Fructose Spike Control: Potential Health Benefits and Plant Naturals

A thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

By

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In dedication to my father, recently deceased, who would have loved to have lived long enough to see this achievement.

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Abstract

Fructose has been linked to, and even claimed, although controversially, to be the cause of the obesity, diabetes and metabolic syndrome global epidemics. However, many early studies have been conducted in animals and humans at very high doses. Following an in-depth literature review, with consideration of fructose intake levels consumed in a normal diet, it is concluded here that there is reasonable evidence that fructose can in particular increase post-prandial triglycerides.

This fits well with dietary fructose, unlike glucose, being relatively unregulated by insulin and ~~is~~ instead mostly and rapidly metabolised in the liver. That metabolism of fructose is not feedback inhibited by energy status has led to suggestions that it is more readily utilised for lipid synthesis. It has also been proposed that only fructose in excess of energy requirements is directed towards or stimulates *de novo* lipogenesis. This thesis therefore hypothesised that flattening the fructose spike reaching the liver from the small intestine would result in reduced triglyceride release from the liver.

Triglyceride release was investigated in primary human hepatocytes treated with high physiological levels of fructose verses and half such levels for twice as long, hence modelling "flattening the fructose spike" at 6, 8 10, 12 and 14 mM glucose, over three applications to represent "meals" during the course of the day. Triglyceride (meal 1- mean of 8 mM fructose for 1 hrs *vs.* 4 mM fructose for 2 hrs- 47%; meal 2- mean of 2 mM fructose for 1 hr *vs.* 1 mM fructose for 2 hrs- 65%; meal 2- mean of 8 mM fructose for 1 hr *vs.* 4 mM fructose 2hrs- 73%; meal 3- 2 mM fructose for 1 hr *vs.* 1 mM fructose for 2 hrs- 58%; meal 3- 8 mM fructose for 1 hr *vs*. 4 mM fructose for 2 hr- 39%) and cytotoxicity (meal 1- 8 mM fructose for 1 hr *vs*. 4 mM fructose for 2 hrs- 17%; meal 2- 8 mM fructose for 1 hr *vs* 4 mM for 2 hrs- 43%; meal 3- 8 mM fructose for 1 hrs *vs.* 4 mM fructose for 2 hrs- 28%) were found to be significantly decreased.

As ~~technology~~ natural plant extracts to potentially flatten the fructose spike and limit triglyceride release, black and green tea were found, in an optimised gut cell-line, to significantly inhibit fructose and glucose transport (60% and 82% respectively at a 1-

in-4 dilution). Synergies with glucose inhibitors (phloretin+quercetin-3-glucoside; luteolin+quercetin-3-glucoside; hesperitin+quercetin-3-glucoside, luteolin+lectin, black tea+phloridzin, green tea+phloridzin and ice tea+phloridzin) were found to be significant for glucose transport inhibition. By slowing down fructose and glucose uptake in the gut such compounds and plant extracts could also activate the ileal brake and reduce appetite and energy intake.

Investigation into further potential health benefits of flattening the fructose spike found that over 72 hrs fructose+glucose induced excess glycogen storage (2.5-fold), over 24 hrs elevated lactate (3-fold) and over 48 hrs intact/total FGF21 (2.2-fold) and greatly more so than isocaloric glucose alone (1.76, 2.88 and 2.1-fold respectively). Over 24 hrs glucose tended to increase intake/FGF21 more so than fructose, which correlated negatively with fibroblast activation protein. Significant lipid accumulation was not seen in these experiments with fructose or glucose.

In conclusion this thesis has: determined that inhibiting fructose transport in the gut has the potential to ~~improve~~ reduce triglyceride release; identified natural plant compounds and extracts to do so; and found excess glycogen, lactate and FGF21 as further potential health markers that may be further investigated in primary human hepatocytes, the gold standard *of in vitro* liver models avoiding the use of animals and expense of human subjects.

Abbreviations

2-NBDG	(2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose)
3D	3-Dimension
Ab	Antibody
ADP	Adenosine d-phosphate
AMP	Adenosine mono-phosphate
ANOVA	Analysis of variance
AP	Acute pancreatitis
Apo	Apolipoprotein
Approx.	Approximately
ASCVD	Atherosclerotic cardiovascular disease
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
BBM	Brush border membrane
BCA	Bicinchoninic acid assay
BCM	Basic culture medium
BK C	Blank control
BK	Blank
BkT C	Black tea non-pH neutralised
BkT N	Black tea pH neutralised
Blk	Blank
BLM	Basolateral membrane
BSA	Bovine serum album
bTEA	basic Triethanolamine-HCL buffer
$CaCl_2$	Calcium Chloride
Caco-2	Colorectal adenocarcinoma
cDNA	Complementary DNA
CHD	Cardiovascular heart disease
ChREBP	Carbohydrate response element binding protein
Cin	Cinnamaldehyde
CKD	Chronic kidney disease
~~CO_2~~	~~Carbon Dioxide~~

Con	Control
cTEA	complete Triethanolamine-HCL buffer
Cu^{+1}	Cupric (copper) ion (one electrons missing)
Cu^{+2}	Cupric (copper) ion (two electrons missing)
DAPI	4′,6-diamidino-2-phenylindole
Decaff	Decaffeinated
DecafT C	Decaffeinated tea non-pH neutralised
DecafT N	Decaffeinated tea pH neutralised
DGAC	Dietary Guidelines Advisory Committee
DGAT2	Diacylglycerol O-Acyltransferase 2
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNL	*de novo* lipogenesis
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EFSA	The European Food Safety Authority
EG	Ethyl gallate
EGC	Epigallo catechin
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (egtazic acid)
ELISA	The enzyme-linked immunosorbent assay
Em	Emission
EPA	Eicosapentaenoic acid
ER	Endoplasm reticulum
Erk1/2	Extracellular signal-regulated kinase 1 and 2
et al	two or more names
Euc	Eucalyptol
Ex	Excitation

F1P	Fructose-1-phophate
F6P	Fructose-6-phosphate
FAP	Fibroblast activation protein
FCS	Foetal calf serum
FDA	Food and Drug Administration
FFA	Free fatty acids
FGF21	Fibroblast growth factor 21 (FGF21
FGFR1	Fibroblast growth factor receptor 1
FITC	Fluorescein isothiocyanate
Flav	Flavour
FRAME	Fund for the replacement of animals in medical experiments
~~Fruc~~	~~Fructose~~
Fruc	Fructose
G+F	Glucose+Fructose
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCG	Gallocatechin-3-gallate
GFP	Green Fluorescent Protein
GIP	Gastric inhibitory polypeptide
GLP-1	Glucagon-like peptide-1
~~Glu~~	~~Glucose~~
Gluc	Glucose
GLUT	Glucose transporter
GLUT5$^{-/-}$	Deletion of Glut5 transporter gene of both alleles
GnT C	Green tea non-pH neutralised
GnT N	Green tea pH neutralised
GPR81	G-protein coupled receptor 81
GRP	Glucokinase regulatory protein
GWAS	Genome-wide association study
H&E	Hematoxylin and eosin
~~H_2O~~	~~Water~~
H3K9	Histone H3 lysine 9 methylation

HBSS	Hanks' Balanced Salt Solution
HCL	Hydrogen chloride
HDL	High-density lipoprotein
HDL-C	High density lipoprotein- cholesterol
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is a zwitterionic sulfonic acid buffering agent.
HepG2	Hepatocellular carcinoma G2
Hesp	Hesperitin
HFCS	High fructose corn syrup
HK	Hexokinase
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPLC	High performance liquid chromatography
Hrs	Hours
hs-CRP	high-sensitivity C-reactive protein
IBMX	3-isobutyl-1-methylxanthine
Ice T C	Ice tea (lemon) control) non-pH neutralised
Ice T L	Ice tea lemon
Ice T M	Ice tea mango
Ice T N	Ice tea (lemon) control) pH neutralised
Ice T P	Ice tea peach
Ice T R	Ice tea raspberry
IcTL	Ice tea lemon
IDL	Intermediate-density lipoprotein
IL-1a	Interleukin 1 alpha
IL-6	Interleukin 6
IL-8	Interleukin 8
~~KCl~~	~~Potassium Chloride~~
~~KH_2PO_4~~	~~Potassium Phosphate~~
KHK	Ketohexokinase
KLB	KlothoB
Km	Body weight (kg) per body surface area (m^2)
K_m	Michaelis constant
KO	Knockout
KRPB	Kreb's ringer phosphate buffer

LCAT	Lecithin–cholesterol acyltransferase
LDH	Lactate dehydrogenase
LDHA	Lactate dehydrogenase A
LDHB	Lactate dehydrogenase B
LDL	Low-density lipoprotein
LDL-C	Low density lipoprotein- cholesterol
Lect Gly	Lectin glycan
Lut	Luteolin
Luteolin-7-G	Luteolin-7-glucoside
M1-d2	Medium one, day two
MBG	Mitel Border Gateway
~~$MgCl_2$~~	~~Magnesium Chloride~~
~~$MgSO_4$~~	~~Magnesium sulphate~~
Min	Minute
mRNA	Messenger RNA
~~Na_2CO_3~~	~~Sodium carbonate~~
~~Na_2HPO_4~~	~~Sodium Phosphate~~
~~NaCl~~	~~Sodium Chloride~~
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate +
NAFLD	Non-alcohol fatty liver disease
~~NaOH~~	~~Sodium Hydroxide~~
NASH	Non-alcohol fatty liver related steatohepatitis
NFK-B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
Nrf2	Nuclear factor erythroid 2-related factor 2
o/n	Overnight
OD	Optical density
P value	Probability of null hypothesis
PAS	Periodic Acid-Schiff
PBS	Phosphate buffered saline
PBS (+)	Phosphate buffered saline (+calcium and magnesium)
PCAF	P300/CBP-associated factor

PCR	Polymerase chain reaction
Pepsi	Diet Pepsi cola
PGC1β	Peroxisome proliferator activated receptor-γ-coactivator-1β
pH	Potential of hydrogen
Phlt	Phloretin
PKC-βII	Protein kinase C-βII
PLIN2	Perilipin 2
PLO	Palmitate, linoleate and oleate
Plz	Phloridzin
PVN	Periventricular nucleus
PYY	Peptide YY
Q3G	Quercetin-3-glucoside
Quercetin-3-G	Quercetin-3-gluocoside
RCT	Randomised controlled trials
Res	Resveratrol
RNA	Ribonucleic acid
rt	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SACN	Scientific Advisory Committee On Nutrition
SD	Standard deviation
SGLT1	Sodium-glucose linked transporter-1
SNP	Single nucleotide polymorphism
SREBP1c	Sterol regulatory element binding protein 1c
SRF	Sugar Research Foundation
SSB	Sugar sweetened beverages
Stds	Standards
TAG	Triacylglycerol
TEER	Trans epithelial electrical resistance
TF	Theoflavone
TMB	3,3’,5,5’-tetramethylbenzidine
TMF	Theomethoxy flavone
TNFalpha	Tumour necrosis factor alpha
Ucp1	Uncoupling protein 1

UPR	Unfolded protein response
USA	United States of America
USDA	United States Department of Agriculture
Van	Vanillin
VH	Ventromedial hypothalamus
VitC	Vitamin C
VLDL	Very-low-density lipoprotein.
vs.	*Verses*
WHB	White Hat bias
WHO	World Health Organisation
WST-1	Water-soluble tetrazolium salts-1

Figures and Tables

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1.0 Introduction

Fructose has received considerable attention in science literature and the media e.g. “The Toxic Truth about Sugar” (Lustig *et al*., 2012), being linked to, and even claimed to be the cause of, obesity, diabetes and metabolic syndrome global epidemics. The obesity epidemic and the proposed link to sugar content in food has led to the World Health Organisation (WHO) in 2015 recommending strongly that free sugar intake should be limited to less than 10% of total energy, with an additional lifespan conditional recommendation of less than 5% (WHO, 2015a). The US Dietary Guidelines Advisory Committee (DGAC) in 2015 also recommended an upper limit of 10% for all added sugars, and the Scientific Advisory Committee on Nutrition (SACN) 2015 has proposed less than 5%. However, with respect to fructose specifically SACN concluded that “…on balance, it is considered that there is insufficient evidence to demonstrate that fructose intake, at levels consumed in the normal UK diet, leads to adverse health outcomes…” More recently though, The European Food Safety Authority (EFSA) concluded that fructose appeared to increase hepatic insulin resistance and uric acid levels more than equivalent amounts of glucose (EFSA, 2021).

Why though would fructose be harmful to health and possibly more so than glucose? Fructose and glucose are isomers with the same molecular weight. Although not fully understood, it is thought that it is partly because fructose spends more time, than glucose, in the free-chain linear form rather than a pyranose ring structure, due ~~perhaps~~ to hydroxyl group orientation, and that its carbonyl group (C=O) is free and more able to react with other molecules (Bun and Higgins, 1981). It is proposed that fructose spending more time in the free-chain linear form is probably why glycation (Motera *et al*, 2019) by fructose is 7-8 times faster than by glucose (Dills 1993). Glycation causes tissue damage by attacking cell proteins and lipids impairing cell function. It is therefore presumed that for this reason fructokinase, rather than glucokinase (for which fructose is a poor substrate), rapidly metabolises fructose in the liver, quickly removing it from the circulation. Unlike glucose metabolism by phosphofructokinase, fructokinase and down-stream metabolism are not feedback-inhibited by energy status and therefore fructose continues to be taken up by the

liver even when in excess. Subsequent metabolism leads to: oxidation for energy; glucose production; glyconeogenesis; lactate production, and possibly increased hepatic lipogenesis with increased triglyceride release into circulation and/or lipid accumulation in the liver and hepatotoxicity (see Figures 1.4 and 1.7). Prolonged fructose exposure is thought to also ~~boost~~ stimulate *de novo* lipogenesis (DNL) at the molecular level (Bizeau & Pagliassotti, 2005). I.E: Pre-sensitisation to fructose stimulates carbohydrate response element binding protein (ChREBP) which activates the enzymes responsible for DNL; Stimulation of peroxisome proliferator activated receptor-γ-coactivator-1β (PGC1β), a transcriptional co-activator for sterol regulatory element binding protein 1c (SREBP1c) may further increase the activity of the enzymes involved in DNL; Elevated malonyl CoA levels increase carbon flux into the lipogenesis pathway leading to inhibition of β oxidation, contributing to increased serum triglycerides or intrahepatic lipid accumulation. An additional health ~~hazard~~ concern caused by fructose specifically could be increased levels of uric acid, which is associated with oxidative stress, production of e.g. tumour necrosis factor-α (Sun & Empie, 2012), hypertension and type II diabetes (Dengham *et al*., 2008), cardiovascular disease (Borghi *et al*., 2014) etc. Increased uric acid can be caused as a metabolic product of built-up ADP and AMP. This may occur as fructose is very rapidly metabolised in the liver by fructokinase which depletes ATP and produces ADP, but is subsequently slowly metabolised by downstream enzymes, e.g. aldolase (Chaudhary *et al*., 2013) (see Figure 1.5).

The main aims of this thesis have been to: A) review the literature and consider which if any of the concerns over fructose with respect to health are justified; B) demonstrate that, if health concerns may be justified, there would be a potential benefit in inhibiting fructose uptake from the gut and flattening the fructose spike reaching the liver; C) develop methodology and test for compounds, that are found naturally in plants, for their potential to inhibit the uptake of fructose from the gut and D) investigate further potential health concerns that flattening the fructose spike may benefit..

1.1. Fructose impacts on health- Literature review

For this work to be of relevance, it is not necessarily needed to demonstrate that fructose has health concerns greater than glucose or starch. A key aim of this thesis is to support the premise that inhibiting the uptake of fructose from the gut would be beneficial. It was therefore only needed to show that fructose has negative health implications, as it is not a role here to propose that fructose be replaced in the diet by, e.g. glucose or starch. Whilst replacing fructose may, or may not, be the best approach, the object of the thesis is to address what can be done about fructose where this is not possible, or difficult, or as an alternative. However, there is much speculation and controversy that fructose is more harmful in some respects than glucose and so this aspect will also be considered in much of the following.

1.1.1 Pre-literature review issues

There are a number of issues that should ~~perhaps~~ be considered when reviewing the literature in this field. For example, many trials have used fructose levels that are much higher than the dietary norms and/or possibly under conditions of negative energy balance. In this section some of these issues are discussed in more detail.

Fructose consumption- physiological levels

In the USA consumption of fructose, according to studies by survey, may be on average around 50 g/day (39-72 g/day) (approximately 10% of total calories) (Vos *et al*., 2008, Marriott *et al*., 2009)).

However, it should also be taken into account that some of this fructose is from fruit and vegetables ~~(18%). and some of the fructose in cakes/pies/snacks, bread/pasta, and ready to eat cereals are from grains (Figure 1.1).~~ To date no studies have shown an association between consumption of fruit and negative health issues (Kitson *et al*., 2014) and many have shown positive health benefits (Hung, H.C., *et al* 2004; Oyebode *et al*., 2014). This is likely due to the fact such contain little free

sugars including added sugar*. Sugars that are added to foods and drinks along with sugars that are 'free' from cell structures like those in fruit juice or honey may have greater detrimental effects on health, whereas this does not appear to be the case for the sugars that are found naturally in milk and within cell structures in fruit and vegetables (WHO, 2015b).

[*The World Health Organisation (WHO) definition for free sugar includes all sugars that are added during food manufacturing and preparation as well as sugars that are naturally present in honey, syrups, fruit juices and fruit concentrates (WHO, 2015b)].

Fructose from fruit juice may therefore be just as harmful as that from table sugar or high fructose corn syrup (HFCS) as many studies arguably show (Bazzano *et al*., 2008), with little or no fibre to slow absorption from the gut. Figure.1.1 shows the percentage contribution of different food groups to free sugars for 19-64yr olds in the UK.

Figure 1.1: The percentage contribution of different food groups to free sugars- ages 19-64yrs.

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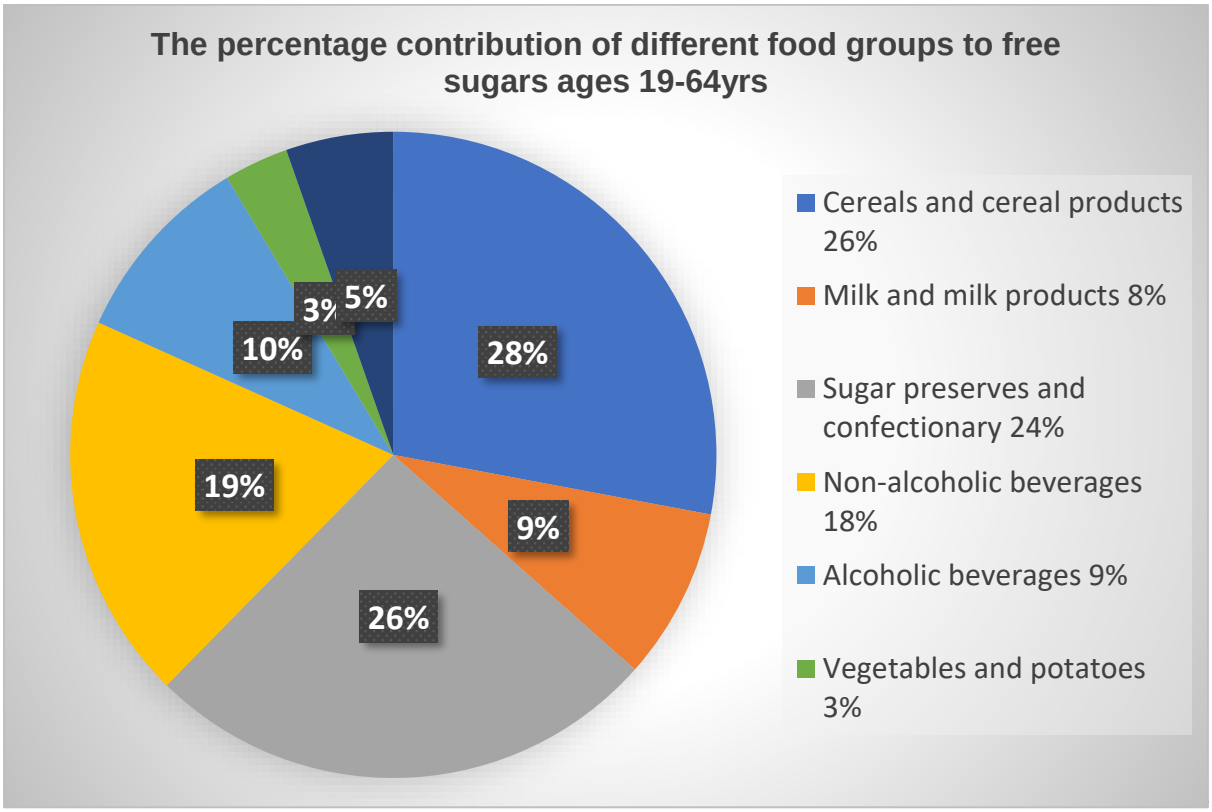

Figure 1.1: The percentage contribution of different food groups to free sugars- ages 19-64 yrs. Adapted from British Nutrition Foundation/Nutrition.gov (https://www.nutrition.org.uk/healthy-sustainable-diets/starchy-foods-sugar-and-

fibre/sugar/?level=Health%20professional#whataresugars): National Diet and Nutrition Survey.

The main sources of free sugars in UK diets are sugary drinks, cereal products sweetened with sugars, confectionery, table sugar and fruit juice. ~~However,~~ Fructose from grains and cereals may also be less harmful ~~(even if harmful otherwise~~) as they are consumed with fibres and phytonutrients which may be protective (Bazzano *et al*., 2008). Fibre is thought to slow down the absorption of fructose (Jenkins *et al*., 2000; Weickert and Pfeiffer, 2008), and therefore reduces the fructose spike experienced by the liver which may be beneficial, although not yet generally demonstrated. Free sugar consumption has been estimated to be approximately 53 g/day in the UK (Young *et al*., 2022), which would equate to approximately 26 g fructose/day. Marriott *et al*, 2009 included an estimate of added fructose in their analysis of fructose intake in the USA which averaged at 41 g/day. However, this did not include fructose in fruit juice which was counted as natural sugars. Some have even higher consumption: boys aged 15-18 yrs and the upper 90th percentile of the overall population sample across all ages were reported to consume 68 g/day fructose, and the upper 95th percentile for males aged 19-22 yrs consumed 122 g/day.

Further still, recent reports~~, however,~~ suggest that people in such self-reported diet datalogs underestimate food intake by up to 50% (Poppitt *et al*., 1998; Trabulsi, *et al*., 2001; Winkler *et al*., 2005; Rennie *et al*., 2007; Devlin *et al*., 2012; Pfrimer *et al*., 2015; Harper and Hallsworth, 2016), and data on national supplies and availability of fructose indicate consumption of approximately 84 g/day in the US (Credit Suisse Research, 2013) (Table 1.1). Some of this sugar may be wasted however, such as during preparation and cooking in the home or in food service establishments, and as such is not a measure of actual consumption (USDA Economic Research Service. 2008). In addition, some research shows that manufacturers often underestimate the fructose content of their products, using a source of HFCS that has a ratio of fructose to glucose of 60:40 rather than the presumed 55:45 (Ventura *et al*., 2011; Walker *et al*., 2014; Olmedo *et al*, 2021).

Taking into consideration under-reporting, production/supply data and underestimation of product content, ~~perhaps~~ a reasonable average of consumption

of added or potentially harmful fructose, in the USA can be estimated to be approximately 50 g/day or more.

Table 1.1: Estimate of Intake of sugars based on "supply chain"/availability by country.

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Country	Sugar availability g/day
United States of America	84
Brazil	79
Australia	76
Argentina	76
Mexico	75
Canada	70
Latin America	68
Malaysia	65
Ukraine	63
Chile	61
Europe	51
Developed world	48
Developing world	28

Table 1.1: Estimate of Intake of sugars based on "supply chain"/availability by country. From the key findings of a study on the dietary impact of "sugar and sweeteners" and their role in the ongoing health debate surrounding obesity and diabetes – (*Credit Suisse Research Institute: Sugar Consumption at a crossroads).* Shows an estimate of sugar intake based on sugar supply chain/availability by country. Kilocalories of sugar consumed/day/person. At 1 g/4 kcals., for the US this equates to approximately 90 g/day fructose from sugar and HFCS combined (Credit Suisse Research 2013).

[Credit Suisse media contacted Will Bowen- but Linkedin post says he may have left?- also emailed his colleague- no reply- and this statement below found in report. so table created from some of the data]]

Energy needs- age and gender considerations

Normal energy needs are generally assumed to be 2000 kcal/day (U.S. Department of Health and Human Services and the U.S. Department of Agriculture, 2015), but this can vary greatly depending on, e.g. age, sex and activity (Figure.1.2) (U.S. Department of Health and Human Services and the U.S. Department of Agriculture, 2015). For example, for active females aged 18-30 yrs, 2400 kcals are recommended and 2800 kcals for moderately active men 18-25 yrs of age (Figure 1.2).

Figure.1.2: Energy requirement by gender, age and activity

	Male				Female		
Age	**Sedentary**	**Moderately Active**	**Active**	**Age**	**Sedentary**	**Moderately Active**	**Active**
18	2,400	2,800	3,200	18	1,800	2,000	2,400
19-20	2,600	2,800	3,000	19-25	2,000	2,200	2,400
21-25	2,400	2,800	3,000	26-30	1,800	2,000	2,400
26-35	2,400	2,600	3,000	31-50	1,800	2,000	2,200
36-40	2,400	2,600	3,000	51-60	1,600	1,800	2,200
41-45	2,200	2,600	2,800	>61	1,600	1,800	2,000
46-60	2,200	2,400	2,800				
61-75	2,000	2,200	2,600				
>76	2,000	2,200	2,400				

Figure.1.2. Energy requirement by gender, age and activity: (data from Trumbo *et al.,* 2002- U.S. Department of Health and Human Services and the U.S. Department of Agriculture, 2015- re-drawn and modified to avoid copyright). Sedentary – physical activity of independent living only
Moderately active- independent living + equivalent to walking 1.5-3 miles/day at 3-4 miles/hr
Active- independent living + 3 miles/day at 3-4 miles/hr

Studies which are based around daily recommended levels should therefore ~~perhaps~~ also take into account the age and activity of subjects. A study insisting on 2000 kcal for all sexes, ages and activity could have such subjects at negative energy balance and would likely struggle to show any effect of fructose (e.g. Stanhope *et al* 2008; Bantle *et al* 2000; Hallfrisch *et al* 1983). Conversely most subjects could be in positive energy balance if for example female, over 26 and sedentary. A key question in this field is whether fructose is only "harmful" when part of a diet which is in excess of daily energy recommendations. In which case it may not be fructose that is the problem, but merely excess energy. Or can fructose also be harmful when consumed as part of a weight maintenance diet that does not provide excess energy?

Pure fructose- is poorly absorbed without glucose

Many studies have involved the application of pure fructose, rather than a source of fructose and glucose which would be the norm *in vivo*. With respect to this it should be noted that fructose is poorly absorbed in the absence of glucose (Rumessen & Gudmand-hoyer, 1986, Truswell *et al*., 1988). E.g. in 10 subjects given a 50g dose of fructose in the absence of glucose, on average only 64% was absorbed, and just 80% of a 25g dose (which may be more physiologically relevant). In the presence of equimolar glucose, 100% of 50g fructose

was absorbed but only 77% with 12.5g glucose. As fructose is generally consumed with approximately equimolar glucose, its potential harm may therefore have been generally underestimated. However, if treating subjects with a source of fructose and glucose some or all of the effects may be due to the glucose which makes choosing the control treatment important too.

Appropriate controls-

The choice of control treatment will depend of course upon what the study is attempting to investigate. When treating with pure fructose, often sucrose or HFCS (which also contains fructose) is used as a control. Although the sugar control contains less fructose than the test fructose intervention, it will be more easily absorbed due to the presence of glucose (see above). In comparison, therefore pure fructose may only have a similar or non-statistically significant greater effect over the control. When using pure fructose as the intervention, glucose would ~~perhaps~~ be the relevant control if the aim is to determine whether fructose is more or less harmful than glucose, while starch would be more appropriate if the aim is to determine if fructose is more or less harmful than complex carbohydrates in the diet. When intervention is with a mixed fructose source (e.g. sucrose or HFCS) the control used in many studies is starch. Any effect seen by the treatment could be due to more readily available glucose, rather than fructose. If the aim of the study is to determine whether replacing a sugar for a complex carbohydrate is more harmful this is ~~perhaps~~ would be the relevant control. However, if wishing to examine whether fructose is the cause of any harmful effects measured, starch is ~~perhaps~~ less useful as a control. In this case glucose would again ~~perhaps~~ be the best control. For the purpose of this thesis, there is a need to know if there is any benefit in controlling the intake of fructose and so starch or glucose as a control would be most useful.

It seems that assessing the effect of any major nutrient in food science has a problem as to what the control should be. Just replacing the test nutrient with water will reduce calories and so therefore adding another important variable.

Industry and White Hat bias (WHB)

There have been numerous reviews and meta-analyses carried out on the subject of the health effects of fructose. However, the interpretation of the available evidence can be biased by subjective perception, as well as arbitrary selection of the data used to draw conclusions. Industry particularly has been accused of such bias.

Historical

Kearns *et al*. (2015) reviewed 350 internal Sugar Research Foundation (SRF) documents from 1959-1971 for relevance to heart disease research and policy (The SRF is the former name of the Sugar Association* and was founded by members of the U.S. sugar industry). Kearns *et al* concluded that the SRF developed a heart disease research program for the purpose of protecting industry interests, designed to influence policymakers evaluating the body of evidence on diet and heart disease. Despite the 2015 U.S. Dietary Guidelines Advisory Committee conclusion that evidence indicates that higher intake of added sugars is associated with increased risk of hypertension, stroke, elevated serum triglycerides and cardiovascular heart disease (CHD), the sugar industry continues to advocate that added sugars are not associated with CHD. As such some industry funded literature may at least historically have been biased.

[*The Sugar Association, headquartered in Washington, D.C., states that its mission "is to promote the consumption of sugar as part of a healthy diet and lifestyle through the use of sound science and research". It engages in public relations on behalf of its member companies, encouraging the use of sugar. Member companies consist of sugar producers in the U.S.]

Recent industry bias

Recently a key investigator in sugar and fructose research, Stanhope (2016) commented that a number of studies that have not shown adverse effects associated with fructose, or even sugar, were conducted by researchers funded by industry or who receive consultancy fees from industry linked to sugar consumption. Some of

the results of these studies conflicted with Stanhope's own findings (Stanhope *et al*., 2016), leading her to suggest that there had been an inappropriate use of controls, statistical models and compliance. Meta-analyses, it is suggested often selects studies that help give the desired conclusion. Choosing several studies for example where the control is sucrose which will also have metabolic effects, tend towards a null conclusion (as discussed above) (Stanhope *et al*., 2016).

Two other studies have suggested industry funding or association has influenced systematic reviews on sugar-sweetened-beverages and weight gain and obesity (Bes-Rastrollo *et al*., 2013; Massougbodji *et al*., 2014). Both reviews concluded that studies found to have conflicting interests were more likely to show no or lesser association between sugar and weight.

Stanhope (2016) suggests that it is in "missing" evidence that industry funded/associated reviews are able to find controversy. To some extent this stance may seem valid, particularly as many early studies were conducted using excessive levels of fructose unlikely to be consumed by the general public. However, latter studies are including physiologically relevant levels of fructose and cannot be so easily dismissed. Stanhope (2016) calls upon governments to fund the relevant studies needed (which will be necessarily large and expensive), in order to obtain conclusive proof of the harmful effects of sugar and fructose.

White hat balance

On the other hand, it should also be noted that the potential for white hat balance (WHB) (i.e. bias leading to distortion of information in the service of righteous ends), with respect to the field of obesity and sugar consumption has also been noted and investigated (Cope and Allison, 2010). Sufficient evidence was found of apparent WHB to mislead readers. It was conjectured that WHB may be due to "feelings of righteous zeal, indignation towards certain aspects of industry or other factors". The authors of this publication declared no conflicting interests.

High Corn Fructose Corn Syrup (HFCS) *vs*. Sucrose

On the controversy over whether fructose is more harmful than glucose we should first consider how this issue has arisen. Many studies have focussed on high fructose corn syrup (HFCS). Health concerns have been focused mostly on HFCS rather than sucrose, as it has or has been perceived to: a) contain higher levels of fructose; b) have fructose and glucose in the free form (i.e. not requiring digestion by sucrase); and c) be consumed in higher amounts than sucrose in the USA (well known for its widespread problems of obesity). However, although HFCS is the primary source of added fructose in the U.S., studies generally show that HFCS globally does not contribute more than sucrose to the increase in fructose consumption, even in the many developed nations where it coincides with increased prevalence of obesity (White, 2008).

In general, meta-analyses and randomised controlled trials (RCTs) conclude that HFCS (55% fructose) and sucrose do not appreciably differ in their physiological effects or outcomes related to: fasting (Bravo *et al* 2013); post-meal blood triglycerides (Le *et al* 2012; Parks *et al* 2008; Stanhope *et al* 2008); fasting lipoprotein cholesterol (Bravo *et al*., 2013*); post-meal de-novo lipogenesis (Parks *et al*., 2008); appetite or energy intake (Akhaven & Anderson, 2007; Akhavan, 2011; Anderson *et al*, 200 38; Bravo *et al*, 2013); Lowndes *et al*; 2012; Monsivais *et al*, 2007; Soenen & Westerterp-Plantenga, 2007); body weight and body fat (Bravo *et al* 2013); ectopic fat deposition ((Bravo *et al* 2013); post-meal blood glucose and insulin (Stanhope *et al*., 2008). This is ~~perhaps~~ not too surprising as HFCS, as used in most dietary circumstances, and sucrose, in fact, have similar amounts of fructose (55% and 45% respectively). From this it can ~~perhaps~~ be suggested that the digestion of sucrose to fructose and glucose is very efficient and not rate limiting with respect to these health concerns. In fact, Rippe *et al*., 2017 have concluded as much showing that the kinetics of fructose and glucose absorption from the gut from sucrose and HFCS are similar. Furthermore, Tappy (2018) surmises that "...disaccharide digestion is a rapid and highly efficient process and is not considered rate-limiting for sugar absorption".

Summary

In reviewing the literature on fructose and its possible negative impacts on health it is ~~perhaps~~ suggested here that it is important to consider the issues discussed in this section (above). For example: were the studies performed using physiologically relevant levels of fructose; were the subjects in positive, neutral or negative energy balance; was fructose included with glucose to aid absorption from the gut and what control intervention was used.

1.1.2 Effects of fructose on de novo lipogenesis

As discussed, a main hypothesis for why fructose may be harmful to health is its potential to be rapidly metabolised to lipid or stimulated lipid syntheis. Studies that have evaluated *de novo* lipogenesis show increases in newly synthesised fatty acids after fructose consumption in comparison with glucose (Chong *et al*., 2007; Parks *et al*., 2008; Hudgins *et al*., 2011), and when consumed with glucose this was true for as little as a 35g dose of fructose (Hudgins *et al*., 2011). The addition of an equal amount of glucose to fructose further increases the lipogenic effect of fructose and is dose dependant (Hudgins *et al*., 2011).

It would seem though (see Figure 1.3), that the contribution of *de novo* lipogenesis to any post-meal hypertriglyceridemia directly from fructose may be relatively small (<1%) based on isotope tracer studies in humans (Sun & Empie, 2012, Tappy. 2018). Chong *et al*., 2007 reported that they found only an estimated 0.4% of labelled circulating VLDL-triglyceride (TAG) 240min after a labelled fructose meal. However, in these studies subjects were fasted and under such circumstances: a) fructose may largely be used for gluconeogenesis (approximately 50%- Delarue, 1993, see Figure 1.3) and b) lactate, another major metabolite of fructose (Dietze *et al*., 1976) may subsequently and only belatedly be converted to lipids in the liver and/or other tissues. Non-fasted studies though, in a gut Caco2 cell-line, rather than liver, also showed DNL from fructose and glucose to be <1% (Steenson *et al*., 2022). And non-fasted studies in five over-weight men showed that although plasma TAG and VLDL-TAG were greater with high fructose (30% energy) *vs*. low fructose (4% energy), the first measurements ever provided of the rate of intestinal DNL in

humans showed fructose consumption had no effect on the rate of intestinal or hepatic DNL (Steenson *et al*., 2020). They also saw lower chylomicron-TAG production rate and chylomicron-TAG fractional catabolic rate with high-fructose consumption which they suggest shows lower clearance of CM, rather than elevated production, may contribute to elevated plasma TAG, possibly due to lower insulin-mediated stimulation of lipoprotein lipase. Chong *et al*., 2007 however, refer to a study by Scwarz *et al*., 1993 in which 30% of circulating TAG-palmitate was from hepatic DNL after fructose ingestion. This study was reported in a meeting extract and can no longer be found. They suggest that the dose of fructose, administration and DNL quantification methodology (12 hr ^{13}C-acetate) may account for the difference.

Chong *et al*., 2007 also found that *de novo* isotope labelled TAG-glycerol (i.e formed from the labelled fructose) made up 38% of the circulating VLDL-TAG 240min after fructose. This they suggest could be due to the activation of hepatic DNL by altering the partitioning of fatty acids by malonyl-CoA, which, as intermediate of the DNL pathway, decreases fatty acid oxidation by inhibiting carrnitine-palmitoyl transferase-1 transportation of long-chain fatty acids into mitochondria. This would lead instead to fatty acids esterification (see 1.2. Potential mechanisms to explain the negative impact of frucrose on health, 1. 2.2 Molecular pathway stimulation- *de novo* lipogenesis and 1.2.3 Energy source shifting- *de novo* lipogenesis). Indeed, in their study net fat oxidation was decreased. Steenson *et al*., 2022 also saw substantial (70%) of *de novo* TAG-glycerol synthesis from glucose or fructose in non-fasted Caco2 cells.

Figure 1.3: Summary of the major metabolic fates of dietary fructose based on the data obtained from the reviewed isotope tracer studies

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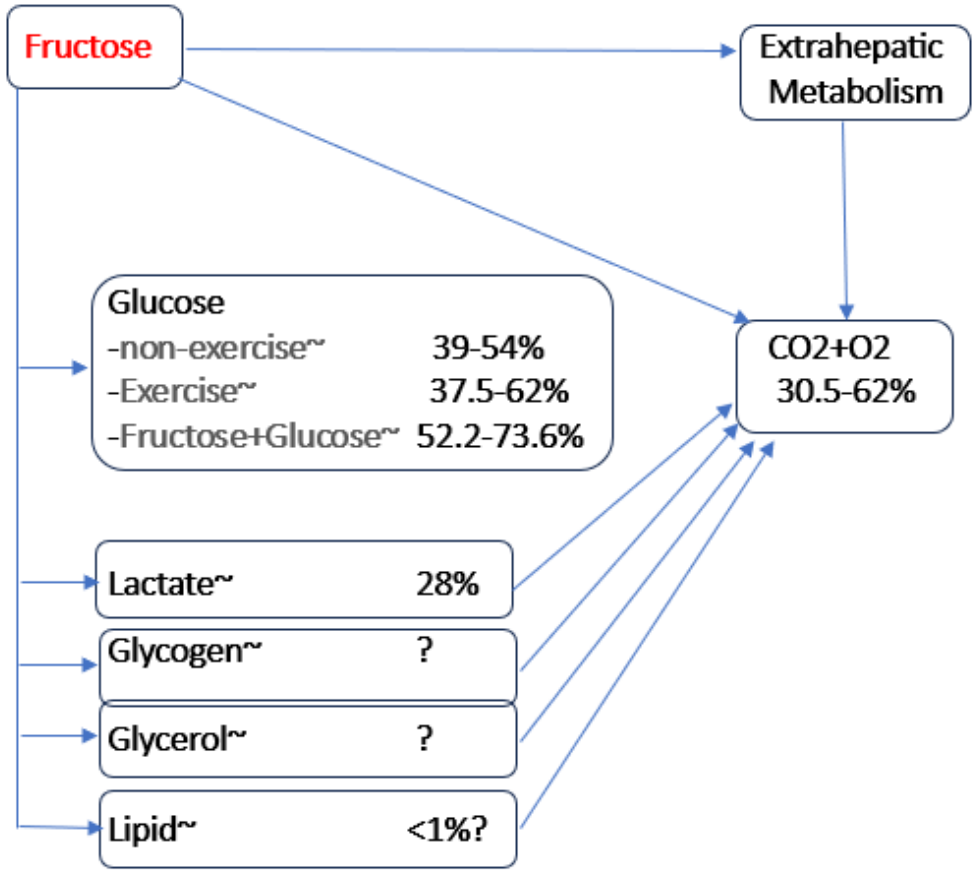

Figure 1.3: Summary of the major metabolic fates of dietary fructose based on the data obtained from the reviewed isotope tracer studies. Metabolic fate of dietary fructose carbons. The data are obtained within study periods less than or equal to 6 hours. After 50–150g fructose ingestion. The percent data are the estimated amounts of ingested fructose doses via the pathway (Sun & Empie, 2012).
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Alternatively, the increased post-meal triglyceride concentrations following fructose consumption (see below 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides) could rather be due to the lower insulin secretion relative to glucose, leading to less activation of adipose tissue lipoprotein lipase, which would result in impaired triglyceride clearance (Chong *et al*., 2007) (see 1.2. Potential Mechanisms to explain the negative impact of fructose on health, 1.2.4 Decreased lipid clearance- contribution to elevated plasma triglyceride).

Such low levels DNL could also be due to such being studies being short term. As discussed, prolonged fructose exposure is thought to also stimulate *de novo* lipogenesis (DNL) at the molecular level (Bizeau & Pagliassotti, 2005). I.E: Pre-sensitisation to fructose stimulates carbohydrate response element binding protein (ChREBP) which activates the enzymes responsible for DNL; Stimulation of peroxisome proliferator activated receptor-γ-coactivator-1β (PGC1β), a transcriptional co-activator for sterol regulatory element binding protein 1c (SREBP1c) may further increase the activity of the enzymes involved in DNL; Elevated malonyl CoA levels increase carbon flux into the lipogenesis pathway leading to inhibition of β oxidation, contributing to increased serum triglycerides or intrahepatic lipid accumulation (see 1.2. Potential mechanisms to explain the negative impact of fructose on health, 1.2.2 Molecular pathway stimulation- *de novo* lipogenesis). As such, effects on DNL are more likely to be seen in longer-term studies.

1.1.3 Effects of fructose on blood lipids

As discussed above, due to the relatively unregulated metabolism of fructose there is the potential for excess metabolites to be utilised for lipid synthesis. The potential for an increase in lipogenesis by fructose obviously gives concerns over possible effects on blood lipids. Epidemiological and interventional studies indicating the possible adverse effects of fructose on blood lipid profiles have been the basis for recommendations discouraging high fructose intakes >10-17% of energy, or >60 g/day (Canadian (Dworatzek *et al*., 2013) and American (Bantle *et al*., 2008) Diabetes Associations, European Association for the Study of Diabetes (Mann *et al*., 2013)).

Measurement of fasted *vs.* post-prandial triglycerides

Generally, triglycerides are measured in the fasted state for two reasons: a) substantial increases in triglycerides after ingestion of food leads to more variability in triglyceride measurements compared to those made in the fasting state; b) historically, before the availability of direct assays for LDL cholesterol, estimations

were calculated using the Friedewald equation, which requires the use of the fasting triglyceride concentration (Ridkera, 2008). The recommendations to measure triglycerides in the fasting state rather than postprandially did not therefore emerge from clinical evidence that fasting concentrations were superior to non-fasting concentrations for the detection of cardiovascular risk.

Overall epidemiological studies do indicate that fasting triglycerides predict risk of vascular disease. Controversy exists, however, regarding the clinical usefulness of fasting triglycerides as an independent predictor of risk, because adjustment for other covariates, in particular high density lipoprotein cholesterol (HDL-C), markedly decreases both the magnitude and significance of observed epidemiologic effects (Sarwar *et al*., 2007).

On the other hand, atherosclerosis has long been hypothesized to be a disorder influenced by postprandial effects (Ridkera, 2008). In a study in which 26,509 initially healthy American women were followed over an 11-year period both fasting and non-fasting triglycerides were associated with future cardiovascular risk (Bansal *et al*. 2007). Among the fasting (but not non-fasting) participants, adjustment for total cholesterol and HDL-cholesterol markedly weakened this association. In a second study of 7,587 women and 6,394 men in Copenhagen with 26 years of follow-up non-fasting triglycerides were also found to significantly predict future vascular events in both sexes (Nordestgaard *et al*., 2007). In this review the effect of fructose on both fasted and non-fasted levels of triglycerides and cholesterols has therefore been considered.

Effects of fructose on fasting triglycerides

Fructose consumed in human studies at doses below 60-100 g/day have not been shown to significantly increase fasting triglyceride concentrations (Sievenpiper *et al*., 2009; Livesey & Taylor, 2008; Stanhope *et al*., 2015). At doses higher than 100 g/day, a dose-response effect has been observed, with increasing doses leading to increases in triglyceride concentrations (Livesey & Taylor, 2008). In subjects with type 2 diabetes a threshold of ≥60 g/day is seen (Sievenpiper *et al*., 2009).

The triglyceride raising effect seems to be more pronounced when fructose replaces starch than when it replaces sucrose (Livesey & Taylor, 2008). This could possibly be because fructose absorption is ~~boosted~~ increased by the presence of glucose, particularly in equimolar amount as in sucrose. A sucrose control treatment in such studies, therefore, although containing less fructose, may provide a similar level of absorbed fructose, or at least weaken the statistics. An effect for fructose *vs*. starch could also be due to the slower digestion and therefore lower availability of monosaccharides for the latter.

However, Johnston *et al* (2013) in a study at 125 g/day saw no increase in fasting triglycerides for fructose or glucose over 2 weeks at 25% of energy requirement. The same study though, after a 6-week washout period and hyper-caloric conditions, showed similar increases in body weight and fasting TAGs for both fructose and glucose. Thus, ~~perhaps~~ suggesting that hypercaloric studies are more relevant.

The fasting triglyceride raising effect of fructose may however be transient (<4 weeks) (Sievenpiper *et al*., 2009), as a longer-term study did not show an effect (Dolan *et al*., 2009). Whether this reflects metabolic adaptation or decreased compliance in the long term remains to be clarified.

Effects on post-prandial triglycerides

Studies with high doses of fructose (≥125 g/day) showed significant increases in post-meal triglycerides in adult volunteers, regardless of whether they were healthy (Stanhope *et al*., 2009), overweight/obese (Teff *et al*., 2004; Stanhope *et al*., 2011), insulin resistant or insulin sensitive (Teff *et al*., 2004). A post-meal triglyceride raising effect was also reported in children (healthy or with non-alcoholic fatty liver disease) who consumed 33% of their daily energy intake (approximately 150 g/d) as fructose compared to glucose (Jin *et al*., 20).

With doses of fructose that are more likely to be consumed by a majority of the population, meta-analyses have found a dose threshold for fructose increasing postprandial triglyceride at ≥50 g/day (Livesey & Taylor, 2008) particularly in sedentary subjects (Tappy & Rosset, 2019). Chong *et al*, 2007 in a single-blinded study showed plasma triglyceride levels increase by approximately 66%, relative to

glucose after 360 minutes (from a baseline of approximately 1.2 mM, to which glucose treatment had returned). Fructose or glucose was delivered via an approximately 500 kcal chocolate drink (0.75 g/kg body weight sugar and 0.5g oil/kg body weight) in overnight fasted subjects. In non-fasted subjects an even greater effect would possibly be seen as fructose is mostly used to store glycogen when low e.g. post exercise and fasting. In a study by Parks *et al*., 2008 subjects consumed carbohydrate boluses of sugar (85 g total sugar, i.e. 0, 42,5 or 63.7 g fructose), followed by a standardized lunch 4 hrs later. Subjects completed a control test of glucose (100:0- glucose: fructose by weight) and a mixture of 50:50 and one of 25:75). Following the morning boluses, triglycerides remained constant for all treatments but appeared to drop for the 100% glucose treatment non-significantly just before lunch (Figure 1.4). Triglycerides rise for all treatments post-lunch and by 4 hrs post-lunch, serum triglycerides were 11-29% greater, and triglyceride-rich lipoprotein-triglyceride concentrations 76-200% greater than 100% glucose after 50:50 and 25:75 ratios were consumed ($P<0.05$), with the high fructose treatment showing the greater effect. By 6 hrs post lunch the difference between glucose control and fructose treatments had widened further as the former begins to drop.

Figure 1.4: Concentrations of serum triglycerides after consumption of CHO solutions and a lunch.

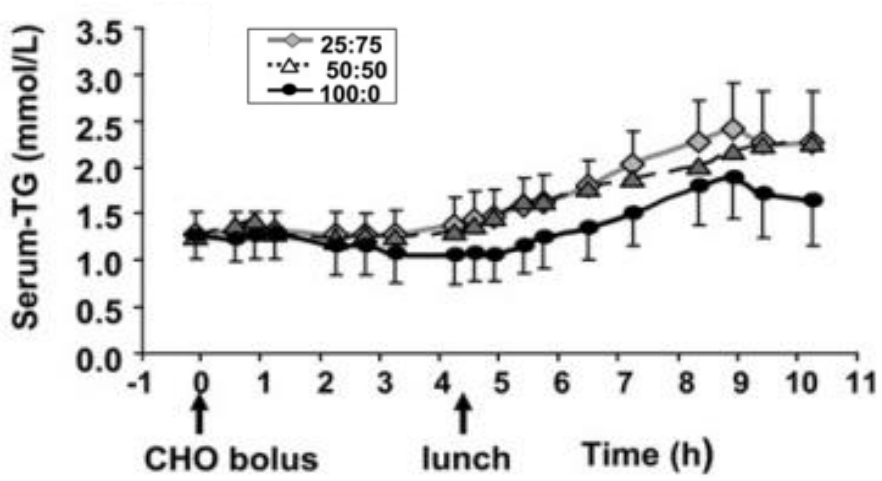

Figure 1.4: Concentrations of serum triglycerides after consumption of sugar solutions and a lunch. Values are means 6 SEM, n=6. Time 0 denotes when the sugar bolus was fed. The solid food lunch was fed at 4.33 h (denoted by arrow). Adapted from Parks *et al*., 2008). [author emailed and permission granted]

A study by Stanhope *et al*. (2015) conducted a parallel-arm, double-blinded diet intervention, study over a number of meals, with physiological levels of fructose plus

glucose, compared with complex carbohydrate. Subjects consuming beverages at 0, 10, 17.5 and 25% energy requirement from HFCS (approximately 0, 25, 44, 62.5g fructose) in place of isocaloric complex carbohydrate, produced a significant effect for post-prandial (PP) triglycerides (0%: 0+/-4; 10%: 22+/- 8; 17.5%: 25+/-5; 25%: 37+/-5 mg/dL, mean delta +/- SE, P<0.0001 effect of HFCS-dose). However, as fructose+glucose mixes were compared to starch, this does not inform as to the potential harmfulness of fructose, as such could just as easily be caused by the glucose being more readily available.

The importance of feeding over more than one meal and monitoring triglycerides over 24 hrs is apparent in the Parks *et al*, 2008 study (Figure 1.4). An increase in triglycerides is not seen until after the second meal of the day. This is consistent with the concept that only excess fructose is metabolised to lipid when energy reserves become sufficient (see 1.2. Potential Mechanisms to explain the negative impact of fructose on health, 1.2.1 Lack of regulation- *de novo* lipogenesis). Many studies may miss this and conclude that fructose does not have an effect on triglycerides. That the impact of sugars on the liver post fasting builds throughout the day is a key part in the design of studies here on attempting to show benefits of flattening the fructose spike.

Overall, from the available data and meta-analyses it can ~~perhaps~~ be suggested that the consumption of fructose at doses greater than or equal to 50 g/day can increase post-meal triglyceride concentrations, and in comparison even with glucose. This is probably the most convincing data for a potentially harmful effect from fructose and has therefore emerged as a key focus of this thesis.

Effects on fasting and post-prandial lipoprotein cholesterol

Triglyceride and cholesterol formed in the liver from dietary fat and *de novo* lipogenesis are secreted by the liver in the form of very-low-density lipoprotein (VLDL). These may combine with other sources (e.g. adipose tissue) of lipid in the blood to form other lipoproteins (e.g. low density and high density lipoprotein) (Cox *et al*., 1990.). In general, lipoprotein cholesterol will likely be influenced by *de novo* lipogenesis in the liver and therefore may originate from fructose or its impact on

other intermediary metabolites (see 1.2. Potential mechanisms to explain the negative impact of fructose on health, 1.2.3 Energy source shifting- de novo).

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High fructose doses (75-150 g/day), compared with glucose or starch, may increase fasting levels of total and LDL-cholesterol both in healthy (Bantle *et al*., 2000; Silbernagel *et al*., 2012) and insulin resistant individuals (Reiser *et al*., 1989) when consumed for up to 4-5 weeks. In the Bantle *et al*., 2000 study 24 healthy adult volunteers (12 men and 12 women; 6 of each sex were aged <40 yrs and 6 of each sex were aged ≥40 yrs), showed higher levels of LDL cholesterol (2.69 mmol/L) after 28 days of high fructose (14% of total energy from added fructose, total fructose=85 g/2000 kcal/day) compared to glucose (2.49 mmol/L) ($p<0.0001$). As for the effect on LDL or HDL-cholesterol concentrations in type I and type II diabetic subjects only a tendency towards an LDL-cholesterol raising effect has so far been seen (+0.25 mmol/L) in type I diabetes in studies ranging from 30-160g fructose *vs*. glucose, sucrose or mixed carbohydrates (Sievenpiper *et al*., 2009).

Fructose in more realistic every-day doses (60-80 g/day) may not increase cholesterol concentrations in healthy subjects when compared with sucrose (Bossetti *et al*., 1984). Although it is not clear whether this study was randomised, and the intervention duration (2 weeks) was relatively short to assess blood lipid effects. Stanhope *et al* (2015) have seen a significant increase in fasting and post-meal non-HDL cholesterol and post-meal LDL-cholesterol at the 17.5% HFCS dose (approximately 48g fructose/39g glucose) compared to complex carbohydrate (white bread, white rice and regular pasta). Although as discussed, a comparison between a fructose+glucose mix (i.e. HFCS) and a complex carbohydrate does not necessarily indicate that fructose is to blame. More readily available glucose of HFCS may possibly be the cause. The outcome of one non-blinded study in type 2 diabetic subjects suggested that a dose of 30 g/day fructose consumed for 2 months may have minimal impact on fasting total and HDL-cholesterol concentrations compared with starch (Grigoresco *et al*., 1988). However, a study comprising both hyper-insulinemic and insulin-sensitive men showed significant increases in total and LDL-cholesterol *vs*. starch after the intake of fructose at 7.5% of total energy (this

would equate in this study to approximately 50 g/d) for 2 weeks (Hallfrisch *et al*., 1983). A total of 2700 kcals were provided each day, which is high compared with most other studies which typically provide 2000 kcals a day. However, as discussed above, this is the normal energy needs for moderately men aged 18-45 yrs as shown in figure 1.2 (U.S. Department of Health and Human Services and the U.S. Department of Agriculture, 2015), which is relevant to this study as the average age of participants was 40 yrs.

Overall, there is so far some evidence of an increase in fasting LDL-cholesterol at moderate-high levels of fructose (>75 g/day) *vs*. glucose and starch, and also at moderate fructose levels when given with glucose in comparison to starch.

Summary- Effects of fructose on blood lipids

With doses of fructose that are more likely to be consumed by a majority of the population, meta-analyses have found a dose threshold for fructose increasing postprandial triglyceride at ≥50 g/day (Livesey & Taylor, 2008; (Tappy & Rosset, 2019). Greater differences were seen after a second meal suggesting fructose is more likely to induce lipid synthesis when energy reserves are satisfied rather than after fasting. It might also be speculated that a large bolus of fructose could raise postprandial triglyceride, even though over the course of a day excessive calories were not consumed.

1.1.4 Effects of fructose on obesity and body fat

Potential for an increase in lipogenesis by fructose obviously gives concerns over effects on obesity and body weight. Epidemiological data focused on High Fructose Corn Syrup (HFCS) have sparked widespread belief that intake of fructose, beyond its natural occurrence in fruits and grain, plays a role in the epidemic of obesity and associated co-morbidities (White, 2008). In the following section the effect of fructose consumption in intervention studies on body: weight; total fat and fat distribution, including visceral, muscle and liver.

Effects of fructose on body weight

There have been a number of systematic reviews and meta-analyses that have investigated the effects of fructose *vs*. other carbohydrates on body weight in humans. Most of these reviews included not only randomised controlled trials (RCT), but also studies not reported to be randomized, i.e. outcomes which may not be fully reliable. Nevertheless, randomised and non-randomised studies led to similar outcomes (Sivenpiper *et al*., 2012a). Overall, none of the meta-analyses showed a differential effect of fructose *vs*. other carbohydrates at doses ≤100 g/day.

Morenga *et al*., in their review of meta-analysis data also concluded that isoenergetic substitution of fructose for other carbohydrates was not associated with weight gain. However, where provided *ad libitum*, increased weight gain could be seen, but as a result of excess rather than any particular attribute of fructose (Morenga *et al*., 2012)

Dolan *et al*., reviewing “normal levels” of fructose intake (approximately 50 g/day) similarly commented that for replacing sugars with other carbohydrates there was not sufficient evidence this would lead to a decrease in weight gain (Dolan *et al*., 2010).

Effects of fructose on total body fat

Only a few studies have investigated the impact of fructose consumption on body fat (Stanhope *et al*., 2009; Ngo *et al*., 2010; Silbernagel *et al*., 2011). All studies were overfeeding studies where healthy volunteers consumed fructose in the form of liquid beverages providing extra calories on top of usual energy needs. In none of these studies did fructose enhance total body fat more than the control sugar (glucose), even when fructose was consumed at very high doses (150-200 g/day) for up to 10 weeks. The available evidence suggests that total amount of body fat is not affected differently by fructose as compared to glucose in humans. This of course does not mean that excess fructose doesn’t increase body fat *per se*, just that it does not seem to do so any more than glucose.

Effects of fructose on body fat distribution

Based on limited evidence it seems fructose may influence body fat distribution in a different way to glucose, although only at very high doses (150 g/day). One study showed minimal changes in visceral fat after 4 weeks of fructose consumption (Sibernagel *et al*., 2011). In another study significant increases in total abdominal fat (+8.6 ± 3.0% *vs*. +4.8 ± 2.1% for glucose) and intra-abdominal (visceral) fat (+14.0 ± 5.5% *vs*. +3.2 ± 4.4% for glucose) were observed with fructose when compared to glucose (Stanhope *et al*., 2009). The longer duration of fructose consumption (10 weeks) in the second study may have allowed for the development of metabolic changes that would not be observed in short-term feeding studies. Overall, the relatively limited evidence available suggests that in the long term, fructose at very high levels may favour more intra-abdominal/visceral fat deposition than glucose. No differential effect of fructose *vs*. glucose was observed on extra-abdominal fat (Stanhope *et al*., 2009) and abdominal sub-cutaneous fat (Silbernagel *et al*., 2011).

Effects fructose on ectopic fat

Intramuscular

A single study that assessed the effects of fructose on ectopic fat accumulation in skeletal muscle (Silbernagel *et al*., 2011) showed a non-significant tendency towards more intramuscular lipid deposition with very high-dose (150 g/day) fructose than glucose (Fructose: +0.97 ± 0.61 units *vs*. Glucose: -0.26 ± 0.54 units, N=20). Despite its limitations (lack of power to detect statistically significant effects and investigators not blinded), this study suggests that a very high fructose diet may increase intramuscular fat deposition compared to glucose. According to the available literature, at the point of conducting this review, there is currently no data on the effect on intramuscular fat of more realistic intakes of fructose (below 150 g/day).

Liver fat- steatosis

Numerous studies have described an increase in newly synthesized fatty acids (*de novo* lipogenesis) after consumption of fructose in comparison with glucose as discussed. Such an increase in *de novo* lipogenesis might be expected to increase

liver fat content, however, in their review Vos & Lavine (2013) concluded that there is currently no direct evidence for increased liver fat deposition with fructose *vs*. glucose intake. Tappy, 2021 in a review concluded that some studies have shown an effect of fructose and glucose on lipid accumulation in the liver at >150 g/day, but that in each case these were as additional high energy in comparison to a weight maintenance diet. One study in an isocaloric control comparison of >150 g/day fructose or glucose saw no increase in intrahepatic fat (Silbernagel *et al*., 2012). Any effect therefore, on lipid accumulation in liver by fructose or glucose ~~may be and perhaps~~ seems likely to be due to excess energy of the diet overall rather than any excess of fructose or glucose overwhelming the liver during a single dose or meal.

Summary- Effects of fructose on obesity and body fat

Meta-analyses have not shown a differential effect of fructose *vs*. other carbohydrates at doses ≤100 g/day for body weight or total body fat. Overall, the relatively limited evidence available suggests that in the long term, fructose at very high levels may favour more intra-abdominal/visceral fat deposition than glucose. An effect on ectopic fat has only been seen at doses >150 g/day.

1.1.5 Effects of fructose on appetite and energy balance

It has been hypothesised that any effect fructose has on weight gain or obesity might be because it does not stimulate insulin secretion or leptin, therefore increasing appetite, energy intake and body weight (e.g. Angelopoulos *et al*., 2009; Harvey 2005). Leptin is made by adipose cells, and along with insulin, helps to regulate energy balance by inhibiting hunger, through action on receptors in the hypothalamus that regulate appetite (Brennan *et al*., 2006). A drop in leptin levels increases appetite. Insulin also initiates storage of lipid and thus potentially weight gain, so a reduction, it could ~~perhaps~~ be hypothesised, might actually be expected to reduce weight gain. Increased appetite, however, would likely increase the intake of carbohydrates and other nutrients that do increase insulin. Fructose may therefore increase insulin secretion indirectly.

As discussed, the evidence that fructose increases body weight at normal-high doses of ≤100 g/day, in an isocaloric setting, is not convincing (Sievenpiper *et al*., 2012) (see 1.1.4 Effects of fructose on Obesity). Therefore, the evidence for an effect of fructose on appetite, energy intake and leptin are covered here only briefly. Other gut hormones potentially affected by fructose are also briefly discussed.

Numerous trials have compared treatments containing predominantly glucose *vs*. fructose with respect to appetite and energy intake. The majority of these reports show no differences (Anderson *et al*., 2002; Bowen *et al*., 2007; Guss *et al*., 1994; Page *et al*., 2013; Rayner *et al*., 2000; Teff *et al*., 2004), and where differences were observed for effects on energy intake, these were equally likely to suggest lower energy intake following fructose intake than following glucose consumption (Rodin *et al*., 1990; Rodin *et al*., 1991; Spitzer *et al*., 1987).

Gut Hormones

Glucose and fructose generate well-known differences in blood glucose and insulin responses. However, data on measures of gastrointestinal physiology and appetite-related hormones are less well defined (see below and Table 1.2).

Leptin (reduction increases hunger)

Page *et al*. 2013 showed no difference between a fructose and glucose 75g dose; whilst Teff *et al*., 2004 at 16.5% total energy (equivalent to approximately 75-100 g/d) showed a reduction in leptin for fructose *vs*. glucose despite having no effect on appetite (area under the curve for leptin during the first 12 hrs (-33 ± 7%; $P < 0.005$) and the entire 24 hrs (-21 ± 8%; $P < 0.02$)).

Ghrelin (promotes hunger)

Ghrelin known as the "hunger hormone", is a peptide hormone produced by ghrelinergic cells in the gastrointestinal tract (Sakata and Sakai, 2010; Inui *et al*., 2004). The receptor for ghrelin, is found on the same cells in the brain as the receptor for leptin, but has opposite effects (Perello *et al*., 2012). When the stomach is empty, ghrelin is secreted. When the stomach is stretched, secretion stops. This is an oversimplification as in reality it is more complicated than simple response to stomach stretching).

Teff *et al*., 2004 at 100 g/d saw less ghrelin suppression for fructose *vs*. glucose, which implies decreased hunger and appetite for fructose, whilst Bowen *et al*., (approximately 50 g/day) and Page *et al*., 2013 saw no difference.

Glucagon-like peptide-1 (GLP-1) (associated with satiety)

GLP-1 secretion by ileal L cells is dependent on the presence of nutrients in the lumen of the small intestine. It is a potent anti-hyperglycemic hormone, inducing glucose-dependent stimulation of insulin secretion while suppressing glucagon secretion (Presswala *et al*., 2015). GLP-1 is therefore associated with satiety.

Teff *et al*. 2004 showed greater levels for fructose *vs.* glucose at 100 g/day, suggesting reduced appetite, whilst Page *et al*., 2013 at a 75 g dose saw lower levels for fructose suggesting increased appetite. Reynor *et al* (45 g infusion at 2 ml/ minutes over 90 minutes) and Bowen *et al*., 2007 saw no change.

Peptide YY (PYY) (reduces appetite)

PYY is secreted by the neuroendocrine cells in the ileum and colon in response to a meal, and has been shown to reduce appetite. It inhibits gastric motility and increases water and electrolyte absorption in the colon. PYY works by slowing the gastric emptying; hence, it increases efficiency of digestion and nutrient absorption after a meal (Liu *et al*., 1996). Page *et al*. 2013 showed levels to be higher with fructose than glucose, suggesting reduced appetite.

GIP (satiety associated)

Gastric inhibitory polypeptide (GIP) is synthesized by K cells, which are found in the mucosa of the duodenum and the jejunum of the gastrointestinal tract. It is believed that the function of GIP is to induce insulin secretion (Thorens 1995).

Rayner *et al*., 2000 saw intraduodenal glucose increased GIP ($P < 0.005$) more than fructose, suggesting increased appetite for fructose, whilst Teff *et al* 2004 showed no difference.

Table 1.2: Fructose vs. Glucose: Effect on gut hormones and the predicted effect on appetite

Hormone- (and effect)	Sugar dose (fructose *vs.* glucose)	No of subjects	Duration	Effect %	Ref	Predicted effect on appetite
Leptin -(reduction increases appetite)	75g	Twenty healthy adult	60min post drink	None	Page *et al.*, 2013	None
	100g/d	12 normal-weight women	12 hrs 24 hrs	(AUC) -33 P < 0.005) -21 (P < 0.02 Compared to glucose	Teff *et al.*, 2004	Increase
Ghrelin -(hunger)	100g/d	12 normal-weight women	23 hrs(1-2 hrs post meal):	Decrease from fasting baseline levels- was 50, 63 and 45% less after breakfast, lunch, and dinner, respectively, compared to glucose. (P<0.05 overall)	Teff *et al.*, 2004	Decrease
	75g	Twenty healthy adult	60min post drink	None	Page *et al.*, 2013	None
	50g	N=28 non-smoking M 20-65, BMI >25 not restrained eaters	2 hrs	None	Bowen *et al.*, 2007	None
GLP-1 -(increase Insulin (satiety))	100g/d	12 normal-weight women	23 hrs	AUC 145% increase compared to glucose P<0.01	Teff *et al.*, 2004	Decrease
	75g	Twenty healthy adult	60min post drink	AUC 26% decreased compared to glucose(p<0.01)	Page *et al*	Increase
	50g	N=28 non-smoking M 20-65, BMI >25 not restrained eaters	2 hrs	None	Bowen *et al.*, 2007	None
	80g Intraduodenal	Ten healthy volunteers	90min	None	Raynor *et al.*, 2007	None
PYY (reduce appetite)	75g	Twenty healthy adult	60min post drink	AUC 35% increase *vs.* glucose (p<0.001)	Page *et al.*, 2013	Decrease
GIP -(increase Insulin	80g Intraduodenal	Ten healthy volunteers	90min	100% increase with glucose compared to	Raynor *et al.*, 2007	Increase

(satiety))				fructose ($P <$ 0.005		
	100g/d	12 normal-weight women	12h entire 24h	None	Teff *et al.*, 2004	None

Summary- Effects of fructose on appetite and energy balance

Taken as a whole the scientific literature evidence so far offers no robust and consistent directional effects of fructose that would lend mechanistic support for any suggested influence on appetite, energy intake or gut hormones at physiological doses (≥50 g/day, see above) under isocaloric conditions. It is not surprising therefore that little or no effect on body weight has been reported for fructose (see above- Effects of fructose on body weight).

1.1.6 Effects of fructose on cardiovascular disease

Cardiovascular diseases are a leading cause of disease globally generally involving the heart or blood vessels. Although there are many contributing factors e.g. diabetes, cholesterol, triglycerides, these are covered in other sections. In this section vascular function and blood pressure are focussed on as ~~perhaps~~ the main cardiometabolic indicators.

Effects of fructose on blood pressure

It has been hypothesised that the increased plasma uric acid (see 1.2. Potential Mechanisms to explain the negative impact of fructose on health, 1.2.6 ATP depletion and uric acid production Oxidative stress and Inflammation), concentrations following the intake of high-dose fructose may raise blood pressure (Perez-Pozo *et al*., 2010). Single intakes of moderate fructose doses (50-60g) (average western consumption is about 50 g/day) have indeed shown raised systolic and diastolic blood pressure in the couple of hours following ingestion (Brown *et al*., 2008; Visvanathan *et al*., 2005).

In contrast, sustained intake (3-4 weeks) of moderate to high fructose doses (40-80 g/day) did not increase blood pressure compared with glucose (Silbernagel *et al*.,

2011), complex carbohydrates (Koivisto & Yki-Järvinen, 1993) and glucose or sucrose (Aeberli *et al*., 2011). In fact, a meta-analysis of 11 studies in non-diabetic subjects with a median intake of 78.5 g/day (range= 53-182 g/day) for 7 days or more (median=4 weeks) showed a significant decrease in diastolic blood pressure of 1.5 mmHg (normal levels are approximately 90 mmHg) relative to starch or glucose (Le *et al*., 2012).

The evidence in pre-diabetic and diabetic subjects is more limited but suggests overall no meaningful effect of fructose on diastolic blood pressure in these subjects (Le *et al*., 2012; Koivisto & Yki-Järvinen, 1993).

Overall, the available evidence suggests that chronic intake of fructose has no sustained effect on blood pressure. However, it may be that both fructose and glucose impact blood pressure. Few studies had a control for sugar to assess this. Even if fructose is no worse than glucose, it still may be of considerable concern compared to starch or other macronutrients.

Effects of fructose on vascular function

Fructose has been hypothesized to have both beneficial and detrimental effects on vascular function. On the one hand, fructose could be preferable to glucose because dietary fructose causes minimal increases in plasma glucose. Post-prandial rises in blood glucose are known to suppress flow-mediated dilation (Kawano *et al*., 1999; Title *et al*., 2000), a measure of vascular endothelial function. On the other hand, the increased plasma uric acid concentrations that follow high-dose fructose consumption have been suggested to be detrimental for endothelial function (Bidwell *et al*., 2010).

Two randomised control trials on this subject suggest that a single moderate dose of fructose (55-75 g/day) has no acute detrimental effect on vascular function in healthy, young individuals, when consumed alone (Mah *et al*., 2011) or in combination with glucose (Bidwell *et al*., 2010).

As discussed above, fructose has been shown to have a significant effect on blood lipids, and there is a considerable association between triglycerides and cardiovascular diseases (Nordestgaard & Varbo, 2014). It may be that longer-term

studies are needed to demonstrate an effect of fructose on cardiovascular health.

1.1.7 Effects of fructose on blood glucose and insulin

Type 2 diabetes is characterized by chronic high blood glucose levels, due to cells becoming less sensitive to insulin, and as the disease progresses, a lack of insulin may also develop. Serious long-term complications include cardiovascular disease, kidney disease, nerve damage etc. Here the effect of fructose on key early indicators of type 2 diabetes are examined: blood glucose; insulin concentrations and sensitivity, and damage to blood haemoglobin.

Effects of fructose on fasting blood glucose

In most studies the effects of chronic (1 to 10 weeks) consumption of moderate doses of fructose on fasting glucose concentrations have not seen an increase and in some cases have shown a decrease. Type II diabetic subjects have shown a strong tendency towards glucose lowering with moderate doses of approximately 60 g/day fructose compared with starch or sucrose (Cozma *et al.*, 2012). Healthy, normal-weight or overweight subjects, have not demonstrated a differential effect of fructose *vs*. glucose or sucrose with doses ranging between 40 and 150 g/day (Aeberli *et al*., 2013; (Bossetti *et al*., 1984; Bantle *et al*., 2000; Silbernagel *et al*., 2011; (Aeberli *et al*., 2013).

Increased fasting glucose concentrations has been reported with very high doses (165 g/day) of fructose or HFCS compared with glucose in groups of volunteers that included overweight and obese subjects (Stanhope *et al*., 2011; Stanhope *et al*., 2011b; Stanhope *et al*., 2009).

With low fructose intakes (≤36 g/day), no effects on fasting glucose have been seen in studies in diabetic and non-diabetic subjects (Sievenpiper *et al*., 2012).

Effects of fructose on fasting insulin concentrations

Numerous studies have concluded that fructose alone or in other foods fed over the dose range of 30-150 g/day over 4-12wks, has no impact on fasting insulin concentrations in diabetic (Cozma *et al* (Cozma *et al.*, 2012); and non-diabetic

(Bossetti *et al*., 1984; Bantle *et al*., 2000; Ngo *et al*., 2011; Silbernagel *et al*., 2011; Aeberli *et al*., 2013); subjects *vs*. glucose, sucrose or starch (Sievenpiper *et al*., 2012).

However, when considering only the comparisons with glucose or the consumption of fructose in liquid form, a significant reduction of fasting insulin concentrations by fructose have been observed (Sievenpiper *et al*., 2012).

Effects of fructose on insulin sensitivity

In healthy subjects, fructose doses comprised between 40 and 150 g/day and consumed for 3-4 weeks did not affect whole body insulin sensitivity of healthy subjects when compared with glucose (Aeberli *et al*., 2013; Silbernagel *et al*., 2011; Aeberli *et al*., 2011).

A study in type 2 diabetic subjects who consumed 45-65 g/day fructose suggested an improvement in insulin sensitivity relative to complex carbohydrates (Koivisto *et al*., 1993).

Exceptionally high fructose intakes (around 165 g/day) consumed for 10 weeks decreased insulin sensitivity in overweight or obese subjects (Stanhope *et al*., 2011; Stanhope *et al*., 2009).

When hepatic rather than whole body insulin sensitivity was measured by fasting glucose and high-sensitivity C-reactive protein (hs-CRP) respectively, deteriorations to the former were observed in healthy subjects at moderate to high doses (80 g/day *vs*. glucose) after only 3 weeks of intake, without noticeable changes in the latter (Aeberli *et al*., 2013). This observation suggests that liver sensitivity to insulin may be adversely affected by fructose at intakes that are within or close to the approximately estimated “normal” range for subjects in the US (50-75 g/d) and realistic in some population groups. The authors hypothesised that the decrease in hepatic insulin sensitivity may involve a stimulation of gluconeogenesis and increased glycogen stores (1.2. Potential Mechanisms to explain the negative impact of fructose on health, 1.2.5 Glucose metabolism dysregulation- insulin insensitivity), or may be related to hepatic lipotoxicity (1.2.6 ATP depletion and uric acid production Oxidative stress and Inflammation).

Effects of fructose on post-prandial blood glucose and insulin

Fructose intake has well-known beneficial (dampening) effects on acute, post-prandial (1-3h post-ingestion) glucose and insulin responses when compared with equivalent (in most cases) single intakes of glucose (40-150 g/day) (Wiebe *et al*., 2011, Bantle *et al*., 2000 etc.*; or sucrose (50-100 g/day) (Bantle *et al*., 2000; Chong *et al*., 2007; Lee *et al*., 1998; Visvanathan *et al*., 2004; or starch (approximately 50-100 g/day) (Bantle *et al*., 1986; Abraha *et al*., 1998). Some of these works involve meta-analysis, e.g. Wiebe *et al*., 2011 reviewed 3,666 citations and identified 53 eligible randomized controlled trials with 1,126 participants. They found that in diabetic participants, fructose reduced 2-hour blood glucose concentrations by 4.81 mmol/L (95% CI 3.29, 6.34) compared to glucose. However, they do not say how much each substrate reduced blood glucose and from what starting point. Such data though can be put into context. Diabetic postprandial blood glucose concentrations may be up to approximately 10 mmol/L so it can be seen that a substantial reduction may be achieved.

[*Parks *et al*., 2008; Teff *et al*., 2004; Bowen *et al*., 2007; Chong *et al*., 2007; Blaak *et al*., 1996; Teff *et al*., 2009; Aro *et al*., 1987; Fukagawa *et al*., 1995; Mah *et al*., 2011; Meyer *et al*., 1971; Van Gaal *et al*., 1999; Visvanathan *et al*., 2004]

Effects of fructose on glycosylated haemoglobin

Fructose at doses up to 88 g/day have been shown to improve long term glycaemic control in subjects with different health status, including diabetic, non-diabetic, and obese individuals. Significant reductions in HbA1c in comparison with other carbohydrates have been shown in three meta-analyses (Cozma *et al*., 2012; Livesey *et al*., 2008; Sievenpiper *et al*., 2012), especially when fructose is compared with glucose (Livesey *et al*., 2008). The effects of fructose were inconsistent when it replaced starch (Cozma *et al*., 2012; Livesey *et al*., 2008; Sievenpiper *et al*., 2012; Grigoresco *et al*., 1988; Koivisto and Yki-Jarvinen., 1993). The improvement in HbA1c concentrations seem to be more pronounced in cases of severe dysglycaemia and with increasing doses of fructose (from 22 to 88 g/day) (Livesey *et al*., 2008). It is not clear, however, whether fructosylation by fructose, rather than glycation by glucose, would have been picked up given the methods used in these studies. Fructose is 7-8 times more reactive than glucose, and this is thought to be due to the higher stability of its open chain form (Motera *et al*, 2019, as it forms a Heyns product when reacting with amino acids rather than the Amadori product. The common methods employed for glucose glycation do not detect the Heyns products and/or other fructose-mediated adducts which has slowed down research on the potential role of fructose glycation in the pathogenesis of chronic disease in humans.

Summary- Effects of fructose on blood glucose and insulin

Fructose (40-150 g/day) has the beneficial effect of reducing acute post-prandial glucose and insulin responses when compared with glucose or sucrose. Only very high doses increase fasting blood glucose. Reduced whole body insulin sensitivity compared to glucose has so far only been observed with very high fructose. However, it is possible that insulin sensitivity of specific tissues (e.g. liver) may be affected by lower (e.g. 80 g/day) doses.

1.1.8. Fructose health effects: literature review conclusions

In this thesis it was first investigated what may be considered to be a moderate to high range of fructose intake. This estimation of approximately 50 g/day has taken into account that a) added fructose is the main concern as intake from fruit and grain may ~~well~~ not be harmful since it is also consumed with fibres and phytonutrients, and b) participants in such studies typically under-report intake by up to 50% (a figure supported by national supplies/availability of fructose consumption data), c) manufacturers typically underestimate the fructose content of their products.

Based on this physiologically relevant dose range of fructose, this thesis has investigated which of the many negative health impacts claimed for fructose maybe more likely or indeed plausible. Of the many health areas investigated, that of the effect of fructose on blood triglycerides seems one for which therapies can be most confidently pursued. The potential health benefits of lowering blood triglycerides are further discussed in appendix I.

1.2. Potential mechanisms to explain the negative impact of fructose on health

The main hypotheses for the effects of fructose on health have been mentioned briefly above. In this section further details are provided along with others that may also be important.

1.2.1 Lack of regulation- de novo lipogenesis

The main mechanism proposed for the potential harmful effects of fructose on health is that of its unregulated metabolism. Unlike dietary glucose, fructose is relatively unregulated by insulin and is instead rapidly metabolised in the liver by fructokinase which binds fructose with high affinity (Michaelis-Menten constant K_m =0.2-0.5 mM- Hers & Kusaka, 1953; Adelman *et al*., 1967). Fructokinase acts 10 times faster than the metabolism of glucose by hexokinase IV (glucokinase, the hexokinase isotype found in the liver), for which fructose is a poor substrate. This rapid metabolism of fructose is believed to help prevent fructosylation of proteins, which occurs several times more rapidly than glycosylation by glucose (Dills 1993). Fructose is

subsequently able to bypass the feedback control mechanisms that operate when glucose is consumed, as fructokinase, unlike phosphofructokinase (PFK), the master regulator of glucose metabolism, is not subject to feedback inhibition by energy status - i.e. concentrations of ATP and citrate (Elliot *et al*., 2002) (see Figure 1.5). As well as a low K_m for fructose, fructokinase is also characterized by a high V_{max} (100 mM, Adelman *et al*, 1967, Heinz *et al*, 1968), and is therefore rarely saturated. Thus, fructose is rapidly absorbed and metabolised by the liver in a largely unregulated manner into triose phosphates whose subsequent metabolism leads to: oxidation for energy; glucose production, glyconeogenesis; lactate and to increased hepatic lipogenesis, leading potentially to steatosis and hepatotoxicity (see Figure 1.5 and also Figure 1.6).

Figure 1.5: Fructose and Glucose differential metabolic pathways

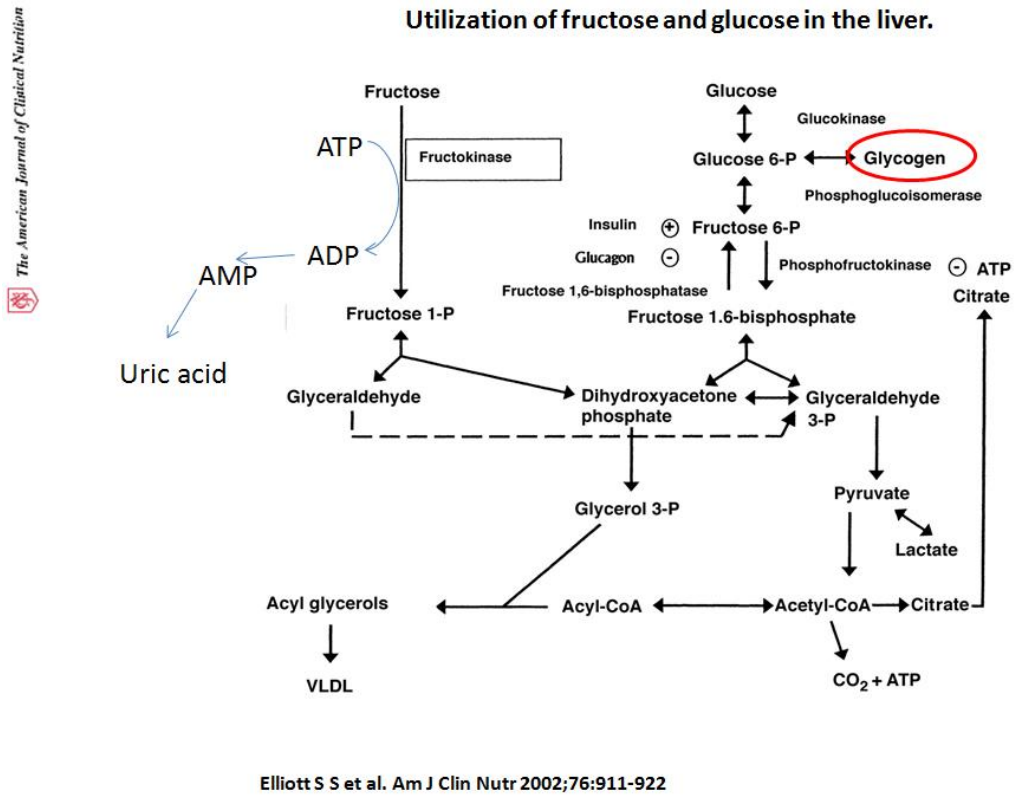


Figure 1.5: Fructose and Glucose differential metabolic pathways: Demonstrates the differential metabolic pathway of fructose and glucose in the liver. Glucokinase in the liver has low affinity for fructose (unlike in other tissues e.g. muscle). Fructose is therefore metabolised rapidly by high affinity fructokinase which is not feedback inhibited by the energy status of the cell. Glucose metabolism via phosphofructokinase is feedback inhibited by citrate and ATP accumulation. Note also the utilisation of ATP by high affinity fructokinase which can result in ATP depletion and conversion of accumulated ADP and AMP to uric acid at high fructose levels.
[author emailed (Peter Harvey)- permission given]

1.2.2 Molecular pathway stimulation- *de novo* lipogenesis

In addition to mechanism 1) above, a number of molecular mechanisms may be involved in the potential development of steatosis (liver lipid accumulation) and hypertriglyceridemia from elevated fructose levels (Lim *et al*., 2010). These include, a) Stimulation of carbohydrate response element binding protein (ChREBP), by fructose and glucose metabolites glucose-6-phosphate or Xylulose-5-P, which activate the enzymes responsible for *de novo* lipogenesis (DNL), b) Stimulation by fructose-1-phosphate of peroxisome proliferator activated receptor-γ-coactivator-1β (PGC1β), a transcriptional co-activator for sterol regulatory element binding protein 1c (SREBP1c) which further increases the activity of the enzymes involved in DNL; c) unrestricted metabolism of fructose to acetylCoA, which if not required for energy accumulates and dimerises and is decarboxylated to form malonylCoA, a key regulatory step in fatty acid synthesis, which also inhibits mitochondrial fatty acid oxidation and increases carbon flux into the lipogenesis pathway.

Fructose therefore is able to activate *de novo* lipogenesis via ChREBP and SREPP1c transcription factors. Glucose activation it seems is restricted to ChREBP. These mechanisms may indicate longer-term effects of fructose. Pre-sensitisation to fructose may therefore be required to observe some health concerns e.g. insulin insensitivity and steatosis. (Bizea & Pagliassotti, 2005) (see Figure 1.6).

1.2.3 Energy source shifting- *de novo* lipogenesis

Surprisingly, isotopic studies show that only a small percentage of ingested fructose (<1%) appears to be directly converted to plasma triglycerol (Sun & Empie, 2012). However, in these studies subjects are fasted and glucose is absent. Under such circumstances: a) fructose may largely be used for gluconeogenesis (approximately 50%- Delarue, 1993) (see Figure 1.3) and b) lactate, another major metabolite of fructose (Dietze *et al*., 1976), which may subsequently and belatedly be converted to lipids in the liver and or other tissues. Hyperlipidaemic effects of large amounts of fructose consumption are observed in studies using infused labelled acetate to quantify longer term *de novo* lipogenesis (Sun & Empie, 2012) suggesting that energy source shifting, or sparing may also play a part in the effects of fructose. This

may occur as fructose metabolites stimulate activation of the enzymes responsible for DNL (see (1.2.2 Molecular pathway stimulation above) (see Figure 1.6).

Further studies in this area should be performed in non-fasted models to determine what happens when fructose is available in excess of energy requirements. Ideally in an *in vivo* model fructose should be supplied with glucose to ensure adequate absorption from the gut.

1.2.4 Decreased lipid clearance- contribution to elevated plasma triglyceride

Observed increases in plasma triglycerides, the main effect by fructose confirmed by studies (see 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides), can also arise as a result of decreased lipid clearance. Fructose results, initially at least, in reduced insulin stimulation, relative to glucose, which will lead to reduced lipoprotein lipase and therefore reduced triglyceride clearance by adipose tissue (Sun & Empie, 2012) (see Figure 1.6).

Figure 1.6: Some of the potential mechanisms to explain the negative impact of fructose on blood triglyceride levels

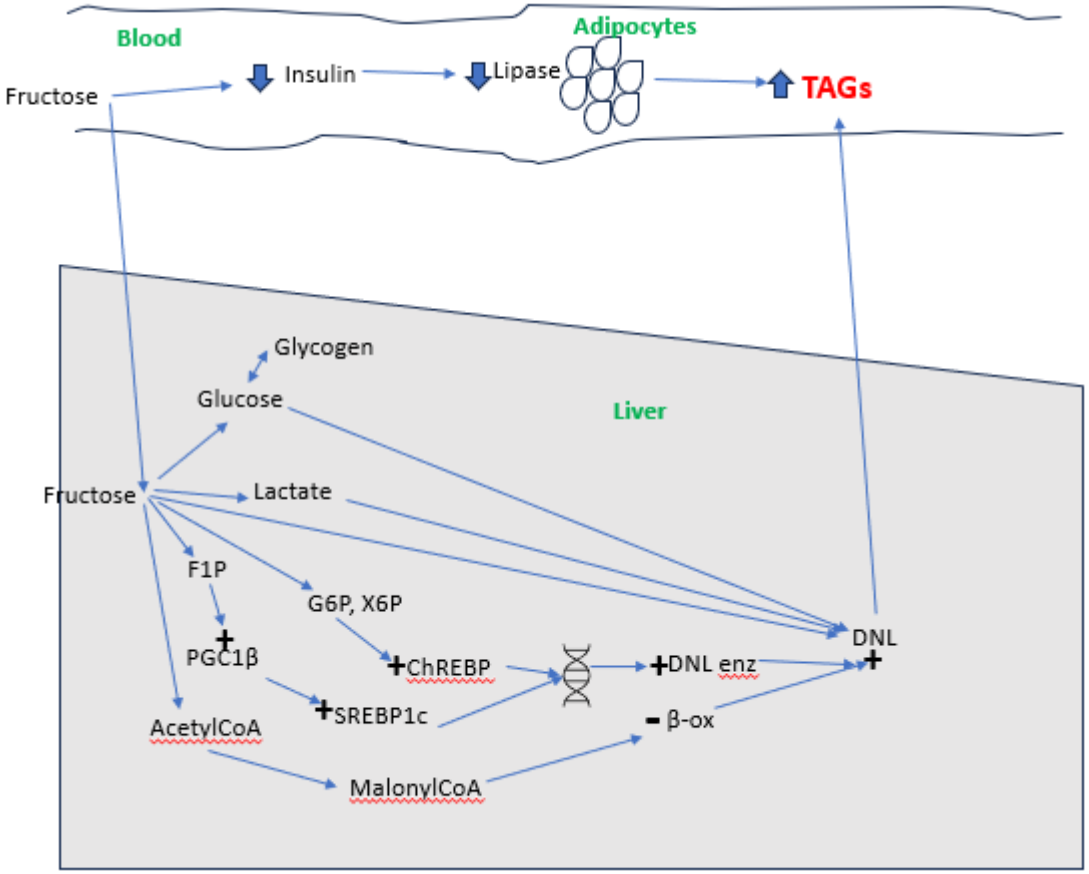


Figure 1.6: Some of the potential mechanisms to explain the negative impact of fructose on blood triglyceride levels. Fructose in the liver may be metabolised to glycogen, or triglycerides by *de novo* lipogenesis (DNL), or metabolites which may also be converted to triglycerides or stimulate activation of the enzymes responsible for DNL potentially leading to other energy source conversion to triglycerides. Fructose in place of glucose also decreases circulating insulin which would decrease lipase and triglyceride breakdown and uptake by adipocytes leading to higher blood levels.

1.2.5 Glucose metabolism dysregulation- insulin insensitivity

Although insulin does not initially greatly rise in response to fructose intake, there may be a belated response. Generally, fructose may be largely converted to glucose in the liver, due to stimulation of gluconeogenesis (Jenssen *et al*., 1990) (Figure 1.3). In fructose infusion studies the glucose produced is generally stored as glycogen rather than released into the blood (Dirlewanger *et al*., 2000). However, insulin is found to rise to some extent in response. It is not well understood but this could be to inhibit glycogenolysis, promote storage and suppress glucose production to regulate glucose levels (Saltiel & Kahn, 2001). Elevated glucose production though is maintained, ~~perhaps~~ possibly by non-hormonal (e.g. neural) control (Moore *et al*., 1998), causing insulin to remain elevated, indicating at least short-term insulin insensitivity (Dirlewanger *et al*., 2000) which might be anticipated could become chronic with repeated fructose exposure.

In summary, excess fructose which continues to enter the liver and be metabolised due to lack of feedback regulation (see above mechanism 1), is converted to glucose for storage as glycogen. The limited research in this area so far suggests that insulin levels rise to regulate glucose production, but fails, leading to insulin insensitivity.

1.2.6 ATP depletion and uric acid production- Oxidative stress and Inflammation-

With respect to hepatic lipid accumulation some commentaries suggest that dietary fat makes the predominant contribution, whereas fructose has a greater role in promoting hepatic oxidative stress, inflammation and fibrogenesis (Neuschwander-Tetri, 2013). One reason for this could be due to increased levels of uric acid which is associated with oxidative stress and production of e.g. tumour necrosis factor-α (Sun & Empie, 2012). Increased uric acid can be caused as a metabolic product of built-up ADP and AMP. This may occur as fructose is very rapidly metabolised in the liver by fructokinase, but is subsequently slowly metabolism by downstream enzymes, e.g. aldolase, leading depletion of ATP (Chaudhary *et al*., 2013) (see Figure 1.5).

1.2.7 Gut microbiome

Increased sugar sweetened beverages (SSBs) and fruit juice (2.0±1.5 servings/day) consumption may negatively alter the gut microbiome and/or gut bacterial translocation (see Appendix II: Fructose metabolism in the intestine) . Studies have shown decreased systemic microbial diversity and increased *Proteobacteria*, potentially contributing to obesity and metabolic disease (Alderete *et al*., 2015).

1.2.8 Obesogen- foetus and child

Emerging studies indicate that consumption of sugars and SSBs is beginning to occur early in life i.e. by the mother during pregnancy and after birth, and this early life exposure is associated with increased risk of obesity by early childhood (Goran, 2015).

1.3. Fructose flattened spike hypothesis

From the potential mechanisms discussed it might seem obvious that reducing fructose uptake from the gut could reduce its potential negative health impacts. It would not be good though, to reduce the total amount of fructose absorbed from the small intestine, as this could lead to carry-over of fructose into the lower gut, potentially causing digestive problems. Attempts to control fructose uptake from the gut would therefore need to involve the same total amount of fructose reaching the liver although more slowly, giving a lower fructose spike over a longer period, i.e. a flattened fructose spike. It is not immediately obvious that this would give a health benefit as the liver would still be exposed to the same quantity of fructose. However, it is generally proposed that only excess fructose is directed towards *de novo* lipogenesis (Lim *et al*., 2010) either directly or indirectly. Lim *et al*., 2010 discussed excess fructose as being when excess “mitochondrial acetyl-CoA exceeds the ability of the tricarboxylic acid cycle to metabolise it. The excess acetyl-CoA is converted to citrate, exits into the cytosol via the citrate shuttle, and serves as the substrate for *de novo* lipogenesis. Acetyl-CoA dimerises and is decarboxylated to form malonyl Coa which inhibits fatty acid β oxidation”. An example of when this would occur is

when “total energy intake from carbohydrate exceeds total energy expenditure.”

In this thesis it is therefore hypothesised, that by inhibiting and slowing the uptake of fructose from the small intestine the reduced fructose spike, although prolonged, (i.e. flattened spike) would result in a reduced excess of fructose (or metabolites) directed toward triglyceride release and cytotoxicity (Figure 1.7).

Figure 1.7: “Flattened fructose spike”- hypothesis

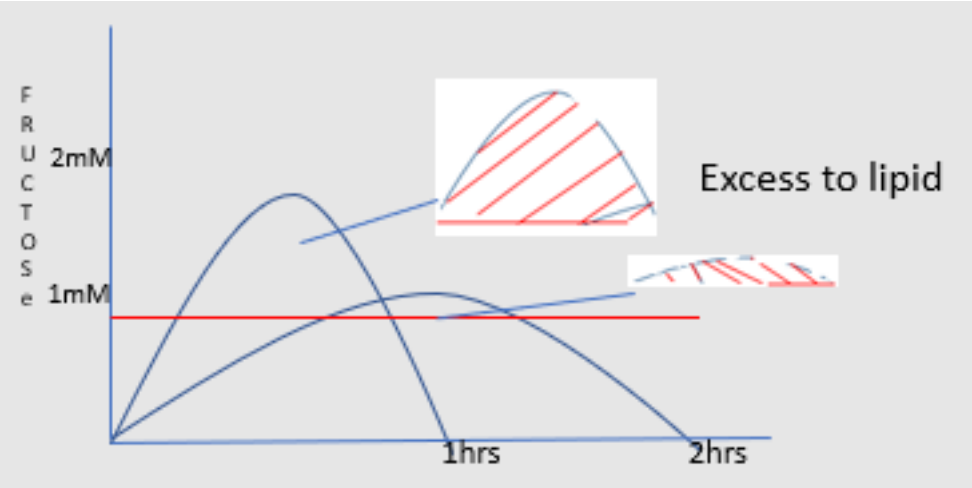


Figure 1.7:“Flattened fructose spike” hypothesis:- only fructose in excess of energy and storage requirements in the liver will be metabolised to lipid. Slowing uptake from the gut will reduce fructose excess leading to reduced lipid synthesis and cytotoxicity either directly or indirectly.

We turn now to the question of how the fructose spike reaching the liver might be flattened.

1.4 Fructose transporters in the gut- as potential targets for inhibition

Sugars are transported into the blood from the gut by a wide group of membrane proteins called glucose transporters (GLUTs) that facilitate, by molecular binding, the transport of monosaccharides across the plasma membrane of gut cells by diffusion. This is a process of passive transport (as opposed to active transport), down a concentration gradient, which does not directly require chemical energy from ATP hydrolysis.

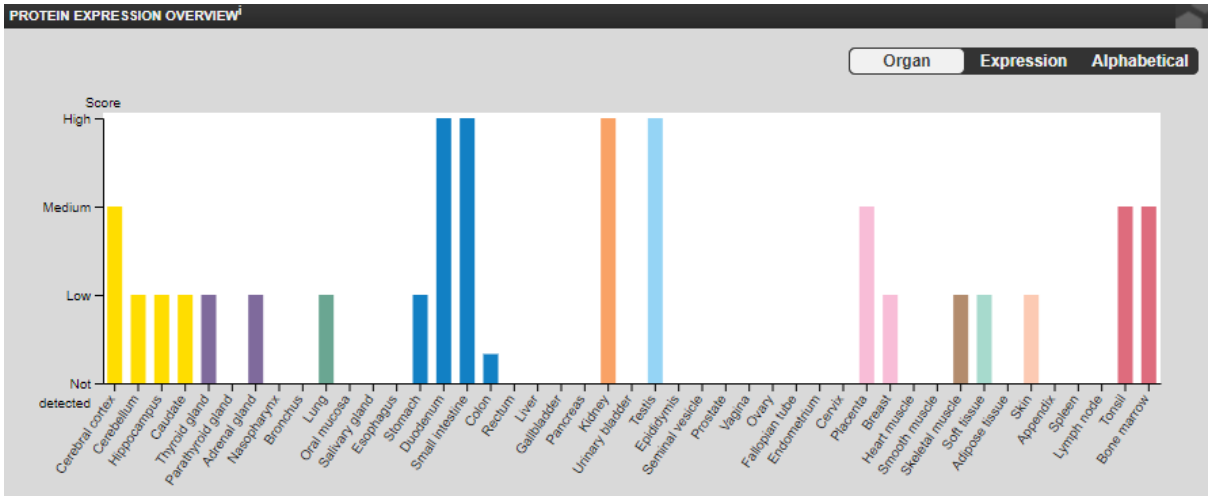
Several GLUT family members are capable of transporting fructose, however, GLUT5 is the only member demonstrating specificity for fructose, i.e. does not show capacity to facilitate transport of galactose, glucose or other monosaccharides.

1.4.1 GLUT5

GLUT5 was cloned over 20 years ago and was initially described as a glucose transporter (Bell *et al*., 1990) until its specificity for fructose in the intestine and sperm was clearly demonstrated (Burant *et al*., 1992). Specificity for fructose was demonstrated as *Xenopus* oocytes injected with synthetic human GLUT5 mRNA showed a stimulation of [^{14}C]-fructose uptake, whilst showing little ability to transport the glucose analogue 2-deoxyglucose. The uptake of fructose could be competed by unlabeled fructose, but not to any significant extent by inhibitors of glucose transporters, glucose, galactose, sucrose, or a-methylglucopyranoside (a specific substrate for the sodium-dependent glucose transporter) and cytochalasin B (GLUT1-4 inhibitor). As further evidence for GLUT5 involvement in the transport of fructose generally, RNA and protein blotting studies showed the presence of high levels of GLUT5 mRNA and protein in human spermatozoa. Human spermatozoa are thought to preferentially utilize fructose for their metabolic needs while in seminal fluid and shift to glucose when in the female reproductive tract (Peterson and Freund, 1975; Rogers and Perreault, 1990). The reason for this is not known. Immunohistochemical and western blot analysis has also localised GLUT5 to the gut lumen side of human jejunal enterocytes (brush border membranes) (Davidson *et al*., 1992).

The biochemical properties and tissue distribution of GLUT5 (see Figure 1.8) therefore seem consistent with a physiological role for this protein as a specific fructose transporter. As such GLUT5 has been generally assumed to be the key fructose transporter although other potential candidates exist.

Figure 1.8: Tissue distribution of fructose transporter GLUT5



https://www.proteinatlas.org/ENSG00000142583-SLC2A5/tissue [they give this info to cite]

GLUT5 homologous transporters

Sequence homology with GLUT5 suggest that GLUT7, GLUT9a/b, GLUT8, GLUT11, and GLUT12 possibly also have the ability to transport fructose: (Manolescu *et al*., 2007; Doujard & Ferris, 2008).

GLUT7 (Cheeseman, 2008), GLUT8 (Romero *et al*., 2009), and GLUT12 (Miller *et al*., 2005) have been shown to be expressed in the gut. Although the specific functions of these transporters might overlap to some extent, the functions of GLUT family members in the gut do not appear to be redundant: GLUT5 is almost exclusively a high-capacity fructose transporter (Ferraris, 2001), GLUT7 is a high-affinity, low-capacity fructose transporter (Thorens & Mueckler, 2010). This might suggest that GLUT7 functions to transport fructose at low concentrations in the gut lumen, and GLUT5 at high levels. GLUT12 transports both fructose and glucose (Rogers *et al*., 2003). There is evidence that GLUT8 attenuates enterocyte fructose transport by regulating GLUT12 protein abundance *in vitro* and *in vivo* and that

disrupted GLUT8 function has deleterious long-term metabolic effects in the high fructose fed rat (DeBosch *et al*., 2012). The purpose of this relationship between GLUT8 and GLUT12 is not understood, but ~~perhaps~~ suggests GLUT12 is more important than so far considered, if there is need for its expression to be regulated by GLUT8.

GLUT5 and possible basolateral transport

GLUT5 has been also identified in the in the basolateral membrane (BLM) from human small intestine (Blackmore *et al*., 1995). Brush border membrane (BBM) (apical) and BLM were isolated by fractionation of human jejunum and by quantitative immunoblotting showed only slightly higher amounts of GLUT5 per mg of membrane protein in the BBM. The localization of GLUT5 in the BLM of the human jejunum may suggest that it participates in the transfer of fructose across the basal membrane of the enterocyte (see Figure 1.9). In testing for potential fructose uptake inhibitors, reagents that are themselves taken up by enterocytes, or their metabolites, may inhibit fructose transport by inhibiting basolateral GLUT5. This offers up greater opportunities for synergy if such reagents operate in concert with others that inhibit apical membrane transport.

1.4.2 GLUT2 and fructose transport

Basolateral

GLUT2 transports glucose across the basolateral membrane of the small intestine to the blood as well as possibly high concentrations of glucose across the apical membrane of the lumen into gut enterocytes (Kellett and Brot-Laroche, 2005). GLUT2 has also been shown to transport fructose across the basolateral membrane (Jones *et al*., 2011).

Apical?

GLUT2 has been proposed to also be effective in the transport of fructose from the gut lumen at the apical membrane (Douard & Ferris, 2008), although it is not certain

to what extent. Studies show the greatest absorption rate occurs when glucose and fructose are administered in equal quantities (Fujisawa *et al*., 1991) as in most normal consumption e.g. when fructose is ingested as part of the disaccharide sucrose or HFCS. It has been suggested that the GLUT5 transfer rate may be saturated at low levels, and absorption is increased through joint absorption with glucose via GLUT2 (Ushijima *et al*., 1991) (Figure 1.9).

Figure 1.9: Fructose and glucose transport across the intestinal epithelium

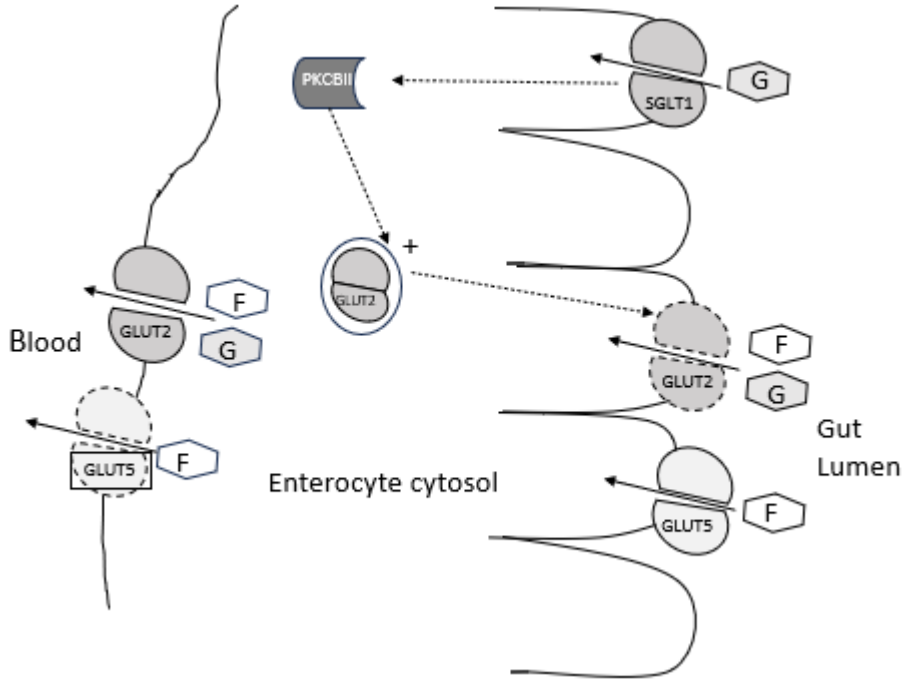


Figure 1.9: Fructose and glucose transport across the intestinal epithelium. GLUT5 has been generally assumed to be the key and specific fructose transporter on the enterocyte apical membrane and the high glucose concentration transporter GLUT2 at the basolateral membrane (BLM). GLUT5 has also been identified in the BLM from human small intestine which may suggest that it also participates in the transfer of fructose across the BLM of the enterocyte. GLUT2 has been proposed to also be effective in the transport of fructose from the gut lumen at the apical membrane, although it is not certain to what extent. It is suggested that preformed intracellular vesicle stored GLUT2 is rapidly translocated to the apical membrane via a SGLT1 (low glucose transporter) activated signalling mechanism.
(Figure replaced and expanded)

SGLT1 activation of GLUT2

Sodium-glucose linked transporter (SGLT1) is responsive to and transports low glucose levels, whilst GLUT2 possibly transports high glucose levels. However, its role seems more complex as it is suggested that preformed, intracellular stores of

GLUT2, are rapidly translocated to the apical membrane via a SGLT1 activated signalling mechanism (Kellet and Brot-Laroche, 2005). As the concentration of free glucose increases, initial transport across the apical membrane occurs through SGLT1, causing activation of protein kinase C (PKC) βII. These events may result in rapid (t1/2~3.5 minutes) activation of apical GLUT2 already in the membrane and further insertion of GLUT2 into the apical membrane from intracellular vesicles underlying the membrane, as seen in immunocytochemistry. In addition to co-transport of fructose and glucose, mentioned above, this might explain how glucose increases the uptake of fructose when fed together as in most diets (Truswell *et al*., 1988), if GLUT2 is involved in the apical transport of fructose.

It may be found therefore that inhibitors of fructose transport, whether via interference with GLUT5, GLUT7, GLUT8, GLUT9, GLUT12 or other transporter(s) may give synergy with inhibitors of GLUT2 and/or SGLT1. In chapter 4 potential inhibitors of fructose uptake were tested with GLUT2 and SGLT1 inhibitors for synergistic activity.

1.5 Physiological fructose levels in the hepatic portal vein

Upon being absorbed from the gut nutrients are first transported to the liver via the hepatic portal vein. To investigate the effect of flattening the fructose spike in a liver model it was needed to consider what a normal range of fructose encountered by the liver might be.

Postprandial fructose concentrations in the hepatic portal vein following 2 g/kg sucrose administered orally in 16 hr fasted rats, were found to peak at 1 mM by 30 minutes (Sugimoto *et al*., 2010). This equates to approximately 10 g fructose dose in a 60 kg human (when adjusted for body surface area (Reagan-Shaw *et al*., 2007)*, about half the quantity of fructose in a 330 ml can of sugar sweetened beverage (SSB) such as cola (typically 39 g sugar/330 ml). A review on the “Hepatic adaptations to sucrose and fructose” (Bizeau & Pagliassotti, 2005) concluded that in humans, physiological postprandial concentrations of fructose in the portal vein range from 0 up to a maximum of 2.2 mM following a high- fructose or high-sucrose meal (50g dose, therefore 25g fructose). However, there is also evidence that

chronic fructose intake elevates uptake in the gut (David *et al.,* 1995; Cui *et al.,* 2004; Patel *et al.,* 2015; Jang *et al*., 2021).

Preliminary testing of the hypothesis that flattening the fructose spike may lead to potential health benefits in human hepatocytes, reported here, have therefore mostly focused on fructose levels of 1-8 mM in order to explore the potential physiological range.

[*Body surface area normalisation has been found to work well in a number of animal species together with biometrics such as calorie expenditure and oxygen utilisation). Body weight (kg) is divided by body surface area (m^2) to give a Km value (same name but not the same the Michaelis constant) for a given species (FDA values for humans and mice adults are 60 kg/1.6 m^2 =37 and 0.02 kg/0.007 m^2=3 respectively. Converting a dose in a mouse to a human equivalent dose is achieved by: Animal dose x Animal Km/Human Km (Reagan-Shaw *et al*., 2007). Jang *et al*. 2018 use a simpler method to estimate human equivalence based on calorie intake. E.g. For a mouse a dose of 0.5 g/kg is approximately 0.5% of daily calorie intake].

1.6 Research Plan

1.6.1 Aims

This research project aimed to study the potential health benefits of reducing the fructose spike reaching the liver by limiting the uptake of physiological levels of fructose and glucose from the diet and identify natural plant compounds for this purpose.

Objectives

1) To investigate the effect of reduced fructose concentration spikes (flattening the fructose spike) reaching the liver, at levels of physiological relevance, on triglyceride release and hepatotoxicity in a model of high physiological relevance i.e. primary human primary hepatocytes. Preliminary work was carried out in the HepG2 cell line.
2) To identify ~~technology~~ natural plant compounds or extracts with the potential to reduce fructose spikes through inhibition of fructose uptake from the gut using

an optimised *in vitro* gut cell line transport model and look for patent protectable synergies.

3) To investigate further potential health benefits of flattening the fructose spike reaching the liver by examining the effects of fructose on lipid accumulation, glycogen storage, lactic acid and uric acid production and the hepatokine FGF21 secretion in primary human hepatocytes.

2.0 Materials and Methodology

2.1 Potential benefits of reduced fructose concentration spikes: on triglyceride release, steatosis, and hepatotoxicity

A key aim of this thesis (see 1.6.1) was to establish potential benefits of controlling fructose uptake. To do this it was planned to test the effect of flattening the fructose spike (i.e. lower concentrations for longer) on primary human hepatocytes.

2.1.1 HepG2 cells

Fresh primary human hepatocytes are often unavailable and so preliminary experiments were initially conducted on cells from the HepG2 cell-line. HepG2 is an immortal cell-line derived human liver hepatocellular carcinoma. They are commonly used as a convenient alternative to primary human hepatocytes. HepG2 cells display robust morphological and functional differentiation with a controllable formation of apical and basolateral cell surface domains that resemble the bile canalicular and sinusoidal domains, respectively, *in vivo*.

Cell culture

The human hepatocellular carcinoma (HepG2) (ECCAC, Salisbury, UK) was maintained in William's E medium, containing 11.1 mM glucose, supplemented with 10% foetal bovine serum (FCS), 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mmol/l l-glutamine, at 37°C in a 5% CO_2:95% air-humidified atmosphere. Cells were passaged as needed using 0.5% trypsin-EDTA. All the cell culture solutions were purchased from Sigma. Typically, cells in T175cm^2 cell culture flask were washed with 20 mls of PBS, followed by addition of 5 mls of 0.5% trypsin-EDTA ensuring the whole cell surface coated surface area was covered. 3 mls were then removed and the flask incubated at 37°C/5% CO_2 for approximately 5 minutes until the cells began to detach. Cells were fully dislodged by gently tapping the sides of the flask. 8 mls of culture medium containing 10% FCS was then added to neutralise the trypsin to avoiding damage to cells. The total volume of 10 mls was then removed pipetting up

and down to ensure that the vast majority of the cells were collected. One third to one fifth of the collected cells was then dispensed to a fresh flask topping up with additional medium to 20 mls and the cells allowed to adhere to the flask surface for at least 4 hrs before replacing with fresh medium. The medium was changed for fresh every 48-72 hrs. The cells were passaged again when 60-70% confluent.

Analysis

Lipid accumulation: Oil Red-O

Lipid accumulation in HepG2 cells was measured by Oil Red-O staining (Steatosis Colourimetric Assay kit Cayman, 10012643). Cells were fixed in formaldehyde and methanol for 15 minutes, then washed and dried in a culture hood. They were then stained with Oil Red-O for 20 minutes, washed with distilled water followed by dilute isopropanol, and dried again in the culture hood. The dye was then extracted with isopropanol for 30 minutes, the dye solution transferred to a 96-well plate, and the absorbance read at 490nm.

Cell viability

Cell viability for the purpose of normalisation was measured using “WST-1 cell proliferation reagent” (product no.11644807001, Roche). The stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bio-reduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. Cells were incubated with the WST-1 reagent for 0.5 - 4 hours. The formazan dye formed during the incubation was quantitated by sampling the cell culture supernatant at 30 minutes intervals and reading the absorbance at 420nm.

As measurement of WST-1 can be affected by cell activity which might be affected by treatment, and some inconsistency was found in incubation time needed, lactate dehydrogenase was used as a measure of cell viability in later experiments- see below.

Triacylglycerol (TAG) release:

Of all the potential harmful effects of fructose proposed in the scientific literature- that of postprandial blood triglycerides seems to have the most supportable evidence at normal levels of consumption (approximately 50 g/day) (see 1.1.8. Fructose health effects literature review conclusions). Therefore, a key part of this work was to investigate the effect of fructose on TAG release. TAG release from cells was measured in the culture medium by analysis using the Triglyceride-Quantification-Kit (Abcam, ab65336) which measures the glycerol released following added assay lipase digestion. Initial levels of glycerol were established by analysis without lipase digestion and deducted from the total found after digestion. Fluorescence at Ex_{535}/Em_{585} was read rather than absorption to enable greater sensitivity.

2.1.2 Primary Human hepatocytes

The use of HepG2 cells was found to be limiting for the reasons discussed (Chapter 3.0 Potential benefits of reduced fructose concentration spikes: on TAG release and hepatotoxicity, 3.3.2 HepG2 cell-line experiments) and cryopreserved primary human hepatocytes were used as an alternative. Human primary hepatocyte frozen cell stocks were obtained from Life Technologies, cat no. HMCPIS. Internal Unilever ethical approval was obtained.

Cell Culture

Upon thawing in recovery medium (CM7000, Life Technologies) cells were centrifuged at 100*g* x 10 minutes, re-suspended in plating medium (CM3000, Life Technologies) and seeded in 100 µl on 96-well plates coated with rat tail type I collagen (Life Technologies, A1048301). Plates were coated by adding 100 µl of 50 µg/ml collagen in 0.02 M acetic acid for one hour at room temperature under sterile conditions. The wells were washed twice in Phosphate Buffered Saline, then used immediately or air dried under sterile conditions and stored at 2°C to 8°C for future use. Cells were seeded at 62,500-87,500 cells/ well. After 6 hrs, the plating medium (100 µl) was replaced with serum free maintenance medium (CM4000, Life Technologies).

Low glucose culture medium

In preliminary experiments using standard 11.1 mM level of glucose in the medium fructose treatments were found to have little effect at physiological levels. This high concentration of glucose may have been leading to high baseline lipid release and a lower level of 4 mM glucose was used with better results. William's-E-medium without glucose was acquired to allow glucose concentration to be adjusted.

William's-E medium without glucose (Genaxxon Bioscience, custom made) was supplemented with glucose at the desired concentration and the following compounds: dexamethasone (0.1 uM), nicotinamide (5 mM), albumen (1 mg/ml), copper (II) sulphate (0.2 mg/L), zinc sulphate (0.75 mg/L), transferren (5 µg/L), sodium selenite (29 nM), insulin (10 nM), hepes (15 mM), glutamine (2 mM, glucose (as required), penicillin and streptomycin (Gibco) (5,000 units/5,000 µg). Except where mentioned supplements were obtained from Sigma.

FRAME lab cocktail

As high levels of background glycerol were found, an alternative supplement regime used at the FRAME lab, Nottingham University was trialled. This comprised of the following: dexamethasone (0.1 µM); nicotinamide (5 mM); 1 mg/ml albumin; penicillin & streptomycin; glutamine (2 mM); copper (II) sulphate (0.2 mg/ml); zinc sulphate 0.75 mg/l; transferrin (5 µg/ml); sodium selenite (30 nM); insulin (10 nM).

Treatment of cells

In this experiment it was attempted to replicate a feeding pattern of distinct meals during a day, with a purpose of providing physiologically relevance. As discussed, (1.13. Effects of fructose on blood lipids, Effects on post-prandial triglycerides) the importance of feeding over more than one meal and monitoring triglycerides over 24 hrs is apparent in e.g. the Parks *et al*, 2008 study (Figure 1.4). In this study an increase in triglycerides was not seen until after the second meal of the day. This is consistent with the concept that only excess fructose is metabolised to lipid when energy reserves become sufficient (see 1.2. Potential Mechanisms to explain the negative impact of fructose on health, 1.2.1 Lack of regulation- *de novo* lipogenesis).

Many studies may miss this and conclude that fructose does not have an effect on triglycerides. Following a meal post fasting, fructose is likely to peak in the blood and therefore liver after approximately 30-60 minutes and lipid release after approximately 3-4 hrs (Parks *et al*., 2008 and 2012) post fasting and continue to rise for several hours after a subsequent meal. A 3-hour time period over which to collect medium represents a snapshot of this profile. "Meals" were modelled by using the following regime:

Cells in 96-well plates were treated over 3 application periods:

"Meal" 1- at 9.00am:

(i) Plate A at a high dose of fructose (8 mM) with collection gently by pipette of conditioned culture media after 1 hr, refreshment using 100 µl culture medium without fructose and 6 mM glucose, and then collection of conditioned culture medium gently by pipette again after 2 hrs.

(ii) Plate B at half the high dose of fructose (4 mM) with collection gently by pipette of conditioned culture medium after 2 hrs, refreshment of 100 µl culture medium without fructose and 6 mM glucose, and then collection of conditioned culture medium gently by pipette again after 1 hr.

(iii) Plate C at a moderate dose of fructose (2 mM) with collection gently by pipette of conditioned culture media after 1 hr, refreshment using 100 µl culture medium without fructose and 6 mM glucose, and then collection of conditioned culture medium gently by pipette again after 2 hrs.

(iv) Plate D at half the moderate dose of fructose (1 mM) with collection of conditioned culture media gently by pipette after 2 hrs, refreshment using 100 µl culture medium without fructose and 6 mM glucose, and collection of conditioned culture medium gently by pipette of again after 1 hr.

This was repeated at 12.00pm ("meal" 2) and 3.00pm ("meal" 3) and at glucose levels ranging from 6-14 mM. In between meals cells were maintained at 6 mM glucose. Samples were snap frozen in liquid nitrogen and stored at -80°C for pooled analysis of collection 1 and 2 for each "meal".

Analysis

Lipid release

Triacylglycerol release into the cell culture medium was measured by lipase digestion and conversion to glycerol as described above for HepG2 cells.

Cytotoxicity by lactate dehydrogenase (LDH) released into the medium.

The effects of fructose on hepatotoxicity were assessed using a cytotoxicity assay based on the release of lactate dehydrogenase (LDH) from the cells (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega- G1780). LDH released into culture supernatant was measured with a 30 minute coupled enzymatic assay resulting in the conversion of a tetrazolium salt into a red formazan product. The amount of colour formed was measured by absorption at 490nm and is proportional to the number of lysed cells.

Cell viability- by intracellular LDH.

Cell viability was measured as a means to normalise TAG release in cell lysates by intracellular LDH (as above). Following a freeze/thaw cycle, and induction of cell lysis using a detergent containing buffer, the amount of colour formed was measured by absorption at 490nm and was proportional to the number of living cells extracted.

Free Fatty Acid:

Free fatty acids were also tested, as measurement of TAGs was found to mostly record glycerol which subsequent experiments indicated was a result of the breakdown of triglycerides by endogenous sample lipase and/or hepatocyte lipase secreted by the primary hepatocytes.

The Fluorometric Assay Kit, (No.700310, Cayman Chemical Company) was used. The assay utilised a coupled enzymatic reaction to determine free fatty acid concentrations. Acyl-CoA synthetase catalyses fatty acid acylation of coenzyme A.

The product acyl-CoA is oxidised by acyl-CoA oxidase and generates hydrogen peroxide. Finally, hydrogen peroxide in the presence of horseradish peroxidase reacts with the reagent O-acyl-3,7-dihydroxyphenoxazine to generate the highly fluorescent product resorufin, measured at Ex_{540}/Em_{585}.

2.2 Identifying plant natural compounds or extracts that can reduce fructose spikes

A key aim of this thesis (see 1.6.1 above) is to identify natural plant compounds or extracts that have the potential to reduce fructose spikes through inhibition of fructose and glucose uptake from the gut, and to look for more effective and patent protectable synergies.

2.2.1 Fructose and glucose assay

A cost-effective colorimetric microtiter plate enzymatic assay for fructose and glucose was based on protocols described by Campbell *et al*.,1999.

Assay Reagents

All the reagents were sourced from Sigma and used at the given concentrations: Basic Triethanolamine-HCL buffer (bTEA) was comprised of: Triethanolamine-HCL (T1502, 50 mM); $MgSO_4.7H_20$ (M9397, 5 mM) adjusted to pH 7.6 using 0.5 M NaOH. Complete Triethanolamine-HCL buffer (cTEA) was freshly prepared by adding BSA to give 0.02% and 5 µl of 1 M dithiothreitol (DTT) to 10 ml of bTEA. To 9 ml cTEA the following freshly prepared reagents were added: NAD (N6522, 19.6 mg); 18 ml dd water; 900 µl of ATP (A26209, 0.05 mg/ml) in 472 mM Na_2CO_3; 50 µl hexokinase (H6380, 1040 U/ml) from frozen stored aliquots (1.5 kU/1.44 2 ml dd water); 2 5 µl G6PDH (G8529, 1040 U/ml) from frozen stored aliquots (1 kU/0.962 ml dd water).

Initial Glucose assay:

5 µl of sample or standard was added to 250 µl of cTEA, mixed by votex and incubated for 15 minutes at room temperature. The absorption of NADH production was read at 340nm.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G6P} + \text{ADP}$$

$$\text{Fructose} + \text{ATP} \xrightarrow{\text{HK}} \text{F6P} + \text{ADP}$$

$$\text{G6P} + \text{NAD} \xrightarrow{\text{G6PDH}} \text{6PGlcU} + \text{NADH}$$

Subsequent Fructose assay:

In the wells above after making the absorption reading at 340nm, 10 µl Phosphoglucose Isomerase (P5381) was added to give 1.4 U/well (5.3 U/ml) mixed by vortex and incubated for 15 minutes at room temperature and the absorption of NADH production read at 340nm.

$$F6P \xrightarrow{PGIuI} G6P$$

$$G6P + NAD \xrightarrow{G6PDH} 6PGlcU + NADH$$

Fructose concentration was derived from total NADH minus glucose NADH. Standards were prepared by mixing fructose and glucose in an appropriate ratio i.e 1:3 as that found in samples post transport assay (see results). These were typically made from 1 M stock solutions of fructose and glucose in PBS to a volume of 10 mls. The combination of glucose with fructose was found to affect the fructose analysis over time, possibly through isomer stability (see Chapter 4.0: Natural plant compounds and extracts to flatten the fructose spike, 4.3 Discussion, 4.2.8 Hypotheses for the effect of glucose on fructose uptake, Mutarotation). Standards were therefore prepared <4 hrs prior to analysis.

2.2.2 Fructose transport model development and inhibition

To test potential natural plant phytochemicals for inhibition of the uptake of fructose, an *in vitro* model was developed. This was achieved using a model established for total cumulative glucose transport, across differentiated Caco-2 monolayers seeded onto transwell permeable inserts, as per the paper “New and better protocols for a short-term Caco-2 cell culture system” Yamashita *et. al*. (2002). Journal of Pharmaceutical Sciences, 91(3), 669-679

The assay required optimisation for GLUT5 expression and fructose transport by experimenting with Caco2 maturation time.

Fructose and glucose transport model

Caco-2 cells were seeded into cell culture inserts (Figure 2.1) ($2.5x10^5$ cells/ well for 24-well plates (BioCoat HTS Fibrillar Collagen Multiwell Inserts [Fisher Scientific UK, # 354804] coated with collagen II matrix) in growth medium (DMEM+Glutamax-1 containing 4.5 g/L D-glucose + 25 mM HEPES, Invitrogen #32430027) + 10% (100x) non-essential amino acids (Invitrogen, #1114035) + 10% 100 mM Sodium pyruvate solution (Sigma S8636) + 10% foetal bovine serum (Sigma F7524). 30 ml of growth medium was also added to the feeder plate below. The cells were left to attach over 24 hrs at 37ºC, 5% CO_2. Following a gentle wash (both inserts and feeder plate) in PBS, the cells were incubated in differentiation medium (BD Entero-STIM™ enterocyte differentiation medium (BD Biosciences #05495+(1000x)) + MITO+™ Serum Extender solution (BD Biosciences, #356007) for a further 2, 5 or 7 days.

Figure 2.1: Fructose and glucose transport model

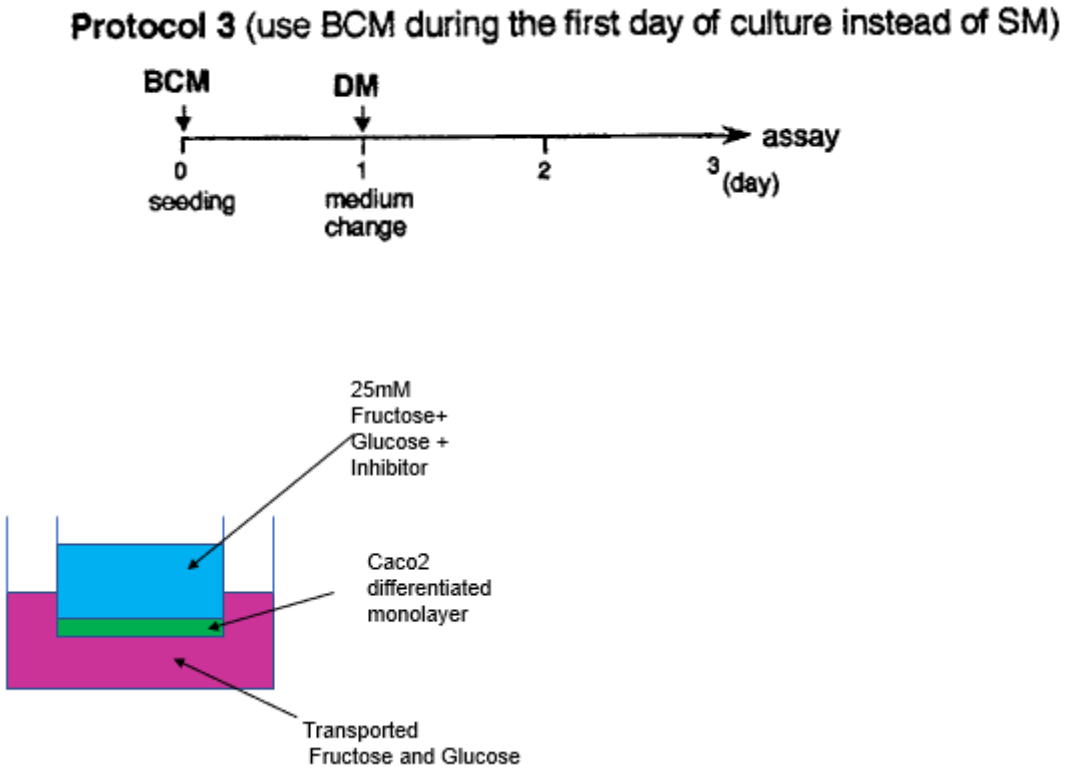


Figure 2.1: Fructose and glucose transport model: Potential inhibitors were added with fructose and glucose in PBS (+) as 0.5 ml to the insert supply well and 1.0 ml PBS (+) were added with fructose (25 mM) and glucose (25 mM) in PBS (+) as 0.5 ml to the upper insert supply well and 1.0 ml PBS (+) added to the bottom collection chamber for 30-240 minutes. PBS (+) from the collection ~~well~~ chamber was then analysed for glucose and fructose.

Trans epithelial electrical resistance (TEER)

TEER was tested to check membrane integrity. TEER is a sensitive and reliable method to confirm the integrity and permeability of the monolayer using an Epithelial Voltohmmeter (Srinivasan *et al*., 2015). The greater the level of resistance the greater the monolayer integrity.

One electrode was placed in the upper compartment (see Figure 2.1) and the other in the lower compartment and the ohmic total resistance measured. Blank resistance of the semipermeable membrane only (without cells) was also measured and deducted from the total resistance. Resistance is inversely proportional to the area of the cells and semipermeable membrane so the cell specific resistance is therefore reported here per $cm^{2.}$

Fructose transport assay

Cell monolayers were washed gently in PBS (+ $MgCl_2$ and $CaCl_2$, Sigma, D8662) (and the inserts transferred to a new standard tissue culture plate. The cells were incubated with fresh PBS (+) for 30-60 minutes at 37°C, 5% CO_2. Cell inserts were then transferred to a new plate, and potential actives or treatments were added with fructose (25 mM) and glucose (25 mM) in PBS (+) as 0.5 ml to the upper insert supply well and 1.0 ml PBS (+) added to the bottom collection chamber for 30-240 minutes (Figure 2.1). PBS (+) from the collection ~~well~~ chamber was then analysed for glucose and fructose- see below.

Transport of fructose or glucose was calculated as a percent sugar transported from the supply well across the cells to the insert collection chamber (% well µmoles transported/hr) E.g. if 0.8 mM fructose was detected in the collection chamber this is equal to 0.4 µmole/hr (i.e. 0.8 mM in 1 ml/1000 ml/2 hrs). This was transported from a pool of 25 mM in 0.5 ml which is equal to 12.5 µmoles (i.e. 25 mM /500ul). 0.4 µmoles fructose transported is 3.2% of 12.5 µmoles.of fructose pool /hr.

Lucifer Yellow transport

As a measure of monolayer and insert membrane integrity Lucifer Yellow transport (Hidalgo *et al*., 1989) was measured by addition of Lucifer yellow (Sigma, L0144). The cell inserts were transferred to a new plate, the supernatant gently aspirated from the cells and replaced with 100 µM Lucifer Yellow solution, PBS (+) was added to the collection well and incubated at 37°C 5% CO_2 for 1 hr. The cell inserts were then discarded and the permeability of the membranes to Lucifer Yellow measured by the fluorescence of the samples at $Ex_{485}Em_{530}$.

Following measurement of fructose and glucose transport, Caco2 cells were lysed and extracted with 200 µl RLT buffer (Qiagen, cat. No. 79216) per 24 well insert seeded at a density of >2.5x105 cells. 4 replicate wells were pooled and frozen at 180°C.

mRNA purification

This was performed using RNeasy Plus kits (Qiagen, cat. no. 73404).
Only RNA strands longer than 200 bases bind to the RNeasy silica membrane, therefore excluding RNAs of less than 200 nucleotides such as ribosomal and transfer RNAs.

Samples were thawed to room temp or 37°C and vortexed for 30 seconds to ensure homogenisation. 700 µl of 70% ethanol (molecular biology standard quality) was added and mixed gently by pipetting. The mixture was then applied to the RNeasy spin column and centrifuged for 15 seconds at >8,000*g*. This was repeated with a second volume of 700 µl. In order to remove DNA, 350 ml RW1 kit buffer was added and incubated at room temperature (rt) for 15 minutes prior to application to a DNase I purification column (Qiagen, cat. No. 79254) followed by centrifugation as above. Columns were then washed thoroughly twice with RPE buffer from the kit, centrifuging as above. The second wash was centrifuged for 2 minutes to ensure full removal of ethanol. RNA was then eluted to fresh RNAase free tubes with 30 ml RNAse free water.

RNA Quality

RNA quality was checked by:

a) Spectrophotometry (NanoDrop ND-1000) to obtain absorption data at 260, 280 and 230nm. As DNA and RNA absorb at 260nm the ratio of absorbance at 260nm and 280nm was used to assess the purity of the RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower than 2.0 for RNA, it may indicate the presence of DNA, protein, phenol or other contaminants that absorb strongly at or near 280nm.

b) The ratio of absorption at 260:230nm was also considered as absorption at 230nm can be caused by contamination with phenolate ions, thiocyanates, and other organic compounds used in the lysis and purification process. Expected 260/230nm values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230nm.

c) Gel electrophoresis (Agilent 2100 Bioanalyser, RNA Nano chips (cat. No. 5067-1511) and RNA 6000 Nano marker, gel matrix and tracker dye)).
On the Agilent chip, the sample moves through microchannels, and sample components are electrophoretically separated. Smaller fragments migrate faster than the large ones. The fluorescent dye molecules intercalate into DNA or RNA strands. They are then detected by their fluorescence and translated into gel-like images (bands) and electropherograms (peaks) from which RNA peaks and contaminants can be identified.

cDNA Synthesis

This was carried out using the two-step RT-PCR qScript cDNA Synthesis kit (Quanta Biosciences, cat no. 95047-100).

Step one- Reverse Transcription:

Complementary double-stranded DNA (cDNA) was synthesized from the single stranded mRNA template in a reaction catalysed by the enzyme reverse transcriptase. A poly -T oligonucleotide primer is hybridized onto the poly-A tail of the mature mRNA template, and random hexamer primers were added which contain every possible 6 base single strand of DNA and therefore hybridized anywhere on the RNA (reverse transcriptase requires this double-stranded segment as a primer to start its operation). Reverse transcriptase was added, along with deoxynucleotide triphosphates (A, T, G, C). This reaction synthesises one complementary strand of DNA hybridized to the original mRNA strand.

Purified mRNA samples were thawed on ice, pipetted to 0.2 ml thin-walled PCR 96 well plates (cut to required size) on ice, to give 1 µg RNA and volumes made up to 15 µl with nuclease free water to which 5 µl of qScript reaction mix containing the oligo(dT), random primers and dNTPs and RNase inhibitor had been added. Plates were centrifuged to collect the contents and placed in a thermocycler (MJ Mini, Biorad) programmed to give 1 cycle at 22°C, 5 minutes; 1 cycle at 42°C, 30 minutes; 1 cycle at 85°C, 5 minutes and hold at 4°C. Upon completion samples were diluted 1/10 and 1/100 for step two- PCR amplification together with specific transporter gene primers of interest (GLUT5, GLUT2, SGLT1).

Step two- PCR:

After digestion of the RNA, a single stranded DNA (ssDNA) was left and because single stranded nucleic acids are hydrophobic, it tends to loop around itself. It is likely that the ssDNA forms a hairpin loop at the 3-prime end. From the hairpin loop, a DNA polymerase was then used as a primer to transcribe a complementary

sequence for the ss cDNA, to give a double stranded cDNA. Double stranded cDNA was then temperature denatured and annealed in presence of specific primers of interest (GLUT5, GLUT2, SGLT1) and amplified over numerous cycles until sufficient levels occur to be detected.

Real-Time PCR:

A DNA-binding dye binds to all double-stranded DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity measured at each cycle (exponential phase) with a detector. The dye only fluoresces when bound to the dsDNA (i.e., the PCR product). However, it should be noted dsDNA dyes such as SYBR Green will bind to all dsDNA PCR products, including nonspecific PCR products (such as primer dimer). This can potentially interfere with, or prevent, accurate monitoring of the intended target sequence. Primer controls (the absence of DNA) were therefore included.
Here a SYBR green supermix (IQTM SYBR Green Supermix- Biorad cat. No. 170-8882) was used containing antibody-mediated hot-start I Taq DNA polymerase, dNTPs and SYBR Green dye.

55.55% supermix was added to 11.12% specific primer (see below), 33.33% nuclease free water. To 63 µl of this reaction mix was added 7 µl cDNA sample which was then split into 20 µl triplicates in 0.2 ml thin-walled PCR 96-well plates (cut to required size) on ice. The plate was loaded onto an Eppendorf Realplex thermocycler programmed for a 95°C, 3 minutes (polymerase activation and DNA denaturation) and then an amplification cycle: 95°C 10-15 seconds (denaturation; 55-60°C, 30-60 seconds (annealing/extension) for 40 cycles.

Primers

GLUT5 primer (SLC2A5- QuantiTect Primer Assay, Product no 249900. cat. No. QT00087766)
GLUT2 primer (SLC2A2)- QuantiTect Primer Assay, Product no 249900. Cat. No. QT01008399)

SGLT1 primer (SLC5A1- QuantiTect Primer Assay, Product no 249900. Cat no. QT00001246)
GAPDH primer (GAPDH-- QuantiTect Primer Assay, Product no 249900. cat. No. QT00079247)

2.2.3 Selection of single actives and beverages

Fructose uptake patent and prior art search

The purpose of this search was to identify compounds or plant extracts which may prevent or improve the possible deleterious effects of fructose uptake through inhibition of gut transporters. When considering plant extracts the main ingredients were identified where possible and selected as being the potential actives.

PatBase and Web of Knowledge were searched using a number of strategies including the key words: fructose, GLUT5, uptake* (and synonyms), and inhib* (and synonyms).

Patents- Search strategies:

1) Fructose and (transport* or uptake or absor*) and (inhib* or anatag* or block*)

2) Fructose and GLUT5

3) GLUT5 and (inhib* or anatag* or block*)

Web of Knowledge- Search strategies:

1)Title= Fructose and (GLUT5 or transport* or uptake* or absor*) and (inhib* or antag* or block*)
-21 hits
Topics= Fructose and (GLUT5 or transport* or uptake* or absor*) and (inhib* or antag* or block*)
>2000 hits- too many to scan- therefore see 2) below-

2)Topic= Fructose and GLUT5 and (inhibit* or antag* or block*)
-69 hits

3) Topic=(flav* and glut5 and (antag* or inhibit* or block*))
-4 hits

4) Topic=flav* and glut
-0 hits

Tea extract preparation

Except where otherwise stated tea extract was prepared by pouring 200 ml of water at 95°C onto a black or green tea bag and leaving for 3 minutes before removing the bag. The extract was then cooled to room temperature. An estimation was made as to what extent such drinks would typically be diluted by the time they reach the small intestine. If the stomach contains 0.5-1.0 litres of fluid and 250 ml of tea is consumed, this equates to a dilution of approximately 1-in-3 to 1-in-5. Alternatively, it has been estimated that a total of 6-8 litres of digestive juices are produced and secreted each day in the adult human gut (Shmidt and Thews, 1983). If 4x250 ml of tea is consumed per day plus other food and drink, this would equate to a dilution of approximately 1 in 8 to 1 in 10.

2.2.4 Polyphenol analysis

Total polyphenol is generally measured using the Folin-Ciocalteu assay (Velioglu *et al*., 1998). As this also detects other antioxidants, ascorbic acid was removed using ascorbate oxidase-based methodology taken from Hewitt and Dickes, 1961 and Foyer *et al*., 1995. The combined Folin- Ciocalteu and ascobate oxidase assays were adapted to a microplate format (see below).

Total polyphenol/antioxidant (minus ascorbic acid) microtiter plate assay

125 µl sodium phosphate buffer (0.1 M pH6.0) was added to 20 µl sample/gallic acid standards. (0-0.3 mg/ml freshly prepared). Of this 135 µl was added to 1.33 µl ascorbate oxidase (400 U/ml) and left for 5-10 minutes at rt. 120 µl of this was added to 12 µl Folin-Ciocalteu reagent (2 M) for 5 minutes exactly (rt). 120 µl of sodium carbonate solution (60 g/L) was added to this and left for 90 minutes at rt. The absorbance was read by spectrophotometer at 765nm.

It should be noted that the Folin-Ciocalteu polyphenol assay measures antioxidants in general- not just polyphenols. Here, ascorbic acid has been removed enzymatically but other antioxidants may also be present in addition to polyphenols. It should also be noted that different polyphenols will have different numbers of phenol rings, phenolic groups (-OH) and arrangements with which the Folin-Ciocalteu reagent will react.

2.2.5 Synergy and product combination benefit testing

Testing of single compounds and beverages for fructose transport inhibition, using the Caco2 glucose transport model adapted for fructose here gave some promising leads although none were significant statistically. These compounds were tested for synergy with the GLUT2 and or SGLT1 inhibitors that may affect fructose transport in the gut.

Synergy testing is complex (Foucquier and Guedj, 2015) and much work has been dedicated to potential solutions (Greco *et al*. 1995; Chou 2006) and their inadequacies (Berenbaum 1977; Caudle and Williams 1993; Nieuwenhuis *et al*. 2011; Ocana *et al*. 2012; Geary 2013). The approaches used here were:

1. Combination Sub-thresholding:
   Two agents that are not significantly effective alone are tested for significance (one-way ANOVA, $p<0.05$) against the untreated control when combined. A substantial difference in effect between the combination and individual single actives should be ensured (Nieuwenhuis *et al*. 2011)

2. Highest Single Agent (Lehar *et al*. 2007):
The combination was compared directly to the individual agent effects rather than the untreated control. A significant difference was required to be shown for the difference between the combination and highest single active effect (One-way ANOVA, $p<0.05$).

3. Response Additivity (also referred to as Linear Interaction Effect (Slinker *et al*., 1998):
Tests for a greater than an additive effect. Synergy was determined as a significant interaction by a two-way ANOVA of the individual and combination effects (Slinker *et al*., 1998).

4. Standard Greater than Additive Effect:
If a combination is significantly different from both single agents alone, and its inhibition adds up to greater than the sum of the single agents.

Generally, a two-tailed statistical test was applied, however where appropriate a one-tail statistical test was applied to potential fructose uptake inhibitors, given that only inhibition is being considered, i.e. a directional hypothesis, (Field, 2018). One-tail tests are of course controversial, however, where a result in the opposite direction would lead to the same conclusion as finding no effect, it is considered a legitimate use (Lombardi & Hurlbert, 2009). If reagents were found to increase fructose transport this would have led to their rejection just as for reagents that showed no effect, as increasing fructose transport in the context of this work is completely undesirable.

2.3 Further potential health benefits of fructose control in fresh primary hepatocytes

A key aim of this thesis (see 1.6.1 above) was to investigate further potential health benefits of controlling fructose uptake. To do this, several markers of the potentially harmful effects of fructose at physiological levels were tested for in primary human hepatocytes.

Fresh primary human hepatocytes were obtained directly by perfusion of liver tissue from surgically removed malignant tumours from Nottingham Hospital, Queen's Medical Centre.

Isolation of human hepatoctyes:

This was kindly performed by the laboratory staff of the FRAME's Lab, School of Life Science, Medical Facility, Queen's Medical Centre, Nottingham University

Procedure:

The tissue was collected in cold Soltran buffer (Baxter Health Care LTD) (diluted 1 in 2 with water to give 300 mOsmol/l) then: the tissue was flushed with the diluted cold Soltran using a syringe; cannulated to give uniform blanching of the lobe using 500 ml perfusion buffer+EGTA without re-circulating (using a Watson-Marlow 323 pump set at 45 rpm); buffer leakage was minimised using superglue to close any vessels; perfusion was continued using fresh perfusion buffer+EGTA solution (1 L). The time of perfusion was and should be around 15-20 minutes. The lobe was flushed with perfusion buffer (without EGTA) prior to collagenase digestion, for 5-6 minutes. The lobe was then perfused with recirculating perfusion buffer+collagenase for approximately 20 minutes until the lobe was soft and tissue marbling was observed. Following perfusion, the lobe was carefully removed and placed into perfusion buffer (without EGTA). The lobe was then minced gently with blunt-ended scissors and then filtered through a 250 µm and then a 100 µm nylon mesh followed with HBSS.

Cells were divided into 50 mL tubes and collected by centrifugation at 60*g* for 4 minutes at rt. (Heraeus Biofuel Primo). The supernatant was removed using a pipette and discarded. The hepatocyte cell pellet was re-suspended in serum-containing

medium and re-centrifuged at 60*g* for 3 minutes. The supernatant was removed by aspiration and discarded. The pellet was re-suspended in serum-containing medium, filtered through a 100 µm nylon mesh and centrifuged again at 60*g* for 3 minutes and the cells re-suspended in plating medium. 35 ml of cell suspension was mixed by vortex with 15 ml neutralised Percoll (1.5 ml 10xHBSS + 13.5 ml Percoll) and centrifuged for 10 minutes at 100*g* to pellet viable hepatocytes. The cells were washed once with serum-containing medium by re-suspending the pellet in plating medium and centrifuging at 60*g* for 4 minutes. The cells were resuspended in approximately 50-100 mL serum-containing medium and counted using 1:1 Trypan Blue. Finally, the cells were seeded $1.2x10^6$ cells per well of a 6-well plate or other.

Perfusion buffer (sterile):
To 500 mL of 1x HH buffer was added 10 mL MBG

Perfusion buffer+EGTA:
1x 500 mL of 1x HH buffer + 10 mL MBG + 10 mL EGTA (25 mM) solution

Perfusion buffer+Collagenase:
1x 500 mL of 1x HH buffer + 10 mL MBG + 10 mL calcium chloride + 65 U collagenase (Serva NB 4G) + 80 mg trypsin inhibitor

MBG solution (HH+):
31 g sodium bicarbonate (Sigma S6297), 25 g glucose (Sigma G7021) and 7.5 g methionine (Sigma M9500) in 500 mL ELGA-purified water, sterilised and store at -20°C in 50 mL aliquots

10x Hanks-Hepes buffer, pH 7.4 (HH):
NaCl 80.0 g, KCl 4.0g, KH_2PO_4 0.6 g, Na_2HPO_4 $12H_20$ 1.2 g, HEPES 47.6 g, phenol red 0.1 g, NaOH approximately 4 g
Components were dissolved in 900 mL of ELGA-purified water, pH adjusted to 7.4 using NaOH pellets (approximately 4 g), then the total was volume adjusted to 1 L and the solution sterilised by autoclaving and stored at 4°C.

90% Percoll/HBSS:

Percoll (Amersham 17-0891-01, density 1.131 g/ml) was diluted 9:1 with 10x HBSS (Sigma, H4641) and stored in 10 mL aliquots at 4°C.

Buffers were placed in 37°C water bath to warm up. Collagenase and trypsin inhibitor was added just prior to use and the solutions oxygenated before use with carbogen (95% oxygen, 5% CO_2).

Perfusion tubing was sterilised before use with perfusion buffer containing EGTA (500 mL). Solutions were run to waste for 30-40 seconds, mainly when changing from perfusion buffer without EGTA to digestion buffer.

Flow rates were (4 cannulas): 50 rpm = 80 ml/min, 60 rpm = 100 ml/min, 70 rpm = 60 mL/30sec.

Culture conditions

Primary human hepatocytes were seeded at $1.2x10^6$ and $2x10^5$ cells per well in collagen-coated 6-well plates and 24-well plates respectively, with serum-containing medium (Williams'E+FBS) to give a total volume of 2 ml, and incubated at 37°C, 5% CO_2 (humidified atmosphere). The medium was carefully changed to 2 ml serum-free medium at 4 hours when cells were observed to be attached. The cells were used the following day.

Serum-containing culture medium- Plating medium:
Williams'E medium with 2 mM L-glutamine (G7513, Sigma), 10% foetal bovine serum, 0.5 mg/ml gentamicin (Lonza), 5.5 mM glucose, 0.225% sodium bicarbonate, 0.5 mg/ml nicotinamide (Sigma N3376), 2.6 µM zinc sulphate (Z4750), 0.11 µM copper (II) sulphate, 22 nM dexamethasone/ SIGMA D4902), 2.7 nM sodium selenite (Sigma S9133 1 mg), 56.4 nM transferrin (Sigma T1428) (approximately 31 µM) and 8.9 nM insulin.

Serum-free culture medium- Maintenance medium:
Plating medium without serum, but with 0.09% albumin solution (Sigma A8412).

Collagen Type I – Solubilisation and Coating:
Calf skin collagen (Sigma C9791) 1 mg/ml in 0.1 M acetic was sterilised by

chloroform diffusion. Approximately10% chloroform was layered at the bottom of the collagen solution in a glass bottle overnight at 4°C. The collagen solution was diluted 1 in 10 with sterile water to obtain a working concentration of 100 µg/ml after aseptically removing the top layer containing the collagen solution. Culture dishes were coated with approximately 10 µg/cm^2 for 4 hours at 37°C in the cell culture incubator, washed with cold sterile PBS then water, allowed to dry in a class II culture cabinet and stored sealed at 4°C.

Plates were washed with appropriate volumes of cold sterile PBS followed by one wash with sterile water.

Treatment of cells

Glucose, fructose, PLO (fatty acid mix (200 µM final concentration- 40% oleate, 40% palmitate, 20% linoleate in 150 mM NaCl, 14% BSA)) were added as dictated by the treatment requirement to serum-free maintenance medium.

Preparation of 10 mM FFA stocks in 24% BSA for steatosis induction:
Palmitic acid: 36 mg, linoleic acid: 40 µl, sodium oleate: 39.6 mg (PLO) were separately dissolved in 7 ml 150 mM NaCl, heated and stirred for 5 minutes, 24% BSA was added to 7 ml + 48 µl 5M NaOH and gently heated and stirred until a solution/emulsion was formed. PLO stocks were made by combining at a ratio of 4:3:3 i.e. 4 ml Palmitic, 3 ml Linoleic and 3 ml Oleic, aliquot and stored at -20°C. For addition to cell culture medium PLO was thawed from -20°C storage at 37°C mixed by vortex until non-hazy),

Analysis

2.3.1 Lipid Accumulation

Nile Red:

Nile red (9-diethylamino-5H-benzo [alpha] phenoxazine-5-one) is a stain commonly used for the detection of lipid droplets.

Cells were seeded in 24-well plates on coverslips and after treatment were fixed in 4% PBS paraformaldehyde for 10-15 minutes, washed twice with PBS, Nile Red 30 µM (diluted from 1 mM stock-in DMSO with 1% pluronic F-127 (polyethylene-polypropylene glycol)) added for 15 minutes in the dark, washed twice and read on a fluorometer at Ex_{530}/Em_{590}*. 1:1000 DAPI nuclear stain was added for 5-10 minutes, the coverslips were mounted on glass slides with 15 µl VectaShield and analysed by fluorescent microscope using a GFP/FITC channel, image analysed using ImageJ software. Nile red staining and nuclei were counted for a field of view area. Four images were taken per slide at a position chosen at random, at x10 magnification, and the data meaned to give one biological replicate.

[*NB: Better selectivity for cytoplasmic lipid droplets is obtained when cells are viewed for yellow gold fluorescence (excitation 450-500nm; emission, >590nm) as red fluorescence tends towards more plasma membrane phospholipid staining (Greenspan *et al*., 1985).

2.3.2 Determination of Glycogen and glucose in cell extracts

As part of the thesis aims was to investigate further potential health benefits of controlling fructose uptake, glycogen levels were examined as fructose is known to stimulate its synthesis This was performed using the Glycogen Assay Kit II (Colorimetric) ab169558 which is suitable for measuring glycogen levels in samples that contain reducing substances (e.g. glucose) that may interfere with oxidase-based assays.

In the assay glycogen is hydrolysed to glucose, which is then oxidized by a hydrolase to form an intermediate that reduces a colourless probe to a coloured product with a strong absorbance at 450nm. Performing the assay in the absence of the hydrolase gives the initial levels of glucose to be deducted from the total. Samples were compared to glycogen standards.

Cells (approximately $1.2x10^6$ per well in 6-well plates) were washed in PBS and harvested by scraping in ice-cold 1 ml dd water and frozen for analysis. Upon

thawing samples on ice, trypsin inhibitor was immediately added then rapidly homogenised using 2x10 sec bursts with a probe sonicator on ice. Samples were then boiled for 10 minutes to inactivate enzymes, centrifuged at 18000 rpm for 10 minutes and the supernatant recovered for assay. Preliminary analysis suggested that a 1:15 dilution was necessary so 4µl was sampled to a 96-well plate and made up to 60 µl with assay glycogen hydrolase buffer. 50 µl were then transferred by multi-pipette to a 96-well plate containing 2 µl of hydrolase enzyme, and one well not containing hydrolase in order to analyse background glucose levels.

2.3.3 Determination of Lactate in Media samples

As part of the thesis aim was to investigate potential health benefits of controlling fructose uptake, lactate levels were examined as fructose in well-known to stimulate its synthesis. Lactate can be exported by the liver providing other tissues with energy but can potentially be converted to lipid leading to a build-up of visceral fat if in excess.

The following lactate determination method is based on an adaption of the Passenou and Lowry method by Arthur PG., *et al*., 1998, an LDH-mediated conversion of lactate to pyruvate, which in the process converts NAD to NADH. Enzymatic rate is quantified by following the increase in NADH at 340nm. This may be measured continuously kinetically or as a timed two-point assay.

Reagents:
Glycine-EDTA-hydrazine buffer pH 9.0, 200 mM glycine, 2 mM EDTA, 1.25% hydrazine hydrate

Reaction mix: Glycine-EDTA-hydrazine buffer, 20 units/ml lactate dehydrogenase, 3 mM NAD

Reaction:
20 µl sample/standard + 100 µl reaction were mixed by vortex in a 96-well plate and absorption was measured at 340nm at time zero and at 30-40 minutes. The reading

at time zero was subtracted from the reading at 40 minutes and referenced to standards of lactic acid (0-10 mM).

2.3.4 Determination of uric acid in cell culture supernatants

Based on methodology by Huang *et al.,* 2014 in “Optimization of pH values to formulate the bireagent kit for serum uric acid assay”.

As fructosylation of peptides etc. by fructose is much more rapid than glycation by glucose fructose is rapidly removed from circulation by high affinity fructokinase and phosphorylated to fructose-1-phophate (F1P) using ATP. As subsequent metabolism of F1P is much slower by comparison, high fructose levels can lead to a depletion of ATP and a build-up of ADP and AMP which can be metabolised to uric acid. Uric acid has been associated with hypertension, gout and liver inflammation. As part of the thesis aims was to investigate potential health benefits of controlling fructose uptake, uric acid levels were examined following fructose treatment. In the assay: “uricase catalyses the selective and complete oxidation of uric acid to produce hydrogen peroxide, which is consumed completely by the action of peroxidase for the oxidization of chromogenic substrates via putative stoichiometry to develop a colour” (Huang *et al*., 2014).

Reagent A:
1.5 kU/L horseradish peroxidase (Sigma, 8250-5KU)
0.25 mM 4-aminoantipyrine (Sigma, 33528-25G-R)
0.67 kU/L ascorbate oxidase (Sigma, A0157-250UN)
3.47 mM sodium benzoate (Sigma, 71300)
in 50 mM sodium phosphate buffer at pH 6.5.

Reagent B:
2.0 kU/L of BF or AG uricase (BF- Sigma, 94310)
2.33 mM TOOS (N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt) (FluoroChem, 045141)
3.47 mM sodium benzoate (Sigma, 1300)

in 50 mM sodium borate (Sigma, S9640) buffer at pH 9.2.

80 µl of reagent A was mixed by vortex with 20 µl sample/standard (uric acid, Sigma, U2625) in a 96-well plate and incubated at 25°C on a rotating shaker for 5 minutes before reading the absorbance at 546nm. 20 µl of reagent B was then added, incubated at 25°C on a rotating shaker and absorbance read at 546nm after 10 minutes. The concentration of uric acid was derived from the difference in absorbance at 546nm at the start and after the addition of Reagent B relative to a uric acid standard calibration curve, minus appropriate cell culture medium blanks. The highest standard concentration of uric acid standard was 297 µM.

Determination of protein by BCA

Protein concentration was measured using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Cat. No. 23225). The assay combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. For the microplate procedure 10 µl of each sample/standard was pipetted into a 96-well plate well followed by 200 µl of the assay reagent and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes. The absorbance at or near 540nm was recorded on a plate reader.

2.3.5 FGF21

Recently it has ~~co~~me ~~light~~ been noted that fructose as well as glucose can stimulate the liver to produce fibroblast growth factor 21 (FGF21) which influences sweet taste preference by acting within the hypothalamus (Jenson- Cody *et al*., 2020).

FGF21 Intact

After release of FGF21 from the liver into the circulation, FGF21 can become truncated leading to some loss of activity and give rise to potent antagonism (Agrawal *et al*., 2018). It is therefore important to measure the active fraction.

Intact FGF21 ELISA, Alpco 43-FGFHU-E01 was sourced via Stratech. This product was chosen as it has been validated in previous work (Cho *et al*., 2020), and looks identical in epitope binding and description to the Eaglebio product SKU: F2131-K01 that was used by Samms *et al*., 2017 but proved difficult obtain.

This human intact FGF-21 assay does not detect human FGF-21 fragments. The assay utilizes a two-site “sandwich” technique with two selected antibodies that bind to different epitopes of human intact FGF-21. One of the antibodies specifically binds to the N-terminal human FGF-21 (amino acids 1-7) and the other is specific to the C-terminal human FGF-21 (amino acids 175-181).

Cell supernatant samples were diluted 1 in 2 (as indicated to be necessary in a preliminary assay) by adding half the recommended volume in the assay and topping up with assay buffer.

FGF21 Total

Total FGF21 was measured using the Human FGF-21 Quantikine ELISA Kit- DF2100. As well as measuring the active intact FGF21 it is also important to measure total FGF21 as the non-active fragments can be inhibitory to the action of FGF21.

This product was chosen as it has been validated in previous work (Samms *et al*., 2017). The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human FGF-21 has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any FGF-21 present are bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human FGF-21 was added to the wells.

Cell supernatant samples were diluted 1 in 4 (as indicated to be necessary in a preliminary assay) by adding one quarter of the recommended volume in the assay and topping up with assay buffer.

Fibroblast activation protein (FAP)

A measure of the enzyme that digests and deactivates FGF21 in circulation (FAP) (Dunshee *et al*., 2016), can also give supportive evidence as to the level of intact and therefore active FGF21.

FAP was measured using the ab25604 Human FAP, SimpleStep ELISA® Kit. This product was chosen as it has been validated in previous work (Samms *et al*., 2017). The SimpleStep ELISA® employs an affinity tag labelled capture antibody and a reporter conjugated detector antibody which immunocaptures the sample analyte in solution. The entire complex (capture antibody/analyte/detector antibody) is in turn immobilised via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards were added to the wells, followed by the antibody mix. After incubation, the wells were washed to remove unbound material. TMB (3,3',5,5'-tetramethylbenzidine) development solution was added and during incubation was catalysed by HRP, generating blue coloration.

Glucose uptake by 3T3 L1 adipocytes

Not all non-active FGF21 is inhibitory or equally so (Agrawal *et al*., 2018). Therefore, it cannot be certain to what extent a ratio of intact/total FGF21 indicates overall FGF21 activity. As FGF21 is known to stimulate the uptake of glucose by 3T3-L1 differentiated adipocytes, the conditioned medium was applied from fructose treated primary hepatocytes to gauge the overall FGF21 activity of the samples.

3T3-L1 fibroblast differentiation to adipocytes

For these experiments 3T3-L1 cells of low passage e.g. P4 post receipt from supplier (ATCC), were found to be necessary to avoid excessive fragility and detachment once differentiated. Approximately 100,000 cells or more were rapidly thawed into 10 ml fibroblast maintenance/growth medium (M1), centrifuged at 124*g,* resuspended in the same medium and seeded into a 75 cm^2 cell culture flask. Upon reaching 60-70% confluence, cells were sub-cultured into 96 or 48-well plates at a seeding density of 75,000/cm^2 for 48 hours or until tightly confluent. The culture medium was then changed to differentiation medium (M1) (see below) containing insulin, dexamethasone and IBMX for 4 days, then a differentiation continuation medium for 3 days. The cells were at this point approximately 80% differentiated showing visible fat droplets in most cells. The cells were washed with PBS twice, serum starved in DMEM containing 0.1% BSA (fatty acid free) for 3 hrs, washed twice in Kreb's ringer phosphate buffer (KRPB) and then incubated with 200 µl (for 48-well plates) blocking antibody (Novus Biologicals, MM0277-6F10) in DMEM 0.1% BSA (fatty acid free) or control for 2 hrs and then 200 µl sample (or positive control e.g. recombinant human FGF21 (Abcam, 238297), in DMEM 0.1% BSA (fatty acid free)) for 24 hrs or insulin for 30 minutes. 2-NBDG (fluorescent glucose analogue) (200 µl) at 600 µM was then added and incubated for 60 minutes. At all steps post differentiation initiation, the cells must be handled very careful otherwise they are easily disrupted and detach from the plate. Cells were then washed three times with ice cold KRPB before the cells were extracted with 200 µl Triton solution (1% Triton-100X, 30% DMSO) and 100 µl sampled to black walled clear bottom 96-well plates and the fluorescence read at Ex_{485}/Em_{520}. The remaining sample was analysed for protein using the BCA assay (see above).

3T3-L1 fibroblast maintenance/growth medium (M1):
DMEM high glucose (25 mM) (Sigma, D5671), 10% new born calf serum, 2 mM glutamine (Sigma, G7513), antibiotic/antimycotic mix (Sigma, A9909), .

3T3-L1 differentiation medium (M2):
DMEM high glucose (25 mM) (Sigma, D5671), 10% foetal calf serum, 2 mM glutamine (Sigma, G7513), antibiotic/antimycotic mix (Sigma, A9909), 250 nM dexamethasone (Sigma, D1756) (from 125 µM stock in ethanol), 500 µM 3-isobutyl-1-methylxanthin (IBMX- Sigma, 1-1-7018) (from 0.25 M stock in 1 M NaOH), 166 nM insulin (Sigma, I9278).

3T1-L1 differentiation continuation medium (M3):
DMEM high glucose (25 mM), 10% foetal calf serum, 2 mM glutamine, antibiotic/antimycotic mix, 166 nM insulin.

3T3-L1 adipocyte maintenance medium (M4):
DMEM high glucose (25 mM), 10% foetal calf serum, 2 mM glutamine, antibiotic/antimycotic mix.

2.3.6. Statistics

Unless otherwise stated in each experimental chapter- where all treatments were compared to the control by one-way ANOVA, multi-comparisons were corrected for using Dunnett's statistical hypothesis testing. Where all treatments were compared to each other by One-way ANOVA, multi-comparisons were corrected for using Tukey's hypothesis testing. Where only appropriate treatments were selected for comparison and analysed by one-way ANOVA, multi-comparisons were corrected for using Šídák's statistical hypothesis testing. Where a a two-way ANOVA was appropriate multiple comparisons testing using Holm-Šídák's statistical hypothesis testing was performed.

Unless otherwise stated central tendency was measured by mean and dispersion was expressed using Standard Error of the Mean (SEM). The number of replicates varied per experiment depending on the number of treatments and available wells per cell culture plate (see legend of each chart). Within a cell culture plate treatments and replicates were randomly and evenly distributed across the plate to

avoid edge and clustering effects.

3.0 Potential benefits of reduced fructose concentration spikes: on triglyceride (TAG) release and hepatotoxicity

3.1 Introduction

A key aim of this thesis (see 1.6.1) was to establish potential benefits of controlling fructose uptake from the gut. In chapter.1 it was investigated as to what may be considered to be a moderate to high range of fructose intake in humans in the US. The estimation reached of approximately ≥50 g/day was based on studies of reported intake (Vos *et al*., 2008, Marriott *et al*., 2009), as well as taking into account that a) added or free fructose is the main health concern (WHO, 2015b), b) participants in such studies typically under-report intake by up to 50% (Poppitt *et al*., 1998; Trabulsi, *et al*., 2001; Winkler *et al*., 2005; Rennie *et al*., 2007; Devlin *et al*., 2012; Pfrimer *et al*., 2015; Harper and Hallsworth, 2016), a figure supported by national supplies/availability of fructose consumption data (Credit Suisse Research, 2013), and c) manufacturers typically underestimate the fructose content of their products (Ventura *et al*., 2011; Walker *et al*., 2014; Olmedo *et al*, 2021). At such doses, of the many negative health impacts claimed for fructose, it was concluded that the effect of fructose on blood triglycerides seems most supported in the scientific literature and in particular meta-analysis studies (Livesey & Taylor, 2008; Tappy & Rosset, 2019).

The aim of this chapter therefore was to investigate the potential effect of reduced fructose concentration spikes (flattening the fructose spike) reaching the liver, at levels of physiological relevance, on triglyceride release and hepatotoxicity in a model of high physiological relevance i.e. primary human primary hepatocytes. Preliminary work was carried out in the HepG2 cell line.

As it is proposed that it is only excess fructose, of that reaching the liver at any given moment, that will be converted to lipid rather than glucose, glycogen and lactate, or stimulate lipid synthesis, it is hypothesised that a flattened fructose spike will result in reduced lipid synthesis and release into the blood. In this context, flattening the fructose spike means lower levels of fructose in the blood at any given moment in time due to a slower uptake from the gut. This might be achieved by plant derived

natural compounds that inhibit or block fructose transporters in the gut. Slowing down the uptake of fructose from the gut, will also prolong the time the liver is exposed to fructose from a given dose. To investigate this, it was planned to model the effect of flattening the fructose spike (i.e. a lower concentration for longer) on primary human hepatocytes. However, as such material is a valuable resource and not always available, preliminary studies were initiated using HepG2 cells (2.0 Materials and Methodology, 2.1.1 HepG2 cells).

Statistics

Treatments were compared by one-way ANOVA and multi-comparisons were corrected for using Dunnett's statistical hypothesis testing. For two-way ANOVAs multiple comparisons were corrected for using Holm-Šídák's statistical hypothesis testing. (see 2.3.6 Statistcs). Within a cell culture plate treatments and replicates were randomly and evenly distributed across the plate to avoid edge and clustering effects.

3.2 Results

3.1.1 Preliminary HepG2 cell line studies: triglyceride release

HepG2 cells were seeded in 12-well plates at 70,000/well and allowed to adhere overnight. The cells were then treated over a period of 24 hours with a 0-8 mM fructose range as this is ~~perhaps~~ what might be expected to be found in the hepatic portal vein supplying the liver following a large meal or bolus of fructose (see 1.0 Introduction, 1.5 Physiological fructose levels in the hepatic portal vein). However, using such doses, little or no increase in triglycerides in the cell culture supernatant was observed. HepG2 cells were therefore treated with much higher doses of fructose (0-80 mM) over a period of 24 hrs. As the assay actually measures glycerol after using a lipase enzyme to convert triglycerides to glycerol, the samples were also analysed without lipase to detect any free glycerol. Triglycerides were estimated by deduction of free glycerol (minus assay lipase) from total glycerol (plus assay lipase).

Free glycerol levels (Figure 3.1) were found to be about four times higher than the estimated triglyceride levels (Figures 3.2).

Figures 3.1 – 3.4: Effect of low to very high fructose doses on HepG2 cells

Fig 3.1: Free Glycerol released by HepG2 cell supernatants treated with fructose for 24hrs

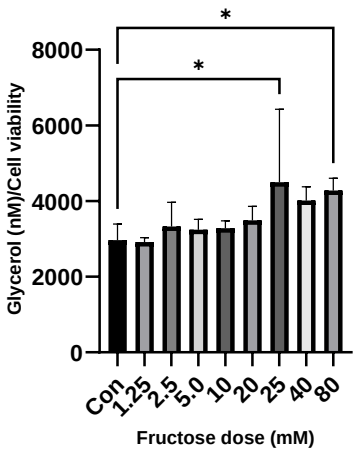


Fig 3.2: Estimated Triglyceride released by HepG2 cell supernatants treated with fructose for 24 hrs

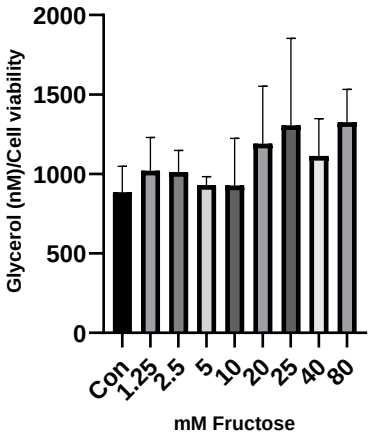


Fig 3.3: Free glycerol + Triglyceride released by HepG2 cell supernatants treated with fructose for 24hrs

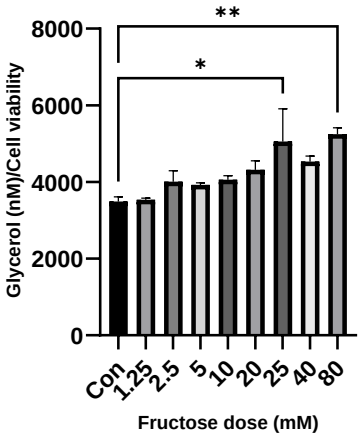


Fig 3.4: Cell viability released by HepG2 cells treated with fructose for 24hrs

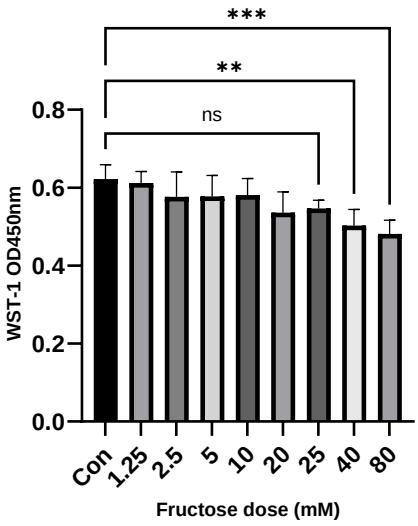


Figure 3.1 – 3.4: Effect of low to very high fructose doses on HepG2 cells. HepG2 cells were treated with fructose 0-80 mM in 24-well culture plates for 24 hrs. Free glycerol level was measured in the absence of the assay lipase. Glycerol nM was normalised to WST-1 as a measure of cell viability. All treatments were compared to the control (Con) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing *= p<0.05, n=5 replicate wells per treatment. Free glycerol levels (Figure 3.1) were found to be about four times higher than the estimated triglyceride levels (Figures 3.2). Significant increases were only seen at the at high non-physiological doses, particularly when free glycerol and triglyceride were combined (Figure 3.3). Fructose treatment showed a dose dependant trend but correlated with reduced cell viability (Figure 3.4)

Triglycerides showed no significant increase even when extremely high levels of fructose were applied (Figure 3.2). Free glycerol levels though did show some increase although only at levels of fructose treatment greater than or equal to 25 mM (Figure 3.1).

Total triglyceride (free glycerol+triglyceride) gave the most convincing (and statistically significant) increase with fructose treatment (Figure 3.3). ~~Perhaps~~ It is considered that triglycerides had been hydrolysed by hepatocyte lipase present in the samples (this was later explored and evidence supporting this demonstrated- see 3.2 Discussion, 3.2.1 Primary human hepatocytes, Glycerol) and therefore the lipase treated analysis without deducting the minus lipase i.e. total glycerol gives the best estimate of ~~"~~triglyceride~~"~~ (Figure 3.3).

Fructose treatment effect on triglycerides showed a dose dependant trend but correlated with reduced cell viability as measured by WST-1 (see 2.0 Materials and Methodology, 2.1.1 HepG2 cells, Cell viability) (Figure 3.4).

No accumulated lipid was seen with Oil Red O staining. The limited success with HepG2 cells may have been compromised by the immortalised cell line having abnormal levels of hexokinase II which may metabolise fructose as well as fructokinase (see 3.0 Discussion, 3.2.2 HepG2 cell-line experiments). More physiologically relevant, cryopreserved primary human hepatocytes were therefore purchased for the following experiments.

3.1.2 Cryo-preserved primary human hepatocytes: triglyceride release, cytotoxicity and flattening the fructose spike

Fructose dose response in primary human hepatocytes

Cells in 24-well plates cell were treated with increasing doses of fructose from low physiological levels to 20 mM for 24 hrs. Glucose was also tested at 20 mM as a direct comparison. Again, as seen using HepG2 cells the cell supernatants consisted of mostly free glycerol with little extra generated by assay lipase.

Glycerol- cell culture supernatant lipase digestion of triglyceride (TAG)

As primary human hepatocytes were giving the same issue as HepG2 cells i.e. high levels of glycerol prior to digestion with assay lipase and low levels of triglycerides (glycerol measured after assay lipase digestion) possible explanations were investigated. On investigation it was noted that triglycerides (Myritol 318) could be reduced to glycerol in the presence of post cell culture medium/supernatant (Figure 3.5) suggesting that the hepatocytes were releasing a lipase into the medium which digests released TAG to glycerol.

Figure 3.5: Conversion of Myritol 318 to glycerol by primary human hepatocyte supernatant vs control media

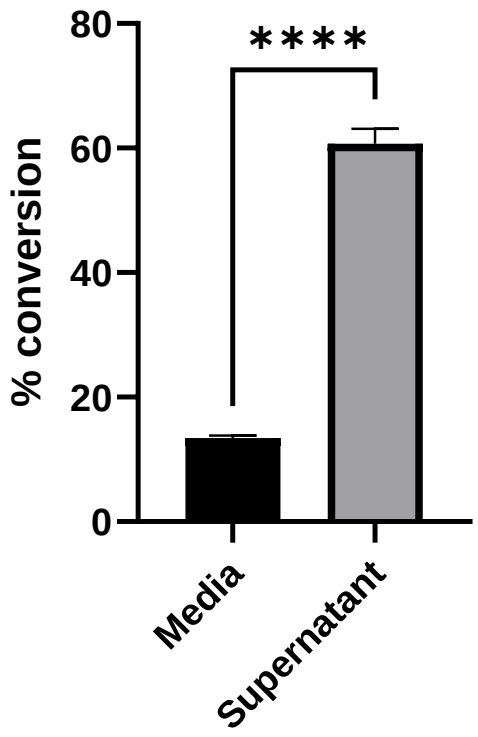


Figure 3.5: Conversion of Myritol 318 to glycerol by primary human hepatocyte supernatant *vs* control media. 70 µM Myritol 318 was added to either 24 hr primary cell culture supernatant or control media for 24 hrs. In the cell culture media treatment a 60% conversion to glycerol was found compared to only 13% with control media. ****= $p<0.0001$, n=3 replicate wells per treatment.

Only 60% conversion of myritol is seen here. As lipoprotein lipase ~~(LPL)~~ shows exponential decay with a half-life of 180 minutes (Yokata *et al*., 2009) it can therefore be reasonably inferred that 100% sample fresh ~~LPL~~ lipase (i.e. as it is secreted)

would be much more active and able to fully convert the TAG that is gradually secreted. (NB: Adding TAGs at 16 µM (the approximate level released over 24 hrs) to living cells was highly cytotoxic, necessitating the use of cell supernatant).

As further verification that the observed glycerol was derived from TAG digestion, fatty acids were also analysed. Free fatty acid levels were found to closely mirror glycerol levels (Pearson product moment correlation r-0.9088, p>0.0001) (Figure 3.7) and as would be expected for digestion from triglycerides, were approximately three times the glycerol level (Figure 3.6). It was also noted in this experiment that after just 2 hrs low level 1.0 mM fructose resulted in a significant increase in lipid (glycerol) (Figure 3.6).

Figure 3.6: Free Fatty Acid and Glycerol released by primary human hepatocytes treated with fructose (2 hrs).

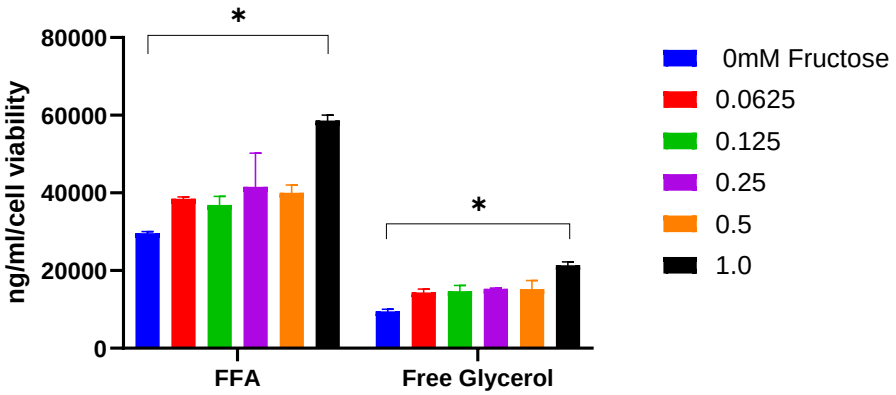


Figure 3.6: Free Fatty Acid and Glycerol ~~in~~ released by primary human hepatocytes treated with fructose (2 hrs). Cells were seeded in 96-well plates treated with 0-1.0 mM doses of fructose for 2 hrs. Free Fatty Acid level was measured as well as free glycerol (i.e. without addition of assay lipase). FFA was approximately three times that of glycerol which is what would be expected if triglycerides are digested by hepatocyte lipase in the samples before analysis. Glycerol and FFA ng/ml were normalised to LDH as a measure of cell viability. After just 2 hrs, 1 mM fructose caused significant lipid synthesis. All treatments are compared to the control (Fructose 0 mM) correcting for multi-comparisons using Dunnett's statistical hypothesis testing *=p<0.05, n=3 replicate wells per treatment.

Figure 3.7: Glycerol vs Free Fatty acid correlation.

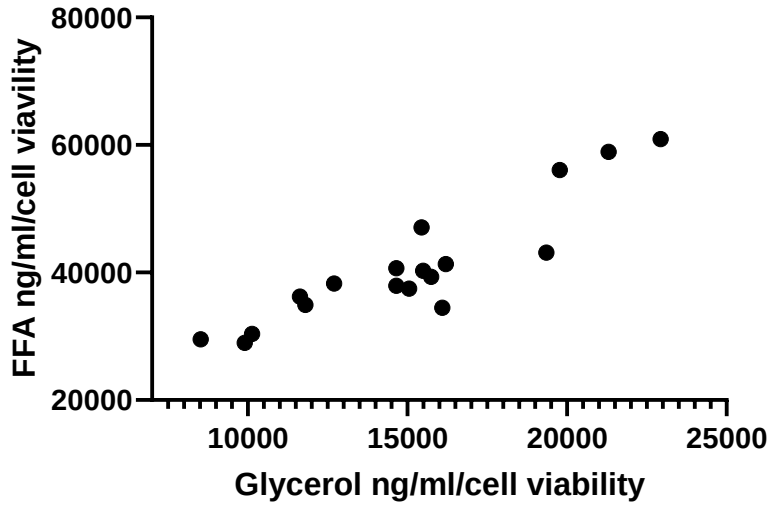


Figure 3.7: Glycerol *vs* Free Fatty acid correlation. Cells were seeded in 96-well plates treated with 0-1.0 mM fructose for 2 hrs. Free Fatty Acid level was measured as well as free glycerol (i.e. without addition of assay lipase). Glycerol and FFA ng/ml were normalised to WST-1 as a measure of cell viability. r=0.908, p<0.0001, n=18.

Total glycerol as a proxy for triglycerides

This data was discussed with thesis secondary supervisor Prof. A. Salter (leading the Future Protein Platform working extensively on lipid metabolism). It was concluded that the reason for the occurrence of high levels of glycerol in samples rather than triglyceride was due to sample lipase released into the culture medium by the primary human hepatocytes. From this point onward total glycerol was therefore accepted as a proxy for triglyceride in these experiments (for further information see 3.2 Discussion, 3.2.1 Primary human hepatocytes, Glycerol).

Using total glycerol as a measure of triglycerides a clear statistically significant dose response with physiologically relevant levels of fructose (1.25-20 mM) was seen (Figure.3.8). Glucose at 20 mM had no effect on triglyceride release.

Figure.3.8: Effect of fructose and glucose on triglyceride released by primary human hepatocytes.

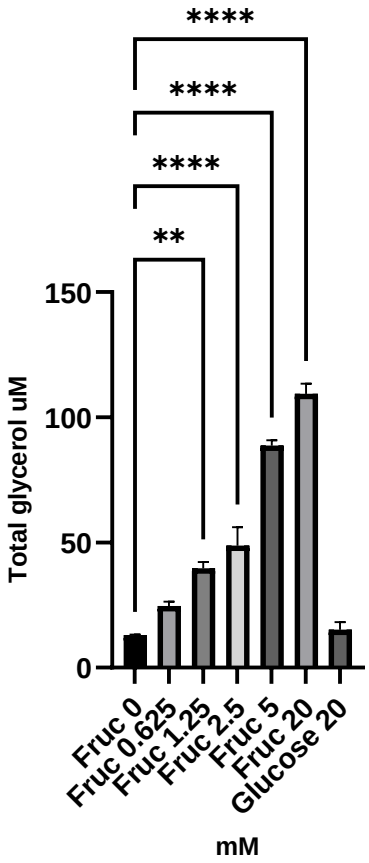


Figure 3.8: Effect of fructose and glucose on Triglyceride released by primary human hepatocytes. Cells were seeded in 24-well plates treated with 0-20 mM doses of fructose from for 24 hrs, with 11 mM glucose. Glucose was also tested at 20 mM as a direct comparison. A clear dose response with fructose was seen. Glucose in comparison resulted in no triglyceride above baseline. All treatments were compared to the (control Fructose 0 mM) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing **=p<0.01 and ****=p<0001, n=3 replicate wells per treatment, except Fruc 20 mM.

3.1.3 Flattening the fructose spike

Having seen a significant increase in triglycerides over 2 hrs with 1 mM fructose, an initial attempt was made to see what effect a model of flattening the fructose spike would have on triglyceride release. Flattening the fructose spike in this study was modelled by treating cells at a high dose of fructose for 1 hr and compared with treating at half the dose for twice as long.

Primary human hepatocytes were seeded into collagen-coated 96-well plates and treated with fructose for 1 hr at a concentration of 2 mM fructose. The cell culture supernatant was then collected after a further 2 hrs post medium change i.e.3 hrs post treatment initiation. Alternatively, cells were treated for 2 hrs at a concentration of 1 mM fructose and supernatant collected after a further 1 hr i.e. 3 hrs post treatment initiation. This was performed for a range of glucose doses from 6-9 mM. Triglycerides were analysed in the cell supernatants as previously described as well as lactate dehydrogenase (LDH) in lysed cells to normalise by cell viability.

The effect of “flattening the fructose spike on triglyceride levels was dramatic. At 6 mM glucose there was a modest 30% reduction in triglycerides, although not statistically significant, increasing steadily to an 83% reduction at the high level 9 mM glucose, which was statistically significant (Figure 3.9). Although any effect on triglyceride release had not previous been seen in these studies with glucose at 20 mM (Figure 3.5), here there was a dose-dependent effect for 6-9 mM glucose (Figure 3.9). This might be associated with the fact that in previous experiments, the basal maintenance glucose level was 11.1 mM, whereas here it was only 6 mM. For each glucose dose triglyceride release appears substantially less for 2 mM fructose for 2 hrs *vs.* 1 mM fructose for 2 hrs, however, this was only statistically significant at 9 mM glucose. Overall, across all glucose treatments there was a statistically significant reduction in triglycerides released by the lower fructose for a longer time (“flattened fructose spike”) by two-way ANOVA (Figure 3.9, Appendix III (a)).

Figure 3.9 Effect of "flattening the fructose spike" on triglyceride ~~in~~ release by primary human hepatocytes

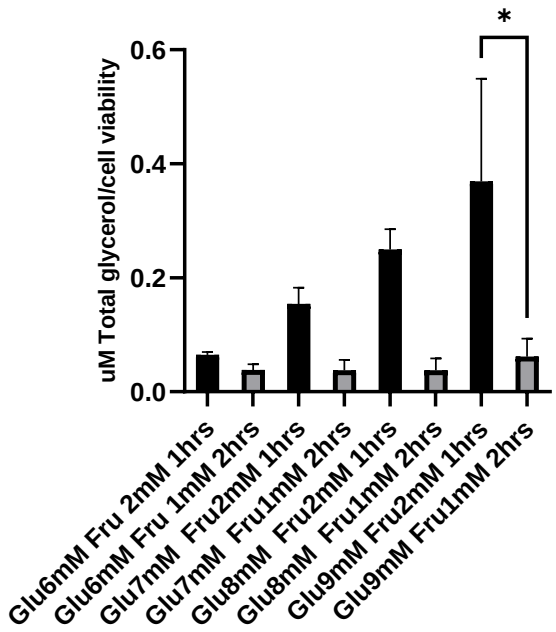


Figure 3.9: Effect of "flattening the fructose spike" on triglyceride ~~in~~ release by primary human hepatocytes. Cells were seeded in 96-well plates and maintained overnight in 6 mM glucose. The cells were treated with fructose for 1 hrs at a concentration of 2 mM fructose and the cell culture supernatant collected after a further 2 hrs post medium change i.e. 3 hrs post treatment initiation. Alternatively, cells treated for 2 hrs at a concentration of 1 mM fructose and supernatant collected after a further 1 hr i.e. 3 hrs post treatment initiation. This was performed for a range of glucose

doses from 6-9 mM. Glycerol µM was normalised to LDH as a measure of cell viability. A two-way ANOVA with Holm-Šídák's multiple comparisons test was performed on 2 mM *vs.* 1 mM for each glucose concentration which showed a significant difference for glucose 9 mM only **=$p<0.01$, n=3 replicate wells per treatment. A two-way ANOVA without comparisons over all glucose treatments showed a significant difference for fructose treatments $p<0.001$ (Appendix III).

A follow up experiment again looked at the effect of modelling "flattening the fructose spike" under more physiological conditions i.e. over a number of modelled "meals" during the day (see figures 3.10-3.15 below, and 2.0 Materials and Methods, 2.1.2 Primary Human Hepatocytes, Treatment of cells for further details).

~~). Cells were treated in 96well plates over 3 "meal" periods:~~

~~"Meal"1- at 9.00am:~~

~~(i) at a high dose of 2 mM fructose with collection of medium after 1hr, refreshment with medium without fructose medium, and then collection again after 2hrs.~~

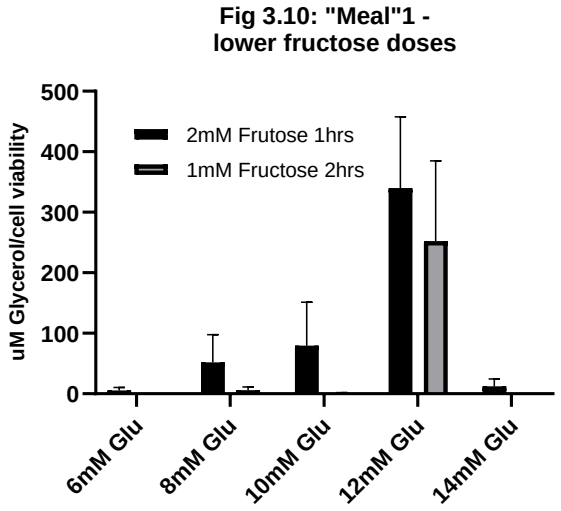
~~(ii) at half the high dose i.e. 1 mM fructose with collection of medium after 2hrs and refreshment with medium without fructose, and collection again after 1hr.~~

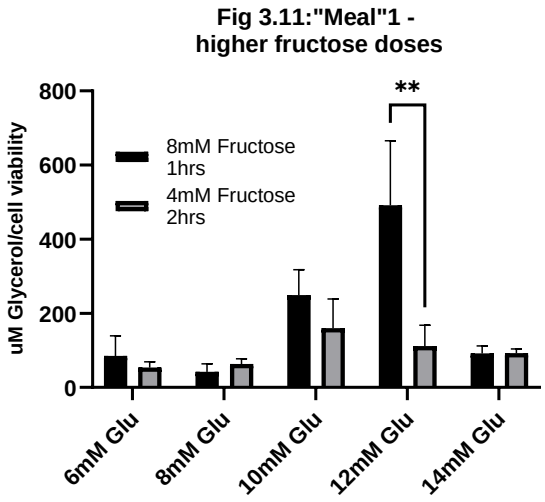
~~This was repeated at 12.00am ("meal" 2) and 3.00pm ("meal" 3). Samples were pooled and snap frozen in liquid nitrogen and stored at -80°C for analysis of triglycerides and cell toxicity.~~

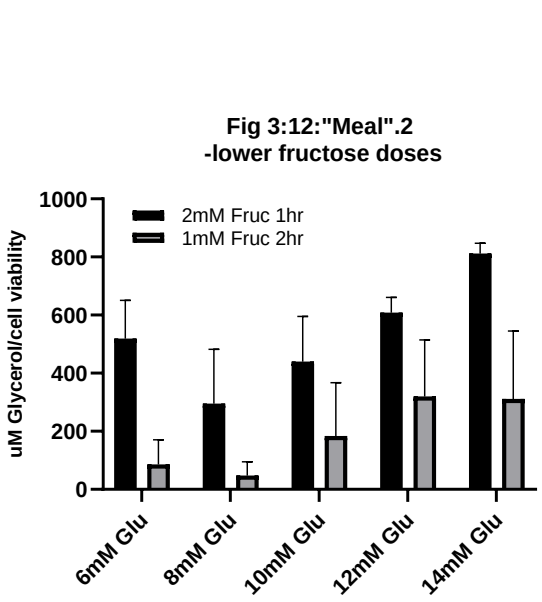
~~The same experiments were performed as above for 8 mM fructose for 1hr *vs.* 4 mM fructose for 2hrs of over 3 "meals".~~

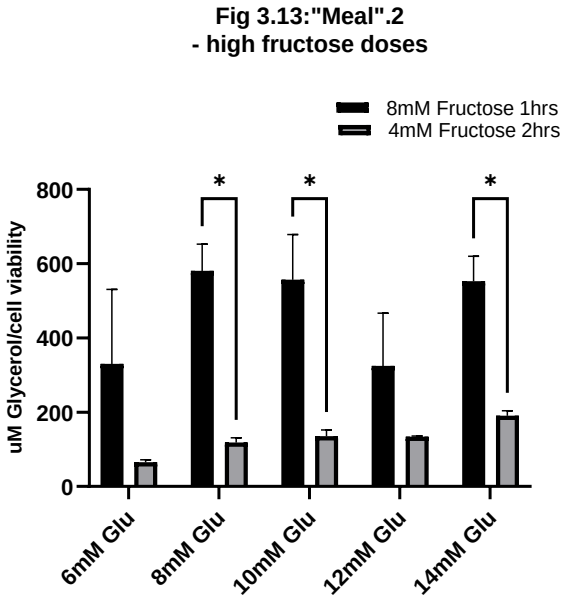
After “meal”1 for 2 mM fructose for 1 hr *vs*. 1 mM fructose for 2 hrs, at just 6 mM glucose there was little or no detectable triglyceride release (Figure 3.10). At 8-, 10- and 12-mM glucose triglyceride release increased dose dependently with lower levels for the lower levels of fructose for twice as long i.e. a “flattened fructose spike”. The differences however were not significant for each of these glucose doses. Overall, across all glucose treatments there was not a statistically significant reduction in triglycerides released by the lower fructose for a longer time by two-way ANOVA (Figure 3.10, Appendix III (b)).

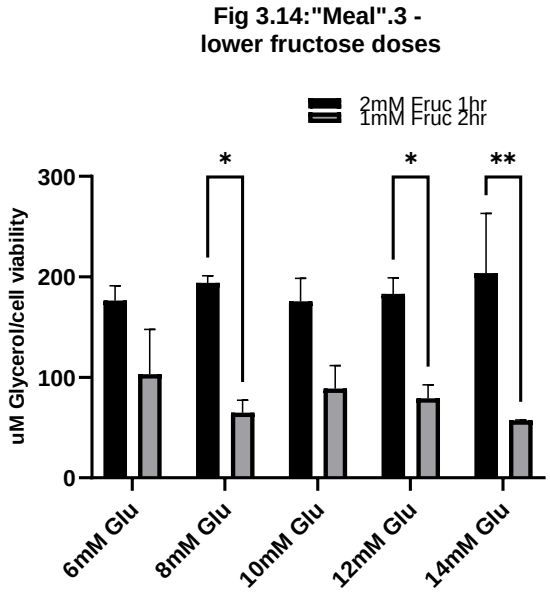
Figure 3.10 – 3.15: Expt.2: Effect of "flattening the fructose spike" on triglyceride release in primary human hepatocytes











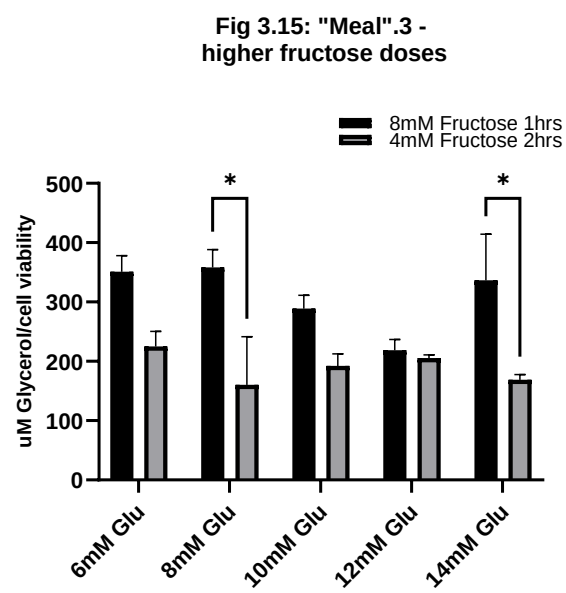


Figure 3.10-15: Expt.2 Effect of "flattening the fructose spike" on triglyceride release by primary human hepatocytes over 3 “meals”. Cells were seeded in 96-well plates and maintained overnight in 4 mM glucose. The cells were treated at 09:00 (“meal”.1), 12.00 (“meal”.2) and 15:00 hrs (“meal”.3) with fructose for 1 hrs at a concentration of 2 or 8 mM fructose and the cell culture supernatant collected after a further 2 hrs post medium change i.e. 3 hrs post treatment initiation, or treated for 2 hrs at a concentration of 1 or 4 mM fructose respectively and supernatant collected after a further 1 hr i.e. 3 hrs post treatment initiation. This was performed for a range of glucose doses from 6-14 mM and triglyceride release from cells was measured in the culture medium. Triglyceride release was normalised by cell viability (LDH 390nm). A two-way ANOVA with Holm-Šídák's multiple comparisons test was performed on the high fructose mM *vs.* the lower fructose mM for each glucose concentration. Triglyceride release decreased as the fructose spike was flattened for each meal. . Overall, a two-way ANOVA without comparisons showed a difference between fructose treatments for 8 mM vs. 4 mM for all meals and 2 mM vs. 1 mM for meals 2 and 3 (see Appendix III). *=p<0.05, **=p<0.01, n=3 replicate wells per treatment.

After “meal”1 8 mM fructose for 1 hr *vs.* 4 mM fructose for 2 hrs, for 6 and 8 mM glucose there was still little detectable triglyceride release for 4 mM fructose (Figure 3.11), but more so than 1 mM (figure 3.10). At 10- and 12-mM glucose the higher fructose dose for 1 hr gave more triglyceride release but this was only substantial and significant for 12 mM glucose. Overall, across all glucose treatments there was a statistically significant reduction in “triglycerides” released by the lower fructose for a longer time by two-way ANOVA (Figure 3.11, Appendix III (c)).

After “meal”2 for 2 mM fructose for 1hr *vs.* 1 mM fructose for 2 hrs, 6 mM -10 mM glucose, unlike “meal”.1, showed detectable levels of triglyceride release for the lower level of fructose of 1 mM (Figure 3.12). However, despite there being a ~~strong trend~~ substantial decrease in triglyceride release for the flattened fructose spike ~~to be lower~~ at each glucose level, the difference was not significant for any comparisons for each glucose level. Levels of “triglycerides” tended to rise as glucose increased. Overall, across all glucose treatments there was a statistically significant reduction in “triglycerides” released by the lower fructose for a longer time by two-way ANOVA (Figure 3.12, Appendix III(d)).

After “meal” 2 for 8 mM fructose for 1 hr *vs*. 4 mM fructose for 2 hrs, 6 mM -10 mM glucose, compared to “meal”.1, showed substantial higher levels of triglyceride release for fructose levels of 4 mM and 8 mM (Figure 3.13). There was also much more triglyceride release for the higher doses at 1 hrs than the lower doses for 2 hrs and this was statistically significant for all but 12 mM glucose. Levels of “triglycerides” tended to rise as glucose increased for 4 mM fructose, but the 8 mM fructose was approximately the same across all glucose doses. Overall, across all glucose treatments there was a highly statistically significant reduction in “triglycerides” released by the lower fructose for a longer time by two-way ANOVA (Figure 3.13, Appendix III (e)).

After “meal” 3 for 2 mM fructose for 1 hr *vs*. 1 mM fructose for 2 hrs, 6 mM -10 mM glucose showed substantially higher levels of triglyceride release for fructose levels of 1 mM than were seen in the first and second meals (Figure 3.14). Triglyceride did not rise as glucose dose increased for either the 2 mM fructose or the 1 mM. For most doses the 2 mM level of fructose for 1 hr *vs.* 1 mM fructose for 2 hrs gave substantially and significantly more triglyceride release. Overall, across all glucose

treatments there was a highly statistically significant reduction in “triglycerides” released by the lower fructose for a longer time by two-way ANOVA (Figure 3.14., Appendix III (f))

After “meal” 3 for 8 mM fructose for 1 hr *vs*. 4 mM fructose for 2 hrs, all glucose doses showed substantially higher levels of triglyceride release for fructose levels of 4 mM fructose than they were in the first and second “meals” (Figure 3.15). Triglyceride did not rise as glucose levels increased for either the 8 mM fructose or the 4 mM fructose. For most doses the 8 mM level of fructose for 1 hr *vs*. 4 mM fructose for 2 hrs showed a substantial ~~trend for~~ non-significant increased ~~d~~ in “triglycerides”. This was significant at 8- and 14-mM glucose. Overall, across all fructose treatments there was a highly statistically significant reduction in “triglycerides” released by two-way ANOVA (Figure 3.15, Appendix III (g)).

Cytotoxicity

After “meal”1 for 2 mM fructose for 1 hr *vs*. 1 mM for 2 hrs there was no difference in cytotoxicity between the treatments at any glucose level. Overall, across all fructose treatments there was no significant reduction in cytotoxicity by the lower fructose for a longer time (Figure 3.16, Appendix IV (a)).

Figure 3.16 – 3.21: Expt.2. Effect of "flattening the fructose spike" on cytotoxicity in primary human hepatocytes- lower fructose doses.

Fig 3.16: "Meal".1 - lower fructose doses

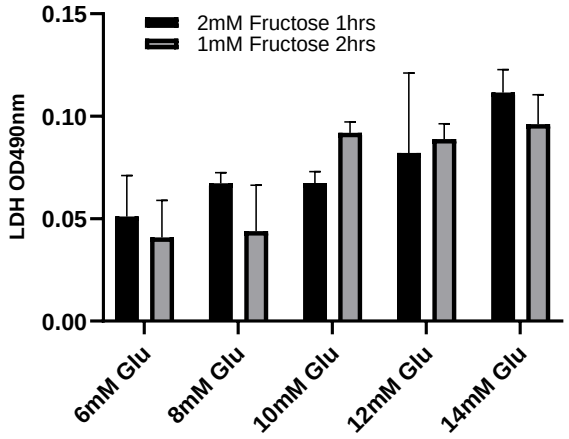


Fig 3.17: "Meal".1 - higher fructose doses

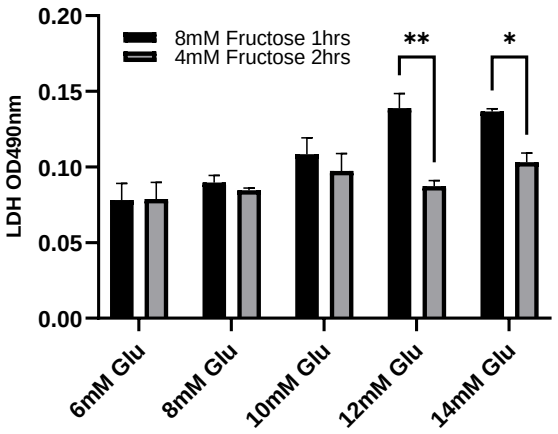


Fig 3.18: "Meal".2 - lower fructose doses

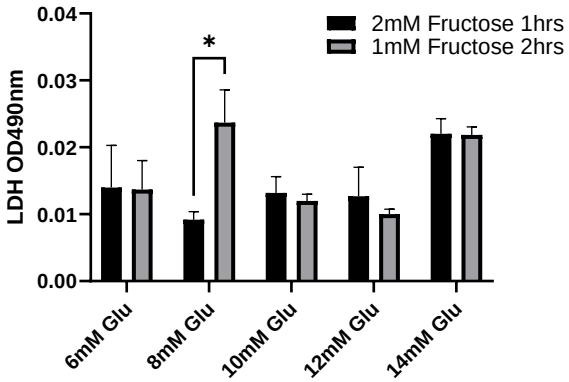
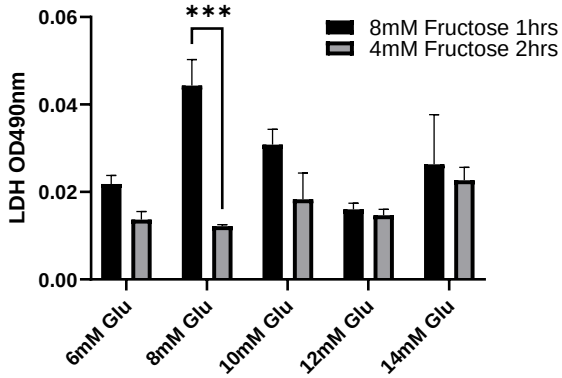
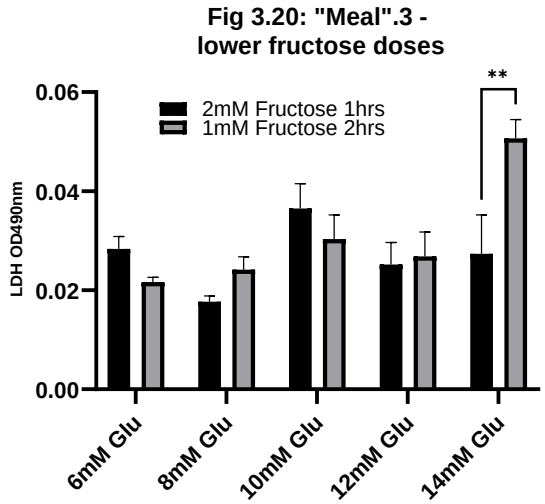


Fig 3.19: "Meal".2 - higher fructose doses





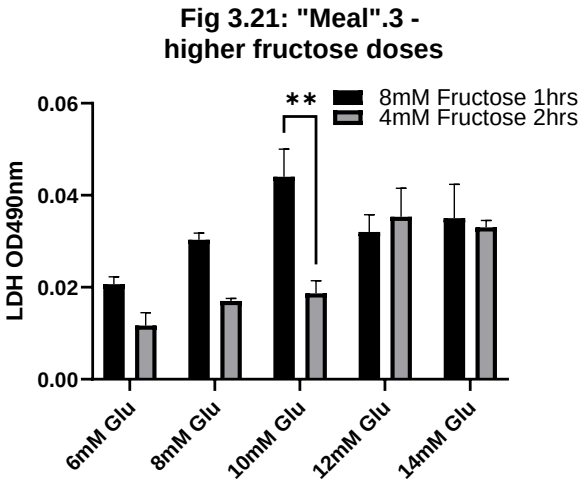


Figure 3.16-3.21: Expt.2. Effect of "flattening the fructose spike" on cytotoxicity in primary human hepatocytes- lower fructose doses. Cells were seeded in 96-well plates and maintained overnight in 4 mM glucose. The cells were treated at 09:00 ("meal".1), 12.00 ("meal".2) and 15:00 hrs ("meal".3) with fructose for 1 hrs at a concentration of 2 or 8 mM fructose and the cell culture supernatant collected after a further 2 hrs post medium change i.e. 3 hrs post treatment initiation, or treated for 2 hrs at a concentration of 1 or 4 mM fructose and supernatant collected after a further 1 hr i.e. 3 hrs post treatment initiation. This was performed for a range of glucose doses from 6-14 mM. A two-way ANOVA with Holm-Šídák's multiple comparisons test was performed on higher fructose mM *vs.* lower mM for each glucose concentration. A two-way ANOVA without comparisons showed a difference between fructose treatments for 8 mM vs. 4 mM fructose for each "meal" but not for 2 mM vs 1 mM (see Appendix IV). *=p<0.05, **=p<0.01, n=3 replicate wells per treatment.

However, after "meal" 1 with 8 mM fructose for 1 hrs *vs* 4 mM for 2 hrs, at higher levels of glucose the higher level of fructose, although for a shorter time, did give significantly greater cytotoxicity (Figure 3.17). Overall, across all glucose treatments there was a highly significant reduction in cytotoxicity by the lower fructose for a longer time by two-way ANOVA (Figure 3.17, Appendix IV (b)).

After "meal" 2 though, with 2 mM fructose for 1 hrs *vs*. 1 mM fructose for 2 hrs, the lower fructose level for longer gave significantly greater cytotoxicity at 8 mM glucose Figure 3.18). However overall, across all glucose treatments there was no significant difference in cytotoxicity between the lower and higher fructose by two-way ANOVA (Figure 3.18, Appendix IV (c)).

After "meal" 2 with 8 mM fructose for 1 hrs *vs*. 4 mM fructose for 2 hrs, the higher fructose level for a shorter time at 8 mM glucose gave significantly greater cytotoxicity (Figure 3.19). Overall, across all glucose treatments there was a

statistically significant reduction in cytotoxicity by the lower fructose for a longer time by two-way ANOVA (Figure 3.19, Appendix IV (d)).

After “meal” 3 with 2 mM fructose for 1 hrs *vs*. 1 mM fructose for 2 hrs, the higher fructose level for a shorter time at 14 mM glucose gave significantly greater cytotoxicity (Figure 3.20). Overall though, across all glucose treatments there was no significant reduction increase in cytotoxicity by the lower fructose for a longer time by two-way ANOVA (Figure 3.20, Appendix IV (e)).

After “meal” 3 with 8 mM fructose for 1 hrs *vs*. 4 mM fructose, at 10 mM glucose, the higher fructose level for a shorter period showed significantly greater cytotoxicity (Figure 3.21). Overall, across all glucose treatments there was a significant reduction in cytotoxicity by the lower fructose for a longer time by two-way ANOVA (Figure 3.21, Appendix IV (f)).

3.2 Discussion

Summary findings:

Supportive evidence has been demonstrated in this study in primary human hepatocytes *in vitro*, that flattening the fructose spike reaching the liver could substantially limit the release of triglycerides into circulation and reduce cytotoxicity. Such was found to increase in magnitude and reliability over the course of three "meals" during a day i.e. from fasted to well fed.

3.2.1 Introduction

The literature review undertaken for this thesis led to a conclusion that postprandial triglycerides may be the most convincing negative health impact of fructose at normal doses of added fructose (1.0 Introduction, 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides). The aim of this chapter was to investigate the effects of a model of "flattening the fructose spike" reaching the liver on triglyceride release and cytotoxicity (see 1.6. Research Plan, 1.6.1 Aims). Many studies have or are investigating compounds, reagents, plant extracts, etc. with the aim to inhibit the uptake of fructose from the gut and limit its possible negative impacts on health (Table 4.2). However, it is not certain that inhibiting fructose uptake will have the desired effect of improving fructose's impact on health. Even if the uptake of fructose from the gut is slowed down it will likely still all reach the liver and potentially be metabolised to or stimulate lipid syntheis. Therefore, whether flattening the fructose spike (lowering levels of fructose reaching the liver, but for longer) can reduce triglyceride release and cytotoxicity was investigated. "Flattening the fructose spike" was modelled in this thesis by treating primary human hepatocytes with high (but physiologically relevant) levels of fructose and comparing to half the dose for twice as long). It has been suggested that only excess fructose at any given moment will be metabolised to triglycerides (Lim *et al*., 2010). If the cell needs energy, the metabolites of fructose will be metabolised to 3-carbon metabolites and oxidised to carbon dioxide and water generating ATP (see 1.0

Introduction, 1.2. Potential mechanisms to explain the negative impact of fructose on health, Figure 1.5). For glucose, when the cell has sufficient energy, citrate and ATP accumulate and feedback-inhibit phosphofructokinase, the rate-limiting enzyme in glycolysis. For fructose, its key enzyme (fructokinase) and down-stream metabolism is not feedback-inhibited by energy status and therefore fructose continues to be taken up by the liver, its main organ of metabolism. It is proposed that this is ~~probably~~ because fructosylation by fructose is 7-8 times faster than glycation by glucose (Dills 1993). Fructose and glucose are isomers with the same molecular weight. Although not fully understood, it is thought that it is partly because fructose spends more time in the free-chain linear form rather than its pyranose form, due ~~perhaps~~ to hydroxyl group orientation, its carbonyl group (C=O) is free and more able to react with other molecules (Bun and Higgins, 1981). As with glucose glycation, fructose fructosylation causes tissue damage by attacking cell proteins amino acids etc. impairing function. It is therefore presumed that fructokinase rapidly metabolises fructose, keeping a strong concentration gradient to quickly remove fructose from the circulation. As such, fructose is mainly metabolised in the liver. If uptake saturates energy needs by the cell, fructose is mostly converted to glucose and stored as glycogen, and lactate. ~~Perhaps~~ It may be considered that only when glycogen stores are full is some fructose converted to lipid, which can be released into the circulation as triglycerides in the form of very-low-density lipoprotein (VLDL) particles or lipoproteins, or stored in the liver as fat droplets, which may be harmful to health. The aim of this chapter therefore was to use *in vitro* primary human hepatocytes to investigate whether a model for flattening the fructose spike reaching the liver would reduce its potentially harmful effects of triglyceride release and cytotoxicity.

3.2.2 HepG2 cell-line experiments

Here, initial studies were performed using HepG2 cells, an immortalised cell line commonly used for studying the liver *in vitro*. However, it was only possible to induce these cells to release substantial levels of triglyceride in response to non-physiological levels of fructose (20-80 mM) (Figures 3.1 and 3.3). This could be due the fact that HepG2 cells have greatly increased hexokinase II (HKII) expression

(Speicher *et al.,* 2010). Rather than HKII, unlike most tissues, normal liver cells have glucokinase (HKIV), a hexokinase that has low affinity for fructose which is therefore metabolised by fructokinase (ketohexokinase) which has high affinity for fructose (K_m =0.5 mM) (Stetten & Goldsmith, 1981). The HKII of most tissues and HepG2 cells has a reasonable affinity for fructose (K_m =7 mM) (Stetten & Goldsmith, 1981) and so in HepG2 cells some fructose will be metabolised by HKII to fructose-6-phosphate and subsequently by phosphofructokinase and therefore will be feedback inhibited by the energy status of the cell. Consequently, it can be more difficult to achieve excess metabolism of fructose in this cell line. When high levels of fructose are metabolised by the liver, ATP can become depleted as it is phosphorylated to form fructose-1-phosphate. This can lead to the build-up of ADP and AMP which may be broken down to uric acid which is potentially a cause of hypertension, joint inflammation and liver cytotoxicity. Studies investigating this pathway have found it difficult to achieve ATP depletion in HepG2 cells without using a HKII inhibitor (Speicher *et al.,* 2010). This may be why it was found to be difficult to induce substantial triglyceride release in HepG2 cells with fructose. It was also found that much higher levels of glycerol in cell supernatants compared to triglyceride were observed. As such, primary human hepatocytes were used in subsequent experiments.

3.2.3 Primary human Hepatocytes

Glycerol

Even using more physiologically relevant cells, high levels of glycerol compared to triglycerides were still observed. *In vivo*, in humans, plasma level of free glycerol is rarely above 280 µmol/L (Jessen *et al*., 1990) and when in obesity are a result of increased adipose tissue mass leading to an increase release of glycerol (Bolinder *et al*., 2000; Janson *et al*, 1992). Triglycerides are typically less than 1.7 mmoles/L (Jessen *et al*., 1990) so we would have expected to see triglycerides in culture medium from liver cells very much higher than glycerol. Upon investigation, therefore, it appeared that triglycerides were being broken down to glycerol as they were released from the cells. Myritol-318, a c8-10 chain length triglycerides which is similar to that produced by human hepatocytes, could be metabolised by conditioned media from hepatocytes in culture (Figure 3.5). Hepatocytes are known to secrete ~~lipolipase and hepatocyte~~ lipase to help the lipid they release become available to other tissues (Hermier *et al.,* 1991). Triglycerides released in culture would be constantly exposed to freshly secreted lipase that had had some time to build up, as neither are able to be transported away from the liver cells into the circulation. That triglycerides can be digested in hepatocyte supernatants has been reported previously (Hemier *et al.,* 1991). Attempts to inhibit the released of lipase activity *in vitro* have been made by others, but this also affects endogenous lipase and therefore TAG release (Hermier *et al.,* 1991). To confirm this hypothesis, free fatty acid levels in the samples were measured and it was found that they mirrored the glycerol levels in profile with a Pearson product moment correlation r-0.9088, $p>0.0001$ (Figure 3.7) and were approximately three-times greater in concentration (Figure 3.6), as would be expected from the molecular structure of triglycerides, i.e. a glycerol backbone bound by esterification to three fatty acids. Total glycerol was therefore accepted as a proxy for triglyceride in these experiments.

Triglycerides

Adding the high free glycerol levels found in the measurement to the small levels of triglyceride to give total glycerol, the data made better sense. It was found that physiological levels of fructose could induce substantial and significant triglyceride

release in primary human hepatocytes in a dose-dependent manner over 24 hrs (Figure 3.8). Whereas glucose, even at 20 mM had no effect. This is what might be expected if the metabolism of excess levels of glucose is feedback inhibited by accumulation of intermediary energy metabolites citrate and ATP and fructose is not (see 1.0 Introduction, (Figure 1.5). Even levels as low as 1.25 mM fructose significantly increased triglyceride release 3-fold (Figure 3.8). Physiological levels of fructose in the hepatic portal vein delivering nutrients straight from the gut to the liver has been recorded at 1-2 mM following a moderate fructose bolus of approximately 20-40g (see 1.0 Introduction, 1.5 Physiological fructose levels in the hepatic portal vein). The average intake of added fructose in the USA can be estimated to be approximately 50 g/day, after consideration of under-reporting in surveys, product content and national supply (production and import) (see 1.0 Introduction, 1.1.1 Pre-literature review issues, Fructose dose- physiological levels). It is also an intake of about 50 g/day fructose that has been reported to increase postprandial triglycerides. This has been shown consistently and confirmed by systematic meta-analyses (Livesey & Taylor, 2008; Tappy & Rosset, 2019) (see 1.0 Introduction, 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides). Half of the population will of course consume greater than 50 g/day.

Having seen a significant increase in “triglycerides” over 2 hrs with 1 mM fructose (Figure 3.6), experiments to model “flattening the fructose spike were explored. A level of 2 mM fructose for 1 hrs was compared with half that fructose dose (1 mM) for twice as long i.e. 2 hrs. This was performed over a range of glucose levels (6-9 mM) as it was anticipated that an ~~boost~~ increase in triglyceride release would not be seen until the energy status of the cells were in excess. The cell supernatant collection 3 hrs after treatment was initiated as a reasonable time window of the expected rise in triglycerides based on lipid profiles reported in the literature (Parks *et al.,* 2008; Stanhope *et al.,* 2015). The effects were dramatic. “Flattening the fructose spike” significantly reduced triglyceride release in the cell supernatant by 83% for the highest levels of glucose (Figure 3.9). As expected, this reduction was small and non-significant at the lowest glucose levels, indicating that excess energy is required to induce lipid synthesis. ~~The trend~~ For each glucose treatment, ~~was for the~~ “flattened fructose” spike ~~to produced~~ gave lower “triglycerides” ~~were measured~~ such that there was a statistically significant reduction overall (Figure 3.9, Appendix III (a)).

Flattening of the fructose spike was then tested at the higher level of 8 mM fructose for 1 hr *vs.* 4 mM and for 2 hrs, and at 2 mM fructose for 1 hr *vs.* 1 mM for 2 hrs, over three periods in succession over the day. This was to represent "meals" starting at 9:00am following a night of low glucose (4 mM) i.e. relatively fasted and at low energy status and glycogen storage. After the first "meal" at 1 mM fructose for 2 hrs, little or no triglyceride release at the lower doses of glucose was observed (Figure 3.10). This is consistent with the cells being in a state of insufficient energy and glycogen storage after an overnight period of low glucose. However, at 2 mM fructose for 1 hr, some triglyceride was detectable even at the lower levels of glucose (Figure 3.10). The data was not statistically significant for any of the glucose doses with respect to high *vs.* low fructose or overall for all glucose doses. ~~but a trend~~ However, generally lower measured triglycerides for lower fructose doses ~~that~~ supports the flattening of the fructose spike hypothesis ~~was evident~~. At the higher levels of fructose (8 *vs.* 4 mM) after "meal" 1, triglyceride release was detectable at the lower 4 mM fructose dose and a clearly significant reduction from 8 mM fructose was seen at the 12 mM glucose dose and overall, across all glucose doses (Figure 3.11, Appendix III (c)). For the second and third "meals" there was substantial triglyceride release at all doses of glucose and fructose, and it was clear that for all doses of glucose the higher fructose gave greater release than the lower level for a longer time (Figures 3.12-15). Overall, this was statistically significant for each "meal" and range of fructose doses (Appendices I (d-g). These data support the hypothesis of this thesis, that flattening the fructose spike reaching the liver i.e. lower levels of fructose for a longer time period, will reduce postprandial lipid.

This is the first time that an attempt has been made to model flattening the fructose spike either *in vitro* or *in vivo*. Few studies can be found investigating triglyceride release in primary hepatocytes or cell-lines at physiological levels of fructose or other (Pinnick *et al*., 2019). Huang *et al*., 2011 treating HepG2 cells with 5.5 mM glucose+5.5 mM fructose *vs.* 11 mM glucose demonstrated a significant increase in triglyceride release over 72 hrs using ^{13}D-glucose by mass isotope distribution analysis. ~~Perhaps~~ Possibly other studies have failed to detect sufficient levels of triglyceride release, particularly in a short time frame as 3 hrs, as it has not been realised that hepatocyte culture medium can contain ~~hepatocyte~~ lipase digesting triglycerides prior to analysis.

Glucose and triglyceride

Over 24 hrs an increase in basal medium level of glucose of 11.1 mM to 20 mM had no effect on triglyceride release, whilst fructose was seen to give a dose dependant manner from 1.25 mM (Figure 3.8). Many studies have reported that fructose is more able to induce lipid synthesis and triglyceride release than glucose, although others have not shown a difference (see 1.0 Introduction, 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides).

In "meal" one at a fixed level of fructose of either 2 mM or 8 mM, increasing glucose increased triglyceride release (Figures 3.11-12). This did not occur in later meals, ~~perhaps~~ possibly because a maximum rate of output of triglyceride is reached. The question therefore arises as to how an increase in glucose leads to an increase in triglyceride release? In theory, if the cells have sufficient energy, citrate and ATP will feedback-inhibit glycolysis and glucose will be stored as glycogen. If glycogen reserves are optimal, one might expect that glucose would accumulate in the cell and no longer be taken up into the cell by GLUT2 which is only a facilitative transporter and needs a concentration gradient. This is an interesting question as many studies that have compared fructose to glucose, or have looked at glucose on its own, show that glucose increases blood triglycerides (see 1.0 Introduction, 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides) but without explaining why, given its theoretical feedback inhibition. One reason could be: as glucose accumulates, it is ~~perhaps~~ converted to fructose via the polyol pathway. Many studies have shown that this pathway is clearly available and utilised in the liver of mice (Park *et al.,* 2017). Fructose conversion to glucose via the polyol pathway has usually been associated with extreme conditions such as hypoxia, etc. However, there is now evidence that production may be elevated by dietary interventions such as high glucose (or complex carbohydrates, high salt, diabetes and alcohol (Nakagawa *et al.,* 2021).

In the polyol pathway glucose is reduced to sorbitol by aldose reductase and NADPH and then oxidised to fructose by sorbitol dehydrogenase and NAD+. Studies have shown that during certain conditions such as hypoxia, glucose is converted to fructose to generate energy via the fructose-driven glycolytic pathway to avoid phosphofructokinase feedback inhibition (Park *et al.,* 2017). To explain the role that

glucose plays in the dyslipidemia aspects of metabolic syndrome Lanaspa *et al.,* hypothesised that aldose reductase could be sufficiently expressed or active in the liver to metabolise at least some glucose to fructose. Having shown ketohexokinase (fructokinase) knockout mice on 10% fructose drinking water were protected from steatosis, using the same knockout *vs.* wildtype on 10% glucose in the drinking water, they showed protection against glucose-induced insulin resistance and fatty liver. In addition, they found fructose to be elevated in wild type liver and blood, as well as higher sorbitol in the blood. By western blot analysis, they found this to be associated with greater levels of ketohexokinase (KHK) and aldose reductase (Lanaspa *et al.,* 2013). They also showed aldose reductase and KHK expression were particularly expressed in areas of severe hepatic steatosis. Aldose reductase knockout mice also had reduced fatty liver.

A by-product of high dietary fructose, uric acid, has been reported to stimulate aldose reductase expression in the polyol pathway and the conversion of glucose to fructose in rats (Sanchez-Lozada *et al.*, 2019). With high glucose they saw an increase in endogenous fructose and accumulation of fat, which was significantly reduced by an inhibitor of xanthine oxidase, the key enzyme in uric acid synthesis.

High salt, which is normally associated with high blood pressure has also been linked to obesity and diabetes. Maeir *et al.,* 2021 provide evidence in mice that this might be via stimulation of the polyol pathway. They showed that blocking the metabolism of fructose blocks high salt's hypertension effects. They note that in adults, in Japan, diabetes and fatty liver are predicted by a high salt diet. ~~Perhaps~~ This gives us an indication that the polyol pathway is also active in human liver.

Further evidence for activity in the human liver comes from an interesting twist on the polyol pathway, which is the possibility of it explaining how ethanol metabolism in liver is accelerated by fructose, the so called "fructose effect" (Tygstrup *et al*., 1965). Studies have suggested that glucose can be synthesised from fructose by the polyol pathway in reverse resulting in mitochondrial NADH oxidation. In a study in which both rats and isolated hepatocytes were treated with fructose, oxidation of ethanol was ~~boosted~~ increased by more than 50% along with glucose and sorbitol production. Blocking the mitochondrial respiratory chain however, had no impact on the result. However, the increase in glucose and sorbitol supports the polyol

pathway hypothesis (Villalobos-Garcia *et al.,* 2021) and also the existence of the pathway in human liver.

If the polyol pathway is active in human liver: (a) slowing glucose uptake might also help flatten the fructose spike; and (b) limiting uric acid and hence stimulation of the polyol pathway by flattening the fructose spike by slowing fructose uptake may have an added benefit by also providing some protection from glucose's adverse effects.

Cytotoxcity

Cytotoxicity follows in a similar pattern to triglycerides – rising throughout the "meals" of the day and generally showing that "flattening the fructose spike" reduces cytotoxicity (Figures 3.16-21). This though, is generally only statistically significant at the higher range of fructose i.e. 8 mM 1 hr *vs.* 4 mM for 2 hrs (Figures 3.17, 3.19, 3.21, Appendices II (b, d, and f). Such levels of hepatic portal fructose might only occur after a large intake of fructose. One 339 ml can of cola would contain 39g of sucrose and therefore 19.5g of fructose, twice the amount leading to 1 mM hepatic portal fructose in rats (Reagan-Shaw *et al*., 2007, and see 1.0 Introduction, 1.5 Physiological fructose levels in the hepatic portal vein). This is consistent with a 25g fructose dose in humans being estimated to give >2 mM hepatic portal fructose Bizeau & Pagliassotti, 2005). So, one might need up to four such doses (e.g. 4 cans of cola) to give 8 mM. This may not be unlikely in the upper quartile of intake in the US but is far from the average of 50 g/day (Vos *et al*., 2008, Marriott *et al*., 2009). However, most such studies are performed over short time periods (1 day- 2 weeks). Regular high fructose consumption ~~boosts~~ stimulates the number of GLUT5-specific fructose transporters in the gut and would lead to greater uptake (Jang *et al*., 2020).

Having provided support for a potential benefit in flattening the fructose spike reaching the liver in reducing triglyceride release, these potential benefits can be explored.

3.3 Summary

The aim of this chapter was to investigate the potential effect of reduced fructose concentration spikes (flattening the fructose spike) reaching the liver, at levels of physiological relevance, on triglyceride release and hepatotoxicity in a model of high physiological relevance i.e. primary human primary hepatocytes. As such, supportive evidence has been demonstrated here in primary human hepatocytes *in vitro*, that flattening the fructose spike reaching the liver could substantially limit the release of triglycerides into circulation and cytotoxicity. Limitation of triglycerides and cytotoxicity was found to increase in magnitude and reliability over the course of three "meals" during a day i.e. from fasted to well fed. These observations also support the ideas that a) fructose needs to be in excess, beyond that needed to sustain cellular ATP, and b) that a single dose can take liver cells into excess and increase triglyceride release, potentially ~~boosting~~ increasing postprandial triglycerides, even if over the course of a longer period the system is in a neutral energy balance. This ~~perhaps~~ could explain why some studies (see meta-analyses: Livesey & Taylor, 2008; Tappy & Rosset, 2019) even show elevated triglyceride for fructose when treated with the same calories as a weight maintenance control, and the same.

This is the first time that an attempt has been made to model flattening the fructose spike either *in vitro* or *in vivo*. Triglyceride release has rarely been reported for primary hepatocytes or cell-lines. ~~Perhaps~~ This ~~is~~ could be because, as indicated here, it is not generally realised that hepatocyte culture medium can contain ~~hepatocyte~~ lipase which can digest triglycerides prior to analysis. As such human primary hepatocytes are potentially a useful model for research programmes to avoid the use of animals and expense of human subjects.

Postprandial triglycerides are considered to be a significant risk factor in a number of major health concerns (see Appendix I). E.g: Atherosclerosis, the leading cause of mortality worldwide and the underlying cause of about 50% of all deaths in Westernized society and 32% globally (Pahwa and Jialal, 2021). This is a massive global burden for which primary prevention therapies are needed; Gestational diabetes has 2-10% prevalence, depending on population group (Rodriguez and Mahdy, 2021); Pre-eclampsia occurring between 2 and 8% of all pregnancies

globally (Karrar and Hong, 2021); Non-alcohol fatty liver disease is the World's most common liver disorder affecting 25% of the population (Marjot *et al.,* 2020); Cancer, in 2018, was the cause of death of 13% of males and 9% of females with 37% of the global population getting some type at some point (WHO, 2018); Kidney disease affects 10% of the population worldwide with millions dying (National Kidney Foundation, 2021); Acute pancreatitis has a population occurrence of 6-7% (Gapp and Chandra, 2021).

Flattening the fructose spike in the small intestine might also make some contribution to limiting the overall level of triglycerides in circulation (see Appendix I). There may also be some potential health benefits in limiting intestinal lipotoxicity, bacterial species disruption and gut barrier leakiness leading to associated pathologies (see Appendix I).

These conditions can be treated with pharmaceutical drugs with varying degrees of effectiveness and side effects. However, ~~perhaps~~ an additional approach ~~w~~could be to invest in prevention. ~~Technology~~ Plant natural compounds and extracts that can flatten the fructose spike reaching the liver and reduce the release of triglycerides into circulation may be just what is needed. In this thesis possible natural technologies from plant sources have been investigated that have the potential to be cheap, safe and cost-effective. This work will be discussed in chapter 4.

4.0 ~~Technology~~ Plant natural compounds and extracts to flatten the fructose spike.

4.1 Introduction

A key aim of this thesis (see Introduction 1.0, 1.6.1 Aims) was to establish potential benefits of controlling the fructose spike. Based on levels of fructose that might be considered to be within a normal dose range i.e. approximately 50 g/day (Vos *et al*., 2008, Marriott *et al*., 2009), it was investigated as to which of the many negative health impacts claimed for fructose may be more likely to impact biomarkers of health. Of these the effect of fructose on blood postprandial triglycerides seemed one that is most supported in the scientific literature and in particular meta-analysis studies (Livesey & Taylor, 2008; Tappy & Rosset, 2019, see Introduction 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides).

As it is proposed that it is only the excess fructose (of that reaching the liver or enterocytes at any given moment) that may be converted to lipid rather than glucose, glycogen and lactate, it is hypothesised here that a flattened fructose spike will result in reduced lipid synthesis and release into the blood. By flattening the fructose spike, the liver or enterocytes~~,~~ receive lower levels of fructose at any given moment due to a slower uptake from the gut, although for a longer time.

A key aim of this chapter therefore was to identify natural plant compounds or extracts that have the potential to reduce fructose spikes through inhibition of fructose uptake from the gut. As such, this was investigated using an optimised *in vitro* gut cell line transport model to test for plant-derived natural compounds that potentially inhibit or block fructose or glucose transporters in the gut and look for patent protectable synergies.

Sugars are transported into the blood from the gut by a wide group of membrane proteins. These in include glucose transporters (GLUTs) which facilitate the transport of monosaccharides across the plasma membrane of gut cells by passive diffusion. The first step involves transport from the gut lumen into the gut cell enterocytes. Several GLUT family members may be capable of transporting fructose; however, GLUT5 is the only member demonstrating specificity for fructose i.e. it does not show

the capacity to facilitate transport of galactose, glucose or other monosaccharides. Sequence homology with GLUT5 suggests that GLUT7, GLUT9a/b, GLUT8, GLUT11, and GLUT12 may also have the ability to transport fructose: (Manolescu *et al*., 2007; Doujard & Ferris, 2008).

GLUT2 is mainly recognised for transporting glucose across the basolateral membrane of the small intestine to the blood as well as high concentrations of glucose across the apical membrane of the lumen into gut enterocytes (Kellett and Brot-Laroche, 2005). However, GLUT2 has also been shown to transport fructose across the basolateral membrane (Jones *et al*., 2011) and has been proposed to be effective in the transport of fructose from the gut lumen at the apical membrane (Douard & Ferris, 2008), although it is not certain to what extent. GLUT5 has a K_m of 15 mM for fructose (Kane *et al*., 1997), whereas levels of fructose in the gut may reach around 25 mM*. It has therefore been suggested that the GLUT5 transfer rate may be saturated at low levels, and uptake increased through joint absorption with glucose via GLUT2 (Ushijima *et al*., 1991). Studies have demonstrated that the greatest absorption rate occurs when glucose and fructose are administered in equal quantities (Fujisawa *et al*., 1991) as in most normal consumption, for example when fructose is ingested as part of the disaccharide sucrose or HFCS.

[*Levels of fructose in the gut probably reach in the region of up to 25 mM, as rats on a 65% of total energy fructose diet had up to 100 mM fructose in the intestinal lumen (Ferraris *et al*. 1990; Jiang & Ferris, 2001- see review Douard and Ferraris, 2013). This is ~~perhaps~~ several times the normal human consumption level even over a whole day. Average consumption in the US is approximately 10% of total energy (Vos *et al*., 008, Marriott *et al*., 2009)].

An aim of this study was to discover inhibitors of fructose transport from the gut without concern for which transporter or transporters were being inhibited. However, as GLUT5 and ~~perhaps~~ possibly GLUT2 are believed to be the main fructose transporters in the small intestine, a well-established *in vitro* glucose transport model for these two transporters was first optimised. Caco2 cell monolayers (a cell line derived from a human colorectal adenocarcinoma) have been widely accepted as a potent *in vitro* model to predict intestinal drug permeability in humans (Karlsson,

1991; Jezyk *et al*., 1993; Yamashita et., 1997). In this thesis a model established for total cumulative glucose transport across differentiated Caco-2 monolayers on transwell permeable inserts (Yamashita *et. al*. (2002) was adapted and used.

Statistics

Treatments were compared by one-way ANOVA and multi-comparisons were corrected for using Dunnett's, Tukey's or Holm-Šídák statistical hypothesis testing where appropriate (see 2.3.6 Statistics). Two-way ANOVA multiple comparisons were corrected for using Holm-Šídák's statistical hypothesis testing. Within a cell culture plate treatments and replicates were randomly and evenly distributed across the plate to avoid edge and clustering effects.

4.2 Results

4.2.1 Fructose assay optimisation

Fructose and glucose were analysed by an enzymatic/spectrophotometric assay based on that of Campbell *et al*., (1999). Briefly, glucose and fructose were phosphorylated by the yeast hexokinase enzyme. Glucose-6-phosphate (G6P) was then oxidized by glucose-6-phosphate dehydrogenase (G6PDH) with the concomitant reduction of $NADP^+$ to form NADH which was measured spectrophotometrically. Fructose-6-phosphate was then isomerised to G6P by the addition of phosphoglucose isomerase, which was oxidized by the G6PDH already present and the resultant total NADH measured. Fructose concentration was derived from deducting the first measurement (glucose concentration) from the second (glucose+fructose) concentration.

Initial fructose and glucose transport experiments with 25 mM of each demonstrated an approximately ratio of 4 glucose molecules to 1 fructose that had been transported across a Caco2 monolayer. A standard curve therefore with glucose and fructose in these proportions was subsequently used.

However, it then became apparent that although full recovery of glucose standards was being achieved compared to glucose standards without fructose, fructose levels were only approximately 50% recovered compared to fructose standards without glucose (Table 4.1).

Table 4.1: Effect of glucose on fructose analysis

Glucose/Fructose ratio mM	Glucose analysis mM	Fructose analysis mM
0.4: 0.1	0.389	0.035
0.8: 0.2	0.833	0.051
1.6: 0.4	1.502	0.195
3.2: 0.8	3.060	0.418

Table 4.1: Effect of glucose on fructose analysis: ~~by enzyme/spectrophotometric methodology~~ A 4:1 glucose to fructose ratio standard curve was generated in phosphate buffered saline and fructose and glucose were analysed. The table shows incomplete recovery of fructose when compared to fructose standards in the absence of glucose.

Recovery of fructose was not a problem when equimolar levels of glucose and fructose were mixed, but was for the higher gluocose:fructose ratio standards necessitated by concentrations found after transportation in the Caco2 monolayer model. At first it was thought that this might be due to inhibition by glucose of fructose conversion to F6P by hexokinase, or conversion to G6P by glucose phosphate isomerase, for which glucose is also a substrate. If just competitive inhibition, this should have been resolved by allowing the reaction to progress for longer, or by adding more reagents. However, neither action gave any improvement in recovery.

For equimolar fructose and glucose at levels of fructose anticipated i.e. 200-1000 µM, little impact on fructose recovery was found up to 4 hrs post mixing. However, overnight there was a tendency for recovery of fructose to drop at higher concentrations (Figure 4.1).

Figure 4.1: Fructose standards- effect of time/freshness

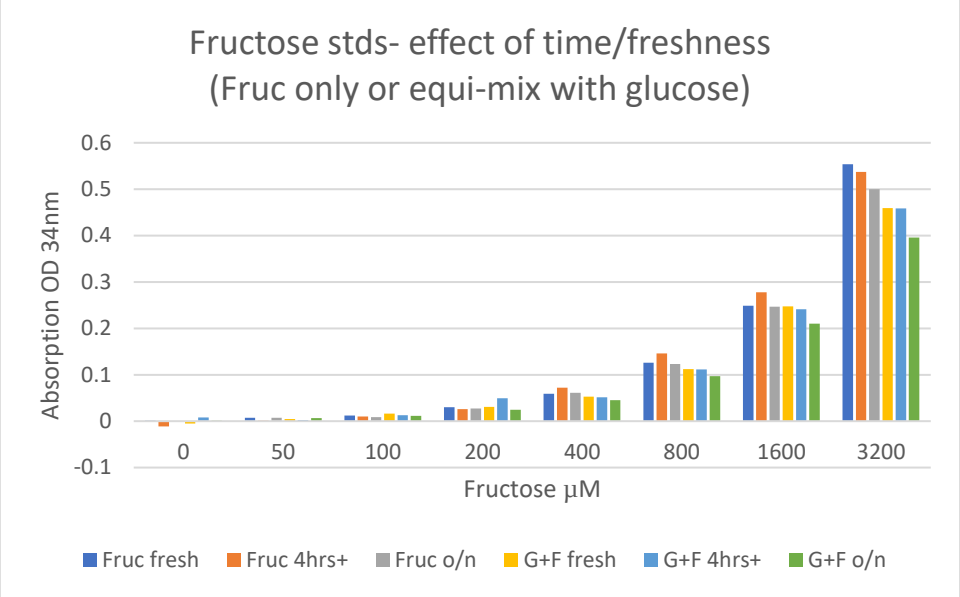


Figure 4.1: Fructose standards- effect of time/freshness: Standard curves for fructose were prepared in phosphate buffered saline either with glucose at equimolar (G+F) or alone (Fruc) and any change in absorption observed overtime at 4 hrs (4 hrs+) and overnight (o/n). Fructose levels were analysed as described by the enzymatic/spectrophotometric assay. For both, a tendency for a slight drop in absorption was see overnight.

For a 4:1 ratio of glucose to fructose, which was approximately what was found in this study after transport across a Caco2 monolayer, a substantial drop in absorbance was seen after 4 hrs and overnight particularly at the higher levels of fructose tested (Figure 4.2). At the 200-800 µM fructose dose range, which were the levels found transported across Caco2 cells for 1-2 hours transport assay, about a 10-30% loss in fructose recovery was seen after 4 hrs (Figure 4.2). This would indicate that the true glucose to fructose ratio after transport in these assays was approximately 3:1. In order therefore to better estimate the levels of fructose transported, standards of a 3:1 ratio were used and allowed to stand for approximately 3-4 hours prior to analysis along-side samples to allow for any loss in recovery.

23 Figure 4.2: Fructose standards- effect of time/freshness of a fructose+glucose 4:1 mix

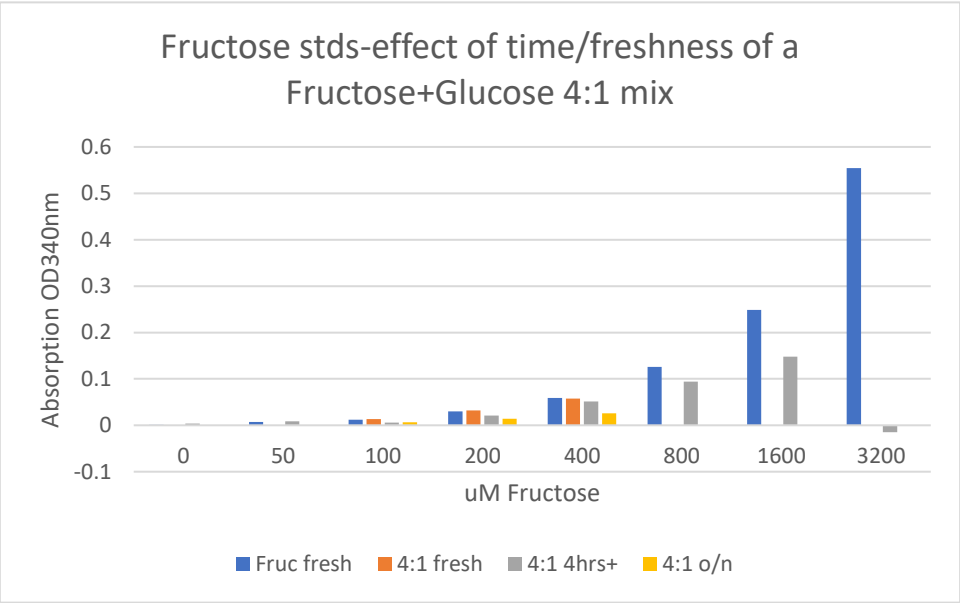


Figure 4.2: Fructose standards- effect of time/freshness of a fructose+glucose 4:1 mix. Standard curves for fructose were prepared in phosphate buffered saline as either fructose alone (Fruc) or with glucose at a 4:1 ratio and any change in absorption observed overtime at 4 hrs (4 hrs+) and overnight (o/n). Data for fresh and overnight were only recorded for 100-400 µM fructose. Fructose levels were analysed as described by enzymatic assay. A substantial drop in absorbance was seen after 4 hrs and overnight particularly at the higher levels of fructose tested.

4.2.2 Fructose and glucose transport assay optimisation

To identify natural plant phytochemicals which have the potential to inhibit the uptake of fructose, there was a need to develop an appropriate *in vitro* model. The basic model used here (Yamashita *et. al*. (2002), originally required 21 days of culture for the cells to achieve sufficient differentiation. As this is incompatible with high through-put screening Becton Dickson Bioscience shortened the process to just 2 days post-seeding of cells using butyric acid to induce growth arrest and differentiation, as demonstrated in several colonic cancer cell lines (Ching *et al*., 1997). Yamashita *et al* 2009, however, have shown that monolayer integrity is compromised using this 2-day protocol compared with 21 days, by measuring trans-epithelial electrical resistance (TEER). TEER was shown to improve greatly with time from between 1 to 5 days post seeding before deteriorating at 6 and 7 days. The effect of these time points on GLUT5 and GLUT2 transporter mRNA expression, as well as fructose transport and monolayer integrity was therefore investigated here.

Caco2 cells were differentiated for 2, 5 or 7 days in insert wells ~~before harvesting, lysed and extracted with RLT~~. TEER as a measure of monolayer integrity was, unlike Yamashiti *et al*., 2009, very similar after 5 days compared to 2 days, but as they had found deteriorated considerably after 7 days (Figure 4.3).

Figure 4.3 - 4.9: Effects of Cacoc2 maturation time on performance as a fructose transport model.

Fig 4.3 Trans epithelial electrical resistance- Caco2 monolayer insert

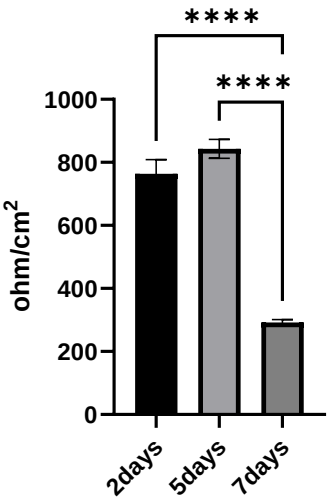


Fig 4.4: Lucifer Yellow- Caco2 monolayer insert

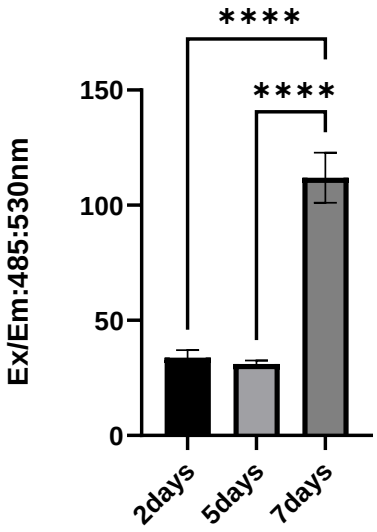


Fig 4.5: GLUT5 mRNA- Caco2 monolayer insert

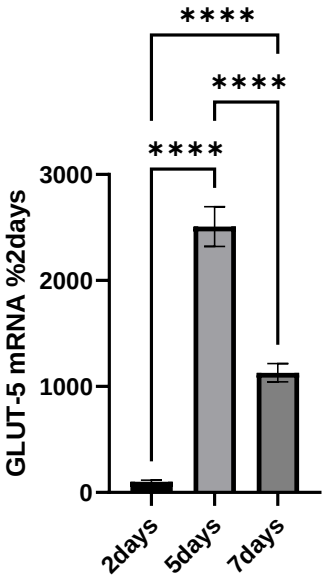


Fig 4.6: Fructose Transport - Caco2 monolayer insert

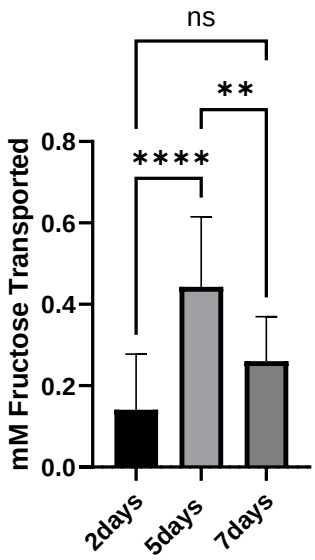


Fig 4.7: SGLT1 mRNA- Caco2 monolayer insert

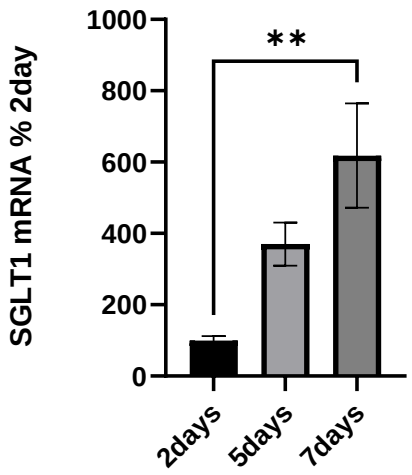


Fig 4.8: GLUT2 mRNA- Caco2 monolayer insert

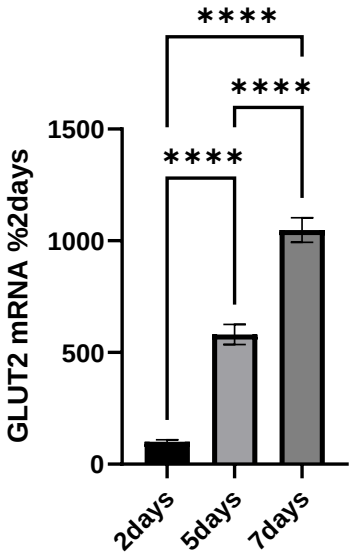
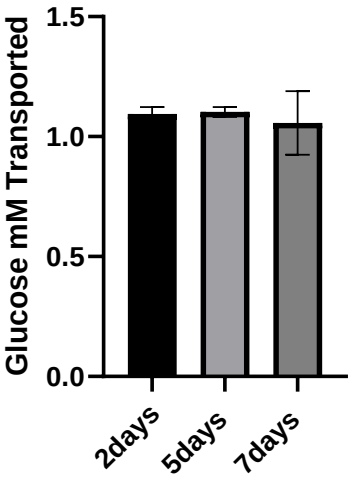


Fig 4.9: Glucose Transport - Caco2 monolayer insert



Figures 4.3 - 4.9: Effects of Caoc2 maturation time on performance as a fructose transport model. Caco2 gut cells were differentiated for 2, 5 or 7 days in insert wells. 25 mM fructose and 25 mM glucose were then added for 30 min, ~~before harvesting, lysed and extracted with RLT buffer~~. Monolayer integrity was measured by Trans epithelial integrity (TEER) and found to be similar at days 2 and 5 but deteriorated by day 7 (Figure 4.3:) and Lucifer Yellow transport (Figure 4.4) was also found to be similar at day 2 and 5 but deteriorated by day 7. GLUT5 mRNA (Figure 4.5) was greatly enhanced after 5 day compared to 2 days and 7 days. Fructose transport (Figure 4.6) was greatly enhanced after 5 day compared to 2 days and 7 days. SGLT1 mRNA (Figure 4.7) was greatly and significantly enhanced after 7 days compared to 2 days, but not significantly greater than 5 days.: GLUT2 mRNA (Figure 4.8) was greatly and significantly enhanced after 7 days compared to 2 days and significantly greater than 5 days. Glucose transport (Figure 4.9) was not affected by despite differences for transporters. All treatments were compared to each other by One -way ANOVA, correcting with Tukey's multi-comparisons hypothesis testing. ** $p<0.01$, ****= $p<0.0001$, n=6

replicate wells per treatment.

The TEER results were replicated using Lucifer Yellow transport as it is also a measure of monolayer integrity. Transport was low for days 2 and 5 days indicating good integrity but showed substantial and significant deterioration by day 7 (Figure 4.4).

The mRNA levels of the specific fructose transporter GLUT5, though, were greatly increased after 5 days compared to 2 days and 7 days post seeding (Figure 4.5).

Upon incubating with 25 mM fructose and 25 mM glucose, fructose transport across the cell monolayer was also found to be substantially and significantly greater after 5 days than 2 days and 7 days (Figure 4.6).

The mRNA levels for the main low glucose concentration transporter SGLT1 were highest at 7 days, significantly greater than at 2 days by several fold, and higher than 5 days, although this was not significantly different (Figure 4.7).

The mRNA expression of the GLUT2 high concentration glucose (and possibly fructose) gut transporter showed a similar pattern as that seen for SGLT1, again being several-fold and significantly greater at 7 days than 2 days, and this time also significantly greater than the 5 days differentiation treatment (Figure 4.8).

Glucose transport however, unlike fructose, did not follow the pattern of its transporters (Figure 4.9). There was no significant difference found between 2, 5 or 7 days. ~~either significantly or in trend,~~ They were all very similar at around 1.0 mM.

Taking the results altogether, subsequent transport studies were performed using 5 days of differentiation.

Fructose transport time course

In the fructose assay, variability was high with coefficients of variance (ratio of standard deviation to the mean) up to 30%, compared with 3-10% for glucose. It was anticipated that this would make it difficult to find a significant difference between treatments without many replicates. Other studies have suggested that the fructose concentration needs to be greater than 0.5 mM to be accurately determined

using the enzyme/spectrophotometric methodology (Douard & Ferris- 2008). At this point in method optimisation the concentration was just over 0.4 mM so the transport assay needed to be optimised further. Fructose and glucose transport were tested over a time course of 30-240 minutes.

Fructose transport was found to increase linearly up to 240 minutes (Figure 4.10). A concentration of 0.5 mM for the amount of transported fructose was achieved at >60 minutes. Variability was notably smaller at two hours but increased again by 4 hrs (Figure 4.10 and 4.12).

Figure 4.10: Fructose and Glucose transport time course.

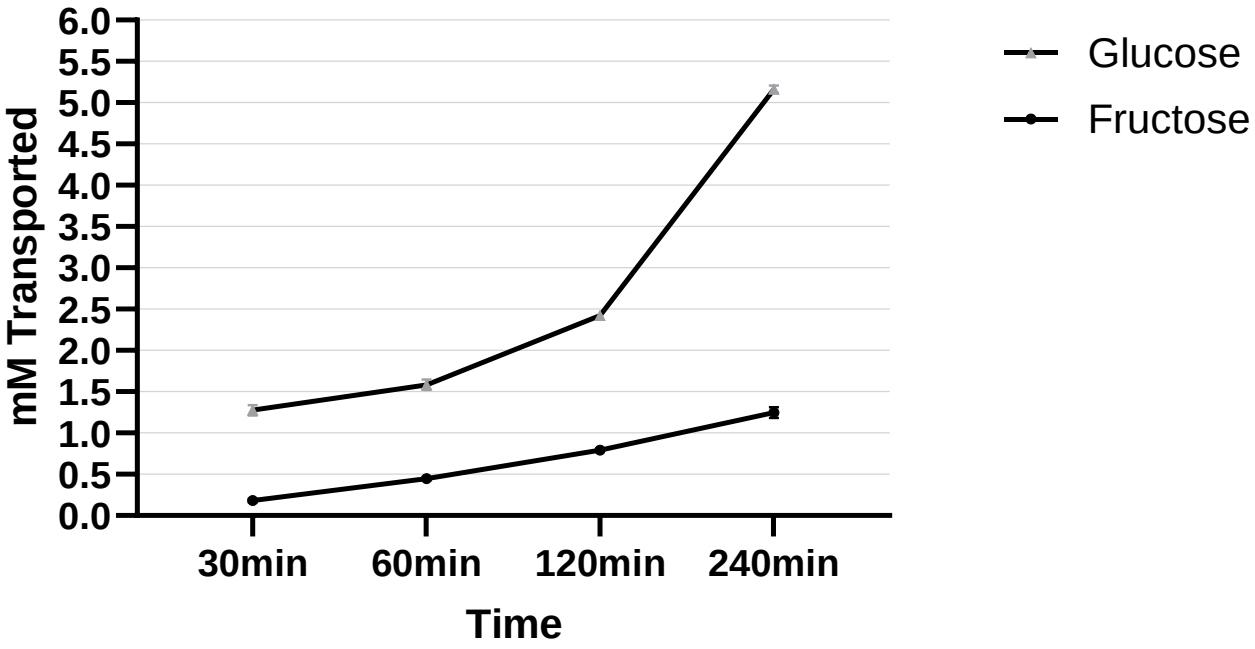


Figure 4.10: Fructose and Glucose transport time course. Caco2 gut cell line cells were differentiated for 5 days in insert wells. In the presence of 25 mM fructose and 25 mM glucose fructose transport increased linearly reaching a level greater than 0.5 mM at just greater than 60 minutes. n=12 replicate wells per treatment.

Monolayer integrity as measured by TEER rises with time peaking significantly at two hours before deteriorating at 4 hrs (Figure 4.12).

Figure 4.12: TEER time course in-Caco2 monolayer inserts

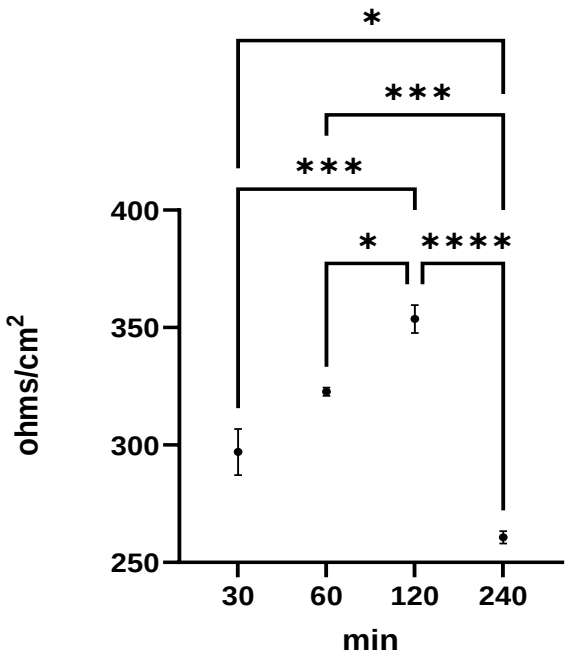


Figure 4.12: TEER time course in-Caco2 monolayer inserts. Caco2 gut cell line cells were differentiated for 5 days in insert wells. In the presence of 25 mM fructose and 25 mM glucose, TEER as a measure of monolayer integrity rose and peaked at two hours significantly and then deteriorated. All treatments were compared to each other by One -way ANOVA, correcting with Tukey's multi-comparisons hypothesis testing. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$, n=3 replicate wells per treatment.

Subsequent transport studies were therefore performed allowing a two-hour duration.

Validating the fructose transport assay with known fructose inhibitors

2,5-anhydromannitol has been identified as a GLUT5 inhibitor in a model of *Xenopus laevis* oocytes transfected with GLUT5 expression vector (Kane *et al*., 1997). Testing this compound in the optimised assay as a means of validation, there was no effect on fructose transport at concentrations up to 100 mM against 25 mM fructose. Its inactivity in the assay was ~~perhaps~~ not surprising as Kane *et al* had used it at 50 mM to inhibit 100 µM fructose in a radioisotope-based assay. Also, no inhibition was observed with L-sorbose-Bn-OZO which has been found to have a K_i (inhibition constant, the concentration required to occupy 50% of the receptor) for fructose

transport inhibition of 3.1 mM (Girniene *et al*., 2003). The assay they used involved the addition of 1 mM [^{14}C]-D-fructose and could have been the maximum they used but the final concentration was not disclosed. Andrade *et al*., 2017 used the same compound at 10 µM and found it an effective inhibitor against 100 nM fructose. Rubusoside has also been reported to inhibit GLUT5 in prepared liposomes with 4 µM fructose (^{14}C-hexose). Again, in the assay used in this study, no inhibition at 300 µM *vs.* 25 mM fructose was observed.

4.2.3 Selection of potential fructose uptake inhibitors

A search of patents and the scientific literature was conducted to identify potential inhibitors (see 2.0 Materials and Methodology, 2.2.3 Selection of single actives and beverages).

For patent activity in this area, in 2009 a patent was granted (JP2009184992A) for a fructose absorption inhibitor which includes as an active ingredient or extract from at least one member selected from the group consisting of banaba, olive, guava, guarana, blueberry, cumin, catechins, evening primrose, orange flower, black tea, *Alpina zerumbet*, Sichuan pepper, bitter orange peel*, Rhodiola sacra*, Western dandelion, and Chinese silk vine. A patent in 2012 (WO12008474A1) required that a fructose absorption inhibitor has a monoterpene as the active ingredient as a preventative or therapeutic agent for symptoms or maladies particularly hyperlipidemia, or a triglyceride increase caused by overconsumption of fructose. In 2013 a patent was filed for an extract of eucalyptus leaf that prevents absorption of fructose but does not prevent absorption of the glucose from intestines and is for prevention or treatment of hyperlipidemia and fatty liver (US2003049337A). Again in 2013, a patent was filed for a fructose absorption inhibitor comprising a hydrolyzable tannin as an active ingredient, preferably having a form composed of a gallic acid derivative and/or an ellagic acid derivative bound to a hydroxy group in glucose via an ester bond and includes ellagitannin and gallotannin (US2014128585A).

Scientific literature in this area includes three papers using eucalyptus leaf extract that specifically refer to the inhibition of fructose uptake for the potential purpose of prevention or reduction of metabolic syndrome, via reduced adiposity (Sugimoto *et*

al., 2005; Sugimoto *et al*., 2010; Sugimoto *et al*., 2010b). The Sugimoto work measured the effect of reduced uptake of fructose on adiposity and triacylglycerol concentrations in the plasma (1·44, SD 0·448 v. 2·79, SD 0·677 nmol/l n=7, $p<0.05$), and liver (19·1, SD 5·07 v. 44·1, SD 16·28 mmol/g, n=7, $P<0·05$) of rats (Sugimoto, 2005). A further study by the same author has demonstrated reduced fructose uptake in humans (Sugimoto *et al*., 2010b), although they didn't demonstrate reduced triglycerides in humans. Direct inhibition of GLUT5 was also not demonstrated but inferred by the fact that glucose uptake was not prevented, but that of fructose was. The next study from this group showed reduced steatosis in rat liver by eucalyptus leaf and banaba extract (Sugimoto *et al*., 2015).

As discussed above (Validating the fructose transport assay with known fructose inhibitors) in a functional study of GLUT5, Kane *et al*., 1997 noted that the locked furan-ring fructose analogue 2.5-anahydomannitol was an effective inhibitor, suggesting that GLUT5 prefers fructose in the furan ring form. Girniene *et al*., 2003 also found fused ring analogues of fructose to be inhibitory of GLUT5 e.g. sorbose-Bn-OZO. Effective inhibition of fructose uptake by sorbose-Bn-OZO was confirmed in a study by Andrade *et al*., 2017. Thompson *et al*., 2015 noted that rubusoside inhibited GLUT5 and GLUT1, whilst astragalin-6-glucoside was only effective against GLUT5. Andrade *et al*. 2017 also reported inhibition of fructose transport for a number of polyphenols at concentrations of around 10-100 μM. These polyphenols were ferulic acid, caffeic acid, coumaric acid, proctocatenoic acid, chrysin and delphinidin.

Many other papers concerned demonstrating the effects of flavanoids, polyphenols, and plant extracts etc. on "metabolic syndrome"-related symptoms and markers in high fructose fed rats. However, none of these papers mention GLUT5 or fructose absorption inhibition. Where a mechanism is discussed, it is usually presumed to be as an antioxidant effect (Santamaria *et al*., 2012; Lee *et al*., 2012; Ahmad *et al*., 2008; Godse *et al*., 2010; Liu *et al*., 2010; Liu *et al*., 2007; Kannappan, 2010; Mohamed *et al*., 2009).

Ultimately the aim here is to discover plant compounds that have the potential to inhibit fructose and glucose uptake, and the use thereof toideally be protected as intellectual property. As such, compounds for which there is prior art were mostly

avoided. Rather, from these searches a number of broad categories have been noted, ~~perhaps~~ suggesting that within each there may be a common mode of action, e.g: gallates and tannins; flavonoids; terpenoids; fructose binders. From these categories, some representative candidates were selected based, to some extent, on ease of acquirement for testing and with natural sources for inclusion in any potential product (Table 4.2). Some compounds were also tested that have been previously shown to inhibit the transport of glucose (demonstrated in a functional assay) as a) a positive control for glucose inhibition and (b) potential fructose transport inhibitors via GLUT2 or SGLT1 (see Discussion). Vanillin and cinnamaldehyde were also selected as they are already in products of the thesis sponsor's supply chain and would therefore be easily sourced for potential future fructose inhibition products (Table 4.2).

Table 4.2: Potential categories and compounds selected for testing as potential fructose transport inhibitors.

Potential active category	**Reason selected**	**Potential active examples**
Gallates/ Tannins Kim *et al*., 2010; Borate *et al*., 2011; Wu *et al*., 2004; Shrestha etal., 2009; Cao *et al*., 2007; Du *et al*., 2021).	Patents-Fructose uptake inhibition; Literature- improves fructose induced metabolic syndrome	**Gallic acid** (Guo *et al*., 2017; Sutra *et al*., 2008; Li *et al*., 2019; Variya *et al*., 2020). **Ellagic acid** (Guo *et al*., 2017). **Pyrogallol** (Choi *et al*., 2019)
Flavanoids (Nayak *et al*., 2014; Wang *et al*., 2013; Arunkuma *et al*., 2103; Chen *et al*., 2011; Hu *et al*., 2012; Ohnog *et al*., 2012; Kannappan *et al*.,	Literature- improves fructose induced metabolic syndrome	**Astibin** (Chen *et al*., 2012). **Quercetin-glucoside** (Ahmad *et al*., 2008).

2010; Liu *et al*., 2007; Chen *et al*., 2004; 2013; Arunkuma *et al*., 2013b; Qin *et al*., 2018; Soni *et al*., 2010; Mehmood *et al*., 2019; Ota-Kontan *et al*., 2020; Mohamed *et al*., 2009).		
Terpenoids (Wang *et al*., 2018; Wang *et al*., 2015; Yang *et al*., 2014; Mohammadi *et al*., 2014; Zhang *et al*., 2011; Paredes-Carbajal *et al*., 1998; Sil and Chakraborti *et al*., 2016; Prabhaka *et al*., 2017; Baka *et al*., 2021; Ajala-Lawal *et al*., 2020; Germoush *et al*., 2020).	Patents – Fructose uptake inhibition/GLUT5; Literature- improves fructose induced metabolic syndrome	**Eucalyptol** (Sugimoto *et al*., 2005; Kawasaki et a., 2008; Sugimoto *et al*., 2010). **Cinnamaldehyde** (Lin *et al*., 2102; Qin *et al*., 2010; Kang *et al*., 2016; Koshovyi *et al*., 202; Rubio-Rodriguez, *et al*., 2021).
Fructose binders protein and polysaccharide	Literature- improves fructose induced metabolic syndrome	**Lectins** (Liu1 *et al*., 2014; Cheung *et al*., 2009). **Arabinogalactan** (Reddy *et al*., 2009).
GLUT2 inhibitors	GLUT2 is also required to transport fructose(?)	**Phloretin** (Kellett and Brot-Laroche 2005) Kwon *et al*., 2007).

	-basolaterally and maybe apically?	**Luteolin** (personal communication, Unilever).
SGLT1 inhibitors	SGLT1 possibly affects GLUT2 activation and translocation	**Phloridzin** (Rossetti *et al*., 1987) **Luteolin-7-glucoside** (personal communication, Unilever)
Unilever supply chain	Ready to use?	**Vanillin** (Castro *et al*., 2012) **Cinnamaldehyde** (see above)

Table 4.2: Plant compounds that occur naturally, that may have the potential to inhibit the uptake/transport of fructose, were selected for screening. The selection process involved searching the literature and patents for actives (see Materials and Methods) which have been reported to inhibit fructose uptake, GLUT5 transporter or to improve metabolic syndrome conditions in an *in vivo* model where animals have been fed a high fructose diet. This led to the identification of broad categories suggesting a common mode of action within each I.E: gallates and tannins; flavonoids; terpenoids; fructose binders. Some compounds were also tested that have been previously shown to inhibit the transport of glucose as a) a positive control for glucose inhibition and (b) potential fructose uptake inhibitors via GLUT2 or SGLT1.

4.2.4 Testing of selected single actives

/Potential actives were tested in a glucose transport model that has been optimised here for fructose based on Yasamati *et al*., 2009 (see optimisation above). Briefly, Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were then treated with potential inhibitors alongside 25 mM fructose+25 mM glucose~~, in a randomised format~~ and the transport of both measured after 2 hrs by an optimised enzymatic/spectrophotometric assay based on Campbell *et al*., 1999 (see optimisation above). Potential actives were included at concentrations of 150 and 300 µM, as amounts that were considered feasible to be included in any future *in vivo* application. These levels had also been found to be effective previously (Personal communication, Unilever sponsor) in the pre-optimised glucose transport

assay. For the initial inhibitor selection four experiments were performed on 4 different days.

Glucose transport data is also given here as glucose might also contribute to the fructose spike via the polyol pathway, either via the liver or within enterocytes of the small intestine (see Chapter 3.0, 3.2 Discussion, 3.2.2 Primary human Hepatocytes, Glucose and triglyceride).

In each experiment glucose transport was inhibited by phloretin, luteolin, phloridzin and quercetin-3-glucoside at 300 µM (Figures 4.13 – 4.26) and less so at 150 µM. As phloretin is a well-known GLUT2 inhibitor and luteolin and quercetin-3-glucoside have also shown good activity in previous experiments by another group (personal communication, Unilever), this was a good indication that the assay had performed as expected.

For fructose transport in Expt.1, only lectin glycan showed significant activity at 150 µM as a potential inhibitor on fructose transport. Vanillin, eucalyptol and ellagic acid showed a tendency for approximately 40 - 50% inhibition (Figure 4.15). At 300 µM, the same compounds showed a greater tendency to inhibit fructose transport, and lectin glycan (61%) again demonstrated a statistically significant inhibition (Figure 4.16). However, a high degree of variation for the control treatment (0.1% DMSO) reduced the ability to find significant inhibition for the other reagents.

In Expt.2 for fructose transport at inhibitor concentrations of 150 µM, lectin glycan and vanillin again showed a tendency for inhibition (Figure 4.19), along with pyrogallol and arabinogalactan. On this occasion, phloridzin significantly inhibited fructose transport by 31%. At 300 µM several actives showed substantial and significant inhibition of fructose transport, although in most cases the effect was only moderate (Figure 4.20). The greatest inhibition was for cinnamaldehyde of approximately 42% *vs* control which was > lectin glycan > gallic acid > arabinogalactan > phloridzin > luteolin.

No fructose transport inhibition was seen for fructose in expt.3 and 4 at 150 µM (Figure 4.23 and 4.27). However, at 300 µM lectin glycan, vanillin, cinnamaldehyde, quercetin-3-glucoside, phloretin, luteolin, ellagic and gallic acid showed some promise over the two experiments (Figures 4.24 and 4.28).

Figure 4.13 – 4.28: Fructose and glucose transport vs potential inhibitors in Caco2 cells.

Fig 4.13: Glucose Transport vs Potential inhibitors- 150uM Expt.1

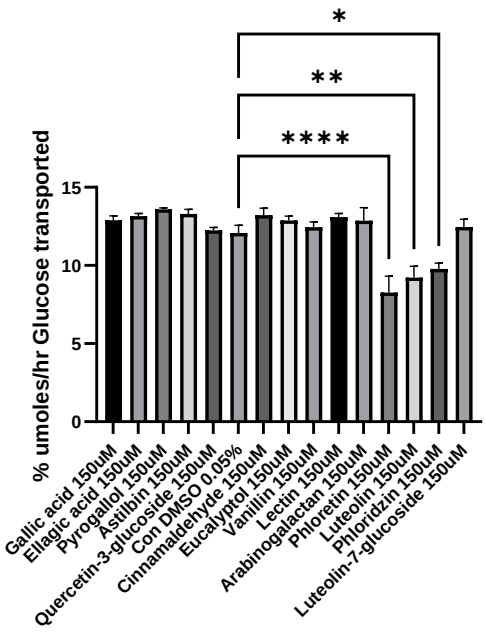


Fig 4.14: Glucose Transport vs Potential inhibitors- 300uM Expt.1

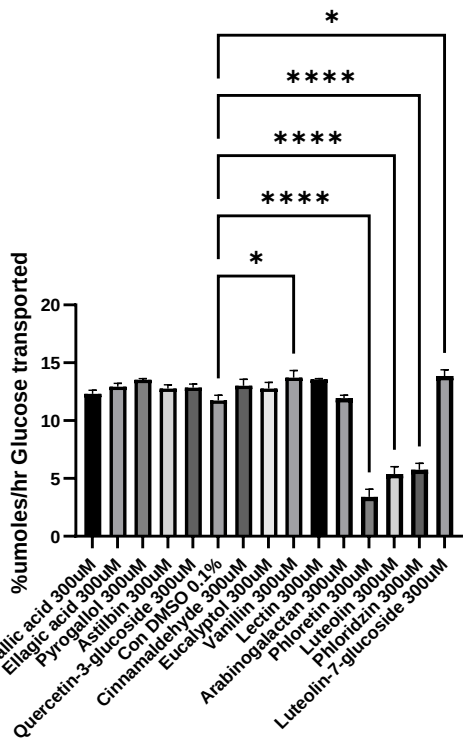


Fig 4.15: Fructose Transport vs Potential inhibitors- 150uM Expt.1

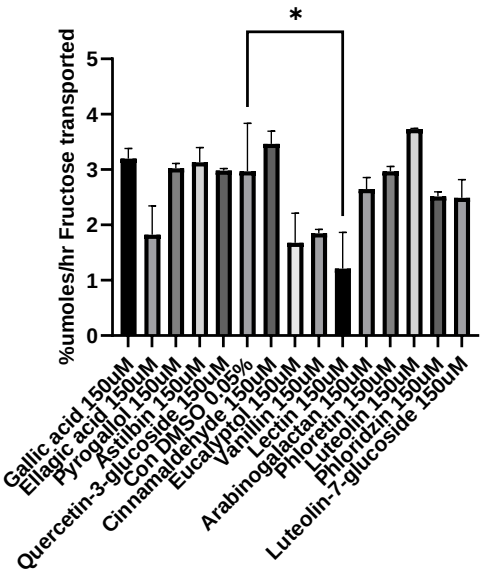


Fig 4.16 Fructose Transport vs Potential inhibitors- 300uM Expt.1

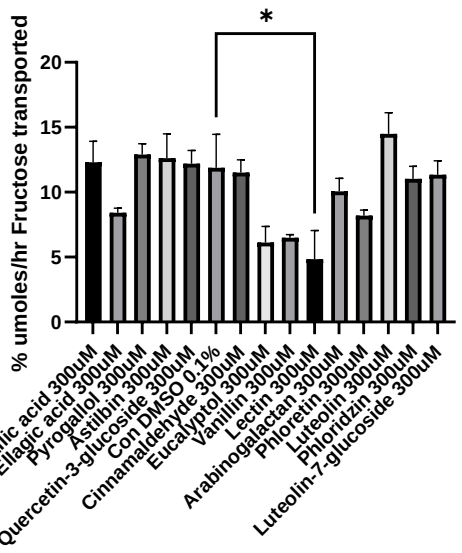


Fig 4.17: Glucose Transport vs Potential inhibitors- 150uM Expt.2

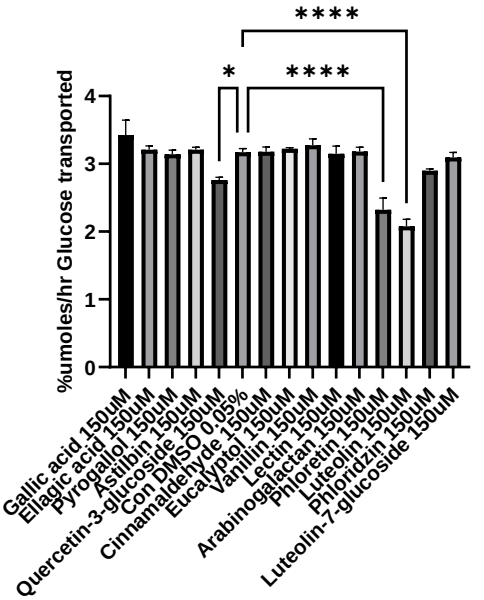


Fig 4.18: Glucose Transport vs Potential inhibitors- 300uM Expt.2

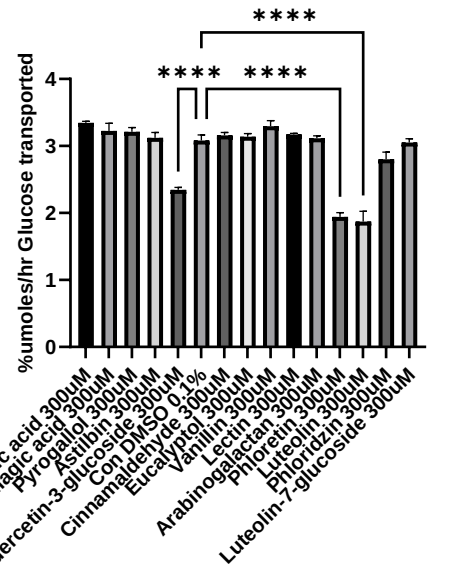


Fig 4.19: Fructose Transport vs Potential inhibitors- 150uM Expt.2

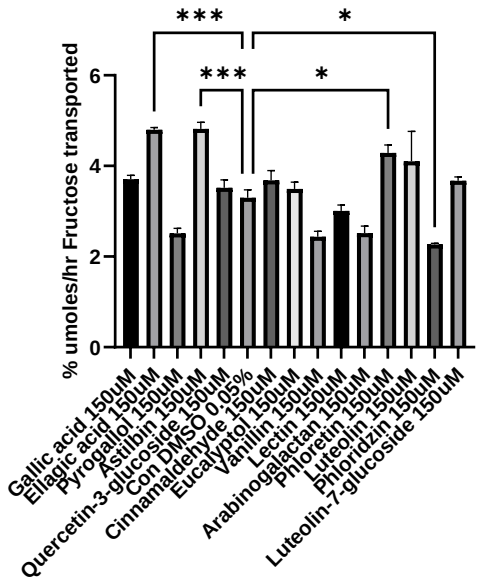


Fig 4.20: Fructose Transport vs Potential inhibitors- 300uM Expt.2

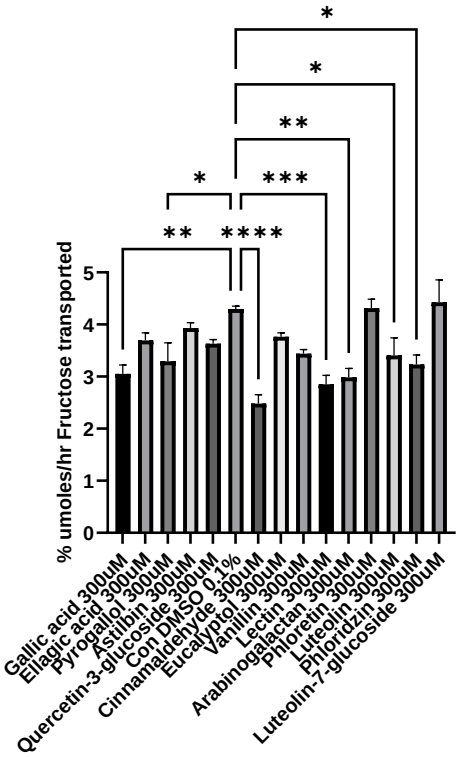


Fig 4.21: Glucose Transport vs Potential inhibitors- 150uM Expt.3

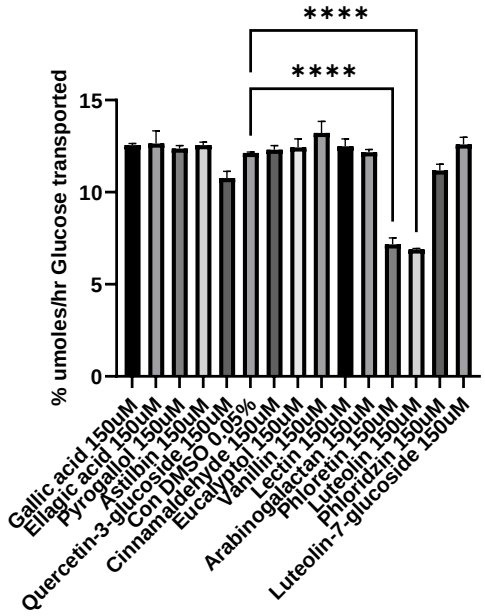


Fig 4.22: Glucose Transport vs Potential inhibitors- 300uM Expt.3

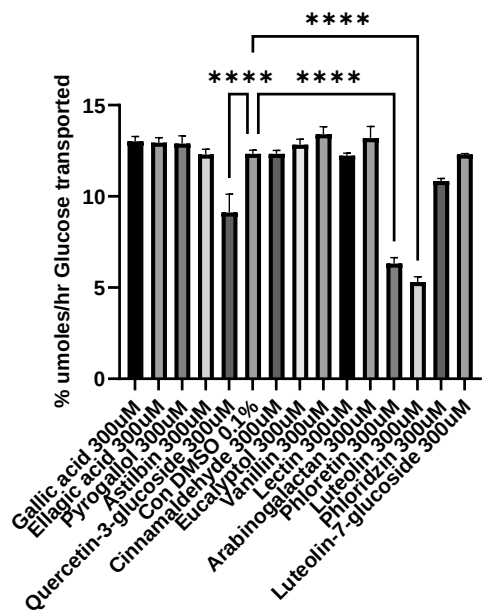


Fig 4.23: Fructose Transport vs Potential inhibitors- 150uM Expt.3

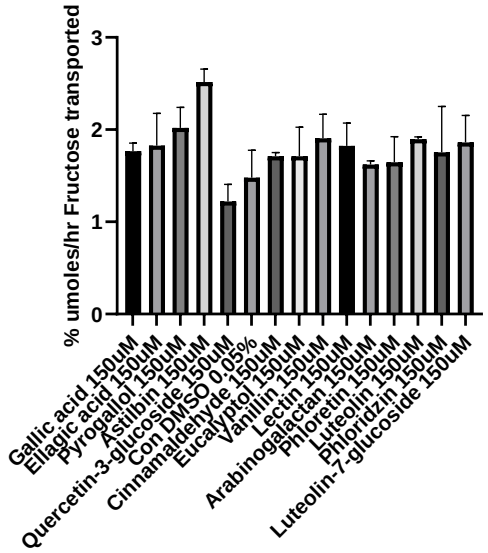


Fig 4.24: Fructose Transport vs Potential inhibitors- 300uM Expt.3

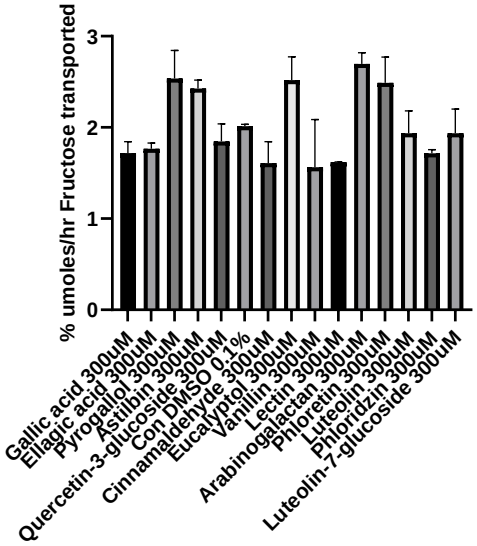


Fig 4.25: Glucose Transport vs Potential inhibitors- 150uM Expt.4

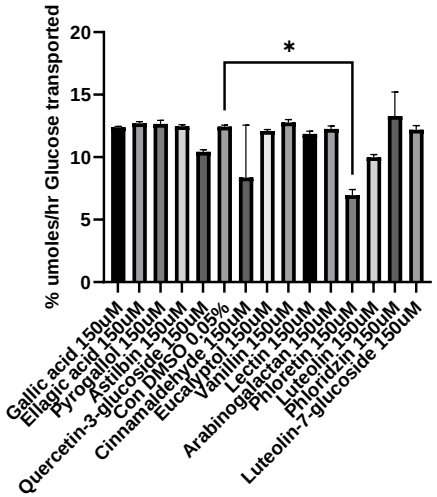
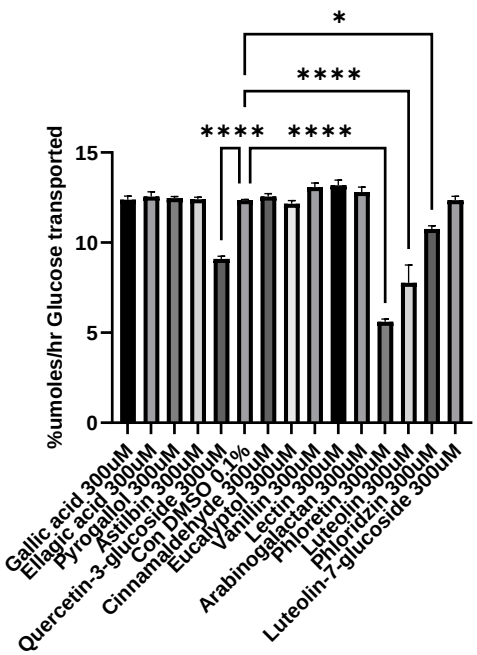
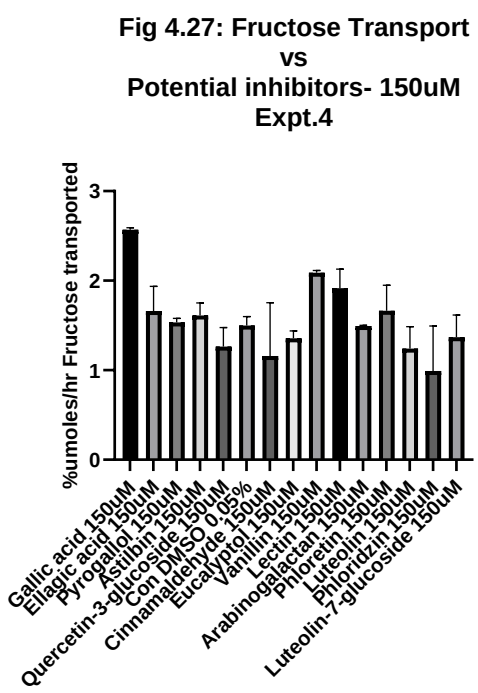


Fig 4.26: Glucose Transport vs Potential inhibitors- 300uM Expt.4





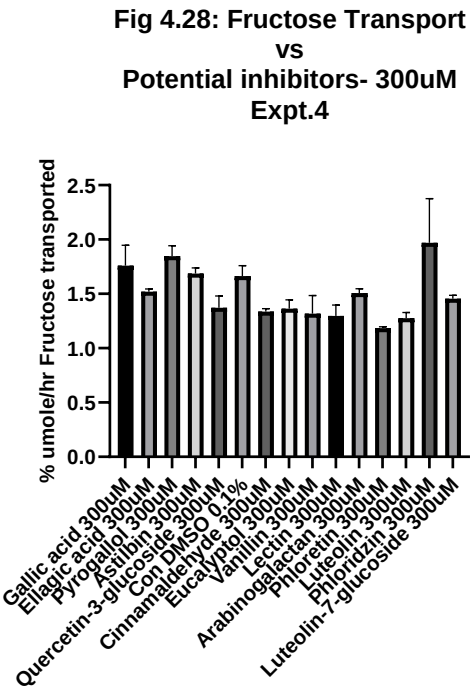


Figure 4.13-28: Glucose transport *vs* potential inhibitors in Caco2 cells. Potential actives were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors (150 µM and 300 µM) in 25 mM fructose + 25 mM glucose in PBS+ for 2 hrs and the transport of both measured after 2 hrs (% well µmoles transported/hr).- All treatments were compared to the control (Con-DMSO 0.05 or 0.1%) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, n=3 replicate wells per treatment.

Over the four experiments, although not statistically significant, lectin glycan showed the greatest tendency for fructose transport inhibition, followed by vanillin, phloridzin, cinnamaldehyde, quercetin -3 -glucoside and eucalyptol (Figure 4.29).

Figure 4.29 Fructose transport vs potential inhibitors. Summary of four expts.

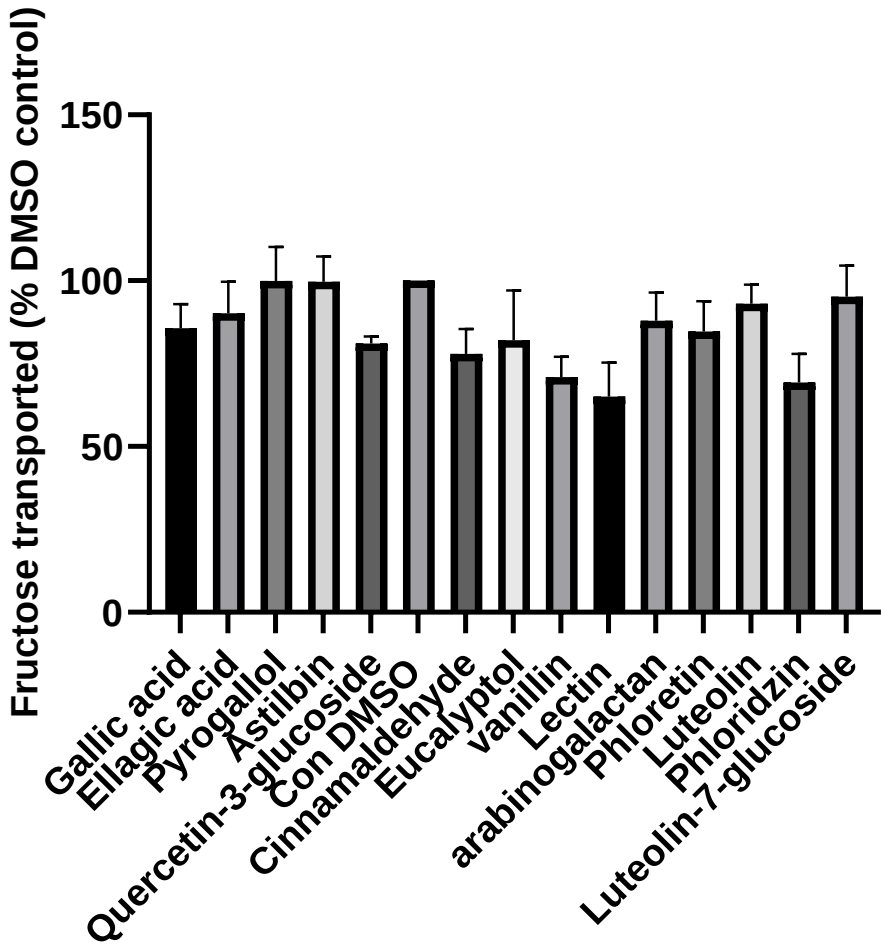


Figure 4.29: Fructose transport *vs* potential inhibitors. Summary of four expts. Over four experiments potential actives were tested in a Caco2 gut cell-line *in* vitro model optimised for fructose transport inhibition. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors (300 µM) in 25 mM fructose + 25 mM glucose in PBS+ for and the transport of both measured after 2 hrs (% well µmoles transported/hr). . Whether statistically significant or not in each expt. the effect on fructose transport was averaged across the four experiments. All treatments were compared to the control (Con-DMSO) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing, n=4 experiments. Over the four experiments, although not statistically significant, lectin glycan showed the greatest tendency for fructose transport inhibition, followed by vanillin, phloridzin, cinnamaldehyde, quercetin -3 -glucoside and eucalyptol.

These compounds were further considered for testing for synergistic activity with GLUT2 inhibitors (Table 4.3).

4.2.5 Synergy testing with selected single actives

Interconnected molecular pathways can be susceptible to interference at many points and junctures. Fructose may have several possible gut transporters in addition to GLUT5 including GLUT2, the main gut high concentration glucose transporter, as well as GLUT7, GLUT9a/b, GLUT8, GLUT11, and GLUT12 which share sequence homology with GLUT5 (Manolescu *et al*., 2007; Doujard & Ferris, 2008).

In this study, some compounds from natural plant sources have been identified that show at least a tendency to inhibit fructose transport, either significantly in one or two experiments.

Table 4.3: Consistency of potential fructose inhibitors over four experiments

	Expt.1	Expt.2	Expt.3	Expt.4	mean
Gallic acid	-19.55	-29	-14.7	+5.77	-14.37
Ellagic acid	-32.46	+14.07	-12.35	-8.68	-9.86
Pyrogallol	-4.69	-23.8	+25.85	+2.23	-0.10
Astilbin	-14.47	-8.54	+20.46	+1.31	-0.31
Quercetin-3-G	-25.02	-15.54	-17.43	-17.55	-18.89
Cinnamaldehyde	-6.14	-42.28	-20.14	-19.64	-22.05
Eucalyptol	-56.71	-12.49	+15.72	-18.1	-17.9
Vanillin	-47.05	-26.04	-22.35	-20.76	-29.05
Lectin Glycan	-64.52	-33.71	-19.62	-22.04	-34.97
Arabinogalactan	-17.88	-30.52	+9.68	-9.37	+12.02
Phloretin	-33.11	+0.33	+0.33	-28.78	+15.31
Luteolin	-3.5	+2.99	-3.85	-23.34	-6.93
Phloridzin	+22.34	-31.24	-14.8	-54.16	-22.68
Luteolin-7-G	-30.9	+2.99	-3.85	+12.6	-4.79

Table 4.3: Consistency of potential fructose inhibitors over four experiments. Over four experiments, potential actives were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport inhibition. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with 300 µM potential inhibitors (150 or 300 µM) in 25 mM fructose + 25 mM glucose in PBS+ and the transport of both measured after 2 hrs (% well µmoles transported/hr). Whether statistically significant or not the effect on fructose transport was averaged across the four experiments. Over the four experiments, although not statistically significant, lectin glycan showed the greatest tendency for fructose transport inhibition, followed by vanillin, phloridzin, cinnamaldehyde, quercetin -3 -glucoside and eucalyptol. All treatments were compared to the control (Con-DMSO) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing, n=4 experiments. None of the treatments were found to give significant transport inhibition.

However, their effect was only moderate (15-35%). As fructose might also be transported across the apical membrane by GLUT2 (see 1.4.2 GLUT2 and

fructose transport), synergies between the compounds that showed greatest consistency for fructose transport inhibition, and some known GLUT2 inhibitors was investigated. The GLUT2 inhibitors selected included phloretin and luteolin, which have consistently and significantly demonstrated inhibition of glucose transport in these experiments. Others, hesperetin and resveratrol were recommended by researchers investigating synergies for glucose inhibition as having consistent effects (personal communication, Unilever).

Synergies tested:

Set.1

Fructose transport inhibitors:	Cinnamaldehyde, Eucalyptol, Vanillin
Vs.	
GLUT2 inhibitors:	Phloretin, Luteolin, Hesperetin, Resveratrol

And

Set.2

Fructose transport inhibitors:	Lectin glycan, Quercetin-3-G, Catechin
Vs.	
GLUT2 inhibitors:	Phloretin, Luteolin, Hesperetin

Catechin was also included as in addition to being mentioned in the patent above (JP2009184992A), green teas have also been reported to perform well in fructose inhibition studies (Villa-Rodriguez *et al*., 2017). Each compound was tested at the lower level of 150 µM in order to give a greater opportunity for synergies to emerge. Three experiments were performed for each set of combinations.

Over three experiments for fructose transport, phloretin+eucalyptol and luteolin+eucalyptol showed a tendency for reduced inhibition relative to phloretin, luteolin and eucalyptol alone (Figure 4.30). However, the differences were not statistically significant.

Figure 4.30-4.33: Fructose + glucose transport inhibitors combined to test for fructose transport inhibition- set.1 and.2

Fig 4.30: Fructose + Glucose transport inhibitors combined to test for Fructose transport inhibition- set.1

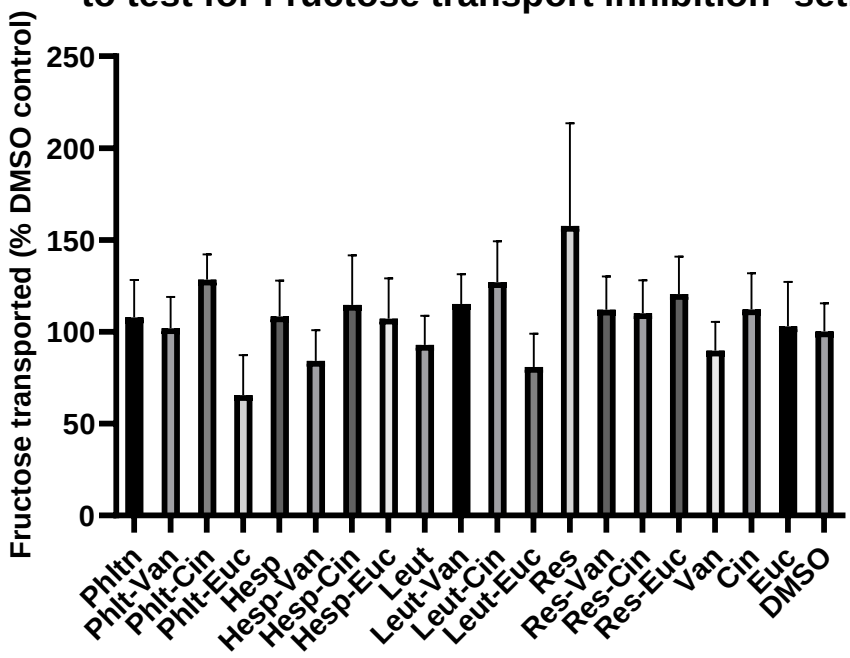


Fig 4.31: Fructose + Glucose transport inhibitors combined to test for Glucose transport inhibition- set.1

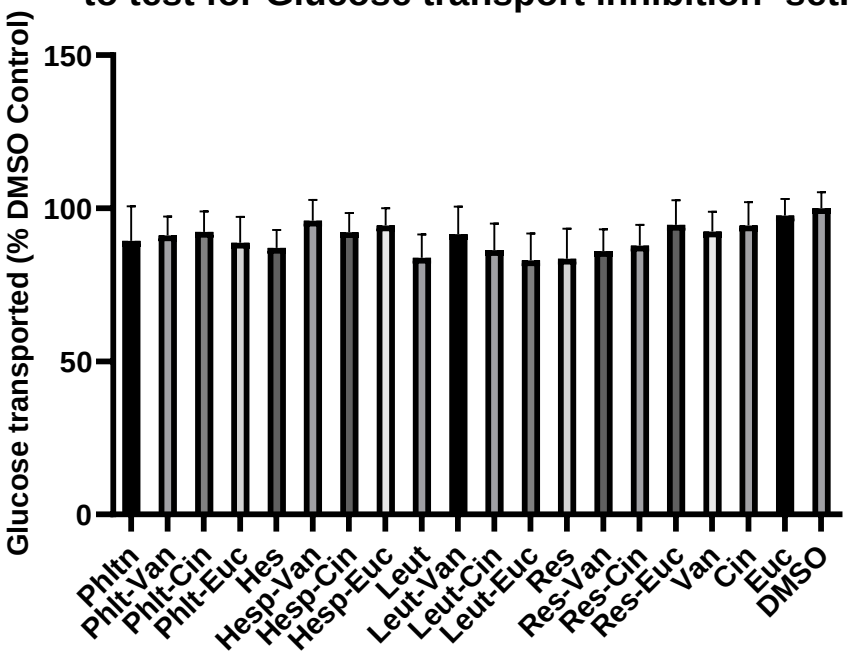


Fig 4.32: Fructose + Glucose transport inhibitors combined to test for Fructose transport inhibition- set.2

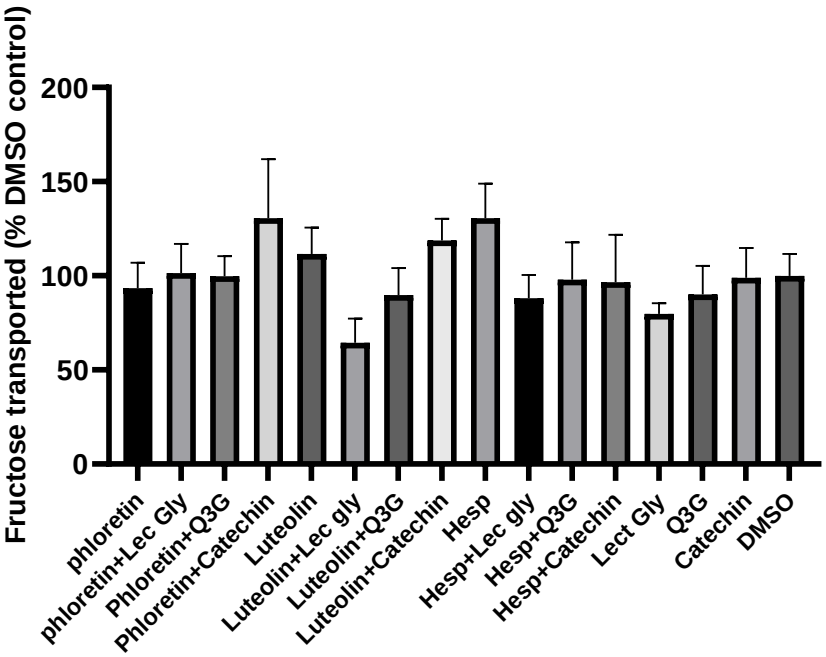


Fig 4.33: Fructose + Glucose transport inhibitors combined to test for Glucose transport inhibition- set.2

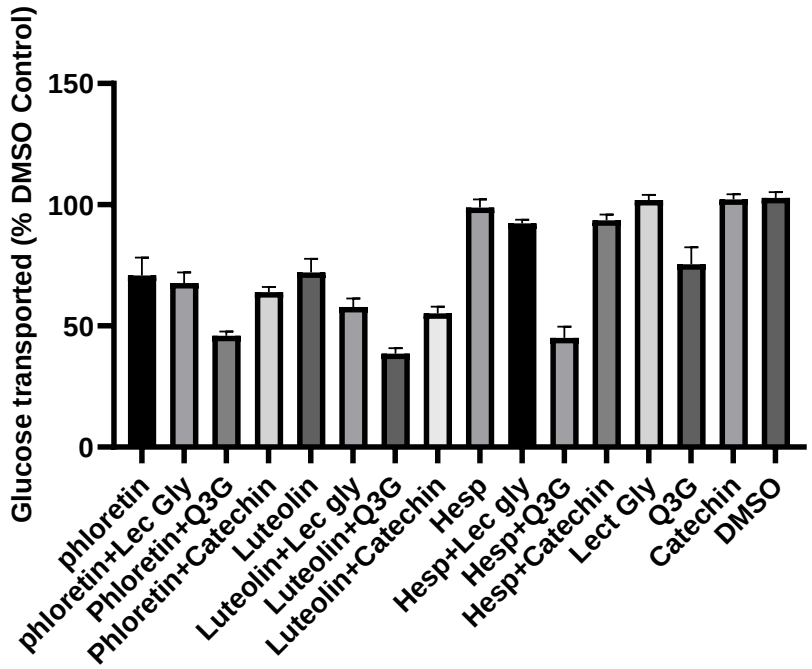


Figure 4.30 – 4.33: Fructose + glucose transport inhibitors combined to test for fructose transport inhibition- set.1 and.2. Potential fructose inhibition actives were tested with glucose inhibitors for synergistic effects. Actives were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport inhibition. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors (150 µM each) in 25 mM fructose + 25 mM glucose in PBS+ and the transport of both glucose and fructose measured after 2 hrs (% well µmoles transported/hr) (% DMSO control shown). Treatments were selected for statistical comparison based on synergy group, e.g: for the vanillin (van) + resveratrol (Res) combination- Van+Res was compared to Res, Van and DMSO control such that for each set there were 36 comparisons (9 groups times four comparisons) + each single active with DMSO control and analysed by one-way ANOVA correcting for multi-comparisons using Holm-Šídák's statistical hypothesis testing, n=9 replicate wells per treatment over 3 experiments. None of the combinations were found to give synergy or significant transport inhibition.

For glucose transport, set.1 showed no tendencies for synergy (Figure 4.31).

For fructose transport set.2, luteolin+lectin glycan showed a tendency for reduction relative to luteolin and lectin glycan alone (Figure 4.32). However, the differences were not statistically significant by one-way ANOVA.

For glucose transport, set.2 phloretin+quercetin-3-glucoside, luteolin+lectin glycan, luteolin+quercetin-3-glucoside, and hesperitin+quercetin-3-glucoside showed substantial reductions in fructose transport (Figure 4.33). In identifying synergistic

effects, “Combination Sub-thresholding” can be tested where two agents not significantly effective themselves, but when combined are significantly different from the control (Nieuwenhuis *et al*. 2011) (see 2.2.5 Synergy and product combination benefit testing).

Combination Sub-thresholding:

(See Table 4.4 for adjusted p values).

Combination	Combination Sub-thresholding?
phloretin+quercetin-3-glucoside	No: The combination is significantly different from the DMSO control (Table 4.4), but so too is phloretin alone and quercetin-3-glucoside alone.
luteolin+lectin glycan	No: The combination is significantly different from the DMSO, but so too is luteolin alone.
luteolin+quercetin-3-glucoside	No: The combination is significantly different from DMSO, but so too is luteolin alone and quercetin-3-glucoside alone.
hesperitin+quercetin-3-glucoside	No: The combination is significantly different from the DMSO, but so too quercetin-3-glucoside alone.

Next, the “Highest Single Agent” was considered. This is where the combination is not compared to the control, but rather is required to be significantly different and more effective than the most effective single active of the pair (Lehar *et al*. 2007) 2.2.5 Synergy and product combination benefit testing)

Highest Single Agent:

(See Table 4.4 for mean differences and adjusted p values).

Combination	Highest Single Agent?
phloretin+quercetin-3-glucoside	Yes: The combination is more effective and significantly different from phloretin which was the more effective agent alone.
luteolin+lectin glycan	No: The combination is not significantly different from luteolin which was the most effective of the pair as a single active.
luteolin+quercetin-3-glucoside	Yes: The combination is more effective and significantly different from luteolin which was the more effective agent alone.
hesperitin+quercetin-3-glucoside	Yes: The combination is more effective and significantly different from quercetin-3-glcuose which was the more effective agent alone

Whether each combination has a greater than additive effect than its single actives alone was then examined (2.2.5 Synergy and product combination benefit testing

Additive effect:

(See Table 4.4 for mean differences and adjusted p values)

Combination	Additive effect?
phloretin+quercetin-3-glucoside	No: The combination had a 56.9% inhibitory effect (Table 4.4) compared to phloretin, which had a 30.8% inhibitory effect and quercetin-3-glucoside which has 27.4% inhibitory effect. There was therefore no additive effect of the combination, as the effect of the single actives alone added up to a greater effect.
luteolin+lectin glycan	No: The combination had glycan had a 45.0% inhibitory effect compared to luteolin which had a 30.8% effect and lectin glycan, which had no effect. The combination did have a greater additive effect, but this was not significant.
luteolin+quercetin-3-glucoside	Yes: The combination had a 64.2% inhibitory effect compared to luteolin, which had a 30.8% effect and quercetin-3-glucoside which had a 27.4% effect. The combination did therefore have a greater than additive effect and

	was significantly different from each active alone
hesperitin+quercetin-3-glucoside	Yes: The combination had a 57.8% inhibitory effect compared to hesperetin, which had a 3.9% effect and quercetin-3-glcuose which had a 30.8% effect. The combination therefore did have a greater than additive effect and was significantly different from both actives of the combination alone.

Table 4.4: Fructose + Glucose transport inhibitors combined to test for Glucose transport inhibition- set.2

Šídák's multiple compar test	Mean 1	Mean 2	Mean Diff.	Adjusted P Value
phloret *vs.* phloret+Lect Gly	70.82	67.65	3.169	>0.9999
phloretin+Lec Gly *vs.* Lect Gly	67.65	101.9	-34.24	<0.0001
phloretin+Lec Gly *vs.* DMSO	67.65	102.8	-35.13	<0.0001
phloretin *vs.* Phloretin+Q3G	70.82	45.9	24.93	0.0005
Phloretin+Q3G *vs.* Q3G	45.9	75.39	-29.5	<0.0001
Phloretin+Q3G *vs.* DMSO	45.9	102.8	-56.89	<0.0001
phloret *vs.* Phloret+Catechin	70.82	63.93	6.893	0.9996
Phloret+Catech *vs.* Catechin	63.93	102.2	-38.32	<0.0001
Phloretin+Catechin *vs.* DMSO	63.93	102.8	-38.86	<0.0001
Luteolin *vs.* Luteolin+Lect gly	72.02	57.75	14.26	0.3041
Luteolin+Lect gly *vs.* Lect gly	57.75	101.9	-44.14	<0.0001
Luteolin+Lect gly *vs.* DMSO	57.75	102.8	-45.03	<0.0001
Luteolin *vs.* Luteolin+Q3G	72.02	38.53	33.49	<0.0001
Luteolin+Q3G *vs.* Q3G	38.53	75.39	-36.87	<0.0001
Luteolin+Q3G *vs.* DMSO	38.53	102.8	-64.26	<0.0001
Luteoli *vs.* Luteolin+Catechin	72.02	55.18	16.83	0.0884
Luteolin+Catech *vs.* Catechin	55.18	102.2	-47.06	<0.0001
Luteolin+Catechin *vs.* DMSO	55.18	102.8	-47.6	<0.0001
Hesp *vs.* Hesp+Lec gly	98.84	92.25	6.591	0.9999
Hesp+Lec gly *vs.* Lect Gly	92.25	101.9	-9.642	0.9431
Hesp+Lec gly *vs.* DMSO	92.25	102.8	-10.53	0.8642
Hesp *vs.* Hesp+Q3G	98.84	45.03	53.81	<0.0001
Hesp+Q3G *vs.* Q3G	45.03	75.39	-30.36	<0.0001
Hesp+Q3G *vs.* DMSO	45.03	102.8	-57.75	<0.0001
Hesp *vs.* Hesp+Catechin	98.84	93.54	5.306	>0.9999
Hesp+Catechin *vs.* Catechin	93.54	102.2	-8.706	0.9838
Hesp+Catechin *vs.* DMSO	93.54	102.8	-9.249	0.9648
phloretin *vs.* DMSO	70.82	102.8	-31.97	<0.0001
Luteolin *vs.* DMSO	72.02	102.8	-30.77	<0.0001
Hesp *vs.* DMSO	98.84	102.8	-3.943	>0.9999
Lect Gly *vs.* DMSO	101.9	102.8	-0.8918	>0.9999
Q3G *vs.* DMSO	75.39	102.8	-27.39	<0.0001
Catechin *vs.* DMSO	102.2	102.8	-0.5436	>0.9999

Table 4.4: Fructose + glucose transport inhibitors combined to test for Glucose transport inhibition- set.2. Experiment set 2, potential fructose inhibition actives were tested with glucose inhibitors for synergistic effects. Actives were tested in an in Caco2 gut cell-line *in vitro* model optimised for fructose transport inhibition. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors (150 µM each) in 25 mM fructose + 25 mM glucose in PBS+ and the transport of both glucose and fructose measured after 2 hrs (% well µmoles transported/hr). Treatments were selected for comparison-based synergy group, e.g: for the luteolin (Lut) + phloretin (Phlt) combination- Lut+phlt was compared to phloretin, luteonin and DMSO control such that there were 33 comparisons (9 groups times three comparisons and each single active *vs* DMSO control) analysed by one-way ANOVA correcting for multi-comparisons using Šídák's statistical

hypothesis testing, n=9 replicate wells per treatment over 3 experiments. Many of the comparisons were significantly different. Some of the combinations were found to be synergistic either as being more effective than the highest effect single active or by having a greater than additive effect of the single active alone.

4.2.6 Catechins

Although gallic acid, ellagic acid and pyrogallol, as representatives of the gallates and tannins, showed little tendency or were inconsistent in their inhibition of fructose transport in the experiments above, reports of the effects of tea catechins to be effective in general (JP2009184992A; Villa-Rodriguez *et al*., 2017) suggested that this category of compounds should be investigated further. A range of catechins and theaflavones therefore were also tested in the fructose inhibition optimised Caco2 model.

Catechin, epicatechin gallate and epicatechin alone gave some of the best effects on fructose transport seen in these experiments showing up to 65% inhibition (Figure 4.34). Ethyl gallate also gave consistent, although not statistically significant, inhibition over the two experiments performed (Table 4.5).

Figure 4.34 and 4.35: Fructose and Glucose transport- effect of tea

Fig 4.34: Fructose Transport- Effect Tea catechins

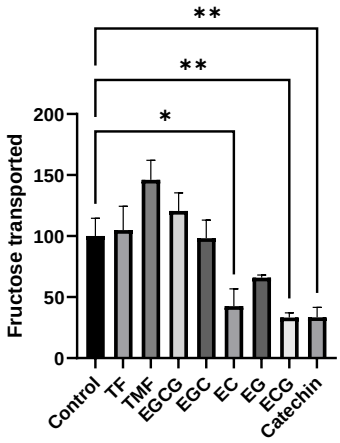


Fig 4.35: Glucose Transport- Effect Tea catechins

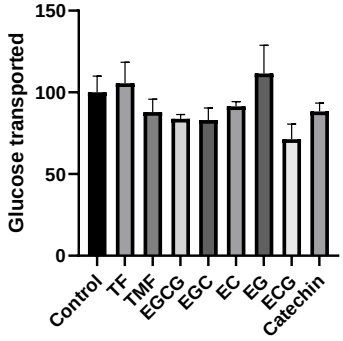


Figure 4.34 and 4.35: Fructose and Glucose transport- effect of tea catechins (% DMSO control). Tea catechins and theaflavones were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport over two experiments. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors (300 µM) in 25 mM fructose+25 mM glucose in PBS+ and the transport of both measured after 2 hrs (% well µmoles transported/hr) (% DMSO control shown). TF= theaflavone; TMF= theamethoxy flavone, EGCG= epigallo catechin gallate, EGC=epigallo catechin, EC=epicatechin, EG= ethyl gallate, ECG=epicatechin gallate. Catechin, ECG and EC showed significant fructose transport inhibition. All treatments were compared to the

control (Control, 0.1% DMSO) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing *p<0.05, **p<0.01, n=6 replicate wells per treatment.

For glucose transport, the tea catechins and theaflavones tested had no significant or substantial effect on transport over the two experiments performed (Figure 4.35).

4.2.7 Tea catechin synergies

The most effective tea catechin inhibitors found were next examined for synergistic activity with GLUT2 inhibitors.

For fructose transport no synergies were seen (Figure 4.36).

Figure 4.36: Tea catechin vs GLUT2 inhibitors potential synergistic fructose transport inhibition.

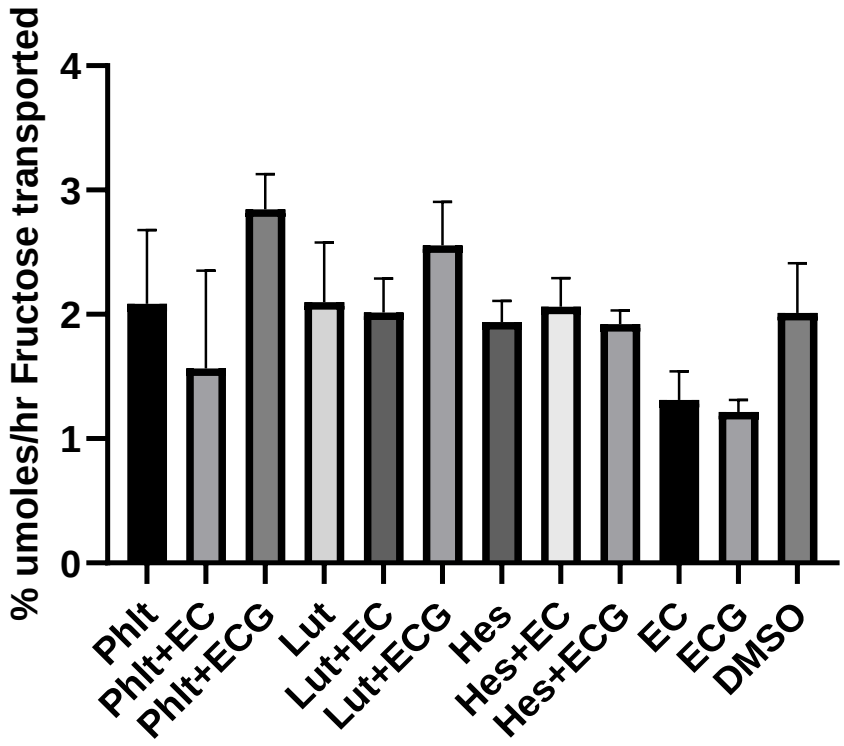


Figure 4.36: Tea catechin *vs* GLUT2 inhibitors potential synergistic fructose transport inhibition. Tea catechin actives were tested with potential glucose inhibition actives for synergy. Actives were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport inhibition. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors (150 µM each) in 25 mM fructose+25 mM glucose in PBS+ for and the transport of both glucose and fructose measured after 2 hrs (% well µmoles transported/hr). Treatments were selected for comparison based on synergy group, e.g: for the EC+phloretin (Phlt) combination- EC+Phlt was compared to EC, Phlt and DMSO control such that there were 23 comparisons (9 groups times three comparisons) + each single active *vs* DMSO control and analysed by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing. None of the combinations were found to give synergy or significant fructose transport inhibition *p<0.05, n=3 replicate wells per treatment.

However, for glucose transport, both catechins tested in combination showed significantly differences from the control (Figure 4.37), opening up the possibility of combination sub-thresholding, highest single active and additive effect synergies. However, upon examination such synergy was not found.

Figure 4.37 and Table 4.5: Tea catechin vs GLUT2 inhibitors potential synergistic Glucose transport inhibition:

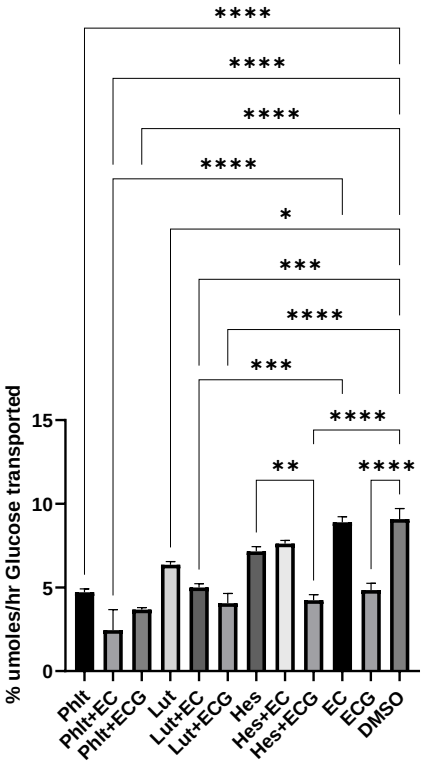


Table 4.5: Tea catechin vs GLUT2 transport inhibitors potential synergistic Glucose transport inhibition

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Phlt *vs.* DMSO	-1.092	-1.517 to -0.6662	Yes	****	<0.0001
Phlt+EC *vs.* DMSO	-1.352	-1.828 to -0.8766	Yes	****	<0.0001
Phlt+ECG *vs.* DMSO	-1.353	-1.779 to -0.9277	Yes	****	<0.0001
Lut *vs.* DMSO	-0.6823	-1.108 to -0.2567	Yes	***	0.0003
Lut+EC *vs.* DMSO	-1.022	-1.448 to -0.5963	Yes	****	<0.0001
Lut+ECG *vs.*DMSO	-1.257	-1.682 to -0.8309	Yes	****	<0.0001
Hes *vs.* DMSO	-0.4824	-0.908 to -0.0568	Yes	*	0.0165
Hes+EC *vs.* DMSO	-0.367	-0.7927 to 0.058	No	ns	0.1473
Hes+ECG *vs.* DMSO	-1.212	-1.637 to -0.7863	Yes	****	<0.0001
EC *vs.* DMSO	0.04721	-0.472 to 0.3784	No	ns	>0.9999
ECG *vs.* DMSO	-1.061	-1.487 to -0.6357	Yes	****	<0.0001
Phlt *vs.* Phlt+EC	0.2606	-0.2153 to 0.736	No	ns	0.8217
Phlt+EC *vs.* EC	-1.305	-1.781 to -0.8294	Yes	****	<0.0001
Phlt *vs.* Phlt+ECG	0.2615	-0.1641 to 0.687	No	ns	0.658
Phlt+ECG *vs.* ECG	-0.292	-0.7177 to 0.133	No	ns	0.4682
Lut *vs.* Lut+EC	0.3396	-0.08606 to 0.76	No	ns	0.2342
Lut+EC *vs.* EC	-0.9747	-1.400 to -0.5491	Yes	****	<0.0001
Lut *vs.* Lut+ECG	0.5742	0.1486 to 0.9998	Yes	**	0.0026
Lut+ECG *vs.* ECG	-0.1952	-0.6208 to 0.230	No	ns	0.9574
Hes *vs.* Hes+EC	-0.1154	-0.5410 to 0.310	No	ns	>0.9999
Hes+EC *vs.* EC	-0.3198	-0.7454 to 0.105	No	ns	0.3186
Hes *vs.* Hes+ECG	0.7295	0.3038 to 1.155	Yes	***	0.0001
Hes+ECG *vs.* ECG	-0.1506	-0.576 to 0.2750	No	ns	0.9979

Figure 4.37 and Table 4.5: Tea catechin *vs* GLUT2 inhibitors potential synergistic Glucose transport inhibition: Tea catechin actives were tested with potential glucose inhibition actives for synergy. Actives were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport inhibition. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors (150 µM each) in 25 mM fructose+25 mM glucose in PBS+ fand the transport of both glucose and fructose measured after 2 hrs (% well µmoles transported/hr). Treatments were selected for comparison based on synergy group, e.g: for the EC+phloretin (Phlt) combination- EC+Phlt was compared to EC, Phlt and DMSO control such that there were 23 comparisons (9 groups times three comparisons) + each single active *vs* DMSO control and analysed by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing. Many of the combinations showed substantial inhibition. However, none were found to give synergy, as single actives were quite effective too *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=3 replicate wells per treatment

Phloretin+EC:

- Significantly different from control- but so too is phloretin so there is no combination sub-threshold
- The highest single active is phloretin- which Phlt+EC is not significantly different from.
- The combination is significantly different from EC but not phloretin so there is no additive effect.

Phloretin+ECG:

- Significantly different from control- but so too is phloretin and ECG so there is no combination sub-threshold.
- The highest single active is phloretin, from which the combination is not significantly different
- The combination is not significantly different from either single active so there is no additive effect

Luteolin+EC:

- Significantly different from control- but so too is luteolin so no combination sub-threshold.
- The highest single active is luteolin, from which the combination is not significantly different
- The combination is not significantly different from either single active so there is no additive effect

Luteolin+ECG:

- Significantly different from control- but so too is luteolin so there is no combination sub-threshold.
- The highest single active is ECG, from which the combination is not significantly different.
- The combination is not significantly different from both single active so there is no additive effect.

Hesperetin+EC:

- Not Significantly different from control- so there is no combination sub-threshold.
- The highest single active is hesperetin, from which the combination is not significantly different
- The combination is not significantly different from either single active so there is no additive effect

Hesperetin+ECG:

- Significantly different from control- but so too is ECG so there is no combination sub-threshold.
- The highest single active is ECG, from which the combination is not significantly different.
- The combination is not significantly different from both single actives so there is no additive effect.

4.2.8 Fructose transport inhibition by tea extracts

As some tea components performed so well as single actives on fructose transport, the effects of aqueous tea extracts directly in the Caco2 fructose transport model was examined. ~~Tea extracts were prepared by pouring 200ml of water at 95^{o}C onto a tea bag and leaving for 3 minutes before removing the bag. The extract was then cooled to room temperature. An estimation was made as to what extent such drinks would be typically diluted by as they reach the small intestine. If the stomach contains 0.5-1.0 litres of fluid and 200ml of tea is consumed, this equates to a dilution of approximately 1-in-3 to 1-in-6 dilution. Alternatively, it has been estimated that a total of 6-8 litres of digestive juices are produced and secreted each day in the adult human gut (Shmidt and Thews, 1983). If 4x250ml of tea/day is consumed plus other food and drink this would equate to a dilution of approximately 1-in-8 to 1-in-10.~~

Preliminary results indicated a significant and substantial 60% inhibition of fructose transport for both brand 1 black and green tea (propriety blends) at dilutions 1-in-2 and 1-in-4 (Figure 4.38).

Figure 4.38 and 4.39: Fructose transport- effects of tea extracts. Tea extracts were tested in a Caco2 gut cell-line in vitro model optimised for fructose transport

Fig 4.38: Fructose Transport- Effects of Tea Extracts

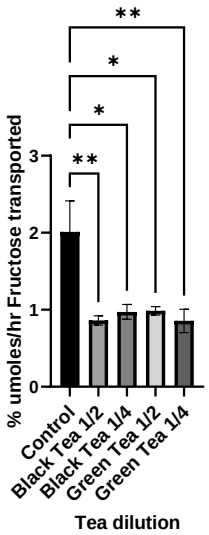


Fig 4.39: Glucose Transport- Effect of Tea Extracts

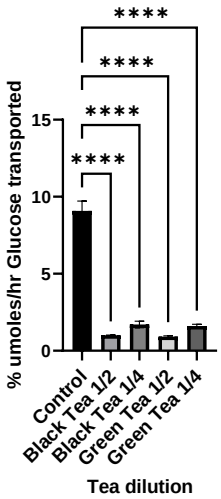


Figure 4.38 and 4.39: Fructose transport- effects of tea extracts. Tea extracts were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with aqueous tea extracts ~~(95°C for 3 minutes)~~ at dilutions considered physiological in 25 mM fructose + 25 mM glucose in PBS+ and the transport of both measured after 2 hrs (% well µmoles transported/hr). Black tea and green tea significantly and substantially inhibited fructose and glucose transport. All treatments were compared to the control by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing. *p<0.05, ** p<0.01 and ****p<0.0001, n=3 replicate wells per treatment.

Glucose transport was also highly significantly inhibited (up to 90%) by tea extracts and in a dose-dependent manner (Figure 4.39).

In a follow up experiment, further dilutions were used to investigate a greater physiological range. For fructose transport inhibition, Brand 1 black tea and green tea aqueous extracts gave largely a dose-dependent response, although not statistically significant (Figure 4.40). Black tea with milk also tended to be inhibitory. Brand 1 ice tea (peach) was shown to give significant and dose-dependent inhibition. At a 1-in-16 dilution, ice tea resulted in 84% inhibition, and 97% at 1-in-8. Brand 2 diet cola and brand 1 decaffeinated tea showed some tendency for inhibition, but this was not statistically significant. Instant Brand 3 coffee had no effect (prepared by adding 200 ml of water at 95°C to one sachet).

Figure 4.40 and 4.41: Effect of tea extracts and other beverages on fructose and glucose transport.

Fig 4.40: Effect of Tea Extracts and other Beverages on Fructose Transport

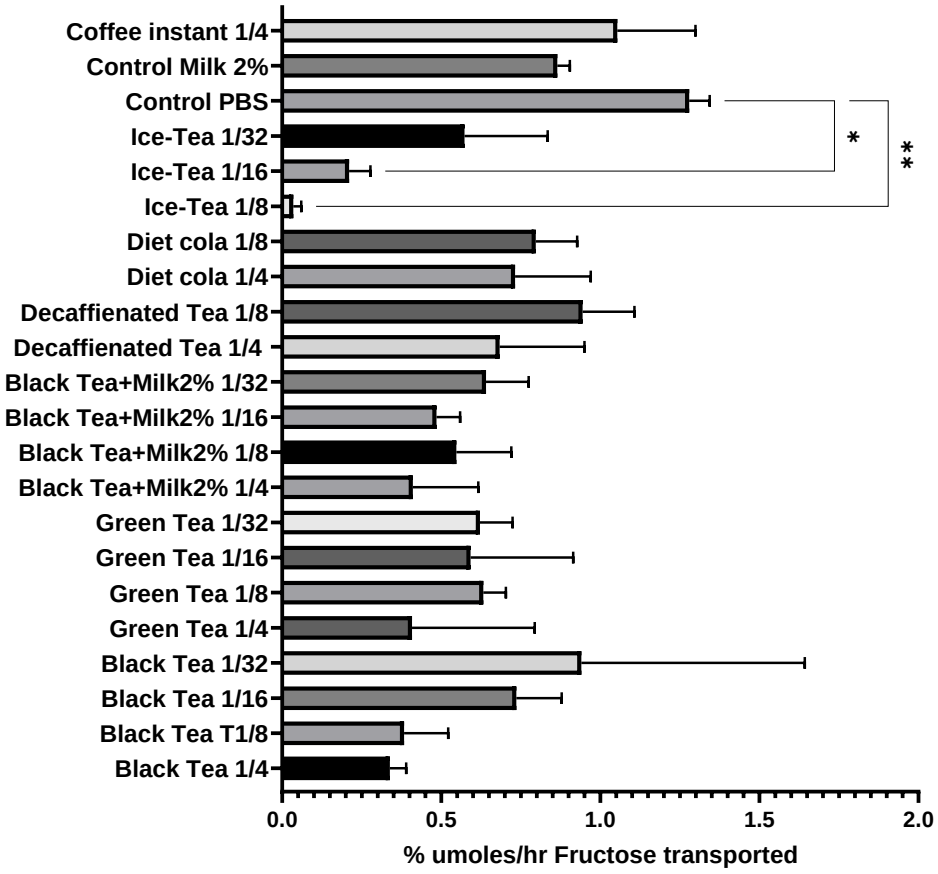


Fig 4.41: Effect of Tea Extracts and other Beverages on Glucose Transport

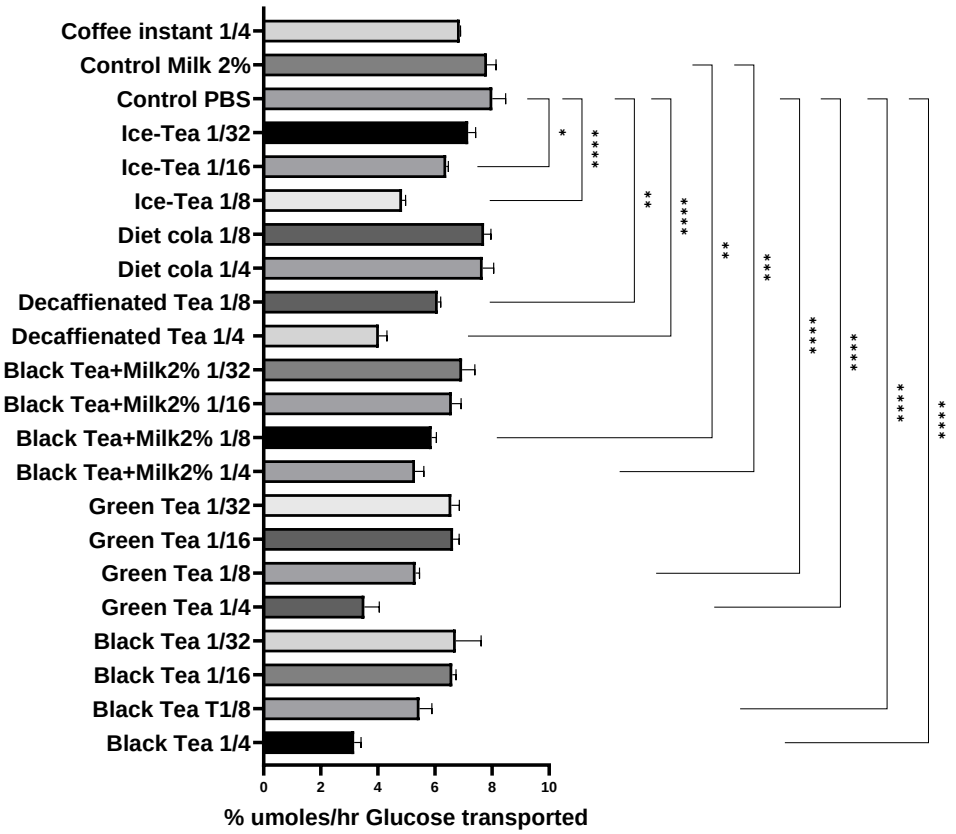


Figure 4.40 and 4.41: Effect of tea extracts and other beverages on fructose and glucose transport. Tea extracts and beverages were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with beverage dilutions considered physiological in 25 mM fructose+25 mM glucose in PBS+ and the transport of both measured after 2 hrs (% well µmoles transported/hr). Ice tea at a 1-in-16 dilution resulted significantly in 84% inhibition, and 97% at 1-in-8. All treatments were compared to the relevant control by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing *p<0.05, **p<0.01, n=3 replicate wells per treatment.

In contrast to its effect on fructose transport, ice tea gave only up to 40% inhibition of glucose transport, although dose-dependently and significantly at the highest dilutions of 1-in-16 and 1-in-8 (Figure 4.41). Black tea, green, black tea with milk and decaffeinated tea gave significant inhibition at the higher concentrations of 1-in-8 and 1-in-4. Diet cola and coffee were not inhibitory.

4.2.9 Ice tea, pH, Ingredients and polyphenols

To investigate whether the acidity of the beverages had any effect on sugar transport in the Caco2 *in vitro* model used here, the pH of each treatment was measured and correlated with the level of transport found for each. The correlation for glucose was weak (Person product moment r^2= 0.1023, but significant (Figure 4.42).

Figure 4.42 and 4.43: pH of beverages show correlation with glucose and transport

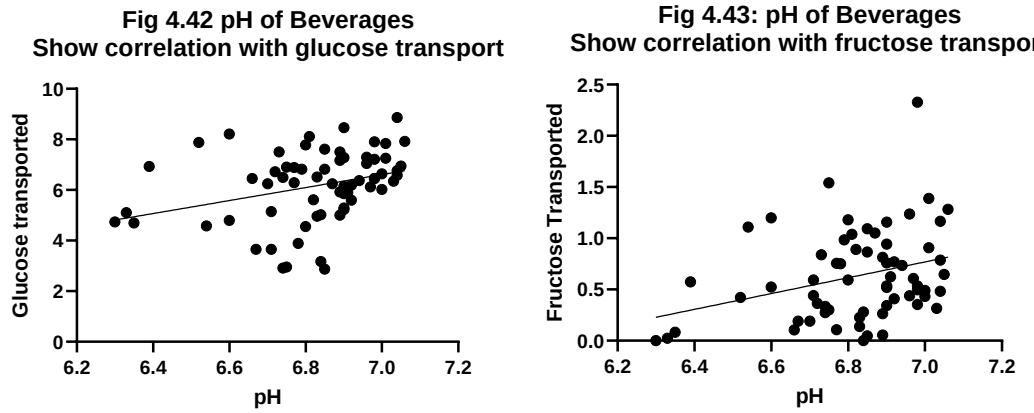


Figure 4.42 and 4.43: pH of beverages show correlation with glucose and transport. Tea extracts and beverages were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with beverage dilutions considered physiological in 25 mM fructose+25 mM glucose in PBS+ and the transport of both measured after 2 hrs (% well µmoles transported/hr). The pH of each beverage dilution was measured. A weak but statistically significant correlation was found for glucose (Person product moment r^2= 0.1023, (p<0.01), n=66 and fructose r^2= 0.09727, (p<0.05), n=66

A similar weak (r^2= 0.09727) but statistically significant correlation was found for fructose transport and pH (Figure 4.43).

The pH was further investigated to confirm that it was not a substantial cause of the inhibitory effects seen on transport. Beverages were adjusted to the pH of the control i.e. pH 7.0. and re-tested alongside the non-neutralised equivalent beverage (Figure 4.44).

For both fructose and glucose transport neutralised black tea, green tea and decaffeinated tea were found to be just as effective as non-adjusted drinks. An acidified PBS control did not cause any reduction in glucose transport. Neither neutralised nor non-adjusted lemon ice tea were effective in this assay. However, freshly opened lemon ice tea was found to be just as effective at a 1-in-16 dilution, or

in fact more so than in the previous experiment. Other flavours of fresh ice tea: mango, lemon and raspberry gave similar levels and significant inhibition Figures 4.44 and 4.45).

Figure 4.44 and 4.45: Effect of tea extracts and other beverages on fructose and glucose transport inc. effect of pH, flavours and freshness:

Fig 4.44: Effect of Tea Extracts and other Beverages on Fructose Transport

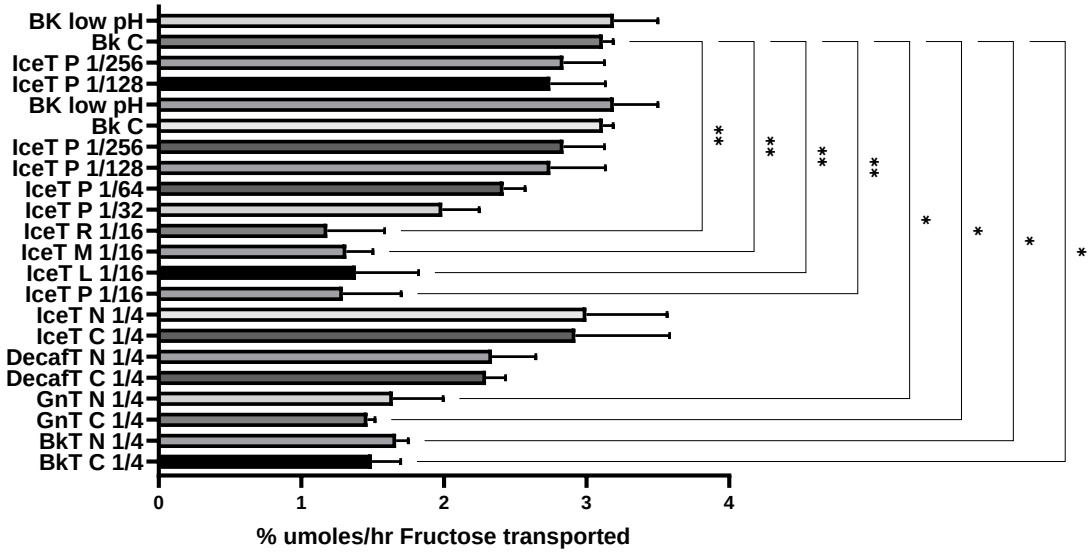
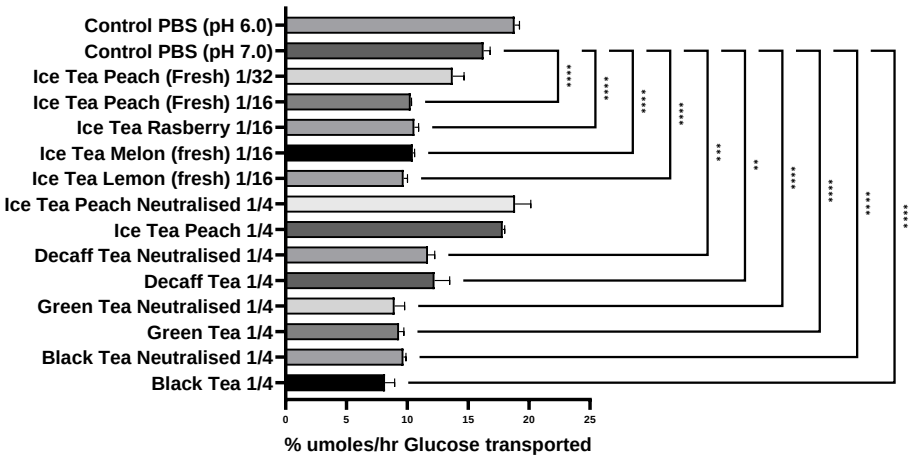


Figure 4.45:

Fig 4.45: Effect of Tea Extracts and other Beverages on Glucose Transport Inc. effect of pH, flavours and freshness.

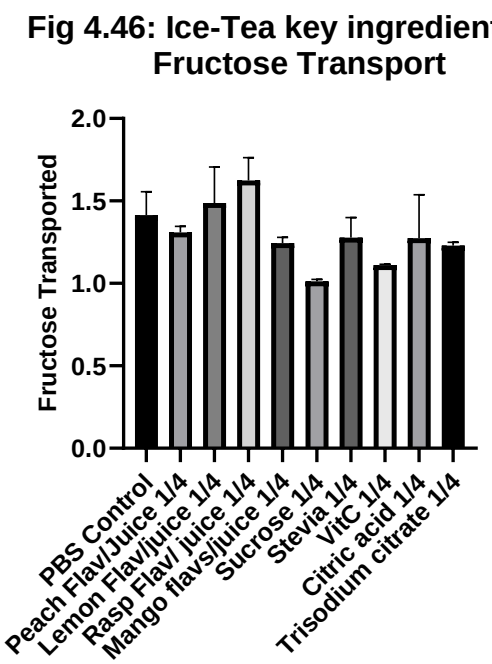


Figures 4.44 and 4.45: Effect of tea extracts and other beverages on fructose and glucose transport inc. effect of pH, flavours and freshness: Tea extracts and beverages were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with beverage dilutions considered

physiological in 25 mM fructose+25 mM glucose in PBS+ and the transport of both measured after 2 hrs (% well µmoles transported/hr). Where beverages were neutralised similar glucose transport inhibition was seen. Different flavours of fresh ice tea were all equally effective at a 1-in-16 dilution. Non-fresh ice tea though had no effect here. All treatments were compared to PBS control by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing **p<0.01, *** p<0.001, ****p<0.0001, n=3 replicate wells per treatment (figure.4.44) and n=3 replicate wells per treatment (figure 4.45).

To further investigate the efficacy of ice tea, the key ingredients of ice tea and the different flavours were tested in the fructose transport model at a 1-in-4 dilution of the concentrations that they are included in ice tea products (proprietary information). None had a substantial or significant effect, although there was ~~perhaps~~ a marginal inhibitory tendency for sucrose and vitamin C (Figure 4.46).

Figure 4.46 and 4.47: Ice-tea key ingredients vs. fructose and glucose transport



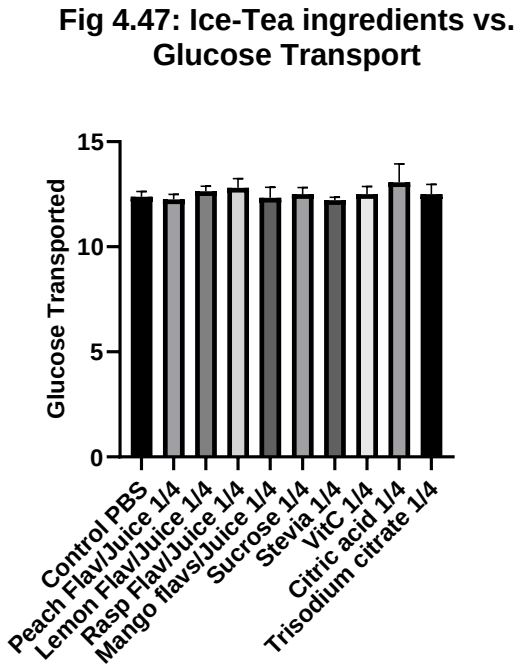


Figure 4.46 and 4.47: Ice-tea key ingredients *vs*. fructose and glucose transport. Ice tea main ingredients were tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with beverage dilutions considered physiological in 25 mM fructose+25 mM glucose in PBS+ and the transport of both measured after 2 hrs (% well µmoles transported/hr). None had a significant effect on fructose transport. All treatments were compared to PBS control by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing, n=3 replicate wells per treatment.

Individual ice tea ingredients also had no effect on glucose transport and showed no tendency for inhibition (Figure 4.47).

As some beverages tested thus far are not high in catechin and yet had significant effects on fructose and glucose transport, the possibility that polyphenols in general might be behind the activity was investigated.

4.2.10 Polyphenols

Total polyphenols in beverages were measured using the Folin-Ciocalteu assay (Velioglu *et al*., 1998). As this is a surrogate assay that basically measures antioxidant activity, ascorbic acid was removed by ascorbate oxidase (Hewitt and Dickes, 1961; Foyer *et al*., 1995) and the assay adapted to a microplate format. Activities measured were related to gallic acid standards.

As anticipated, the beverages that were most effective at inhibiting fructose transport were found to be high in polyphenols (antioxidant activity); i.e. black tea, green tea and decaffeinated tea, with the noticeable exception of ice tea (Figure 4.48). Beverages with high polyphenol (antioxidant activity) however, did not necessarily have high inhibitory activity, e.g. coffee. The beverages: diet cola, lemonade, energy-drink, soda water, tonic-water, and mineral water showed no fructose or glucose transport inhibition (data not shown) and little or no polyphenol content. Vitamin-water contained a small amount of polyphenol, but still showed no inhibitory activity.

Figure 4.48: Polyphenol content of beverages and ice tea.

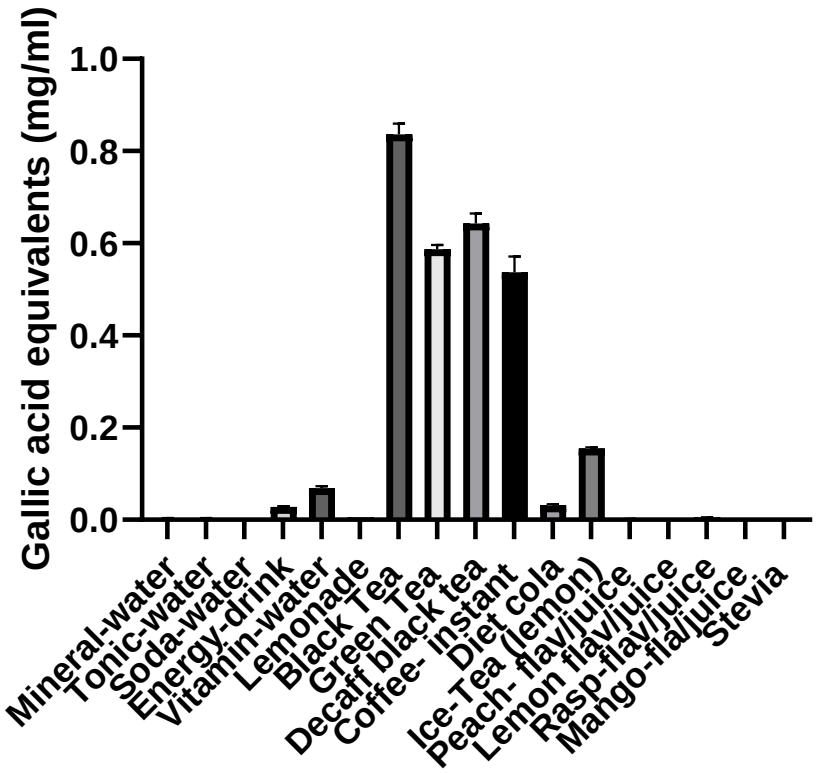


Figure 4.48: Polyphenol content of Beverages and ice tea ingredients. Total polyphenols were measured in duplicate using the Folin-Ciocalteu assay (Velioglu *et al.*, 1998) after the removal of the antioxidant ascorbic acid (Hewitt & Dickes, 1961; Foyer *et al.*, 1995) and the assay adapted to a microplate format. Activities measured were related to gallic acid standards. In general, those beverages with high antioxidant/polyphenol content showed substantial fructose and/or glucose transport inhibition, with the exception of coffee. Those with little or no polyphenol/antioxidant content showed little or no inhibitory activity, with the exception of ice tea. n=3 replicate wells per treatment.

4.2.11 Beverages and synergy with phloridzin

Having demonstrated that tea extracts can inhibit fructose and glucose transport, synergy with phloridzin was then investigated. Phloridzin was chosen to look for potential synergies, as it is an inhibitor of SGLT1 (Rossetti *et al*., 1987) which possibly stimulates GLUT2 translocation to the apical (gut lumen side) membrane (Kellet and Brot-Laroche, 2005), and fructose might also be transported by GLUT2 (Jones *et al*., 2011). Synergy was determined by demonstrating either response additivity, combination sub-thresholding or highest single agent or product benefit (see 2.0 Materials and Methodology, 2.2 Identifying plant natural compounds or extracts that can reduce fructose spikes, 2.2.5 Synergy and product combination benefit testing).

For the effects of black tea+phloridzin on glucose transport, a two-tailed significance test showed no combination sub-thresholding or highest single activity effect (Figure 4.49).

Figure 4.49 and 4.50: Black tea synergy with phloridzin vs. glucose and transport.

Fig 4.49: Black Tea synergy with Phloridzin *vs.* Glucose Transport

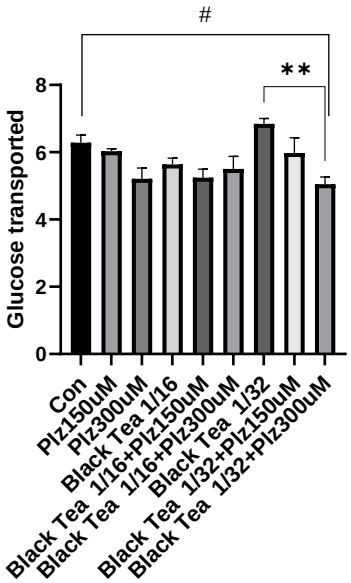


Fig 4.50: Black Tea synergy with Phloridzin *vs*. Fructose Transport

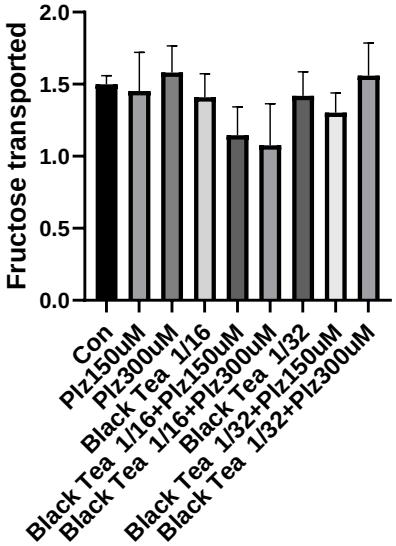


Figure 4.49 and 4.50: Black tea synergy with phloridzin *vs*. glucose and transport. Black tea (1-in-32 and 1-in-16 dilution and phloridzin (Plz, 150 and 300 µM) were tested for synergistic effect on glucose

transport in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors in 25 mM fructose+25 mM glucose in PBS+ (0.5 ml to the insert supply well and 1.0 ml PBS (+) added to the bottom collection chamber) and the transport of both measured after 2 hrs (% well µmoles transported/hr). All treatments were compared to the control and all combinations with respective single actives (e.g. black tea 1/32+Plz 300 µM with black tea 1/32 and Plz 300 µM by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, and a two-way ANOVA to test for interaction ** $p<0.01$, # $p<0.1$, n=3 replicate wells per treatment. Although showing significant inhibition, black tea 1-in-32+Plz 300 µM does not show combination sub-thresholding or highest single active synergy.

However, if a one-tail test is considered, the combination does demonstrate combination sub-thresholding, as black tea1/32+Plz 300 µM shows significant inhibition, whereas black tea 1/32 or Plz 300 µM alone do not ($p<0.1$) (Table 4.6).

Table 4.6: Black Tea+Phloridzin Vs. Glucose Transport- One-way ANOVA

Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value
Con *vs.* Plz150µM	0.06379	-0.2651 to 0.3927	No	ns	>0.9999
Con *vs.* Plz300µM	0.2687	-0.06022 to 0.5975	No	ns	0.1816
Con *vs.* Black tea 1/16	0.1611	-0.1677 to 0.4900	No	ns	0.8536
Con *vs.*Blk tea 1/16+Plz150µM	0.2603	-0.06858 to 0.5892	No	ns	0.2144
Con *vs.* Blk tea 1/16+Plz300µM	0.1947	-0.1341 to 0.5236	No	ns	0.6244
Con *vs.* Blk tea 1/32	-0.1405	-0.4694 to 0.1883	No	ns	0.9427
Con *vs.*Blk tea 1/32+Plz150µM	0.07612	-0.2528 to 0.4050	No	ns	>0.9999
Con *vs.* Blk tea 1/32+Plz300µM	0.3088	-0.02010 to 0.6376	No	ns	0.078
Blk tea 1/16 *vs.* Blk tea 1/16+Plz150µM	0.09915	-0.2297 to 0.4280	No	ns	0.9979
Plz150µM *vs.* Blk tea 1/16+Plz150µM	0.1965	-0.1324 to 0.5254	No	ns	0.6111
Blk tea 1/16 *vs.* Blk tea 1/16+Plz300µM	0.0336	-0.2953 to 0.3625	No	ns	>0.9999
Plz300µM *vs.* Blk tea 1/16+Plz300µM	-0.07391	-0.4028 to 0.2550	No	ns	>0.9999
Blk tea 1/32 *vs.*Blk tea1/32+Plz150µM	0.2166	-0.1122 to 0.5455	No	ns	0.4623
Plz150µM *vs.* Blk tea 1/32+Plz150µM	0.01233	-0.3165 to 0.3412	No	ns	>0.9999

Blk tea 1/32 *vs.* Blk tea 1/32+Plz300µM	0.4493	0.1204 to 0.7782	Yes	**	0.0032
Plz300µM *vs.*Blk tea 1/32+Plz300µM	0.04012	-0.2888 to 0.3690	No	ns	>0.9999

Table 4.6: Black Tea+Phloridzin *Vs.* Glucose Transport- One-way ANOVA. All treatments were compared to the control (Figure 4.49) and all combinations with respective single actives (e.g. black tea 1/32+Plz 300 µM with black tea 1/3 and Plz 300 µM– by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing * $p<0.05$, ** $p<0.01$, n=3 replicate wells per treatment. Although showing significant inhibition black tea 1-in-32+Plz 300 µM does not show combination sub-thresholding or highest single active synergy.

Although needing to be carefully considered, it may be appropriate to apply a one-tail statistical test here to potential glucose and fructose transport inhibitors, as only inhibition is being considered, i.e. a directional hypothesis, (Field, 2018). Of course, one-tail tests are controversial; however, where a result in the opposite direction would lead to the same conclusion as finding no effect, it is considered a legitimate use (Lombardi & Hurlbert, 2009). In this study reagents found to increase fructose transport would have led to their rejection, just as for reagents that showed no effect, as increasing fructose transport in the context of this work is completely undesirable. However, with combination sub-thresholding it is important to check that there is a substantial difference between the reagent combination from the single actives it is compared with. In this case, black tea1/32+Plz 300 µM did not substantially show less glucose transport than phloridzin 300 µM (Plz 300 µM). Highest single active synergy also did not apply, as although black tea1/32+Plz 300 µM is significantly different from black tea 1-in-32, this was not the highest inhibitory single active.

Two-way ANOVA however, shows significant interaction between Black tea and Plz- demonstrating response additive synergy (Table 4.7).

Table 4.7: Black Tea+Phloridzin vs. Glucose Transport- Two-way ANOVA

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	22.24	**0.019**	*	Yes
Row Factor	11.42	0.0367	*	Yes
Column Factor	40.62	0.0002	***	Yes

Table 4.7: Black Tea+Phloridzin *vs.* Glucose Transport- Two-way ANOVA. 2-way ANOVA shows significant interaction between black tea and phloridzin- demonstrating response additive synergy.

For fructose with black tea and phloridzin, no statistically significant differences were seen between treatments (Figure 4.50 above), and no interaction was seen between black tea and phloridzin by two-way ANOVA (see alsoTable 4.8 below), n=3 replicate wells per treatment.

Table 4.8: Black Tea + Phloridzin vs. Fructose Transport- Two-way ANOVA

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	7.325	0.7619	ns	No
Row Factor	17.11	0.144	ns	No
Column Factor	4.388	0.5837	ns	No

Table 4.8: Black Tea + Phloridzin *vs.* Fructose Transport- Two-way ANOVA. 2-way ANOVA shows no significant interaction between black tea- therefore not demonstrating response additive synergy, n=3 replicate wells per treatment.

For the green tea+phloridzin mixture, combination sub-thresholding was found *vs.* glucose transport as green tea 1/16+phloridzin 150 µM is significantly reduced, whilst the actives alone at these doses did not show a significant reduction (Figure 4.51). Similar results were seen for green tea 1/16+phloridzin 300 µM and green tea 1-in-16 and Phloridzin 300 µM. However, the difference between the combination and the single active phloridzin 300 µM was not substantial.

Figure 4.51 and 4.52: Green tea synergy with phloridzin vs. glucose and fructose transport

Fig 4.51: Green Tea synergy with Phloridzin *vs*. Glucose Transport

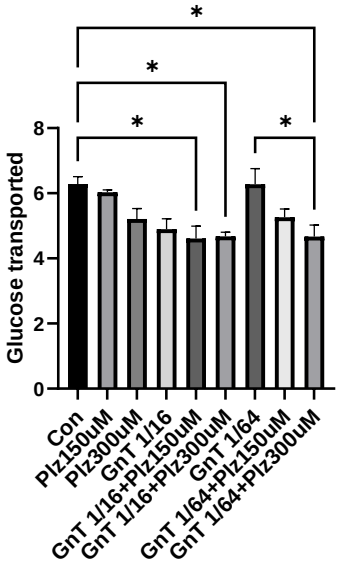


Fig 4.52: Green Tea synergy with Phloridzin *vs*. Fructose Transport

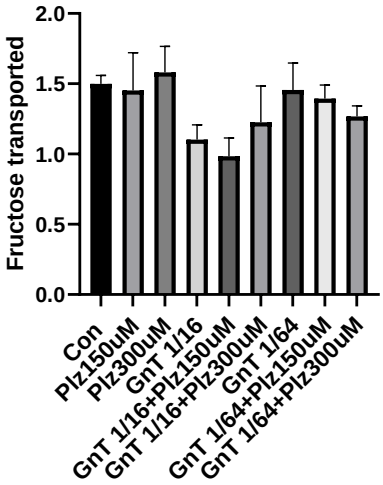


Figure 4.51 and 4.52: Green tea synergy with phloridzin *vs*. glucose and fructose transport. Green tea (1-in 64 and 1-in-16 dilution and phloridzin (Plz, 150 and 300 µM) were tested for synergistic effect on glucose transport tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors in 25 mM fructose+25 mM glucose in PBS+ (0.5 ml to the insert supply well and 1.0 ml PBS (+) added to the bottom collection chamber) and the transport of both measured after 2 hrs (% well µmoles transported/hr). All treatments were compared to the control and all combinations with respective single actives (e.g. green tea 1/32+Plz 300 µM with green tea 1/32 and Plz 300 µM_ by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, and a two-way ANOVA to test for interaction *p<0.05, n=3 replicate wells per treatment. No substantial synergy was seen for any combination.

Two-way ANOVA also does not show a significant interaction between green tea and phloridzin and therefore not demonstrating Response additive synergy for glucose (Table 4.9).

Table 4.9: Green Tea+Phloridzin s. Glucose Transport- Two-way ANOVA

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	10.59	0.2254	ns	No
Row Factor	33.8	0.0012	**	Yes
Column Factor	25.23	0.0043	**	Yes

Table 4.9: Green tea+phloridzin *vs.* glucose transport- Two-way ANOVA. 2-way ANOVA shows no significant interaction between green tea and phloridzin therefore not demonstrating response additive synergy, n=3 replicate wells per treatment.

For the effect of green tea+phloridzin combinations on fructose transport, no synergy was seen either by one-way ANOVA (Figure 4.52) or by interaction between green tea and phloridzin by two-way ANOVA (Table 4.10).

Table 4.10: Green tea+phloridzin vs. fructose transport- Two-way ANOVA

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	5.296	0.8177	ns	No
Row Factor	31.03	0.0262	*	Yes
Column Factor	1.494	0.8076	ns	No

Table 4.10: Green tea+phloridzin *vs*. fructose transport- Two-way ANOVA. 2-way ANOVA shows no significant interaction between green tea and phloridzin therefore not demonstrating response additive synergy, n=3 replicate wells per treatment.

For the effect of diet cola and phloridzin combinations on glucose and fructose transport, no significant differences were found between treatments by one-way ANOVA (Figure 4.53 and 4.54) or by two-way ANOVA for treatment interaction (Table 4.11 and 4.12).

Figure 4.53 and 4.54: Diet cola synergy with phloridzin vs. glucose and fructose transport

Fig 4.53: Diet cola synergy with Phloridzin Vs. Glucose Transport

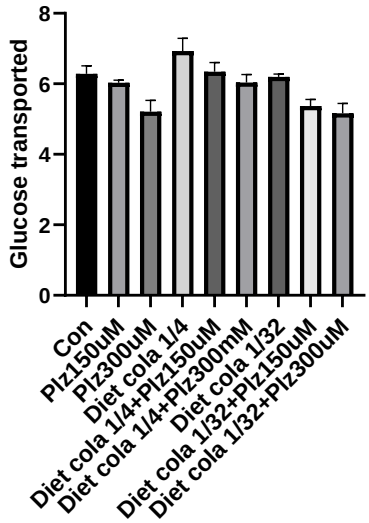


Fig 4,54: Diet cola synergy with Phloridzin Vs. Fructose Transport

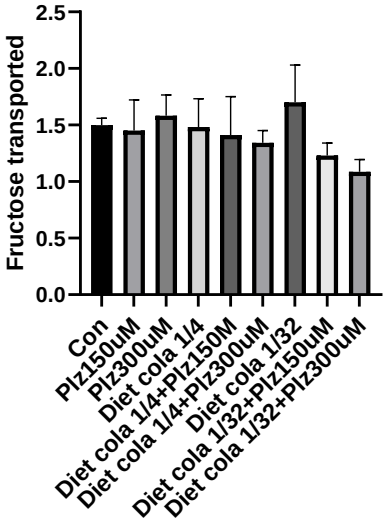


Figure 4.53 and 4.54: Diet cola synergy with phloridzin *vs*. glucose and fructose transport. Diet cola (1-in-4 and 1-in-32 dilution and phloridzin (Plz, 150 and 300 µM) were tested for synergistic effect on glucose transport tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors in 25 mM fructose+25 mM glucose in PBS+ for 2 hrs (0.5 ml to the insert supply well and 1.0 ml PBS (+) added to the bottom collection chamber) and the transport of both measured after 2 hrs (% well µmoles transported/hr). All treatments were compared to the control and all combinations with respective single actives (e.g. diet cola 1/32+Plz 300 µM with diet cola 1/32 and Plz 300 µM_ by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, and a two-way ANOVA to test for interaction * $p<0.05$, n=3 replicate wells per treatment. For the effect of diet cola and phloridzin combinations on glucose transport, no significant differences were found between treatments by one-way ANOVA.

Table 4.11: Diet cola+phloridzin vs. glucose transport- Two-way ANOVA

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	3.475	0.6864	ns	No
Row Factor	30.15	0.0012	**	Yes
Column Factor	39.03	0.0003	***	Yes

Table 4.11: Diet cola+phloridzin *vs*. glucose transport- Two-way ANOVA. 2-way ANOVA shows no significant interaction between diet cola and phloridzin therefore not demonstrating response additive synergy, n=3 replicate wells per treatment.

Table 4.12: Diet cola+phloridzin vs. fructose transport- Two-way ANOVA

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	12.03	0.5951	ns	No
Row Factor	3.913	0.637	ns	No
Column Factor	7.896	0.4116	ns	No

Table 4.12: Diet cola+phloridzin *vs*. fructose transport- Two-way ANOVA. 2-way ANOVA shows no significant interaction between diet cola and phloridzin therefore does not demonstrate response additive synergy, n=3 replicate wells per treatment.

For ice tea+phloridzin combinations on glucose transport, there was combination sub-thresholding for ice tea 1-in-32 dilution+phloridzin 300 µM which was significantly different from the control, whereas the actives alone showed no significant inhibition (Figure 4.55). On this occasion, the combination is substantially different from both single actives.

Figure 4.55 and 4.56: Ice-tea synergy with phloridzin vs. glucose and fructose transport

Fig 4.55: Ice-Tea synergy with Phloridzin vs. Glucose Transport

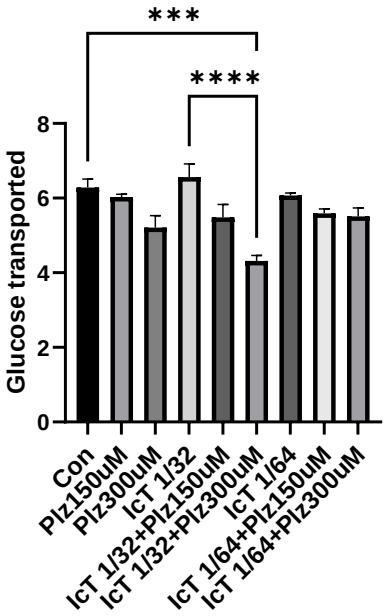


Fig 4.56: Ice-Tea synergy with Phloridzin vs. Fructose Transport

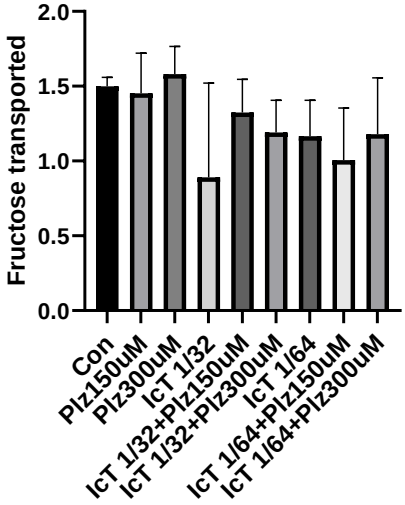


Figure 4.55 and 4.56: Ice-tea synergy with phloridzin *vs*. glucose and fructose transport. Ice tea (peach) (1-in-32, 1-in-64 and 1-in-128 dilutions and phloridzin (Plz, 150 and 300 µM) were tested for synergistic effect on glucose transport tested in a Caco2 gut cell-line *in vitro* model optimised for fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with potential inhibitors in 25 mM fructose+25 mM glucose in PBS+ (0.5 ml to the insert supply well and 1.0 ml PBS (+) added to the bottom collection chamber) and the transport of both measured after 2 hrs (% well µmoles transported/hr). All treatments were compared to the control and all combinations with respective single actives (e.g. Iced tea 1/32+Plz 300 µM with ice tea 1/32 and Plz 300 µM_ by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, and a two-way ANOVA to test for interaction ***p*<0.01, ****p*<0.001, *****p*<0.0001, n=3 replicate wells per treatment. Combination sub-thresholding for ice tea 1-in-32 dilution+phloridzin 300 µM is shown and is substantially different from both single actives.

There was also response additive synergy for the effect of ice tea+phloridzin on glucose transport, as there was a significant interaction between the ice tea and phloridzin by two-way ANOVA (Table 4.13).

Table 4.13: Ice tea+Phloridzin Vs. Glucose Transport- Two-way ANOVA

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	18.1	**0.0223**	*	Yes
Row Factor	5.073	0.1528	ns	No
Column Factor	54.97	<0.0001	****	Yes

Table 4.13: Ice tea+Phloridzin *Vs*. Glucose Transport- Two-way ANOVA. 2-way ANOVA shows a significant interaction between Ice tea and phloridzin therefore demonstrating response additive synergy, n=3 replicate wells per treatment.

For fructose transport, Ice tea and phloridzin showed no significant treatment effects (Figure 4.56 above) and no synergistic interaction was found (Table 4.14).

Table 4.14: Ice tea+Phloridzin vs. Fructose Transport- Two-way ANOVA

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	6.197	0.9169	ns	No
Row Factor	17.42	0.1686	ns	No
Column Factor	0.2616	0.9597	ns	No

Table 4.14: Ice tea+phloridzin *vs*. fructose transport- Two-way ANOVA. 2-way ANOVA shows no significant interaction between ice tea and phloridzin and therefore does not demonstrate response additive synergy, n=3 replicate wells per treatment.

4.2.12 Effect of glucose on fructose transport

It has previously been noted that the presence of glucose enhances fructose transport (Rumessen & Gudmand-hoyer, 1986, Truswell *et al*., 1988). The main hypothesis for this phenomenon is that in response to high glucose SGLT1 stimulates GLUT2 translocation to the apical (gut lumen side) membrane ((Rossetti *et al*., 1987); Kellet and Brot-Laroche, 2005), which may then transport glucose and fructose (Jones *et al*., 2011).

As the SGLT1 inhibitor, phloridzin, had little effect on fructose transport in these studies ((Figures 4.19 and 4.20) the effect of glucose on fructose transport was investigated and a reduction in the fructose:glucose ratio was also found to reduce fructose transport (Figure 4.57).

Figure 4.57: The effect of glucose on fructose transport

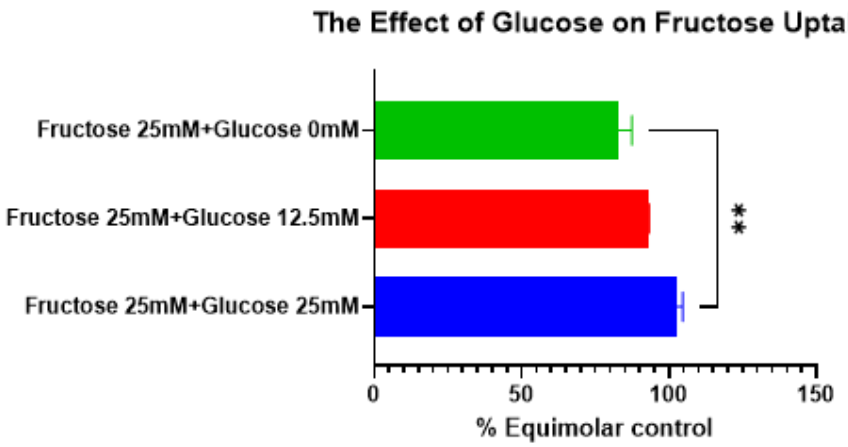


Figure 4.57: The effect of glucose on fructose transport. Caco2 gut cells were seeded in 96-well plate inserts and differentiated for 5 days. Cells were treated with fructose at 25 mM mixed by vortex with glucose at 25 mM, 12.5 mM and 0 mM in PBS+ (0.5 ml to the insert supply well and 1.0 ml PBS (+) added to the bottom collection chamber) and the transport of both measured after 2 hrs (% well µmoles transported/hr). In the absence of glucose there was significant reduction in fructose. All treatments were compared to all treatments by one-way ANOVA correcting for multi-comparisons using Tukey statistical hypothesis testing, $^{**}p<0.01$, n=3 replicate wells per treatment.

4.3. Discussion

Summary key findings:

By adapting an established *in vitro* model for testing intestinal drug permeability in humans, a tool has been developed for investigating fructose transport that is arguably more physiologically relevant than other models commonly used. By increasing the responsiveness of the model to fructose, the levels of fructose transported fall into a range that can be reasonably detected by an enzymatic/spectrophotometric analysis. Black tea, green tea and ice tea were found to be potent inhibitors of both fructose and glucose transport. Synergies found were generally for inhibiting glucose rather than fructose uptake.

4.3.1 Introduction

In the previous chapter, data was presented that supports the "flattening the fructose spike" hypothesis of this thesis. Some of the potential benefits of flattening the fructose spike reaching the liver in the hepatic portal vein, or ~~perhaps~~ even enterocytes of the small intestine and microbiome of the lower gut, were discussed. In this chapter, the aim was to identify ~~technology~~ plant natural compounds and extracts that has the potential to flatten the fructose spike by inhibiting/slowing the uptake of fructose from the small intestine where the transporters for fructose reside. It was also discussed in the previous chapter that glucose might also contribute to the fructose spike in the liver by being converted to fructose via the polyol pathway. Therefore, it has also been important in this chapter, where possible, to consider ~~technology~~ plant natural compounds and extracts that can also inhibit/slow the uptake of glucose from the gut.

4.3.2 Fructose transport model optimisation

To identify potential natural plant phytochemicals that inhibit the transport of fructose, the development of an appropriate *in vitro* model was needed. Initially, fructose uptake assays used by others in the field were considered. Kane *et al*., 1997 adapted *Xenopus laevis* oocytes to express GLUT5, the main gut fructose transporter. Andrade *et al*., 2017 expressed GLUT5 protein in liposomes. GLUT5

though, may not be the only fructose transporter in the small intestine. GLUT7, GLUT9a/b, GLUT8, GLUT11, and GLUT12 share sequence homology with GLUT5 and therefore possibly also have the ability to transport fructose (Manolescu *et al*., 2007; Doujard & Ferris, 2008). Caco2 cells have been reported to express GLUT8, GLUT9b, GLUT2 (DeBosch *et al*., 2012) and GLUT7 (Gauer et 2012). GLUT7 (Cheeseman, 2008), GLUT8 (Romero *et al*., 2009), and GLUT12 (Miller *et al*., 2005) have been shown to be expressed in the gut. Although the specific functions of these transporters might overlap to some extent, the functions of GLUT family members in the gut do not appear to be redundant: GLUT7 is a high-affinity, low-capacity fructose transporter (Thorens & Mueckler, 2010). This might suggest that GLUT7 functions to transport fructose at low concentrations in the gut lumen, and GLUT5 at high levels. GLUT12 transports both fructose and glucose (Rogers *et al*., 2003). There is evidence that GLUT8 attenuates enterocyte fructose transport by regulating GLUT12 protein abundance *in vitro* and *in vivo* and that disrupted GLUT8 function has deleterious long-term metabolic effects in the high fructose-fed rat (DeBosch *et al*., 2012). The purpose of this relationship between GLUT8 and GLUT12 is not understood, but ~~perhaps~~ suggests GLUT12 is more important than so far considered, if indeed there is need for its expression to be regulated by GLUT8. The Caco2 cell line was therefore selected as the basis for the model used in this study as it may represent more of the complex interactions possibly occurring for fructose uptake. As it was not known to what extent GLUT7, GLUT8, GLUT9a/b and GLUT12 are involved in fructose transport, optimising GLUT5 was focussed on as it is almost exclusively a high-capacity fructose transporter (Ferraris, 200), possibly along with GLUT2, which is classically thought to be the primary enterocyte glucose transporter and might also transport fructose.

The models of fructose transport mentioned above (Kane *et al*., 2012; Andrade *et al*., 2017) were based on the transport of radiolabelled fructose. These seem to necessitate very low levels of fructose being used, e.g. 100 nM-1 mM. Concentrations of fructose of this order are much lower than would be anticipated to occur in the gut lumen *in vivo* following a substantial fructose dose, and therefore are less physiologically relevant. ~~Perhaps~~ If high levels of unlabelled fructose were to be used alongside the very low concentration (necessary due to cost) of labelled, the sensitivity of the assay would be lost and therefore also the advantage of using such

hazardous radioactive reagents. For this study it was therefore elected to detect the transport of fructose by enzymatic and spectrophotometric methodology.

Caco2 (a cell-line derived from human colorectal adenocarcinoma) cell monolayers have been widely accepted as a potent *in vitro* model to predict intestinal drug permeability in humans (Karlsson, 1991; Jezyk *et al*., 1993; Yamashita *et al.,* 1997). The model, however, requires 21 days of culture for the cells to achieve sufficient differentiation. As this is incompatible with high through-put screening Becton Dickson Bioscience have shortened the process to just 2 days post-seeding using butyric acid, which has been shown to induce growth arrest and differentiation in several colonic cancer cell lines (Chong *et al*., 1997). However, Yamashita *et al* 2002, have shown that monolayer integrity is compromised using this 2 day protocol compared with 21 days by measuring trans-epithelial electrical resistance (TEER). In their study TEER was shown to improve greatly with time from between 1 to 5 days post seeding before deteriorating at 6 and 7 days. Therefore, the effect of using these time points on GLUT5 and GLUT2 transporter mRNA expression, as well as fructose transport and monolayer integrity was investigated here. It was found that GLUT5 mRNA expression was greatly improved after 5 days differentiation and less so after 7 days (Figure 4.5), with fructose transport following the same pattern (Figure 4.6). The mRNA of key glucose transport transporters GLUT2 (Figure 4.8) and SGLT1 (Figure 4.7) was also greatly increased at 5 days and also 7 days, whereas glucose transport was unchanged (Figure 4.9). Monolayer integrity as measured by trans-epidermal electrical resistance (Figure 4.3) and non-specific Lucifer Yellow dye transport by leakage (Figure 4.4) was only slightly improved at 5 days and was diminished at 7 days.

It was particularly important to take advantage of the improvement in fructose transport at 5 days, as using the enzymatic/spectrophotometric methodology for fructose measurement can be insufficiently sensitive at low fructose concentrations (Douard & Ferris- 2008). Differentiating cells for 5 days rather than 2 days helped bring the detected levels of fructose up towards 0.5 mM (Figure 4.6). However, studies have suggested that to be determined accurately using an enzyme/spectrophotometric method, levels need to be above 0.5 mM (Douard & Ferris- 2008). At the levels of fructose determined to have crossed the Caco2 monolayer after the 30 minutes in the Yamashita *et al* 2002 protocol, there was also

still quite a high coefficient of variation found (approximately 6-31%) (Figure 4.6) compared with that for glucose transport (typically 3-10%) (Figure 4.9). In an attempt to ~~boost~~ elevate levels of fructose transported and reduce variation, a time course was performed. This showed a linear increase in fructose transport with time, giving greater than 500 µM transported after 1-2 hours (Figures 4.10 and 4.11), and monolayer integrity as measured by TEER was found to be optimal after 2 hrs (Figure 4.12). Therefore, further studies were carried out using an optimised format of the model, differentiating for 5 days and allowing 2 hrs for potential actives to inhibit fructose transport. This not only enabled the testing of *in vivo* relevant levels of fructose and glucose, but also for testing to be done in a healthy intestine relevant cell layer and at levels of fructose and glucose that enable more reproducible results due to lower measurement variability.

4.3.3 Validating the fructose and glucose transport assay with known inhibitors

Unlike for glucose, there are no well-known inhibitors of fructose transport. In a functional study of GLUT5, Kane *et al*., 1997 noted that the locked furan-ring fructose analogue 2.5-anahydomannitol was an effective inhibitor, suggesting that GLUT5 prefers fructose in the furan ring form. Searching for a specific inhibitor of GLUT5, Girniene *et al*., 2003 also found fused ring analogues of fructose to be effective, e.g. sorbose-Bn-OZO. Effective inhibition of fructose transport by sorbose-Bn-OZO was confirmed in a study by Andrade *et al*., 2017 who, based on the work by Girniene *et al*., 2003, used sorbose-Bn-OZO as a “specific” inhibitor of GLUT5 and therefore a tool to identify other potential GLUT5 inhibitors. However, it is not clear why they considered sorbose-Bn-OZO to be a specific inhibitor, as such a claim had not been made previously nor by Gimiene *et al*., 2003. Merino *et al*., as recently as 2020, have stated that “So far, no potent and specific inhibitors of GLUT5 have been discovered”. Again, in an effort to investigate GLUT5 interactions and, frustrated by the lack of known fructose transport inhibitors, Thompson *et al*., 2015, noted that rubusoside inhibited GLUT5 and GLUT1, whilst astragalin-6-glucoside was only effective against GLUT5. However, although able to source 2.5-anahydomannitol, sorbose-Bn-OZO and rubusoside here, none showed significant effects on fructose in this optimised assay. This may in part be due to the fact that in each of these previous studies, high concentrations of each compound were used

against very low levels of fructose, as they were based on detecting radiolabelled fructose transport. However, in these experiments here, phloretin, luteolin and to some extent phloridzin were consistently effective at inhibiting glucose transport. Phloretin is a well-known inhibitor of GLUT2 and phloridzin of SGLT1 glucose transporters (Kellett, and Brot-Laroche, 2005). Luteolin has been a consistent inhibitor of glucose transport using a similar non-fructose transport optimised model by another group (personal communication, Unilever).

4.3.4 Patent and Literature search

A search in the scientific literature and patents for other plant compounds that have shown some fructose inhibitory activity was performed for two purposes. Firstly, for evidence for which compound structures/types are effective and, secondly, to see what should be avoided for patent purposes due to prior art. Andrade *et al*., 2017 reported some inhibition for a number of polyphenols at concentrations of around 10-100 µM: ferulic acid, caffeic acid, coumaric acid, proctocatenoic acid, chrysin and delphinidin. Such doses seemed reasonably related to that which might result from a potential practical oral dose of the compounds up to approximately 500 mg, diluted by passage through the stomach and small intestine with a drink or food: Effects though were very modest, and in a radiolabel-based assay against just 100 nM fructose, and are therefore ~~perhaps~~ not comparable to a likely physiological dose following a sizable fructose meal or drink. Thompson *et al,* 2015, tested some 36 plant natural compounds in their model up to 20 mM, before finding only rubusoside and astraglin-6-glucoside effective. Patent searches suggested a number of categories of compounds of relevance, namely; gallates and tannins (US2014128585A), monoterpenes/eucalyptus leaf (WO12008474A1, US2003049337A) and catechins (JP2009184992A). In the scientific literature, many papers concerned demonstrating the effects of flavanoids, polyphenols, and plant extracts etc. on “metabolic syndrome” related symptoms and markers in high fructose-fed rats. In only a couple of these papers is GLUT5, or fructose absorption inhibition mentioned. Where a mechanism is discussed, it is usually presumed to be an antioxidant effect (Santamaria *et al*., 2012; Lee *et al*., 2012; Ahmad *et al*., 2008; Godse *et al*., 2010; Liu *et al*., 2010; Liu *et al*., 2007; Kannappan, 2010; Mohamed *et*

al., 2009). However, it is possible ~~and perhaps likely~~ that they may have some effect on the absorption of fructose from the gut, rather than being taken up systemically themselves in high enough concentrations to have had any other effect such as an antioxidant. Therefore, the compounds selected for initial testing reflected these search results, as well as the ease and cost of supply (Table 4.2).

4.3.5 Single compound effects and Synergies

Over four experiments lectin glycan, cinnamaldehyde, eucalyptol and vanillin showed a tendency to inhibit fructose transport, and quercetin-3-glucose in 3 of the 4 experiments (Table 4.3). However, these were not statistically significant overall despite being so for lectin in two experiments and cinnamaldehyde and quercetin-3-glucoside in one. Inhibition of glucose transport was only seen for the previously validated phloretin, phloridzin and luteolin compounds, as well as for quercetin-3-glucoside (Figures 4.13-26).

As interconnected molecular pathways can be susceptible to influence at numerous points and junctures, synergistic effects were considered next. In addition to the constitutive GLUT5 transporter, fructose might also be transported from the gut lumen by GLUT2, the main gut glucose transporter at high concentrations. The next investigation, therefore, examined synergies between some of those compounds that showed greatest consistency over four experiments, and some established GLUT2 inhibitors (personal communication, Unilever). No synergistic effects were seen for fructose transport (Figures 4.30-32). However, some synergy was seen for glucose transport i.e.: phloretin+quercetin-3-glucoside by combination sub-thresholding highest single agent and additive effect; luteolin+quercetin-3-glucoside by highest single agent and additive effect; hesperitin+quercetin-3-glucoside by highest single active and additive effect; and luteolin+lectin glycan by additive effect (Figure 4.33), and can therefore be considered for patenting and further study such as *in vivo* testing.

Although not directly able to flatten the fructose spike as glucose uptake inhibitors, these combinations may be able to achieve a similar effect during high glucose intake by reducing the conversion of glucose to fructose via the polyol pathway (see

chapter 3.0, 3.2 Discussion, 3.2.2 Primary human Hepatocytes, Glucose and triglyceride). Indirectly, this may help flatten the fructose spike in the liver and hence possibly reduce triglyceride release.

4.3.6 Catechins, tea extracts and other beverages

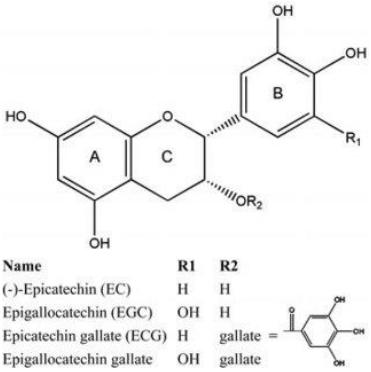
Catechins have also been reported to inhibit fructose uptake (Slavic *et al*., 2009; Villa-Rodriguez *et al*., 2017) and alleviate the metabolic symptoms caused by high fructose diets in rats (Sutra *et al*., 2008) as well as limiting fructose absorption (JP2009184992A). In the first experiment here, catechin, epicatechin gallate (ECG) and epicatechin (EC) gave some of the best effects found here on fructose transport, delivering up to 65% inhibition (Figure 4.34), but not in the case of glucose (Figure 4.35).

Interestingly EC and ECG have no R1 hydroxyl group on benzene ring B (Figure 4.58), suggesting that this might be a key feature. However, catechin (Figure 4.59) does have a hydroxyl group at this position and as such it may ~~so perhaps it is~~ not be so important.

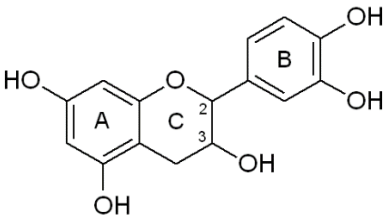
Figures 4.48 and 4.59: Chemical structure of catechins and catechin

Figure 4.58: Chemical structure of catechins

Figure 4.59: Chemical structure of catechin



Name	R1	R2
(-)-Epicatechin (EC)	H	H
Epigallocatechin (EGC)	OH	H
Epicatechin gallate (ECG)	H	gallate
Epigallocatechin gallate	OH	gallate



Figures 4.58 and 4.59: Chemical structure of catechins (Figure 4.58) and catechin (Figure 4.59). EC and ECG have no R1 hydroxyl group on benzene ring B (Figure 4.58), suggesting that this might be a key feature. However, catechin (Figure 4.59) does have a hydroxyl group at this position so therefore, ~~perhaps~~ it may not be so important.

Follow-up testing of aqueous tea extracts directly in the Caco2 fructose transport model showed significant and substantial (60%) inhibition of fructose transport for

both black and green teas at dilutions of 1-in-2 (p<0.01) and 1-in-4 (p<0.05) (Figure 4.38) and by 90% in glucose transport (Figure 4.39). Green tea has been shown to inhibit glucose and fructose transport previously (Villa-Rodriguez *et al*., 2017) with IC50 values of 0.26 ± 0.04 and 0.8 ± 0.09 mg extract/ml *vs*. 1 mM and 5 mM respectively. As tea contains approximately 400 mg solids per 100 ml (personal communication, Bangalore Unilever) a 1-in-4 dilution used in this study to inhibit 25 mM glucose and fructose would have contained approximately 1 mg extract/ml. Black tea though has not previously been shown to inhibit the transport of glucose or fructose. As such, the effect of black tea, which is more commonly available than green tea and cheaper, is a novel observation and patentable applications are in progress and further study such as in-vivo testing can be considered.

In a follow up experiment testing several other beverages, black tea and green tea again substantially and significantly inhibited fructose (Figure 4.40) and glucose transport (Figure 4.41). Ice tea gave 97% fructose transport inhibition at a 1-in-4 dilution and a dose-dependent effect up to a 1-in-32 dilution (Figure 4.40). Ice tea was much less effective, though, on glucose transport (Figure 4.41). The effect of ice teas on fructose transport is surprising as it contains only 0.12-0.14% powdered black tea and therefore much less catechin content. As tea contains approximately 400 mg solids per 100 ml (personal communication, Bangalore Unilever), ice -tea is approximately one-third the strength of typical black tea. Black tea was also tested with milk in the assay as milk can often bind factors in tea and therefore might reduce its effectiveness (Tewari *et al*., 2000; Xiao *et al*., 2011., Rashidinejad *et al*., 2017). However, black tea with milk was also highly effective against glucose transport (Figure 4.41) and also showed ~~a~~ substantial non-significant ~~trend~~ inhibition against fructose transport (Figure 4.40). Decaffeinated black tea also significantly inhibited glucose transport (Figure 4.41), although less substantially than non-decaffeinated. This might suggest that caffeine is an effective ingredient and may explain why black tea was similarly effective as green tea. However, instant coffee contains similar caffeine level (57-65 mg/serving) yet had no effect on fructose (Figure 4.40) or glucose transport (Figure 4.41).

In considering why black tea was similarly effective as green tea it is interesting to note that Henning *et al*., 2003 found that black tea had a much lower total catechin content than green tea (34.8 ± 1.9 mg/100 ml *vs.* 196.6 ± 5.2). The catechins

though, that were most effective in this thesis, catechin (2.7 ± 0.3 *vs*. 5.8 ± 0.9), EC (5.3 ± 0.4 *vs.* 11.9 ± 0.1) and ECG (4.5 ± 0.1 *vs.* 13.7 ± 0.3) were not so different in Henning *et al*'s analysis. Black tea contained more gallic acid than green tea (6.5 ± 0.1 *vs.* 1.2 ± 0) and GCG (4.2 ± 0.5 *vs.* 1.1 ± 0.1), but much less EGC (6.2 ± 0.9 *vs.* 76.4 ± 1.8), and EGCG (8.9 ± 0.6 *vs.* 83.9 ± 2.8), and similar levels of catechin gallate (3.0 ± 0 *vs.* 3.1 ± 0.1) (Henning *et al*., 2003). The catechins that were greatly higher for green tea (EGC and EGCG) i.e. the main catechins in green tea, were not effective inhibitors in this thesis (Figure 4.34). Gallic acid wasn't particularly effective at inhibiting fructose transport (Table 4.3). GCG (gallocatechin-3-gallate) was not tested, but if highly effective could explain black teas efficacy. Black tea also contained theaflavins (14.2 ± 1.0), whereas green tea did not (Henning *et al*., 2003). Theaflavins were not effective inhibitors in this thesis (Figure 4.34) but it is possible that together they may have a synergistic effect. Caffeine levels in black and green tea were found to be very similar (36.2 ± 1.6 *vs.* 33.1 ± 0.7) (Henning *et al*., 2003). It should be remarked upon though that the analysis by Henning *et al*., were of course not on the tea samples used in this thesis and were in fact some years ago. The teas used in this study may be very different. Season (Bhandari and Goswami, 2019; Deka *et al*., 2021), weather (Wakamatsu *et al*., 2019), location, altitude (Chen *et al*., 2014), processing (Shimamura *et al*., 2008; Lee *et al*., 2013, Zaiter, *et al*., 2016; Donlao and Ogawa, 2019; Anggraini *et al*., 2021), storage condition (Bazine *et al*., 2010), brewing conditions (Saklar *et al*., 2015), variety (Henning *et al*., 2003), breed (Jiang *et al*., 2020) etc. can have substantial effects on tea catechin and other polyphenol content.

The surprising effect by ice tea particularly on fructose transport, given that it has so much less tea and therefore catechins, raised the possibility that pH may have had an effect

4.3.7 Effect of pH

When the pH of the beverage samples was measured, a statistically significant correlation was found with both glucose (Figure 4.42) and fructose transport (Figure 4.4.3). The association though was very weak with Pearson product moment correlations of just 0.102 and 0.097 respectively. That pH is unlikely to explain the effects seen was also demonstrated by the observation that, for instance, ice tea at a dilution of 1-in-8 and a pH of 6.7 had a very substantial effect on fructose transport and diet cola had none at a pH of 6.8 (Figure 4.40). Ice tea also had a much greater effect on fructose transport than glucose despite each treatment being the same pH (Figures 4.40 and 4.41 respectively). As a further check that pH was not driving the inhibition, some of the samples were neutralised and re-tested. At a neutral pH 7.0, black tea, green tea and decaffeinated tea were still as effective as the non-neutralised samples (Figure 4.44 and 4.45).

Ice tea (peach), whether neutralised or not had no effect in that experiment. Fresh samples though, i.e. opened that day, of peach flavoured ice tea were just as effective as they had been in the previous experiment (Figure 4.44 and 4.45). This might suggest that volatiles or non-oxidised compounds in the ice tea products were responsible for their inhibitory action. Freshly opened mango, lemon and raspberry flavours were equally as effective in the assay as the fresh peach. Such flavours consist of volatile compounds such as terpenes, that have been found to inhibit the transport of fructose (see above Patent and Literature search). However, after obtaining the formulations of ice tea products and ingredients, each were tested in the Caco2 model at a 1-in-4 dilution of their quantity as formulated in the product. None had a significant effect on either fructose or glucose transport, although sucrose and vitamin C showed a small non-significant tendency (Figure 4.46 and 4.47). The flavours as ingredients had no effect, potentially ruling out the volatiles as inhibitory actives hypothesis. One possibility that remains is that a few of the ingredients act together in synergy. However, the number of combinations and permutations to test this would be huge. An alternative hypothesis is that some ingredients are oxidised and are only effective in low oxygen and/or extremely fresh conditions.

4.3.8 Polyphenols/antioxidant activity

To investigate whether polyphenols/antioxidant activity is a common and key feature of beverage effects on inhibition, polyphenol/antioxidant activity was measured. In general, beverages high in polyphenols had the most inhibitory effect, and those with very little polyphenol content had no effect on fructose or glucose transport (Figure 4.48). One exception was ice tea, with low polyphenol/antioxidant activity but very high inhibitory activity, as discussed. Having investigated whether the lower pH of ice tea was the cause of its surprising inhibitory activity (Figures 4.44 and 4.45), and after testing all of the ingredients separately (Figure 4.46), it is still not clear why such a beverage with such low polyphenol content should be so active. Another exception was instant coffee, which was very high in polyphenol/antioxidant activity, but had little or no inhibitory activity (Figure 4.40 and 4.41). Such an observation suggests that "polyphenol/antioxidant activity" is unlikely the only key feature giving the teas their ability to inhibit fructose and glucose transport.

Other studies have also reported inhibitory effects of plant polyphenols on both glucose transport (Araujo *et al*., 2011; Johnston *et al*., 2005; Kwon *et al*., 2007; Williamson, 2013) and fructose transport (Andrade *et al*., 2017). Such observations have led to the suggestion that the array of individual polyphenols in plants might act additively or synergistically to have a greater effect (Lee, Lim, & Kwon, 2015). The antioxidant capacity of plant polyphenols has been proposed to be important in reducing the metabolic syndrome symptoms caused by the feeding of a high fructose diet in animals via such modes of action as regulation of lipogenesis, repair of mitochondrial dysfunction, inhibition of inflammatory pathways and improvement of insulin sensitivity. In the regulation of lipogenesis, polyphenols are suggested to suppress over-expression of key genes involved such as SREBP-1c (sterol regulatory element-binding transcription factor-1c), fatty acid synthase, acetyl-CoA carboxylase-1 and stearoyl-CoA desaturase-1 (Shrestha *et al*., 2009). Repair of mitochondrial dysfunction has been linked to a ~~boost~~ stimulation of ~~in~~ endogenous antioxidant activity by plant naturals targeting Nrf2 (nuclear factor erythroid 2-related factor 2) (Chen *et al*., 2016). Nrf2 binds to the antioxidant response element in the nucleus, leading to the transcription of protective antioxidant genes. Inhibition of inflammatory pathways by polyphenols though inactivation of the NFK-B (nuclear factor kappa-light-chain-enhancer of activated B cells), a protein involved in the

cellular stress response pathway which regulates inflammatory mediators such as TNFα and IL-6 has also been suggested (Maithilikarpagaselvi *et al*., 2016). However, whether polyphenols are absorbed by the gut into the blood at sufficient concentrations to have any of these effects directly is unclear. Following ingestion both within the gut and after absorption, polyphenols are readily metabolised by intestinal and liver cells (Rothwell *et al*., 2016). Even with very high intakes, intracellular levels could be many times lower than other antioxidants, e.g. glutathione and ascorbic acid (Lotito *et al*., 2006). Cell culture studies suggest that much of the benefits of polyphenols, e.g. anti-inflammatory are due to cell-signalling pathway modulation (Williams *et al*., 2004), which requires much lower polyphenol concentrations (Spencer *et al*., 2001; Spence *et al*., 2003). However, ~~perhaps~~ a ~~more likely~~ highly plausible mode of action in cases such as excess fructose or glucose feeding might, given that the concentration of such antioxidants in the gut are much higher than that of the blood or tissues, be the inhibition of the transport of these nutrients into the body before they can have any of their proposed harmful effects.

4.3.9 Beverages and phloridzin synergies

Phloridzin is a plant polyphenol which is well known to reduce glucose uptake from the intestine by inhibiting the high affinity, low glucose level transporter SGLT1 (sodium-dependent glucose transporter 1) (Rossetti *et al*., 1987). It has been suggested that activation of SGLT1 leads to the translocation of GLUT2 to the enterocyte apical membrane to transport higher levels of glucose (Kellet and Brot-Laroche, 2005). GLUT2 may also be responsible for the transport of fructose when GLUT5 is saturated. The inhibition of SGLT1 might therefore also inhibit fructose uptake, as well as glucose uptake. Significant fructose transport inhibition was seen here with phloridzin in one experiment (Figures 4.19 and 4.20), but not consistently. A number of beverages were tested for synergy with phloridzin to look for greater effect and to allow for potential intellectual protection on such technology.

Black tea and phloridzin combination showed combination sub-threshold synergy (Figure 4.49) and response additivity for glucose (Table 4.7). Combination sub-thresholding was also found for green tea+phloridzin for glucose transport (Figure

4.51) and ice tea+phloridzin, which also demonstrated response additivity (Figure 4.55) and response additivity (Table 4.13). The sizes of the effects seen here were only around 25%. This is because test doses were selected to give a good chance of showing a synergy. If individual doses cause too much of an effect on their own, it becomes difficult to see a combination benefit. These combinations now warrant further follow-up and confirmation.

4.3.10 Best methodology?

In this study, synergies for test compounds on fructose transport were not seen. Although inhibitory t~~rends~~ tendencies were seen, the fructose data were too variable to see any significant differences. This raises the question as to whether a better method for analysing fructose transport could be used. That variability for fructose was much higher than for glucose is not surprising as the fructose quantity is derived from subtracting glucose from glucose+fructose, therefore the variability of both measures contributes to the statistical analysis. Other methods have used less physiologically relevant *in vitro* models, e.g., frog gametes (Kane *et al*., 1997) or liposomes (Andrade *et al*., 2017) induced to express GLUT5. Such models were avoided here as fructose uptake in the gut may be via a number of different transporters (GLUT5, GLUT2, GLUT7, GLUT9a/b, GLUT8, GLUT11, and GLUT12 (Manolescu *et al*., 2007; Doujard & Ferris, 2008)) which can possibly interact giving greater chance of synergy between compounds. Radiolabelling methods for measuring fructose transport require very low levels of fructose, presumably to avoid signal dilution (Kane *et al*., 1997; Girniene *et al*., 2003; Andrade *et al*., 2017). However, such levels do not equate to the levels of fructose expected in the gut lumen following a substantial fructose bolus (Ferraris *et al*. 1990; Jiang & Ferris, 2001, Douard and Ferraris, 2013). The levels of fructose used in such radiolabel methods are probably small enough to be safely metabolised by the enterocytes of the small intestine and unlikely to cause any serum fructose spikes. I.e. studies have shown that only fructose levels above approximately 5 g/dose would overwhelm the small intestines capacity such that fructose would reach the hepatic portal vein and hence the liver (Jiang *et al*., 2021). Even higher doses would be required to overwhelm the capacity of the liver to metabolise fructose safely for

energy, or to synthesise glucose for glycogen storage and lactate. An increase in postprandial triglycerides is generally not seen below fructose intakes of 50 g/day, cholesterol below 100 g/day and lipid accumulation (steatosis) below 150 g/day (Tappy *et al*., 2021). Another potential problem with using radiolabelled fructose is that the products of fructose metabolism would also be detected in addition to fructose. An alternative method of analysis of transported fructose would be high-performance liquid chromatography (HPLC). Campbell *et al*., 1999 however, compared enzymatic assays, as used here, with HPLC methods for glucose and fructose and concluded that precision and accuracy was similar, with no difference in sensitivity. They concluded that the enzymatic assay had greater throughput and was much more cost effective. In checking for precision, accuracy and sensitivity however, they used standard curves of glucose and fructose separately rather than together. Here though, when the two were mixed together, particularly at ratios that were representative of samples (approximately 3:1 glucose to fructose), recovery of fructose was found to be poor (Table 4.1). It would be useful to do a comparison between enzymatic and HPLC methods under these circumstances before future work is carried out.

4.3.11 Phloridzin and GLUT2 transport of fructose?

The lack of synergies found for fructose transport could also be due simply to the limited effect phloridzin tended to have on fructose transport in these studies. In fact, it has long been considered that phloridzin blocks glucose but not fructose transporters (Fridhandler *et al*., 1955) and is still used as a tool in studies as such (Abu *et al*., 2018). Andrade *et al*., 2017 have shown inhibition by 1 mM phloridzin against 1 mM ^{14}C-fructose, however, as discussed this is a very low level of fructose. In one of the experiments in this study, fructose transport was significantly inhibited in the presence of phloridzin at 150 and 300 µM (Figures 4.19 and 4.20) and showed ~~a downward~~ a non-significant trend in 3 out 4 experiments. However, in the synergy experiments with beverages, phloridzin had no effect on its own (Figures 4.49-56). The rational for combining phloridzin with other potential uptake inhibitors was that it may ~~boost~~ induce GLUT2 (the proposed secondary main transporter of fructose) re-localisation, via its interaction with SGLT1, which is thought to ~~boost~~ increase GLUT2 levels at the apical membrane (Kellet and Brot-Laroche, 2005). However, the lack of

consistent effect found here by phloridzin tends to support the suggestion that the hypothesis that fructose is transported by GLUT2 at the apical membrane is controversial (Ferraris *et al*., 2018; and see below-“Hypotheses for the effect of glucose on fructose uptake”).

4.3.12 Hypotheses for the effect of glucose on fructose uptake

Observations of the enhancing effect of glucose on fructose transport (Rumessen & Gudmand-hoyer, 1986, Truswell et al., 1988) led to an early hypothesis that in the gut, fructose was absorbed through a disaccharidase (sucrase)-related transport system which simultaneously transported glucose and fructose (Riby *et al*., 1993). This was supported by the observation that acarbose, a blocker of brush border (gut lumen) sucrase, had shown inhibition of glucose-facilitated fructose absorption in rats (Fujisawa *et al*., 1991). However, in children, others showed that acarbose pre-treatment impeded the absorption of sucrose but had no effect on fructose absorption from a fructose-glucose mixture (Hoekstra and van den Aker, 1996). Alternatively, it has been hypothesised that the glucose effect on fructose absorption is a result of nonspecific movement of fructose across the intestinal epithelium by solvent drag or passive diffusion (Corpe *et al*., 1999, Fine *et al*., 1994, Riby *et al*., 1993). This hypothesis was supported by the observation that other actively transported nutrients, e.g. amino acids (Hoekstra *et al*., 1993) and galactose (Fujisawa *et al*., 1991) also ameliorated fructose uptake. However, other facilitated compounds did not affect fructose transport, e.g. 3-*O*-methylglucose (a non metabolisable glucose analogue), sorbitol (Fujisawa *et al*., 1991) and urea (Kneepkens *et al*., 1984). As glucose and galactose upregulate GLUT2 mRNA, and 3-O-methylglucose was reported not to (Miyamoto *et al*., 1993), a direct transport mechanism involving GLUT2 mRNA upregulation was therefore hypothesised to explain the ameliorative effect of glucose on fructose uptake (Cheeseman *et al*., 1993, Gibson *et al*., 2007). However, it is noticeable in the Miyamoto study, that although their densitometry data did indeed show 3-*O*-methylglucose to have had no effect on GLUT2 mRNA (Miyamoto *et al*., 1993, Table 1), their autoradiogram appears to show a substantial increase (Miyamoto *et al*., 1993, Figure 4), although not to the same degree as glucose and galactose. If 3-*O*-methylglucose does also

increase GLUT2 mRNA, this would undermine the proposal that glucose enhancement of fructose uptake is due to an upregulation of GLUT2 as 3-*O*-methylglucose does not increase fructose uptake. It would be interesting to see what effect amino acids, urea and sorbitol, which as mentioned above also increase fructose uptake, have on GLUT2 mRNA. In addition, it would be interesting to see whether galactose and fructose, which increases GLUT2 mRNA (Miyamoto *et al*., 1993), also increase glucose transport?

Other evidence though has been suggested to support the involvement of GLUT2 in the transport of fructose in mice (Gouyon *et al*., 2003). On a high fructose diet, it was found that 60% of fructose uptake could be inhibited with the GLUT2 inhibitor Cytochalastin B. Mice on a previously high glucose diet also demonstrated ~~boosted~~ enhanced fructose uptake, whereas on a low carbohydrate diet, GLUT2 knock out had no effect on fructose uptake. These results are consistent with the hypothesis that preformed intracellular stores of GLUT2 are rapidly translocated to the apical membrane via a glucose induced SGLT1-activated protein kinase C (PKC) signalling mechanism (Heliwell *et al*., 2000, Kellet and Brot-Laroche, 2005).

In humans however, no apical GLUT2 has yet been found. These observations have only been tested in either fasted (Dyer *et al*., 2002) or deceased (Blackmoore *et al*., 1995) subjects, in which non-translocation of GLUT2 to the apical membrane is ~~perhaps~~ not surprising. Others have reported no increase in GLUT2 immunoreactivity in the apical membrane after glucose administration to mice by gavage and suggest that the GLUT2 detected by Western blotting in isolated brush border (apical) membrane fractions may be due to contamination with that from the basolateral membrane (Rhee *et al*., 2015). The claim that PKC mediates translocation of GLUT2 to the brush border membrane (Helliwell *et al*., 2000) has also been challenged by a study using PKC inhibitors that showed only non-specific effects on glucose uptake in excised rat jejunum (Scow *et al*., 2011). However, the absence of GLUT2 in the apical membrane in *ex-vivo* studies to date could be due to the fact that PKC is inactivated soon after the tissue is excised (Heliwell *et al*., 2000). The Scow *et al*. study does provide evidence for the constitutive presence of GLUT2 *ex-vivo* in the apical membrane, explaining the partial inhibition of glucose uptake with the GLUT2 inhibitor phloretin in mice.

Ferraris *et al*., 2018 discuss that there has been a “decade of controversy” with respect to GLUT2 replacing GLUT5 as the main fructose transporter. They outline a number of issues with the apical GLUT2 hypothesis. Firstly, the possibility of transport of fructose by GLUT2 is significantly reliant upon the apical GLUT2 model (i.e. glucose levels stimulate SGLT1 to upregulated GLUT2 at the apical membrane). The apical GLUT2 model is based on the observation that glucose transport is inhibited by phloretin and that phloretin does not inhibit SGLT1. However, it has been reported that phloretin can inhibit SGLT1 with a K_i (dissociation constant) of 50 μM (Cui *et al*., 2004), and therefore phloretin inhibition does not directly demonstrate GLUT2 transport. Ferraris *et al*., 2018 also argue that GLUT2 may have little relevance, as it has been shown that high fructose perfusion of the small intestine upregulates only GLUT5 and not GLUT2 in weening rats (Hirayama *et al*., 2001). Although, this latter observation does not seem to challenge the hypothesis that glucose might increase pre-formed GLUT2 re-location to the apical membrane. However, in a recent study it was found that specific deletion of GLUT2 in the small intestine of mice only restricted glucose uptake by 20% (Schmitt *et al*., 2017). In another study involving GLUT2$^{-/-}$ mice, labelled glucose was able to enter the intestinal cells at the apical membrane (presumably via SGLT1), but not the basolateral membrane (Röder *et al*., 2014). The researchers concluded that GLUT2 is not likely to be involved in the apical transport of glucose, irrespective of concentration (Röder *et al*., 2014). Moreover, computer model simulated GLUT2 increase in the apical membrane predicts a downhill glucose reflux from cytosol to lumen (Naftalin, 2014). With respect to fructose, GLUT5$^{-/-}$ mice show no fructose absorption (Patel *et al*., 2014). Ferraris *et al*., 2018 also speculate that other transporters, GLUTs 7,8,9 and 12, that have been proposed to have dual specificity for glucose and fructose (Manolescu *et al*., 2007; Ebert *et al*., 2017), play little or no role in fructose transport. GLUTs 7, 8 and 9 have low intestinal expression relative to GLUT5 (Patel *et al*., 2014), and as mentioned only GLUT5 is upregulated by fructose, and there was no fructose transport in GLUT5$^{-/-}$ mice (Patel *et al*., 2014).

In the adapted Caco2 model used in this study, a reduction in the fructose:glucose ratio was, as per other studies, also found to reduce fructose transport (Figure 4.57). And as discussed above, in this thesis it was also seen that phloridzin, a well-known SGLT1 inhibitor for glucose and phloretin, a well-known GLUT2 inhibitor,

had little or no effect on fructose transport (Figures 4.15-29) suggesting that neither GLUT2 nor SGLT1 are involved in fructose transport in the Caco2 cell line model, as used here at least. In these experiments therefore, how has glucose ~~boosted~~ elevated fructose transport, as glucose is increased from 0 mM to 25 mM (Figure 4.57)? One explanation could be that although glucose does not alter GLUT5 mRNA and protein levels in enterocytes *in vivo*, both glucose and fructose are potent activators of GLUT5 mRNA in Caco2 cells (Mahraoui *et al*., 1994, Mahraoui *et al*., 1994b, Matosin-Matekalo *et al*., 1997; Mesonero *et al*., 1995). Therefore, in the model developed here, glucose may be increasing fructose transport by ~~boosting~~ increasing GLUT5, not GLUT2. This seems a plausible explanation as, in fasted mice, upon feeding, GLUT5 mRNA peaked within 4 hours (Shu *et al*., 1998). However, an intriguing observation in this thesis suggests another possible mechanism.

4.3.13 Mutarotation

During assay development, it was noted that the presence of high levels of glucose interfered with fructose analysis such that there was a substantial loss in fructose recovery. One reason it was thought, might be because glucose inhibits fructose phosphorylation by hexokinase (Slein *et al.,* 1950), which is the initial reaction in the assay used here. However, when levels of enzyme and ATP in the assay were increased and a longer time for fructose phosphorylation allowed, no improvement in fructose recovery was seen. ~~Perhaps~~ Another possible reason for reduced recovery could be that glucose affects fructose isomeric stability. There are five tautomeric chemical structures of fructose (Figure 4.60).

Figure 4.60: Tautomeric forms of D-fructose in solution

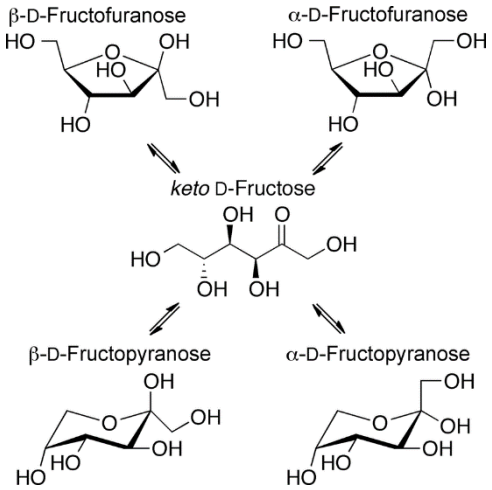


Figure 4.60: Tautomeric forms of D-fructose in solution. Barclay *et al.,* 2012.

Freshly solubilised fructose exists almost entirely in the pyranose form (chemical structure that includes a six-membered ring consisting of five carbon atoms and one oxygen atom) and only 5% in the furanose form (5-membered ring) (Cagan, 1981). However, despite pyranose being more thermodynamically stable (Ma *et al*., 1988), generally fructose is found in aqueous solution to be 22% furanose and 70% pyranose (Funcke *et al*., 1979). With respect to fructose, hexokinase can only convert the furanose form (Gottschalk, 1945; Slein *et al*., 1950; Burt, 1959). The distribution of d-fructose tautomers in solution is related to several variables, such as solvent and temperature (Schneider *et al*., 1985). Therefore, could the reduced recovery of fructose in the assay be because the presence of glucose alters the ring structure of fructose, reducing the ratio of furanose to pyranose, reducing the

availability of fructose for the hexokinase? Hexokinase also requires fructose to be in the beta-D- anomeric formation (Gottschalk, 1945). ~~Perhaps~~ It could be that the presence of high glucose also affects the ratio of these forms and/or furanose/pyranose.

~~With this in mind~~, It is therefore of note that GLUT5 also has a preference for the furanose isomer of fructose, as does GLUT2 (Kane *et al*., 1997). However, if glucose were to increase pyranose fructose, hence explaining its poor performance in the hexokinase-based assay, this would diminish, not improve, its transport by GLUT5. ~~Perhaps, therefore,~~ Alternatively, glucose could increase~~s~~ the ratio of alpha-fructose thereby lowering its affinity for hexokinase. It is not known whether GLUT5 has preference for alpha or beta anomeric fructose, but if it favours the alpha this could explain the glucose effect on increased fructose uptake. Is it reasonable though to suggest that glucose affects the isomeric form of fructose? It has been noted that amino acids can affect the mutarotation of glucose in solution (Shallenberger *et al*., 1984) and sucrose has been reported to affect the mutarotation of fructose (Nelson & Beegle, 1919) and is known to increase the sweetness of fructose by shifting conformation towards the pyranose form (Mathlouthi, 1984). It does therefor at least seem plausible that glucose could affect fructose mutarotation. Studies would need to investigate whether or not GLUT5 has a preference for the alpha form. However, if so, ~~technology~~ natural plant compounds or extracts that maintain fructose in the alpha or pyranose format might also reduce fructose uptake and help flatten the fructose spike reaching the liver.

4.3.14 Altering the fructose to glucose ratio

Whether glucose increases fructose uptake by increased solvent drag, elevating GLUT2 or by altering its mutarotation, a further potential route to reducing the uptake of fructose might be to actually increase the fructose to glucose ratio, by either increasing fructose, decreasing glucose or both. Increasing the level of fructose in a product initially seems counter-intuitive ~~in light of~~ given the potential detrimental effects on health by fructose as discussed in this thesis. However, if doing so were to reduce the uptake of fructose, such that there was little or no change in total fructose uptake, there could be a net benefit in other parameters. Fructose is 1.7,

and glucose 0.7, times sweeter than sucrose (Hanover and White., 1993) and therefore, the overall load of fructose+glucose could be reduced whilst maintaining sweetness, reducing total calories and ~~perhaps even~~ potentially reducing total fructose uptake, or at least keeping it constant.

From the *in vitro* data above (Figure 4.57), a fructose to glucose ratio of 2.3 would give an approximately 10% reduction in fructose uptake. As such a dose of 50 g sugar will have a: sweetness factor of 60 ((25 x1.7) + (25 x 0.7)), 200 kcal and 100% fructose (25 g) absorption. A 30 g fructose to 12.9 g glucose (total 42.9 g sugar) dose would also have a sweetness factor of 60 ((30 x 1.7) + (12.9 x 0.7)), 172 kcal and approximately 90% fructose uptake i.e. 27g. Reduced glucose would also give a reduced insulin spike. Using *in vivo* data e.g. a study by Rumessen & Gudmand-hoyer, 1986 which found that 100% of 50 g of fructose with equimolar glucose was absorbed, but only 77% with 12.5 g glucose. I.e. a ratio of fructose to glucose of 4, gave 77% absorption and a ratio of 1 gave 100%. Extrapolating therefore, a 2.3 ratio based on this *in vivo* data would also gives approximately 90% fructose absorption. More *in vivo* data would be needed to confirm the accuracy of these calculations. Further research would also be needed to establish if a 28% drop in calories and 48% drop in glucose stimulating insulin would be significantly disadvantaged by an 8% increase in fructose uptake.

4.4 Summary

By adapting an established *in vitro* model for testing intestinal drug permeability in humans, a tool has been developed for investigating fructose transport that is arguably more physiologically relevant than other models commonly used. By increasing the responsiveness of the model to fructose, the levels of fructose transported fall into a range that can be reasonably detected by an enzymatic/spectrophotometric analysis. This makes it possible to (a) avoid radioisotope methodology that uses very low non-physiological levels of fructose and detects unknown fructose metabolites, and (b) avoid GLUT5 only expression in models such as frog gametes and liposomes that lack the transport complexity and interaction of pathways of a gut cell model. Hence this model has the potential to

discover more effective fructose and glucose transport inhibiting compounds and synergies.

The well-known SGLT1 inhibitor phloridzin and the GLUT2 inhibitor phloretin did not inhibit fructose transport in the model developed here. This may not be surprising, as the widely accepted assumption that fructose can be transported by GLUT2 at the apical membrane is still controversial. Following an observation that glucose at high levels interferes with fructose recovery in the enzymatic assay, an alternative hypothesis is proposed for the well-known stimulatory effect of the presence glucose on fructose transport. I.e. the possibility that glucose alters the mutarotation of fructose to an isomer for which GLUT5 has greater preference. An appreciation of the effect glucose has on fructose uptake could lead to the formulation of products that limit fructose uptake, calories and/or insulin spikes without affecting sweetness or the need to add any other ingredients.

A key aim of this chapter was to identify natural plant compounds or extracts that have the potential to reduce fructose spikes through inhibition of fructose uptake from the gut. As such, this study is the first to demonstrate that black tea and ice tea can be potent inhibitors of both fructose and glucose transport. As others have previously, this study also shows green tea to be highly effective.

The addition of such teas to, for example, high sugar soft drinks or food may potentially have the benefit of preventing, or at least reducing, postprandial hypertriglycaemia. For example, if a litre of black, green or ice tea were condensed to 100 ml or a freeze-dried powder and added to 900 ml of cola creating effectively a 1-in2 dilution, this could potentially reduce sugar uptake substantially, including both fructose and glucose. Synergies found here were generally for inhibiting glucose rather than fructose uptake, however, via the polyol pathway, inhibition of glucose uptake might also help flatten the fructose spike in both liver and enterocytes.

5.0 Further potential health benefits of Flattening the Fructose Spike

5.1 Introduction

In the first chapter of this thesis, based on levels of fructose that might be considered to be within a normal dose range, i.e. approximately 50 g/day (Vos *et al*., 2008, Marriott *et al*., 2009), we investigated which of the many negative health impacts claimed for fructose maybe more likely. Of these, the effect of fructose on postprandial blood triglycerides seemed one that is most supported in the scientific literature and in particular meta-analysis studies (Livesey & Taylor, 2008; Tappy & Rosset, 2019, see Introduction 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides). The potential impact of flattening the fructose spike, i.e. lower levels for a longer time period *vs*. higher levels for a short time period, and ~~technology~~ plant natural compounds and extracts to achieve this was investigated in chapters 3 and 4.

This research project aimed to study the potential health benefits of controlling the uptake of physiological levels of fructose from the diet and identify natural plant compounds and extracts for this purpose. The main aim of this chapter therefore was to investigate further potential health benefits of flattening the fructose spike reaching the liver. This was investigated by examining the effects of fructose on lipid accumulation, glycogen storage, lactic acid and uric acid production and the hepatokine FGF21 in primary human hepatocytes.

~~In this chapter, we investigated whether there were any other potentially harmful effects of fructose that may benefit from further investigation namely: lipid accumulation, glycogen storage, lactic acid production and the hepatokine FGF21~~ (see 1.6.1, Research Plan, Aims).

Statistics

Treatments were compared by one-way ANOVA and multi-comparisons were corrected for using Dunnett's or Holm-Šídák statistical hypothesis testing where appropriate (see 2.3.6 Statistics). Within a cell culture plate, treatments and

replicates were randomly and evenly distributed across the plate to avoid edge and clustering effects.

5.2 Lipid accumulation in the liver

Many summaries of the potential harmful effects on health by fructose conclude or make the assumption that lipid accumulation in the liver is one of the most well established (e.g: Zhang *et al*., 2020; Ahadi *et al*., 2020; Buziau *et al*., 2021; Maier *et al*., 2021; Alessandro *et al*., 2021; Hydes *et al*., 2021). Such summaries though tend not to mention the fact that the effects on lipid accumulation are generally only seen at levels of fructose consumption of >150 g/day (Tappy *et al*., 2021). However, there is little research on the effect of fructose at more normal levels of consumption (see 1.0 Introduction, 1.1.4 Effects of fructose on Obesity, Liver steatosis). Large clinical trials are expensive, particularly for end points such as fatty liver as this is difficult to measure non-invasively. Techniques such as ultrasound, which is the most commonly used to measure liver fat *in vivo* has poor accuracy (Dewdney *et al*., 2020b). Magnetic resonance methods are more accurate but not so commonly available (Ko *et al*., 2029). We therefore investigated lipid accumulation *in vitro*, in response to fructose, glucose and fat, and compared isocaloric levels of fructose and glucose treatments. Although the cellular effects of fructose on hepatocytes have been investigated using human cell lines, there have been few studies using primary hepatocytes (Pinnick *et al*., 2019). Human primary hepatocytes were used as they are considered to be the “gold standard” *in vitro* model for liver function studies (Green *et al*., 2014).

Initial experiments investigated the effects of fat (palmitic acid: linoleic acid: oleic acid (PLO) in a ratio of 4:3:3, fructose and glucose on lipid accumulation in primary hepatocytes by Nile Red staining.

Adding a range of PLO over 72 hrs to a high glucose concentration gave a clear increase in lipid accumulation. A dose-dependent increase in stained lipid was seen for 0.2 and 0.5-mM PLO, but a large drop was seen for 1 mM PLO. Adding 5 mM fructose to 11 mM glucose showed no increase lipid staining (Figure 5.1).

Figures 5.1 – 5.3: Effect of fat or frutcose on lipid accumulation in primary hepatocytes- nile red spectrophotometric assay.

Fig 5.1: Effect of Fat on lipid accumulation in primary hepatocytes Nile Red spectrophotometric assay

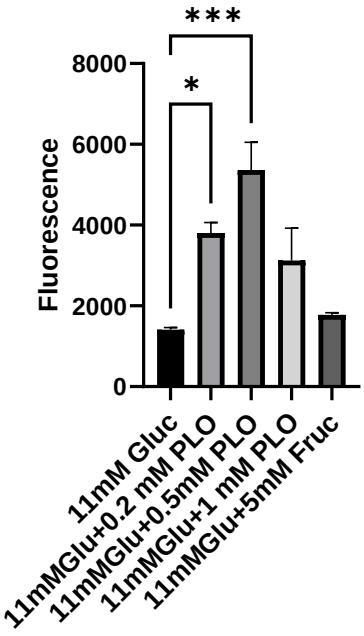


Fig 5.2: Effect of Fat on Cell Survival in primary hepatocytes

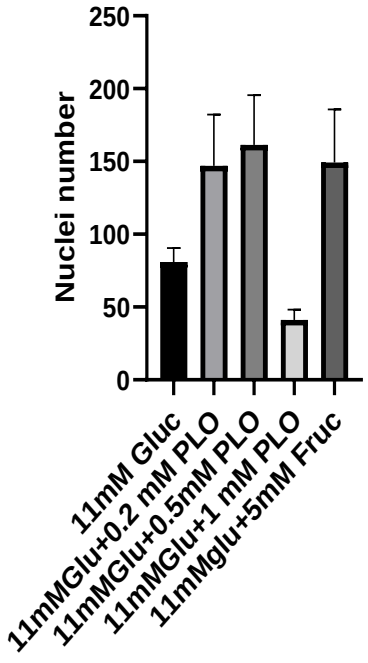


Fig 5.3: Effect of Fat on lipid accumulation in primary hepatocytes
Nile Red spectrophotometric assay- normalised to nuclei number

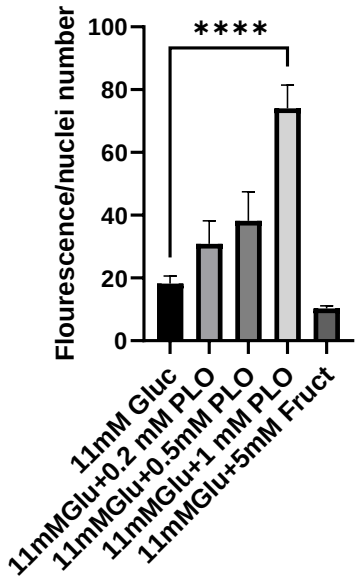


Figure 5.1-5.3: Effect of fat or fructose on lipid accumulation in primary hepatocytes-nile red spectrophotometric assay. Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs. After treatment, cells were fixed ~~in 4% paraformaldehyde~~ and stained with Nile Red and the fluorescence read ~~at Ex.530nm, Em590nm.~~ , DAPI nuclear stain was added, coverslips mounted on glass slides and analysed by fluorescent microscope ~~using a blue/UV channel. Nuclei were counted for a field of view area~~. ~~Four images were taken per slide at a position chosen at random, at x10 magnification, and the data meaned to give one biological replicate.~~ All treatments were compared to the control (11 mM Glucose) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing, **P*<0.05, ****P*<0.001, *****P*<0.001, n=4 replicate wells per treatment.

The drop in lipid accumulation for 1.0 mM PLO suggested there might be a problem with cytotoxicity. DAPI nuclear stain was added to the cells and nuclei counted for a set area (field of view x10 magnification) on a fluorescent microscope using a blue/UV channel. Estimation of cell number by nuclei number tended to drop greatly, although not significantly, for 1 mM PLO explaining its drop in staining (Figure 5.2). Nuclei number unexpectedly tended to be increased by the addition of 0.2 and 0.5 mM PLO in a dose-dependent, although not significantly. This could have been due to the high level of glucose present.

Normalizing Nile Red lipid staining with nuclei number, a clear dose-dependent effect of PLO on lipid accumulation was seen. However, the only dose that was statistically significant was the 1 mM dose that reduced cell survival (Figure 5.3).

Having seen reduced cell survival at high fat levels at 11 mM glucose and increased cell survival at lower levels of fat (Figure 5.2), it was concluded that an effect on lipid accumulation may require image analysis rather than a simple spectrophotometric reading. A lower level of glucose was also tested. There was a tendency for greater staining with 11 mM glucose+5 mM fructose compared with just 11 mM glucose (Figure 5.4 and 5.5), although the difference was not statistically different. However, there tended to be less cells estimated by the count of DAPI-stained nuclear number at all levels PLO for 4 mM glucose (Figure 5.6 above). In the absence of PLO, 4 mM glucose and 11 mM glucose were not statistically different. A substantial drop in cell nuclei was also seen at the highest fat level (1.0 mM) for 11 mM glucose (Figure 5.6) again. As primary hepatocytes do not replicate and grow in culture, this ~~perhaps~~ suggests a cytotoxic effect by fat at both high and low levels of glucose.

Figures 5.4 – 5.6: Effect of fat, glucose and fructose on lipid accumulation in primary hepatocytes- normalised to nuclei number.

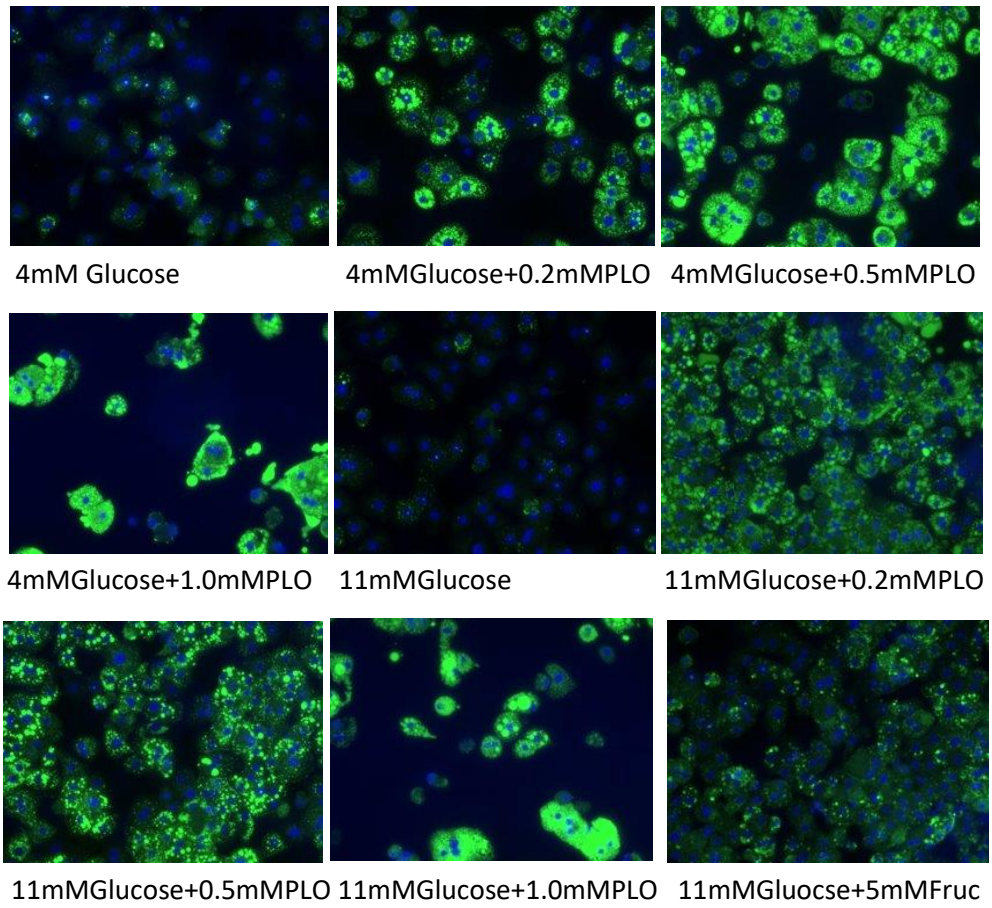


Fig 5.5: Effect of Fat, Glucose and Fructose on lipid accumulation in primary hepatocytes- normalised to nuclei number

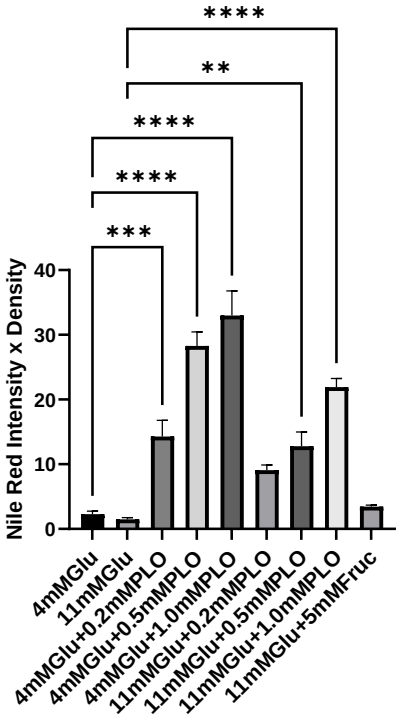
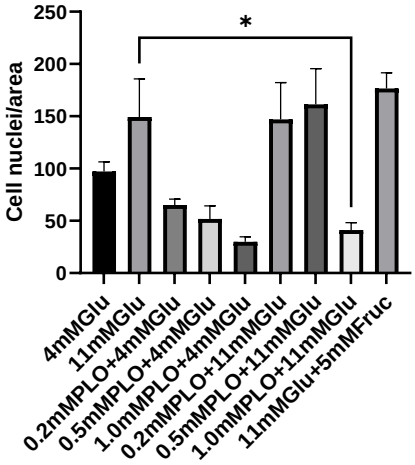


Fig 5.6: Effect of Fat and Glucose on Cell Survival in primary hepatocytes



Figures 5.4-5.6: Effect of fat, glucose and fructose on lipid accumulation in primary hepatocytes- normalised to nuclei number. Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs. After treatment, cells were fixed ~~in 4% paraformaldehyde~~ and stained with Nile Red and the fluorescence read ~~at Ex.530nm, Em590nm.~~ DAPI nuclear stain was added, coverslips mounted on glass slides and analysed by fluorescent microscope ~~using a GFP/FITC channel, image analysed using ImageJ software and normalised to nuclear number~~. ~~Four images were taken per slide at a position chosen at random, at x10 magnification, and the data averaged to give one biological replicate.~~ In figure 5.4 x10 magnification photos were obtained. In figure 5.5 and 5.6 all treatments were compared to the relevant control (4 mM or 5 mM glucose) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing *P<0.01, **P<0.01, ***P<0.01 and ****P<0.0001, n=4 replicate wells per treatment.

There was tendency for adding 5 mM fructose to 11 mM glucose to increase lipid accumulation. However, this was not statistically significant and may simply be due to increased total sugar or calories.

In the follow up experiment 16 mM glucose with 11 mM glucose+5 mM fructose were compared, such that each had an equimolar concentration of monosaccharide and was isocaloric. Glucose at 16 mM showed no increase in lipid staining over glucose at 5.5 mM (Figure 5.7). Although not statistically significant, 11 mM glucose+5 mM fructose showed a tendency to give twice the lipid staining that was seen with 16 mM glucose. In the presence of 0. 2 mM PLO, lipid accumulation was generally twice as

high. However, under such circumstances fructose gave rise to no more lipid accumulation than glucose.

Figure 5.7: Effect of fructose and glucose and fat on lipid accumulation in primary hepatocytes.

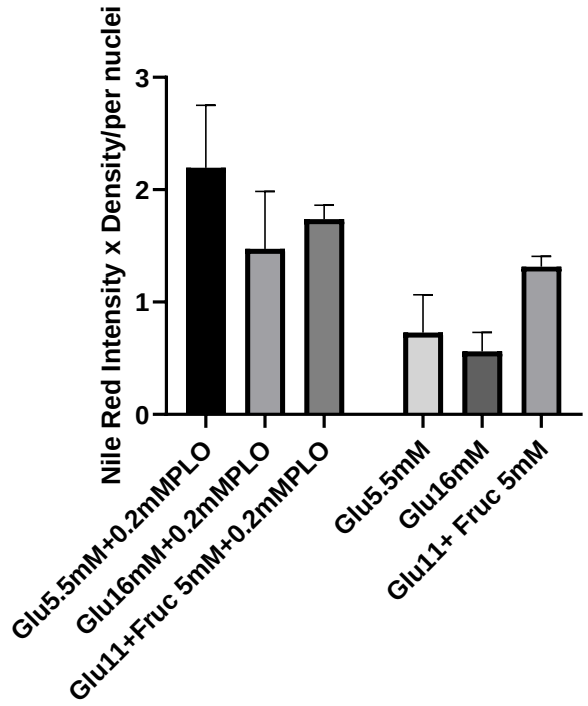


Figure 5.7: Effect of fructose and glucose and fat on lipid accumulation in primary hepatocytes. 16 mM isocaloric comparison Cells were seeded in 24-well plates on coverslips and treated for 48 hrs with refreshment every 24 hrs. After treatment, cells were fixed ~~in 4% paraformaldehyde~~ and stained with Nile Red and the fluorescence read ~~at Ex.530nm, Em590nm~~. 1:1000 DAPI nuclear stain was added, coverslips mounted on glass slides and analysed by fluorescent ~~microscope using a GFP/FITC channel, image analysed using ImageJ software and normalised to nuclear number. Four images were taken per slide at a position chosen at random, at x10 magnification, and the data averaged to give one biological replicate.~~ Isocaloric pairs were compared and all treatments to the relevant Glu5.5 mM control, by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, n=3 replicate wells per treatment. Although not statistically significant, glucose 11 mM+ 5 mM fructose showed a tendency to give twice the lipid staining that was seen with glucose 16 mM. In the presence of 0.2 mM PLO lipid accumulation was generally twice as high. However, under such circumstances fructose gave rise to no more lipid accumulation than glucose.

5.3 Fructose *vs* isocaloric glucose dose range on lipid accumulation and other metabolites.

Having seen a tendency for 5 mM fructose+11 mM glucose to increase lipid accumulation (Figure 5.7), although not significantly, for the following experiment the number of replicates was doubled to 6 and the time period increased from 48 to 72 hrs. A greater range of fructose concentrations were tested to establish a dose-response for impact on lipid accumulation. As less differences between fructose and glucose had been previously seen in the presence of fat, PLO was not further used.

It is generally proposed that only excess fructose is directed towards *de novo* lipogenesis (Lim *et al*., 2010) either directly or indirectly. Lim *et al*., 2010 discussed excess fructose as being when "total energy intake from carbohydrate exceeds total energy expenditure." However, the metabolites of fructose are also known to be used for gluconeogenesis and glycogen storage. As such, It is ~~perhaps~~ a legitimate question as to whether *de novo* lipid synthesis would occur before full glycogen storage is reached, and also whether excess metabolites would more likely be directed towards lactate production. As such, these metabolites were also analysed.

Treatment media was refreshed, and the conditioned medium collected for analysis of lactate every 24 hrs. After 72 hrs, cells were fixed and stained for lipid with Nile Red and a parallel set of cells identically treated were harvested to measure glycogen. Ideally, cells would have been collected at 24 and 48 hrs for lipid and glycogen analyses, however, numbers of fresh primary hepatocyte on the day did not allow for this.

For lipid staining with Nile Red, no treatments showed a significant increase when compared with the 5.5 mM glucose control. There were no differences between isocaloric pairs e.g. 11 mM glucose+8 mM fructose and 19 mM glucose (Figure 5.8 and 5.9).

Figure 5.8 and 5.9: Fructose vs isocaloric glucose dose range on lipid accumulation and primary hepatocyte nuclei

Figure 5.8:

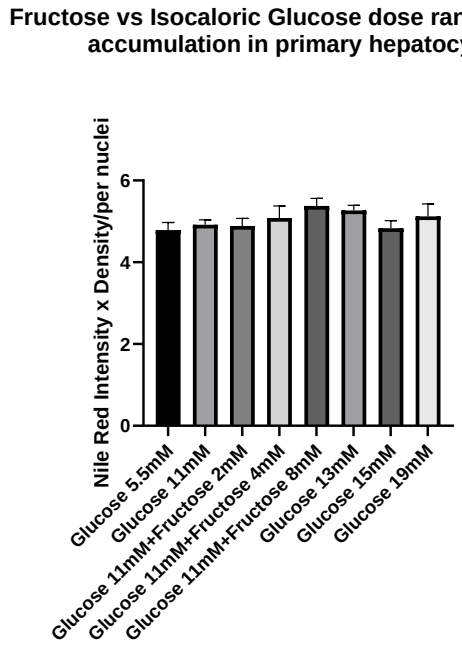
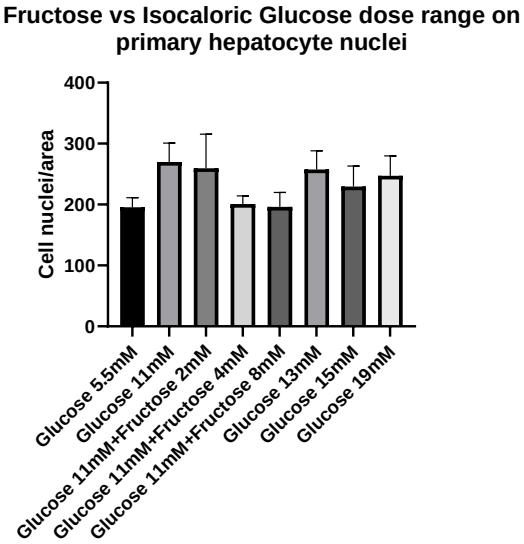


Figure 5.9:



Figures 5.8 and 5.9: Fructose *vs* isocaloric glucose dose range on lipid accumulation and primary hepatocyte nuclei: Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs. After treatment, cells were fixed in 4% paraformaldehyde and stained with Nile Red and the fluorescence read at Ex.530nm, Em590nm. 1:1000 DAPI nuclear stain was added, coverslips mounted on glass slides and analysed by fluorescent microscope using a GFP/FITC channel, image analysed using ImageJ software and normalised to nuclear number. Four images were taken per slide at a position chosen at random, at x10 magnification, and the data averaged to give one biological replicate. All isocaloric treatments were compared to the 5.5 mM glucose treatment, as well as between isocaloric pairs, by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, n=6 replicate wells per treatment. No treatments showed a significant effect on lipid accumulation or cell nuclei number, when compared with the 5.5 mM glucose control, and here were no differences between isocaloric pairs e.g. 11 mM glucose+8 mM fructose and 19 mM glucose.

5.3.1 Glycogen.

In the fasted state, some 30-50% of fructose reaching the liver has been reported to be converted to glucose by gluconeogenesis (Delarue, 1993; Sun & Empie, 2012). This is usually stored as glycogen rather than released into the blood (Dirlewanger *et al*., 2000). A similar proportion of fructose may be metabolised to carbon dioxide and water to generate energy or lactate (Sun & Empie, 2012) (see Figure 1.5, 1.0 Introduction, 3.1.2 Effects of Fructose on *de novo* lipogenesis). It may depend therefore, to a large extent on the levels of glycogen stored and the energy requirements of the cell at any given point, as to how much fructose or glucose may be directed to lipid synthesis and storage. As such, the effect on glycogen metabolism of a range of isocaloric fructose and glucose doses (as above for lipid accumulation) were investigated.

All fructose+glucose treatments increased glycogen levels substantially and significantly in a dose-dependent manner (Figure 5.10). Glucose did not significantly increase the amount of glycogen even at the highest dose of 19 mM, although it did show a tendency to rise. Each fructose treatment raised glycogen levels substantially and significantly more than its isocaloric glucose pair, i.e. 11 mM glucose+2 mM fructose increased glycogen significantly by approximately 30% more than 13 mM glucose; 11 mM glucose+4 mM fructose increased glycogen significantly by approximately 50% more than 15 mM glucose; and 11 mM glucose+8 mM fructose increased glycogen significantly by approximately 40% more than 19 mM glucose (Figures 5.10-5.13).

Figures 5.10-5.13: Fructose vs isocaloric glucose dose range on glycogen and intracellular glucose in primary hepatocytes.

Figure 5.10:

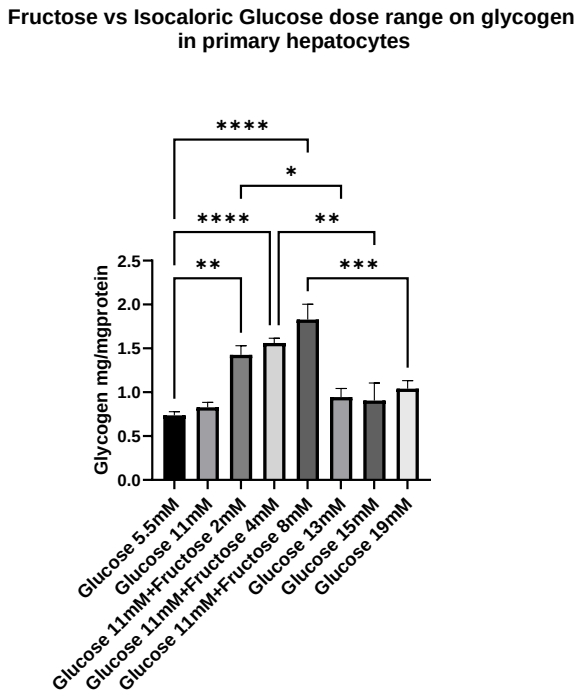


Figure.11

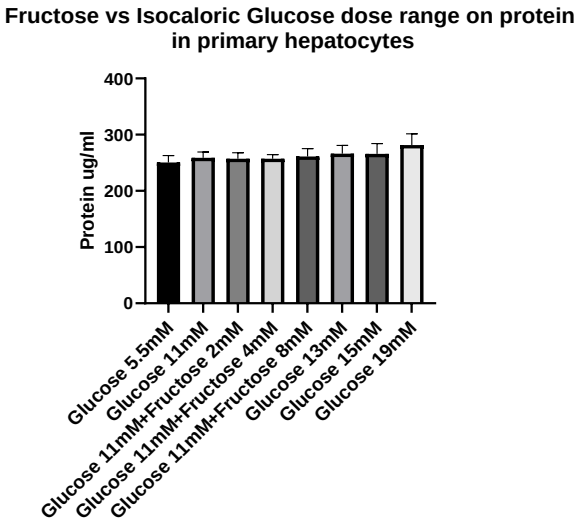


Figure 5.12:

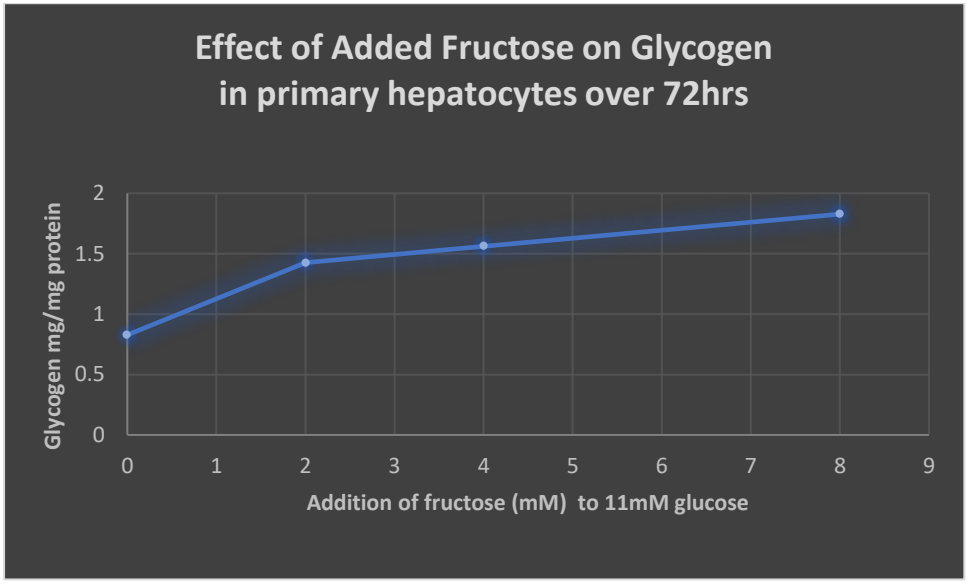

Effect of Added Fructose on Glycogen in primary hepatocytes over 72hrs
Glycogen mg/mg protein
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
8
9
Addition of fructose (mM) to 11mM glucose

Figure 5.13:

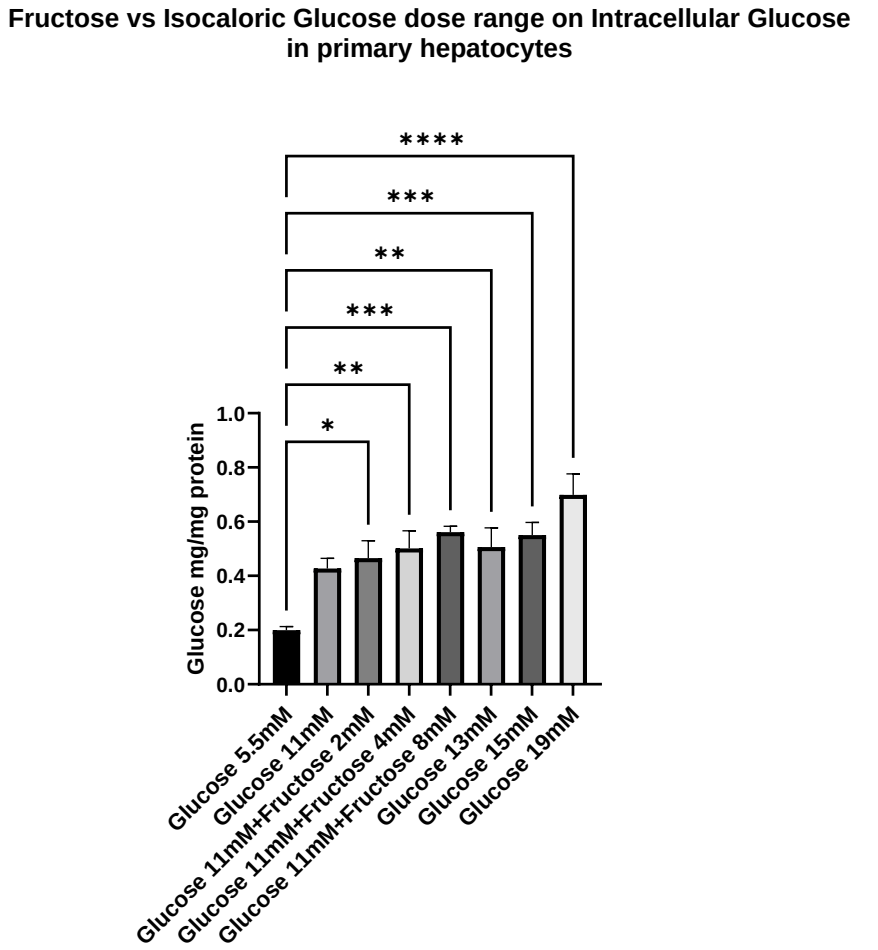


Figure 5.10-5.13: Fructose *vs* isocaloric glucose dose range on glycogen and intracellular glucose in primary hepatocytes. Cells were seeded in 6-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs. Cells were harvested by scraping in ice-cold 1 ml dd H_2O and frozen for glycogen analysis~~. Upon thawing samples on ice, trypsin inhibitor was added immediately then rapidly homogenised on ice using 2x10sec bursts by probe sonication and boiled to inactivate enzymes. The supernatant was hydrolysed to form glucose, which was oxidized by a hydrolase to form an intermediate that reduces a colourless probe to a coloured product with strong absorbance at 450nm.~~ Performing the glycogen assay in the absence of the hydrolase gives the initial levels of glucose (figure.5.13) to be deducted from the total. ~~Samples were compared to glycogen standards. For initial glucose analysis hydrolase was not added~~. All isocaloric treatments were compared to the 5.5 mM glucose treatment, as well as between isocaloric pairs, by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n=6 replicate wells per treatment.

There was a tendency for protein levels to rise slightly with glucose treatment (Figure 5.11) however, no significant differences were found on comparing all treatments to the 5.5 mM glucose control, or between isocaloric pairs. Without normalisation, glucose still did not significantly increase glycogen, and each fructose treatment still raised glycogen substantially and significantly more than its isocaloric glucose pair.

On a linear scale, it can be seen more clearly that although a sharp increase in glycogen was seen for the first additional 2 mM fructose treatment, which becomes less steep after further addition, the rise though had not plateaued at even the highest addition of fructose of 8 mM (Figure 5.12).

Glycogen is analysed by converting to glucose with hydrolase. By not including the enzyme in parallel samples, the background glucose in the sample is analysed, which is then deducted from the total. All fructose treatments increased this intracellular glucose in a dose-dependent manner (Figure 5.13). Glucose only treatments tended to increase intracellular glucose in a dose-dependent manner and tended to do so more than their fructose isocaloric pairs, but not significantly.

5.3.2 Lactate

In the fasted state, some 30% of fructose reaching the liver has been reported to be converted to lactate (Dietze *et al*., 1976; Sun & Empie, 2012) (see Figure 1.3, 1.0 Introduction, 3.1.2 Effects of Fructose on *de novo* lipogenesis), and may subsequently be converted to lipids in the liver and or other tissues.

Over 24, 48 and 72 hrs, fructose treatments increased lactate production and release into the culture medium substantially in a dose-dependent manner (Figures 5.14, 5.15 and 5.16). This was statistically significant for all the fructose doses at 24 hrs (Figure 5.13), the highest two doses at 48 hrs (Figure 5.15), but only the highest dose at 72 hrs (Figure 5.16). Glucose only treatments had no effect on lactate production at any time point. For all treatments, lactate increased two-fold between 24 hrs and 48 hrs and remained at the same level from 48 hrs to 72 hrs. There were significant differences between isocaloric treatments for the two higher doses at 24 and 48 hrs, but only for the highest dose at 72 hrs.

Figures 5.14-5.16: Fructose vs isocaloric glucose dose range on extracellular lactate released by primary hepatocytes over three days

Figure 5.14:

Fructose vs Isocaloric Glucose dose range on extracellular Lactate released by primary hepatocytes over the first day of three (24 hrs)

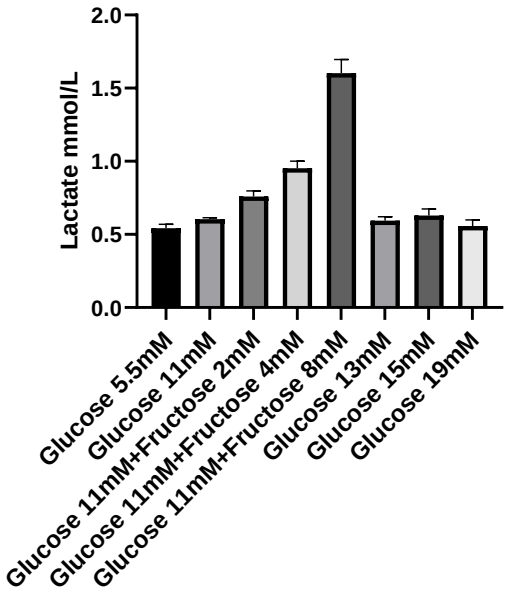


Figure 5.15:

Fructose vs Isocaloric Glucose dose range on extracellular Lactate released by primary hepatocytes over the second day three (48 hrs).

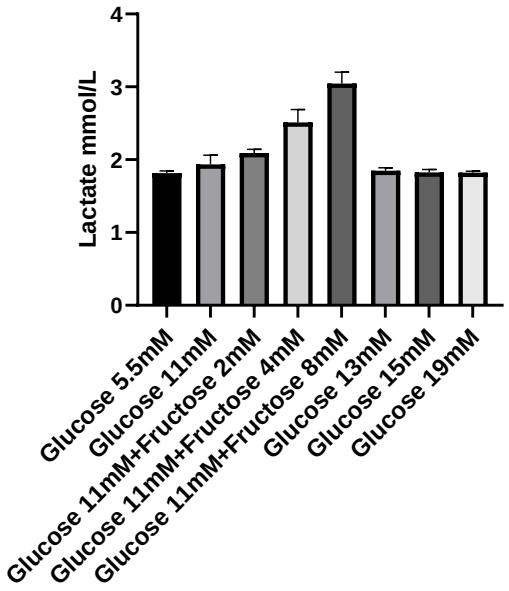
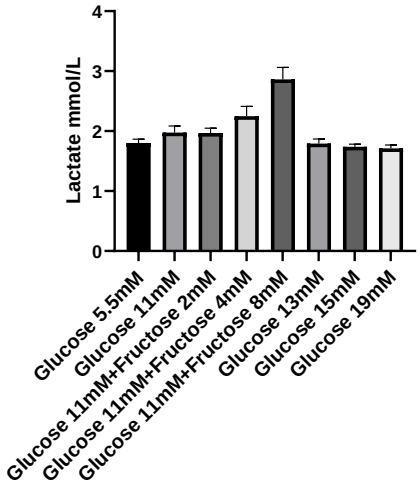


Figure 5.16:

Fructose vs Isocaloric Glucose dose range on extracellular Lactate released by primary hepatocytes over the third day of three (72 hrs).



Figures 5.14-5.16: Fructose *vs* isocaloric glucose dose range on extracellular lactate ~~in~~ released by primary hepatocytes over three days. Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs and lactate measured in the conditioned medium. ~~Lactate was measured by lactate dehydrogenase-mediated conversion to pyruvate, which in the process converts NAD to NADH which is quantified by absorption at 340nm.~~ For each time point all isocaloric treatments were compared to the 5.5 mM glucose treatment, as well as between isocaloric pairs, by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing as, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, n=6 replicate wells per treatment.

5.3.3 Uric acid

An additional health issue ~~hazard~~ specifically caused by fructose could be elevated levels of uric acid, which is associated with oxidative stress, production of, for instance, tumour necrosis factor-α (Sun & Empie, 2012), hypertension and type II diabetes (Degham *et al*., 2008) and cardiovascular disease (Borghi *et al*., 2014). Increased uric acid can be caused as a metabolic by-product from a build-up ADP and AMP. This may occur as fructose is very rapidly metabolised in the liver by fructokinase which depletes ATP to produce ADP, but is subsequently slowly metabolised by downstream enzymes, e.g. aldolase (Chaudhary *et al*., 2013) (see 1.2. Potential mechanisms to explain the negative impact of fructose on health, 1.2.6 ATP depletion and uric acid production- Oxidative stress and Inflammation). Therefore, whether fructose and glucose have different effects on uric acid release in

the experiments as described above was investigated. Levels, however, were very low and no significant differences, trends, or tendencies of any notable interest were found (Figures 5.17, 5.18 and 5.19).

Figures 5.17-5.19: Fructose vs Isocaloric Glucose dose range on Uric acid ~~in~~ released by primary hepatocytes over three days

Figure 5.17:

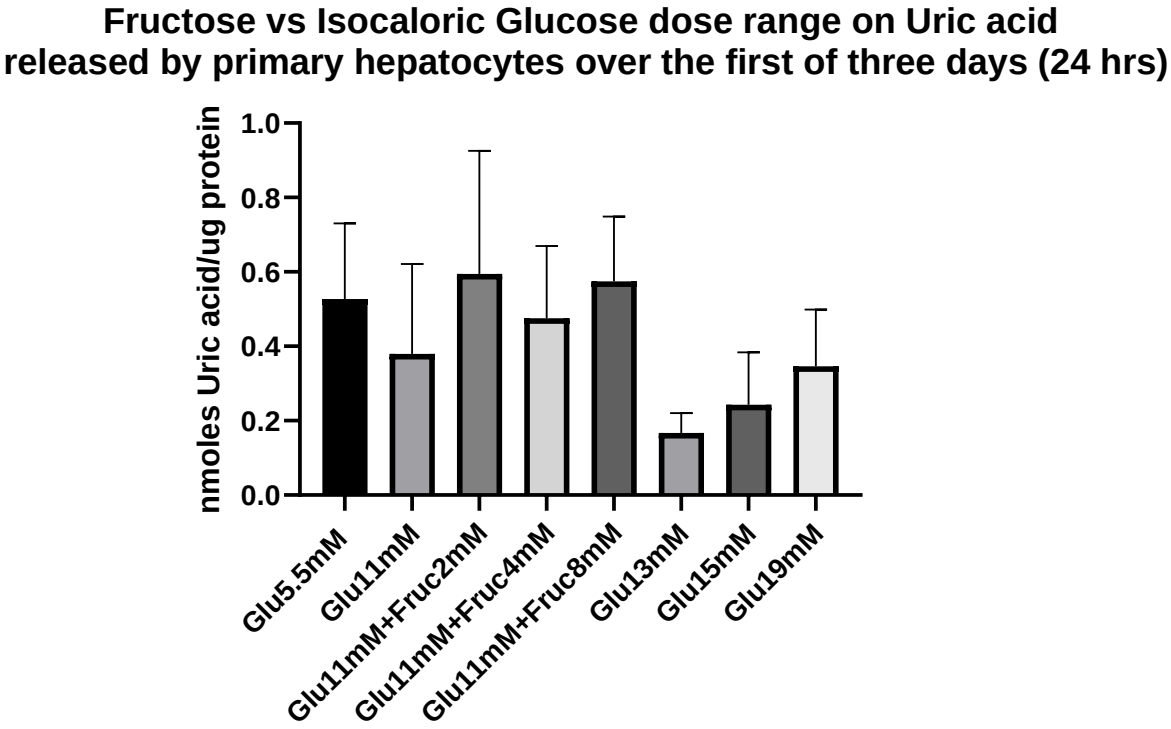


Figure 5.18:

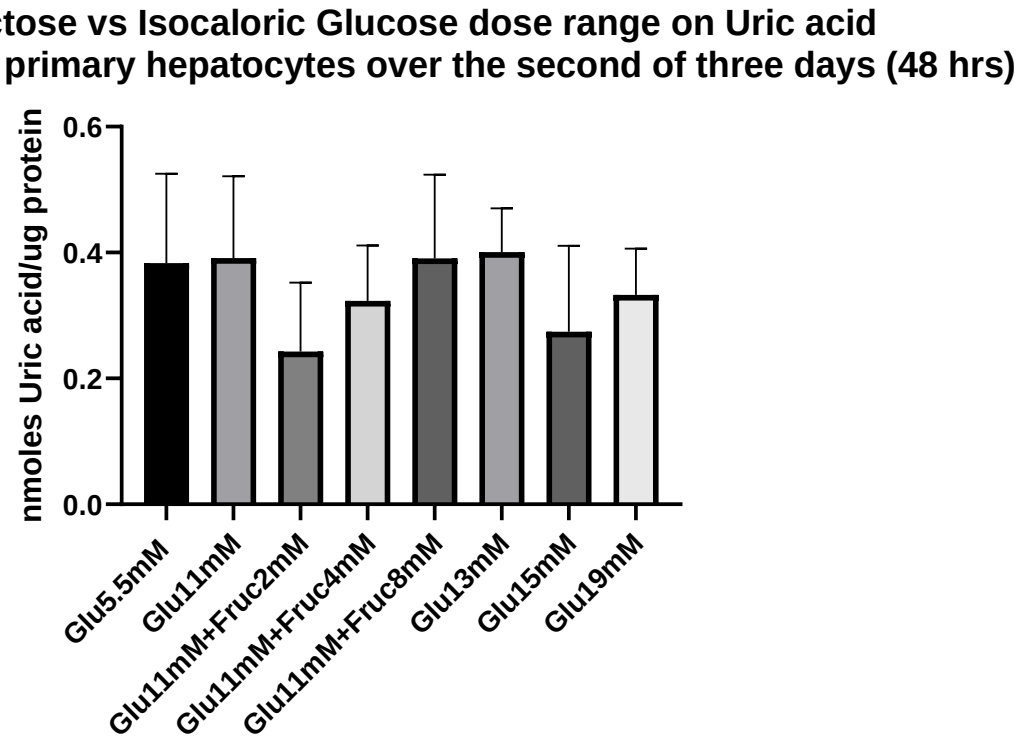
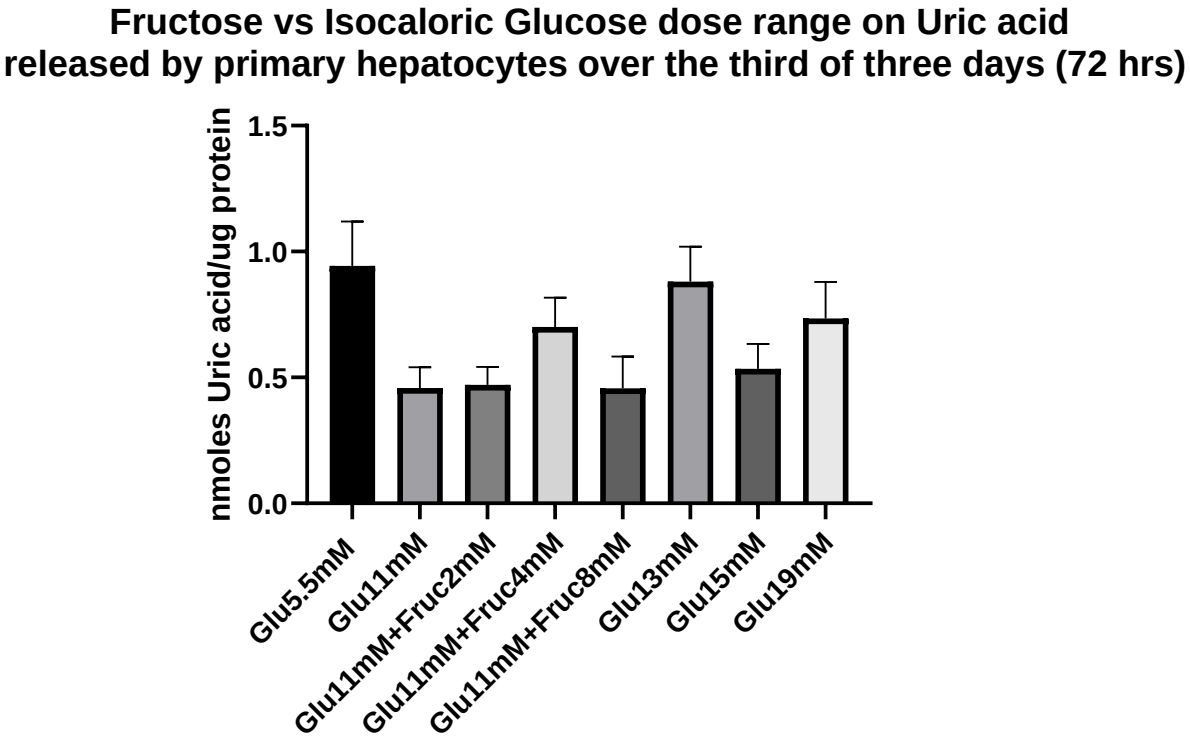

Fructose vs Isocaloric Glucose dose range on Uric acid released by primary hepatocytes over the second of three days (48 hrs)
nmoles Uric acid/ug protein
0.6
0.4
0.2
0.0
Glu5.5mM
Glu11mM
Glu11mM+Fruc2mM
Glu11mM+Fruc4mM
Glu11mM+Fruc8mM
Glu13mM
Glu15mM
Glu19mM

Figure 5.19:



Figures 5.17-5.19: Fructose *vs* Isocaloric Glucose dose range on Uric acid ~~in~~ released by primary hepatocytes over three days. Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs. Uric acid was measured in the conditioned medium ~~by oxidation with uricase to produce hydrogen peroxide, then by the formation of coloured products using peroxidase.~~ For each time point all isocaloric treatments were compared to the 5.5 mM glucose treatment, as well as between isocaloric pairs, by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, n=6 replicate wells per treatment.

5.4 Fibroblast Growth Factor 21 (FGF21)

Circulatory levels of fibroblast growth factor 21 (FGF21), a recently discovered endocrine hormone, are mostly produced by the liver in response to an imbalance of major nutrients such as sugars. In most reports, circulating FGF21 levels rise after consumption of simple sugars, but not of complex carbohydrates (von Holstein-Rathlou *et al*., 2016). Via the hypothalamus, FGF21 signals to the brain to reduce sugar intake by suppressing sweet-taste preference (Jenson- Cody *et al*., 2020). As such, FGF21 has been hypothesised to function to protect the liver from damage (Jensen-Cody *et al*., 2020). In the studies presented in his thesis, fructose appears to have a differential effect to glucose on parameters such postprandial triglycerides (Chapter 3.0, Figures 3.9-15), glycogen (Figure 5.10) and lactate (Figure 5.14-16).

Therefore, whether fructose and glucose have different effects on FGF21 release by the liver was investigated.

In the experiment described above (see 5.1 Lipid accumulation in the liver, Fructose dose response on lipid and other metabolites, Figures 5.7-19) testing isocaloric treatments of fructose and glucose, total and intact FGF21 was measured by ELISA. Intact FGF21 is fully active. However, cleavage at its N- and C- termini can reduce activity and give rise to potent antagonism (Agrawal *et al*., 2018). Truncation at the N-terminus impairs the interaction of FGF21 with its receptor (FGFR1c), and progressive shortening of the C-terminus weakens binding to co-receptor b-klotho protein. The Alpco intact FGF21 assay used here uses a sandwich protocol; one antibody (coated to the plate) binds the N-terminus, and a second antibody binds the C-terminus, so if either terminus is truncated, no detection occurs. As non-intact FGF21 may be inhibitory, total FGF21 was also measured to ascertain a ratio. Fibroblast inactivation protein (FAP), which digests both FGF21 C- and N-termini (Agrawal *et al*., 2018) was also measured as a guide to sample FGF21 activity.

5.4.1 Intact FGF21

After 24 hrs, only the 11 mM glucose+8 mM fructose, the highest of the fructose doses, had a significant impact on intact FGF21, giving a substantial 2-fold increase over the 5.5 mM glucose control (Figure 20). There were no significant differences between fructose and glucose isocaloric pairs, although there was a tendency for the fructose treatments to give slightly higher results. After 48 hrs (Figure 5.21), all treatments had tended to increase intact FGF21 by at least 2-fold compared to 24 hrs. The FGF21 levels had all risen by at least 5-fold after all fructose treatment doses, and significantly compared to the 5.5 mM glucose control, and to a substantially and significantly greater level than their glucose isocaloric pairs. The second highest fructose dose (1 mM glucose+4 mM fructose) gave the greatest intact FGF21 level, rather than the highest dose. The glucose only treatments tended to increase intact FGF21 in a dose-dependent manner, with only 19 mM glucose giving significantly greater levels than the 5.5 mM glucose control. After 72 hrs (Figure 5.22), levels of intact FGF21 had tended to drop for all treatments compared to 48 hrs but were still generally higher than at 24 hrs. For the fructose

treatments, the two lower doses still gave significantly greater levels than the 5.5 mM glucose control, whilst the highest dose did not, giving the lowest level of intact FGF21 of the three. Fructose treatments still tended to give higher levels than glucose for all doses, but only the lowest dose pair gave significantly different results. As for 48 hrs glucose, doses still tended to give a dose-dependent increase in intact FGF21.

Figures 5.20-5.22: Fructose vs isocaloric glucose dose range on intact FGF21 ~~in~~ released by primary hepatocytes over three days.

Figure 20:

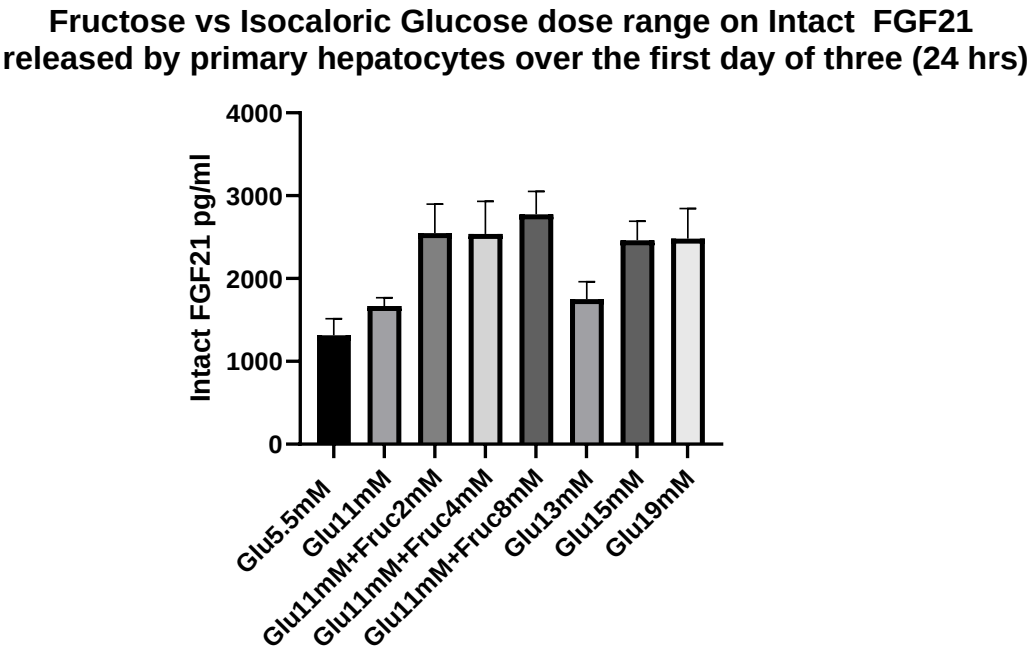


Figure 5.21:

Fructose vs Isocaloric Glucose dose range on Intact FGF21 released by primary hepatocytes over the second day of three (48 hrs)

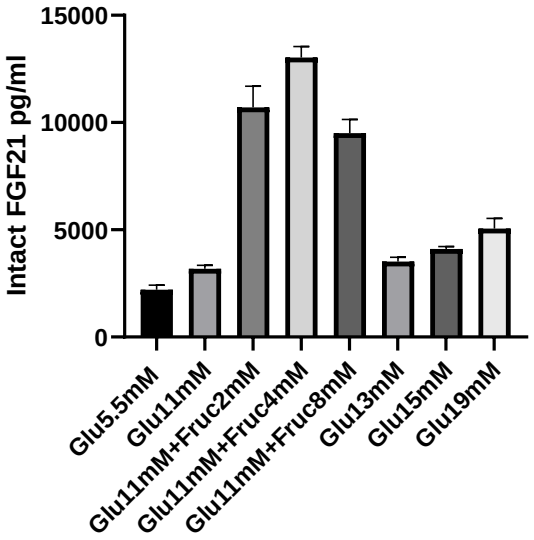
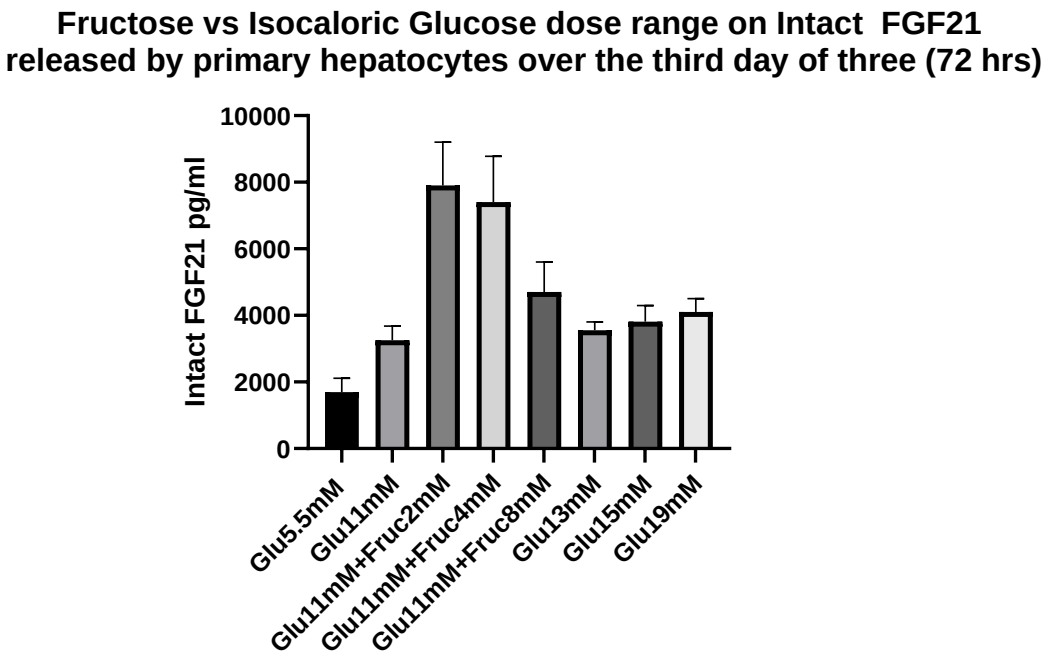


Figure 5.22:



Figures 5.20-5.22: Fructose *vs* isocaloric glucose dose range on intact FGF21 ~~in~~ released by primary hepatocytes over three days. Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs. Intact human FGF21 was measured in the conditioned medium ~~using an ELISA that does not detect FGF-21 fragments. The assay utilizes a two-site "sandwich" technique with two selected antibodies that bind to different epitopes of human intact FGF-21. One of the antibodies specifically binds to the N-terminal human FGF-21 (1-7) and the other is specific to the C-terminal human FGF-21 (175-181).~~ For each time point all isocaloric treatments were compared to the 5.5 mM glucose treatment control, as well as between isocaloric pairs, by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing as, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, n=3 replicate wells per treatment.

5.5.2 Total FGF21 *vs*. intact FGF21

Total FGF21 levels at 24 hrs (Figure 5,23) show a greater difference between fructose and glucose isocaloric treatments than the little or no difference seen for intact FGF21 (Figure 5.24). After 48 hrs, fructose treatments gave much greater total FGF21 than isocaloric glucose treatments (Figure 5.23), as was the case for intact FGF21 (Figure 5.24), however, the difference was less. The same was seen after 72 hrs (Figures 5.23 and 5.24). Statistical analysis was not performed on total FGF21 as the key measure is intact/total FGF21.

Figures 5.23 and 5.24: Fructose vs Isocaloric Glucose dose range on Total FGF21 in released by primary hepatocytes over three days compared with Intact FGF21.

Figure 5.23:

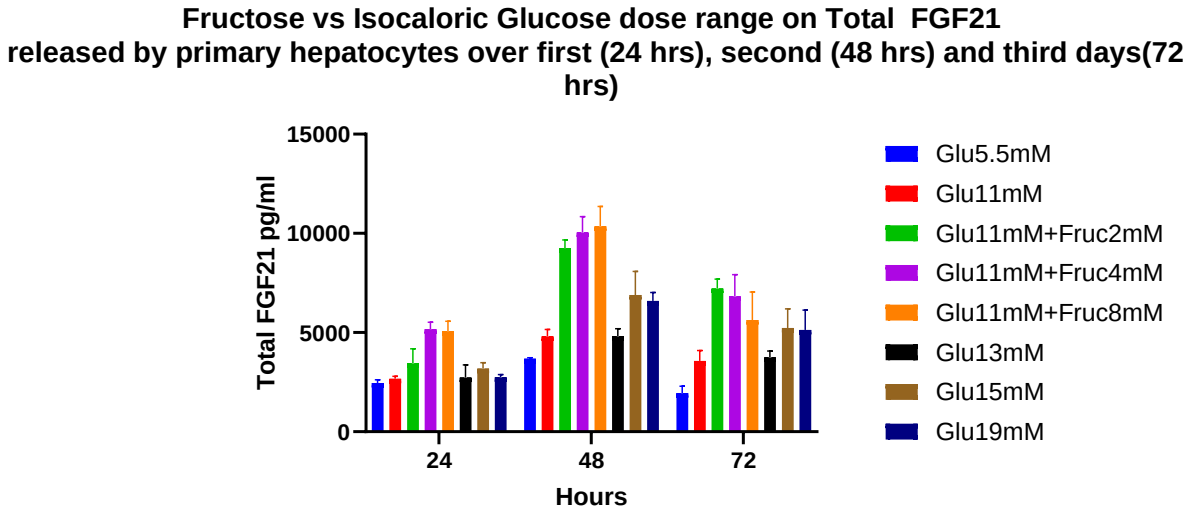
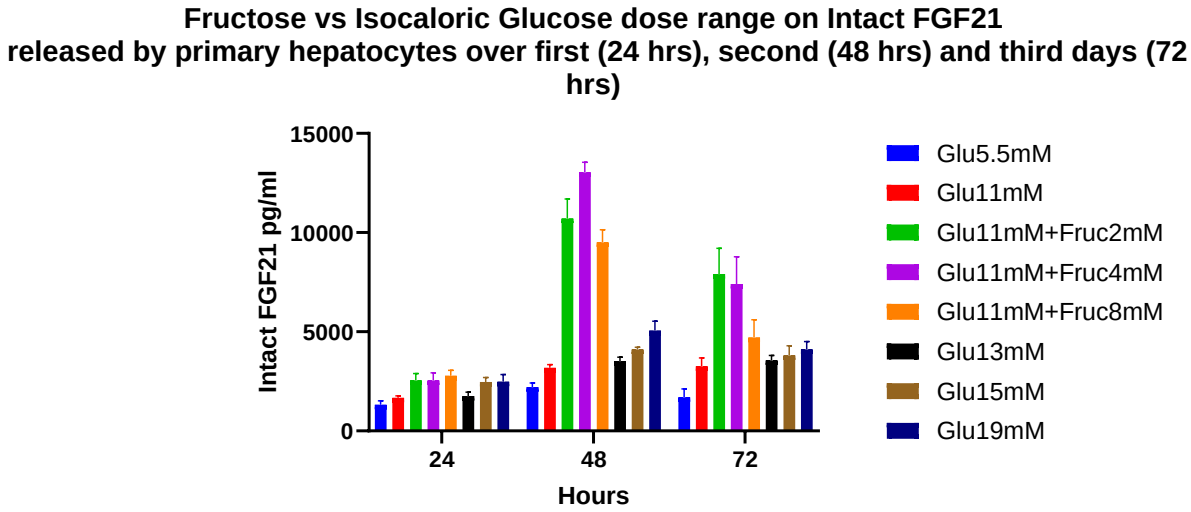


Figure 5.24:



Figures 5.23 and 5.24: Fructose *vs* Isocaloric Glucose dose range on Total FGF21 ~~in~~ released by primary hepatocytes over three days compared with Intact FGF21. Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs in triplicate. Intact human FGF21 was measured in the conditioned medium ~~using an ELISA that does not detect FGF-21 fragments. The assay utilizes a two-site "sandwich" technique with two selected antibodies that bind to different epitopes of human intact FGF-21. One of the antibodies specifically binds to the N-terminal human FGF-21 (1-7) and the other is specific to the C-terminal human FGF-21 (175-181).~~ There were 3 replicates per treatment. Total FGF21 was measured by a quantitative sandwich enzyme immunoassay. Statistical analysis was not performed on total FGF21, (see intact/total FGF21 below).

5.5.3 Intact/Total FGF21

For the ratio of intact FGF21 to total FGF21 after 24 hrs only, 19 mM glucose is significantly different, rising substantially by approximately 40% (Figure 5.25), although in the glucose treatments, levels rose in a dose-dependent manner. Contrary to that seen for intact (Figure 5.20 and 5.24) and total FGF21 (Figure 5.23), their ratio tended to be greater for fructose, at least for the highest doses. 19 mM glucose treatment led to a significantly and substantially greater ratio than its isocaloric pair of 11 mM glucose+8 mM fructose. With the exception of the lower dose, fructose treatments did not affect the intact/total FGF21 (Figure 5.25). However, 11 mM glucose+2 mM fructose did give a significant increase. After 48 hrs, the pattern of data for intact/total (Figure 5.26) FGF21 is similar to that which was seen for intact (Figure 5.21) and total (Figure 5.23) after 48 hrs. Only fructose treatments showed a significant increase in ratio compared with the 5.5 mM glucose control. For the two lower doses (11 mM glucose+2 mM fructose and 11 mM glucose+4 mM fructose), there is a significant increase in the ratio compared with

their isocaloric glucose pairs. Glucose treatments tended to increase the intact/total FGF21 ratio in the control. After 72 hrs (Figure 5.27), a similar pattern to 48 hrs was seen, but any differences from the 5.5 mM glucose control and between isocaloric fructose and glucose treatments were not significant. However, the difference between 11 mM glucose+4 mM fructose and 15 mM glucose was close to significance (P=0.0579).

Figures 5.25-5.27: Fructose vs isocaloric glucose dose range on Intact/Total FGF21 ~~in~~ released by primary hepatocytes over three days.

Figure 5.25:

Fructose vs Isocaloric Glucose dose range on Intact/Total FGF21 released by primary hepatocytes over the first of three days (24 hrs)

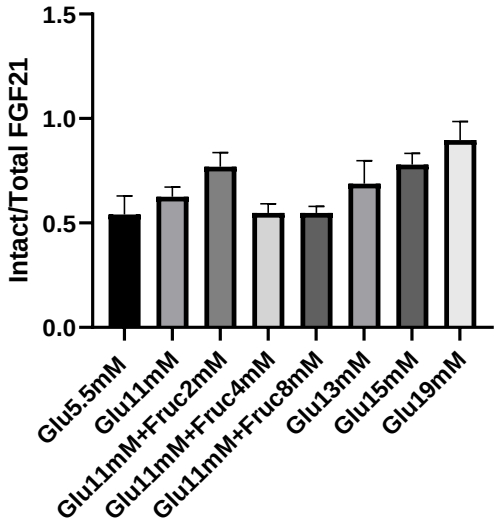


Figure 5.26:

Fructose vs Isocaloric Glucose dose range on Intact/Total FGF21 released by primary hepatocytes over the second of three days (48 hrs)

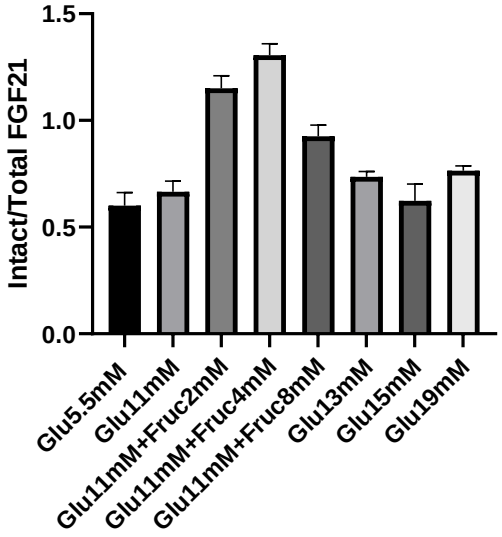
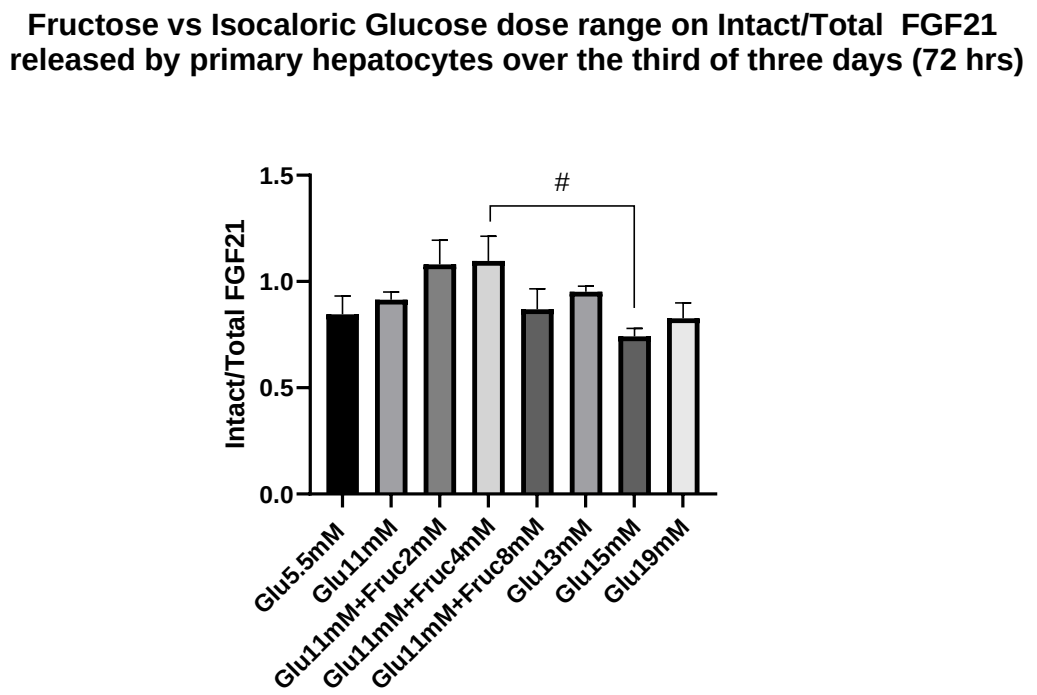


Figure 5.27:



Figures 5.25 5.27: Fructose *vs* isocaloric glucose dose range on Intact/Total FGF21 ~~in~~ released by primary hepatocytes over three days. Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs. Intact human FGF21 was measured in the conditioned medium ~~using an ELISA that does not detect FGF-21 fragments. The assay utilizes a two-site "sandwich" technique with two selected antibodies that bind to different epitopes of human intact FGF-21. One of the antibodies specifically binds to the N-terminal human FGF-21 (1-7) and the other is specific to the C-terminal human FGF-21 (175-181). Total FGF21 was measured by a quantitative sandwich enzyme immunoassay.~~ For each time point all isocaloric treatments were compared to the 5.5 mM glucose treatment control, as well as between isocaloric pairs, by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, #P=0.0579, n=3 replicate wells per treatment.

5.5.4 Fibroblast Activation Protein (FAP)

Total FGF21 includes both intact-FGF21 which is fully active and non-intact FGF21. The non-intact FGF21 may be less active, non-active or even potently inhibitory (Agrawal *et al*., 2018). We cannot be certain as to the inhibitory or merely non- or less-active effect of the portion of total FGF21 that is not intact. This may be different for fructose and glucose. For instance, most of the non-active fragments for fructose may be potently inhibitory, whilst for glucose just non-active. In order to examine this further FAP, which digests both FGF21 C- and N-termini and has also been associated metabolic disease (Agrawal *et al*., 2018), was also investigated by measurement in the conditioned medium.

After 24 hrs 11 mM glucose+4 mM fructose and 19 mM glucose had significantly decreased FAP (Figure 5.28). Of the isocaloric pairs there was a significant difference between 11 mM glucose+8 mM fructose and 19 mM glucose. In general, there was a ~~trend~~ tendency for glucose treatments on FAP to be lower than for the isocaloric fructose treatments. After 48 and 72 hrs, there were no differences in FAP between the treatments (Figures 5.29 and 5.30).

Figures 5.28-30: Fructose vs Isocaloric Glucose dose range on Fibroblast Activation Protein (FAP) ~~in~~ released by primary hepatocytes over three days.

Figure 5.28:

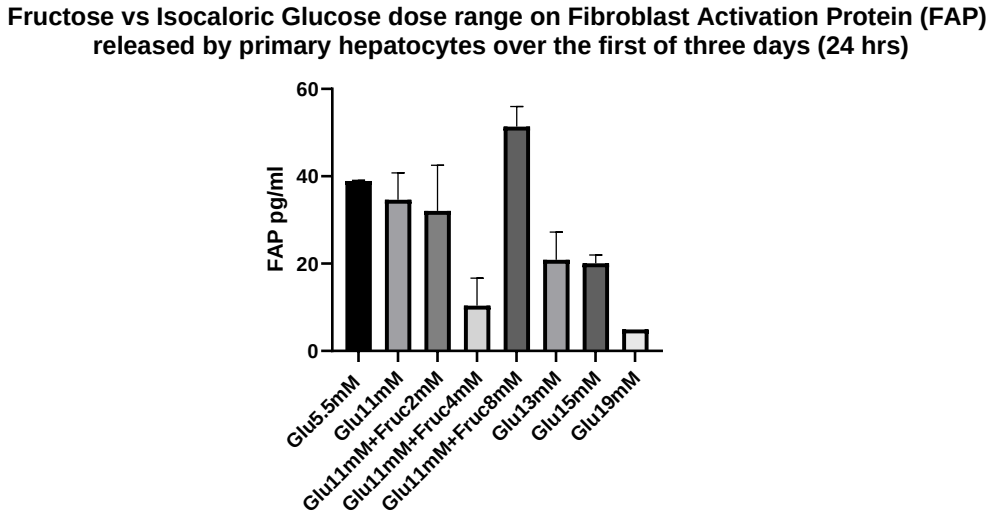


Figure 5.29:

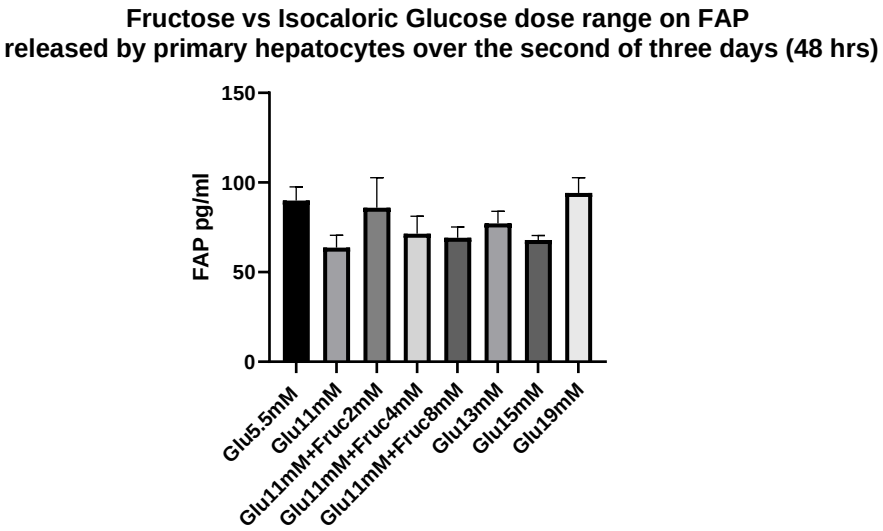
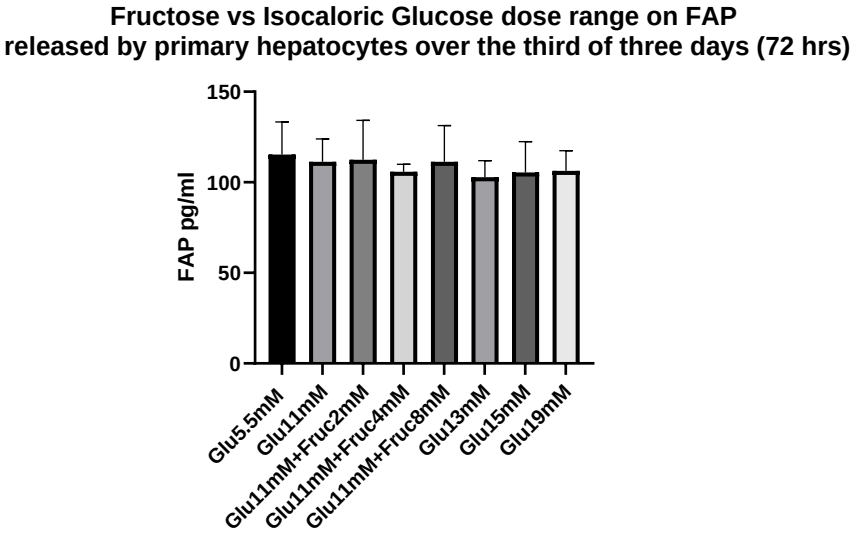


Figure 5.30:



Figures 5.28-30: Fructose *vs* Isocaloric Glucose dose range on Fibroblast Activation Protein (FAP) ~~in~~ released by primary hepatocytes over three days. Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs. FAP was measured in the conditioned medium ~~by ELISA.~~ For each time point, all isocaloric treatments were compared to the 5.5 mM glucose treatment control, as well as between isocaloric pairs by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, *P<0.05, **P<0.01, n=3 replicate wells per treatment.

For the 24 hrs data there was a significant correlation between FAP and intact/total FGF21 (Pearson correlation r= -0.5154, *P*=0.0141) (Figure 5.31). The corelation was not significant but strongest for glucose (r=0.5502, *P*=0.0795) (Figure 5.32), compared to fructose (r=0.2498, *P*=0.4587) (Figure 5.33).

Figures 5.31-5.33: Fructose vs isocaloric glucose dose range on FAP vs Intact/Total FGF21 in released by primary hepatocytes over three days.

Figure 5.31:

Fructose vs Isocaloric Glucose dose range on FAP vs Intact/Total FGF21 released by primary hepatocytes over the first of three days (24 hrs)

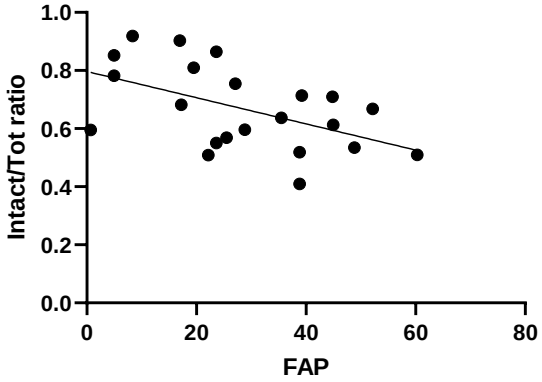


Figure 5.32:

Fructose vs Isocaloric Glucose dose range on FAP vs Intact/Total FGF21 for glucose only released byprimary hepatocytes over the first of three days (24 hrs)

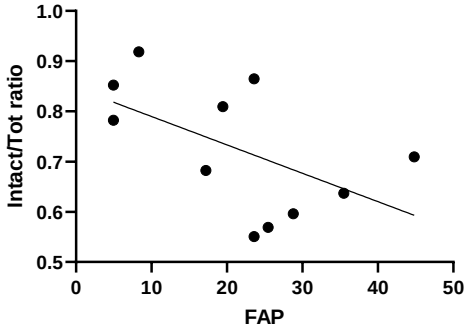
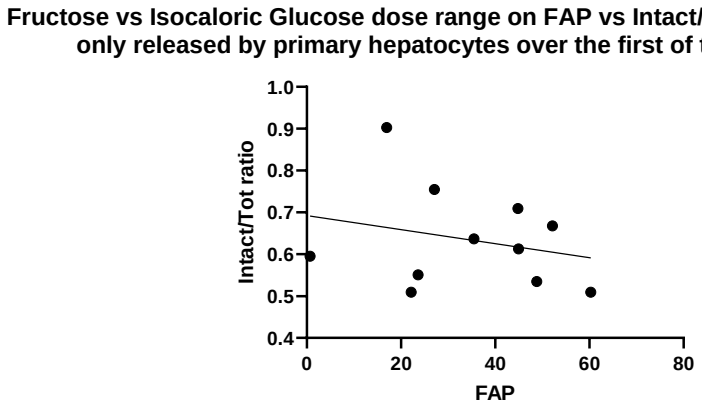


Figure 5.33:



Figures 5.31-5.33: Fructose *vs* isocaloric glucose dose range on FAP *vs* Intact/Total FGF21 ~~in~~ released by primary hepatocytes over three days. Cells were seeded in 24-well plates on coverslips and treated for 72 hrs with refreshment every 24 hrs. FAP, intact FGF21 and total FGF21 were each measured in the conditioned medium ~~by ELISA~~. There were 3 replicates per treatment. For the 24 hrs data there was significant correlation between FAP and intact/total FGF21 (Pearson correlation r= -0.5154, *P*=0.0141) (Figure 5.31), n=23. The corelation was not significant but strongest for glucose (r=0.5502, *P*=0.0795) (Figure 5.32), n=11, compared to fructose (r=0.2498, *P*=0.4587) (Figure 5.33), n=11.

5.5.5 Effect of fructose and glucose isocaloric treated primary hepatocyte conditioned medium on glucose uptake in 3T3 L1 differentiated adipocytes.

Total FGF21 includes both intact-FGF21 which is fully active and non-intact FGF21. The non-intact FGF21 may be less active, non-active or even potently inhibitory (Agrawal *et al*., 2018). As we cannot be certain as to the inhibitory or merely non- or less-active effect of the portion of total FGF21 that is not intact, and this may be different for fructose and glucose treatments. In order to examine this further therefore, the conditioned medium from the hepatocytes was applied to 3T3 L1 adipocytes and glucose uptake examined, an increase in uptake being a well-established effect of FGF21 (Kharitonenkov, *et al*. 2005).

Time course- 4, 7, 11 days

The differentiation protocol for 3T3 L1 fibroblasts used in most published methods involves differentiation with insulin, IBMX and dexamethasone for 2 days, then with insulin for 2 days and then maintenance medium every 2 days for up to 9-11 days post-initiation (Kharitonenkov, *et al*. 2005).

This regime was often not possible to follow due to travel issues. A 4-, 7- and 11-days regime was therefore tested. Optimal lipid staining was found after 7 days (Figures 5.34 and 5.35).

Figures 5.34 and 5.35: 3T3 L1 Fibroblast differentiation to adipocytes over 4, 7 and 11 days- Nile Red lipid staining.

Figure 5.34:

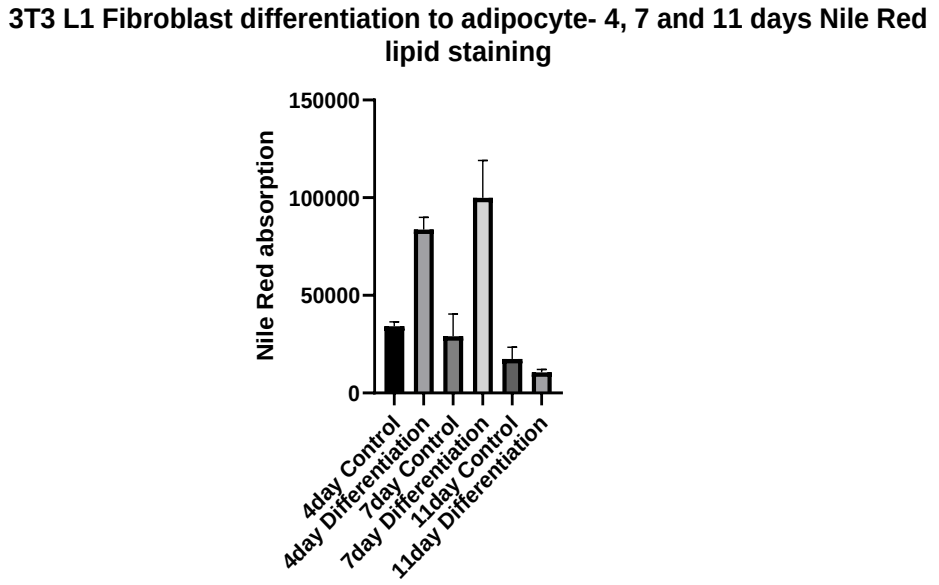
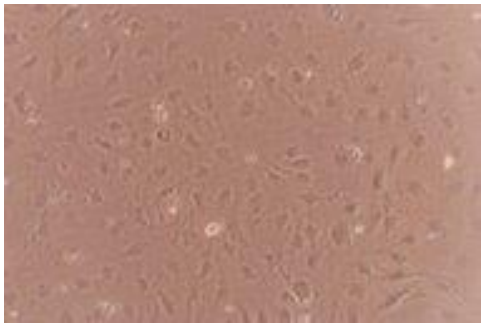
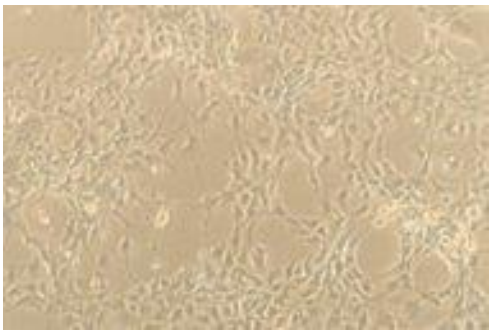


Figure 5.35:

3T3 L1 Fibroblast differentiation to adipocyte- 4, 7 and 11 days time course- Nile Red lipid staining x10mag images



0 days differentiation



4th day insulin, IBMX and dexamethzone

7th day following 3 days in insulin

11th day following 4 days DMEM only

Figures 5.34 and 5.35: 3T3 L1 Fibroblast differentiation to adipocyte over 4, 7 and 11 days- Nile Red lipid staining. Cells were seeded in 96-well plates, in triplicate, ~~at 25,000/well~~ in growth medium to reach confluence in 2 days, switched to differentiation medium 1 containing insulin, IBMX and dexamethasone for 4 days, then medium 2 containing insulin for 3 days and then maintenance medium for 4 days. For lipid detection, cells were fixed ~~in 4% paraformaldehyde~~ and stained with Nile Red, and the fluorescence read ~~at Ex.530nm, Em590nm~~. Figure 5.34: Using a 4+3+4 day strategy, an optimum in lipid staining was reached after 7 days rather than 11 days. Figure 5.35: Visually by phase contrast light microscopy x10, greater than 80% of cells could be seen to contain fat droplets after 7 days. This was much reduced after 11 days.

Comparing with the standard 2 day strategy, similar levels of lipid staining were achieved, and again optimal staining was achieved before 11 days (Figure 5.36).

Figure 5.36: 3T3 L1 Fibroblast differentiation to adipocytes over 2, 4, 6, 8, 11 days -Nile Red lipid staining

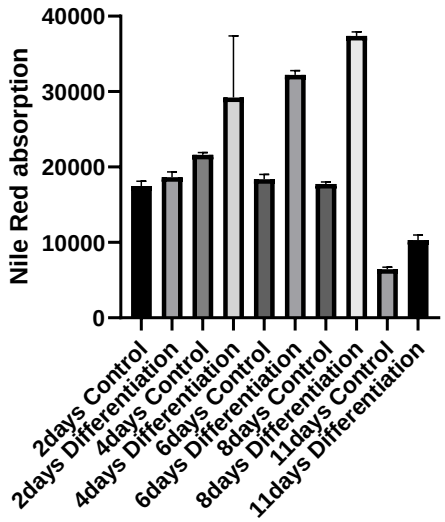


Figure 5.36: 3T3 L1 Fibroblast differentiation to adipocytes over 2, 4, 6, 8, 11 days- Nile Red lipid staining. Cells were seeded, in triplicate in, in 96-well plates ~~at 25,000/well~~ in growth medium to reach confluence in 2 days, switched to differentiation medium 1 containing insulin, IBMX and dexamethasone for 2 days, then medium 2 containing insulin for 2 days and then maintenance medium for up to 7 days. For lipid detection cells were fixed ~~in 4% paraformaldehyde~~ and stained with Nile Red and the fluorescence read ~~at Ex.530nm, Em590nm.~~ Using a 2-day strategy, an optimum in lipid staining was reached after before 11 days.

A study has shown that different culture volumes on 3T3 L1 cells affect their differentiation (Sheng *et al*., 2015). Therefore, 100 µl and 300 µl cultures were compared on the seven-day differentiation strategy (a) medium.2 (insulin, IBMX and dexamethasone) (M2) for two days; medium.3 (insulin) (M3) for two days; medium.4 (maintenance medium i.e FBS only) (M4) for three days; and (b) M2 for four days and M3 for three days. Little or no difference was found between the standard two days for each differentiation medium and the four-and three-day method using 100 µl culture medium volume (Figure 5.37). However, using 300 µl with the method of M2 for four days and M3 for three days gave the highest Nile Red lipid staining compared to controls (maintained on 3T3 L1 fibroblast medium).

Figure 5.37: 3T3 L1 Fibroblast Differentiation- effect of day strategy and culture volume Nile red lipid staining

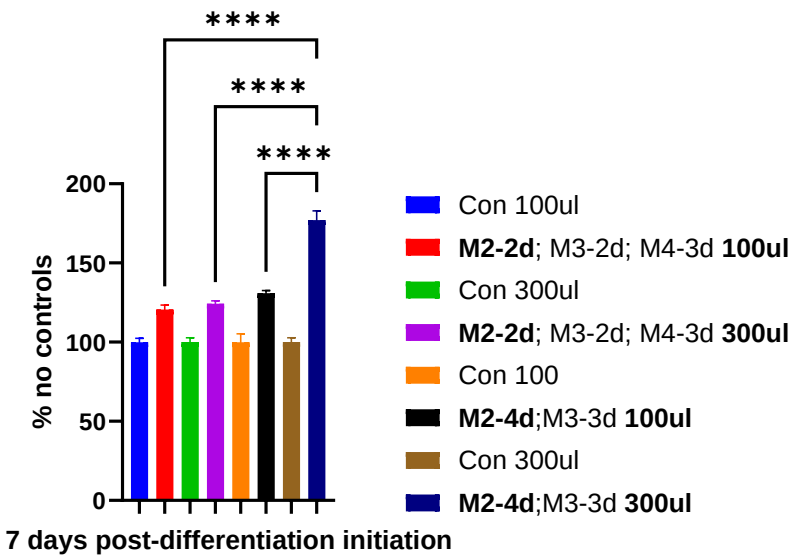


Figure 5.37: 3T3 L1 Fibroblast Differentiation- effect of day strategy and culture volume- Nile red lipid staining. Cells were seeded in 96-well plates ~~at 25,000/well~~ in growth medium to reach confluence in 2 days, followed by 100 µl or 300 µl on a seven day differentiation strategy of either: medium.2 (insulin, IBMX and dexamethasone) (M2) for two days; medium.3 (insulin) (M3) for two days; medium.4 (maintenance medium) (M4) for three days; or M2 for four days and M3 for three days. For lipid detection, cells were fixed ~~in 4% paraformaldehyde~~ and stained with Nile Red, and the fluorescence read ~~at Ex.530nm, Em590nm~~. Using 300 µl with the new method of M2 for four days and M.3 for three days gave the highest Nile Red lipid staining compared to controls (maintained on 3T3 L1 fibroblast medium). Each treatment was compared with each other treatment (not controls) by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, ****P<0.0001, n=3 replicate wells per treatment.

Having optimised culture conditions by Nile Red lipid staining, the effect of culture well size on glucose uptake was investigated using the fluorescent dye 2-NBDG. A dose-dependent response was seen for insulin tested at 10 and 100 nM for each well size (Figures 5.38, 5.39 and 5.40). 10 nM Human recombinant FGF21 gave a glucose uptake response similar in magnitude to the 10 nM insulin in each well size. Glucose uptake increased with increasing well size, however only in 48-wells was 100 nM insulin significantly distinguished from 10 nM (Figure 5.39).

Figures 5.38 -5.40: Effect of Insulin and FGF21 on Glucose uptake ~~in a~~ by 3T3 L1 Adipocytes in a 96, 48 and 24-well plate.

Figure 5.38:

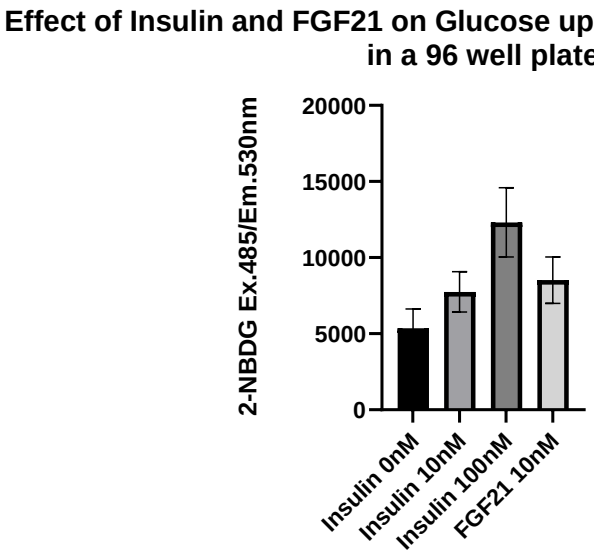


Figure 5.39:

Effect of Insulin and FGF21 on Glucose uptake by 3T3 L1 Adipocytes in a 46 well plate

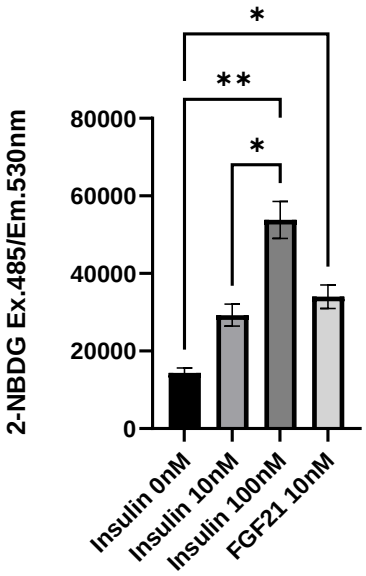
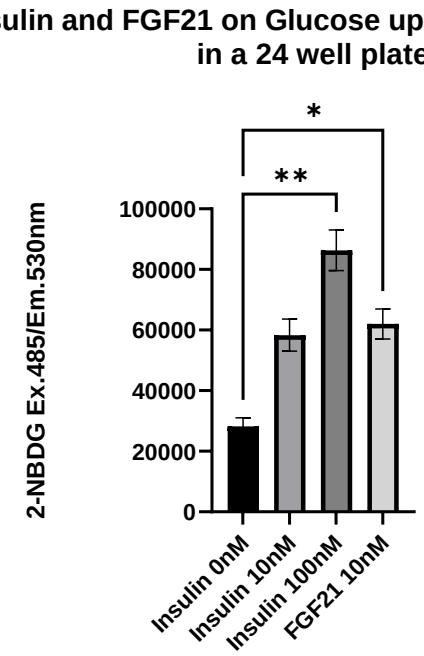


Figure 5.40:



Figures 5.38 -5.40: Effect of Insulin and FGF21 on Glucose uptake ~~in a~~ by 3T3 L1 Adipocytes in a 96, 48 and 24-well plate. 3T3 L1 pre-differentiated fibroblasts were seeded in either 96, 48 or 24-well plates ~~at a density of 78,000/cm^2~~ for 2 days, then differentiated for 4 days with insulin, IBMX and dexamethasone, and 3 days with insulin. Cells were serum-starved ~~in DMEM 0.1% BSA~~ for 3 hrs and then treated with human recombinant FGF21 for 24 hrs, or insulin for 30 minutes before adding ~~600µM~~ 2-NBDG for 1 hr. Cells were then washed and scraped off in 1% Triton (X-100?) in 30% DMSO and the fluorescence read ~~in a 96 well clear bottom, black walled plate at Ex.485/Em.530nm~~. All treatments were compared to the control (0 nM Insulin) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis, testing, *P<0.05, **P<0.01, n=3 replicate wells per treatment.. A dose-dependent response was seen for insulin tested at 10 and 100 nM for each well size. 10 nM Human recombinant FGF21 gave a glucose uptake response similar in magnitude to 10 nM insulin in each well size. Glucose uptake increased with increasing well size, however only in 48-wells was 100 nM insulin significantly distinguished from 10 nM (Figure 5.39).

Blocking antibody to FGF21 receptor FGFR1 (Novus Biologicals, MM0277-6F10) was found to block induction of glucose uptake by human recombinant FGF21 in a dose-dependent manner, although not significantly (Figure 4.41). A blocking antibody concentration of 4 µg/ml can be seen to be sufficient.

Figure 5.41: FGF21 stimulated glucose uptake blocked by FGFR1 Neutralising antibody.

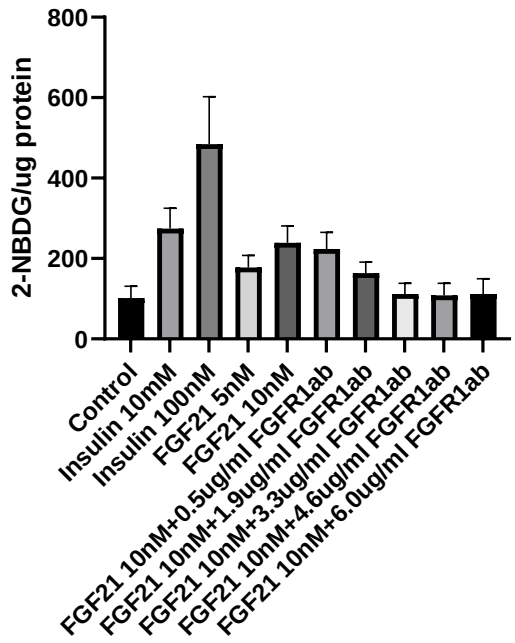


Figure 5.41: FGF21 stimulated glucose uptake blocked by FGFR1 Neutralising antibody. 3T3 L1 pre-differentiated fibroblasts were seeded in 48-well plates ~~at a density of 78,000/cm^2~~ for 2 days, then differentiated for 4 days with insulin, IBMX and dexamathazone, and 3 days with insulin. Cells were serum-starved ~~in DMEM 0.1% BSA~~ for 3 hrs and then treated with blocking antibody for 2 hrs, then with human recombinant FGF21 for 24 hrs (or insulin for 30 minutes) before adding ~~600µM~~ 2-NBDG for 1 hr. Cells were washed and scraped off in 1% Triton (X-100?) in 30% DMSO and the fluorescence read ~~in at Ex.485/Em.530nm in a 96 well clear-bottom, black-walled plate~~. Extracts were analysed for protein ~~by BCA assay.~~ All treatments were compared to the control (0 nM Insulin) by one-way ANOVA correcting for multi-comparisons using Dunnett's statistical hypothesis testing, *P<0.05, **P<0.01, ****P<0.0001, n=3 replicate wells per treatment. The FGFR1 neutralising antibody was found to block induction of glucose uptake by human recombinant FGF21 in a dose-dependent manner, although not significantly. A blocking antibody concentration of 4 µg/ml can be seen to be sufficient.

Conditioned cell culture media from primary hepatocytes treated with a dose range of isocaloric fructose and glucose concentrations (see Figures 5.8-19), cultured over a 24 hr period on the second day of three (48 hrs time point) were applied to 3T3 L1 adipocytes that had been prepared in the same way as those above (Figure 5.41) and in the same way as FGF21 above (Figure 4.41). FGFR1 blocking antibody was

applied in parallel to cells prepared and treated with conditioned medium as above (Figure 5.41).

Glucose 11 mM+Fruc 4 mM showed a significant increase in glucose uptake *vs*. Control (5.5 mM glucose) (Figure 5.42). This was the most effective dose for FGF21 intact (Figure 5.20) and intact/total ratio (Figure 5.26 above and below for ease of comparison). Otherwise for fructose treatments, there was tendency for a dose-dependent effect on glucose uptake, except for the highest dose, which was also the case for the FGF21 intact and intact/total. The increase for 11 mM glucose+Fruc 4 mM was significantly blocked by pre-incubation with the FGFR1 antibody, suggesting that the increase seen was due to FGF21. It is also interesting to note that any tendency for a dose-dependent increase in glucose uptake for fructose showed an inclination to be blocked by the FGFR1 antibody, whilst any tendency for glucose was not blocked.

Glucose uptake measured in 3T3 L1 adipocytes shown in Figure 5.42 was normalised to the protein content of these cells, rather than that measured in the primary hepatocytes that the conditioned used to treat them. Protein concentrations in the 3T3 adipocytes were found to be consistent for all samples (Figure 5.43).

Figure 5.42: Figure 5.42: Effect of primary hepatocyte medium conditioned over the second day (48 hrs) of treatment, with isocaloric fructose and glucose, on Glucose uptake in by 3T3 L1 Adipocytes

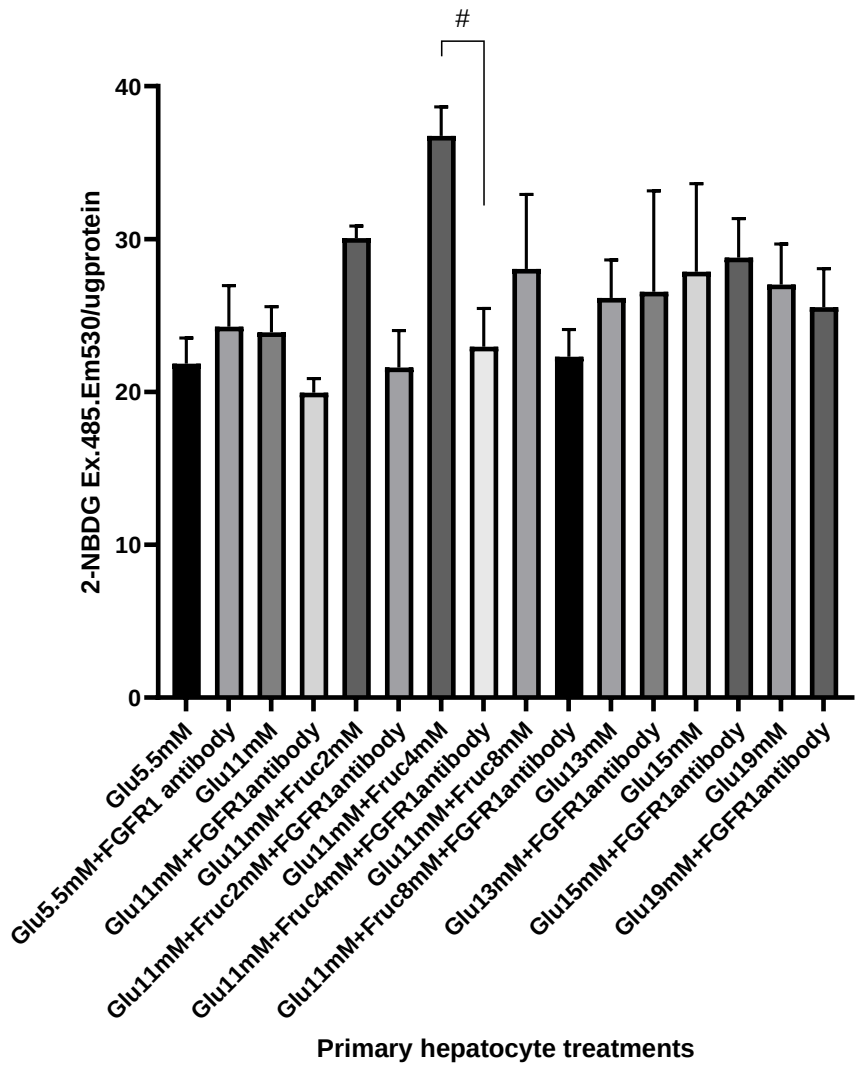


Figure 5.42: Effect of primary hepatocyte medium conditioned over the second day (48 hrs) of treatment, with isocaloric fructose and glucose, on Glucose uptake ~~in~~ by 3T3 L1 Adipocytes. 3T3 L1. Pre-differentiated fibroblasts were seeded~~, in triplicates,~~ in 48-well plates ~~at a density of 78,000/cm^2~~ for 2 days then differentiated for 4 days with insulin, IBMX and dexamethasone, and 3 days with insulin. Cells were serum-starved ~~in DMEM 0.1% BSA~~ for 3 hrs, treated with or without FGFR1 blocking antibody for 2 hrs, then treated for 24 hrs with conditioned medium from human primary hepatocytes that had been treated with a dose range of isocaloric glucose and fructose before ~~600µM~~ 2-NBDG was added for 1 hr. Cells were washed and scraped off in 1% Triton X-100 in 30% DMSO and glucose uptake measured by fluorescence ~~at Ex.485/Em.530nm in a 96 well clear-bottom, black-walled plate.~~ Extracts were analysed for protein by BCA assay. All isocaloric treatments were compared to the 5.5 mM glucose control, as well as isocaloric pairs by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, *P<0.05, n=3 replicate wells per treatment.

Figure 5.43: Effect of primary hepatocyte medium conditioned over the second day (48 hrs) of treatment with isocaloric fructose and glucose, on protein in 3T3 L1 Adipocytes.

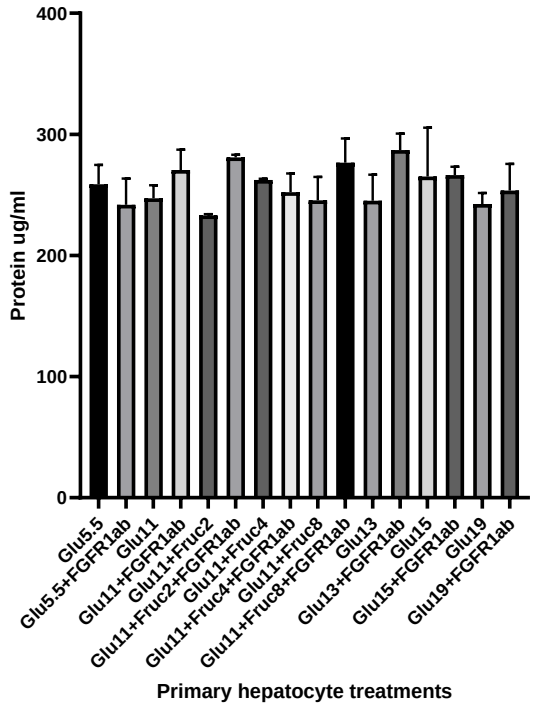


Figure 5.43: Effect of primary hepatocyte medium conditioned over the second day (48 hrs) of treatment with isocaloric fructose and glucose, on protein in 3T3 L1 Adipocytes. 3T3 L1 pre-differentiated fibroblasts were seeded in 48-well plates ~~at a density of 78,000/cm^2~~ for 2 days then differentiated for 4 days with insulin, IBMX and dexamethasone, and 3 days with insulin. Cells were serum-starved ~~in DMEM 0.1% BSA~~ for 3 hrs, treated with or without FGFR1 blocking antibody for 2 hrs, then treated for 24 hrs with the conditioned medium from human primary hepatocytes that had been treated with a dose range of isocaloric glucose and fructose. After extraction in 1% triton cells were analysed for protein. ~~Upon thawing the sample on ice, trypsin inhibitor was added immediately then rapidly homogenised on ice by probe sonication and boiled to inactivate enzymes. Protein was measured by Thermo Scientific™ Pierce™ BCA Protein Assay~~. All isocaloric treatments were compared to the 5.5 mM glucose control, as well as isocaloric pairs by one-way ANOVA correcting for multi-comparisons using Šídák's statistical hypothesis testing, n=3 replicate wells per treatment.

5.6 Discussion

Summary key findings:

Using a primary human hepatocytes in this study difficulty was found inducing lipid accumulation and uric acid at physiological levels of fructose and glucose. However, glycogen (Figures 5.10-13), lactic acid (Figures 4.14-16) and active FGF21 (Figures 5.2-43) responded well and were all greatly increased by fructose rather than glucose.

5.6.1 Lipid Accumulation

Clearly, primary hepatocytes can be made to accumulate lipids in the presence of fats in the form of palmitate, linoleic acid and oleic acid (Figures 5.3-7). Here this served as a useful positive control showing that under such *in vitro* conditions, the human primary hepatocyte model was able to respond to changes in macronutrients. However, either in the presence of fats or not, fructose and glucose in these studies did not have a significant effect on lipid accumulation (Figures 5.3-7).

That an effect with glucose was not seen is not surprising. Glucose metabolism in the liver is well-known to be feedback-inhibited by ATP and citrate action on phosphofructokinase. Once the cell has adequate levels of glucose for energy, metabolism to pyruvate, and hence potential substrates for fatty acid synthesis, is supressed. In such cases, glucose is stored as glycogen, although under some circumstances, glucose can be converted to fructose via the polyol pathway (Park *et al*., 2017). At what concentrations and metabolic situations this may happen is not clear. Other studies by other workers in this area have also found difficulty in seeing an effect of glucose on lipid accumulation with monolayer primary hepatocytes. Lu *et al*., 2012 saw no effect on Nile Red staining up to a glucose dose of 44.8 mM. In general glucose at high levels has been found to increase triglyceride secretion in *in vitro* models rather than stimulated lipid accumulation (Green *et al*., 2015).

Fructose at a physiological level of ≤ 8 mM has been reported to increase lipid accumulation in primary hepatocytes, however, the effects were highly variable, occurring for only 1-in-5 subjects (Huggett, 2020). *In vivo*, the best animal models for studying steatosis use fat and fructose (Gunawan *et al*., 2021). However, *in vitro*,

the effects of fructose and glucose at more physiological levels have mostly been in isolation, in the absence of fatty acids (Pinnick *et al*., 2019). A study in HepG2 cells using oleic acid at 0.5 mM saw no effect in PLIN2 mRNA or protein when glucose was increased from 5.5 mM to 30.6 mM (much higher than levels of glucose and fructose used here) (Richte *et al*., 2019). PLIN2 (perilipin 2) functions in the formation of lipid droplets by increasing fatty acid uptake in fatty liver (Conte *et al*., 2016). However, the study did see a substantial increase in DAGT2. DGAT2 (acyl-CoA: diacylglycerol acyltransferase 2) catalyses the terminal step in triglyceride biosynthesis and also plays a key role in hepatic steatosis (Choi *et al*., 2007; Yu *et al*., 2005). Such a marker may be indicative of potential steatosis, which only materialises over much longer treatment. Green *et al*., 2016 comment that "…studying hepatic processes and the etiology and progression of disease …. in humans is challenging, not least as NAFLD may take years to develop". As such, in animal models, effects on dyslipidaemia even at very high levels of fructose and fat can take longer than 4-8 weeks (Gunawan *et al*., 2021).

In the experiments presented here, we have used fatty acids concentrations ranging from 0.2-1.0 mM, which is a similar range found in human plasma (0.2-0.7 mM) (Duncombe *et al*., 1963; Husek *et al*., 2002). In just 48 or 72 hours, there was a several-fold increase in accumulated lipid (Figures 5.5). Therefore, primary cells in culture seem much more sensitive to their fatty acid environment than liver cells *in vivo*. It may be difficult to induce much greater lipid accumulation than already seen at these levels. Any change induced by fructose or glucose is likely to have been small in comparison and therefore not detected. Further experiments might be more sensitive to addition of fructose and glucose at much lower levels of added fatty acids. Such further experiments in these studies have not been possible due to the cessation of the supply of liver material from the Nottingham Hospital.

Lipid release *vs*. Lipid accumulation

In chapter 3.0 (Potential benefits of reduced fructose concentration spikes: on TAG release and hepatotoxicity), at similar physiological levels of fructose and glucose used in this chapter, effects on triglycerides released into the culture medium were seen in only a few hours. Clearly it is more difficult to affect lipid accumulation than it

is to affect triglyceride release. The secretion of lipid as triglycerides as very low-density lipoproteins (VLDLs) requires one apoB100 molecule (Ipsen *et al*., 2018). Fatty acids at moderate levels can increase secretion of apoB100 increasing the capacity for secretion. However, prolonged exposure can lead to endothelial reticulum (ER) stress and apoB100 degradation, leading to reduced secretion (Koo *et al*., 2013; Falcon *et al*., 2010). As VLDL secretion requires apopB100, it might be that only prolonged high levels of fatty acid inhibit VLDL secretion, causing intracellular accumulation. Insulin also stimulates storage of lipid by the liver partly by inducing aopoB100 degradation (Kawano *et al*., 2013), although reduced apoB100 does not necessarily lead to reduced VLDL release, as additional triglyceride can be secreted by larger VLDL particles (Fabbrini *et al*., 2008). However, if VLDL particle size becomes too large, such that they exceed the pore size of the sinusoidal endothelium, secretion cannot occur and the lipid can only accumulate in the hepatocyte (Horton *et al*., 1999). Therefore, lipid secretion under some circumstances would seem to be biphasic, increasing under low levels of rising fatty acid and then reaching a plateau and possibly even decreasing, leading to accumulation (Ipsen *et al*., 2018). The key step to this biphasic pattern appears to be ER stress or insulin leading to apoB100 degradation. Although apoB mRNA levels in subjects with a fatty liver (NAFLD) have been found to be higher than in non-NAFLD controls (Higuch *et al*., 2011; Nakamuta *et al*., 2009), apoB synthesis rates in subjects with inflamed fatty liver (non-alcoholic steatohepatitis (NASH) have been found to be lower than controls (Charlton *et al*., 2002).

Fructose, and therefore glucose via the polyol pathway, under some circumstances, can lead to ER stress either through increasing lipid *de novo* lipogenesis (DNL), increasing intracellular triglyceride (Dewdney *et al*., 2020) (see Appendix I: Triglycerides and health concerns), or uric acid through depletion of ATP (Sun & Empie, 2012). Radioisotope studies have reported increased DNL in patients with non-alcoholic fatty liver disease (NAFLD) (Diraison *et al*., 2003), and in the overweight/obese with fatty liver (Lambert *et al*., 2013) compared to controls. In another study obese subjects with NAFLD were shown to derive a quarter of the triglycerides in their liver from DNL. These subjects even showed elevated DNL during the fasted as well as the fed state (Donnelly *et al*., 2005). The evidence is therefore growing that DNL may be a key feature of NAFLD lipid accumulation.

Lipid accumulation conclusions

Although no significant effect by fructose or glucose on lipid accumulation has been seen in these experiments, it might be possible that either: (a) studies with lower levels of fatty acid would be more sensitive to fructose and glucose treatment; and/or (b) longer-term experiments allowing for the possible need for DNL to cause ER stress may be required. The latter would be limited with primary hepatocytes, as after more than around a week they begin to de-differentiate (Green *et al*., 2016).

5.6.2 Glycogen

Whilst fructose treatments had a dramatic effect on glycogen, glucose treatments without fructose had little or no effect (Figure 5.10). That glucose had no significant effect suggests that glycogen levels were optimal after 72 hrs of culture post-liver extraction. This is not surprising. In an experiment on fasted subjects, the rate of glycogen repletion in the liver after a mixed macronutrient meal (carbohydrate, protein and fat, containing 1.82 g/kg body weight of glucose) was found by carbon-13 nuclear magnetic resonance spectroscopy to be approximately 6 g/hr. This reached a peak after 5 hrs (Taylor *et al*., 1996). Consuming only polymeric glucose, glycogen repletion rates have been reported by several groups to be closer to more like 4 g/hr (review: Gonzalez *et al*., 2016). Repletion rate will also depend on the initial level of glycogen (Fleig *et al*., 1987). Liver glycogen has been found to be reduced to 44% of peak concentration by cycling to exhaustion at 70% maximal oxygen consumption (VO2max). If it is assumed that cells are completely depleted of glycogen prior to plating, then it might be expected that repleting by glucose alone would take approximately 15 hrs. Therefore, unless glycogen synthesis is very much slower in primary hepatocytes in culture, it would be expected that complete repletion would be easily reached well before 72 hrs exposure in high levels of glucose, such as 11-19 mM. Rat primary hepatocytes have been reported to show peak glycogen storage in culture after just 4 hrs (Salhanick *et al*., 1989).

Fructose is well-known to show greater rates of glycogen repletion than glucose *in vivo*, and particularly when fed together (Casey *et al*., 2000; Decombaz *et al*., 2011; Fuchs *et al*., 2016). Fuchs *et al*., 2016 found the rate doubled from 4 g/hr to 8 g/hr. However, the work presented here is the first time that such has been demonstrated in human primary hepatocytes. Although the cellular effects of fructose in

hepatocytes have been investigated using human cell lines, there have been few studies using primary hepatocytes (Pinnick *et al*., 2019). It is also of particular interest to notice that fructose with glucose would seem to lead to not only double the glycogen concentration but may also double the peak glycogen concentration. This too, is the first time that such has been reported in human primary hepatocytes. A typical full concentration of glycogen in human liver is on average around 8% of total liver weight (Cole and Kramer, 2016). It was found here that for a 5.5 mM glucose treatment, cell protein was approximately 0.6 mg/mg protein. If a cell contains approximately 18% protein (Cooper *et al*., 2021), then we can estimate that this is approximately 10% of the cell mass. Hepatocytes make up around 80% of the cells in the liver, which suggests that these experiments represent approximately 8% glycogen in a whole liver. In the fructose- and glucose-treated cells, there was 1.4-1.8 mg/mg protein, equating to 19-24% glycogen, and from Figure 5.12, it can be seen that glycogen synthesis still may not have plateaued at the highest fructose treatment level.

An increase glycogen storage capacity has also been shown *in vivo*. Rats fed on a high fructose diet for 4 weeks resulted in an approximately 20% increase in liver size (Maslak *et al*., 2015). Liver histopathology showed excessive glycogen storage by H&E staining in hepatocytes, using the Periodic Acid-Schiff (PAS) method for glycogen visualization, relative to controls without evidence of steatosis or inflammation. Glycogen hepatopathy (GH) has also been described in type 1 diabetic humans and is considered to be a glycogen storage disease (Azhar *et al*., 2021). The cause can be genetic or an acquired overactivation of glycogen synthesis enzymes and is frequently misdiagnosed as non-alcoholic fatty acid disease (NAFLD). More recently excess hepatic glycogen storage (glycogenosis), defined as excess hepatocyte glycogen visible by routine H&E light microscopy), has been noted as common in adults and children with NAFLD (Allende *et al*., 2021), and associated with lower levels of steatosis and fibrosis, but increased liver cell injury.

However, in another study that investigated glycogen in mice given high levels of fructose (Softic *et al*., 2017), they found that glycogen levels were not affected. The fructose though was delivered as a 30% water solution without glucose, and although the food contained carbohydrates, it may not have contained

monosaccharide glucose. It may be that for fructose to induce excessive glycogen storage, free glucose is needed that is absorbed at the same as the fructose.

High liver glycogen levels, it is suggested, inhibits glycogen synthesis in muscle (Fleig *et al*., 1987) and stimulates glycogenolysis (Roden *et al*., 2001). How then is glycogen able to continue to accumulate beyond the typical full concentrations of glycogen in human liver of around 8% of total liver weight (Cole and Kramer, 2016)? A number of possible regulatory effects of fructose on glucose metabolism and glycogen synthesis have been proposed: (1) Fructose-1-phosphate (F1P) stimulates the rate of glucose phosphorylation to glucose-6-phosphate by releasing the inhibition of glucokinase regulatory protein (GRP) on glucokinase. This occurs as F1P antagonises a complex between fructose-6-phosphate (F6P), GRP and glucokinase (review: Schaftingen *et al*., 2017); (2) Glycogen synthase catalyses the rate-limiting step in glycogen synthesis. It is activated by dephosphorylation when phosphorylase drops below a threshold concentration. F1P increases the conversion of inactive glycogen synthase to active glycogen synthase possibly as a competitive inhibitor of phosphorylase (Gergely *et al*., 1985); (3) Glucose-6-phophate is ~~perhaps the~~ suggested to be the key regulator of glycogen synthesis (Jensen *et al*., 2012) as an allosteric activator of phosphorylated glycogen synthase (Stalmans *et al*., 1987), and also promotes dephosphorylation by glycogen-associated protein phosphatase-1 (Cadefau *et al*., 1997; Bollen *et al*., 2003). Both glucose and fructose increase G6P concentration. The G6P:ATP ratio seems to correlate with the activation state of hepatic glycogen synthase. As fructose is rapidly metabolised to F1P by fructokinase, ATP can become depleted, potentially increasing the activity of glycogen synthase (Ciudad *et al*., 1988); (4) The breakdown of glycogen is partly regulated by glycogen phosphorylase which removes glucose residues from glycogen polymer by breaking the terminal α-1,4-gycosidic link. F1P may also enhance net glycogen levels by allosterically inhibiting glycogen phosphorylase (Hannou *et al*., 2018).

These proposed mechanisms are not completely clear as glycogen synthase regulation is highly complex, with numerous kinases targeting at least nine phosphorylation sites (Nielsen & Woitaszewski, 2004; Jenson & Lai, 2009; Jensen *et al*., 2012). With respect to proposal (1) above, although a radiotracer study has shown that carbon from fructose alone is incorporated into glycogen more so than

glucose alone, when supplied together glucose utilisation increases 3 fold, whilst that of fructose increases by only 50% (Parniak and Kalant, 1988)~~.~~. With respect to mechanism (4) above for fructose ~~boosting~~ stimulating glycogen synthesis, although it might seem surprising, it has been estimated that during glycogen synthesis, glycogenolysis can occur at greater than 57% the rate of net synthesis (Magnusson *et al*., 1994). Such a glycogen-cycle, it is suggested may allow rapid changes in glucose flux (Gonzalez *et al*., 2016).

Both glucose and fructose increased intracellular glucose content dose-dependently in this study (Figure 5.13). For glucose treatment, this is easily explained as the greater the concentration, the more will be transported into the cell down a concentration gradient by the passive glucose transporter GLUT2. For fructose, this is confirmation of gluconeogenesis despite the presence of a high concentration of added insulin in the culture medium (see 1.0. Introduction, 1.2. Potential Mechanisms to explain the negative impact of fructose on health, Glucose metabolism dysregulation- insulin insensitivity). *In vivo*, although fructose does not stimulate the secretion of insulin, it will normally be fed in the presence of similar levels of glucose which will. Elevated glucose production is maintained, it is suggested, ~~perhaps~~ by non-hormonal (e.g. neural) control (Moore *et al*., 1998), causing insulin to remain elevated, indicating at least a short-term insulin insensitivity (Dirlewanger *et al*., 2000) which might be anticipated to become chronic with repeated fructose exposure. It may be asked, though, how is glucose production from fructose maintained *in vitro*, in an enclosed system without input from other organs? Do hepatocytes secrete a paracrine-acting factor or factors? Alternatively, does F1P largely increase glucose via (1) above i.e. stimulating the rate of glucose phosphorylation to glucose-6-phosphate by releasing the inhibition of glucokinase regulatory protein (GRP) on glucokinase? However, as mentioned, fructose administered with glucose ~~boosts~~ elevates glucose carbon utilisation-3 fold and fructose carbon inclusion was increased by about 50% (Parniak and Klant, 1988). It would seem therefore, that gluconeogenesis is significantly maintained in the presence of high insulin, leaving open the question as to how.

Glycogen conclusions

It has been shown in this study that fructose with glucose can ~~boost~~ stimulate glycogen storage 2.5-fold above levels which are typically seen to be fully replete. Others have shown this too *in vivo* and in rat primary hepatocytes, but this has not been shown previously in primary human hepatocytes. Although the cellular effects of fructose on hepatocytes have been investigated using human cell lines, there have been few studies using primary hepatocytes (Pinnick *et al*., 2019). As such, this study has shown that human primary hepatocytes are potentially a useful model for research programmes and could limit the use of animals and expense of human subjects. At the highest levels of fructose used here, the rise in glycogen storage had not plateaued, suggesting even higher levels of glycogen storage may be possible. The potential health implications for excessive glycogen storage are largely unknown. In clinical studies, excess glycogen i.e. glycogenosis, is frequently misdiagnosed as non-alcoholic fatty acid disease (NAFLD). This could mean that some studies that have apparently shown an increase in NAFLD in response to high fructose, may actually have shown glycogenosis. On the one hand, this might mean there is less to be concerned about with respect to fructose and metabolic disease. On the other hand, as little is known about the health implications of dietary-induced excessive glycogen, further research in this area is needed (see further discussion in Chapter 6).

5.6.3 Lactate

In human primary hepatocytes, this study found that fructose treatments increased lactate released into the culture medium in a dose-dependent manner over a 24 hrs period, after 24, 48 and 72 hrs of treatment (Figures 5.14-16). There was no effect on lactate for glucose, and for the two highest dose isocaloric pairs, fructose treatment was significantly and substantially greater at all three time periods. There was also an increase in lactate output for the 48 hr and 72 hr time period *vs*. the 24 hr time period (Figures 5.15 and 5.16 *vs* 5.14). This would suggest that during the first 24 hr period, energy or intermediate metabolite requirement was not fully met and glycolysis was required. After this point, the lack of effect of glucose treatments on lactate production is consistent with the feedback inhibition hypothesis, where citrate and ATP feedback and inhibit phosphofructokinase (see 1.2. Potential

Mechanisms to explain the negative impact of fructose on health, 1.2.1 Lack of regulation- *de novo* lipogenesis) (Elliot *et al*., 2002). That fructose showed such a strong dose-dependent effect on lactate is consistent with the hypothesis that fructose metabolism in the liver is not feedback-inhibited. Such an effect on lactic acid has not been demonstrated before in human primary hepatocytes. Although the cellular effects of fructose on hepatocytes have been investigated using human cell lines, there have been few studies using primary hepatocytes (Pinnick *et al*., 2019). As such, this study has shown that human primary hepatocytes are potentially a useful model for such research programmes and could limit the use of animals and expense of human subjects.

In vivo, lactate may also be used for glycogen synthesis by gluconeogenesis. This has been termed the indirect pathway of glycogen synthesis, as opposed to the direct pathway of glucose conversion to glucose-6-phophate then directly added to glycogen polymers (Bollen *et al*., 1998). Such direct and indirect glycogen synthesis pathways have been associated with liver zonation (reviewed- Jungermann *et al*., 1996). Zonation results from local oxygen and nutrient concentration differences as blood reaches the liver, leading to differential gene expression and metabolism. Cells that receive more oxygen and nutrients, due to their location are termed periportal cells, exhibit more aerobic metabolism. Glycogen synthesis mainly occurs in cells termed perivenous. Normally *in vivo*, when glycogen is fully restored, lactate is released systemically to be converted to glycogen by the periportal cells. *In vitro* we presumably have a population of both cell types, or ~~perhaps~~ it could be that the high oxygen used in cell culture affects gene expression such that periportal cells are favoured? Jungermann *et al*., 1996 were able to favour a periportal phenotype in isolated hepatocytes with glucagon, and perivenous with insulin. In the experiments presented here with 10 nM insulin, it could be that a perivenous phenotype was favoured and hence a substantial build-up of lactate.

It is curious that at 48 hrs both glycogen synthesis and lactate production were increased for glucose relative to 24 hrs. It would seem that glucose was degraded to lactate even though glycogen levels were not fully restored. However, at some point over a 24 hrs time period, glycogen storage may have become full such that degradation of glucose was initiated, or ~~perhaps~~ a mixed population of cells means

that perivenous cells fully are glycogen replete produce lactate, whilst periportal cells convert lactate to glycogen?

In addition to lactate being available for glycogen synthesis within the liver, high fructose diets can lead to large postprandial increases in blood lactate which is available to peripheral tissues e.g. muscle for energy (Tappy, 2021), particularly when supplied with glucose (Hengist *et al*., 2020; Watkins *et al*., 2020). Some studies, although not all, have reported that high fructose intake, rather than glucose, can increase visceral adiposity (Stanhope *et al*., 2009).

Lactate has for many decades been thought of as a waste by-product. However, it has now become viewed as an important source of energy and a possible signalling molecule involved in metabolic regulation (Sola-Penna *et al*., 2008). In a recent study, lactate was found to inhibit lipolysis in adipocytes (Liu *et al*., 2008). Niacin, which has long been used as a treatment for dyslipidaemia (Garg and Grundy, 1990), has been found to be a ligand of the once orphan G-protein coupled receptor GPR81 (Tunaru *et al*., 2003; Wise *et al*., 2003). As niacin tends to have the significant side-effect of causing flushing, Lui *et al*, 2008 searched for other ligands and found lactate at physiological concentrations to be an effective inhibitor of lipolysis in human, mice and rat adipocytes. Mice deficient in GPR81 do not respond to lactate, and other lipolytic agents do not lose activity in GPR81 deficient mice. The group also demonstrated that lactate specifically induces GPR81 internalisation after receptor activation, and that site-directed mutagenesis of GPR81, coupled with homology modelling that classically reserved residues in the transmembrane binding domains, are responsible for interacting with lactate. As such, the group have concluded that GPR81 is a sensor for lactate and is considered a target for drug development.

Lactic acid conclusions

Fructose, compared to glucose, greatly increases lactate production in primary hepatocytes. This is consistent with *in vivo* observations. However, rather than being a harmless waste product, lactate may exacerbate excess glycogen storage or lead to lipid synthesis in the liver. Lactate may also provide additional unnecessary energy to peripheral tissue potential leading to a build-up of visceral fat. As a

signalling molecule it may further promote increased body fat by inhibiting lipolysis, and as such its specific receptor is a target for treatment therapy.

This study is the first time that this has been seen in human primary hepatocytes. This is consistent with *in vivo* observations and as such, this study has shown that human primary hepatocytes are potentially a useful model for such research programmes and could limit the use of animals and expense of human subjects.

5.6.4 FGF21

After 48 hrs, fructose+glucose treatments were seen here to dramatically increase intact (active) FGF21 secreted into the cell culture medium by 5-6-fold (Figure 5.21). In contrast, glucose was only seen to significantly increase intact FGF21 at this time point, at its highest dose of 19 mM and only by a factor of two. A similar pattern was seen for total FGF21, however to a lesser extent (Figure 5.23). Total FGF21 includes truncated fragments that are either not active or even potently inhibitory (Agrawal *et al*., 2018). As the difference between fructose and glucose treatments was less for total FGF21 than for intact FGF21, this would suggest that fructose treatments gave rise to less inactive or inhibitory fragments than glucose. And as a guide to the overall activity of the FGF21 secreted, intact/total FGF21 (Parmar e*t al*., 2016) showed fructose as more effective with a two-fold increase and with glucose showing no significant effect (Figure 5.26). It might be concluded therefore, that consumption of a mixture of fructose and glucose rather than glucose on its own, if replicated *in vivo*, would lead to a greater FGF21 response and therefore a reduced preference for sweetness and reduced sugar intake. As we cannot be certain as to the inhibitory or merely non- or less-active effect of the portion of total FGF21 that is not intact, this may be different for fructose and glucose. For instance, most of the non-active fragments for fructose may be potently inhibitory, whilst for glucose just non-active. Fibroblast activation protein (FAP), an enzyme which digests FGF21 in the plasma (Dunshee *et al*., 2016), showed no particular trend at the 48 hrs time period (Figure 5.29). This might suggest that there is little difference in inhibitory or non-active profile between fructose and glucose here. However, in order to examine this further, we applied the conditioned medium from the hepatocytes to 3T3 L1 adipocytes and tested for effect on glucose uptake. This assay has a high degree of

variability, however the fructose treatment that gave the greatest increase in intact FG21 and intact/total FGF21 (11 mM glucose+4 mM fructose) was also the only treatment that showed a significant increase in glucose uptake (Figure 5.42). This effect was significantly reduced in the presence of a neutralising antibody to the FGF21 receptor FGFR1, suggesting the activity here was due to the active FGF21 in the medium. Although not significantly different from the 5.5 mM glucose control, the 11 mM glucose+2 mM fructose and 11 mM glucose+8 mM fructose treatments showed a tendency to increase glucose uptake and also an inclination to be blocked by the antibody to FGFR1. The glucose treatments showed a tendency to increase glucose uptake, but no inclination to be blocked.

After 72 hours the effect of fructose treatments on intact FGF21 and intact/total FGF21 had become less dramatic which might suggest that the effect seen at 48 hrs is transient and in the long-term fructose is not so effective at reducing sweetness preference and sugar intake(Figures 5.22 and 5.27).

In the shorter-term, after only 24 hrs, a rather different picture was seen. For intact FGF21, fructose and glucose treatments looked very similar, although only the 11 mM glucose+4 mM fructose gave a significant increase from the 5.5 mM glucose control (Figure 5.19). Total FGF21 was substantially greater for the fructose treatments than for glucose (Figure 5.23). As such, for intact/total FGF21, glucose treatments tended to be greater than for the fructose treatments (Figure 5.25). The 19 mM glucose treatment increased intact/total FGF21 significantly by approximately 50% and was significantly different from its isocaloric fructose pair, which had no effect. From this, it might be predicted that after 24 hrs, glucose would reduce sweetness preference and sugar intake more than fructose. Again, the degree of inhibitory or non-activity of the non-intact portion of each sample is not known.

The 24 hrs hepatocyte-conditioned medium was not tested in the 3T3 L1 adipocyte glucose uptake assay, as the difference between treatments and the control and between fructose and glucose at 24 hrs for intact FGF21 was much less than at 48 hrs. As such, an effect would difficult detect given that the glucose uptake assay ~~is perhaps~~ may only be semi-quantitative. In preliminary tests, a ten-fold increase in insulin from 10 to 100 nM gave only an approximately two-fold increase in glucose

uptake (Figures 5.39 and 5.41). An increase in recombinant human FGF21 from 5 to 10 nM gave only a non-significant 25% increase in glucose uptake (Figure 5.41). This is similar to that seen by other groups reporting in the scientific literature (Kharitonenkov *et al*., 2005). The somewhat modest effect seen in this study on glucose uptake for the samples taken after 48 hrs will therefore also have been affected by the low sensitivity of the glucose uptake assay.

FAP at 24 hrs however, was not significantly different for fructose and glucose treatments (Figure 5.28), but both showed a dose-dependent decrease in FAP, with 19 mM glucose falling significantly more than its 11 mM glucose+8 mM fructose isocaloric pair. Overall, there was a significant negative correlation between treatment and FAP (Figure 5.31) i.e. higher levels of intact/total FGF21 tend to lead to lower levels of FAP. This ~~is perhaps~~ may therefore be what might be expected to be seen in order to protect the activity of the FGF21 secreted into circulation. It has been proposed that FAP plays an important function in attenuating levels of active FGF21 (Dunshee *et al*., 2016; Zhen *et al*., 2016; Parmar *et al*., 2018). As the correlation is greatest for glucose treatments (Figures 5.31 and 32), it might be tempting to conclude that this helps confirm a higher level of FGF21 activity for the glucose-only treatments in the shorter-term. The effect of most fructose meals or drink consumption could be argued to be short-term, often repeated two or three times a day every day. On the other hand, if repeated daily might equate to chronic fructose and glucose exposure and would more parallel the findings made after 48 hrs *in vitro*. If that is the case, such continued chronic sugar consumption might begin to have a less of an impact on FGF21 in the longer-term, as was seen at 72 hrs in this study.

To further investigate, a more sensitive assay than glucose uptake in 3T3 L1 adipocytes would ~~perhaps~~ need to be found. One alternative might be to look at downstream markers of FGFR1 activation. Such surrogate targets used by other workers include Erk1/2 phosphorylation (Kharitonenkov *et al*., 2005; Geng *et al*., 2019; Guo *et al*., 2019; Lewis *et al*., 2020; Liu *et al*., 2020), cFOS (Jensen-Cody *et al*., 2020; von Holstein-Rathlou *et al*., 2015; Song *et al*., 2015; Geng *et al*., 2019), GLUT1 and GLUT4 (Kharitonenkov *et al*., 2005). Where cFos was evaluated, this was generally using cell staining rather than Western blot. However, dose

responses have not been reported for these, and so it may be that they are just as semi-quantitative as using glucose uptake. Jenson-Cody *et al*. 2020 noted that the few cells that showed cFos staining also showed KlothoB staining, an essential co-receptor membrane protein in successful FGF21 receptor binding and activation. Only neurons of the ventromedial hypothalamus also showed KLB (KlothoB) staining. In this way, they were able to get around the problem of cFos being a surrogate marker, (which could be activated by numerous pathways as it plays an important role in many cellular functions). A similar problem ~~perhaps~~ potentially exists with using phosphorylated Erk1/2 as a marker. Analysis to help in determining overall FGF21 activity in samples such as in this study would not be an exercise in investigating localisation of FGF21 binding, and so this would not be a solution that could be used in this case. With respect to sweet taste preference, one target of FGF21 has been identified as glutamatergic cells in the periventricular nucleus (PVN) of the ventromedial hypothalamus (VH) (Jensen-Cody *et al*., 2020). Ideally further studies would be best using these cell types, however, although they are commercially available, they are quite expensive.

Circulatory levels of FGF21 are mostly produced by the liver in response to an imbalance of major nutrients such as sugars. In most reports, circulating FGF21 levels rise after consumption of simple sugars, but not complex carbohydrate (von Holstein-Rathlou *et al*., 2016). FGF21, via the hypothalamus, signals to the brain to reduce sugar intake by suppressing sweet-taste preference (Jenson- Cody *et al*., 2020). As such, FGF21 has been hypothesised to function to protect the liver from damage (Jensen-Cody *et al*., 2020).

In a study in ten healthy subjects comparing 75g fructose and glucose, fructose was seen to raise FGF21 levels 3-fold in 2 hrs, returning to baseline in 5 hrs (Dushay *et al*., 2015). Glucose caused only a 2-fold increase after 3 hrs returning to baseline in 5 hrs. In a similar acute study, glucose has been shown to have a biphasic effect on FGF21 in the liver (Lin *et al*., 2012b). After 1 hour, a 70 g glucose dose showed FGF21 levels to be decreased, rising to higher than fasting levels after 3 hours (i.e. a drop then a rise). Dushay *et al*., 2015 also saw a similar significant dip in FGF21 level for glucose treatment in the first 60 minutes. For fructose they saw a non-significant decrease in FGF21 after 30 minutes, returning to baseline by 60 minutes,

before rising steeply. However, the number of participants was ~~l~~ow, with a high degree of subject variability. Further studies would be needed to see if there is a consistent difference between glucose and fructose in the first 60 minutes. In subjects that had had a gastric bypass and subjected to a hyper-insulinemic-euglycemic clamp, a study using fructose has also shown an increase in FGF21 after 120 minutes, returning to baseline after 4-5 hrs, with a significant decrease at 30 minutes (ter Horst *et al*., 2017). If confirmed, glucose and fructose might be expected to ~~boost~~ exacerbate sweet preference and possibly intake during the course of a 1 hr meal, but to reduce intake after the meal. Studies *in vitro* could investigate whether the biphasic effect of glucose and fructose on FGF21 occurs in human primary hepatocyte culture conditions and investigate the effect of flattening the fructose spike (for example, 1 mM fructose for 2 hrs has more or less of an effect on FGF21 than 2 mM for 1 hr).

Some subjects possess a particular FGF21 single nucleotide polymorphism (SNP) associated with greater reduction in carbohydrate intake (Heianza *et al*., 2016). It is not known whether these subjects have a greater FGF21 response to sugar intake by the liver, but if so, this would suggest that ~~boosting~~ increasing FGF21 may be useful in helping people to lose weight. In a study on innate preferences, subjects were rated on their liking of sweets. Those with a lesser liking had baseline levels of FGF21 1.5-fold higher than those with a lower liking (Søberg *et al*.2017). Treatment of animals with FGF21 have shown improved effects on metabolic conditions (Xu *et al*., 2009), including reduction of body weight and improvements of glucose and lipid metabolism (Kharitonenkov *et al*., 2014). However, it should be noted that high FGF21 levels correlate with poor metabolic health in humans and animal studies (ter Horst *et al*., 2017). This may be a consequence rather than a cause of such conditions, in that subjects with poor metabolic health may have over many years consumed high carbohydrate diets leading to sustained FGF21 levels. Dushay *et al*., 2015 however, saw that as well as baseline FGF21 being higher in subjects with metabolic syndrome, glucose and particularly fructose stimulated a greater FGF21 response in such subjects. In “The good, the bad and the unknown” Hoffman and Havel., 2015 consider this seemingly “paradoxical” situation where “the metabolic effects of FGF-21 appear to be mainly beneficial while those of dietary fructose are generally

deleterious." One possible explanation they draw attention to is that subjects of metabolic syndrome become FGF21 resistant and that the elevated response may be compensatory (Dushay *et al*., 2015).

A longer-term study on the effect of fructose has reported firstly that baseline levels of both intact and total FGF21 were 2-3 times higher for women than men (Rodgers *et al*., 2019). After two weeks on a 75 g dose of fructose daily, women's levels had decreased to a level similar to that of the males. Stimulated levels were also 3-fold higher in women than men, both at the start and end of the study. In another longer-term study in mice, feeding fructose at 30% of total energy (equivalent to approximately 150 g/day in humans), FGF21 had increased by approximately 20-fold after 5 weeks, but had fallen to show only an approximately 2.5-fold increase by 12 weeks. Although difficult to compare between models, the study in mice and the data for women ~~perhaps~~ shows a similar pattern to that seen here, where FGF21 rose steeply after 48 hrs and dropped off after 72 hrs.

FGF21 knockout (KO) mice have been reported to show a greater preference for fructose or glucose in drinking water rather than plain water, and mixed glucose and fructose (i.e. sucrose) in drinking water over fructose or glucose alone (von Holstein-Rathlou *et al*., 2016). Intake of fat, protein and more complex carbohydrates were unaffected. Low protein diets with either high carbohydrate or high fat have also been found to drive FGF21 elevation independently of energy content (Lundsgaard *et al*.2017; Vinales *et al*., 2019). Interestingly, the highest rises in FGF21 led to the lowest risk of weight gain over 6 months and correlated with increased energy expenditure. A further study showed that whilst inhibiting sucrose intake, FGF21 promoted the rejection of low-protein diets, and in effect stimulated protein intake when low (Larson *et al*. 2019). It is suggested that the direct effect of FGF21 is to decrease the intake of sugars, which leads to increases in other macronutrients such as protein (Von Holstein-Rathlou *et al*., 2019).

FGF21 has also been shown to decrease alcohol consumption in transgenic mice over-expressing FGF21 (Talukdar *et al*.2016; Desai *et al*.2017). In humans, an FGF21 SNP has been associated with alcohol intake (Schumann *et al*.2016; Søberg *et al*.2017; Frayling *et al*.2018; Merino *et al*.2018; Clarke *et al*.2017), and alcohol

consumption has been shown to increase FGF21 by up to 40-fold (Desai *et al*., 2017; Søberg *et al*., 2018; Song *et al*., 2018; Søberg *et al*., 2018). The evidence again points to a protective effect, as FGF21 KO mice experience greater liver damage after 16 weeks of drinking water containing 30% ethanol (Desai *et al*., 2017).

Carbohydrate-responsive element-binding protein (ChREBP), a key regulator of carbohydrate metabolism in the liver, has been shown to regulate FGF21 expression in mice after sugar intake (von Holstein-Rathlou *et al*., 2016; Fisher *et al*., 2017). However, it not yet known whether ChREBP regulates FGF21 in humans, although it is increased by glucose treatment in human primary hepatocytes. It has been established by euglycemic and hyperinsilinaemic clamps that in response to glucose, insulin regulates FGF21 rather than glucose directly (Samms *et al*., 2017). Fructose and alcohol do not elicit an immediate insulin response, suggesting an alternative mechanism. In a study where high sucrose increased FGF21 mRNA in mice, raised ChREBP in the liver and brown adipose tissue (BAT) was seen (Maekawa *et al*., 2017). Interestingly, uncoupling protein 1 was also elevated in white and brown adipose tissues, ~~perhaps~~ possibly leading to the increased energy expenditure found, protecting the mice from increased body weight. It is suggested that FGF21 has evolved as protection against toxicity from over-consumption of high fructose and alcohol in fermented and non-fermented fruit, and ~~perhaps~~ could also ~~to~~ promote higher protein intake (von Holstein-Rathlou *et al*., 2019).

FGF21 Conclusion

The key observation made in this study was that after 48 hrs, fructose+glucose increased intact and intact/total FGF21 substantially more than glucose alone. This is consistent with both short-term, e.g. several hours post meal, and longer-term studies, e.g. 2 weeks. If the hypothesis is that FGF21's role is to protect the liver by limiting exposure to toxic levels of sugars, these results are ~~perhaps~~ a further indication that fructose is a greater danger at high levels than glucose. Flattening the fructose spike reaching the liver with ~~technology~~ plant natural compounds such as polyphenols and extracts may allow the liver to metabolise fructose more safely and with less toxic effects. As such, FGF21 may be seen to increase less in

response. Although FGF21 is considered to be protective, ~~perhaps~~ this should not be seen to be a problem as it will ~~possibly~~ indicate less harm. Some studies have seen a transient drop in in FGF21 after just 30-60 minutes post-feeding for glucose and fructose, which, it may be speculated, ~~might~~ encourages greater sugar consumption during a meal. Flattening the fructose spike may help alleviate this dip.

Based on the current scientific literature, this is the first time that a fructose+glucose mixture has been shown to increase FGF21 levels more than glucose in human primary hepatocytes. As this is consistent with *in vivo* observations, this study has shown that human primary hepatocytes are potentially a useful model for such research programmes and could limit the use of animals and expense of human subjects.

5.7 Summary

The main aim of this chapter was to investigate further potential health benefits of flattening the fructose spike reaching the liver. This was investigated by examining the effects of fructose on lipid accumulation, glycogen storage, lactic acid and uric acid production and the hepatokine FGF21 in primary human hepatocytes. As such, it was found to be difficult to induce lipid accumulation and uric acid at physiological levels of fructose and glucose. However, glycogen (Figures 5.10-13), lactic acid (Figures 4.14-16) and active FGF21 (Figures 5.2-43) responded well and were all greatly increased by fructose rather than glucose, indicating the potential for flattening the fructose spike to provide further health benefits, and for the first time the potential of human primary hepatocytes as model to study this further.

6.0 Discussion

6.1 Summary of findings

This thesis has considered the scientific literature and evaluated the various alleged impacts of fructose on health problems at levels typically consumed in the USA. From this work, it has become apparent that increases in postprandial triglyceride release is a health concern that is supported by meta-analysis and other scientific literature (Livesey & Taylor, 2008; Tappy & Rosset, 2019). The aims of this thesis therefore, have been to investigate the potential effect of flattening the fructose spike reaching the liver, as a means to reduce postprandial triglyceride release form the liver; identify ~~technology~~ plant natural compounds and extracts to achieve this; and investigate further potential health benefits. The phrase "flattening the fructose spike" here means to lower the concentration of fructose reaching the liver at any given time, but over a longer time period. It is proposed that only excess fructose is directed towards *de novo* lipogenesis (Lim *et al*., 2010) either directly or indirectly. In this thesis therefore, it is hypothesised that by inhibiting the uptake of fructose from the small intestine, a reduced and prolonged fructose spike, (i.e. a flattened spike) would result in a reduced excess of fructose (or metabolites) directed toward triglyceride release and cytotoxicity. As such, it was found that a model of "flattening the fructose spike" in primary human hepatocytes reduced triglyceride release over successive applications during the course of the day, representing "meals" (Figures 3.9-15). Cytotoxicity was also decreased (Figures 3.17-21). Natural plant compounds were investigated as possible ~~technology~~ means to flatten the fructose spike by inhibiting fructose uptake in the intestine, and a number of promising leads were found. Investigating further potential health benefits, the accumulation of lipid (Figures 5.3-5.8) and uric acid (Figures 5.17-19) in primary human hepatocytes was found to be difficult to induce at physiological levels of fructose and glucose. However, glycogen (Figures 5.10-13), lactic acid (Figures 4.14-16) and active FGF21 (Figures 5.2-43) responded well and were all greatly increased by fructose rather than glucose, indicating the potential to provide further health benefits. This chapter futher discusses the results of this study, work published relating to these findings, and suggestions for future investigations.

6.1.1 “Flattening the fructose spike”.

To model “flattening the fructose spike”, fructose at a concentration of 8 mM or 2 mM was applied to human primary hepatocytes over a short period of 1 hr and compared to half the dose (4 mM or 1 mM respectively) over a period twice as long, i.e. 2 hrs. The doses chosen were considered to be within physiological range based on hepatic portal levels measured in animals and humans (Bizeau & Pagliassotti, 2005), and the evidence that chronic fructose intake elevates uptake in the gut (David *et al.,* 1995; Cui *et al.,* 2004; Patel *et al.,* 2015; Jang *et al*., 2021). Glycerol was measured as a proxy for triglycerides, as triglycerides were found to be mostly broken down in conditioned culture medium (Figures 3.5-7), most likely by extracellular lipase, which is known to be secreted from primary hepatocytes (Hermier *et al.,* 1991). It was found that only low levels of triglyceride were released after the cells had been fed low levels of fructose overnight (Figures 3.10 and 3.11). This was as expected, as it has been hypothesised that only excess fructose is directed towards lipogenesis (Lim *et al*., 2010) and after an overnight “fast” the cells need to replete energy and glycogen stores. For subsequent applications throughout the day by modelling “meals”, greater levels of triglycerides were released even at lower levels of fructose and a substantial and significant reduction in proxy triglycerides was found when “flattening the fructose spike” (Figures 13-15).

6.1.2 ~~Technology~~ Plant natural compounds and extracts to flatten the fructose spike

An *in vitro* gut cell model for glucose transport (Campbell *et al*.,1999) was optimised for fructose analysis here by improving expression of the main fructose transporter GLUT5 mRNA. This, and extending the assay time course, elevated fructose transport in the model to levels which could reasonably be detected. A range of natural plant compounds representing potentially effective categories based on literature and patent searches, i.e.: gallates and tannins; flavonoids; terpenoids; fructose binders; and GLUT2 and SGLT1 transport inhibitors, were tested for their effectiveness at inhibiting the transport of fructose and glucose. Lectin glycan, vanillin, cinnamaldehyde, quercetin-3-glucoside and eucalyptol showed a tendency for moderate and consistent fructose transport inhibition (Table 4.3). These were

tested for synergistic activity with GLUT2 inhibitors, with phloretin+quercetin-3-glucoside, luteolin+quercetin-3-glucoside and hesperitin+quercetin-3-glucoside showing successful glucose transport inhibition (Figure 4.33). The catechins epicatechin, epicatechin gallate; and catechin itself showed more potent fructose transport inhibitory activity (Figure 4.34). As such brand 1 black and green tea (proprietary blends) aqueous extracts were tested and also found to be very effective for both fructose and glucose transport suppression (Figures 4.38-45). Despite containing much less tea extract or catechins, ice tea was found to be the most effective beverage tested on fructose transport, but less so on glucose transport (Figures 4.40-45). Investigations into whether this was due to low pH or particular ingredients did not lead to any positive conclusions (Figures 4.42-45). Brand 2 diet cola had a tendency to be moderately effective, but brand 3 instant coffee was not (Figures 4.40 and 4.41). Black tea, green tea, ice tea and diet cola were combined with phloridzin to look for potential synergies. Phloridzin was tested as it is a well-known SGLT1 low concentration glucose transport inhibitor, and as some evidence has suggested that, in response to SGLT1 activation, GLUT2 localises to the apical (gut lumen side) membrane of enterocytes (Kellet and Brot-Laroche, 2005). GLUT2, a high concentration glucose transporter, is also reported to transport fructose into the cell (Jones *et al*., 2011). Significant synergistic activity was found for black tea+phloridzin (Figure 4.49, Table 4.7), green tea+phloridzin (Figure 4.51) and brand 1 ice tea+phloridzin (Figure 4.55, Table 4.13) on glucose transport. For fructose, although there was a tendency for synergy, this was not statistically significant (Figures 4.50 and 4.56).

6.1.3 Further potential health benefits of flattening the fructose spike

As there is limited data on the effect of fructose at normal levels of consumption on lipid steatosis and non-alcoholic fatty acid disease in human studies (see 1.0 Introduction, 1.1.4 Effects of fructose on obesity and body fat, Liver fat), fresh primary human hepatocytes were used in this study to investigate intracellular lipid accumulation. No significant effect was seen at physiological doses of fructose and glucose, either with or without the added fatty acids palmitate, linoleate and oleate (Figures 5.1-8). Isocaloric mixtures of fructose+glucose were compared directly to

glucose alone (i.e. 11 mM glucose+2 mM fructose *vs* 13 mM glucose; 11 mM glucose+4 mM fructose *vs*.15 mM glucose and 11 mM glucose+8 mM fructose *vs*. 19 mM glucose). Over 72 hrs, a much more substantial effect was seen by fructose+glucose, than glucose treatments on glycogen storage (Figure 5.10). In a dose-dependent linear manner, glycogen was increased by up to 2.5-fold for the highest fructose dose (Figure 5.10), with no sign of a plateau having been reached (Figure 5.12). Glucose on its own did not significantly affect glycogen storage, suggesting that after 72 hrs in well-nourishing medium, the cells had reached optimal glycogen levels (Figures 5.10). This might indicate that fructose intake can lead to excess glycogen storage. Intracellular glucose levels were found to increase dose-dependently for glucose alone and fructose+glucose treatments (Figure 5.13). Together, the intracellular glycogen and glucose data suggest fructose stimulated gluconeogenesis and glycogen synthesis. Lactate levels were measured in the same experiment in medium conditioned over 24, 48 and 72 hrs time points. At each time point, lactate increased substantially in a dose-dependent manner for fructose+glucose mixtures, but not for glucose alone (Figures 5.14-16). This supports the hypothesis that glycolysis is feedback-inhibited by cell energy status and that fructolysis is not (Elliott *et al*., 2002). Uric acid levels were measured in conditioned medium in samples taken after 24, 48 and 72 hrs, but were not seen to be affected by fructose or glucose treatments (Figures 5.17-19).

In the same experiment, it was also seen that the hepatokine FGF21 increased substantially in a dose-dependent manner for fructose+glucose treatments (Figures 5.21-27). Intact (active) FGF21 increased several-fold for fructose treatments at the 48 and 72 hrs time points, whilst isocaloric glucose treatments tended to have only a modest affect (Figures 5.21 and 5.22). For total FGF21, which includes non-active and inhibitory truncated fragments of FGF21 (Agrawal *et al*., 2018), the effect was similar but less dramatic (Figures 5.23). The intact/total FGF21 ratio, representing overall FGF21 activity (Dunshee *et al*., 2016), still showed a greater effect on fructose+glucose treatments than glucose alone (Figure 5.26 and 5.27). However, at the 24 hrs time point, intact/total FGF21 tended to be higher for glucose than fructose (Figure 5.25). As it is not known how much of non-active FGF21 is inhibitory in each sample, one of the main digestive enzymes of FGF21, fibroblast activation protein (FAP) (Dunshee *et al*., 2016), was also measured. At the 48 hrs

and 72 hrs time points, no treatment effect was seen (Figures 5.29 and 5.30). However, at the 24 hrs time point FAP correlated negatively with intact/total FGF21 (Figures 5.28 and 5.31) and mostly with that of glucose (Figure 5.32), ~~possibly~~ supporting a conclusion that active FGF21 was greater for glucose at this shorter time point. The 48 hr time point conditioned media from this experiment were applied to 3T3-L1 fibroblasts differentiated to adipocytes. They showed a significant increase in glucose uptake by fructose+glucose treatments for the second highest fructose dose, 11 mM gucose+4 mM fructose, which had also given the highest FGF21 response (Figure 5.42). This effect was significantly blocked by a neutralising antibody to the FGF21 receptor FGFR1. Each of the other fructose treatments tended to be blocked by the antibody, whilst glucose treatments showed only a tendency to increase glucose transport and no indication that this might be blocked (Figure 5.42). Therefore, the data tended to confirm that the greater intact/total FGF21 ratio seen for fructose treatments at the 48 hrs time point was due to greater overall FGF21 activity. Circulating levels of FGF21 mainly produced by the liver, interact with the hypothalamus to decrease the sweet taste preference in response to sugars rather than complex carbohydrates (Jenson-Cody *et al*. 2020). It is thought that this might be a feedback mechanism to protect the liver from toxicity due to excessive intake (von Holstein-Rathlou *et al*., 2019). The greater levels of FGF21 for fructose at the 48 and 72 hrs time points in this study may indicate, therefore, a greater level of potential harm by fructose on the hepatocytes.

6.1.4 Limitations

In vitro

Isolated and cultivated primary cells usually differ strongly from the corresponding cell type in an organism. For example, when primary hepatocytes are isolated from their normal microenvironment hundreds of genes are up or down regulated (Zellmer *et al*., 2010). 3D *in vitro* models have been shown to be more responsive in some studies (Green *et al*., 2015). The Caco2 cells used in this study although shown to express GLUT5, GLUT2 and SGLT1 transporters may not express other possible transporters of fructose and glucose e.g. GLUT7, GLUT9a/b, GLUT8, GLUT11, and GLUT12 (Manolescu *et al*., 2007; Doujard & Ferris, 2008). Using either cell type

primary hepatocytes or Caco2 cells in isolation it is obviously not possible to capture interactions between different cell types or extrapolate from *in vivo* doses to *in vitro* concentrations.

Short-term studies and treatment regime

Fructose and glucose are obviously consumed over many years. As in most studies a limitation here is that only short-term consequences have been considered.
If glucose was being converted to fructose in these experiments, it may be difficult to see a difference between fructose and glucose over such short time periods. This ismgenerally unavoidable *in vitro* as e.g. primary human hepatocytes in culture can begin to de-differentiate after about a week and develop myoblast-like features (Rowe *et al*., 2013).

In chapter 3.0 an attempt was made to model feeding cells as “meals” by treating cells for 1 or 2 hours then over three periods during the day.. However, in chapter 5.0 cells were treated solidly over 24, 48 and 72 hrs i.e in a way that does not simulate typical feeding. As discussed below further studies should follow up the observations made here with a similar meal type modelling to that used in chapter 3.0.

WST-1 *vs* LDH

Preliminary experiments normalised glycerol and triglyceride measurerments to WST-1 a reagent provided by Roche (product no.11644807001) as a measure of cell viability. The stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bioreduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. As such, the measurement of WST-1 can be affected by cell activity which might be affected by treatment. Lactate dehydrogenase was therefore used as a measure of cell viability in later experiments.

Fructose analysis high variability

Despite optimising fructose transport in this work in preliminary experiments such that it was the recommended minimum levels for detection by the colourimetric/enzymatic assay used, results were still highly variable at times. This made it difficult at times to show significant decreases in fructose transport. The use of radiolabelling methods for measuring fructose transport were discussed above (4.3.3). These generally require very low levels of fructose to avoid signal dilution which do not equate to the levels of fructose expected in the gut lumen following a substantial fructose bolus (Ferraris *et al*. 1990; Jiang & Ferris, 2001, Douard and Ferraris, 2013). The levels of fructose used in such radiolabel methods are probably small enough to be safely metabolised by the enterocytes of the small intestine and unlikely to cause any serum fructose spikes. I.e. studies have shown that only fructose levels above approximately 5 g/dose would overwhelm the small intestines capacity such that fructose would reach the hepatic portal vein and hence the liver (Jiang *et al*., 2021). Even higher doses would be required to overwhelm the capacity of the liver to metabolise fructose safely for energy, or to synthesise glucose for glycogen storage and lactate. An increase in postprandial triglycerides is generally not seen below fructose intakes of 50 g/day, cholesterol below 100 g/day and lipid accumulation (steatosis) below 150 g/day (Tappy *et al*., 2021). The use of radiolabelled substrates would also likely be compromised by the emergence and measurement of radiolabelled metabolites which would be mistakenly assumed to be transported substrate.

An alternative method of analysis of transported fructose would be high-performance liquid chromatography (HPLC). Campbell *et al*., 1999 however, compared enzymatic assays, as used here, with HPLC methods for glucose and fructose and concluded that precision and accuracy was similar, with no difference in sensitivity. They concluded that the enzymatic assay had greater throughput and was much more cost effective. In checking for precision, accuracy and sensitivity however, they used standard curves of glucose and fructose separately rather than together. Here though, when the two were mixed together, particularly at ratios that were representative of samples (approximately 4:1 glucose to fructose), recovery of fructose was found to be poor (Table 4.1). It would be useful to do a comparison

between enzymatic and HPLC methods under these circumstances before future work is carried out.

This issue in follow up studies could however be possibly resolved as a fructose assay by the company “abcam” is now available which can be used with serum samples and has two advantages over the assay used here so far. Firstly, interfering glucose can be removed with just 1 µl of “Sample Cleanup Mix”, which means that data for fructose does not need to be generated by deducting a glucose reading, which it has been found to greatly increases variability. Secondly, the assay binds end-point metabolites to a fluorescent probe which will have greater sensitivity than the colourimetric assay used here.

Limited supply of fresh human primary hepatocytes

In chapter 5 investigation into the effects of fructose and glucose lipid accumulation, glycogen, lactate, uric acid and FGF21, in isocaloric doses were only performed on the fresh human primary hepatocytes of one subject. A high degree of variability in response by different subjects has previously been reported (Huggett *et al*., 2020) where fructose at a physiological level of ≤ 8 mM was seen to increase lipid accumulation in primary hepatocytes in only 1-in-5 subjects. Limited cells were available as the usual supply from Nottingham hospital became disrupted. These experiments should be followed up and confirmed in several subjects when the supply of fresh human primary hepatocytes can be restored or in albeit expensive frozen hepatocytes

FGF21/3T3-adipocyte glucose uptake assay- high variability- semi-quantitative

In this study the 3T3-adipocyte glucose uptake assay that was used to investigate FGF21 activity may only be semi-quantitative. In preliminary tests, a ten-fold increase in insulin from 10 to 100 nM gave only an approximately two-fold increase in glucose uptake (Figures 5.39 and 5.41). An increase in recombinant human FGF21 from 5 to 10 nM gave only a non-significant 25% increase in glucose uptake (Figure 5.41). This is similar to that seen by other groups reporting in the scientific literature (Kharitonenkov *et al*., 2005). The somewhat modest effect seen in this

study on glucose uptake for the samples taken after 48 hrs will therefore also have been affected by the low sensitivity of the glucose uptake assay.

6.2 Implications

6.2.1 Excess fructose: Triglyceride release and glycogen

These *in vitro* results support the scientific literature, that fructose treatments can more easily increase triglyceride release than lipid accumulation (see 1.0 Introduction, 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides). In human studies, increases in postprandial triglycerides have generally been seen at a fructose intake of approximately. ≥50 g, which is the approximate average daily consumption of fructose in the USA (Vos *et al*., 2008, Marriott *et al*., 2009). Lipid accumulation, on the other hand, tends to require approximately.150 g/day (Tappy *et al*., 2021). Also, little or no lipid accumulation in response to fructose was seen in this study (Figures 5.1-8), ~~perhaps~~ possibly because fructose was clearly still able to increase glycogen storage. It has been hypothesised that fructose is only likely to be directed towards lipid synthesis when it is in excess (Lim *et al*., 2010). However, Lim *et al*., only implied “excess” fructose as that which led to excess energy availability. It might therefore be asked whether full glycogen storage would be necessary before lipid synthesis is incurred. That significant lipid accumulation by fructose was not seen in these experiments, ~~perhaps~~ suggests that this is restricted whilst glycogen storage is still an option. The large up to 2.5-fold build-up of glycogen that was seen with fructose treatment (Figure 5.10) would ~~perhaps~~ indicate that fructose over-stimulates glycogen synthesis to an excessive level. Fructose has been proposed to ~~boost~~ elevate glycogen synthesis through a build-up of fructose-1-phosphate (F1P), due to the rapid activity of hexokinase and slower activity of downstream enzymes such as aldolase B (Chaudhary *et al*., 2013). F1P is proposed to ~~boost~~ increase glycogen synthesis in a number of ways, but at least partly by increasing the G6P:ATP ratio by lowering ATP as a result of the rapid phosphorylation of fructose (Ciudad *et al*., 1988). A build-up of F1P and lowering of ATP has also been suggested to lead to uric acid synthesis, as ADP and AMP accumulate and are metabolised (Chaudhary *et al*., 2013). However, no effects were seen by any treatments on uric acid levels

(Figures 5.17-19). This may simply be because AMP and ADP have not accumulated to such a level that a stage of breakdown has been reached. From this data, it can p~~erhaps~~ be concluded that without this stage being reached, build-up of F1P and possibly the lowering of ATP is still sufficient to ~~boost~~ enhance glycogen synthesis and to excess.

6.2.2 FGF21, excess glycogen storage and hepatocyte harm

That FGF21 is substantially increased in these experiments (Figures 5.20-22), might suggest though that the cells have experienced some levels of harm. FGF21 paradoxically seems to be beneficial when used to treat animals with metabolic syndrome symptoms yet is ~~boosted~~ elevated by fructose and correlates with metabolic syndrome (Hoffman and Havel., 2015). As such, it has been hypothesised that FGF21 is released to give protection to the liver under harmful circumstances by interacting with the hypothalamus to reduce sweet preference and sugar intake (von Holstein-Rathlou *et al*., 2019). However, fructose treatments in this study have not induced significant levels of lipid accumulation (Figures 5.3-8), the loss of cell nuclei as a result of cytotoxicity (Figures 5.2, 5.6 and 5.9), or uric acid synthesis (Figures 5.17-19). Either FGF21 does not signal cell harm in this way, or there is a low-level cell harm that is not detected by these outcomes. As any cause of harm in these cells does not seem to be due to lipid accumulation, which may cause endothelial reticulum stress, or uric acid which has been proposed to cause oxidative stress (Sun & Empie, 2012), ~~perhaps~~ the idea that the build-up of excess glycogen (Figure 5.10) should be considered as potentially harmful.

In this study, similar doses of fructose and glucose that increased triglycerides (Chapter.3, Figures 3.9-15), also caused 2.5-fold excess glycogen (Chapter.5, Figure 5.10). If as hypothesised by Lim *et al*., 2010, only excess fructose beyond energy requirements would lead to lipid synthesis, then this is no surprise. However, if full glycogen storage also needs to be reached, then the increased triglyceride release seen would ~~perhaps~~ be surprising. ~~Perhaps~~ That both occurred at similar doses could mean~~s~~ that full glycogen storage is not required. However, these were different experiments and therefore hepatocytes from different subjects. The same two events, lipid and glycogen synthesis, might still not be likely to happen to any

great degree in the same cells or experiment. Alternatively, ~~perhaps~~ due to zonation within the liver, periportal cells ~~can~~ could produce lipid ~~and~~ whilst perivenous cells are still able to synthesise glycogen from lactic acid (reviewed- Jungermann *et al*., 1996).

Flattening the fructose spike that reaches the liver could potentially reduce the triglyceride release seen, but whether it could also reduce excess glycogen storage is ~~perhaps~~ more debatable. However, if by not overwhelming the liver with fructose, more is metabolised aerobically in periportal cells, ~~perhaps there would be~~ this might lead to less lactate being produced for perivenous cells to produce glycogen.

6.2.3 How does glucose increase uptake of fructose? - new proposal

Studies have shown that glucose can increase the uptake of fructose (Rumessen & Gudmand-hoyer, 1986; Truswell *et al*., 1988). In this study, it was also found that a reduction in the fructose:glucose ratio reduced fructose uptake (Figure 4.57). As discussed above, it is generally thought that glucose enhances fructose uptake by increasing GLUT2 translocation to the apical membrane (Kellet and Brot-Laroche, 2005) and that GLUT2 also transports fructose into the cell (Jones *et al*., 2011). Ferraris *et al*. 2018 have recently published a review discussing that there has been a “decade of controversy” with respect to GLUT2 replacing GLUT5 as the main fructose transporter. They conclude that GLUT2 may play little or no role in fructose transport (Ferraris *et al*., 2018). It was also seen in this study that phloridzin, a well-known SGLT1 inhibitor for glucose, and phloretin, a well-known GLUT2 inhibitor, have little or no effect on fructose transport in most experimrnts (Figures 4.13-29, Table 4.3), suggesting that neither GLUT2 nor SGLT1 is involved in fructose transport, at least in the Caco2 cell line model, used here. One explanation might be that glucose alters fructose mutarotation. It was noticed in these studies that high levels of glucose interfered with fructose analysis, such that there was a substantial loss in fructose recovery. Hexokinase, a key enzyme in the assay, can only convert the furanose and beta-D- isomeric formation of fructose (Preedy, 2012). GLUT5 also shows stereoisomer specificity (Kane *et al*., 1997). Why then might it be expected that glucose could affect the isomeric form of fructose? It has been seen that amino acids can affect the mutarotation of glucose in solution (Shallenberger *et al*., 1984)

and sucrose has been reported to affect the mutarotation of fructose (Nelson & Beegle, 1919). ~~Technology such as~~ Natural plant compounds that maintain fructose in the optimal stereoisomer format might also reduce fructose uptake and help flatten the fructose spike that reaches the liver and intracellular enterocytes.

6.3 Recent published works and relevance

6.3.1 Polyol pathway

Recently studies have suggested that the polyol pathway may be more prevalent than previously thought. In the polyol pathway, glucose is reduced to sorbitol by aldose reductase and then oxidised to fructose by sorbitol dehydrogenase. Many studies have shown that this pathway is clearly available and utilised in the liver of animals (Lapansa *et al*., 2013; Park *et al*., 2017). Lapansa *et al*. provided evidence that the pathway was essential for mice on high glucose drinking water to develop a fatty liver. For humans, in the region of the 30% glucose metabolised has been found to be directed via the polyol pathway in subjects with diabetes (Yan, 2018). Inhibiting aldose reductase, a key enzyme in the polyol pathway, in these subjects reduced oxidative stress and inflammation. The glucose transport inhibitors that have been identified in this study (Figures 4.33, 4.37-45, 4.49 and 4.55) may therefore also be important in controlling the fructose spike within the liver. Uric acid has been reported to stimulate aldose reductase expression in the polyol pathway and the conversion of glucose to fructose in rats (Sanchez-Lozada *et al*, 2019). With high glucose intake, they saw an increase in endogenous fructose, and an accumulation of fat that was reduced significantly with an inhibitor of xanthine oxidase, the key enzyme in uric acid synthesis. In this thesis, uric acid was not seen to be affected by fructose or glucose treatments (Figures 5.17-19), therefore this might only be important at doses higher than typically consumed. Perhaps more significantly, metabolic syndrome and obesity have been associated recently with high salt diets (Kuwabara *et al*., 2017; Lapansa *et al*., 2018) and activation of the aldose reductase–fructokinase pathway in the liver (Lapansa *et al*., 2018). Fructokinase knockout mice given drinking water that was mildly elevated in salt were protected from developing features of metabolic syndrome (Lapansa *et al*.,

2018). Inhibiting glucose transport with the ~~technology~~ plant natural compounds and extracts identified in this thesis (Figures 4.33-4.55) might also flattening the fructose spike reaching the liver, particularly in subjects with high salt diets.

6.3.2 Fructose metabolism in the intestine

(Discussed in greater detail in Appendix II)

The liver has the highest expression of active fructokinase. However, it has recently been shown that the small intestine also has considerable levels too (Lapansa *et al*., 2013; Jiang *et al*., 2021). Lapansa had demonstrated in knockout studies that frucokinase activity in the intestine could protect the liver from lipid accumulation. In a mouse study, Jang *et al*., 2021 applied equal amounts of ^{13}C-fructose and unlabelled glucose and saw that gluconeogenesis began to plateau at a fructose dose of 0.5 g/kg (approximately. 2-5-3 g/60kg human equivalent based on body surface area or calorie intake), at which point fructose was seen to rise rapidly in the portal vein. It can be concluded that only when fructose intake overwhelms the metabolic capacity of the intestine will the liver be exposed to significant levels of fructose. In conclusion, Jang *et al*. suggested that "…slower fructose intake to match the intestinal capacity can alleviate [liver lipogenesis] syndromes." ~~Perhaps~~ Slowing uptake by the cell using ~~technology~~ the plant natural compounds and extracts identified in this thesis (Figures 4.33-4.55) ~~w~~could allow greater clearance of fructose within the enterocyte, limiting transport of fructose into the blood and flattening the fructose reaching the liver.

6.3.3. Chylomicrons

In the intestine, both fructose and glucose have been reported to stimulate *de novo* lipogenesis, increase triglyceride synthesis and lead to the release of lipoprotein into circulation as chylomicrons (Hoffman, 2019). In a review, Tappy, 2021 speculates that "…[chylomicrons] may be associated with chronic elevation of triglyceride-rich lipoproteins and of their remnants in the blood, which may… be responsible for initiating atherosclerosis". Studies have demonstrated an increase in the specific serum chylomicron apolipoprotein, ApoB48, in response to fructose feeding, distinct

from ApoB100 of VLDL and LDL particles from the liver (Hoffman *et al*., 2019, review). However, Xaio *et al*., 2013 showed that with an infusion of fructose+lipid over several hours in healthy subjects, there was only a small increase in ApoB48 triglyceride compared to ApoB100 triglyceride. It should also be taken into consideration that the VLDL and LDL triglycerides carry a higher risk of atherosclerosis than chylomicrons. Mounting evidence now suggests that ApoB100 is the greater predictor of vascular/heart disease than LDL cholesterol or total cholesterol (Jacobson, 2011; Lim *et al*., 2011).

As such, whether flattening the fructose spike in the enterocyte substantially reduces the release of chylomicron triglyceride into the blood or not, it ~~perhaps~~ may make~~s~~ little difference in comparison to the quantity of triglyceride from the liver and its inherently greater risk.

6.3.4 Intestinal damage and gut microbiome

In the liver, lipid accumulation in the long-term can lead to non-alcoholic fatty acid disease and ultimately inflammation and non-alcohol fatty liver related steatohepatitis (NASH). Tappy, 2021 hypothesises "…that the same process could occur in enterocytes leading to lipotoxicity". Intestinal lipid accumulation has been reported following a high fat diet in mice (Guozhu *et al*., 2019). Of the tissues that can metabolise fructose, enterocytes are exposed to the highest levels by far; up to ~~perhaps~~ approximately 25 mM, compared to 1-8 mM for the liver (see 1.0 Introduction, 1.5 Physiological fructose levels in the hepatic portal vein). Therefore, it is ~~perhaps~~ not surprising that fructose has been suggested as a main dietary cause of intestinal damage and leaky barrier function (Overview by Binienda, *et al*., 2020).

High levels of fructose intake have been seen in a number of studies to impair the gut barrier, leading to changes in the gut bacterial species profile and in toxins leaking into the circulation (Cho *et al*., 2019), and to a greater extent than glucose (Cros *et al*., 2020). The small intestine is now thought to metabolise the majority of a dose of fructose up to approximately. 5-6 g (Jang *et al*., 2021). In these studies, if intestinal metabolism was saturated, some fructose passed through to the lower gut where it is likely to be metabolised to short chain fatty acids such as acetate.

Antibiotics have been shown to limit liver steatosis in high fructose-fed mice (Bergheim *et al*., 2008; Brandt *et al*., 2017). Also, germ-free mice fed high fructose diets showed reduced hepatic triglycerides compared to the same diet in control mice (Kaden-Volynets *et al*., 2019), also suggesting that gut microbes may be at least partly responsible for liver steatosis and hypertriglycemia. Therefore, it has been proposed that high fructose feeding can lead to the production of short chain fatty acids such as acetate that are absorbed by the gut and become a source of fuel for lipid synthesis in the liver (Zhou and Zhong, 2017). Gut barrier damage has been associated with a number of other pathologies, including inflammation of joints, cardiometabolic disorders, inflammatory bowel disease, irritable bowel disease (Bischoff *et al*., 2021), chronic fatigue, schizophrenia and depression (Simeonova *et al*., 2018). As such, flattening the fructose spike (Figures 3.10-15) in the small intestine, thereby reducing the spill-over of fructose into the lower gut and by not promoting the “bad” bacteria that produce endotoxins, and in limiting gut membrane damage, may have the potential to protect from a number of these health issues.

6.3.5 Glycogenosis

Studies in rats fed on a high fructose diet have been shown by liver histopathology to exhibit excessive glycogen storage (glycogenosis), but with no evidence of steatosis or inflammation (Maslak *et al*., 2015). More recently, glycogenosis has been noted as common in adults and children with NAFLD (Allende *et al*., 2021), and associated with lower levels of steatosis and fibrosis, but with increased liver cell injury. Hepatocellular injury was identified as having a higher ballooning grade, presence of acidophil bodies, Mallory-Denk bodies and megamitochondria. In histopathology, ballooning degradation is a form of apoptosis (Yip and Burt, 2006), and is found in inflamed fatty liver (steatohepatitis) (Liangpunsakul and Chalasani, 2003). Acidophil bodies are necrotic, often with periportal distribution and are typical of viral hepatitis. They have been found in association with ballooned hepatocytes and the diagnosis of non-alcoholic steatohepatitis and apoptosis (Yeh *et al*., 2016). Mallory bodies are damaged intermediate filaments within liver cells, most common in alcoholic hepatitis (with a prevalence of 65%) and alcoholic cirrhosis (51% prevalence) (Jensen *et al*., 1994), but also in other diseases e.g. non-alcoholic cirrhosis (24% prevalence).

Megamitochondria are extremely large and abnormally shaped mitochondria seen in hepatocytes in alcoholic liver disease and in nutritional deficiencies. Free radicals are reported to play a crucial role in the mechanism of their formation (Wakabayashi, 2002), either caused by toxins or as a consequence of high fat Western diets (Shami *et al*., 2021).

Although little is known about the health implications of dietary induced excessive glycogen, it would seem that it is associated with significant damage in the liver. In this thesis, fructose was found to substantially increase glycogen storage to levels 2.5-fold that of glucose, and estimated normal levels of glycogen i.e. approximately 8% of total liver weight (Cole and Kramer, 2016) (Figures 5.10-13). Further research into the health implications of this effect by fructose is needed, but if flattening the fructose spike can reduce glycogenosis, it may also have the potential to protect from a number of these health issues.

6.3.6 Lactate

In this thesis, lactate production was found to be much greater in response to fructose than to glucose (Figures 5.14-16). Therefore, it is interesting to note that in a recent study, lactate dehydrogenase B (LDHB), which converts lactate to pyruvate, showed the greatest protein acetylation in a mouse non-alcoholic fatty liver disease (NAFLD) model, leading to impaired lactate clearance and to further NAFLD progression (Wang *et al*., 2021). LDHB, but not LDHA (which converts pyruvate to lactate), acetylation was significantly changed depending on the severity of liver disease ranging from steatosis (fatty liver) to non-alcoholic steatohepatitis (NASH). Lactate was also seen to accumulate in the serum and liver in both human and mouse models where NAFLD was mediated by high fat diet. They found that acetylation was due to acetyltransferase P300/CBP-associated factor (PCAF). When they overexpressed PCAF, LDHB activity was inhibited, and lactate clearance was reduced. In another recent study on acute liver failure, lactate increased nuclear histone H3K9 hyperacetylation and damage-response gene expression (Ferriero *et al*., 2018). It has been suggested that H3K9 may aggravate lipid accumulation by enhancing SREBP1 (Sterol regulatory element-binding transcription factor 1) which plays a key role in the induction of lipogenesis by the liver, and

steatosis (Tao *et al*., 2013). Wang *et al*. 2021, suggested therefore that lactate plays a key role in the onset of steatosis. When they fed a high fat diet and acetylated LDHB, this resulted in increased H3K9 acetylation and increased SREBP1, lipid accumulation and substantial inflammation. When PCAF was inhibited, progression to NASH was attenuated. They concluded that a potential therapeutic approach to NAFLD could involve targeting PCAF-dependent LDHB acetylation. In previous work, Wang *et al*., 2018 showed that inflammatory stress raised lactate levels in the liver. Other studies have also shown damaging effects of lactate in liver diseases. Blood and liver levels of lactate increase in liver disease with increasing severity from NAFL to NASH (Jeppesen *et al*., 2013; Ha *et al*., 2016). Lactate was also found to be a possible cause of histone lactylation in inflammation and cancer (Zhang *et al*., 2019).

Although further research is needed on the health implications of excessive lactate in serum and liver, if flattening the fructose spike can reduce lactate, it may also have the potential to protect from a number of health concerns.

6.3.7 Fructose, glycogen, lactate, steatosis and NASH

The steatosis/fatty liver early stages of non-alcoholic fatty liver disease (NAFLD) are thought to be relatively benign. However, 20-30% of NAFLD patients develop inflammation, fibrosis, hepatocyte necrosis and eventually non-alcoholic steatohepatitis (NASH), a serious health condition, leading to complications such as cirrhosis and hepatocellular carcinoma (Chalasani *et al*., 2018; Younossi *et al*., 2016). Progression from NAFL to NASH is poorly understood, with oxidative stress, hormone imbalances and mitochondrial abnormalities amongst the potential causes in a two-hit or even multi-hit phenomenon (Friedman *et al*., 2018). NAFLD at various stages can include inflammation, ballooning degeneration polymorphonuclear cell infiltrates, Mallory bodies, apoptotic bodies, vacuolated nuclei, megamitochondria, fibrosis, etc. (European Association for the Study of the Liver, *et al*., 2016). For NASH, increased hepatocyte death via apoptosis or necrosis and inflammation are hallmarks (Wong *et al*., 2018).

Taken together, there are numerous interconnecting phenomena with respect to fructose, glycogen, lactate, the onset of steatosis and progression to NASH. High fructose diets may induce lipid accumulation directly through providing excess metabolites beyond that needed for energy and glycogen storage. In parallel, excessive glycogen may create the conditions for progression from steatosis to NASH, such as apoptosis and necrosis from ballooning, acidophil bodies, Mallory-Denk bodies and megamitochondria. Also, high lactate levels may be involved in inducing lipid accumulation through H3K9 acetylation and SREBP stimulation. Fructose and not glucose has been shown to increase SREBP1 (Softic *et al*., 2017). ~~Perhaps~~ This may occur~~s~~ directly, or via excessive lactate H3K9 acetylation, and ~~perhaps~~ high lactate-mediated histone lactylation and inflammation maybe key to progression from NAFL to NASH.

6.3.8 FGF21 and plasma triglyceride

In a recent study investigating the regulatory roles of carbohydrate response element-binding protein (ChREBP), it was found that overexpression in mice decreased plasma triglycerides, whilst increasing lipogenesis and lipid accumulation in the liver (Iizuka *et al*., 2018). ChREBP is the route by which fructose increases FGF21 (von Holstein-Rathlou *et al*., 2016; Fisher *et al*., 2017), so not surprisingly, they also found FGF21 levels raised. FGF21 has previously been shown to reduce plasma triglycerides by increasing their disposal in adipose tissue by accelerating catabolism and decreased VLDL secretion in the liver (Schlein *et al*., 2016). As such, FGF21 did not prevent lipid synthesis and steatosis, ~~perhaps~~ most likely as this has been found to occur at much higher fructose intakes of ≥ 150 g/day (Tappy *et al.,* 2021). Uncoupling protein 1 (Ucp1) and peroxisome proliferator-activating protein gamma (Pparg) mRNA were also increased in this study (Iizuka *et al*., 2018), both also previously found to be induced by FGF21 (Dutchak *et al*., 2011; Fisher *et al*., 2012). Uncoupling protein 1 may be linked to the increased energy expenditure found in other studies in mice on high carbohydrates, but low fat or protein (Lundsgaard *et al*.2017; Vinales *et al*.2019), protecting the mice from increased body weight. Peroxisome proliferator-activating protein gamma increases insulin sensitivity by enhancing the storage of fatty acids in fat cells and reducing circulating levels, by inducing FGF21 (Ahmadian *et al*., 2013). Therefore, FGF21 may not only

have a protective effect of changing sweetness preference and reducing sugar intake (Jenson- Cody *et al*., 2020), but may also help limit or reduce hypertriglyceridemia.

In this thesis, fructose+glucose ~~boosted~~ increased FGF21 after 48 and 72 hrs treatment, but ~~perhaps~~ less than for glucose after just 24 hrs. Some studies have seen a transient drop in FGF21 after just 30-60 minutes post-feeding for glucose and fructose (Lin *et al*., 2012; Dushay *et al*., 2015; ter Horst *et al*., 2017), which, it may be speculated, might encourage greater sugar consumption during a meal. As fructose increases ChREBP, increasing fructose might arguably and paradoxically be expected to have the same effect as overexpressing ChREBP. However, it is generally accepted that moderate fructose consumption can lead to increased postprandial triglycerides. The paradox ~~perhaps~~ possibly arises from the fact that FGF21 is part of a feedback mechanism which is activated to protect the potentially harmful effects of fructose (von Holstein-Rathlou *et al*., 2019). FGF21 may be able to reduce rising levels plasma triglycerides, but overall if due to high enough levels of fructose, an increase in triglycerides would seem still occur (Livesey & Taylor, 2008; Tappy & Rosset, 2019). As such, reducing fructose intake or its spike reaching the liver, and therefore ChREBP, would not be expected to increase triglyceride release. Flattening the fructose spike may help to alleviate the dip in FGF21 seen during a meal with fructose and glucose, thereby maintaining FGF21 levels during a meal or snack, potentially reducing postprandial triglycerides and ~~boosting~~ enhancing energy expenditure, as well as influencing sweet taste preference and sugar intake.

6.3.9 Ileal Brake

Inhibiting and therefore slowing down the uptake of fructose and glucose by the gut could have another benefit. Studies have shown that although all macronutrients in general entering the small intestine tend to reduce appetite and energy intake there is a proximal to distal gradient with ileum > jejunum > duodenum (Wilbrink *et al*., 2021- Review). This differential effect is likely due to the fact that negative feedback from the duodenum (duodenal brake) and jejunum (jejunal brake) only regulate gastric acid secretion and gastric emptying of nutrients to the small intestine. Whereas the negative feedback from the ileum (ileal brake) results in a reduction of

gastric acid, biliary and pancreatic secretion, with inhibition of gastric emptying, intestinal motility and transport (Layer, P *et al*., 1990). The duodenal/jejunal and ileal brakes are thought occur to be due to the interaction of nutrients with G-protein coupled receptors on enteroendocrine cells inducing gastrointestinal peptide secretion e.g. cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1). These peptide mediators induce their effects either via entering the bloodstream, acting as hormones (endocrine effect), via activation of vagal afferents (neuronal effect) or via an effect on neighbouring cells (paracrine effect).

Although the "intestinal brake" effect on appetite and energy appears largely not to be macronutrient specific i.e. at equi-caloric amounts, the inhibition on energy intake and appetite is in the same range for fat, protein and carbohydrate, the effect has been seen to be greatest for sucrose rather glucose alone. Chaikomin *et al*., 2000 found that an ileum infusion rate of glucose of 0.66 kcal/min resulted in on average a 10% reduction in energy intake, and a 0.57 kcal/min of sucrose resulted in a reduction of 32%. This may indicate that fructose or glucose+fructose are the most effective macronutrients at inducing the ileal brake.

It is also interesting to note that of the range of tastants (bitter, sweet, salt, sour and umami) bitter tastants have been found to be the most effective at inducing the ileal brake (Klaassen *et al.,* 2021). This is of particular interest as the compounds (catechins) and beverages (teas) found here to be most effective at slowing the uptake of fructose and glucose from the gut are known to be bitter in taste (Xu *et al*., 2018). This makes sense as a) it is considered that these prototypical tastes exist in order to predict the type of food that will be ingested e.g. bitter for potentially toxic substances (Wilbrink *et al*., 2021- Review), b) taste receptors, are expressed, not only on the tongue but, throughout the entire human gut, in particular on EECs (Van Der Wielen, N *et al*., 2014; Gu, *et al*., 2014) and c) activation of taste receptors can result in the release of GI peptides such as CCK, PYY and GLP-1.

6.4 Future work

6.4.1 Modelling the effect of "meals" and "flattening the fructose spike": on FGF21, glycogen and lactate in primary human hepatocytes.

In this thesis, glycogen (Figures 5.10-13), lactate (Figures 5.15-16) and FGF21 (Figures 5.21-27) responded to fructose+glucose treatments substantially and significantly over 48 and/or 72 hrs, relative to glucose alone. Having established that primary human hepatocytes are a useful tool for these outcomes, there is a reasonable probability that these potential health markers will show detectable changes over a shorter period. In chapter 3 "Flattening the fructose spike", significant differences were found for triglycerides with models of "meals" and "flattening the fructose spike" reaching the liver (Figures 3.9-15). Flattening the fructose spike was modelled by applying 2- or 8-mM fructose over 1 hr *vs*. half the concentration of fructose for twice as long, i.e. 1- or 4-mM, respectively, for 2 hrs. Meals were modelled by applying treatments for a short period (1-2 hrs), then refreshing with media without added fructose, and repeating every 3 hrs for 3 "meals". I such studies samples of culture media would need to be taken regularly, e.g. every 15-30 minutes in order to investigate whether there is a dip for FGF21 (intact/total) in the first 30-60 minutes. Subsequent medium samples could be taken every 30-60 minutes. Lactate could also be measured in the conditioned medium. For glycogen analysis, cells would need to be collected for extraction. It would be useful to do this for each meal initially, and over several days to see at what time point and at what fructose and glucose concentration the maximum storage of glycogen is reached.

If significant effects are not seen in one day over the course of 3 applications ("meals"), this could be repeated for as long as needed, up to 1-2 weeks. As primary human hepatocytes in culture can begin to de-differentiate after about a week and develop myoblast-like features (Rowe *et al*., 2013), specialised markers for hepatocyte differentiation (Rowe *et al*., 2013) could be checked daily by mRNA analysis. Cells could be collected for histological analysis to see if excess glycogen is leading to such features as ballooning, acidophilic bodies, Mallory-Denk bodies and megamitochondria (Yip and Burt, 2006), although expertise in this might be

difficult to acquire. Alternatively, or in addition, lactate dehydrogenase released into the culture medium as cells become necrotic, or cytokines e.g. IL-6, IL-1a and IL-8, or ~~perhaps~~ a cytokine array would give some indication of early inflammation.

Triglycerides and free fatty acids could be measured in the culture medium focusing on fatty acid length and degree of saturation. Some studies, particularly recently, have seen differences in fatty acid profile as well as saturation with fructose *vs*. glucose treatment (Silva *et al*., 2019; Chen *et al*., 2020; Belew *et al*., 2021; Jones *et al*., 2021) and *de novo* lipogenesis (Krause,*et al*., 2020; Federico *et al*., 2021). Some data show that cholesterol may also be increased by fructose at normal doses (7.5% of 2700 kcal energy intake, i.e approximately. 50 g/day) *vs*. starch in hyper-insulinemic and insulin-sensitive men (Hallfrisch *et al*., 1983). In animal models, a high fat+fructose diet tends to give greater dyslipidaemic responses than fructose alone (e.g. Gunawan *et al*., 2019). In this thesis, it was found to be difficult to increase lipid accumulation with either fructose or glucose over and above that found with 200 µM fatty acids (palmitic acid: linoleic acid: oleic acid (PLO) (Figures 5.1-7). Lower concentrations of added fatty acid could be tested to ~~perhaps~~ improve sensitivity of the model to added carbohydrates.

If glucose was being converted to fructose in these experiments, it may be difficult to see a difference between fructose and glucose over such short time periods. It would be interesting to see whether a greater difference in outcomes would be seen if the polyol pathway is inhibited. Ultimately, if it remains difficult to detect changes over such short-time periods, different *in vitro* models could be tried. 3D *in vitro* models have been shown to be more responsive in some studies (Green *et al*., 2015).

Insulin sensitivity is another possible health concern that could be worth investigating further in primary hepatocytes. Although whole body insulin insensitivity is only seen at high fructose doses, hepatic insulin insensitivity has been found to occur at a moderately high intake of 80 g/day (Aeberli *et al*., 2013). Higher doses of glucose than examined so far could also be tested. A study in rat primary hepatocytes has shown that at 15 mM glucose, uric acid production is increased at 2 mM fructose

after a 1-hour incubation (Petrie *et al*., 2013).

6.4.2 ~~Technology optimisation:~~ Tea Catechin profile optimisation

In this thesis, both brand 1 black and green teas were highly effective at inhibiting both fructose and glucose transport in the optimised Caco2 *in vitro* model (Figures 4.38-45). EC, ECG and catechin were also found to be effective as single actives. Teas of varying catechin profiles particularly high in EC, ECG and catechin would be important to focus on to find optimal varieties. Testing many varieties for inhibition with known polyphenol profiles would likely offer more insight into the features that are most desirable for fructose and glucose transport inhibition. Ultimately teas could ~~perhaps~~ be bred to give the best possible profile.

It was surprising that ice tea gave even greater inhibitory activity against fructose transport than black and green tea (Figures 4.40 and 4.44), as it contains much lower amounts of tea. Testing other branded ice teas may help elucidate what it is about rand 1 ice tea that makes it so effective.

6.4.3 Clinical study

A clinical study would be important to determine whether teas are also effective *in vivo*. This is an expensive option and would ~~perhaps~~ best be performed when optimised varieties have been selected. Short-term studies, i.e. over one drink or application could be exercised firstly to determine if the uptake of fructose can be reduced. It might be difficult to detect fructose in the blood/plasma as most of this sugar is taken up by the liver. Ideally, fructose would be measured in the hepatic portal vein, but this would be difficult to access as levels in circulation would likely be less than approximately 500 µM and the enzyme/spectrophotometric assay used in this thesis requires levels of fructose greater than 500 µM (Douard & Ferris- 2008). Campbell *et al*., 1999 did not find high performance liquid chromatography methodology to be any more sensitive than the enzymatic/spectrophotometric method. However, a fructose assay by the company “abcam” is now available which can be used with serum samples and has two advantages over the assay used here so far. Firstly, interfering glucose can be removed with just 1 µl of “Sample Cleanup

Mix", which means that data for fructose does not need to be generated by deducting a glucose reading, which has been found to greatly increase variability. Secondly, the assay binds end-point metabolites to a fluorescent probe which will have greater sensitivity than the colourimetric assay used here. Postprandial triglycerides could be tested for up to 12 hrs (Park *et al*., 2008), and on each day over several days. Fructose upregulates GLUT5 (Hirayama *et al*., 2001) and lipid synthesis enzymes (Lim *et al*., 2010) and so it might take a few days to see an effect. FGF21 and lactate in the plasma would also be key metabolites to measure. As slowing down the uptake of fructose and glucose could potentially activate the ileal brake it would also be useful to investigate any effects on appetite by questionnaire.

6.4.4 Mutarotation

~~Perhaps a little ambitious unless,~~ It would be interesting, if adequate NMR, mass spectrophotometry or HPLC facilities are available, to investigate what stereoisomeric form fructose is in in the presence of, and at different ratios of glucose. This might help to illuminate how it is that glucose is able to affect fructose uptake in the gut. The pyranose stereoisomer of fructose is more thermodynamically stable (Ma *et al*., 1988), so generally fructose is found in solution to comprise 30% furanose and 70% pyranose (Preedy, 2012). GLUT5 has been found to prefer the furanose form (Kane *et al*., 1997). It would not be surprising if glucose also interfered with stereoisomeric forms of fructose as proposed (see 4.0 ~~Technology~~ Plant natural compounds and extracts to flatten the fructose spike, 4.2.8 Hypotheses for the effect of glucose on fructose uptake, Mutarotation). Sucrose has been reported to affect the mutarotation of fructose (Nelson & Beegle, 1919). Other potential members of a gut lumen environment could also be tested such as amino acids, fats, fibre, acidity, etc. If analysis is sufficiently sensitive, further potential inhibitors could be discovered in this way and then further tested in the Caco2 model to confirm or test the concept that mutarotation affects fructose uptake. To test whether GLUT5 prefers the stereoisomeric alpha or beta form, a GLUT5 transport model only would need to be established, e.g. a liposome (Andrade *et al*., 2017) or oocyte model (Kane *et al*., 1997) overexpressing GLUT5. Also, models for the other GLUTs: 7, 8, 9a/b and 12 could be set up, although recent research suggests that they are not likely to be involved (Ferraris *et al*., 2018).

6.5 Conclusions

The aims of this thesis have been to determine whether flattening the fructose spike reaching the liver could potentially deliver health benefits, to identify ~~technology~~ plant natural compounds and extracts to achieve this and to investigate further possible health benefits. Through analysis of the scientific literature, postprandial triglycerides were identified as a health concern that was relevant at the typical levels of fructose intake in the diet. By modelling “meals” and “flattening the fructose spike” in primary human hepatocytes, evidence has been found to support the hypothesis that lower levels of fructose over a longer period of time can lead to reduced triglyceride release and cytotoxicty. In an *in vitro* gut cell line model, black, green and ice teas were found to strongly inhibit fructose transport. For black tea and ice-tea, this has not been shown before. Also, synergies have been found which will help such ~~technology~~ plant natural compounds and extracts to be patented. Further potentially harmful effects of fructose, relative to glucose, i.e. excessive glycogen and lactate accumulation have been found to be greatly increased in primary human hepatocytes, warranting further investigation as to their response to flattening the fructose spike. FGF21, a marker of liver harm as well as protection, has also been shown to respond to fructose, more so than glucose, in primary hepatocytes and can therefore be investigated further in this model.

This is the first time that these outcomes have been demonstrated in human primary hepatocytes. As they are consistent with *in vivo* observations, this study has shown that human primary hepatocytes are potentially a useful tool for research in these areas, and as such could limit the use of animals and the expense of human subjects.

Appendices

Appendix I Triglycerides and health concerns

Introduction

Significantly elevated triglycerides (hypertriglyceridemia) is a highly prevalent issue, occurring in 10% of the US population (Laufs *et al.,* 2020). Triglycerides are the main lipid component of lipoproteins particles such as chylomicrons (transporting lipid from the intestines) and very low-density lipoprotein (VLDL) particles (transporting lipid from the liver), which also transport cholesterol. Triglyceride concentrations measured in the blood or cell culture supernatant is reflective of lipoproteins rich in triglycerides and their remnants (partially lipolysed particles). ~~Lipoprotein~~ Lipase (hepatic, pancreatic and endothelial) hydrolyses triglycerides contained in these lipoproteins to release free fatty acids which then become available for tissue (adipose, liver, heart pancreas etc.) uptake (Jacobsen *et al.,* 2020).

Atherosclerosis

Most studies record fasting triglyceride level rather than postprandial because variability is less and also for the historical reason of using the Friedewald equation which requires the use of the fasting triglyceride concentration (see 1.0 Introduction, 1.1.3 Effects of fructose on blood lipids, Measurement of fasted *vs.* post-prandial triglycerides). Atherosclerosis has long been hypothesised to be a disorder influenced by postprandial triglycerides (Ridkera, 2008). For example, in a study in which 26,509 initially healthy American women were followed over a 11-year period, both fasting and non-fasting triglycerides were associated with future cardiovascular risk (Bansal *et al.,* 2007). In another study of 7,587 women and 6,394 men in Copenhagen with 26 years of follow-up (Nordestgaard *et al.,* 2007) non-fasting triglycerides were also found to significantly predict future vascular events in both sexes. The association is now well established, and The International Diabetes Federation defines metabolic syndrome by waist size plus any two of a number of biometrics including a raised triglyceride level (>150 mg/dL) (Russo *et al.,* 2019).

Atherosclerosis is where artery walls narrow and harden as a result of the build-up of plaques made up mostly of cholesterol and fat, obstructing blood flow. By the age of 65 most people will be affected at least to certain extent (Aronow *et al.,* 2013), and in Western society it is the biggest cause of mortality and disability (Topol and Califf, 2007).

One of the main triggers of atherosclerosis is considered to be high cholesterol, which aids the uptake of other lipids, particularly low density lipoprotein cholesterol (LDL-C) particles by the arterial walls via increasing endothelial permeability. Once in the subendothelial space LDL particles become oxidised and chemoattractant, encouraging monocytes to adhere which then convert to macrophages. Together, these processes enhance the uptake of further lipoprotein particles leading to a build-up of intracellular cholesterol and layers of smooth muscle cells. Eventually plaques become unstable and frequently rupture. Triglyceride as well as LDL-C and high-density lipoprotein cholesterol (HDL-C) have been shown to be independent predictors of atherosclerosis. Low rather than high HDL-C levels associates with premature atherosclerosis and high HDL-C has been found to be protective. High triglyceride levels are often correlated with low HDL-C (Jacobsen *et al*., 2020).

Although atherosclerosis and cardiovascular health are the main concern with elevated serum triglycerides there are other health issues associated with hypertriglyceridemia that might also benefit from flattening the fructose spike.

Non-alcohol fatty liver disease or Liver Steatosis

Non-alcohol fatty liver disease, although so far in limited studies, not demonstrated to be caused by fructose at normal levels of consumption (see 1.0 Introduction, 1.1.4 Effects of fructose on obesity, Liver fat- steatosis), may ~~perhaps~~ be prevented to some extend by a reduction in plasma triglycerides. Even where lipid accumulation in the liver (steatosis) is not noted, an increase in triglyceride synthesis in the cell can promote endothelial reticulum (ER) stress, as the ER becomes overloaded with lipids during the synthesis of VLDLs (Dewdney *et al*., 2020). ER stress has been associated with non-alcoholic fatty liver disease through the induction of SREBP1-1c (sterol regulatory element-binding protein 1c) (Kammoun *et al*., 2009), which is central to hepatic lipogenesis induction (Shimano *et al*., 1999) as well triglyceride

storage (Brown *et al*., 2010). Even if fructose is found not to cause steatosis at moderate levels, ~~perhaps~~ it could stress the condition into becoming more serious and progressing to a disease status.

The protein Apolipoprotein C-III (ApoCIII) found in triglyceride-rich proteins such as VLDLs has also been found to rise in response to fructose and may be causally linked to lipid dysregulation of the liver and ultimately to increased steatosis (Hieronimus *et al.,* 2020). However, as discussed short-term studies have not shown lipid accumulation at normal doses of fructose. ~~Perhaps~~ This could be ~~is~~ because dysregulation rather than simple storage of lipid is a longer-term process.

It has also been noted that, contrary to fructose, glucose does not induce a rise in ApoCIII. It is speculated that this might be because fructose partly causes an increase in triglycerides through a lack of insulin secretion (Sun & Empie, 2012, Steenson *et al*., 2020) . Reduced insulin levels lead to reduced lipoprotein lipase and therefore reduced triglyceride clearance by adipose tissue (Sun & Empie, 2012) (see 1.0 Introduction, 1.2. Potential mechanisms to explain the negative impact of fructose on health, 1.2.4 Decreased lipid clearance- contribution to elevated plasma triglyceride). Glucose shows a strong insulin response which supresses ApoCIII (Hieronimus *et al.,* 2020). That fructose increases ApoCIII, Hieronimus *et al* suggest, is evidence of the involvement of a “reduced insulin” mechanism for some of the negative impacts of circulating triglyceride and lipid dysregulation. Flattening the fructose spike will not affect insulin levels and will therefore not help where this is the mode of action.

Cancer

As often a component of metabolic syndrome, triglycerides are considered to be linked and possibly even causal to the risk of developing cancer (Ulma *et al.,* 2009). A number of studies have revealed associations between high triglycerides levels and several cancers: colorectal , thyroid cancer, cervix, endometrial, and bladder (Tulinius *et al.,* 1997; Ulma *et al.,* 2009); rectal (Kono *et al.,* 1990; Bird *et al.,* 1996; Otani *et al.,* 2006; Tabuchi *et al.,* 2006; Yamada *et al.,*1998); thyroid ((Tulinius *et al.,* 1997; Ulma *et al.,* 2009); prostate (Wuemli *et al.,* 2005); and gynaecological cancers (cervix, ovary, endometrial) (Tulinius *et al.,* 1997; Cust *et al.,* 2007).

Correlation though of course is not necessarily evidence of cause, although it may be an indication that closer examination is needed. Elevated triglycerides may be just a symptom of other pro-carcinogenic metabolic aspects e.g. high glucose levels are linked to a number of cancers. However, Ulma *et al.,* 2009 in their studies, controlled for such confounding factors. It is not clear how triglycerides might be a causal factor in cancers. One possible mechanism is through inflammation (Esteve *et al.,* 2005; Kundu and Surh, 2008). Others include bile acid excretion, hormones or supply of energy which may have an involvement in colorectal cancers (MckKeown-Eyssen,1994).

Alternatively, as discussed above for steatosis, increase in triglyceride synthesis in the cell can promote ER stress as the ER becomes overloaded with lipids during the synthesis of VLDLs (Dewdney *et al.,* 2020). Cells that proliferate rapidly have evolved an unfolded protein response (UPR) as an ER stress response. ER stress is dampened by increasing expression of protein-folding chaperones and limiting protein translation. Long-term stress though, can lead to the response inducing programmed cell death, which contributes to a number of pathological outcomes, including cancer. Cancer cells have high growth rates for their cell type, so also need a higher activity of their protein folding assembly. Studies suggest the UPR is a key survival mechanism in cancer cells, as they express a number of UPR proteins to an elevated level (Ozcan and Tabas, 2012). Over-expression of such markers has been shown to induce myeloma growth in transgenic mice (Carrasco *et al.,* 2007).

Pancreatitis

High triglycerides may also be associated with organ failure in acute pancreatitis (AP) (Nawaz *et al.,* 2015). AP is the most common gastrointestinal disorder requiring hospitalisation in the US, and severe AP generally involving persistent organ failure develops in 15-20% of patients. The mechanism by which triglycerides could cause such organ failure is not clear, but in the main it is thought that biotoxicity may be involved, as during AP injury, pancreatic lipases are released into the blood and digest circulating triglycerides and adipose tissue, releasing fatty acids. Where triglycerides are already high, free fatty acids overwhelm disposal

mechanisms leading to the free fatty acid concentration reaching levels which cause tissue damage through mitochondrial stress (Zhang *et al.,* 2012; Chang *et al.,* 2008; Chang *et al.,* 2009) and a storm of cytokines induced by an inflammatory cascade resulting in multiple organ failure (Navina *et al.,* 2011). Some have suggested that the rise in triglycerides occurs following the onset of AP and is therefore as a result of lipase hydrolysis of adipose tissue (Dominguez-Munoz et a., 1995). However, in the study by Nawaz *et al.*, 2015, patients were enrolled and assessed early in their diagnosis helping to negate this challenge and no significant difference in clinical outcomes was found between AP patients grouped by the timing of the triglyceride level measurement. They also found that the association between organ failure and triglycerides was independent of other relevant clinical factors such as obesity and diabetes, and ~~showed a proportional trend i.e.~~ the higher the serum triglycerides, the greater the likelihood for persistent organ failure tended to be. In fact, hypertriglyceridemia has been recognised as the third leading cause of pancreatitis in Western countries and the second in China (Chen *et al*., 2021). As the prevalence of blood triglyceride increases so does that of AP (Petro *et al.,* 2019).

Internationally a standard therapy for hypertriglyceridemic pancreatitis has not been established (Lee *et al*., 2019). As the greater the triglyceride level the greater the incidence of AP, early suppression of circulating triglycerides in the blood has become the major therapy approach (Sandhu *et al*., 2011) (Chen *et al*., 2021). Recent studies have suggested that even mild to moderate levels of triglycerides can give an increased risk of AP (Jacobsen *et al*. 2020). To assess whether interventions such as flattening the fructose spike would be beneficial in such circumstances, further studies would be necessary.

Gestational diabetes and pre-eclampsia

Abnormal levels of TAGs have been found to be associated with pregnancy complications (Wiznitzer *et al*., 2009). In a study of 9911 healthy women assessed, both before and after pregnancy, 1209 suffered gestational mellitus or preeclampsia. The occurrence in those with higher triglyceride (upper quartile) at 20% was substantially greater than those with lower quartile triglyceride levels with only 7% occurrence. In a review by Ray *et al*., 2006, of a total 22 studies it was also

concluded that hypertension during pregnancy is associated with elevated triglyceride levels.

Gestational diabetes (where women without diabetes develop high blood sugar during pregnancy) leads to an increased risk of pre-eclampsia, depression and the requirement of Caesarean section (Jameil *et al.,* 2014). There is also a risk that the baby will be born too large, have jaundice and low blood sugar and may even be still born (Jameil *et al.,* 2014). In the longer term, type 2 diabetes and being overweight are also risks for the resultant children (Jameil *et al*., 2014). Pre-eclampsia is characterized mainly by the onset of high blood pressure in the mother during pregnancy, and effects up to 8% of pregnancies worldwide (WHO, 2011). The condition puts those who have suffered at an increased risk of heart disease and stroke in later life (Steegers *et al.,* 2010). Lipid levels during gestation normally vary substantially, nearly quadrupling by the end of pregnancy. However, initially levels of triglycerides decrease in the first trimester of pregnancy and then increase gradually but substantially during the second, peaking before delivery. The mechanisms involved are not fully understood, but the estrogen/progesterone ratio during gestation is thought to be an important factor in lipoprotein metabolism. Estrogens have been shown to increase triglyceride concentrations through upregulation of production by the liver (Mazurkiewicz *et al.,* 1994 and Satter *et al.,* 1997). Whilst progesterone action on lipoprotein has been shown to oppose that of estrogen (Desoye *et al.,* 1987). The ratio is low in the first trimester leading to low triglycerides. Three possible mechanisms have been proposed for an association between pre-eclampsia and dyslipidaemia. Oxidative stress caused by elevated blood lipid may induce endothelial dysfunction leading to a decreased release of prostacyclin is one hypothesis (Kaaja *et al.,* 1995). Dysregulation of lipoprotein lipase leading to a lipidemic profile is the basis of a second. This latter is supported by the observation that serum from women with pre-eclampsia had both a higher ratio of free fatty acids to albumin and increased lipolytic activity, resulting in enhanced endothelial uptake of free fatty acids, which are further esterified to triglycerides (Endresen *et al.,* 1992 and Lorentzen *et al.,* 1995). Thirdly, metabolic syndrome may play a role, as hyperinsulinemia has also been shown to associate with pre-eclampsia (Kaaja *et al.,* 1997).

It would seem that studies that have found an association between triglyceride levels and such gestational complications (see above) have only monitored triglyceride levels during pregnancy, at the earliest after 10 weeks. It is not known therefore whether pre-pregnancy triglyceride and therefore dietary levels associate with such complications, or, based on the possible mechanisms discussed above, whether the highly variable triglyceride levels during pregnancy are influenced by diet. To assess whether interventions such as flattening the fructose spike would be beneficial in such circumstances, further studies would be necessary.

Kidney disease

Kidney disease and blood triglycerides have also been linked. Studies have shown that in chronic kidney disease (CKD), triglyceride levels are raised and rise further as the disease advances (Wang *et al.,* 2015). Possible mechanisms include a reduction in the activity of enzymes involved in the metabolism of the triglyceride-rich lipoproteins, resulting in a longer circulatory life span; such has been proposed and supported with evidence (Soohoo *et al.,* 2019). It would seem therefore that as CKD is thought to be a cause of increased triglycerides rather than triglycerides a risk factor for CKD, there would be no advantage in flattening the fructose spike to reduce triglycerides in this disease case. However, there is evidence suggesting that blood lipid-lowering interventions found to help reduce cardiovascular disease risk, may also be beneficial in patients with CDK and prevent progression of renal disease as a result of the reduced cardiovascular disease risk (Jun *et al.,* 2012). This would suggest that other triglyceride-lowering methods ~~technology~~ e.g. plant compounds and extracts might also help prevent CDK and progression of renal disease.

Gout

Many studies have reported an association between gout and dyslipidaemia such as hypertriglyceridemia (Matsubara *et al.,* 1989; Emmerson *et al.,* 1998; Chen *et al.,* 2013; Peng *et al.,* 2015). However, as to whether the relationship between such dyslipidaemia and gout is causal has been in doubt. Recently through a Mendelian randomisation (see below) of a large-scale genome-wide association study (GWAS)

was performed to explore this issue (Yu *et al.,* 2021). They found that HDL was negatively associated with gout, and independently, triglycerides positively associated with serum urate. The analysis indicated that HDL, in addition to having a direct effect on gout, also did so by reducing urate. Triglycerides affected gout by elevating urate levels, and the analysis confirmed that urate directly increased the risk of gout. They conclude that "…triglyceride levels can directly and indirectly elevate the risk of gout.

The Mendelian randomization methodology used in this work has become a powerful and potent tool for identifying causal relationships otherwise not possible from observational studies. A GWAS on its own can only establish an association, not a causal link. Randomized controlled trials are generally used to delineate causality, however these are expensive, time-consuming laborious and often difficult to justify ethically. Mendelian randomisation allocates variants in a way analogous to a randomised controlled trial, and as such is less susceptible to confounders than non-randomised observational studies (Yu *et al.,* 2021). As strong evidence has been presented as to the causality of triglycerides for gout, direct or indirect, triglyceride lowering ~~technology~~ plant natural compounds and extracts for flattening the fructose spike may ~~perhaps~~ be considered as a potential preventative measure.

Cost burden

In addition to the health concerns of high triglycerides there is also a substantial cost burden and pressure on health services. A study estimated the cost of high triglyceride-related cardiovascular disease or diabetes in adults on statins in the US. They found a projected annual incremental cost burden associated with high triglycerides in patients with diabetes or CVD on statins of $10.7 billion.

Flattening the fructose spike would ~~perhaps~~ not be a recommended therapeutic strategy in extreme cases but could ~~perhaps~~ contribute to a prevention-based approach in the general diet before triglycerides become elevated to such levels, particularly as high blood triglycerides often may not cause any symptoms.

Treatment of hypertriglyceridemia

Changes in lifestyle are the first steps in any therapy aiming to reduce triglycerides, including avoidance of sugar. Medication such as niacin has shown limited benefit (Jacobsen at al., 2020). A number of potentially beneficial drugs are currently under development with further trials. Statins have become a strong preference as they can give up to 27% reduction in triglyceride levels by inhibiting HMG-CoA reductase and are found to reduce cardiovascular risk (Catapano *et al.,* 2016; Adams *et al.,* 2014). Fibrates have also demonstrated some potential, reducing both fasting and postprandial triglyceride-rich lipoproteins by greater than 50%. However, the latter have not performed well in clinical studies *vs.* statins (Catapano *et al.,* 2016). The omega-3 fatty acid icosapentyl ethyl, at a very high dose of 4 g has shown success in some studies, used in conjunction with significant and existing statin treatment (Bhatt *et al.,* 2019). Possibly via a mechanism involving interaction with PPARs, the *n*-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA have been shown to reduce triglyceride concentrations by up to 45%. However, such omega-3-fatty acids under meta-analysis have not shown a benefit on a number of cardiovascular outcomes. Further studies are on-going in randomised placebo-controlled trials. If successful, omega-3 fatty acids could be combined with other therapies e.g. statins and fibrates, which are tolerated well and are safe (Kotwal *et al.,* 2016; AstraZeneca study, 2016; Amarin Pharma Inc study, 2016). IONISANGPTL3-LR is a new drug that targets a reduction in triglycerides by action on lipoprotein lipase to presumably improve clearance and is still going through trial tests (Berghean *et al.,* 2017).

Such drug-orientated treatments however, could suffer from side effects. Statins for example, have given concern over side effects such as muscle pain, diabetes and abnormal blood levels of liver enzymes (Naci *et al.,* 2013). Rhabdomyolysis (a form of muscle damage) is a rare but severe side effect, involving destruction of muscle cells. Although only occurring in 0.1% of patients, if not treated, it can result in fatalities due to kidney disease (Baigent *et al.,* 2010). Older age and interaction with medications such as fibrates has been shown to increase the risk of rhabdomyolysis (Valiyil *et al.,* 2010). As such, preventative therapies or measures that are safe, such as plant naturals with some history of use, have featured in sourcing potential actives to flatten the fructose curve in this thesis.

Appendix II Fructose metabolism in the intestine

Fructokinase exists in two alternatively splice forms, ketohexokinase-C (KHK-C) and KHK-A. Whereas both KHK-C and KHK-A can metabolize fructose, KHK-C is considered the primary enzyme involved in fructose metabolism due to its lower Km of 0.8 mM. (Asipu *et al*., 2003; Khitan & Kim, 2013). The liver has the highest expression of KHK-C, however, the small intestine also has considerable expression (Jang *et al*., 2020). In a study by Lapansa *et al*., 2013 using small intestinal KHK-C knockout mice, they saw increased uptake of fructose by the liver leading to lipid accumulation. Where KHK-C was overexpressed in mice intestine, liver steatosis was reduced. These experiments suggest that fructose is metabolised by intestine enterocytes, and only when fructose intake overwhelms the intestine's capacity for metabolism will the liver be exposed to significant levels of fructose. As well as a low Km for fructose, fructokinase is also characterized by having a high Vmax occurring at approximately 100 mM (Adelman *et al*., 1967, Heinz *et al*., 1968), and therefore, it might be expected that the enzyme is rarely saturated. However, fructose clearly does reach the liver in sufficient quantities when fructose intake is high. So how does fructose metabolism become overwhelmed in the small intestine? In liver studies, ATP has been seen to be depleted by high fructose levels as fructose is phosphorylated to fructose-1-phosphate rapidly by high affinity fructokinase (see 1.0 Introduction, 1.2. Potential mechanisms to explain the negative impact of fructose on health, 1.2.6 ATP depletion and uric acid production- Oxidative stress and Inflammation). As fructose-1-phosphate builds up, it ~~perhaps~~ could as a product inhibit~~s~~ fructokinase allowing some fructose to be transported into the circulation, rather than be metabolised. ATP depletion is commonly proposed to lead to uric acid synthesis as ADP and AMP levels rise and are then broken down. Uric acid has been associated with oxidative damage in the liver, hypertension and gout where serum levels rise (Sun & Empie, 2012).

Flattening the fructose spike in intestine enterocytes, as well as the liver, may therefore also have significant potential benefits to health. Slowing uptake by the cell would allow greater clearance of fructose within the enterocyte at a rate that does not lead to the build-up of ATP and hence uric acid, as well as limiting transport of fructose into the blood and to the liver. However, if the enterocyte is also a

significant site of fructose metabolism, will slowing down the uptake of fructose by transporters in the intestine lead to a flattened fructose spike reaching the liver?

The level of fructose needed to overwhelm enterocyte metabolic capacity is a key question. Jang *et al*., 2020 in mice applied equal amounts of ^{13}C-fructose and unlabelled glucose at doses from 0.25 g/kg to 2 g/kg of each. Labelled glucose produced from fructose metabolism and gluconeogenesis begins to plateau at a fructose dose of 0.5 g/kg, at which point fructose is seen to rise rapidly in the portal vein. Whilst intestinal labelled fructose-1-phosphate peaked at 0.5 g/kg, in the liver although a lot lower than the intestine (i.e about one twentieth), continued to rise following the 1 g/kg fructose dose. Saturation of gluconeogenesis occurs at about 1 g/kg. They also saw fructose beginning to plateau at 1 g/kg in the portal vein although not quite saturating by 2 g/kg. The explanation offered is that there has been a "saturation of fructose absorption from the gut lumen". This they point out is consistent with the high levels of fructose found in the faeces. This to me seems to be a somewhat surprising observation. It would mean that doses of fructose no greater than 2 g/kg would increase the fructose spike experienced by the liver in the hepatic portal vein. Based on a conversion using relative *body surface areas this equates to an approximately 9.7 g dose for a 60 kg human adult (Reagan-Shaw et a., 2009).

[*Body surface area normalisation has been found to work well in number of animal species and biometrics such as calorie expenditure and oxygen utilisation). Body weight (kg) is divided by body surface area (m^2) to give a Km value (kg per m2, not the Michaelis constant of substrate affinity) for a given species (FDA values for humans and adult mice are 60 kg/1.6m^2 =37.5 and 0.02 kg/0.007m^2 =2.8 respectively. Converting a dose in a mouse to a human equivalent dose is achieved by: mouse dose x mouse Km (3)/Human Km (37).

Jang *et al.,* 2020 use a simpler method to estimate human equivalence based on calorie intake. For a mouse, a dose of 0.5 g/kg is approximately 0.5% of daily calorie intake. In a human that would be about 3 g fructose. 2 g/kg would therefore be approximately 12 g fructose i.e. of a similar order to that estimated by body surface area.]

Doses ≥50 g/day fructose have been consistently found to lead to raised levels of triglycerides in humans (see 1.0 Introduction, 1.1.3 Effects of fructose on blood lipids, Effects on post-prandial triglycerides). Consumed over three portions a day of approximately 17 g/dose it can be seen that intestinal metabolism of fructose is saturated for each dose and the liver would therefore receive significant levels of fructose per dose. In addition, it would also suggest that significant quantities of fructose would not be absorbed and reach the microbiome of the lower gut, in mice at least. However, studies in mouse pups have shown that previous exposure to fructose ~~boosts~~ enhances absorption of fructose and genes associated with its metabolism and thus clearance (David *et al.,* 1995; Cui *et al.,* 2004; Patel *et al.,* 2015). Jang *et al.,* 2020 therefore looked to see if such also occurred in adult mice. They fed mice once a day 2 g/kg for 5 days and sampled on days 1, 3 and 5. On the third day elevated levels of circulating fructose and glucose were seen compared to day 1. No further increase occurred on day 5. Faecal levels of fructose were much lower.

They did not establish saturation levels of fructose conversion to glucose or for fructose emergence in the portal vein, although levels of fructose in the portal vein substantially increased after the first day, suggesting that levels greater than 2 g/kg would be required to saturate the fructose spike reaching the liver. This would equate to greater than 10-12 g/dose in humans (by body weight area). Some data though suggests that the situation in humans might be quite different. With fasting triglyceride, at fructose doses higher than 100 g/day, a dose-response effect has been observed, with increasing doses leading to increases in triglyceride concentrations (Livesey & Taylor, 2008). Dose dependency has also been noted with respect also to the type of outcome measured. In several controlled intervention studies at ≥50 g/day, increases in postprandial triglyceride are seen, and at ≥150 g/day, increases in hepatic lipid accumulation are seen (Tappy *et al.,* 2021).

So, in answer to the question - will slowing the uptake of fructose from the gut flatten the fructose spike reaching the liver? Jang *et al.,* 2020 suggested that "…slower fructose intake to match the intestinal capacity can alleviate [liver lipogenesis] syndromes." As such, it does~~, perhaps,~~ seem reasonable to propose that if the rate of uptake of fructose by the enterocyte does not exceed the cell's capacity to metabolise fructose, then less fructose will be transported into the blood and reach

the liver. Some of the fructose will have been converted to glucose, which can also cause lipogenesis in the liver. However, as discussed, glucose uptake by the liver is feedback-inhibited and thus uptake and metabolism by the liver will be limited, as studies tend to show.

Chylomicrons

In the intestine, both fructose and glucose have been reported to stimulate *de novo* lipogenesis (DNL), increase triglyceride synthesis and lead to the release of lipoprotein into the circulation as chylomicrons (Hoffman, 2019).

The idea that enterocytes have the enzymes needed to synthesis and release chylomicron triglycerides-rich lipoprotein particles into circulation is not new (Haidari *et al.,* 2002). However, the role of chylomicrons in elevated triglyceride levels with respect to health concerns has not been significantly considered until recently (Tappy, 2021). As discussed above, the vast majority of a small single dose of fructose i.e. <5-6 g estimated human equivalent dose, is metabolised in the intestine (Jang *et al.,* 2018). Enterocytes have also been reported to store lipid droplets after a meal for a number of hours and release the lipid as chylomicrons into the circulation after a following meal. This it is suggested, can substantially add to the overall quantity of triglyceride (Robertson *et al.,* 2003). Tappy (2021) speculates that "…[chylomicrons] may be associated with chronic elevation of triglyceride-rich lipoproteins and of their remnants in the blood, which may… be responsible for initiating atherosclerosis". In particular, enterocyte chylomicron and triglyceride production is seen in patients with symptoms of metabolic syndrome in comparison to non-over weight controls (Shojaee-Moradie *et al.,* 2013).

Studies have demonstrated an increase in the specific serum chylomicron *apolipoprotein ApoB48, in response to fructose feeding, distinct from ApoB100 of VLDL and LDL particles (Hoffman *et al.,* 2019, review). However, the proportion of triglycerides from the intestine relative to total triglycerides in circulation following a fructose or other meal does not appear to have been estimated. Using healthy subjects, Xaio *et al.,* (2013) showed that with fructose+lipid compared to saline+lipid (constant infusion of 20%, 60 ml/hr for 10 hrs), after several hours there was significantly higher (approximately +1 mg/L, i.e. approximately +50%) level of

triglyceride-rich lipoprotein ApoB48, and a significantly higher (approximately +20 mg/L, i.e. approximately +25%) level of triglyceride-rich lipoprotein ApoB100. Overall, there was approximately 80-100 mg/L ApoB100 and 2-4 mg/L ApoB48. The intestinal chylomicron contribution was therefore very small compared to liver triglyceride both for the increase induced by fructose and in the circulation in total. Glucose+lipid (constant infusion of 20%, 60 ml/hr for 10 hrs) caused a similar increase in ApoB48 triglyceride but only showed a non-significant increase in ApoB100 triglyceride.~~, despite showing a similar trend~~. A "following meal" peak was not seen as meals were by continuous infusion, ~~perhaps~~ possibly explaining the overall low ApoB48 triglyceride levels compared to ApoB100. However, it would have to be a very large post second meal chylomicron surge to compete with the reported 20-fold higher ApoB100 triglyceride. It should also be taken into consideration that the VLDL and LDL triglyceride carry a higher risk of atherosclerosis than chylomicrons. As VLDL distributes triglycerides to tissues around the body, it increases in density and becomes an LDL particle; the so-called "bad" particle that is mostly cholesterol. ApoB, particularly with LDL particles, is considered to be the main cause of plaques and atherosclerosis. Mounting evidence now suggests that ApoB100 is the greater predictor of vascular/heart disease than LDL cholesterol or total cholesterol (Jacobson, 2011; Lim *et al.,* 2011).

Whether flattening the fructose spike in the enterocyte substantially reduces the release of chylomicron triglyceride into the blood or not, it ~~perhaps~~ would seem, given the above, to make little difference anyway in comparison to the quantity of triglyceride from the liver and its inherent greater risk.

[*Apolipoprotein B is the main lipoprotein of triglyceride rich lipoprotein particles such as VLDL, LDL and chylomicrons. It carries lipids (triglyceride and cholesterol) which are otherwise insoluble in the blood to where they are needed in the body].

It should though be mentioned that a study, although in only five over-weight men, showed that in the first measurements ever provided of the rate of intestinal DNL in humans fructose consumption had no effect on the rate of intestinal or DNL (Steenson *et al*., 2020). They also saw lower chylomicron-TAG production rate and chylomicron-TAG fractional catabolic rate with high-fructose consumption which they

suggest shows lower clearance of CM, rather than elevated production may contribute to elevated plasma TAG, possibly due to lower insulin-mediated stimulation of lipoprotein lipase.

Intestinal damage

In the liver, lipid accumulation in the long-term can lead to non-alcoholic fatty acid disease and ultimately, if provoked, inflammation and non-alcoholic steatohepatitis (NASH). Tappy, (2021) hypothesises "…that the same process could occur in enterocytes leading to lipotoxicity, the consequences of which remain to be assessed." One major cause of the progression of non-alcoholic fatty liver disease and steatosis in the liver is over-nutrition, particularly with fructose, according to the Asian-Pacific Working Group on NAFLD (Wee *et al.,* 2013; Lee *et al*., 2014). Of the tissues that can metabolise fructose, enterocytes are exposed to very much the highest levels, ~~up to perhaps~~ which could equate to approximately 25 mM, compared to 1-8 mM for the liver and up to approximately 0.5 mM in the kidney (see 4.0 ~~Technology~~ Plant natural compounds and extracts to flatten the fructose spike, 4.1 Introduction). It is ~~perhaps~~ not surprising therefore that fructose has been suggested as a main dietary cause of intestinal damage and a leaky barrier (Overview by Binienda, *et al.,* 2020) as this could happen in a number of ways. One might be lipid accumulation in the intestine due to fructose-induced lipid synthesis. A second may be inflammation due to ATP depletion leading to uric acid synthesis, which has been associated with oxidative damage in the liver (see above 3.2.4 Fructose metabolism in the intestine; Sun & Empie, 2012) . Another possibility might be through interaction with the gut microbiome, discussed below.

Gut Microbiome-

Acetate and liver steatosis

High fructose intake has been seen by a number of studies to impair the gut barrier function leading to gut bacteria and toxins leaking into the circulation (Bergheim *et al.,* 2008; Cho *et al.,* 2019). Glucose has been found to cause less impairment despite being responsible for greater weight gain, suggesting that such damage is independent of energy intake (Bergheim *et al.,* 2008; Cros *et al.,* 2020; Tappy and

Rosset, 2019). High levels of fructose have also been associated with the occurrence of short-chain fatty acids in the liver (Zhou and Zhong, 2017). As discussed above, the small intestine is now thought to metabolise the majority of a dose of fructose up to 5-6 g (Jang *et al.,* 2018) (i.e. about one-third of each dose that might contribute to a daily intake of 50 g over for instance three courses (50 g/day being the average added fructose intake in the USA, see 1.0 Introduction, 1.1.1 Pre-literature review issues, Fructose dose- physiological levels). In these studies, if intestinal metabolism is saturated, some fructose passes through to the lower gut where it is likely to be metabolised to short-chain fatty acids such as acetate by the gut flora and is absorbed into the circulation. This suggests an alternative way that fructose can induce hepatic lipid synthesis and accumulation. Antibiotics have been shown to limit liver steatosis in high fructose-fed mice (Bergheim *et al.,* 2008; Brandt *et al.,* 2017). Germ-free mice fed high fructose diets showed reduced hepatic triglycerides compared to the same diet in control mice (Kaden-Volynets *et al.,* 2019), also suggesting that gut microbes are at least partly responsible for liver steatosis and hypertriglyceridemia. Flattening the fructose spike such that enterocyte fructose metabolism is not overwhelmed may reduce fructose overspill into the lower gut and hence limit short-chain fatty acids such as acetate from fuelling lipid synthesis.

Endotoxins

High fructose intakes have been shown to disrupt the gut microbiome, promoting bacterial species that produce the endotoxins found in blood circulation (Volynets *et al.,* 201)**.** Such endotoxins are also suspected of damaging the gut barrier. As mentioned above antibiotics have been shown to limit gut barrier damage in high fructose-fed mice (Bergheim *et al.,* 2008; Brandt *et al.,* 2017).

Gut barrier damage has been associated with a number of pathologies including inflammation of joints, cardiometabolic disorders, inflammatory bowel disease, irritable bowel disease (Bischoff *et al.,* 2021), chronic fatigue, schizophrenia and depression (Simeonova *et al.,* 2018). As such, flattening the fructose spike in the small intestine, reducing spill-over of fructose into the lower gut, not promoting “bad” bacteria that produce endotoxins, and limiting gut membrane damage, may have the

potential to protect from a number of these health issues.

Appendix III Two-way ANOVA stats "Triglycerides"

Table Analysed: **(a) Figure 3.9 Expt1 2 mM Fruc *vs*. 1 mM, for 6-9 mM glucose**

Two-way ANOVA	Ordinary			
Alpha	0.05			

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	12.29	0.2266	ns	No
Row Factor	16.41	0.1343	ns	No
Column Factor	30.55	0.0032	**	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.06619	3	0.02206	F (3, 16) = 1.609	P=0.2266
Row Factor	0.08834	3	0.02945	F (3, 16) = 2.147	P=0.1343
Column Factor	0.164	1	0.1645	F (1, 16) = 12.00	P=0.0032
Residual	0.2194	16	0.01371		

Difference between column means

Mean of Fru 2mM 1hrs	0.2092
Mean of Fru 1mM 2hrs	0.04364
Difference between means	0.1656
SE of difference	0.04780
95% CI of difference	0.06423 to 0.2669

Data summary

Number of columns (Column Factor)	2
Number of rows (Row Factor)	4
Number of values	24

Table Analysed: **(b) Figure 3.10 Expt.2 "meal" 1: 2 mM Fruc *vs*. 1 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	1.326	0.9467	ns	No	
Row Factor	59.10	0.0005	***	Yes	
Column Factor	2.505	0.2587	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	8364	4	2091	F (4, 20) = 0.1788	P=0.9467
Row Factor	372928	4	93232	F (4, 20) = 7.973	P=0.0005
Column Factor	15804	1	15804	F (1, 20) = 1.352	P=0.2587
Residual	233867	20	11693		
Difference between column means					
Mean of 2mM Fructose 1hrs	97.64				
Mean of 1mM Fructose 2hrs	51.73				
Difference between means	45.90				
SE of difference	39.49				
95% CI of difference	-36.46 to 128.3				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row Factor)	5				
Number of values	30				

Table Analysed: **(c) Figure 3.11 "meal" 1: 8 mM fruc *vs.* 4 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	20.37	0.0572	ns	No
Row Factor	33.89	0.0088	**	Yes
Column Factor	8.640	0.0433	*	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	161931	4	40483	F (4, 20) = 2.745	P=0.0572
Row Factor	269508	4	67377	F (4, 20) = 4.568	P=0.0088
Column Factor	68698	1	68698	F (1, 20) = 4.658	P=0.0433
Residual	294993	20	14750		

Difference between column means

Mean of 8mM Fructose 1hrs	191.7
Mean of 4mM Fructose 2hrs	95.98
Difference between means	95.71
SE of difference	44.35
95% CI of difference	3.201 to 188.2

Data summary

Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	30

Table Analysed: **(d) Figure 3.12 "meal" 2: 2 mM fruc *vs.* 1 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	2.792	0.8719	ns	No	
Row Factor	19.64	0.1137	ns	No	
Column Factor	31.64	0.0014	**	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	78940	4	19735	F (4, 20) = 0.3039	P=0.8719
Row Factor	555263	4	138816	F (4, 20) = 2.138	P=0.1137
Column Factor	894581	1	894581	F (1, 20) = 13.78	P=0.0014
Residual	1298713	20	64936		
Difference between column means					
Mean of 2mM Fruc 1hr	534.7				
Mean of 1mM Fruc 2hr	189.3				
Difference between means	345.4				
SE of difference	93.05				
95% CI of difference	151.3 to 539.5				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row Factor)	5				
Number of values	30				

Table Analyzed: **(e) Figure 3.13 “meal” 2: 8 mM fruc *vs.* 4 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	4.653	0.5843	ns	No	
Row Factor	9.377	0.2504	ns	No	
Column Factor	53.94	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	75004	4	18751	F (4, 20) = 0.7263	P=0.5843
Row Factor	151151	4	37788	F (4, 20) = 1.464	P=0.2504
Column Factor	869416	1	869416	F (1, 20) = 33.68	P<0.0001
Residual	516324	20	25816		

Difference between column means	
Mean of 8mM Fructose 1hrs	468.8
Mean of 4mM Fructose 2hrs	128.3
Difference between means	340.5
SE of difference	58.67
95% CI of difference	218.1 to 462.9

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	30

Table Analysed(f) Figure 3.14 “meal” 3: 2 mM fruc *vs*. 1 mM, 6 – 14 mM glucose

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	3.916	0.6651	ns	No	
Row Factor	0.2979	0.9957	ns	No	
Column Factor	63.30	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	5406	4	1352	F (4, 20) = 0.6027	P=0.6651
Row Factor	411.3	4	102.8	F (4, 20) = 0.04585	P=0.9957
Column Factor	87385	1	87385	F (1, 20) = 38.97	P<0.0001
Residual	44853	20	2243		
Difference between column means					
Mean of 2mM Fruc 1hr	186.4				
Mean of 1mM Fruc 2hr	78.49				
Difference between means	107.9				
SE of difference	17.29				
95% CI of difference	71.87 to 144.0				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row Factor)	5				
Number of values	30				

Table Analysed: **(g) Figure 3.15 "meal" 3: 8 mM fruc *vs*. 4 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	11.93	0.2252	ns	No	
Row Factor	7.190	0.4630	ns	No	
Column Factor	42.49	0.0001	***	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	30481	4	7620	F (4, 20) = 1.554	P=0.2252
Row Factor	18371	4	4593	F (4, 20) = 0.9365	P=0.4630
Column Factor	108570	1	108570	F (1, 20) = 22.14	P=0.0001
Residual	98084	20	4904		
Difference between column means					
Mean of 8mM Fructose 1hrs	310.5				
Mean of 4mM Fructose 2hrs	190.2				
Difference between means	120.3				
SE of difference	25.57				
95% CI of difference	66.98 to 173.7				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row Factor)	5				
Number of values	30				

Appendix IV Two-way ANOVA stats cytotoxicity

Table Analysed: **(a) Figure 3.16 "meal" 1: 2 mM fruc *vs.* 1 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	6.349	0.6884	ns	No	
Row Factor	37.55	0.0293	*	Yes	
Column Factor	0.2691	0.7594	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.002209	4	0.0005522	F (4, 20) = 0.5686	P=0.6884
Row Factor	0.01306	4	0.003266	F (4, 20) = 3.363	P=0.0293
Column Factor	9.363e-005	1	9.363e-005	F (1, 20) = 0.09641	P=0.7594
Residual	0.01942	20	0.0009712		
Difference between column means					
Mean of 2mM Fructose 1hrs	0.07580				
Mean of 1mM Fructose 2hrs	0.07227				
Difference between means	0.003533				
SE of difference	0.01138				
95% CI of difference	-0.02020 to 0.02727				
Data summary					
Number of columns (Column Factor)	2				
Number of rows (Row Factor)	5				
Number of values	30				

Table Analysed: **(b) Figure 3.17 "meal" 1: 8 mM fruc *vs*. 4 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	16.70	0.0242	*	Yes	
Row Factor	42.03	0.0003	***	Yes	
Column Factor	17.71	0.0009	***	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.002876	4	0.0007191	F (4, 20) = 3.546	P=0.0242
Row Factor	0.007239	4	0.001810	F (4, 20) = 8.924	P=0.0003
Column Factor	0.003050	1	0.003050	F (1, 20) = 15.04	P=0.0009
Residual	0.004056	20	0.0002028		
Difference between column means					
Mean of 8mM Fructose 1hrs			0.1103		
Mean of 4mM Fructose 2hrs			0.09013		
Difference between means			0.02017		
SE of difference			0.005200		
95% CI of difference			0.009320 to 0.03101		
Data summary					
Number of columns (Column Factor)			2		
Number of rows (Row Factor)			5		
Number of values			30		

Table Analysed: **(c) Figure 3.18 "meal" 2: 2 mM fruc *vs*. 1 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				

Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	20.47	0.1166	ns	No	
Row Factor	29.03	0.0432	*	Yes	
Column Factor	2.135	0.3586	ns	No	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.0002973	4	7.432e-005	F (4, 20) = 2.116	P=0.1166
Row Factor	0.0004216	4	0.0001054	F (4, 20) = 3.000	P=0.0432
Column Factor	3.101e-005	1	3.101e-005	F (1, 20) = 0.8828	P=0.3586
Residual	0.0007025	20	3.513e-005		

Difference between column means	
Mean of 2mM Fructose 1hrs	0.01420
Mean of 1mM Fructose 2hrs	0.01623
Difference between means	-0.002033
SE of difference	0.002164
95% CI of difference	-0.006548 to 0.002481

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	30

Table Analyzed: **(d) Figure 3.19 "meal" 2: 8 mM fruc *vs*. 4 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				

Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	22.73	0.0326	*	Yes	
Row Factor	17.24	0.0775	ns	No	
Column Factor	25.18	0.0011	**	Yes	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.0009059	4	0.0002265	F (4, 20) = 3.262	P=0.0326
Row Factor	0.0006868	4	0.0001717	F (4, 20) = 2.473	P=0.0775
Column Factor	0.001003	1	0.001003	F (1, 20) = 14.45	P=0.0011
Residual	0.001389	20	6.943e-005		

Difference between column means	
Mean of 8mM Fructose 1hrs	0.02787
Mean of 4mM Fructose 2hrs	0.01630
Difference between means	0.01157
SE of difference	0.003042
95% CI of difference	0.005220 to 0.01791

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	30

Table Analysed: **(e) Figure 3.20 “meal” 3: 2 mM fruc *vs.* 1 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				

Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	26.78	0.0141	*	Yes	
Row Factor	37.30	0.0032	**	Yes	
Column Factor	3.098	0.1847	ns	No	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.0009034	4	0.0002258	F (4, 20) = 4.078	P=0.0141
Row Factor	0.001259	4	0.00031	F (4, 20) = 5.682	P=0.0032
Column Factor	0.0001045	1	0.0001045	F (1, 20) = 1.888	P=0.1847
Residual	0.001108	20	5.538e-005		

Difference between column means

Mean of 2mM Fructose 1hrs	0.02700
Mean of 1mM Fructose 2hrs	0.03073
Difference between means	-0.003733
SE of difference	0.002717
95% CI of difference	-0.009401 to 0.001935

Data summary

Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	30

Table Analysed: **(f) Figure 3.21 "meal" 3: 8 mM fruc *vs*. 4 mM, 6 – 14 mM glucose**

Two-way ANOVA	Ordinary				
Alpha	0.05				

Source of Variation	% of total variation		P value	P value summary	Significant?
Interaction	19.22	0.0213	*	Yes	
Row Factor	37.60	0.0009	***	Yes	
Column Factor	16.97	0.0018	**	Yes	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.0007295	4	0.0001824	F (4, 20) = 3.668	P=0.0213
Row Factor	0.001427	4	0.0003566	F (4, 20) = 7.173	P=0.0009
Column Factor	0.0006440	1	0.0006440	F (1, 20) = 12.95	P=0.0018
Residual	0.0009943	20	4.972e-005		

Difference between column means	
Mean of 8mM Fructose 1hrs	0.03240
Mean of 4mM Fructose 2hrs	0.02313
Difference between means	0.009267
SE of difference	0.002575
95% CI of difference	0.003896 to 0.01464

Data summary	
Number of columns (Column Factor)	2
Number of rows (Row Factor)	5
Number of values	30

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