added and the mixture stirred to dissolve all the monosaccharides. Lemon juice (Tesco, UK) was added into the low or high fructose drink before it is refrigerated to be served cold.

C) Lemon-flavoured sugar drink (with [U^{13}C]fructose isotope)

The following recipe was used to make the lemon-flavoured high fructose drink used in Chapter 4.

Appendix Table 4: Ingredients needed for the lemon-flavoured high fructose drink and their respective energy percentages in the drink

Ingredient	Amount	Energy content (kcal)	% contribution
Glucose	40 g	160	39.6
Fructose	60 g	240	59.4
[U^{13}C]fructose	0.5 g	2	0.5
Lemon juice	10 ml	2	0.5
Boiled heavy drinking water	180 ml	N.A.	0
	Total energy content:	404	100%

First, the amount of glucose and fructose needed were measured into a 250 ml glass. Boiled heavy drinking water was then added and the mixture stirred to dissolve all the monosaccharides. Next, [U^{13}C]fructose (Merck, UK) and lemon juice (Tesco, UK) was added into the low or high fructose drink before it is refrigerated to be served cold.

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