Fructose consumption aggravates dysregulation of postprandial lipid metabolism in obese hypertriglyceridemic men with high cardiometabolic risk profile and associates with liver fat deposition

Short title: Visceral/liver fat, postprandial lipemia, de novo lipogenesis and fructose-containing beverages

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Objectives

This project focuses on the deleterious effects of fructose-containing beverages on lipid metabolism. Our hypothesis is that consumption of fructose-containing beverages increases *de novo* lipogenesis (DNL) with numerous consequences on lipid and glucose metabolism, insulin resistance, inflammation and blood pressure. We further propose that fructose also decreases the removal of triglyceride-rich lipoproteins (TRLs) from the circulation by inhibiting the activity of lipoprotein lipase (LPL), a key enzyme in lipoprotein metabolism. We therefore focus our study on the effects of fructose on postprandial lipemia and features of ectopic fat deposition including visceral adipose tissue and liver fat content which are key features of individuals with metabolic syndrome and type 2 diabetes. So far, little data exist on the patophysiological responses to fructose-containing beverages in obese subjects with multiple cardiometabolic risk factors. We will test the hypothesis that hypertriglyceridemic (HTG) obese subjects are particularly prone to the deleterious effects of fructose-containing beverages compared to normolipidemic (NTG) obese subjects. We have therefore designed our study to differentiate the weight-dependent *vs.* weight-independent effects of fructose-rich diet in HTG and NTG obese subjects.

Background

Obesity has become a contemporary global health hazard affecting not only adults but also adolescents and children. The prevalence of obesity in children as well as in adults is rising rapidly both in USA and Europe. In parallel with obesity, the prevalence of type 2 diabetes and cardiovascular disease (CVD) is increasing dramatically and this portends a new wave of CVD. In the face of this major health problem a better understanding of factors contributing to a positive energy balance and to body fat gain is necessary, particularly regarding factors related to the development of visceral obesity associated with excess ectopic and liver fat, which is the most dangerous form of obesity.

Notably, consumption of sugar-sweetened beverages (SSB) has increased steadily over the past decades and is identified as the major source of added sugar intake accounting for a major part of excess energy intake. A major dietary change over the past decades has in fact been the dramatic increase of fructose intake due to increased consumption of soft drinks and juice beverages. The fructose intake has increased by about 20 % per capita in USA between 1970 and 2005 and the present daily intake (approximately 55 g) accounts for about 10 % of the total daily caloric intake.[1] Recently, large epidemiological studies have documented the association between SSB consumption and increased risk for obesity, type 2 diabetes and CVD.[2-4] Furthermore, recent meta-analyses reported that the risk to develop type 2 diabetes was increased by 26 % in the highest quintile of SSB intake which corresponds to the consumption of 1-2 soft drinks per day.[4]

Strong evidence indicates that added fructose, a component of sucrose and high fructose corn syrup, has specific deleterious cardiometabolic effects as compared to glucose.[5] A high fructose diet is linked with dyslipidemia, insulin resistance/glucose intolerance, visceral obesity and inflammation independently of body weight changes.[6] In addition, fructose has been reported to raise blood pressure when consumed as SSBs whereas glucose does not appear to have such deleterious effect.[7] Thus, all sugars are not equal in respect to their adverse metabolic effects. Of note, fructose derived from fruits and high-fiber cereal products are considered beneficial.[8] It is critically important to recognize the SSBs as the major source of not only extra calories but also of excess fructose. Excessive intake of SSB has turned out to be a serious public health problem and a restriction of SSBs should be a major target in the prevention against obesity, type 2 diabetes and CVD. To succeed in this fight it is critical to better understand the mechanisms by which excess fructose consumption contributes to the health hazards of obesity, particularly in the etiology of the related atherogenic dyslipidemia.

Dietary sugar intake and cardiometabolic health at the population level

Recently, the association of blood lipid levels and consumption of added sugars was studied in the adult population in the National Health and Nutrition Examination Survey (NHANES) (*n*= 6113).[9] In this American cross-sectional study higher fructose consumers had more unfavorable lipid levels, namely significantly lower HDL cholesterol, higher triglycerides and a high ratio of triglycerides to high density lipoproteins (HDL) whereas women also had higher low density lipoprotein (LDL) cholesterol levels. Likewise, in the Framingham study daily soft drink consumers had higher incidence of elevated triglycerides and low HDL cholesterol than non-consumers (relative risk: 1.22 and 1.22, respectively).[10]

Several studies have consistently reported increased responses of fasting and postprandial triglyceride levels and 24 hr profiles to short term feeding of fructose as compared to glucose in both lean and obese subjects.[2, 11] These perturbations directly lead to other lipid abnormalities including elevation of apo-B levels, accumulation of small dense LDL, and increased remnant lipoproteins, combined with reduced HDL cholesterol which all are components of the atherogenic lipid triad, a strong risk factor for CVD. Interestingly, the deleterious effect of fructose on lipid metabolism is directly linked to the daily intake; a fructose intake > 50 g/ day is associated with postprandial hyperlipemia whereas intake above 100 g/day also results in elevation of fasting serum triglycerides.[5] ***Thus, fructose intake is directly linked to an atherogenic dyslipidemia.***

Metabolic consequences of fructose feeding

Fructose is rapidly taken up by the hepatocytes from the portal circulation and found only in micromolar concentrations in the systemic circulation. Therefore, high fructose intake primarily alters hepatic glucose and lipid handling. The low glycemic index of fructose has been considered beneficial especially for diabetic subjects, but recent evidence suggest that the harms of added fructose outweigh its glycemic benefits. Although fructose does not stimulate acute insulin excursion like glucose, fructose has adverse effects on glucose metabolism and insulin sensitivity.[12] In addition, fructose seems to trigger deleterious endocrine signals that are reflected in energy metabolism.

Furthermore, fructose (in contrast to glucose) does not stimulate ghrelin secretion which together with low postprandial insulin response contributes to lower satiety and possibly overeating after fructose *vs.* glucose.[13] Fructose is also associated with a blunted leptin response. ***In summary, fructose intake is linked with an endocrine profile potentially favoring extra energy intake and weight gain.***

POTENTIAL MECHANISMS UNDERLYING FRUCTOSE INDUCED HYPERTRIGLYCERIDEMIA

Regulation of very density lipoprotein (VLDL) metabolism

Serum triglyceride concentration reflects the balance between the secretion and removal of triglyceride-rich particles (TRLs). We have recently reported that overproduction of large $VLDL_1$ particles is the hallmark of dyslipidemia in the metabolic syndrome and type 2 diabetes. We also reported that liver fat is one driving force that aggravates overproduction of VLDL particles in insulin resistance.[14] Our results also provided the first *in vivo* evidence that insulin down regulates $VLDL_1$ secretion in subjects with low liver fat while the suppression of $VLDL_1$ secretion by insulin is hampered in subjects with high liver fat.[14]

We recently reported that hypertriglyceridemia in abdominally obese subjects is due to dual metabolic defect, namely the combination of increased secretion and severely impaired clearance of triglyceride-rich $VLDL_1$ particles. Increased liver and subcutaneous abdominal fat were linked to overproduction of $VLDL_1$ particles whereas increased plasma levels of apo-CIII associated with impaired catabolic rate of $VLDL_1$ particles in obese HTG compared to NTG subjects.[15] The disturbed hepatic lipid metabolism is also critical in the pathogenesis of hepatic steatosis, and it has been suggested that the inability of the liver to secrete triglyceride-rich VLDL particles causes hepatic steatosis.[16] This concept considers VLDL-secretion as a way to protect liver against excess lipid accumulation and lipotoxicity.

In clinical practice, it has been suggested that the presence of an elevated waist circumference together with increased fasting triglyceride concentrations, the so-called *hypertriglyceridemic waist phenotype*, could represent a useful and simple screening approach to identify individuals likely to be characterized by excess ectopic fat deposition and the related dysmetabolic profile.[17, 18]

Regulation of *de novo* lipogenesis (DNL) in the liver

The liver X receptor (LXR) and the farnesoid receptor (FXR) play critical roles in the regulation of DNL and triglyceride synthesis via the transcription factors sterol response element-binding protein 1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP) that are major regulators of DNL via activation of fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD). The SREBP-1 is a master regulator of hepatic lipogenesis[19] (**Figure 1**). The peroxisome proliferator-activator receptor gamma

coactivator-1β (PGC-1β) coactivates SREBP-1c. ChREBP is also considered to be a hepatic carbohydrate sensor and it is activated by both glucose and fructose. An intriguing observation is that SREBP1-c seems also to be activated by fructose independently of insulin.[20] Knockdown of PGC-1β resulted in reduced expression of SREBP-1, and downstream lipogenic genes in the liver prevent both fructose-induced hypertriglyceridemia and hepatic insulin resistance.[19, 21] Collectively these actions mediate the lipogenic effects of fructose in the liver by enhancing its lipogenic potential and deposition of fat in the liver.[12, 23] Notably, DNL has been reported to be enhanced in subjects with NAFLD.[24-26]

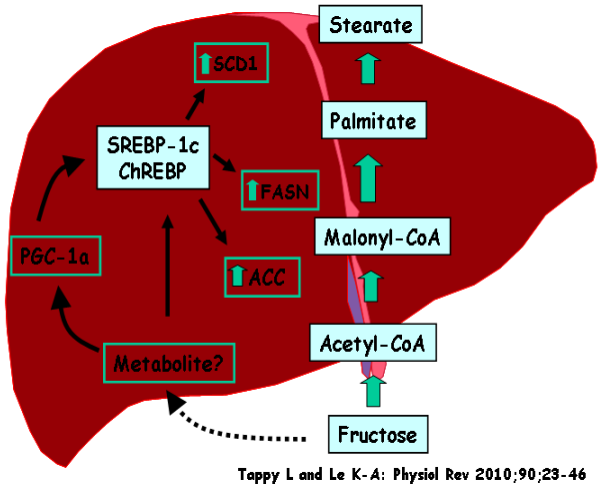


Fig 1. *Mechanisms for fructose-induced hepatic de novo lipogenesis*

The final step in triacylglycerol synthesis is regulated by diacylglycerol transferase-2 (DGAT2)[24-25] that transfers FA from fatty acyl CoA to diacylglycerol (DAG). Knockdown of DGAT2 expression *in vivo* associates with reduction of liver fat.[27-29] The accumulation of DAG in liver triggers the activation of protein kinase C (PKC) serine-threonine kinase. PKC is highly expressed in the liver with subsequent impairment of insulin signaling and is a link between insulin resistance and fatty liver.[30] An intriguing observation is that DAGs comprised of unsaturated acyl chains are more potent activators of PKC than those containing saturated acylCoAs.[31] Steatoryl-CoA desaturase (SCD)-1 is a key enzyme converting saturated FAs to monosaturated FAs and it is reported to be a good surrogate marker for DNL.[32] Recently hepatic DAG concentration measured from liver biopsies was reported to correlate with liver fat percent.[33]

In addition to glycerolipids another candidate to interfere with insulin signaling in the liver is the accumulation of ceramides molecules of sphingolipids family.[34] Ceramides not only interfere with insulin signaling but also interact with inflammation and stress signaling and mitochondrial function. Both increases of DAGs and ceramides are enhanced upon fatty acid oversupply and reflect a shift of fatty acyl CoAs between different metabolic pathways.[35] To elucidate the role and impact of these lipids on metabolism request new technology utilizing specific platforms of ultra-performance liquid chromatography (UPLC)/ mass spectrometry (MS) based lipidomic analyses.[36] In this protocol we will track the responses of major lipid components in TRL fraction to the dietary changes. **In summary, the data support strongly the concept that the effects of glucose and fructose on lipid metabolism are not equal with respect to lipid metabolism and that hepatic insulin resistance covers not only glucose pathway but also pathways of lipid metabolism.**

Apo-CIII and VLDL assembly

Apo-CIII is an apolipoprotein carried not only in TRLs and HDL particles but also in LDL in circulation.[37, 38] Several studies have shown that apo-CIII is a determinant of serum and VLDL triglycerides (TG) as well as of VLDL catabolic rate since it functions as an inhibitor of lipoprotein lipase (LPL), the rate limiting enzyme of TG lipolysis.[39] Interestingly, VLDL apo-CIII production rate correlates closely with that of VLDL-TG in centrally obese men.[40] Notably, the apo-CIII activity is influenced the dietary composition, and unsaturated fats have been shown to decrease apo-CIII as well as serum triglycerides [41]. Recent data suggest that apo-CIII is not only linked to the turnover of TRLs but may also be linked to the biosynthesis of VLDL particles.[38, 42] The observation that VLDL-apoCIII production is stimulated by plasma free fatty acids in humans[43] implicate that apo-CIII may enhance the production of large VLDL particles in the setting of excess TG availability as seen in NAFLD. This mechanism may also explain why carriers of specific apo-CIII variants (C-482™and T-455C) have elevated serum triglycerides and apo-CIII that associated with an increased prevalence of non-alcoholic fatty liver disease (NAFLD).[44] Intriguingly, a novel mechanism has recently been indentified that links glucose and plasma TG metabolism via the regulation of apo-CIII expression by glucose (Caron S *et al.* submitted). This mechanism involves the transcription factors ChREBP and hepatocyte nuclear factor receptor α (HNF-4α). As ChREBP is activated by both glucose and fructose this is a novel mechanism explaining how fructose may enhance lipogenesis in the liver**. In summary, emerging results clarify how glucose influences apo-CIII expression, but no data exists on the response of apo-CIII to fructose intake.**

Genes influencing TRL metabolism

Common genetic variations predisposing to dyslipidemia among subjects with and without type 2 diabetes include genes regulating lipid metabolism in the liver and VLDL clearance from circulation.[45-47] Overall genetic variants seem to account for one-third of the susceptibility to high TG.[48] Recently, allelic variants of the MLX interacting protein-like (MLXIPL) gene were reported to associate with high triglyceride levels. Interestingly, this gene controls hepatic energy metabolism, specifically lipogenesis from sugars through ChREBP.[46-47, 49] Other genes regulating different steps in VLDL synthesis include microsomal triglyceride transfer protein (MTP), APO-B, glucokinase hexokinase 4 regulatory protein (GCKR), polypeptide N-acetylgalactosaminyltransferase 2 (GALNT2) and DGAT2. Recent studies have found that common variants in the APO-CIII gene and palatin-like phospholipase domain containing 5 (PNPLA5) contributed to fatty liver.[44, 50] APO-CIII and APO-A5 are a part of gene cluster on chromosome 11. Both APO-CIII, APO-A5 and angiopoietin-like 3 (ANGPTL3) genes are important regulators of serum TG level by LPL mediated clearance of TRLs.[51]. Other genes regulating clearance capacity include LIPG (endothelial lipase), hepatic lipase (HL) and glycosylphosphatidylinositol (GPI)- anchored HDL-binding protein 1 (GPIHBP1). If fructose consumption results to more pronounced DNL, to storage of TAG in the liver and postprandial lipemia in carriers of these common variants has not been studied.

This study aims to examine the diet-gene interactions by comparing the differences in dietary responses of postprandial lipemia between carriers and non-carriers of common variants of approximately 40 genes shown to associate with triglyceride metabolism (in particular variants in MLXIPL, GCKR, GALNT2, APOB, APOA5, LIPG, HL, LPL and ANGPTL3 as well as combined effects of many risk variants in all 40 genes).[46] We will further study expression quantitative trait loci (eOTLs) comparing mRNA transcript levels and their changes in response to glucose load in adipose tissue biopsies in hyper- *vs.* hyporesponders to fructose.

Hepatic heparan-sulfate proteoglycans (HSPGs) are important to mediate TRL clearance by the liver, and recent studies have shown that changes in the composition of hepatic HSPGs resulted in profoundly reduced clearance of TLRs. HSPG synthesis is regulated by >35 genes, of which syndecan-1 and genes associated with initiation (exostosin: EXT1/2) or sulfation (N-deacetylase sulfotransferase: NDST1 as well as 2-O-sulfotransferase: HS2ST1) of HSPG have all been associated with TRL uptake in vitro. Degradation of HSPG is regulated by 2 enzymes; heparanase (HPSE) and sulfatase (SULF). In humans, little is known on the 'metabolism' of HSPGs in health or disease, whereas data on the effect of changes in HSPG metabolism on cardio-metabolic homeostasis is entirely unknown. We will therefore study the impact of genetic variations in HSPG-related genes and triglyceride metabolism.

Fructose metabolism in the liver

High fructose diets have been shown to induce insulin resistance and hypertriglyceridemia in several animal models.[52] It should be recognized that the liver is the major site of fructose metabolism.[53] Importantly the metabolism of fructose differs fundamentally from that of glucose **(Figure 2)**. The metabolism of fructose by fructokinase results in the release of three-carbon molecules substrates for DNL which are utilized for the synthesis of glycerol and free fatty acids. In contrast to glucose metabolism, fructose conversion to three carbon molecules occurs independently of regulation by insulin and there is no negative feedback via cell energy status to control this pathway resulting in increased DNL and consequently VLDL synthesis and secretion. Insulin action in the liver involves insulin receptor kinase which phosphorylates insulin receptor substrate-1 (IRS1) and IRS2 resulting in activation of PI3K (phosphatidylinositol 3-kinase) and the protein-serine/threonine kinases Akt2. Activation of Akt2 enhances glycogen synthesis and inhibits gluconeogenesis. Defects in insulin signaling cascades have been observed in animal models on high fructose diet including increased activity of protein tyrosine phosphatase 1B (PTP-1B), phosphatase and tensin homolog deleted on chromosome ten (PTEN), SH2 domain-containing inositol phosphatase (SHIP2) and c-Jun amino terminal kinase (JNK1) resulting in hepatic insulin resistance.[12]

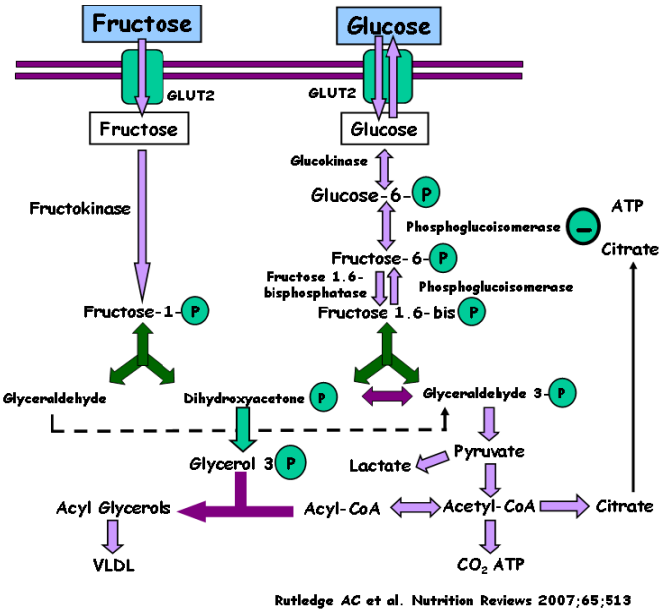


Fig 2. *Metabolic pathways of fructose and glucose in the liver*

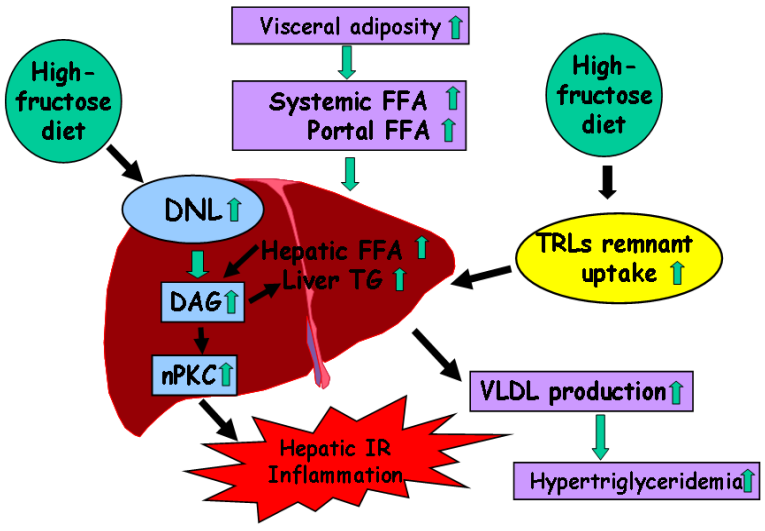
High fructose feeding and DNL

The available evidence that fructose increases DNL from animal models in particular from fructose fed golden hamster are plenty and convincing.[52] The key question is whether fructose consumption increases DNL *in vivo* in man to the extent that is reflected in significant excess liver fat accumulation and VLDL overproduction resulting in atherogenic dyslipidemia and increased CVD risk. Presently, scientific evidence is limited because of small studies with short follow-up periods. Acute oral fructose load increased DNL in healthy men but not in women in one recent study.[54] The recent data by Parks *et al.* confirmed that acute consumption of fructose enhanced lipogenesis and increased postprandial TG levels.[55] In contrast, the contribution of newly synthesized fatty acids to VLDL-TG remained minimal after acute fructose load despite increases of postprandial TG levels in another study.[56] A short term high fructose feeding (3 g/kg BW corresponding to 800-1000 extra calories per day) on top of isocaloric diet in lean healthy men increased hepatic DNL by six-fold and fasting triglycerides by 80%.[57] Recently Stanhope *et al.*[6] reported that fructose -sweetened beverages providing 25 % of daily energy intake over 10 weeks increased significantly DNL and postprandial TG response but not fasting TG levels. Subjects consuming fructose exhibited significant increase in visceral fat but no changes was seen in control subjects consuming glucose. Notably, both groups showed a small but significant weight gain. Fructose consumption, but not glucose consumption, was associated with significant decrease of insulin sensitivity and increases of fasting glucose and insulin levels. Overfeeding of fructose on top of isocaloric diet over 7 days lead to increased liver fat in healthy lean men.[58] So far this is the only study suggesting that fructose may enhance fat deposition in the liver. However, it is still unknown whether this is due to calorie excess or fructose per se. **In summary, acute fructose consumption in quantities similar to average American daily fructose intake stimulate DNL and enhance postprandial lipemia. The effect of chronic fructose intake and the direct contribution of DNL to liver fat, VLDL production and postprandial TG kinetics are not established in humans and research in this area is urgently needed.**

Fructose and inflammation

Recently, fructose feeding has been reported to activate inflammatory pathways **(Figure 3)**.[59] Fructose feeding in rats is associated with increased inflammatory proteins and markers of oxidative stress.[60] In two animal models fructose feeding was associated with increase of tumor necrosis factor α (TNFα) levels in plasma associated with activation of the proinflammatory transcription factor NF-κB. Interestingly, TNFα has been reported to stimulate hepatic VLDL apo-B production accompanied by increases of MTP and hepatic insulin resistance.[61]

Accumulation of TG in the liver leads to increased synthesis of the proinflammatory lipid family ceramide and reactive oxygen species (ROS) generation.[62] Recently, fructose has been reported to increase ceramide concentration.[63] In the liver accumulation of diacylglycerol (DAG) and generation of ROS activates protein kinase C delta (PKC-δ), IκB kinase (IKK-β) and NF-κB pathways.[64] IKK-β seems to act as a mediator of TNFα induced insulin resistance[65, 66] IKK-β activation results in increased expression of sterol regulatory element-binding protein 1 (SREBP-1) and induction of DNL. Increased NF-κB activation promotes JNK-1 activation via inhibition of serine phosphorylation of IRS-1 resulting in defective insulin signaling and insulin resistance. Fructose has been also reported directly to activate JNK-1.[66-68]



Given that fructose induces hepatic lipogenesis and associates with inflammatory processes it is plausible that these mechanism act in concert to further aggravate the lipogenesis pathway. To the best of our knowledge no data exist on markers of inflammation and oxidative stress in man during fructose feeding.

Fig 3. *Consequences of high fructose diet on lipid metabolism in the liver.*

Specific Aims

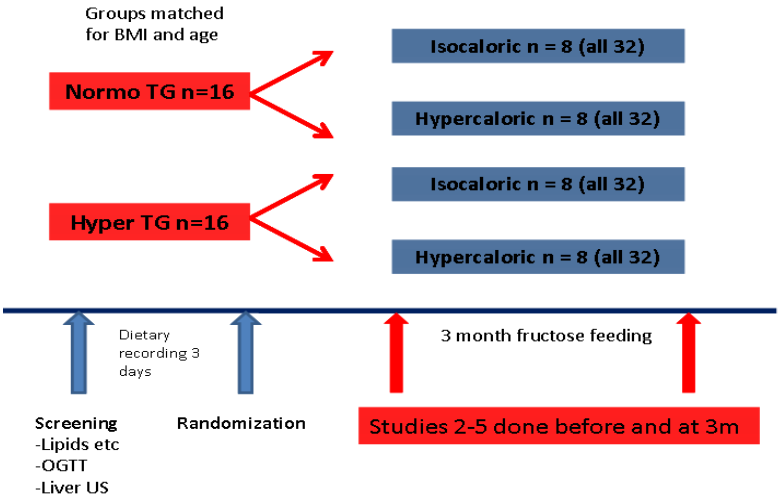
Our hypothesis is that consumption of fructose-containing beverages aggravates postprandial lipemia by dual mechanisms; by an increased *de novo* lipogenesis reflected by increased liver fat content and VLDL production; and by a decrease in the clearance rate of TRLs linked to reduction in LPL activity.

This project addresses the following specific questions:

1. Does a diet supplemented with fructose-containing beverages increase postprandial lipemia independently of body weight gain due to extra calories?
2. Does a diet supplemented with fructose-containing beverages increase DNL, VLDL production and liver fat deposition more in high risk HTG obese subjects than in NTG obese individuals?
3. Is the clearance of TRLs in the postprandial state impaired in response to a diet supplemented with fructose containing beverages?
   A. Is a potential clearance defect of TRLs linked to responses of apo-CIII and apo-A5 to a diet with high fructose?
   B. Are LPL mass and activity responses different in NTG *vs*. HTG subjects during fructose diet?
4. Is blood pressure raised by a diet supplemented with fructose-containing beverages?
5. Is the acute inflammatory response to a fructose-rich diet measured in the fasting state or the postprandial period exacerbated in obese subjects with HTG?
6. Do common variants in genes affecting lipid metabolism (individually and/or when combined) modify the effect of a diet enriched in fructose-containing beverages on lipid metabolism (in particular hypertriglyceridemia), and gene expression in adipose tissue?
7. Is gut microbiota modified by a diet enriched in fructose-containing beverages and are these changes linked to metabolic dysfunction?

Study design, subjects and methods

This international, multi-center, randomized study has four centers: Helsinki University, Finland; University of Gothenburg, Sweden; Napoli University, Italy; and Laval University, Canada. Each centre enrolls 32 subjects (see footnote below).[i] Thus, total number of subjects will be *n*=128 **(Figure 4).**



Study groups (*n*=16 in every centre)

- **Obese normolipidemic** healthy men with big waist (>96 cm, BMI <40 kg/m^2), normal lipid values (TG <1.7 mmol/l and LDL <3.5 mmol/l), and normal liver fat.

- **Obese hypertriglyceridemic** men with big waist (waist > 96 cm, BMI <40 kg/m^2), hypertriglyceridemia (TG >1.7 mmol/l and LDL <3.5 mmol/l), with increased liver fat.

Fig 4. *Study design and numbers of subjects in each centre.*

Experimental diets

Subject eligible for the study **will be randomly assigned to one of the two diet groups for 12 weeks:**

A) "Hypercaloric fructose diet group"

Subjects will be instructed to drink 990 ml of a beverage sweetened with 7.6 % fructose per day while consuming a self-selected *ad libitum* (usual) diet. No advice is given to reduce the energy content of their usual diet. Subjects will be in positive energy balance and therefore subjected to a weight gain of up to 2.5 kg in the 3 month study period. The subjects will be followed by the trained dietician 6 weeks after the study to ensure that the subjects return to a BW of maximum 1 kg above their baseline BW.

[i] Helsinki University plans to perform a kinetic study as an extension of the main study. The aim of the substudy is to elucidate the effects of fructose diet on lipoprotein metabolism using stable isotopes. This substudy replaces study visit 3 and is described on page 9.

B) "Isocaloric fructose diet group"
Subjects will be instructed to drink 990 ml of a beverage sweetened with 7.6 % fructose per day while consuming an isocaloric diet. This group will be instructed by a trained dietitian to reduce their carbohydrate intake from other source by 75 g in order to remain in neutral energy balance. Subjects are required to keep their weight stable during the 3 months and their daily energy intake will be adjusted by the dietitian if the body weight deviates more than 1 kg at the weekly check-ups.

Dietary protocol

The beverages will be prepared as 7.6 % (w/w) solutions flavored with unsweetened drink mix. 750 ml of fructose sweetened beverage provides 75 g of fructose and 304 kcal corresponding to 15 % of energy intake for a person with a daily caloric intake of 2000 kcal. Subjects will be instructed to consume the beverages together with the main meals. During traveling the fructose drink may be replaced by crystalline fructose (75 g, same brand in each center) during weeks 1-10 but not during the last two weeks of fructose consumption for maximum of 5 days during the whole study.

Assessment of dietary intake

To assess habitual food and beverage consumption, subjects will participate in a baseline evaluation that includes a dietary record on 3 consecutive days including 2 weekdays and 1 weekend day. The subjects will be carefully instructed not to change their usual eating habits. Further, subjects are asked to repeat the 3-day food diaries at week 4, and 11 to obtain a total of 9-day diaries during the intervention period. The mean of the ~~6~~ 9 days will be used to estimate energy and macronutrient intake from the diet during the intervention period. Participants are not required to weight foods but are asked to measure the volume of foods consumed with household measurements (cups, tablespoons) or to indicate the weight of the products. After completing the food records, participants will meet with the dietitian to review the food records for completeness. Energy and macronutrient intake will be calculated by the dietician using using local dietary programs, which is based on a national Finnish database for food composition.[69] Subjects carry a pedometer during the days of dietary recordings.

Subjects are required to be in contact with the dietitian once per 1-3 weeks to control beverage supply, to monitor weight and compliance. The home scale is checked at the first visit.

Power calculations

Primary endpoints in the current study are 1) change in plasma triglyceride (TG) area under curve (AUC) between the NTG and HTG during fructose feeding (between groups) and 2) change in TG AUC in HTG subjects during isocaloric fructose *vs*. hypercaloric fructose feeding (within group). The calculations are based on following assumptions: TG AUC increases from 7 to 10.5 mmol/l×hrs in normotriglyceridemic and from 7-9 to 14 mmol/l×hrs in hypertriglyceridemic subjects during fructose. Standard deviation is assumed to be 4.3 based on previous studies in similar groups of patients. 1) To detect significant difference (>3.5 mol/l×hrs) between the groups (*n*=25) with unpaired t-test. 2) To detect significant increase (>3.5 mol/l×hrs) within the group (*n*=15) with paired t-test. These calculations assume that alpha = 0.05 and power = 0.80 in all tests.

Inclusion criteria

Male subjects with body mass index (BMI) 27-40 kg/m^2, waist >96 cm will be recruited. The weight must have been stable (+- 1 kg during the last 6 monts). Each participant will undergo a physical examination and laboratory tests to exclude hepatic diseases (other than hepatic steatosis) and renal, thyroid and hematological abnormalities. Subjects with total cholesterol <6.2 mmol/l, serum triglycerides <5.0 mmol/L, BMI <40 kg/m^2, non-smoking and regular daily alcohol consumption less than 2 doses (*i.e.,* 5 g pure alcohol, maximum alcohol consumption is 2 portions per day or 14 portions per week) are included to the study. Any lipid medication (indicated for primary prevention) or other medication known to affect lipid levels is discontinued at least a month before the screening visit and reintroduced after the completion of the study. Type 2 diabetes is excluded with an oral glucose tolerance test.

Exclusion criteria: Age < 20 years or >65 years, BMI or lipid levels outside the inclusion range, smoking, alcohol consumption over 2 doses/day, type 2 diabetes, cardiovascular disease, hormonal therapy, any chronic disease requiring medication except for controlled hypertension.

The reasons for focusing on males are two-fold: previous studies demonstrate an effect of gender to fructose feeding.[70] Furthermore, as there are major gender differences in body fat distribution, visceral and ectopic fat deposition (women having, on average less visceral and liver fat than men), the study size in a study with both men and women would have been forced to be significantly larger. This wouldn't be compatible with the budget limitations for this grant application. However, the gender difference to fructose feeding is exceedingly interesting and our goal is to perform a follow-up study with both males and females. That study will be designed by the results from this study.

Study visits The number of visits is about 3-5 depending on the local circumstances to allocate imaging studies; the kinetic study will have a separate schedule for visits.

Please see specific instructions in excel: Clinical data files_040612 and Fructose_sample handling_fatload_ogtt_heparin_TIMEPOINTS_040612 and Fructose Fat load bleeding schedule 040612.

Record all results of patients included to study in: **Fructose_data_**040612.

1. Screening

Subjects will undergo physical examination (cardiorespiratory status, blood pressure, BMI, waist-hip-ratio and recording of their medical history. A blood sample is drawn after an overnight fast.

1.1 The screening measures: serum triglycerides, LDL cholesterol and HDL cholesterol, blood count, creatinine, sodium, potassium, thyroid stimulating hormone, glucose, and liver enzymes.

1.2 An oral glucose tolerance test (see below).

Studies 2-5 for screen positives are performed twice: before and at the end of the three month diet period.

2. Lipolytic enzymes and genetic studies

2.1 Subjects will receive 75 IU/kg heparin *i.v.* to determine lipoprotein lipase and hepatic lipase masses and activities.[71]

2.2 A blood sample for DNA extraction is drawn. Single nucleotide polymorphisms (SNPs) in genes involved in regulation of lipid metabolism, insulin resistance, inflammation or oxidative stress will be analyzed using Taqman and Sequenom methods. In addition, among hyper- and hyporesponders to fructose, expression quantitative trait loci (eOTL) analysis will be performed comparing mRNA transcript levels and their changes during the intervention from adipose tissue biopsies and relating this to genetic variation.

2.3 Since diet may lead to low-grade inflammation and insulin resistance due to changes in gut microbial community, the subjects will also provide a stool sample (using specific tubes and instructions) for analysis of gut microbiota profile (future analysis) and answer a health questionnaire. A blood sample for lipopolysaccharide (LPS) and angiopoietin-related protein 4 (Angptl4/fait) analyses is drawn for future analyses.[72-74]

2.4 The questionnaire of physical activity is done twice: before fructose period and during weeks 10-12

3. Oral fat load study

After a 12-h fast, subjects will receive a mixed meal consisting of bread, butter, cheese, low fat milk and tea or coffee (63 g carbohydrates, 56 g fat (P/S ratio 0.11), 39.9 g protein) in the morning. Blood samples are drawn before and up to 8 hours after the meal. During this time only water will be served *ad libitum* and the subjects will remain physically inactive.

To study *de novo* lipogenesis during postprandial period the subjects will receive deuterated water (^2H_2O) 2 g per kg body weight. In the evening before the oral fat load study a blood sample (background sample) (Taken DURING PRECEEDING week before: see sample handling) will be drawn. The subjects will sip deuterated water (2 dl) between 18.00 and 22 hrs together with 5 dl of mineral water.

3.1 Blood samples (For all sampling: please see Exel: Fructose_sample handling_fatload_ogtt_heparin_TIMEPOINTS_040612 and Fructose Fat load bleeding schedule 040612) are taken the night before (background serum sample) for DNL analysis and before and during the fat load. Lipoprotein fractions will be isolated using density gradient ultracentrifugation.[75]

Concentrations of lipids, apolipoproteins, other biochemical variables will be performed in whole plasma and/or chylomicron (Sf > 400), $VLDL_1$ (Sf 60-400) and $VLDL_2$ (Sf 20-60) fractions. Analysis will include:

3.1.1 Triglycerides, cholesterol, apo-B100, apo-B48, markers of energy balance (FFA, β-OHB), glucose and insulin are studied in PLASMA.

3.1.2 Enrichment of deuterated water to measure DNL in TRL- fractions are measured.[76]

3.1.3 Apo-CIII, apo-A5, uric acid, markers of inflammation (high-sensitive CRP, TNF-α, interleukin-6) are measured. Samples for other biomarkers (serum amyloid A [SAA], IL-8, paraoxonase [PON1], myeloperoxidase [MPO], monocyte chemotactic protein-1 [MCP-1]), markers of coagulation activity (plasminogen activator inhibitor-1) and incretins (glucagon-like peptide-1 [GLP-1] and gastric inhibitory polypeptide [GIP]) are collected for future analyses.

3.1.4 Lipidomics profiling utilizing the state of the art GC/MS technology to map a large and comprehensive spectrum of lipid and lipid intermediate species including SCD desaturation index (*i.e.,* the ratio of 16:1n-7/16:0 that is used as an index of SCD enzyme activity) and their responses to diets in serum and VLDL fractions are measured at two time points.[77]

3.1.5 Fasting samples: apo-B, apo-AI, apo-AII, adiponectin, ghrelin, leptin, LDL size and HDL size.

3.1.6 Needle fat biopsies from subcutaneous abdominal fat are taken fasting and 4 hrs postprandially. Adipose tissue mRNA levels will be profiled to detect which genes are upregulated *vs*. downregulated in response to fructose and how this relates to genetic variation.

3.1.7 Four spare samples (two plasma and two serum samples) of each time-point.

3.1.8 In fasting state and during the postprandial period (0 and 4 hrs)[78, 79] blood pressure measures will be performed using a standardized system (BP Tru 200 manometer) and heart rate variability will be tested by ECG recording.

4. Determination of liver, subcutaneous and intra-abdominal fat

On a separate visit the amount of liver, subcutaneous and visceral fat are determined. Proton magnetic resonance spectroscopy is performed with a 1.5 T whole-body device[80] to determine liver fat content. Magnetic resonance imaging is used to determine subcutaneous abdominal and intra-abdominal fat as previously described.[80] A standardized protocol will be used at all centers.

5. Oral glucose tolerance test

An oral glucose tolerance test (For all sampling: please see Excel: Fructose_sample handling_fatload_ogtt_heparin_TIMEPOINTS_040612) will be performed in the morning after an overnight fast at screening as well at the end of the 3 month diet period. Sampling time will be at 0, 5, 10, 30, 60, 90, 120, 180 and 240 minutes for determination of glucose, insulin, C-peptide and gut incretins to allow modeling oral glucose insulin sensitivity index (OGIS).[81]

SUMMARY:

1. Measurements of routine laboratory lipid and biomedical parameters will be centralized and workload allocated to the expertise of the centers (Helsinki, Laval, Gothenburg, and Naples).
2. DNL measurements and lipidomic analyses will be done in Gothenburg (Prof. Jan Borén).
3. SNP genotyping by Taqman and Sequenom and adipose tissue mRNA profiling by Affymetric microarray will be performed in Malmö (Prof. Marju Orho-Melander).
4. Studies of microbiota will be performed in Gothenburg (Dr. Fredrik Bäckhed).

Substudy (in Helsinki)

Kinetic protocol visit (replacing study visit 3)

This study will replace study visit 3 and is performed before and at 3 months after the diet period. The aim of the study is to elucidate the effects of fructose diet on triglyceride and apo-B non-fasting kinetics using stable isotopes. We will use d3-leusine to study apo-B kinetics and d5-glycerol to study triglyceride kinetics.[82] Simultaneously, hepatic DNL is calculated from enrichment of deuterated water ingested during the kinetic study. The study outline is shown in **figures 5 and 6**. Study subjects will consume ^2H_2O during the kinetic protocol as described in visit 3. At 8:00 the subjects will receive a bolus injection of $^2H_3$-leucine (7 mg/kg) and $^2H_5$-glycerol (500 mg) as in our previous kinetic protocol.[83] The subjects will be served an oral fat load described in Visit 3. ***Urine samples of minimum 100 ml will be collected in the morning of***

kinetic protocol visit and 4 hours after serving the mixed meal. The urine samples will be used to measure the microvesicles containing multiple protein and nucleic acid classes (mRNA, miRNA, siRNA).

Dietary triglyceride tracers will be administered through test meal served as outlined in Figure 6 and containing 1,2,3,4-$^{13}C_{16}$ –palmitate (1-2 g). Blood samples are drawn according to the figure 6 (red dots). Other biochemical, physiological, lipid and apolipoprotein analysis are done as described in visit 3.

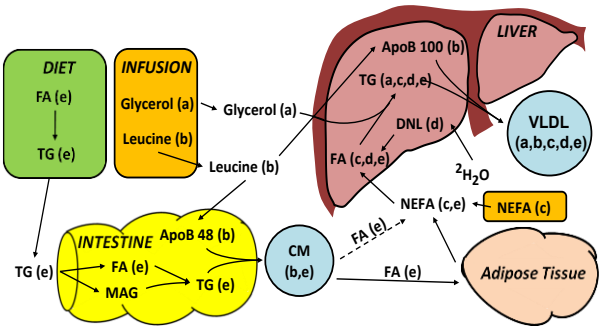


Fig 5. *Summary on TRLs metabolism*

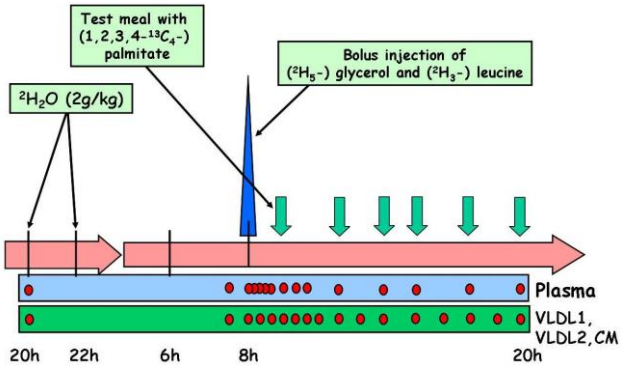


Fig 6. *Study design*

Kinetic modeling

Times series data from enrichments of plasma leucine, leucine in apo-B100 from $VLDL_1$ and $VLDL_2$, and glycerol from triglycerides from $VLDL_1$ and $VLDL_2$ are used as input to the kinetic model together with pool size measurements to simultaneously determine the kinetics of VLDL-triglycerides and apo-B100.[83]

Novelty and importance

This study provides a multidisciplinary *state-of-the-art* program to elucidate the deleterious effects of fructose on metabolism using a novel multidisciplinary approach in which clinical studies and molecular medicine are combined. The participating investigators, who all are at the scientific forefront in their respective fields, have different but highly complementary knowledge and technical competence. This enables us to perform innovative and cutting-edge science to dissect how fructose influences dyslipidemia, insulin resistance and inflammation utilizing novel imaging technology, lipidomics and genomics. The study focuses on abdominally obese subjects with hypertriglyceridemia to furthering evidence that these individuals are not only prone to type 2 diabetes but also to the development of related cardiometabolic abnormalities. The study is therefore designed to identify fructose mediated effects that are independent of weight gain. Overall, this research program has relevance to our understanding of factors involved in the development of type 2 diabetes and related metabolic complications including CVD risk factors.

Lipoprotein metabolism is a complex system in which abnormal concentrations of various lipoprotein particles can result from alterations in their rates of production, conversion and/or catabolism. Traditional methods that measure plasma lipoprotein concentrations only provide static estimates of lipoprotein metabolism and hence limited mechanistic information. By contrast, we will use tracers labeled with stable isotopes, which provide us with a powerful and cutting-edge tool for probing lipid and lipoprotein kinetics *in vivo* and furthering our understanding of the pathogenesis of dyslipoproteinemia. Thus, the combination of stable isotopes, studies of postprandial lipid metabolism and measurements of hepatic DNL will for the first time allow us to explore the dynamic changes leading to both postprandial accumulation of chylomicrons and elevated VLDL secretion. The great synergism and excellence of the network guarantees success of the proposed study.

The microvesicles measured in urine samples are rapidly emerging as valuable, non-invasive ways to identify biomarkers of kidney metabolism. Consumption of fructose leads to increasing urate concentrations in plasma. The measurement of urine microvesicles offers a unique opportunity to study the effect of fructose consumption on novel biomarkers of kidney metabolism.

Collaborative partners

- *Helsinki University Central Hospital, Biomedicum, Helsinki, Finland*. **Principal Investigator Marja-Riitta Taskinen**, MD, PhD, professor. Team members: **Niina Matikainen**, MD, PhD, scientist, endocrinologist, coordinator of the clinical studies; **Kirsi Pietiläinen**, MD, PhD, senior lecturer, internist and nutritionist; **Leonie Bogl**, PhD, nutritionist; and **Nina Lundbom**, MD, PhD, senior lecturer, radiologist and imaging expert.
- *Quebec Heart and Lung Institute (QHLI), Quebec, Canada*. **Jean-Pierre Després**, PhD, professor, local PI. Team members: **Natalie Alméras**, PhD, the coordinator of clinical studies with great expertise in metabolic studies; **Eric Larose**, MD, chief of the Cardiovascular Imaging Center and responsible for the imaging; and **Caroline Rhéaume,** MD, PhD, a primary care physician and scientist responsible for the medical supervision of the subjects.
- Sahlgrenska *Academy at University of Gothenburg and Sahlgrenska University Hospital, Gothenburg, Sweden.* **Jan Borén**, MD, PhD. professor, the local PI. Team members: **Björn Eliasson**, MD, PhD, adjunct professor, clinical studies; **Fredrik Bäckhed**, PhD, lecturer, studies of microbiota and its metabolic consequences; **Martin Adiels**, PhD, scientist, modeling and biostatistics and **Marcus Ståhlman**, PhD, scientist, advanced lipidomics analysis.
- *University of Naples, Federico II, and Faculty of Medicine, Naples, Italy.* **Angela A. Rivellese**, MD, associate professor in Internal Medicine, local PI. Team members: **Gabriele Riccardi**, MD, professor in endocrinology; **Giovanni Annuzzi**, MD; and **Lidia Patti**, biologist.
- *Lund University Diabetes Center, Malmö, Sweden*. **Marju Orho-Melander**, PhD, professor of genetic epidemiology, local PI.
- ***Urine microvesicle analysis (Helsinki substudy): Harry Holthöfer MD, PhD, Professor, Director, National Centre for Sensor Research/Bioanalytical Sciences, Dublin City University, Ireland.***

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