# Combatting the Fructose Epidemic

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Fruitful or Fruitless?

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# Combatting the Fructose Epidemic

## Fruitful or Fruitless?

PROEFSCHRIFT

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volgens het besluit van het College van Decanen,
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# Chapter 1

General introduction

# The fructose epidemic

In the last half-century, enhanced industrial processes increased the availability of sugar and, consequently, the intake of added sugars in Western society.[1] In the Netherlands, only ~30% of the adult population adheres to the 2015 World Health Organization recommendation of a free sugar intake (i.e. added sugars as well as sugars that are naturally present in honey, syrup, fruit juices, and fruit concentrates) of less than 10% of total energy intake.[1] This is alarming since evidence is compelling that added sugars play an important role in the current epidemic of non-communicable diseases, including obesity, non-alcoholic fatty liver disease (NAFLD), dyslipidemia, type 2 diabetes (T2DM), cardiovascular disease (CVD), and colorectal cancer (CRC).[2-6] Fructose – which is the principal component of simple sugars, together with glucose – has been postulated to be the most detrimental carbohydrate.[7,8] This may be explained by differences in metabolic pathways and target organs.[9]

Fructose is preferentially metabolized in the gut, liver, and kidney by a cascade of fructolytic enzymes which efficiently convert fructose into triose-phosphates that subsequently can enter the glycolytic and gluconeogenic pathways.[9]
First, ketohexokinase (KHK) is responsible for the rapid and irreversible phosphorylation of fructose to fructose 1-phosphate (F1P) (**Figure 1.1**).[10] Of importance, this metabolite is specific to the fructolytic pathway and is not shared with glycolysis or gluconeogenesis.[10] Furthermore, as opposed to the tight regulation of glucose metabolism[9], fructose lacks regulation since KHK is not inhibited allosterically by adenosine triphosphate (ATP) (or other signals of cellular energy state), nor by its immediate product (i.e. F1P).[9,10] In addition to KHK's insensitivity to cellular energy status, the low Km and high activity of KHK for fructose allow the liver to efficiently clear most fructose, consequently, leaving only minute amounts of fructose to reach the systemic circulation.[9,10] The liver metabolizes approximately 70% of the ingested fructose compared to only ~15-30% of the ingested glucose.[11,12] Furthermore, in case of large fructose loads, KHK-mediated fructose phosphorylation consumes ATP so substantially that it results in acute lowering of hepatocellular ATP and free phosphate (as the latter is sequestered in F1P).[13,14] F1P can rapidly accumulate to millimolar concentrations in hepatocytes because the activity of KHK is higher than that of downstream enzymes.[15]
Second, aldolase B (ALDOB) cleaves F1P into dihydroxyacetone phosphate (DHAP) and glyceraldehyde (GA) (**Figure 1.1**).[10] The latter is subsequently phosphorylated by a triose-kinase to glyceraldehyde 3-phosphate (GA3P). Both DHAP and GA3P can enter the glycolytic and gluconeogenic pathways. The metabolic fates include conversion to

glucose (gluconeogenesis), oxidation, and formation of intrahepatic lipid (IHL) via hepatic *de novo* lipogenesis (DNL; **Figure 1.1**).[16-18]

As a result of these metabolic differences, fructose has potentially more lipogenic effects than glucose and, therefore, may be an important modifiable risk factor of NAFLD.[9]

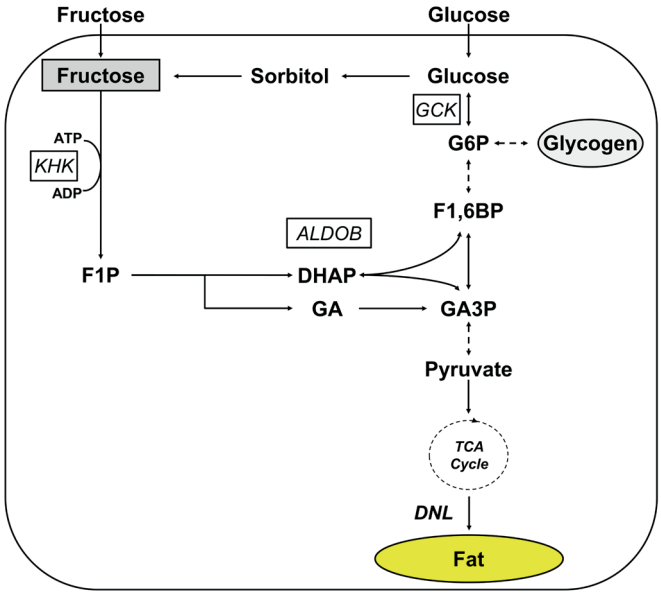


**Figure 1.1. Fructose metabolism.**

Upon entering hepatocytes, fructose is phosphorylated by ketohexokinase (KHK) to fructose 1-phosphate (F1P). Next, F1P is cleaved by aldolase B (ALDOB) to dihydroxyacetone phosphate (DHAP) and glyceraldehyde (GA). The latter is phosphorylated by triose-kinase to glyceraldehyde 3-phosphate (GA3P). Both fructose-derived DHAP and GA3P enter the glycolytic/gluconeogenic pathways. The metabolic fates include conversion to lipids via hepatic *de novo* lipogenesis (DNL).

Dashed lines indicate simplified pathways.

Abbreviations: ADP, adenosine diphosphate; ALDOB, aldolase B; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; DNL, *de novo* lipogenesis; F1,6BP, fructose 1,6-biphosphate; F1P, fructose 1-phosphate; GA, glyceraldehyde; GA3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; GCK, glucokinase; KHK, ketohexokinase; TCA, tricarboxylic acid cycle.

# Non-alcoholic fatty liver disease: epidemiology and (health) burden, definition, pathophysiology, and risk factors

NAFLD has become a major health problem in the 21st century. The worldwide prevalence of NAFLD is ~25% in adults (affecting approximately 1.25 billion people)[19], with wide geographical variation ranging from ~6% to 33% depending on the population, ethnicity, lifestyle, and method of diagnosis.[20-23] The global NAFLD prevalence is gradually increasing with age and is more common in males than in females.[24,25] NAFLD is the principal cause of liver transplantation in Western society and an important cause of liver-related mortality.[26,27] NAFLD is highly prevalent among people with T2DM and is currently viewed as a risk factor of T2DM.[28,29] In addition, in the last decade, several studies have identified NAFLD as a risk factor of other non-communicable diseases including chronic kidney disease, cancer, and CVD.[19,30-33] In fact, CVD is the primary cause of death in individuals with NAFLD.[34] The clinical, psychosocial, and economic burden of the NAFLD is enormous and is forecasted to grow.[35-38] The corresponding healthcare expenditures are alarming, with an estimated combined cost of about €35 billion per year (between €354 and €1163 per patient) in four European countries (i.e. Germany, France, Italy, and the United Kingdom).[38,39]

NAFLD comprises a histological spectrum of abnormalities associated with an excess of IHL accumulation in the absence of excessive alcohol consumption or other underlying hepatic disease.[40] Hepatic steatosis, the first stage of NAFLD, is defined as the accumulation of IHL in more than 5% of hepatocytes (detected by either imaging or histology).[40] Hepatic steatosis can progress to more advanced stages of NAFLD, including lobular or portal inflammation and ballooning with or without peri-sinusoidal fibrosis (i.e. non-alcoholic steatohepatitis) (**Figure 1.2**), and can even result in liver failure and hepatocellular carcinoma.[40]

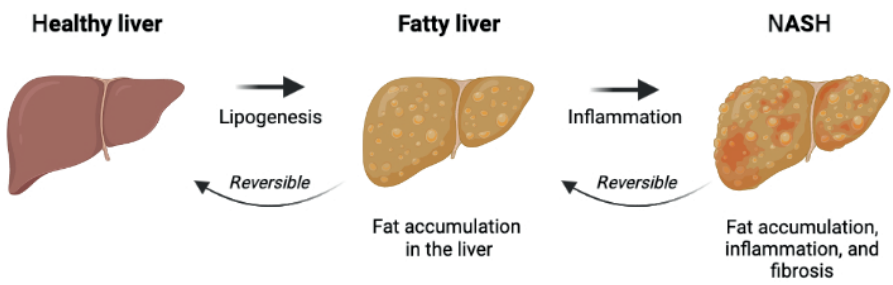


**Figure 1.2. Stages of non-alcoholic fatty liver disease.**
Abbreviation: NASH, non-alcoholic steatohepatitis. Created in BioRender.com.

Hepatic steatosis is caused by a disbalance between the influx and the efflux of lipids.[41,42] The following four pathways may contribute to the accumulation of IHL:

1) increased influx of free fatty acids derived from the adipose tissue or dietary intake, including saturated fat;
2) increased hepatic DNL (i.e. the biochemical process by which new fatty acids are synthesized from non-lipid precursors) from simple sugars, including glucose and fructose;
3) decreased hepatic fatty acid oxidation;
4) decreased secretion of triglyceride-rich very-low-density lipoproteins (VLDL) particles.[41,42]

The contribution of each pathway to the accumulation of IHL depends on multiple factors including genetic background, environment, physical condition, and nutritional status.[43,44] In particular pathway two (i.e. newly synthesized fatty acids from simple sugars via DNL) is increasingly recognized as a contributor to IHL deposition associated with obesity and NAFLD.[44-46]

The etiology of NAFLD is the result of complex relationships between genetic and environmental factors.[47,48] Multiple NAFLD susceptibility genes have been identified.[49-51] In addition to genetic predisposition for NAFLD, other risk factors include an unhealthy lifestyle. The lifestyle of Western society has drastically changed over the past decades. People have become more sedentary and less physically active. In addition, the current Western diet is characterized by a high intake of added sugars, (saturated) fat, and energy-dense food items due to enhanced industrial processes. This imbalance between energy requirements and energy intake causes (visceral) adipose tissue expansion and dysfunction, insulin resistance, and DNL, which are established precursors of NAFLD.[52] Besides a high-energy intake *per se*, certain individual macronutrients may in particular contribute to the development of NAFLD. As aforementioned, simple sugars (i.e. glucose and fructose) stimulate DNL and saturated fat (as opposed to polyunsaturated fat) stimulates adipose tissue lipolysis, which both accelerate IHL deposition.[41,42,53]

Furthermore, insulin resistance is a central feature in the pathophysiology of hepatic steatosis. IHL accumulation follows from insulin resistance in several ways.[54] First, under normal conditions, insulin suppresses adipose tissue lipolysis and hepatic glucose and triglyceride-rich VLDL production[55], and stimulates glycogen and lipid synthesis. However, in the setting of insulin resistance, impaired suppression of lipolysis in the adipose tissue results in an increased flow of free fatty acids (FFAs) towards the liver and the consequent accumulation of IHL.[35] Moreover, increased circulation of FFAs itself further inhibits the antilipolytic-effect of insulin, causing a vicious circle.[56,57]

Second, hyperinsulinemia also activates transcription factors, including sterol regulatory element binding protein 1 (SREBP1c) and liver X receptor (LXR), which induce expression of genes involved in DNL and, subsequently, contribute to IHL accumulation.[58] Third, hyperglycemia can contribute to increased DNL by providing more substrate and by inducing the lipogenic transcription factor carbohydrate response element binding protein (ChREBP), that coordinates the upregulation of the enzymes involved in DNL.[59]
Insulin resistance not only causes hepatic steatosis, but could also be the consequence of IHL content.[60] NAFLD may contribute to the excess circulation of fatty acid metabolites in peripheral tissue which results in insulin resistance.[61,62] Also, previous studies have shown that hepatic steatosis is positively associated with endogenous glucose production (as a result of a reduced ability to suppress gluconeogenesis[63]) which further impairs adequate insulin and glucose handling.[44,45] Last, proinflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), are elevated in NAFLD, which are associated with insulin resistance.[60] These data collectively highlight the bidirectional relationship between insulin resistance and hepatic steatosis.[46]

## The conundrum of the lipogenic effects of fructose

Despite the ample biochemical rationale as outlined above (**Figure 1.1**)[9], empirical evidence is lacking that fructose is truly more lipogenic than glucose. Although animal studies have convincingly demonstrated that fructose causes hepatic steatosis[64,65], previous meta-analyses of controlled intervention studies[66] and epidemiological studies[67] addressing the role of fructose in IHL accumulation are inconclusive. In addition, recent lessons from inborn errors of fructose metabolism further contribute to the conundrum.[68,69]

Hereditary fructose intolerance (HFI; OMIM #229600) is an autosomal recessive metabolic disorder, which arises from a deficiency of aldolase B (EC 4.1.2.13; encoded by the *ALDOB* gene).[70,71] HFI patients need to follow a lifelong fructose-restricted diet since they do not tolerate fructose.[72,73] Additionally, HFI patients should avoid sorbitol-containing and high-glycemic foods products since fructose can also be synthesized endogenously from sorbitol (via the polyol pathway; **Figure 1.1**).[74-76]
Remarkably, Simons and colleagues recently showed that HFI patients on a fructose-restricted diet were not protected from IHL accumulation.[68] In fact, when compared to age, sex, and BMI-matched healthy controls, HFI patients accumulated more IHL as

quantified by magnetic resonance spectroscopy.[68] Furthermore, despite adhering to a fructose-restricted diet, HFI patients accumulated hepatocellular F1P, as approximated by the presence of hypoglycosylated transferrin.[54] In agreement, the unexpected observation of hepatic steatosis in HFI patients has been confirmed in a mouse model with global knockout of aldolase B (i.e. ALDOB-KO mice) that phenocopies HFI.[69] Similar to HFI patients[68], ALDOB-KO mice fed a fructose-free diet accumulated hepatocellular F1P and developed hepatic steatosis.[69] However, these ALDOB-KO mice were protected from the accumulation of both hepatocellular F1P and IHL when treated with a KHK inhibitor (i.e. blocking the first committed step in fructose metabolism, and, thus, preventing the formation of F1P; **Figure 1.1**).[77]
These data suggest that the direct pathway of fructolysis (i.e. the downstream metabolism of fructose to trioses; **Figure 1.1**) *per se* is not necessary for IHL deposition. Moreover, they suggest that hepatocellular F1P (or concomitant hepatocellular ATP and phosphate depletion) acts as a key signalling molecule in the pathogenesis of hepatic steatosis in HFI.

## Possible explanations for the conundrum

The following biological and/or methodological explanations can be put forward for the discrepancies between textbook biochemistry, the hitherto reported inconclusive observations of fructose's lipogenic effects in intervention and epidemiological studies, and the striking observations in HFI.

Although the committed phosphorylation steps in glucose and fructose metabolism are catalyzed by distinct enzymes (**Figure 1.1**)[78], fructose and glucose metabolism appear to be closely linked, amongst others, by glucokinase regulatory protein (GKRP) (**Figure 1.3**).[79-81] GKRP is a liver-specific protein that resides in the nucleus.[80,81] In the fasting state, it sequesters and inhibits glucokinase (GCK), which limits the net hepatic glucose uptake and glucose metabolism.[80,81] In the postprandial state, the GKRP-GCK complex dissociates and allows GCK to translocate from the nucleus to the cytosolic space where it facilitates the conversion of glucose to glucose 6-phosphate (G6P), hence hepatic glucose uptake.[80,81] Increased hepatic glucose disposal via activation of GCK generates substrate and reducing equivalents supporting DNL (and glycogen synthesis).[79] In agreement, the relevance and pathogenesis of dysfunctional GKRP-GCK binding has also been demonstrated by genetic studies. Variants in the gene encoding GKRP – resulting in a protein with reduced ability to bind GCK – are associated with increased DNL and IHL content as well as dyslipidemia.[82]

A potent disruptor of the GKRP-GCK complex is F1P, the product of KHK-mediated fructose phosphorylation (**Figure 1.3**).[79,83] Previous studies have shown that already trace amounts of fructose can markedly enhance hepatic glucose uptake and metabolism.[81,84-90] In addition, Moore and colleagues simulated this process by performing oral glucose tolerant tests (OGTTs) with and without addition of fructose in healthy adults and patients with T2DM.[91,92] In these experiments, participants underwent a standard 75 g OGTT, resulting in high plasma glucose excursions.[91,92] However, addition of 7.5 g fructose to a 75 g OGTT significantly decreased the plasma glucose excursions.[91,92] In theory, these observations can be explained by F1P-induced dissociation of the GKRP-GCK complex resulting in more cytosolic GCK and, thus, enhanced glycolysis, which accounts for the decreased plasma glucose excursions.

Taken together, these data propose a possible role for F1P-induced disruption of the GKRP-GCK complex in IHL deposition and could account for the conflicting observations of fructose's lipogenic effects as outlined above. Furthermore, since high hepatocellular F1P concentrations and hepatic steatosis are the hallmark of HFI[68,77], constant F1P-induced dissociation of the GKRP-GCK complex could also explain the paradoxical fatty liver phenotype seen in HFI.[79] This assumption is supported by detailed phenotyping of the ALDOB-KO mice (typified by high hepatocellular F1P levels) that showed a higher cytosolic-to-nuclear ratio of GCK expression – indicative of increased dissociation of GKRP-GCK – which was associated with hepatic steatosis.[77]

Last, methodology could, at least in part, account for the conflicting results from epidemiological studies on the lipogenic effects of fructose. Nutritional epidemiology suffers from numerous limitations, including: 1) reporting bias (underreporting bias by specific subgroups, including individuals with obesity or T2DM[93]), 2) use of various definitions of the determinant (fructose as monosaccharide versus sucrose-bound fructose as disaccharide), 3) use of food-frequency questionnaires (FFQs) that are not properly validated for certain fructose-containing food items, 4) imprecise measurement of the determinant, the outcome, or both, 5) insufficient adjustment for potential confounding factors (in particular lifestyle and its collinearity with dietary patterns).

ChREBP is another candidate in fructose-mediated IHL deposition (**Figure 1.3**). ChREBP is a carbohydrate-sensing transcription factor that is highly expressed in key metabolic cell types, including hepatocytes, enterocytes, and proximal tubule cells of the kidney.[94-96] ChREBP is activated by depletion of intracellular ATP and phosphate and its key function is to maintain phosphate homeostasis.[97] In turn, ChREBP upregulates fructolytic, glycolytic, gluconeogenic, and lipogenic enzymes in the liver.[94,95,98,99]

ChREBP may play a role in the fructose-induced pathogenesis of IHL accumulation and could explain the hitherto reported discrepancies of fructose's lipogenic effects. First, as aforementioned, robust KHK-mediated fructose phosphorylation results in a marked decline in intracellular ATP and phosphate levels (secondary to its sequestration as F1P).[13,14] In turn, fructose-mediated activation of ChREBP could account for the deleterious effects on hepatic lipogenic targets in humans.[100] Second, intracellular features of HFI are, among others, F1P build-up and ATP and phosphate depletion[15,101], which possibly activate ChREBP and consequently stimulate DNL. Consistently, ALDOB-KO mice showed pronounced intrahepatic ATP depletion (presumably due to inhibited regeneration of ATP from F1P) and decreased phosphate levels, which was associated with high expression of DNL genes and hepatic steatosis.[77]

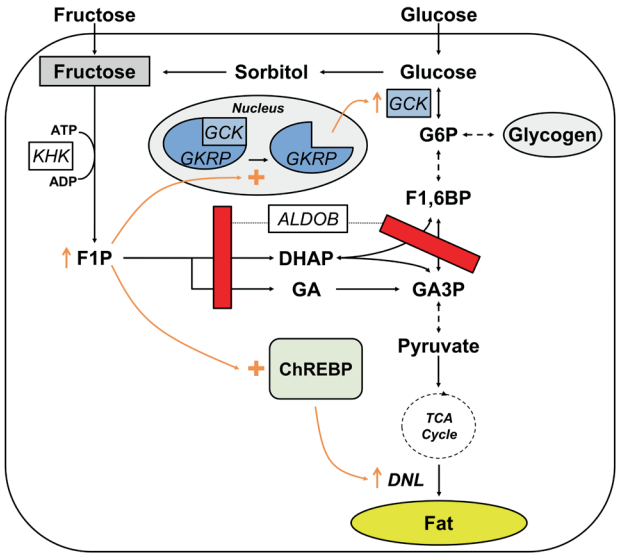


**Figure 1.3. The interaction between fructose and glucose metabolism.**
Hepatocellular fructose 1-phosphate (F1P) – as metabolic signal – may indirectly promote *de novo* lipogenesis (DNL) and consequently intrahepatic lipid (IHL) accumulation via multiple mechanisms. First, in the liver, F1P potently relieves the inhibitory effect of glucokinase regulatory protein (GKRP) on glucokinase (GCK), allowing the latter to translocate from the nucleus to the cytosolic space where it catalyzes the phosphorylation of glucose, which increases hexose- and triose-phosphate carbon pools and consequent DNL. Second, hepatocellular F1P and concomitant declined ATP and phosphate levels activate carbohydrate responsive element binding protein (ChREBP), a transcription factor with multiple downstream effects including induction of DNL enzymes and DNL activity.
Dashed lines indicate simplified pathways.
Abbreviations: ADP, adenosine diphosphate; ALDOB, aldolase B; ATP, adenosine triphosphate; ChREBP, carbohydrate response element binding protein; DHAP, dihydroxyacetone phosphate; DNL, *de novo* lipogenesis; F1,6BP, fructose 1,6-biphosphate; F1P, fructose 1-phosphate; GA, glyceraldehyde; GA3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; GCK, glucokinase; GKRP, glucokinase regulatory protein; KHK, ketohexokinase; TCA, tricarboxylic acid cycle.

## Aims and outline of this thesis

Based on the hitherto presented literature, several questions arise concerning the causal role of fructose in the pathogenesis of IHL accumulation, and its underlying mechanism.
First, recent studies have convincingly identified F1P (or concomitant intracellular ATP and phosphate depletion) as the key driver of IHL accumulation in HFI.[68,69,77] However, the underlying mechanism of F1P-mediated hepatosteatosis remains unknown. Although results from these studies imply a role for GKRP-GCK disruption and/or ChREBP in the pathogenesis HFI, this is based on observational findings only, which in themselves do not infer causality. Therefore, the exact roles of the GKRP-GCK complex and ChREBP as potential mediators in the pathogenesis of IHL accumulation need to be determined. Furthermore, previous OGTT experiments with and without addition of 7.5 g fructose demonstrated the interaction between fructose and glucose metabolism in humans.[91,92] Similarly, numerous experimental studies demonstrated that trace amounts of fructose – possibly by the GKRP-GCK complex – can already markedly enhance hepatic glucose uptake and metabolism.[81,84-90] However, the threshold at which fructose potentially disrupts the GKRP-GCK complex and consequently enhances glycolysis needs to be determined in humans.
Second, despite convincing evidence of fructose's lipogenic effects in animal models[64,65], epidemiological studies failed to replicate these findings.[67] Therefore, there is a critical need to assess the relationship between fructose intake and IHL content at the population level. In particular, the causal nature of this relationship remains to be established.

Therefore, the two research questions of this thesis are:

1) Which are the key molecular mechanisms by which fructose participates as a signalling molecule in the pathogenesis of IHL accumulation and what is the role of F1P herein?
2) What is the (causal) relationship between fructose intake and the risk of non-communicable disease at the population level?

**Part I of this thesis provides an overview of fructose metabolism in HFI and NAFLD and a new method to quantify fructose concentration.**
First, in **chapter 2** of this thesis, we reviewed the most recent advances in the pathogenesis of HFI, in particular the liver phenotype, and the implications for its treatment and the understanding of fructose-induced NAFLD.
Second, in order to properly study the (causal) role of fructose in non-communicable disease in our follow-up studies described in this thesis, we developed and validated an

Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (UPLC-MS/MS) method to accurately and precisely quantify fructose in serum and urine (described in **chapter 3**).

**In part II of this thesis, we investigated the role of fructose as a signalling molecule and the interaction between fructose and glucose metabolism both in mice and humans.**

First, we aimed to determine the key molecular mechanisms by which fructose participates as signalling molecule in the pathogenesis of IHL accumulation and the role of F1P herein. Inevitably, animal models are required to demonstrate the underlying mechanism in the development of hepatic steatosis. Although human stable isotope studies or deep-phenotyping of rare inborn errors of metabolism (i.e. individuals carrying mutant *GCK* or *ALDOB*) can provide a glimpse of the clinical relevance of F1P-induced disruption of the GKRP-GCK complex, they will not prove causality since the GKRP-GCK complex cannot be directly measured with these methods. For this, liver biopsies are required, which is unethical since human liver tissue is not easily accessible. Furthermore, there is no good *in vitro* model to study the GKRP-GCK complex (apart from hepatocytes isolated from rats[102]). Therefore, in **chapter 4**, we performed experiments in ALDOB-KO mice (characterized by high hepatocellular F1P) to determine the role of the GKRP-GCK complex and ChREBP as potential mediators in the pathogenesis of IHL accumulation.

Second, in **chapter 5**, we aimed to study the interaction between fructose and glucose metabolism in humans, in particular the threshold at which fructose – possibly by disrupting the GKRP-GCK complex – decreases plasma glucose excursions. Therefore, we performed multiple OGTTs with different quantities of fructose (i.e. 0, 1, 2, 5, 7.5, 15 g) to study the acute effects of fructose on the glucose response in healthy participants.

**In part III of this thesis, we examined the (causal) relationship between fructose and risk of non-communicable disease at the population level.**

First, in **chapter 6**, we aimed to assess the relationship between fructose intake and IHL content at the population level. We examined the cross-sectional association between total fructose and fructose from different sources (i.e. fruit, fruit juice, and sugar-sweetened beverages) and IHL content. For this we used a large extensively phenotyped cohort study, the Maastricht Study (n~7,000), in which IHL content was quantified by magnetic resonance imaging.[103]

Second, we aimed to assess the causal nature of the relationship between fructose and non-communicable disease at the population level (**chapter 7** and **chapter 8**). Although

traditional epidemiology provides insight into the clinical relevance of the observational relationship, it is unfortunately prone to residual confounding (due to measured and unmeasured confounders), and, hence, does not infer causality. To overcome these limitations a Mendelian randomization (MR) study can be performed to determine whether fructose is causally involved in the pathogenesis of non-communicable disease. MR is based on the concept that genetic variants are randomly distributed among haploid cells at meiosis and they, therefore, can be used as instruments to study the effects of lifelong exposure to fructose on non-communicable disease risk without the disruptive effects of confounding factors.[104] Although two functional mutations in *KHK* (responsible for the first committed step in fructose metabolism) have been described[105], these are too rare to be used as instruments in genetic epidemiology.
Therefore, in **chapter 7**, we first assessed the functionality of a common missense variant in the gene encoding *KHK* (i.e. rs2304681:G>A [p.Val49Ile]; minor allele frequency: 0.37). Impaired KHK function results in reduced fructose metabolism, and consequently the unphosphorylated fructose escapes intracellular entrapment and is eventually excreted via the kidneys in the urine (similar to essential fructosuria [OMIM #229800], an inborn error of fructose metabolism in those who have a loss of *KHK* [EC 2.7.1.3][106]). Based on the premise that fructosuria reflects impaired KHK function[106], we first examined the association between the rs2304681 minor A allele and urinary fructose levels in the Maastricht Study (n~1,500).[103] Next, we performed a two-sample MR analysis to examine the potential causal association between genetically proxied impaired KHK function (reflected by urinary fructose levels derived from the Maastricht Study, as determinant) and risk of CRC (as outcome). For the outcome we used data on the genetic association between the rs2304681 minor A allele and CRC risk, obtained from a large case-control study on CRC (n~100,000).[107]
In addition, in **chapter 8**, we subsequently performed multiple two-sample MR analyses to determine if genetically proxied impaired fructose metabolism (reflected by urinary fructose levels derived from the Maastricht Study as determinant; **chapter 7**) is causally associated with the risk of clinically relevant cardiometabolic endpoints (as outcomes). For the outcomes we used data on the genetic associations between the rs2304681 minor A allele and cardiometabolic traits including IHL content (n~37,000), T2DM (n~1,332,000), hypertension (n~440,300), and myocardial infarction (n~583,200), derived from multiple open access databases.[108-112]

Last, in **chapter 9**, the key findings of the thesis are discussed as well as the methodological considerations of the reported studies. In addition, conclusions based on this thesis and suggestions for future research are reported.

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# PART I

## Background

## Overview of fructose metabolism and a new method to quantify fructose concentration

# Chapter 2

## Recent advances in the pathogenesis of hereditary fructose intolerance: implications for its treatment and the understanding of fructose-induced non-alcoholic fatty liver disease

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# Abstract

Hereditary fructose intolerance (HFI) is a rare inborn disease characterized by a deficiency in aldolase B, which catalyzes the cleavage of fructose 1,6-bisphosphate and fructose 1-phosphate (Fru 1P) to triose molecules. In patients with HFI, ingestion of fructose results in accumulation of Fru 1P and depletion of ATP, which are believed to cause symptoms, such as nausea, vomiting, hypoglycemia, and liver and kidney failure. These sequelae can be prevented by a fructose-restricted diet.

Recent studies in aldolase B deficient mice and HFI patients have provided more insight into the pathogenesis of HFI, in particular the liver phenotype. Both aldolase B deficient mice (fed a very low fructose diet) and HFI patients (treated with a fructose-restricted diet) displayed greater intrahepatic fat content when compared to controls. The liver phenotype in aldolase B deficient mice was prevented by reduction of intrahepatic Fru 1P concentrations by crossing these mice with mice deficient for ketohexokinase, the enzyme that catalyzes the synthesis of Fru 1P. These new findings not only provide a potential novel treatment for HFI but lend insight into the pathogenesis of fructose-induced non-alcoholic fatty liver disease (NAFLD), which has raised to epidemic proportions in Western society. This narrative review summarizes the most recent advances in the pathogenesis of HFI and discusses the implications for the understanding and treatment of fructose-induced NAFLD.

# Introduction

Hereditary fructose intolerance (HFI; OMIM 22960), an inborn error of fructose metabolism, was first reported in 1956 by Chambers and Pratt.[1] A 24-year old woman was admitted for evaluation of faintness, abdominal pain, and nausea upon fruit or sugar ingestion. The physicians subjected her to systematic, single-blinded exposure to a variety of oral sugars. Administration of solely fructose and sucrose, not glucose, galactose or lactose, provoked symptoms of nausea in a dose-dependent manner. Based on these findings, the patient was diagnosed with 'idiosyncrasy to fructose'.[1] Six years later, Hers and Joassin identified the enzymatic defect of HFI in two liver biopsy specimens as a 'functional deficiency of fructose-1-aldolase activity', i.e. aldolase B.[2]
Recent experimental and clinical studies have provided more insight into the pathogenesis of HFI, in particular its liver phenotype. In the present narrative review, we will give an overview of these studies and subsequently elaborate on the implications, not only for the treatment of HFI, but also for the current epidemic of fructose overconsumption.

# Background

## Clinical manifestations

The first symptoms of HFI appear when a neonate is exposed to fructose-containing infant formulas[3] or when fructose-containing foods, such as fruits and vegetables, are introduced to young infants.[4,5] Signs of acute intoxication are vomiting, abdominal pain, lactic acidosis, hyperuricemia, hypoglycemia, and acute liver failure. Persistent fructose ingestion can lead to failure to thrive, liver disease (i.e. hepatic steatosis, fibrosis, and cirrhosis), signs of proximal renal tubular dysfunction (i.e. Fanconi syndrome), and eventually death. These sequelae can be prevented when treated with a fructose-restricted diet. Further, since fructose can also be synthesized endogenously from sorbitol (via the polyol pathway, **Figure 2.1**), HFI patients additionally should avoid sorbitol-containing food products and high-levels of high-glycemic foods.[4,5] When adhering to these dietary restrictions, the prognosis of HFI appears excellent, although little is known about the long-term pathology of adults with HFI.[6-9]

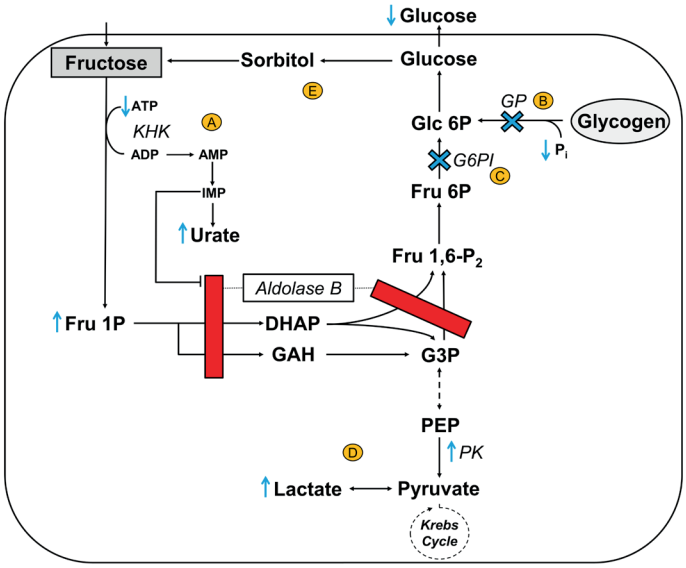


**Figure 2.1. Metabolic consequences of aldolase B deficiency in the liver after an oral fructose load.**
In physiological states, fructose is rapidly phosphorylated by KHK and subsequently converted by aldolase B to trioses (DHAP and GAH) that enter the glycolytic/gluconeogenic pathways. Aldolase B also catalyzes the conversion of Fru 1,6-$P_2$ to triose phosphates (DHAP and G3P). In aldolase B deficiency, the catabolism of Fru 1P is impaired, and the metabolism of Fru 1,6-$P_2$ is blocked (red bar). Accumulation of Fru 1P has several acute downstream effects denoted in yellow circled letters as follows: (1) depletion of intracellular inorganic phosphate ($P_i$) and ATP, and consequently formation of IMP and urate (A); (2) impairment of glycogenolysis (by inhibition of GP and loss of $P_i$) (B) and gluconeogenesis (by inhibition of G6PI) (C), resulting in hypoglycemia; and (3) stimulation of PK activity that—in combination with an impaired gluconeogenesis—promotes hyperlactatemia (D). Further, fructose, which can be produced endogenously from sorbitol (via the polyol pathway), may contribute to the accumulation of Fru 1P (E).
Blue cross indicates blocked pathway as a consequence of Fru 1P accumulation. Dashed arrow indicates multiple intermediate enzymatic steps that have not been visualized for simplicity purposes.
Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; Fru 6P, fructose 6-phosphate; Fru 1P, fructose 1-phosphate; Fru 1,6-$P_2$, fructose 1,6-biphosphate; G3P, glyceraldehyde 3-phosphate; Glc 6P, glucose 6-phosphate; G6PI, glucose-6-phosphate isomerase; GAH, glyceraldehyde; GP, glycogenphosphorylase; IMP, inosine monophosphate; KHK, ketohexokinase; PEP, phosphoenolpyruvate; Pi, inorganic phosphate; PK, pyruvate kinase.

## Genetics and epidemiology

The human gene for aldolase B *(ALDOB)* has been mapped to chromosome 9q22.3.[10,11] At present, over 40 causative mutations of the *ALDOB* gene have been documented, of which c.448G>C (p.A149P), c.524C>A (p.A174D), c.357delAAAC (Δ4E4), and c.1005C>G (p.N334K) account for 59% and 86% of HFI mutations in North Americans and Europeans, respectively.[12-17] Based on the carrier frequency of the most common mutations in neonates, it has been estimated that the incidence of HFI is 1:18,000-20,000 in live births.[18,19]

*Metabolic derangements*

The metabolic derangements of aldolase B deficiency have been the scope of previous, high-quality review papers.[9,20,21] Briefly, fructose-1,6-bisphosphate aldolase (aldolase; EC 4.1.2.13) is responsible for the reversible conversion of fructose 1,6-bisphosphate (Fru 1,6-$P_2$) or fructose 1-phosphate (Fru 1P) to the triose phosphate dihydroxyacetone phosphate (DHAP) and either glyceraldehyde 3-phosphate (G3P) or glyceraldehyde, respectively, which are intermediates of the glycolytic/gluconeogenic pathway (**Figure 2.1**).[22] At least three aldolase isozymes (A, B, and C) have been described which differ in tissue expression and activity for the substrates Fru 1,6-$P_2$ and Fru 1P. Aldolase B is expressed in the liver, kidney, and small intestine, and has activity for both Fru 1,6-$P_2$ and Fru 1P. This is in contrast to both aldolase A (predominantly expressed in skeletal muscle) and aldolase C (predominantly expressed in brain and smooth muscle) which have the highest efficiencies for Fru 1,6-$P_2$ as a substrate[23,24], although aldolase C may perform fructose metabolism in the brain.[25]

Liver biopsies of HFI patients show substantially reduced Fru 1P aldolase activity (0-15%), but preserved Fru 1,6-$P_2$ aldolase activity (5-30%) leading to a marked increase in the ratio of Fru 1,6-$P_2$ : Fru 1P activities, which was used as a diagnostic before the introduction of genetic testing.[26] This remains the only definitive diagnostic test as so many HFI-causing mutations remain unknown or variants found by DNA testing have unknown consequences.[15] The relatively preserved Fru 1,6-$P_2$ aldolase activity could theoretically be explained by residual aldolase A activity in the liver that compensates for the defect in aldolase B activity for the substrate Fru 1,6-$P_2$, but not for Fru 1P[20], or, alternatively, aldolase A activity in erythrocytes, which are also present in liver lysates.

As a consequence of the catalytic deficiency of aldolase B, a fructose load in HFI patients results in the rapid accumulation of Fru 1P and, hence, intracellular phosphate and adenosine triphosphate (ATP) depletion[27,28]. Reduced intracellular concentrations of inorganic phosphate lead to an increased rate of degradation of AMP.[29] As a result, adenosine deaminase and xanthine oxidase activities are increased and inosine monophosphate (IMP) and urate are rapidly formed (**Figure 2.1**).[29] The specific inhibition of aldolase B by the increased IMP further accentuates the increase in Fru 1P.[28]

High levels of intrahepatic Fru 1P – in combination with the loss of intracellular inorganic phosphate ($P_i$) – inhibit glycogenolysis by impairment of glycogen phosphorylase (GP).[30-33] This is also illustrated by the failure of exogenous glucagon to correct for the fructose-induced hypoglycemia in HFI patients.[34,35] Further, high levels of Fru 1P impair gluconeogenesis by competitive inhibition of glucose-6-phosphate isomerase (G6PI).[36,37] The rate of gluconeogenesis may also depend on the intrahepatic concentration of ATP[38], which is low in case of HFI following fructose ingestion. The

impaired gluconeogenesis is evidenced by the inability of dihydroxyacetone administration (which enters the gluconeogenic pathway) to prevent fructose-induced hypoglycemia in HFI patients.[35] In conclusion, fructose-induced impaired glycogenolysis and gluconeogenesis both result in a decreased hepatic glucose production and, consequently, the rapid development of hypoglycemia. Of note, in the absence of fructose, gluconeogenesis is not impaired in HFI.[39]

In addition, an impaired gluconeogenesis together with Fru-1P-induced activation of pyruvate kinase (PK) promote accumulation of lactate and, consequently, hyperlactatemia[40,41] (**Figure 2.1**). Notably, these metabolic defects do not only occur after oral intake of fructose, but also upon sorbitol consumption.[4,42] This is due to the oxidation of sorbitol to fructose via the polyol pathway (**Figure 2.1**). This pathway of endogenous fructose production can be activated through dehydration and hyperosmolarity as well as high glycemic foods.[43-46]

## Recent advances from animal studies

### The phenotype of aldolase B knockout mice resembles the human HFI phenotype

Recent work has demonstrated that aldolase B knockout (ALDOB-KO) mice exhibit similar metabolic features as HFI patients.[47,48] In these mice, chronic exposure to fructose resulted in growth retardation and death.[47,48] An acute, oral fructose load caused a rise in serum liver enzymes and intestinal jury, characterized by the destruction of apical villi and the presence of apoptotic cells in the duodenum and jejunum.[48] In addition, ALDOB-KO mice exposed to an oral fructose load showed decreased hepatic ATP and phosphate levels, and elevated serum urate concentrations.[48] Finally, oral fructose provoked severe hypoglycemia in a dose-dependent fashion.[48] Exploration of the gluconeogenic pathway by a pyruvate tolerance test revealed a reduced, but not absent ability for gluconeogenesis.[48] This is remarkable given the absence of aldolase B, which not only affects fructolysis but also glycolysis/gluconeogenesis (**Figure 2.1**). Furthermore, there was no residual aldolase A or C expression in the liver (Lanaspa, personal communication) and suggests that gluconeogenesis occurs in other tissues.[49] Some key enzymes of gluconeogenesis (i.e., phosphoenolpyruvate carboxykinase and glucose-6-phosphatase) were found to be upregulated in the livers of ALDOB-KO mice.[48]

Although Fru-1P-mediated impairment of glycogenolysis was not specifically studied, the ALDOB-KO mice were characterized by an increased hepatic glycogen content after an oral fructose load.[48] Of interest, glycogen synthase activity – determined by the ratio

of phosphorylated to total glycogen synthase – was increased[48], suggesting an enhanced glycogenesis. Of additional interest, the increased hepatic glycogen content and decreased serum glucose and insulin were also observed in ALDOB-KO mice that were not exposed to an acute oral fructose load.[48] This chronic feature could be due to the endogenous fructose production via the polyol pathway[4,42], or alternatively, an increased hepatic glucose uptake (see below).



## Aldolase B knockout mice are characterized by an increased intrahepatic triglyceride content

In addition to the above-described metabolic features, ALDOB-KO mice chronically exposed to small amounts of fructose in the chow (~0.3%) displayed an increased amount of hepatic triglycerides, hepatic inflammation – characterized by the presence of apoptotic and necrotic cells, and diffuse macrophage infiltration –, and signs of periportal fibrosis.[47,48] Hepatic expression of enzymes involved in *de novo* lipogenesis (DNL), i.e. ATP-citrate lyase (ACL), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), was greater in ALDOB-KO mice, suggesting that this pathway accounts, at least in part, for the increased hepatic triglycerides levels.[48] In addition, cytosolic glucokinase (GCK) was more abundant in ALDOB-KO mice when compared to wildtype mice.[48]

GCK converts glucose to glucose 6-phosphate (Glc 6P) in the liver, pancreas, and pituitary, and is the first step in glycolysis. Thanks to its unique kinetic properties, GCK is a major regulator of hepatic glucose uptake and pancreatic insulin secretion.[50] In the post-absorptive state, hepatic GCK is bound to glucokinase regulatory protein (GKRP), a liver specific protein. The GKRP-GCK complex resides in the nucleus and thus inactivates GCK.[51,52] In the postprandial state, a rise in intracellular glucose facilitates the dissociation of GCK from GKRP and migration of GCK to the cytosolic space where it facilitates phosphorylation and, hence, storage of glucose. Of interest, Fru 1P is a very potent disruptor of the GKRP-GCK complex. Experimental studies have shown that only trace amounts of Fru 1P are required to dissociate GCK from GKRP.[52-57] Notably, intrahepatic Fru 1P concentrations in ALDOB-KO mice were also elevated after chronic exposure to only small amounts of fructose in the chow.[48] From these studies, it can be speculated that accumulation of Fru 1P in ALDOB-KO mice chronically fed small amounts of fructose induces dissociation of the GKRP-GCK complex, which would explain the greater cytosolic GCK activity in ALDOB-KO mice. Consequently, hepatic glucose uptake is stimulated, thereby contributing to the reduced serum glucose and insulin levels in these mice. The metabolic fate of the glucose taken up by the liver can be several fold, amongst others an enhanced storage of glycogen and fat. Although the latter requires glycolysis (which appears to be blocked in case of aldolase B deficiency) and subsequent DNL, the pentose phosphate pathway (PPP) – a metabolic pathway

that parallels glycolysis – may serve as an alternative pathway to convert Glc 6P to G3P (**Figure 2.2**). Of interest, a previous experimental study has shown that the PPP increases in parallel to DNL in rat fatty livers.[58]

There are other biologically plausible mechanisms that could explain the upregulated DNL pathway leading to hepatic fat accumulation in ALDOB-KO mice. First, experimental studies have shown that activation of the AMP-deaminase pathway and formation of urate (**Figure 2.1**) induce mitochondrial dysfunction, which results in downregulation of fatty acid oxidation and stimulation of DNL.[59] Second, carbohydrate-responsive element-binding protein (ChREBP) is activated upon intracellular phosphate depletion and stimulates expression of glucose-6-phosphatase and DNL genes[60,61], all in accordance with the observations in ALDOB-KO mice.[48]

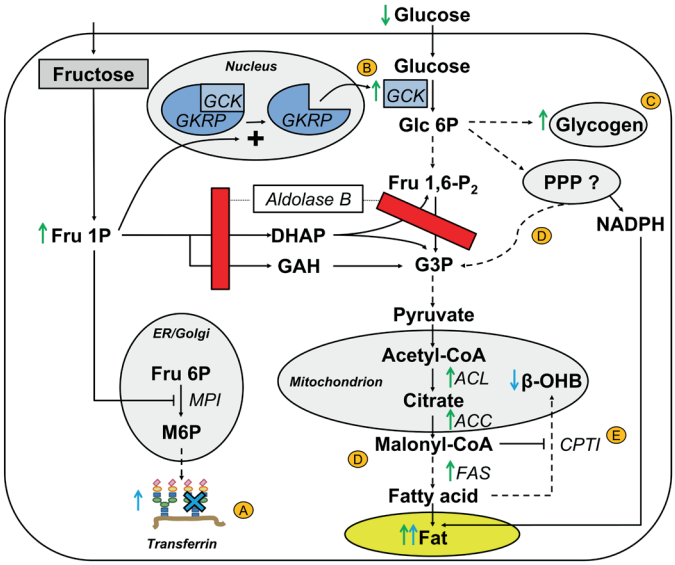


**Figure 2.2. Hypothesized pathogenesis of hepatic fat accumulation in aldolase B deficiency.**
Accumulation of Fru 1P has several chronic downstream effects leading to fat accumulation denoted in yellow circled letters. ALDOB-KO mice fed a low-fructose diet (~0.3%) display increased hepatic Fru 1P concentrations. This also seems to be the case in adult HFI patients treated with a fructose-restricted diet, as can be deduced from an abundancy of circulating hypoglycosylated transferrin. Hepatic Fru 1P inhibits glycosylation of transferrin by impairment of MPI (A). Catalytic amounts of Fru 1P dissociate GCK from GKRP in the nucleus, which allows migration of GCK toward the cytosolic space where it converts glucose to Glc 6P and, as a consequence, facilitates hepatic glucose uptake (B). The metabolic fates of an increased hepatic glucose uptake can be: (1) storage as glycogen (C) and (2) storage as fat via DNL with carbons and electrons derived from possibly the pentose phosphate pathway (PPP) (D). Malonyl-CoA, an intermediate of DNL, inhibits fatty acid beta-oxidation (and formation of β-OHB) through impairment of the mitochondrial fatty acid transporter CPTI (E). Of note, alternative mechanisms may contribute to the development of hepatic fat accumulation in aldolase B deficiency as well, such as Fru 1P-induced formation of urate and activation of ChREBP, which both stimulate DNL (see text).

Green arrows indicate observations in ALDOB-KO mice. Blue arrows and blue cross indicate observations in HFI patients. Dashed arrow indicates multiple intermediate enzymatic steps that have not been visualized for simplicity purposes.
Abbreviations: ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; ALDOB, aldolase B; β-OHB, beta-hydroxybutyrate; CPTI, carnitine palmitoyltransferase I; DHAP, dihydroxyacetone phosphate; ER, endoplasmic reticulum; Fru 6P, fructose 6-phosphate; FAS, fatty acid synthase; Fru 1P, fructose 1-phosphate; Fru 1,6-$P_2$, fructose 1,6-biphosphate; G3P, glyceraldehyde 3-phosphate; Glc 6P, glucose 6-phosphate; GAH, glyceraldehyde; GCK, glucokinase; GKRP, glucokinase regulatory protein; M6P, mannose 6-phosphate; MPI, mannose-6-phosphate isomerase; NADPH, nicotinamide; adenine dinucleotide phosphate; PPP, pentose phosphate pathway.



## Inhibition of ketohexokinase protects ALDOB-KO mice from metabolic derangements

The importance of Fru 1P in the pathogenesis of the metabolic derangements as observed in ALDOB-KO mice was unequivocally demonstrated by inhibition of ketohexokinase (KHK), the enzyme that catalyzes the first step in fructose metabolism: the phosphorylation of fructose to yield Fru 1P. In most mammals, including humans, KHK exists as two isoforms, A and C.[62] KHK-C has high affinity for fructose and is abundant in the liver, intestine, and kidney. In contrast, KHK-A has much lower affinity for fructose and is more ubiquitously expressed.[63] Nearly all of the aforementioned metabolic abnormalities in ALDOB-KO mice ameliorated when they were crossed with KHK knockout (KHK-KO) mice, i.e. both KHK-A and KHK-C.[48] Further, similar results were observed after treatment with osthole, a natural KHK inhibitor.[64] Fructose-loaded ALDOB-KO mice treated with osthole were protected from intrahepatic ATP depletion, hyperuricemia, rise in liver enzymes, and severe hypoglycemia.[48] In addition, osthole treatment resulted in a decrease of the GCK cytosol/nucleus ratio, indicative of more GCK bound to GKRP in the nucleus.[48]
Importantly, ALDOB-KO mice were not protected from the above mentioned metabolic abnormalities when crossed with KHK-A specific knockout mice.[48] In fact, the mice possessing only KHK-C resulted in an exacerbated phenotype.[48] This observation is likely explained by the fact that inhibition of KHK-A results in reduced metabolism of fructose in peripheral tissues and, hence, a greater supply to the liver, which is detrimental in case of aldolase B deficiency. These findings suggest that inhibition KHK-C may serve as a therapeutic target that could make the fructose-restricted diet redundant in HFI patients.

# Recent advances in humans

## Patients with HFI are characterized by an increased intrahepatic triglyceride content

Until recently, only anecdotal reports suggested that hepatic fat accumulation persists in HFI patients, despite a fructose-restricted diet.[6] A recent cross-sectional observational study including 16 genetically diagnosed HFI patients reported a high prevalence of fatty liver, as assessed by ultrasound or hepatic magnetic resonance imaging.[65] This issue was recently more structurally addressed in 15 adult HFI patients who were on a life-long fructose-restricted diet, ranging from 0.3 to 7.0 grams of fructose per day (the average fructose intake of American adults ranges from 32 to 75 grams per day[66]). Magnetic resonance imaging spectroscopy of the liver revealed that intrahepatic triglyceride (IHTG) content was higher in HFI patients in comparison to 15 healthy age-, sex-, and BMI-matched individuals.[67] Although liver stiffness, a non-invasive marker of liver fibrosis, was not significantly different between both groups, one HFI patient displayed a liver stiffness measurement compatible with liver fibrosis stage 3 or higher. Metabolic profiling revealed that HFI patients were more glucose-intolerant, as reflected by higher plasma glucose excursions during a standard 75-gram oral glucose tolerance test.[67]

Further investigations to delineate the underlying mechanism that leads to an increased IHTG content in HFI patients were limited due to the non-invasive nature of human studies. Nevertheless, the use of liver-specific plasma biomarkers allowed some insight. First, hypoglycosylated transferrin, a liver-specific protein, was more abundant in HFI patients, which is in line with previous studies.[68,69] Experimental studies have shown that Fru 1P inhibits mannose-6-phosphate isomerase (MPI) activity, one of the first enzymes involved in the glycosylation process (**Figure 2.2**).[70] The higher levels of hypoglycosylated transferrin (yet within the normal range) therefore suggest that intrahepatic Fru 1P concentrations are higher in HFI patients than in controls, even on a fructose-restricted diet. This may be explained by the minute levels of ingested fructose (blocked by aldolase B) or, alternatively, by endogenous fructose production via the polyol pathway. Despite the suggestion of higher intrahepatic Fru 1P levels in HFI patients on a fructose-restricted diet, plasma uric acid concentrations were not different between both groups.[67] This finding is consistent with observations in ALDOB-KO mice, which only displayed increased plasma uric acid levels after an oral fructose load.[48]

Second, plasma beta-hydroxybutyrate levels, a liver-specific biomarker of beta-oxidation, was significantly lower in HFI patients compared to healthy individuals.[67] Notably, DNL and beta-oxidation are reciprocally regulated. Malonyl-CoA, a precursor

of *de novo* synthesized fatty acids, inhibits the activity of the long-chain fatty acid transporter carnitine-palmitoyltransferase I (CPTI). Consequently, the transport of long-chain fatty acids over the mitochondrial membrane is hampered and beta-oxidation is impaired.[71] It can therefore be concluded that the biomarker patterns in HFI patients are similar to the in-depth phenotyping of the ALDOB-KO mice (as illustrated in **Figure 2.2**).



## Variants in the GKRP gene show phenotypic similarities with ALDOB-KO mice and HFI patients

Unfortunately, it is not possible to non-invasively measure the GKRP-GCK interaction as a potential explanation for the increased IHTG content in HFI patients since this would require liver biopsies. Nevertheless, genetic epidemiology is a valuable tool in predicting the metabolic consequences of increased GKRP-GCK disruption in humans.[72] Rs1260326 and rs789004 are common variants in the GKRP gene (*GCKR*), which are in strong linkage disequilibrium. The former is a functional variant that encodes a GKRP protein that dissociates from GCK more easily[73], comparable to the effect of Fru 1P on the GKRP-GCK complex. The previously reported associations of these common gene variants with cardiometabolic traits in the general population show some striking similarities with the metabolic abnormalities observed in ALDOB-KO mice and HFI patients (**Table 2.1**). First, variants in *GCKR* have been associated with reduced beta-hydroxybutyrate levels, pronounced DNL, and a greater IHTG content.[74-77] Further, these variants have been associated with lower fasting insulin concentrations[78,79] and higher 2-h post glucose load glucose levels[80], the former in agreement with ALDOB-KO mice[48] and the latter with HFI patients.[67] Of note, despite the consistently reported association between *GCKR* variants and increased plasma triglycerides[78,79], HFI patients were characterized by normal plasma triglycerides levels.[67] This discrepancy may be explained by the fact that HFI patients were (relatively) metabolically healthy, i.e., non-(abdominally) obese.[67] We previously reported that *GCKR* interacts with metabolic health on plasma triglycerides, i.e., the unhealthier the greater the effect on plasma triglycerides levels.[81] Finally, a recent meta-analysis suggested that the common variants in *GCKR* protect against chronic kidney disease, but predisposes to cardiovascular disease (CVD).[82] These relevant clinical endpoints have not been addressed in HFI patients chronically treated with a fructose-restricted diet and therefore deserve further study.

**Table 2.1. Cardiometabolic features in ALDOB-KO mice, HFI patients, and human carriers of common variants in the *GCKR* gene.**

| | ALDOB-KO mice[a] | HFI patients[b] | *GCKR*[c] | References |
|---|---|---|---|---|
| Intrahepatic triglycerides | ↑ | ↑ | ↑ | 48, 65, 67, 83 |
| Serum AST/ALT[d] | ↑ | ↑ | ↑ | 48, 65, 84 |
| DNL[e] | ↑ | ? | ↑ | 48, 76 |
| Serum beta-hydroxybutyrate | ? | ↓ | ↓ | 67, 74 |
| Intrahepatic glycogen | ↑ | ? | ? | 48 |
| Serum glucose | ↔ | ↔ | ↓ | 48, 67, 78-80 |
| Serum glucose, 2-h post glucose load | ? | ↑ | ↑ | 48, 67, 80 |
| Serum insulin | ↓ | ↔ | ↓ | 48, 67, 79, 80 |
| Serum urate | ↔ | ↔ | ↑ | 48, 67, 85, 86 |
| Serum triglycerides | ? | ↔ | ↑ | 65, 67, 78, 79, 81 |
| eGFR[f] | ? | ? | ↑ | 82 |
| Coronary artery disease | ? | ? | ↑ | 82 |

Arrows indicate the direction of association, not the effect size

[a] Observations in ALDOB-KO mice fed a low fructose diet (~0.3%)

[b] Observations in adult HFI patients chronically treated with a fructose-restricted diet

[c] Common variants in rs1260326 and rs780084, that encode a GKRP protein that binds glucokinase less effectively

[d] Aspartate transaminase (AST) and alanine transaminase (ALT)

[e] *De novo* lipogenesis (DNL) is assessed by hepatic expression of key enzymes (ALDOB-KO mice) and stable isotopes (*GCKR*)

[f] eGFR: estimated glomerular filtration

## Implications for the current epidemic of fructose overconsumption

Since the industrial revolution, the intake of fructose in the United States has risen dramatically.[66] Fructose – which has a sweeter taste than glucose – is often added as a sweetener (e.g., as high fructose corn syrup) to processed foods. Given the parallel increase in fructose consumption and the current obesity epidemic and its sequelae (dyslipidemia, type 2 diabetes mellitus [T2DM], gout, and CVD) in Western society, fructose has been implicated as a major contributing factor.[87-90]

Non-alcoholic fatty liver disease (NAFLD), a histological spectrum ranging from simple steatosis, to steatohepatitis, fibrosis, and cirrhosis, is another frequently encountered phenomenon in obese individuals.[91] NAFLD may not only progress to end-stage liver failure and hepatocellular carcinoma, it has also been associated with new-onset T2DM and CVD.[92,93] The pathogenesis of NAFLD involves a complex interaction between genetic factors and unhealthy lifestyle habits.[94]

Experimental studies in rodents and humans have unequivocally demonstrated that fructose overfeeding leads to an increased hepatic fat content[95-99] and many symptoms of the metabolic syndrome.[100] The mechanism by which fructose causes hepatic fat

accumulation can be directly by serving as a substrate for DNL. Further, fructose can also indirectly enhance DNL via the hitherto mentioned mechanisms: 1) Fru 1P-induced disruption of the GKRP-GCK complex, which facilitates hepatic glucose uptake and consequently DNL (**Figure 2.2**); 2) Fru 1P-induced ATP depletion and urate formation, which stimulates DNL[27-29]; and 3) Fru 1P-induced intracellular phosphate depletion, which activates ChREBP, a transcription factor with multiple downstream effects, amongst other stimulation of DNL.[60,61] Of note, these processes have been observed in humans with normal aldolase B function.[101-103]

The recent studies in ALDOB-KO mice and HFI patients suggest that the direct lipogenic effects of fructose do not necessarily play a role in the pathogenesis of fructose-induced NAFLD.[48,67] Moreover, they suggest that the accumulation of intermediates of fructolysis, i.e., Fru 1P, is a key element in the pathogenesis of fructose-induced NAFLD.

From these findings, it can also be deduced that inhibition of Fru 1P formation by blocking upstream KHK activity may be a novel therapeutic modality, not only for HFI, but also for fructose-induced NAFLD. Indeed, the fatty liver phenotype in fructose-fed mice improved after treatment with liver-specific small interfering RNA (siRNA) targeting *KHK* expression.[104] Further, previous experimental studies have demonstrated that fructose-fed KHK-KO mice were protected from hepatic fat accumulation and other metabolic abnormalities, such as obesity and hyperinsulinemia, when compared to wildtype mice.[105-107] Again, analogous to the observations in ALDOB-KO[48], specific knockout of KHK-A resulted in an exacerbation of the metabolic abnormalities, including increased hepatic fat accumulation.[106,107] Of interest, in humans, a loss of KHK results in essential fructosuria (OMIM #229800).[39] This benign condition is not known to provoke any clinical symptoms[39] and, hence, emphasizes the therapeutic potential of KHK inhibition.

## Future perspectives

The recent studies in ALDOB-KO mice and HFI patients have contributed to our understanding of the pathogenesis of HFI and fructose-induced NAFLD.[48,67] There are, however, several issues that deserve further study.

First, experimental studies are warranted to establish the exact roles (and their relative contributions) of the GKRP-GCK complex, urate, and ChREBP as potential mediators in the pathogenesis of hepatic fat accumulation in aldolase B deficiency. Furthermore, although the recent studies have convincingly identified Fru 1P as the key driver behind hepatic fat accumulation in aldolase B deficiency, the exact contribution of endogenous fructose production (via the polyol pathway) to the accumulation of intrahepatic Fru 1P

remains to be elucidated. Future studies are warranted to determine to what extent gluconeogenesis and glycolysis are functional in aldolase B deficient livers, and which alternative pathways (e.g. PPP) are involved. Long-term follow-up of a large cohort of HFI patients is needed to study whether these patients are protected from chronic kidney disease and predisposed to CVD, similar to individuals carrying common variants in *GCKR.*[82] Finally, clinical studies are required to demonstrate whether KHK inhibition will: a) replace the fructose-restricted diet as a treatment for HFI and b) be efficacious in the treatment of fructose-induced NAFLD in the general population. Interestingly, Huard et al.[108] recently reported the discovery of a small molecule that selectively inhibits KHK activity *in vitro* and *in vivo* more effectively than osthole.

## Concluding remarks

HFI is a rare inborn error of fructose metabolism. Recent studies in ALDOB-KO mice and HFI patients have proposed a prominent role for Fru 1P in the pathogenesis of hepatic fat accumulation, and suggest that an increased dissociation of GCK from GKRP is involved. These findings have therapeutic implications for not only HFI, but also for fructose-induced NAFLD in the general population. These studies clearly demonstrate that fructose-induced NAFLD can benefit from the insight gained from rare inborn errors of metabolism, and *vice versa*.

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# Chapter 3

## Development and validation of a UPLC-MS/MS method to quantify fructose in serum and urine

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# Abstract

**Background**

The study of the involvement of fructose in the pathogenesis of cardiometabolic disease requires accurate and precise measurements of serum and urinary fructose. The aim of the present study was to develop and validate such a method by Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (UPLC-MS/MS).

**Methods**

Fructose was quantified using hydrophilic interaction UPLC-MS/MS with a labelled internal standard. Serum fructose levels were determined in healthy individuals (n=3) after a 15-gram oral fructose load. Twenty-four hours urinary fructose levels were determined in individuals consuming low (median: 1.4 g/day, interquartile range [IQR]: 0.9-2.0; n=10), normal (31 g/day, 23-49; n=15) and high (70 g/day, 55-84; n=16) amounts of fructose.

**Results**

The calibration curves showed perfect linearity in water, artificial, serum, and urine matrices ($r^2$ >0.99). Intra- and inter-day assay variation of serum and urinary fructose ranged from 0.3 to 5.1% with an accuracy of ~98%. Fasting serum fructose levels (5.7±0.6 µmol/L) increased 60 min after a 15-gram oral fructose load (to 150.3±41.7 µmol/L) and returned to normal after 180 min (8.4±0.6 µmol/L). Twenty-four hours urinary fructose levels were significantly lower in low fructose consumers when compared to normal and high fructose consumers (median: 36.1 µmol/24 h, IQR: 26.4-64.2; 142.3 µmol/24 h, 98.8-203.0; and 238.9 µmol/24 h, 127.1-366.1; p=0.004 and p<0.001, respectively).

**Conclusion**

Fructose concentrations can be measured accurately and precisely with this newly-developed UPLC-MS/MS method. Its robustness makes it suitable for assessing the value of fructose in clinical studies.

# Introduction

The rise in intake of simple sugars has been associated with the current epidemic of cardiometabolic disease.[1-3] Fructose – which is the principal component of simple sugars, together with glucose – has been postulated to be the most detrimental carbohydrate.[1] This may be explained by differences in metabolic pathways and target organs, which is primarily the liver in case of fructose.[1]

Studies addressing the role of fructose in the pathogenesis of cardiometabolic disease require accurate and precise determination of fructose in different body fluids. Currently available methods, including enzymatic methods[4] and methods based on liquid chromatography (LC)[5,6], gas chromatography mass spectrometry (GC-MS)[7], and LC-MS[5], however, have some limitations. Enzymatic methods[4], for example, have high inter-batch variation and lack sensitivity and selectivity.[8,9] In addition, GC-MS requires elaborate sample preparation[7,8,10] and high sample volumes[8], and is insufficient sensitive to measure fructose in the micromolar range.[7] Therefore, the quantification of serum fructose was challenging or not possible with these methods. In addition, robust chromatographic separation between glucose and fructose is needed to determine fructose without interference of the high glucose concentrations.

To study the impact of fructose in cardiometabolic disease we need a well-validated and robust technique for the quantification of fructose. Therefore, the aim of the present study was to develop and validate a new Ultra Performance Liquid Chromatography–tandem Mass Spectrometry (UPLC-MS/MS) method with good validity and precision as a means of quantifying serum and urinary fructose. We describe a newly developed specific and highly sensitive method for determination of fructose in serum and urine. This method is state-of-the-art, fully validated according to international guidelines[11-13], and an improvement compared to above previous described methods.

# Materials and methods

## Materials

D-fructose (≥99%), $^{13}C_6$-D-Fructose (99%), cesium acetate (99.9%) and triethylamine (TEA, ≥99%) were obtained from Sigma-Aldrich (Steinheim, Germany). Sulfosalicylic acid (SSA, 98%) was obtained from Acros (Geel, Belgium). Water and acetonitrile (ULC/MS quality) were obtained from Biosolve Chimie (Dieuze, France).

## Sample preparation

Serum blood was collected and stored at -20°C until analysis (further described in more detail). Before analysis, defrosted serum samples were thawed and mixed thoroughly. One hundred µL serum was mixed with 10 µL $^{13}C_6$-fructose (3063 µmol/L) and deproteinized with 5 mg SSA in an Eppendorf cup. After centrifugation (24000 g, 4°C, 10 minutes), 50 µL supernatant was mixed with 150 µL acetonitrile in an injection vial.
Twenty-four hours urine was collected and stored at -20°C until analysis (further described in more detail). Before analysis, defrosted urine samples were thawed and mixed thoroughly. Fifty µL urine was mixed with 10 µL $^{13}C_6$-fructose (3063 µmol/L) and 200 µL acetonitrile. After centrifugation (24000 g, 4°C, 10 minutes), the supernatant was transferred to an injection vial.

## UPLC tandem MS analysis

Fructose was chromatographically separated from other sugars by ultra-performance liquid chromatography (Acquity UPLC, Waters, Milford, USA) and detected in ESI positive multiple reaction monitoring (MRM) mode using a Xevo TQ-XS (Waters, Milford, USA). Hydrophilic Interaction chromatography (HILIC) was performed on an Acquity UPLC BEH Amide, 150 x 2.1 mm, 1.7 µm (Waters, Milford, USA) with a binary gradient of 30 µmol/L cesium acetate and 0.02% (v/v) TEA in MS water (solvent A) and 30 µmol/L cesium acetate and 0.02% (v/v) TEA in acetonitril (solvent B). A linear gradient was started at 15% solvent A, which was changed within 6.5 minutes to 24% solvent A. After cleaning the column with 45% solvent A for 2 minutes, the column was equilibrated for 2 minutes at the initial conditions. The injection volume was 4 µl and the column temperature was 75°C at a flow rate of 200 µl/min. Quantification of fructose was performed by calculating the peak area ratio of the fructose peak area to the $^{13}C_6$-fructose peak area. The MRM transitions for fructose and $^{13}C_6$-fructose were respectively 313.2>133.1 and 319.2>133.1. Electrospray ionization was performed at a capillary voltage of 3.5 kV, a source temperature of 150°C, and a desolvation temperature of 150°C. For qualitative and quantitative analysis, Masslynx software (V4.1, SCN 644, Waters, Milford, USA) was used.

## Method validation

The validation protocol was based on international guidelines[11-13], and covered all necessary parameters including selectivity, recovery, matrix effect, specificity, sensitivity, linearity, precision, accuracy, and stability.

*Selectivity, recovery, and matrix effect*

Selectivity can be verified by the absence of a chromatographic peak at the expected retention time of the analyte in a "blanc" matrix. However, for endogenous compounds, like fructose in serum or urine, this is not possible. Therefore, recovery was used as an approximation of selectivity. The relative recovery was determined by spiking fructose to six different serum (0, 50 and 100 µmol/L) and urine (0, 100 and 200 µmol/L) samples. Spiked and non-spiked samples were analyzed in triplicate and recovery (%) was calculated by subtracting the basal value from the spiked value divided by the spiked concentration. The internal standard normalized matrix factor (IS-norm MF) was used to evaluate the matrix effect.[14,15] Six different post-extracted serum and urine matrices were spiked and analyzed as described above. Fructose standards and IS were prepared in solvent accordingly (neat solution). The matrix factor for fructose (MF-F) and IS (MF-IS) was calculated by the ratio of the peak area in the presence of matrix (post-extracted), to the peak area in the absence of matrix (neat solution) after correction of the endogenous fructose concentration in that matrix. IS-norm MF was calculated by dividing the MF-F by the MF-IS. The coefficient of variation (CV, %) of the IS-norm MF should not exceed 15%.

*Specificity*

Specificity is defined as the ability to distinguish and measure the analyte in the presence of structurally similar moieties in the intended matrix. Therefore, a standard mixture of glucose, mannose, and galactose was spiked at four concentration levels (0, 100, 1000 and 5000 µmol/L) to two different pooled serum and urine samples. All samples were analyzed in triplicate. Responses detected and attributable to the interfering components should not be more than 20% of the fructose response.

*Sensitivity*

Sensitivity was defined as the lower limit of quantification (LLOQ) which is the lowest nonzero standard on the calibration curve. A quality control sample at this low level is not available because fructose is always present in serum and urine. Therefore, an artificial serum and urine matrix was prepared as described previously.[16] The artificial serum matrix was spiked at a target concentration of 5 µmol/L fructose and the artificial urine matrix was spiked at a target concentration of 20 µmol/L fructose. QC samples were analyzed in five-fold on three separate days. Accuracy (ratio of the determined concentration and the nominal concentration, %) should be ± 20% and precision should be ± 20% CV.

*Linearity*

Linearity was determined by adding a standard solution of fructose to water, serum, urine, and artificial matrices. Concentration ranges were chosen based on physiological concentrations. A zero calibrator, and six calibrators were prepared for serum (5-150 µmol/L), urine (20-300 µmol/L), water and corresponding artificial matrices. The peak area ratio of fructose and $^{13}C_6$-fructose multiplied by the concentration of $^{13}C_6$-fructose was plotted as a function of the concentration fructose. For each calibration curve slope, the correlation coefficient ($r^2$) of 0.99 or better was accepted.

*Precision and accuracy*

Intra-day precision and accuracy was determined by the analysis of low, middle, and high QC samples (n=6). Therefore, artificial serum was spiked with 25, 75 and 150 µmol/L fructose and artificial urine was spiked with 50, 100 and 300 µmol/L fructose. Inter-day precision and accuracy was determined by repeating these analytical runs on three different days. In addition, these experiments were done with two different serum and urine samples to address variability due to the biological matrix. Analysis of these biological QCs will give the mean endogenous background concentrations and only precision (and no accuracy) can be determined for these QCs. The mean value for precision (CV, %) and accuracy (%) should be within ±15%.

*Stability*

Freeze/thaw and auto-sampler stability were measured at three levels of QCs and in two different serum and urine samples. The QC concentration was determined in triplicate and compared with the theoretical concentration. QC Accuracy should be within ±15% and biological matrix was used to investigate possible changes after freeze/thaw cycles and/or over time. Freeze/thaw stability of QCs and biological matrix was determined by three cycles of freeze/thaw. Samples were stored at −80°C for 24 h and subsequently thawed at room temperature. When completely thawed, samples were refrozen for 24 h. Auto-sampler stability was evaluated by keeping samples in the auto-sampler at 6°C after 20 h followed by their re-injection.

*Method comparison*

For method comparison, an enzymatic-spectrometric method based on the formation of reduced nicotinamide-adenine dinucleotide phosphate via phosphorylation of D-fructose by hexokinase and adenosine-5'-triphosphate was used.[17]

## Clinical validation studies

*Serum fructose levels after an oral fructose load*

Fasting and post load serum fructose levels were determined in a pilot study including healthy individuals (n=3). Participants received a 15-gram oral fructose load after an overnight fast of at least eight hours. Serum fructose concentrations were measured at baseline (t=0), t=60, t=120, and t=180 minutes.

*Urinary fructose levels in individuals consuming different amounts of fructose*

Urinary fructose levels were measured in three study groups of participants with a wide, distinctive range of average daily fructose intake. First, patients with hereditary fructose intolerance (HFI), an inborn error of fructose metabolism that is treated with a fructose-restricted diet, were included as a group reflective of a low daily fructose intake (further referred to as low fructose consumers). The characteristics of these patients (n=15) have been reported previously.[18] Second, these HFI patients were compared to healthy individuals matched for age, sex, and body mass index (BMI; n=15). Since these individuals did not follow a specific diet, they are included in the present study as a group reflective of a normal daily fructose intake (further referred to as normal fructose consumers). Third, participants of the FRUITLESS study (Effects of Fructose Restriction on Liver Steatosis; ClinicalTrials.gov Identifier: NCT03067428) were included as a group reflective of a high daily fructose intake (further referred to as high fructose consumers). The FRUITLESS study is a double-blind randomized placebo-controlled study, in which participants are randomized to a six-week fructose-restricted diet with either glucose or fructose supplementation. Inclusion criteria for study participation were: 1) a daily fructose intake exceeding the Dutch average daily fructose intake of 45 gram/day[19], 2) a fatty liver index ≥60 (which is compatible with fatty liver disease[20]), and 3) a BMI ≥28 kg/m$^2$. In the present study, the baseline measurements of the first 17 consecutive participants are included, i.e. before initiation of the fructose-restricted diet and glucose/fructose supplementation.

All participants provided written informed consent prior to study inclusion. Both studies were carried out according to the Declaration of Helsinki[21], and approved by the medial ethical committee of Maastricht University Medical Center (study numbers 162003 and 162034).

The study protocols of both studies (HFI study and FRUITLESS study) are equivalent with regard to all measurements, except for glucose measurements (which was done in whole blood and plasma in the HFI and FRUITLESS study, respectively). The measurements conducted in the HFI study have been described in detail previously.[18] In short, all participants visited the research ward after an overnight fast of at least eight

hours to undergo, amongst others, anthropometric measurements (height, weight, and waist and hip circumference), assessment of systolic and diastolic blood pressure, and blood withdrawal for determination of whole blood/plasma glucose, and serum lipids, phosphate, and creatinine levels. Whole blood was collected in a covered serum test tube (BD Vacutainer, Plymouth, UK) and was allowed to stand for 20 minutes at room temperature and subsequently centrifuged (1800 g, room temperature, 15 minutes). The resulting supernatant was transferred into a cryotube and stored at -20°C until analysis. Daily fructose intake was assessed using a three-day food journal and a recently developed, extensive sugar composition table.[22] Further, at the third (corresponding the final) day of dietary intake recording, urine was collected for 24 hours in 2L containers with 12 ml HCL added as a preservative. Prior to urine collection, all participants received instructions on how to collect 24h urine samples. They were asked to discard their first urine sample in the morning and from then onwards to collect all samples for 24 hours including the first sample of the following day. Participants recorded in the case of missing a urine collection. After delivery of urine collections to the research unit, samples were measured and stored at −20°C upon further analysis. Urinary phosphate and creatinine levels were determined (enzymatic colorimetric assay, Cobas 8000 instrument, Roche Diagnostics, Mannheim, Germany). The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.[23] Proximal tubular function was determined by the ratio of tubular maximum reabsorption of phosphate (TmP) to GFR (TmP/GFR).[24] Only those urine samples that were collected for ≥20 hours were included in the present analyses.

## Statistical analyses

*Method validation*

Data are presented as mean with standard deviation. We compared the performance of a UPLC-MS/MS method quantifying serum and urinary fructose concentrations with that of an enzymatic-spectrometric method (n=55 paired samples). However, it was not possible to analyze serum fructose with the enzymatic-spectrometric method due to its low sensitivity. Therefore, the validation between the two methods was limited to urinary fructose. Pearson correlation coefficient was used to assess whether the ranking of urinary fructose concentrations was similar between the two methods. In addition, a Bland-Altman plot of the differences between the two methods' data *vs.* their mean was obtained to examine the levels of agreement and verify the absence of systematic error.[25] The Bland-Altman plot was drawn on $\log_e$ transformed data because the distribution of the differences was skewed.[25] Last, two-way mixed

effects models (absolute agreement) was used to calculate the intra-class correlation coefficient (ICC), indicating similarity in individuals' rank and similarity in absolute urinary fructose concentrations as obtained by two methods.[26]

*Clinical validation studies*

Data are presented as mean with standard deviation or median with interquartile range (IQR) for normally and non-normally distributed, continuous variables, respectively. Data are presented as number for categorical variables. Mann Whitney U tests were used to compare baseline characteristics between the three study groups of distinctive fructose intake (with low fructose consumers as the reference group). P-values were Hochberg-adjusted to correct for multiple testing.
Linear regression was used to assess determinants of (log-transformed) 24h urinary fructose concentrations. Fructose intake, age, sex, BMI, eGFR, and TmP/GFR were entered as independent variables. In a sensitivity analysis, (log-transformed) 24h urinary fructose concentrations were adjusted for urinary creatinine levels.
All analyses were carried out with the IBM Statistical Package of Social Sciences (SPSS) version 25 for Windows (SPSS inc. Chicago, IL; www.spss.com). Results were considered statistically significant at $p<0.05$.



# Results

## Hydrophilic interaction chromatography

Fructose was separated from other sugars with a HILIC-UPLC column with a retention time of 4.53 minutes and detected in MRM-mode for specific Cs+ daughter-ions. Representative chromatograms of a standard solution of fructose, serum, and urine sample are shown in **Supplemental Figure S3.1**.

## Method validation

*Selectivity, recovery, and matrix effect*

Recovery was used as an approximation of selectivity. The mean recovery of fructose in serum, spiked with 50 and 100 µmol/L fructose, was 102.5% and 101.2%, respectively (**Table 3.1a**). The mean recovery of fructose in urine, spiked with 100 and 200 µmol/L fructose, was 89.4 and 93.8%, respectively (**Table 3.1b**). The matrix factor for the individual serum and urine samples are summarized in **Table 3.1a and 3.1b**. The CV% of the IS-norm MF was below 15% demonstrating that there is no significant influence of the matrix on fructose analysis.

**Table 3.1a. Recovery and matrix factor of fructose in serum.**

| | Nominal conc. (µmol/L) 0 | | Nominal conc. (µmol/L) 50 | | | | Nominal conc. (µmol/L) 100 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Serum** | **Found conc. (µmol/L)** | **Precision (%)** | **Found conc. (µmol/L)** | **Precision (%)** | **Recovery (%)** | **IS-norm MF** | **Found conc. (µmol/L)** | **Precision (%)** | **Recovery (%)** | **IS-norm MF** |
| A | 7.4 ± 1.4 | 18.2 | 57.5 ± 1.0 | 1.7 | 100.1 | 0.91 | 104.4 ± 0.7 | 0.7 | 97.0 | 0.92 |
| B | 77.8 ± 1.4 | 1.8 | 127.2 ± 2.4 | 1.9 | 98.8 | 1.08 | 175.1 ± 2.6 | 1.5 | 97.3 | 0.80 |
| C | 7.1 ± 0.5 | 7.0 | 56.1 ± 2.1 | 3.8 | 97.9 | 1.14 | 103.4 ± 2.5 | 2.5 | 96.2 | 0.87 |
| D | 62.7 ± 4.1 | 6.5 | 116.7 ± 2.8 | 2.4 | 107.9 | 0.96 | 170.4 ± 1.7 | 1.0 | 107.7 | 1.00 |
| E | 7.45 ± 0.3 | 4.0 | 58.2 ± 2.7 | 4.6 | 101.6 | 1.03 | 113.1 ± 6.1 | 5.4 | 105.6 | 1.02 |
| F | 7.6 ± 0.3 | 3.5 | 61.9 ± 2.2 | 3.7 | 108.6 | 1.01 | 110.8 ± 0.9 | 0.8 | 103.1 | 1.04 |
| | | | | **Mean** | **102.5** | **1.02** | | **Mean** | **101.2** | **0.94** |
| | | | | **VC%** | **4.5** | **8.0** | | **VC%** | **4.9** | **9.9** |

Data are presented as mean (n=3) ± standard deviation.

**Table 3.1b. Recovery and matrix factor of fructose in urine.**

| | Nominal conc. (µmol/L) 0 | | Nominal conc. (µmol/L) 100 | | | | Nominal conc. (µmol/L) 200 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Urine** | **Found conc. (µmol/L)** | **Precision (%)** | **Found conc. (µmol/L)** | **Precision (%)** | **Recovery (%)** | **IS-norm MF** | **Found conc. (µmol/L)** | **Precision (%)** | **Recovery (%)** | **IS-norm MF** |
| A | 25.5 ± 1.2 | 4.5 | 118.5 ± 2.9 | 2.4 | 92.9 | 1.17 | 221.0 ± 8.6 | 3.9 | 97.7 | 1.08 |
| B | 16.1 ± 2.1 | 13.1 | 102.0 ± 11.2 | 11.0 | 85.9 | 1.28 | 204.6 ± 13.9 | 6.8 | 94.2 | 0.97 |
| C | 8.3 ± 1.1 | 13.0 | 98.0 ± 2.0 | 2.0 | 89.7 | 0.93 | 193.8 ± 3.8 | 2.0 | 92.7 | 0.99 |
| D | 4.8 ± 0.3 | 6.2 | 100.7 ± 1.4 | 1.4 | 95.9 | 1.00 | 208.1 ± 8.8 | 4.2 | 101.6 | 1.03 |
| E | 120.3 ± 1.4 | 1.2 | 198.4 ± 1.9 | 0.9 | 78.0 | 0.91 | 282.3 ± 6.2 | 2.2 | 81.0 | 0.82 |
| F | 5.8 ± 1.2 | 21.3 | 99.7 ± 11.7 | 11.7 | 93.9 | 1.22 | 197.1 ± 4.3 | 2.2 | 95.6 | 0.96 |
| | | | | **Mean** | **89.4** | **1.09** | | **Mean** | **93.8** | **0.97** |
| | | | | **VC%** | **7.4** | **14.6** | | **VC%** | **7.5** | **9.0** |

Data are presented as mean (n=3) ± standard deviation.

*Specificity*

No significant influence of structurally similar moieties in serum or urine was demonstrated. Precision (CV%) of two different serum and urine pool samples, spiked with three levels of standard mix, ranged from 1.8 to 7.2% (**Table 3.2a and 3.2b**).

**Table 3.2a. Specificity of fructose in serum.**

| Added std Mix* (µmol/L) | Serum pool A Found conc. (µmol/L) | Precision (%) | Serum pool B Found conc. (µmol/L) | Precision (%) |
|---|---|---|---|---|
| 0 | 9.2 ± 0.43 | 4.7 | 35.4 ± 0.9 | 2.5 |
| 100 | 10.8 ± 1.0 | 9.6 | 35.8 ± 0.6 | 1.7 |
| 1000 | 10.0 ± 0.8 | 7.5 | 35.1 ± 0.6 | 1.8 |
| 5000 | 9.3 ± 0.8 | 9.0 | 36.6 ± 1.2 | 3.4 |
| Mean ± SD | 9.8 ± 0.7 | 7.2 | 35.7 ± 0.7 | 1.8 |

Data are presented as mean (n = 3) ± standard deviation.
*Standard mixture of glucose, mannose, and galactose.



**Table 3.2b. Specificity of fructose in urine.**

| Added std Mix* (µmol/L) | Urine pool A Found conc. (µmol/L) | Precision (%) | Urine pool B Found conc. (µmol/L) | Precision (%) |
|---|---|---|---|---|
| 0 | 17.0 ± 0.3 | 1.9 | 114.7 ± 2.9 | 2.5 |
| 100 | 18.5 ± 0.5 | 2.5 | 118.7 ± 5.5 | 4.6 |
| 1000 | 18.0 ± 0.7 | 3.9 | 119.2 ± 3.1 | 2.6 |
| 5000 | 16.5 ± 0.4 | 2.5 | 115.3 ± 6.2 | 5.3 |
| Mean ± SD | 17.5 ± 0.9 | 5.2 | 117.0 ± 2.3 | 2.0 |

Data are presented as mean (n=3) ± standard deviation.
*Standard mixture of glucose, mannose, and galactose.

*Sensitivity*

The LLOQ of fructose in serum and urine was 5 and 20 µmol/L, respectively. The accuracy, intra- and inter-day precision (CV%) are summarized in **Table 3.3a and 3.3b**. Accuracy for the LLOQ in serum and urine ranged from 82.8 to 113.0%. Intra- and inter-day precision for the LLOQ in serum and urine ranged from 2.2 to 9.7%.

**Table 3.3a. Intra- and inter-day precision and accuracy of fructose in serum and QC samples.**

| Sample | Nominal conc. (µmol/L) | Measured conc. (µmol/L) | Precision (CV, %) | Accuracy (%) |
|---|---|---|---|---|
| Intra-day (n=6) | | | | |
| Serum A | - | 9.6 ± 0.2 | 2.0 | - |
| Serum B | - | 24.8 ± 0.7 | 2.0 | - |
| LLOQ | 5 | 5.5 ± 0.1 | 2.2 | 109.9 |
| Low QC | 25 | 25.7 ± 1.3 | 5.1 | 102.6 |
| Medium QC | 75 | 74.0 ± 1.7 | 2.3 | 98.6 |
| High QC | 150 | 147.8 ± 0.4 | 0.3 | 98.6 |
| Inter-day (n=3) | | | | |
| Serum A | - | 10.1 ± 0.4 | 4.2 | - |
| Serum B | - | 33.9 ± 0.8 | 2.3 | - |
| LLOQ | 5 | 5.65 ± 0.16 | 2.9 | 113.0 |
| Low QC | 25 | 26.7 ± 0.9 | 3.4 | 106.8 |
| Medium QC | 75 | 76.3 ± 2.1 | 2.7 | 101.7 |
| High QC | 150 | 151.0 ± 3.0 | 2.0 | 100.7 |

Data are presented as mean ± standard deviation.

**Table 3.3b. Intra- and inter-day precision and accuracy of fructose in urine and QC samples.**

| Sample | Nominal conc. (µmol/L) | Measured conc. (µmol/L) | Precision (CV, %) | Accuracy (%) |
|---|---|---|---|---|
| Intra-day (n=6) | | | | |
| Urine A | - | 17.7 ± 0.9 | 4.8 | - |
| Urine B | - | 114.5 ± 2.9 | 2.5 | - |
| LLOQ | 20 | 16.6 ± 0.8 | 4.9 | 82.8 |
| Low QC | 50 | 44.2 ± 1.3 | 3.0 | 88.3 |
| Medium QC | 100 | 93.6 ± 1.5 | 1.6 | 93.6 |
| High QC | 300 | 283.0 ± 4.2 | 1.5 | 94.3 |
| Inter-day (n=3) | | | | |
| Urine A | - | 17.4 ± 0.4 | 2.0 | - |
| Urine B | - | 114.1 ± 0.9 | 0.8 | - |
| LLOQ | 20 | 18.6 ± 1.8 | 9.7 | 93.1 |
| Low QC | 50 | 45.7 ± 1.7 | 3.8 | 91.4 |
| Medium QC | 100 | 92.8 ± 1.0 | 1.1 | 92.8 |
| High QC | 300 | 281.4 ± 2.2 | 0.8 | 93.8 |

Data are presented as mean ± standard deviation.

*Linearity*

Calibration curves for fructose were linear over the concentration ranges in water, artificial, serum, and urine matrix ($r^2$>0.99; **Figure 3.1**). Mean slope (response factor, [Rf]) for fructose as tested in water, artificial serum, artificial urine, two different urine samples, and two different serum samples was 0.9325±0.022 (2.4%, CV).

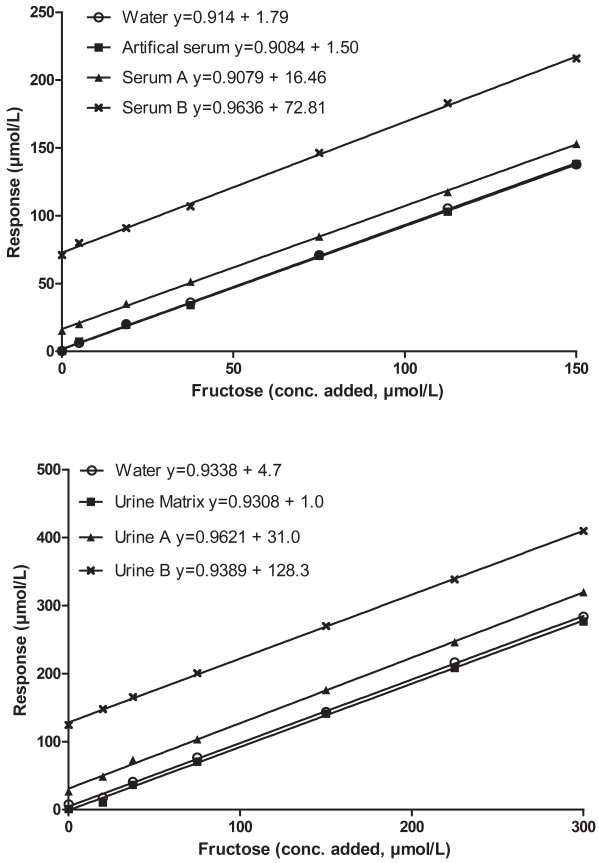


**Figure 3.1. Calibration curves of water and serum; and water and urine.**
Linearity was determined by adding a standard solution of fructose to water, serum, and urine. The peak area ratio of fructose/$^{13}C_6$-fructose multiplied by the concentration of $^{13}C_6$-fructose (response, Y) was plotted as a function of the added fructose concentration (X).

*Precision and accuracy*

The intra- and inter-day precision and accuracy of all QC samples are summarized in **Table 3.3a and 3.3b**. The intra- and inter-day precision and accuracy were between 0.3 and 3.8% and, 88.3 and 106.8%, respectively.

*Stability*

QC samples, and two different serum and urine samples were stable for at least 3 freeze/thaw cycles and when stored at 6°C for at least 20 h in the auto-sampler (**Table 3.4a and 3.4b**). The stability results were within the set criteria, indicating that fructose was stable under the test conditions.

**Table 3.4a. Stability of fructose in serum.**

| Test | Nominal conc. (µmol/L) | Measured conc. (µmol/L) | Precision (%) | Accuracy (%) |
|---|---|---|---|---|
| Auto-sampler | 25 | 27.1 ± 1.1 | 4.1 | 108.6 |
| | 75 | 76.0 ± 0.8 | 1.1 | 101.3 |
| | 150 | 152.9 ± 2.5 | 1.6 | 101.9 |
| | Serum pool A | 10.8 ± 1.3 | 12.3 | - |
| | Serum pool B | 37.1 ± 1.1 | 2.9 | - |
| Freeze/thaw | 25 | 26.4 ± 1.4 | 5.3 | 105.6 |
| | 75 | 74.2 ± 1.2 | 1.7 | 99.0 |
| | 150 | 149.2 ± 1.6 | 1.1 | 99.4 |
| | Serum pool A | 9.5 ± 0.6 | 6.0 | - |
| | Serum pool B | 36.2 ± 1.1 | 3.1 | - |

Data are presented as mean ± standard deviation.

**Table 3.4b. Stability of fructose in urine.**

| Test | Nominal conc. (µmol/L) | Measured conc. (µmol/L) | Precision (%) | Accuracy (%) |
|---|---|---|---|---|
| Auto-sampler | 50 | 44.6 ± 1.1 | 2.5 | 89.3 |
| | 100 | 93.1 ± 1.2 | 1.3 | 93.1 |
| | 300 | 282.9 ± 4.9 | 1.8 | 94.3 |
| | Urine pool A | 17.1 ± 0.3 | 1.8 | - |
| | Urine pool B | 116.1 ± 3.9 | 3.4 | - |
| Freeze/thaw | 50 | 45.7 ± 1.7 | 3.8 | 91.4 |
| | 100 | 93.0 ± 1.1 | 1.2 | 93.0 |
| | 300 | 282.8 ± 0.4 | 0.1 | 94.3 |
| | Urine pool A | 18.3 ± 1.7 | 9.0 | - |
| | Urine pool B | 115.1 ± 0.9 | 0.8 | - |

Data are presented as mean ± standard deviation.

*Method comparison*

The Pearson correlation coefficient for urinary fructose concentrations as obtained by a UPLC-MS/MS method and an enzymatic-spectrometric method was high ($r^2$: 0.913; n=55; **Supplemental Figure S3.2a**). Further, the Bland-Altman plot showed no systematic differences between both methods (**Supplemental Figure S3.2b**). Last, the ICC between the UPLC-MS/MS method and the enzymatic-spectrometric method was 0.963 for urinary fructose concentrations.

## Clinical validation studies

*Serum fructose after a 15-gram oral fructose load*

Serum fructose levels increased from 5.7±0.6 µmol/L at baseline to 150.3±41.7 µmol/L at t=60 minutes after a 15-gram oral fructose load in healthy individuals (n=3; all men; age: 29±15 years; BMI: 22.1±1.9 kg/m$^2$). Serum fructose levels subsequently decreased over the following 2-hour time course to 23.5±10.4 µmol/L and 8.4±0.6 µmol/L at t=120 and 180 minutes, respectively (**Figure 3.2a**).



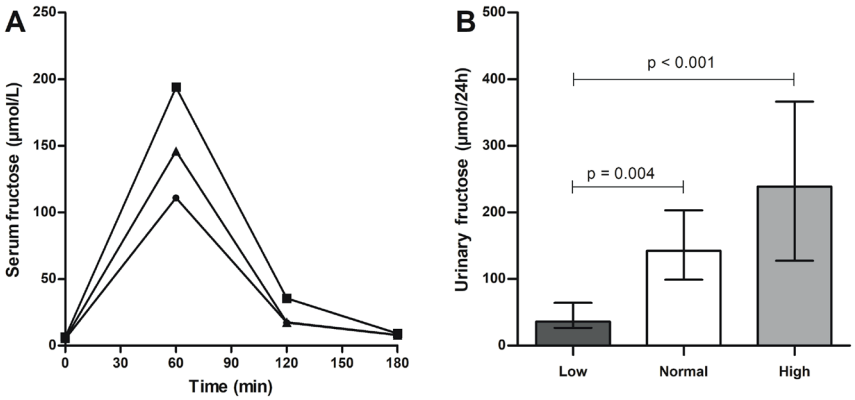


**Figure 3.2. Individual serum fructose levels after a 15-gram fructose load in three healthy individuals (A); and 24 h urinary fructose levels in low, normal, and high fructose consumers (B).**
Data are presented as median (IQR). Analyzed with a Mann Whitney U test (reference group low fructose consumers). P-values are Hochberg-adjusted to correct for multiple-testing.

*Urinary fructose in individuals consuming different amounts of fructose*

Six individuals that were included in the original study populations were excluded from the present study because of incomplete (<20 hours) urine collection (low fructose consumers n=5; high fructose consumers n=1).
In the final study population, normal fructose consumers (median: 31 g/day, IQR: 23-49; n=15) did not significantly differ for age, sex, anthropometrics, and serum lipids from low fructose consumers (median: 1.4 g/day, IQR: 0.9-2.0; n=10; **Table 3.5**), which can largely be explained by the fact that they were originally matched for age, sex and BMI.[18] High fructose consumers (median: 70 g/day, IQR: 55-84; n=16) had a significantly higher BMI, waist circumference, and serum triglycerides concentration, and lower serum HDL-cholesterol levels than low fructose consumers (**Table 3.5**). These differences may, at least in part, be explained by the original inclusion criteria of the high fructose consumers, i.e. a high fatty liver index, which includes, amongst others,

BMI, waist circumference, and serum triglycerides.[20] Low fructose consumers had significantly lower 24h urinary fructose levels in comparison to both normal and high fructose consumers (median: 36.1 µmol/24h, [IQR: 26.4-64.2] in low versus 142.3 µmol/24h [98.8-203.0] in normal and 238.9 µmol/24h [127.1-366.1] in high consumers, p=0.004 and p<0.001, respectively; **Figure 3.2b**). Univariate regression analysis in the three groups combined revealed that fructose intake (as a continuous variable) is a determinant of (log-transformed) 24h urinary fructose concentration (r=0.61, β=0.008; 95%CI: 0.005-0.012). Age, sex, BMI, eGFR, and TmP/GFR were no significant determinants of 24h urinary fructose concentrations in multivariate regression analyses. Last, the results remained similar when (log-transformed) 24h urinary fructose concentrations were adjusted for urinary creatinine levels (data not shown).

**Table 3.5. Baseline characteristics of the three study groups.**

| | Low fructose consumers (n=10) | Normal fructose consumers (n=15) | High fructose consumers (n=16) |
|---|---|---|---|
| Fructose intake (g/day) | 1.4 (0.9 - 2.0) | 31 (23 - 49)* | 70 (55 - 84)* |
| Sex (M/F) | 6/4 | 11/4 | 5/11 |
| Age (y) | 29 (23 - 63) | 28 (25 - 52) | 53 (39 - 61) |
| Smoker (yes/no) | 2/8 | 0/15 | 0/15 |
| BMI (kg/m$^2$) | 19.9 (19.0 - 21.8) | 21.8 (21.0 - 23.3) | 31.4 (29.8 - 37.5)* |
| Waist circumference (cm) | 76.5 (74.9 - 89.8) | 87.0 (84.5 - 91.3) | 108.8 (103.9 - 126.8)* |
| SBP (mmHg) | 129.9 (124.5 - 142.1) | 121.5 (112.0 - 130.7) | 131.5 (120.5 - 141.5) |
| DBP (mmHg) | 78.2 (62.6 - 84.5) | 69.3 (63.5 - 77.0) | 86.0 (77.5 - 88.0) |
| Total cholesterol (mmol/L) | 5.0 (4.3 - 5.5) | 4.1 (3.9 - 5.2) | 4.5 (3.9 - 6.0) |
| HDL-cholesterol (mmol/L) | 1.6 (1.6 - 2.3) | 1.4 (1.1 - 1.8) | 1.2 (1.0 - 1.4)* |
| LDL-cholesterol (mmol/L) | 2.6 (2.0 - 3.7) | 2.4 (1.8 - 3.3) | 2.9 (2.2 - 4.0) |
| Triglycerides (mmol/L) | 0.8 (0.7 - 1.1) | 0.9 (0.5 - 1.2) | 1.3 (1.0 - 1.8)* |
| Glucose (mmol/L)[a] | 4.4 (4.3 - 4.9) | 4.5 (4.2 - 4.7) | 5.1 (4.8 - 5.7) |

Data are presented as medians (interquartile range) for continuous variables and numbers for categorical variables. Analyzed with a Mann Whitney U test with low fructose consumers as a reference. P-values are Hochberg-adjusted to correct for multiple-testing.

* p<0.05 versus low fructose consumers.

[a] Glucose was measured in whole blood in low and normal fructose consumers and in plasma in high fructose consumers; as a consequence, statistical analysis was not performed to detect any potential difference between low and high fructose consumers.

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure.

## Discussion

We describe here a rapid, specific, and sensitive UPLC-MS/MS method for the determination of serum and urinary fructose in the micromolar range. We used HILIC in combination with a specific and sensitive MS/MS detection and demonstrated good validity and precision.

Our UPLC-MS/MS method has two distinct advantages, when compared to previously described methods which inherit limitations such as elaborate sample preparation for GC-MS methods[7,8,10], high sample volumes[8], and low sensitivity.[7-9] First, our current method is rapid in use. Sample pretreatment is straightforward since it does not require pre-column derivatization since cesium acetate is used as a solvent additive which makes it possible to form a specific cationic adduct of $[\text{fructose} + Cs]^+$ that can be detected in ESI+. Consequently, hands-on time for a series of 100 samples is only 90 minutes. The method permits high throughput analysis because of a run-to-run time of only 10 minutes and the UPLC-MS/MS system can run continuously, unattended, overnight. Second, the current method is sensitive and specific since it has the ability to quantify serum fructose in the micromolar range without interference of other sugars in the sample. Further, the urinary fructose concentrations measured with our UPLC-MS/MS method are in good agreement with data we obtained by an enzymatic-spectrometric method. This method validation could not be made for serum fructose because the enzymatic-spectrometric method is not sensitive to detect serum fructose in low concentrations.

We performed a number of exploratory experiments to demonstrate the clinical relevance and utility of our UPLC-MS/MS method. First, we showed that it was able to detect serum fructose concentrations in the micromolar range both in the fasting state and postprandial after a 15-gram oral fructose load. On the basis of the half-life of serum fructose (~30 minutes in this study) and the fact that participants visited the metabolic ward after an overnight fast, it can be concluded that serum fructose levels in the fasting state cannot be derived from an exogenous source, i.e. diet. Indeed, recent stable isotope studies have shown that serum fructose in the fasting state is derived from endogenous sources.[27] Second, our UPLC-MS/MS method was able to quantify fructose concentrations in 24h urine collections. Tasevska and colleagues previously showed that urinary sucrose and fructose levels can be used as objective biomarkers of dietary sugar intake.[28] In a non-controlled setting, we showed that individuals consuming low amounts of fructose also excrete low amounts of fructose in their urine. Although the study groups were highly selected, age, sex, and BMI were no determinants of 24h urinary fructose concentrations. In addition, glomerular and proximal tubular function, estimated by eGFR and TmP/GFR, respectively, were no

determinants either. It should be taken into account that it is uncertain how the proximal tubular function affects urinary fructose excretion. Further, it should be noted that this study included relatively healthy individuals with an eGFR ranged from 55.1 to 137.2 ml/min/1.73m$^2$. It can, therefore, not be excluded that at a more advanced stage of renal insufficiency fructose excretion will not be affected.

In conclusion, in the presented study we developed and validated a UPLC-MS/MS method as a means of quantifying serum and urinary fructose. The method is precise to detect fructose concentrations in the micromolar range. We demonstrated the clinical relevance of measuring serum and urinary fructose using this newly-developed method.

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# Supplemental material

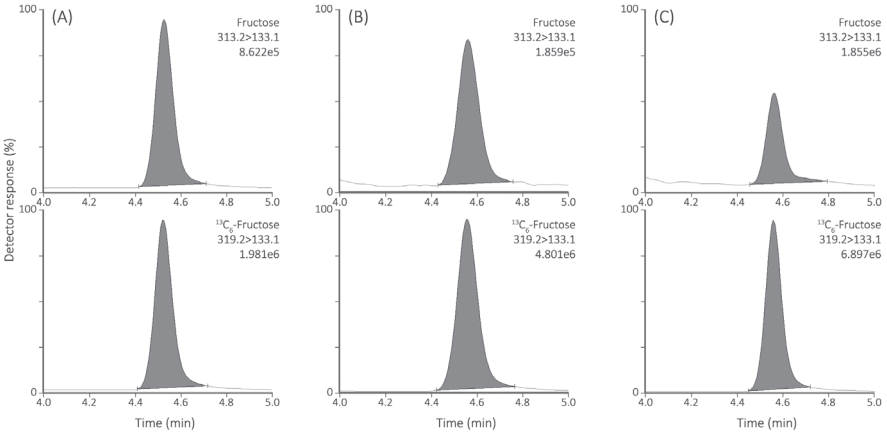


**Figure S3.1. Representative chromatogram of: a standard solution of fructose (260 µmol/L) and $^{13}C_6$-fructose (600 µmol/L) (A); a serum sample (fructose: 12 µmol/L; $^{13}C_6$-fructose: 306 µmol/L) (B); and a urine sample (fructose: 165 µmol/L; $^{13}C_6$-fructose: 612 µmol/L) (C).**

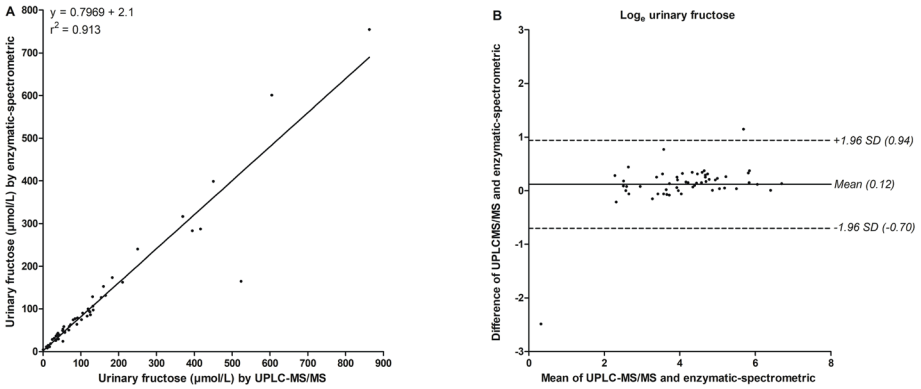


**Figure S3.2. Pearson correlation for urinary fructose concentrations as obtained by a UPLC-MS/MS method and an enzymatic-spectrometric method (A); and a Bland-Altman plot of the differences between the two methods (B).**
Horizontal dashed lines indicate 95% confidence intervals of the difference between both methods.

# PART II

## Fructose as a signalling molecule

## Interaction between fructose and glucose metabolism: from mice to men

# Chapter 4

## Hepatic glucokinase regulatory protein and carbohydrate response element binding protein do not explain the fatty liver phenotype in aldolase B deficiency



Amée M. Buziau, Maaike H. Oosterveer, Kristiaan Wouters, Dean R. Tolan, Coen D.A. Stehouwer, Casper G. Schalkwijk, Martijn C.G.J. Brouwers

*Manuscript in preparation*

# Chapter 5

## Effects of fructose added to an oral glucose tolerance test on plasma glucose excursions in healthy adults

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*Metabolism Open 2023;18:100245*

# Abstract

**Background and objective**

Previous experimental studies have shown that fructose interacts with glucose metabolism by increasing hepatic glucose uptake. However, human studies investigating the effects of small ('catalytic') amounts of fructose, added to an oral glucose load, on plasma glucose levels remain inconclusive. The aim of this study, therefore, was to repeat and extend these previous studies by examining the plasma glucose response during a 75 g oral glucose tolerance test (OGTT) with the addition of different doses of fructose.

**Methods**

Healthy adults (n=13) received an OGTT without addition of fructose and OGTTs with addition of different doses of fructose (1, 2, 5, 7.5 and 15 g) in a random order, on six separate occasions. Plasma glucose levels were measured every 15 minutes for 120 minutes during the study**.**

**Findings**

The plasma glucose incremental area under the curve (iAUC) of the OGTT without addition of fructose was not significantly different from any OGTT with fructose ($p \geq 0.2$ for all fructose doses). Similar results were observed when these data were clustered with data from a similar, previous study (pooled mean difference: -10.6; 95%CI: -45.0; 23.8 for plasma glucose iAUC of the OGTT without addition of fructose versus an OGTT with 5 g fructose; fixed-effect meta-analysis, n=38). Of interest, serum fructose increased from 4.8 µmol/L (interquartile range: 4.1–5.9) at baseline to 5.3 µmol/L (interquartile range: 4.8-7.5) at T=60 minutes during an OGTT *without* addition of fructose (p=0.002).

**Conclusion**

Low doses of fructose added to an OGTT do not affect plasma glucose levels in healthy adults. The role of endogenous fructose production, as a potential explanation of these null-findings, deserves further investigation.

# Introduction

The rise in intake of added sugars has been associated with the current epidemic of obesity, type 2 diabetes mellitus (T2D), dyslipidemia, and cardiovascular disease.[1-4] Recent studies have shown that fructose, more than glucose, is disadvantageous for cardiometabolic health[5-7], which may be explained by the fact that fructose is preferentially metabolized in the liver resulting in, among others, intrahepatic lipid accumulation and hepatic insulin resistance.[1-4]

Of interest, findings of previous experimental studies suggest that fructose also interacts with glucose metabolism by increasing hepatic glucose uptake (**Supplemental Figure S5.1**).[8-12] First, *in vitro* studies have shown that small ('catalytic') amounts of fructose dissociate glucokinase from glucokinase regulatory protein, a liver-specific protein, which results in more free, cytosolic glucokinase that facilitates the conversion of glucose to glucose-6-phosphate.[8] Second, experimental studies in dogs and humans have shown that these 'catalytic' amounts of fructose increase hepatic glucose uptake.[9,10] Third, Moore et al elegantly showed that adding 7.5 g of fructose to a 75 g oral glucose tolerance test (OGTT) reduced plasma glucose excursions, most likely due to increased hepatic glucose uptake, in both healthy adults and individuals with T2D.[11,12] However, it is currently not known at what threshold fructose interacts with glucose *in vivo*. In fact, a more recent study[13], could not replicate the findings reported by Moore et al.[11]

Therefore, the aim of the present study was to repeat and extend the original study by Moore et al by studying the plasma glucose response during an OGTT with and without the addition of different doses of fructose (ranging from 1 gram to 15 grams).



# Research design and methods

## Participants and experimental design

Thirteen healthy adults were studied on six separate occasions with at least a four-day interval (**Supplemental Figure S5.2 and S5.3**).

Participants visited the research ward in the morning after an overnight fast (10:00 PM) and remained fasted prior to the OGTT. All participants completed a health questionnaire regarding, among others, medical history and medication use. Height was determined using a stadiometer. Weight was measured by using electronic scales. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was determined using a measuring tape at the level of the umbilicus, measured while participants were in a standing position.

A 20-gauge intravenous cannula was inserted on the dorsal side of the hand for blood sampling at baseline and after ingestion of the carbohydrate solution every 15 minutes for a total of 120 minutes during the study. The hand was placed in a thermostatically controlled warmed box throughout the study to obtain arterialized venous blood samples.[11,12]

Participants were instructed to ingest 82.5 g dextrose monohydrate (=75 g glucose; fructose content $\leq$0.15%; Tereos, Aalst, Belgium), with or without addition of different fructose doses (Nutricia, Scholten, the Netherlands), dissolved in 250 mL water over the course of 5 minutes. Participants were blinded and randomly received in total six different carbohydrate solutions dissolved in 250 mL water during each study visit, including: 1) OGTT without addition of fructose, 2) OGTT with 1 g fructose, 3) OGTT with 2 g fructose, 4) OGTT with 5 g fructose, 5) OGTT with 7.5 g fructose, and 6) OGTT with 15 g fructose (**Supplemental Figure S5.2 and S5.3**).

The study was carried out according to the Declaration of Helsinki[14] and approved by the medial ethical committee of Maastricht University Medical Center+. All participants provided written informed consent.

## Laboratory measurements

Plasma glucose levels were determined every 15 minutes by using the YSI2300 STAT Plus Glucose Lactate Analyser (YSI, Yellow Springs, OH). Serum fructose concentrations were measured with a recently developed and validated Ultra Performance Liquid Chromatography–tandem Mass Spectrometry method.[15] Serum lipids were measured by an enzymatic colorimetric assay (Cobas 8000 instrument, Roche Diagnostics, Mannheim, Germany). Low-density lipoprotein cholesterol was calculated using the Friedewald formula.

## Statistical analyses

Data are presented as median with interquartile range or as frequencies in case of continuous and categorical variables, respectively (unless stated differently).

The trapezoidal rule was used for the calculation of the incremental area under the curve (iAUC). Wilcoxon signed-rank tests (unless stated differently) were used to compare between the plasma glucose iAUC during an OGTT without addition of fructose and plasma glucose iAUC during the OGTTs with different doses of fructose.

Statistical analyses were performed with the use of the Statistical Package for Social Sciences (Version 25.0; IBM, Chicago, IL) and the 'R' statistical software (R Developmental Core Team) using the metaphor package.[16] Results were considered statistically significant at $p<0.05$.

# Results

## Population characteristics

Participants were predominantly male and, on average, not overweight (**Table 5.1**). None of them were diagnosed with T2D.

**Table 5.1. Baseline characteristics.**

| | Overall population (n=13) |
|---|---|
| Sex (M/F) | 11/2 |
| Age (y) | 24 (21 - 45) |
| Smoker (yes/no) | 0/13 |
| BMI (kg/m$^2$) | 23.5 (22.0 - 25.8) |
| Waist circumference (cm) | 84.0 (76.6 - 89.5) |
| Systolic blood pressure (mmHg) | 121 (111 - 133) |
| Diastolic blood pressure (mmHg) | 70 (68 - 78) |
| Total cholesterol (mmol/L) | 3.7 (3.5 - 4.4) |
| HDL-cholesterol (mmol/L) | 1.4 (1.2 - 1.8) |
| LDL-cholesterol (mmol/L) | 2.0 (1.6 - 2.6) |
| Triglycerides (mmol/L) | 0.7 (0.5 - 0.9) |
| Glucose (mmol/L) | 5.2 (5.0 - 5.4) |

Categorical data presented as frequencies and continuous data as median (IQR).
Abbreviations: HDL: high-density lipoprotein; LDL: low-density lipoprotein.



## Plasma glucose response

Plasma glucose levels were not statistically significantly lower at any time point during an OGTT with 7.5 g fructose when compared to an OGTT without addition of fructose (**Figure 5.1A**), nor during an OGTT with 1 g, 2 g, 5 g, 15 g fructose when compared to an OGTT without addition of fructose (**Supplemental Figure S5.4**). Moreover, the plasma glucose iAUC was not significantly different between an OGTT without addition of fructose and OGTTs with addition of different doses of fructose ($p \geq 0.2$ for all fructose doses versus OGTT without addition of fructose; **Figure 5.1B**).

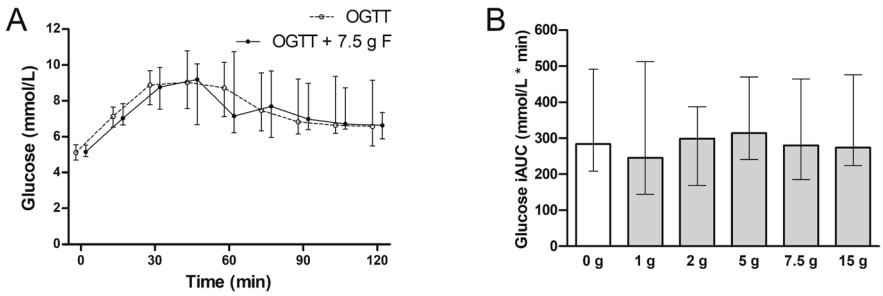


**Figure 5.1. Glucose response during an OGTT without addition of fructose and OGTTs with different doses fructose (n=13).**
Panel A: Plasma glucose concentrations during a 75 g oral glucose tolerance test (OGTT) with addition of 0 g and 7.5 g fructose (F).
Panel B: Plasma glucose incremental area under the curve (iAUC) during a 75 g OGTT with addition of 0 g, 1 g, 2 g, 5 g, 7.5 g, and 15 g fructose.
Data are presented as median (IQR).

## Sensitivity analyses

To gain more insight into these null-findings, we performed additional analyses.
First, we clustered our data (n=13) with the individual data (n=25) that were kindly provided by Braunstein et al.[13] Unfortunately, individual data from the experiments by Moore et al were no longer available (Moore; personal communication).[11,12] A fixed-effect meta-analysis of the available data (n=38) did not show a significantly lower plasma glucose iAUC after 5 g fructose added to an OGTT when compared to an OGTT without addition of fructose (pooled mean difference: -10.6; 95% CI: -45.0; 23.8; **Supplemental Figure S5.5**).
Second, other experimental studies have shown that fructose is also metabolized in the intestines, thereby preventing fructose spill over to the liver.[17-19] To gain insight into the degree of spill over (from intestines *and* liver), we measured the serum fructose response (T=0 and T=60 minutes) during the OGTTs with the different fructose doses. An exponential relationship was observed between the different doses of fructose added to an OGTT and the serum fructose response (**Figure 5.2**).

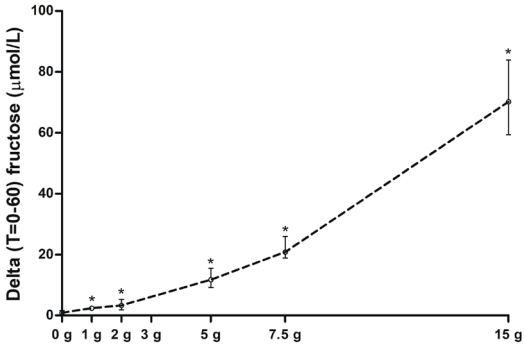


**Figure 5.2. Fructose response during OGTTs with addition of different doses of fructose (n=13).**
Delta serum fructose (from baseline to T=60 minutes) during a 75 g oral glucose tolerance test (OGTT) with addition of 0 g, 1 g, 2 g, 5 g, 7.5 g, and 15 g fructose.
Data are presented as median (IQR). *, p<0.05 versus OGTT without addition of fructose.
Analyzed with Wilcoxon signed-rank tests.



Third, previous experimental studies have shown that high intracellular glucose concentrations stimulate endogenous fructose production via the polyol pathway (**Supplemental Figure S5.1**).[20] We observed a small, statistically significant increase in serum fructose (from baseline to T=60 minutes) during the OGTT *without* addition of fructose (p=0.002; **Figure 5.3**).

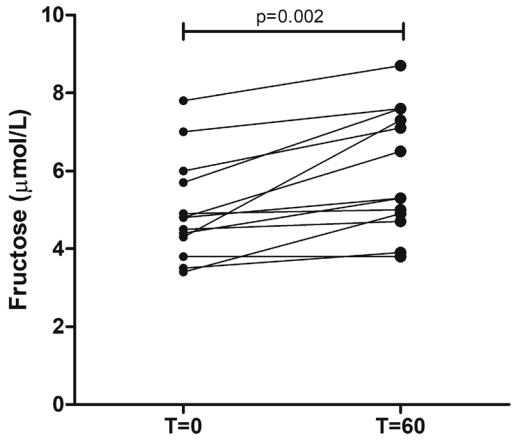


**Figure 5.3. Fructose response during an OGTT without addition of fructose (n=13).**
Serum fructose concentrations at T=0 and T=60 minutes during a 75 g oral glucose tolerance test (OGTT) without addition of fructose.
Data are presented as median (IQR). Analyzed with Wilcoxon signed-rank tests.

## Discussion

In the present study, we did not find an effect of oral fructose on the plasma glucose response during an OGTT in healthy adults.

Our findings are in contrast with those reported by Moore et al who showed that adding 7.5 g of fructose to an OGTT reduced plasma glucose excursions in both healthy adults (n=11) and individuals with T2D (n=5).[11,12] However, our findings are in agreement with a recent study from Braunstein et al who also could not replicate Moore's findings by adding 5 g or 10 g fructose to an OGTT in healthy adults (n=25).[13] Although there were some subtle differences in the study design of these four OGTT studies[11-13], including blinding, the total number of the OGTTs, wash-out period between the OGTTs, and the utility of a thermostatically controlled heated box, it is unlikely that these could account for the observed discrepancy.

Of interest, Braunstein et al found that self-reported ethnicity was a significant effect modifier for the effect of fructose on the plasma glucose iAUC (p=0.04), i.e. the plasma glucose iAUC was higher during an OGTT with fructose in three individuals who self-reported their ethnic category as 'other'.[13] Similarly, Moore et al reported a higher plasma glucose iAUC with addition of 7.5 g fructose to an OGTT in two Asian males.[11] However, the present study included only Caucasians and, therefore, ethnicity cannot explain the higher plasma glucose iAUC during some OGTTs with addition of fructose in our study.

Finally, statistical power could be an issue. The reproducibility (and accuracy) of a glucose response during an OGTT is in general poor and dependent on numerous variables.[21] Therefore, in a sensitivity analysis, we clustered our data with the data reported by Braunstein et al.[13], which did not materially alter the results.

In order to gain more biological insight into these null-findings and explain the discrepancy with other *in vivo* studies (in dogs and humans) showing that fructose favors hepatic glucose uptake[8-10], we performed additional sensitivity analyses. First, since animal studies have shown that intestinal fructose metabolism scavenges fructose away from the liver (and peripheral circulation)[17-19], we studied the serum fructose response during an OGTT with addition of different doses of fructose. We observed a non-linear relationship between the fructose dose and the serum fructose response, indeed suggesting that at lower doses less fructose escapes the intestinal (and hepatic) fructose metabolism. On the other hand, we did detect a statistically significant increase in serum fructose after fructose doses even as little as 1 gram and 2 grams. This suggests that at least some fructose passes the small intestine and reaches the liver favoring dissociation of glucokinase from glucokinase regulatory protein.

Second, in another sensitivity analysis, we studied the serum fructose response during an OGTT *without* addition of fructose. We found a small, statistically significant increase in serum fructose levels. Although the oral glucose might have contained trace amounts of fructose (maximum 0.15% [according to the manufacturer] * 82.5 g = 0.1 g), we believe that this amount is too low to explain the observed increase in serum fructose. It is, therefore, more likely that the increase in serum fructose during an OGTT *without* addition of fructose is explained by endogenous fructose production via the polyol pathway.[20] Indeed, Francey et al performed a stable isotope study and showed that endogenous fructose production was increased 60 minutes after oral intake of 30 g of glucose.[22] Hence, it possible that the amount of endogenously produced fructose is already sufficient to maximally dissociate glucokinase from glucokinase regulatory protein, explaining why the addition of exogenous fructose to an OGTT did not affect plasma glucose excursions (**Supplemental Figure S5.1**). This would imply that repeating the OGTT with lower doses of glucose – below the threshold of endogenous fructose production – might yield different results.



## Conclusions

We did not find an interaction between oral glucose and low doses of fructose on plasma glucose excursions in healthy adults. The potential role of endogenous fructose production deserves further investigation.

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# Supplemental material

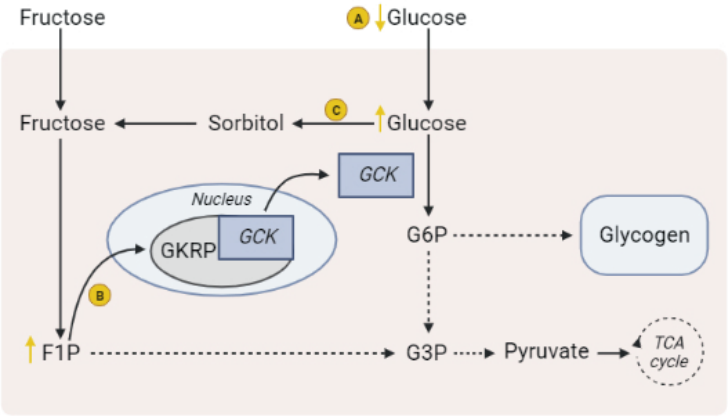


**Figure S5.1. Overview of fructose and glucose metabolism in the liver.**
(A) Findings of previous experimental studies have shown that fructose interacts with glucose metabolism by increasing hepatic glucose uptake, i.e. fructose 1-phosphate (F1P) dissociates glucokinase (GCK) from glucokinase regulatory protein (GKRP), which results in more free, cytosolic GCK that facilitates the conversion of glucose to glucose-6-phosphate (G6P). (B) Increased cytosolic GCK favors hepatic glucose uptake, resulting in lower plasma glucose levels. (C) Previous studies have shown that high intracellular glucose concentrations stimulate endogenous fructose production via the polyol pathway.
Dashed arrow indicates multiple intermediate enzymatic steps that have not been visualized for simplicity purposes.

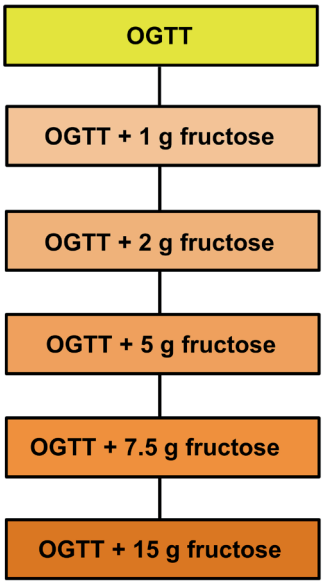




**Figure S5.2. OGTT experiments in healthy adults (n=13).**
A 75 g oral glucose tolerance test (OGTT) without addition of fructose and OGTTs with addition of 1 g, 2 g, 5 g, 7.5 g, and 15 g fructose.

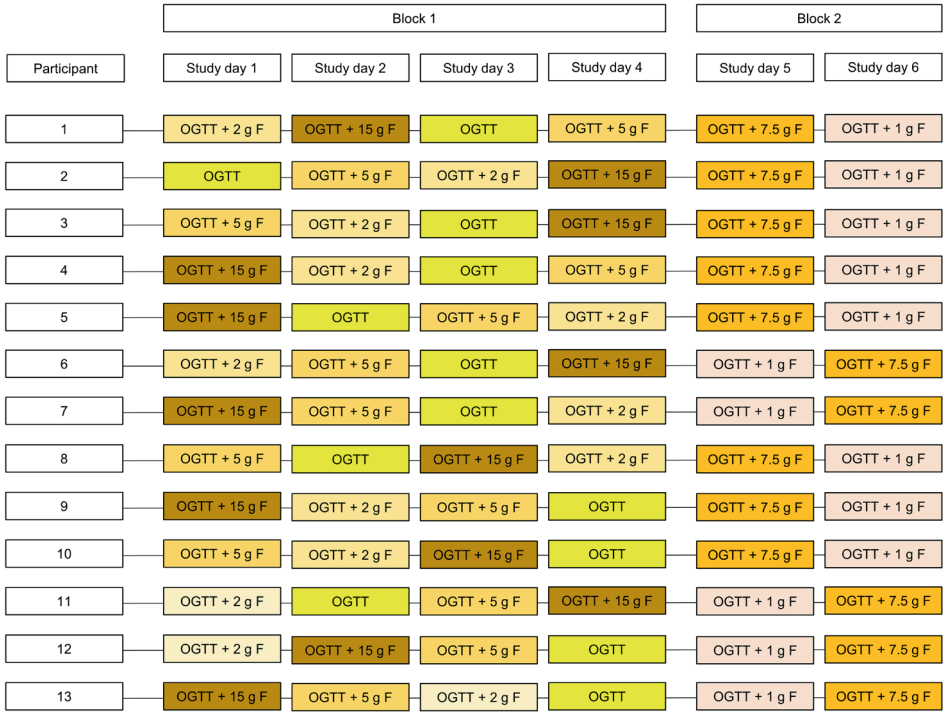


**Figure S5.3. Block randomization of the OGTT experiments in healthy adults (n=13).**
Block 1 includes a 75 g oral glucose tolerance test (OGTT) without addition of fructose (F) and three OGTTs with addition of the three most distinctive doses of fructose (i.e. 2 g, 5 g, and 15 g).

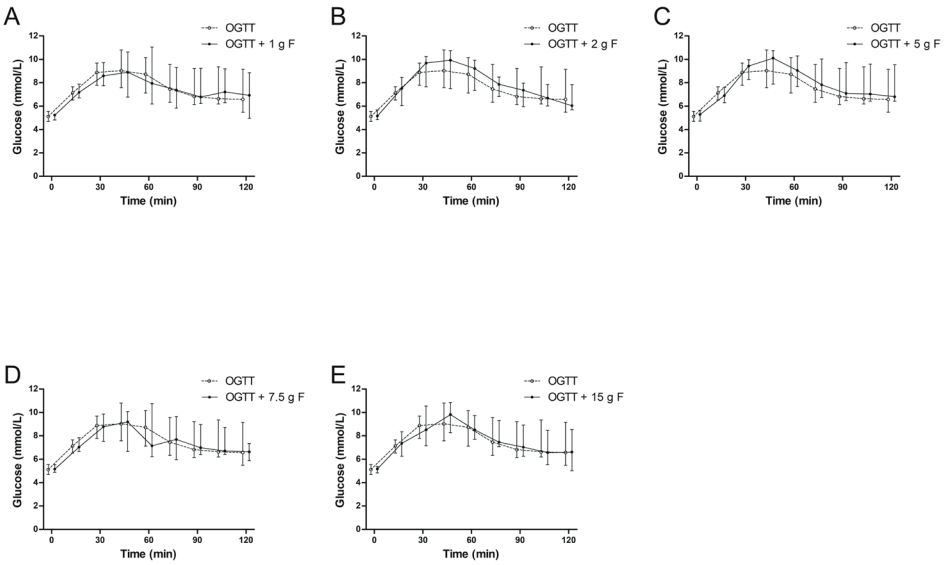


**Figure S5.4. Glucose response during an OGTT without addition of fructose and OGTTs with different doses fructose (n=13).**
Plasma glucose concentrations during a 75 g oral glucose tolerance test (OGTT) with addition of 0 g (reference) and 1 g (A), 2 g (B), 5 g (C), 7.5 g (D), and 15 g (E) fructose (F).
Data are presented as median (IQR).

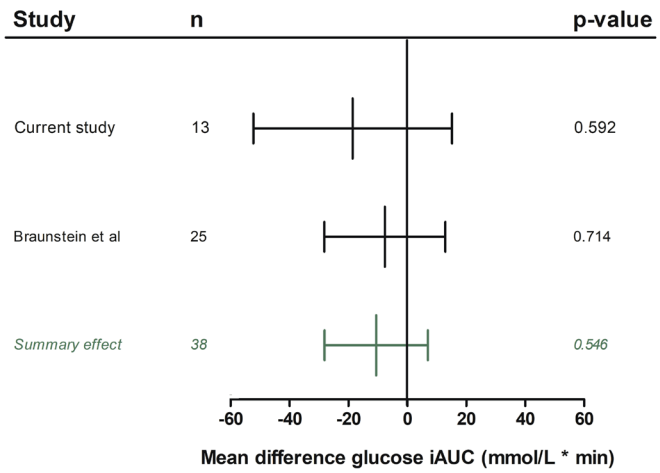


**Figure S5.5. Mean difference in glucose response between an OGTT without addition of fructose and an OGTT with 5 g fructose.**
Mean difference in plasma glucose incremental area under the curve (iAUC) during a 75 g oral glucose tolerance test (OGTT) without addition of fructose versus an OGTT with 5 g fructose.
Data are presented as mean difference ± SEM.
Analyzed with paired T-tests for individual data and a fixed-effect meta-analysis for summary effect.

# PART III

## Fructose intake and non-communicable disease at the population level

# Chapter 6

## Fructose intake from fruit juice and sugar-sweetened beverages is associated with higher intrahepatic lipid content: the Maastricht Study

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*Diabetes Care 2022;45(5):1116-23*

# Abstract

**Objective**

Epidemiological evidence regarding the relationship between fructose intake and intrahepatic lipid content (IHL) is inconclusive. We, therefore, assessed the relationship between different sources of fructose and IHL at the population level.

**Research design and methods**

We used cross-sectional data from the Maastricht Study, a population-based cohort (n=3,981; 60±9 years; 50% women). We assessed the relationship between fructose intake (assessed by a food frequency questionnaire) – total and derived from fruit, fruit juice and sugar-sweetened beverages (SSB) – and IHL (quantified by 3T-Dixon-MRI) with adjustment for age, sex, type 2 diabetes, education, smoking status, physical activity, and intakes of total energy, alcohol, saturated fat, protein, vitamin E, and dietary fiber.

**Results**

Energy-adjusted total fructose intake and energy-adjusted fructose from fruit was not associated with IHL in the fully adjusted model (p=0.647 and p=0.767).

In contrast, energy-adjusted intake of fructose from fruit juice and SSB was associated with higher IHL in the fully adjusted models (p=0.019 and p=0.009). Individuals in the highest tertile of energy-adjusted intake of fructose from fruit juice and SSB had a 1.04-fold (95%CI: 0.99;1.11) and 1.09-fold (95%CI: 1.03;1.16) higher IHL, respectively, when compared to the lowest tertile in the fully adjusted model. Finally, the association for fructose from fruit juice was stronger in individuals with type 2 diabetes (p for interaction=0.071).

**Conclusions**

Fructose from fruit juice and SSB is independently associated with higher IHL. These cross-sectional findings contribute to current knowledge in support of measures to reduce the intake of fructose-containing beverages as a means to prevent non-alcoholic fatty liver disease at the population level.

# Introduction

Non-alcoholic fatty liver disease (NAFLD) is highly prevalent among people with type 2 diabetes and emerging as the principal cause of liver transplantation in Western society.[1,2] Furthermore, epidemiological evidence is accumulating that NAFLD *per se* is a risk factor for type 2 diabetes.[2-4] Currently, a myriad of pharmacological agents that target NAFLD have entered phase II-III clinical trials.[5] However, given the high global prevalence of NAFLD (~25%[6]), it is also desirable to dispose of non-pharmacological measures to reduce the burden of NAFLD and its sequela at the population level.

There has been a long debate on whether dietary fructose is a modifiable risk factor of NAFLD. Despite convincing evidence derived from animal studies[7], there have been inconsistent experimental data in humans.[8,9] Furthermore, the findings from observational studies that addressed the relationship between fructose intake and intrahepatic lipid accumulation (IHL), the first stage of NAFLD, vary from positive[10,11], inverse[12], and divergent associations.[13] Of note, some of these studies have been conducted in selected (pediatric) groups[10], did not use histology or imaging to quantify IHL,[12,13] or did not sufficiently adjust for potential confounders.[10-13] In addition, only one of these studies made a distinction between multiple sources of dietary fructose.[13]

The aim of the present study was, therefore, to assess the independent relationship between habitual fructose intake – total and derived from fruit, fruit juice and sugar-sweetened beverages (SSB) – and IHL, quantified by using 3T-Dixon-MRI, in the Maastricht Study, an extensively phenotyped population-based cohort.[14]



# Research design and methods

## Study population

The Maastricht Study is a population-based cohort study with an oversampling of individuals with type 2 diabetes.[14] In brief, the Maastricht Study focuses on the etiology, pathophysiology, complications, and comorbidities of type 2 diabetes. All individuals between 40 and 75 years old who lived in the southern part of the Netherlands were eligible for participation.

The present study includes cross-sectional data from 7,689 participants who completed the baseline measurements from November 2010 until December 2017. MRI measurements of the liver were implemented from December 2013 onwards (available in n=5,180). Participants with invalid MRI measurements, missing data on dietary intake, implausible energy intake, and missing data on covariates were excluded from all analyses, resulting in a study population of 3,981 participants (**Supplemental Figure S6.1**).

The Maastricht Study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

## Assessment of dietary intake

Assessment of dietary intake has been reported in detail before.[15] In brief, habitual dietary intake over the past 12 months was estimated by using a tailor-made and validated food frequency questionnaire (FFQ), which assessed the frequency of the consumption and the amount of consumed food and nutrients. Intakes of total energy and individual mono- and disaccharides were calculated using the Dutch food composition database (and, in the case of missing values in this composition database, complemented with values obtained from other relevant food composition databases).
For the present study, fructose intake (g/day) was defined as the sum of 50% sucrose intake plus free fructose intake. Further, fructose-containing food items were categorized as follows: 1) total fructose (g/day); 2) fructose from fruit (fresh and dried fruit; g/day), 3) fructose from fruit juice (g/day), and 4) fructose from SSB, including sugar-containing fruit drinks and syrups (g/day).

## Assessment of intrahepatic lipid content

IHL was assessed through Dixon-MR imaging using a 3.0 Tesla MRI system (MAGNETOM Prismafit, Siemens Healthineers, Erlangen, Germany) with body matrix and supine radiofrequency coils. After a scout scan, transversal two-dimensional T2-weighted True Fast Imaging with Steady-State Free Precession (T2w TRUFI) images were acquired through the liver with the following parameters: voxel size: 1.2×1.2×5.0 $mm^3$, repetition time (TR): 422 ms, echo time (TE): 1.65 ms, flip angle: 60°, number of signal averages: 1, parallel imaging (GRAPPA) factor: 2. Next, transversal two-dimensional turbo spin echo Dixon-MR images were acquired through the liver during a breathhold using the following parameters: voxel size: 2.0×2.0×6.0 $mm^3$, number of slices: 4, TR: 500 ms, TE: 31 ms, turbo factor: 5, number of signal averages: 1, parallel imaging (GRAPPA) factor: 3. Three regions-of-interest (ROIs) were drawn in the liver by trained observers on the T2w TRUFI images. Subsequently, these ROIs were copied to the water and fat Dixon-MR images to calculate the intrahepatic lipid fraction.
This method was validated and calibrated against proton magnetic resonance spectroscopy ($^1$H-MRS), the gold standard to non-invasively quantify IHL, in 36 participants. After calibration, the intra-class correlation coefficient between Dixon-MRI and $^1$H-MRS was 0.989 (95%CI: 0.979;0.994). IHL was expressed as the ratio $CH_2/H_2O$ (*100%).

## Measurement of covariates

All participants completed questionnaires regarding age, sex, educational level (low, medium, high), smoking status (never, former, current smoker), and history of cardiovascular disease (CVD).[14] Use of medication was assessed during medication interviews. Weight, height, waist circumference, and office systolic and diastolic blood pressure were measured during a physical examination. Fasting levels of glucose, HbA1c, and lipid profile were measured in venous blood samples. Daily physical activity levels were measured during 8 consecutive days using the activPAL3 physical activity monitor (PAL Technologies, Glasgow, UK).[16]

Alanine aminotransferase (ALT) was measured enzymatically on a Roche Cobas 8000 Modular Analyser (Roche Diagnostics, Basel, Switzerland).

All participants underwent a standardized 2-h 75 g oral glucose tolerance test (OGTT) after an overnight fast.[14] Use of insulin and/or fasting capillary glucose levels >11.0 mmol/L were considered as contraindications for an OGTT. Participants fulfilling either of these criteria were automatically classified as having diabetes. Glucose metabolism status, i.e., normal glucose metabolism, prediabetes (i.e., impaired fasting glucose and/or impaired glucose tolerance) and diabetes, were based on venous plasma glucose levels obtained during OGTT according to the World Health Organization 2006 criteria in all other participants.

The Matsuda index ($10{,}000/\sqrt{G0 \times I0 \times \text{mean } G \times \text{mean } I}$; where G0=fasting glucose, I0=fasting insulin, mean G=mean glucose during OGTT, and mean I=mean serum insulin levels during OGTT) was used as a measure of insulin sensitivity.[14]



## Statistical analyses

Continuous data are presented as mean±standard deviation, or as median (interquartile range) in case of non-normal distribution. Categorical data are presented as n (%).

All nutrient variables were adjusted for total energy intake by using the residual method.[17]

Multivariable linear regression models were constructed to study the associations between the energy-adjusted intake of 1) total fructose, 2) fructose from fruit, 3) fructose from fruit juice, and 4) fructose from SSB and the IHL, independent of confounders. Energy-adjusted fructose intake was entered as either a continuous variable (to derive a p for trend) or as a category. Based on the distribution of energy-adjusted intake of fructose from fruit, fruit juice and SSB, which contained a large number of low-consumers in the latter two groups (**Supplemental Figure S6.2**), we decided to categorize the participants according to tertiles of energy-adjusted fructose

intake to obtain discriminative categories of intake. The tertiles of energy-adjusted fructose intake were entered in the models as independent variables (with the lowest tertile as a reference). The following regression models were used: model 1: crude; model 2 was adjusted for age, sex, and type 2 diabetes, the latter because of the oversampling of type 2 diabetes in the Maastricht Study; model 3 was additionally adjusted for (proxies of) lifestyle, i.e., educational level, smoking status, physical activity, and total energy intake; model 4 was additionally adjusted for nutritional factors that have been associated with IHL in randomized controlled trials, i.e., intakes of alcohol, saturated fat, protein, and vitamin E[18-21]; and model 5 was additionally adjusted for dietary fiber, which has been associated with IHL in observational studies.[22]

IHL was $^{10}$log transformed to fulfill the assumption of normality for linear regression. To obtain interpretable results we back-transformed the regression coefficients, which should be interpreted as the fold change (and not the additive change) in IHL that is associated with the difference between the tertile of fructose intake and the reference group (i.e., lowest tertile of energy-adjusted fructose intake), as can be deduced from:

$Log(y)=\beta_0+\beta_1 x$
$Exp(log(y))=exp(\beta_0+\beta_1 x)$
$y=exp(\beta_0)exp(\beta_1 x)$

For instance, a regression coefficient of 0.019 implies that for every unit increase in fructose intake, ($^{10}$log) IHL increases with 0.019. After back-transformation, the interpretation should be that for every unit increase in fructose intake, IHL increases 1.04-fold (=10^0.019), i.e., by 4%.

Additional analyses were performed to test for an interaction between energy-adjusted fructose intake and type 2 diabetes or sex on IHL in the fully adjusted model.

Several sensitivity analyses were performed. First, multivariable logistic regression analyses were conducted with hepatic steatosis as a dependent, dichotomous variable which was defined as IHL ≥5.56%.[23] This cut-off value, originally expressed as ($CH_2/(H_2O+CH_2)$)[23], corresponds to 5.89% (=0.0556/(1-0.0556)) when IHL is expressed as $CH_2/H_2O$, as was done in the present study. Second, the original analyses were repeated with replacement of: 1) the covariate type 2 diabetes in models 2-5 by the Matsuda index (available in n=1,415) to explore the role of insulin sensitivity in the relationship between fructose intake and IHL; and 2) IHL by ($^{10}$log transformed) serum ALT levels (available in n=1,602).

Statistical analyses were performed with the use of the Statistical Package for Social Sciences (Version 25.0; IBM, Chicago, IL). A *P* value of <0.05 was considered statistically

significant in all analyses, except for interaction tests where a less stringent significance threshold of p <0.10 was applied.

# Results

## Study population

**Table 6.1** shows the characteristics of the overall population and stratified according to IHL tertiles. The mean age of the study population was 60±9 years, 50% were female, 20% were diagnosed with type 2 diabetes, and the median IHL was 3.2% (IQR: 2.0-6.1). Participants in the highest IHL tertile were more often men, were older, had a lower educational level, were less physically active, and had a higher BMI compared to those in the lowest IHL tertile. Compared with participants in the lowest IHL tertile, those in the highest were metabolically unhealthy, as reflected by lower HDL cholesterol, and higher serum triglycerides, HbA1c, prevalence of prediabetes, and systolic and diastolic blood pressure. Further, the prevalence of CVD and the use of medication (including lipid-modifying, glucose-lowering, and antihypertensive medication) were higher in the highest IHL tertile. Finally, intakes of total fructose, fructose from fruit, and dietary fiber were lower, while intakes of total energy and saturated fat were greater in the highest IHL tertile.

**Table 6.1. Characteristics of the overall population and stratified according to intrahepatic lipid content (IHL) tertiles.**

| Characteristic | Total (n=3,981) | First tertile (n=1,327) | Second tertile (n=1,327) | Third tertile (n=1,327) |
|---|---|---|---|---|
| IHL, % | 3.2 (2.0-6.1) | 1.7 (1.3-2.0) | 3.2 (2.7-3.9) | 8.3 (6.1-12.6) |
| ALT, (U/L) | 26.0 (21.0-34.0) | 22.0 (19.0-28.0) | 26.0 (21.0-33.0) | 31.0 (24.0-42.0) |
| Age, y | 60 ± 9 | 57 ± 9 | 60 ± 8 | 61 ± 8 |
| Women, % | 50 | 62 | 46 | 40 |
| Education, % low/medium/high | 32/28/40 | 26/30/44 | 32/27/41 | 38/27/35 |
| Smoking, % never/former/current | 40/49/12 | 44/44/12 | 39/49/12 | 35/53/12 |
| Physical activity, min/d | 51.4 (36.6-69.6) | 56.0 (40.6-73.6) | 52.7 (38.6-72.0) | 45.1 (31.9-62.1) |
| Body mass index, kg/m$^2$ | 26.5 ± 4.1 | 24.3 ± 3.0 | 26.3 ± 3.6 | 28.9 ± 4.1 |
| Waist circumference, cm | 93.7 ± 12.6 | 85.6 ± 9.6 | 93.6 ± 11.0 | 101.8 ± 11.4 |
| Total cholesterol, mmol/L | 5.3 ± 1.1 | 5.3 ± 1.0 | 5.3 ± 1.1 | 5.2 ± 1.2 |
| HDL cholesterol, mmol/L | 1.6 ± 0.5 | 1.8 ± 0.5 | 1.6 ± 0.5 | 1.4 ± 0.4 |
| LDL cholesterol, mmol/L | 3.1 ± 1.0 | 3.1 ± 0.9 | 3.1 ± 1.0 | 3.0 ± 1.1 |
| Triglycerides, mmol/L | 1.2 (0.9-1.7) | 1.0 (0.8-1.2) | 1.2 (0.9-1.6) | 1.5 (1.1-2.1) |
| Lipid-modifying medication, % | 28 | 17 | 28 | 39 |
| HbA1c, % (mmol/mol) | 5.5 (5.2-5.9)37 | 5.4 (5.1-5.6) 36 | 5.5 (5.3-5.8) 37 | 5.7 (5.4-6.4) 39 |
| Matsuda index of insulin sensitivity | 3.56 (2.14-5.34) | 4.87 (3.63-6.72) | 3.81 (2.60-5.42) | 2.31 (1.52-3.40) |
| GMS, % NGM/prediabetes/type 2 diabetes/other type of diabetes | 65/15/20/1 | 82/9/8/1 | 71/13/15/1 | 41/21/38/0 |
| Glucose-lowering medication, % | 15 | 7 | 12 | 27 |
| Office SBP, mmHg | 133 ± 17 | 128 ± 17 | 133 ± 17 | 138 ± 16 |
| Office DBP, mmHg | 75 ± 10 | 73 ± 10 | 76 ± 9 | 78 ± 9 |
| Antihypertensive medication, % | 33 | 20 | 31 | 48 |
| History of CVD, % | 13 | 10 | 13 | 16 |
| Total fructose, g/day | 35.9 (26.2-47.5) | 36.7 (27.0-47.6) | 36.3 (26.3-47.6) | 34.8 (25.1-47.2) |
| Fructose from fruit, g/day | 9.1 (4.7-14.8) | 9.6 (5.3-15.5) | 9.1 (5.0-15.6) | 8.2 (3.9-13.4) |
| Fructose from fruit juice, g/day | 0.9 (0.1-3.8) | 1.0 (1.2-3.8) | 0.9 (0.1-3.8) | 0.9 (0.1-3.9) |
| Fructose from SSB, g/day | 0.4 (0.0-2.8) | 0.3 (0.0-2.1) | 0.3 (0.0-2.4) | 0.6 (0.0-3.8) |
| Total energy, kcal/day | 2074 (1721-2486) | 2027 (1699-2446) | 2087 (1748-2515) | 2105 (1703-2500) |
| Alcohol, g/day | 8.6 (1.8-18.7) | 7.8 (1.5-15.7) | 9.6 (2.5-19.5) | 8.3 (1.6-21.0) |
| Carbohydrates, g/day | 222 (179-272) | 223 (179-273) | 224 (181-273) | 219 (178-269) |
| Saturated fat, g/day | 27.3 (20.5-35.3) | 26.2 (20.2-34.3) | 27.6 (20.4-35.8) | 28.1 (20.8-36.1) |
| Protein, g/day | 82.0 (68.9-96.9) | 80.8 (68.0-94.8) | 82.7 (69.5-98.1) | 82.5 (69.3-97.6) |
| Vitamin E, mg/day | 12.5 (9.7-16.0) | 12.5 (9.8-16.0) | 12.5 (9.8-16.2) | 12.5 (9.6-15.8) |
| Dietary fiber, g/day | 26.1 (21.3-31.8) | 26.4 (21.4-32.0) | 26.6 (21.7-32.2) | 25.5 (21.1-30.9) |

Data are reported as mean ± standard deviation, median (interquartile range), or number (%) as appropriate. Nutrient variables represent absolute intake values.

Abbreviations: ALT, alanine aminotransferase; CVD, cardiovascular disease; DBP, diastolic blood pressure; GMS, glucose metabolism status; HbA1c, glycated hemoglobin A1c; IHL, intrahepatic lipid content; NGM, normal glucose metabolism; SBP, systolic blood pressure; SSB, sugar-sweetened beverages.

## Relationship between fructose intake and intrahepatic lipid content

Total fructose intake was associated with lower IHL (p<0.001; **Table 6.2, models 1-3**), but this association was lost after adjustment for nutritional factors that are associated with IHL (p=0.903; **Table 6.2, model 4**).

When fructose intake was categorized according to different sources of fructose, a similar association was observed between intake of fructose from fruit and lower IHL (p<0.001; **Table 6.2**). Again, the strength of association was attenuated after adjustment for nutritional factors that are associated with IHL (p=0.044; **Table 6.2**, **model 4**), and was completely lost after additional adjustment for dietary fiber (p=0.767; **Table 6.2, model 5**).

In contrast, intake of fructose from fruit juice was associated with higher IHL, also after full adjustment for potential confounders (p=0.019; **Table 6.2**). Individuals in the highest tertile of energy-adjusted intake of fructose from fruit juice had a 1.04-fold (95%CI: 0.99;1.11; **Table 6.2**) higher IHL when compared to the lowest tertile in the fully adjusted model.

Similarly, intake of fructose from SSB was associated with higher IHL in the fully adjusted model (p=0.009; **Table 6.2**). Individuals in the highest tertile of energy-adjusted intake of fructose from SSB had a 1.09-fold (95%CI: 1.03;1.16; **Table 6.2**) higher IHL when compared to the lowest tertile in the fully adjusted model.

**Table 6.2. Multivariable-adjusted associations of energy-adjusted fructose intake and intrahepatic lipid content (IHL) (n=3,981).**

| | Energy-adjusted fructose intake tertiles | | | |
|---|---|---|---|---|
| Total fructose* | T1 | T2 | T3 | |
| Median g/day | 24.4 | 35.1 | 47.6 | *P* for trend |
| Model 1 | 1 | 0.89 (0.84; 0.95) | 0.83 (0.78; 0.88) | <0.001 |
| Model 2 | 1 | 0.94 (0.89; 0.99) | 0.90 (0.85; 0.96) | <0.001 |
| Model 3 | 1 | 0.95 (0.90; 1.00) | 0.91 (0.86; 0.97) | <0.001 |
| Model 4 | 1 | 1.01 (0.95; 1.07) | 1.01 (0.95; 1.08) | 0.903 |
| Model 5 | 1 | 1.01 (0.95; 1.07) | 1.02 (0.95; 1.09) | 0.647 |
| Fructose from fruit* | T1 | T2 | T3 | |
| Median g/day | 3.1 | 9.0 | 17.8 | *P* for trend |
| Model 1 | 1 | 0.88 (0.83; 0.94) | 0.82 (0.77; 0.87) | <0.001 |
| Model 2 | 1 | 0.90 (0.85; 0.95) | 0.84 (0.80; 0.90) | <0.001 |
| Model 3 | 1 | 0.91 (0.86; 0.97) | 0.87 (0.82; 0.92) | <0.001 |
| Model 4 | 1 | 0.94 (0.89; 1.00) | 0.91 (0.86; 0.97) | 0.044 |
| Model 5 | 1 | 0.96 (0.90; 1.01) | 0.95 (0.89; 1.02) | 0.767 |
| Fructose from fruit juice* | T1 | T2 | T3 | |
| Median g/day | 0.1 | 0.9 | 5.3 | *P* for trend |
| Model 1 | 1 | 0.95 (0.90; 1.02) | 0.96 (0.90; 1.02) | 0.512 |
| Model 2 | 1 | 1.01 (0.96; 1.08) | 1.02 (0.96; 1.08) | 0.078 |
| Model 3 | 1 | 1.02 (0.96; 1.08) | 1.03 (0.97; 1.09) | 0.082 |
| Model 4 | 1 | 1.03 (0.97; 1.09) | 1.05 (1.00; 1.12) | 0.008 |
| Model 5 | 1 | 1.02 (0.96; 1.08) | 1.04 (0.99; 1.11) | 0.019 |
| Fructose from SSB* | T1 | T2 | T3 | |
| Median g/day | 0.0 | 0.5 | 4.5 | *P* for trend |
| Model 1 | 1 | 1.00 (0.94; 1.07) | 1.09 (1.02; 1.16) | <0.001 |
| Model 2 | 1 | 1.02 (0.96; 1.08) | 1.11 (1.04; 1.17) | 0.001 |
| Model 3 | 1 | 1.02 (0.96; 1.08) | 1.08 (1.02; 1.14) | 0.024 |
| Model 4 | 1 | 1.03 (0.97; 1.09) | 1.12 (1.06; 1.19) | <0.001 |
| Model 5 | 1 | 1.02 (0.96; 1.08) | 1.09 (1.03; 1.16) | 0.009 |

* Energy-adjusted fructose by means of the residual method.
Regression coefficients should be interpreted as the fold change in IHL that is associated with the difference between the tertile of fructose intake and the reference group (see methods section).
P for trend values were obtained from linear regression with fructose as continuous variables (see methods section).
Model 1: energy-adjusted intake of fructose.
Model 2: + age, sex, and type 2 diabetes.
Model 3: + educational level, smoking status, physical activity, and intake of total energy.
Model 4: + energy-adjusted intakes of alcohol, saturated fat, protein, and vitamin E.
Model 5: + energy-adjusted intake of dietary fiber.
Abbreviations: SSB, sugar-sweetened beverages; T, tertile.

There was a statistically significant interaction between type 2 diabetes and total fructose, fructose from fruit, and fructose from fruit juice on IHL (p for interaction=0.089, 0.058, and 0.071, respectively), and the associations were more pronounced in individuals with type 2 diabetes (**Supplemental Table S6.1**). Furthermore, individuals with type 2 diabetes in the second and third tertile of intake of fructose from SSB had a statistically significantly higher IHL when compared to

individuals without type 2 diabetes (p=0.001 and p=0.020; **Supplemental Table S6.1**). Of note, the strength of these associations did not differ between individuals with newly diagnosed type 2 diabetes (based on an OGTT) and individuals with prior diagnosed type 2 diabetes (data not shown).
Sex did not modify the association between fructose intake and IHL (p for interaction >0.10; data not shown).

## Sensitivity analyses

First, when hepatic steatosis, defined as IHL ≥ 5.56% ($CH_2/(H_2O+CH_2)$), was considered as a dichotomous variable, associations were generally similar (**Figure 6.1**). In the fully adjusted model, individuals in the highest tertile of intake of fructose from SSB were more likely to have hepatic steatosis when compared to the lowest tertile (OR: 1.37; 95%CI: 1.12;1.68; **Figure 6.1**). Again, individuals with type 2 diabetes in the highest tertile of intake of fructose from fruit juice had a numerically higher risk of hepatic steatosis when compared to individuals without type 2 diabetes (OR: 1.33; 95%CI: 0.93;1.90 versus OR: 1.06; 95%CI: 0.85;1.34, respectively; p for interaction=0.097; **Figure 6.1**).

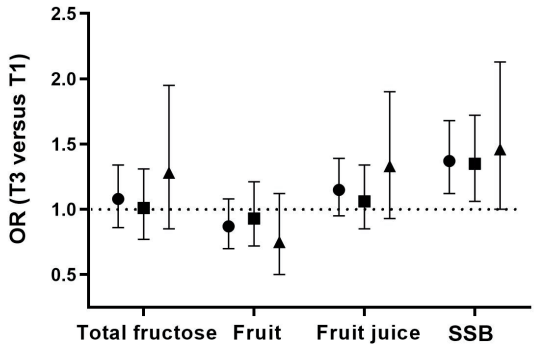


**Figure 6.1. Association between energy-adjusted fructose intake (highest vs. lowest tertile) and hepatic steatosis among the overall population (n=3,981) (●), individuals without type 2 diabetes (n=3,171) (▪), and individuals with type 2 diabetes (n=810) ( ▲ ).**
Data are presented for the fully adjusted model. Abbreviation: T, tertile.

Second, replacement of the covariate type 2 diabetes by the Matsuda index in the fully adjusted model showed a robust, positive association between fructose from SSB and IHL, whereas the association between fructose from fruit juice and IHL was attenuated towards the null (**Supplemental Table S6.2**).
Last, repeated analyses with ALT as an outcome variable resulted in null associations (**Supplemental Table S6.3**).

## Conclusions

In the present study, we found that the intake of fructose from fruit juice and SSB is independently associated with higher IHL in a large, extensively phenotyped, population-based cohort. The strength of the association between fructose from fruit juice and IHL appeared to be stronger in individuals with type 2 diabetes.

Only few studies have addressed the relationship between dietary fructose and IHL at the population level. Kanerva and colleagues previously reported a surprisingly inverse association between total fructose intake and the prevalence of NAFLD in a Finnish population-based cohort.[12] However, this study was limited by the use of surrogate outcome measures, i.e., the fatty liver index and the NAFLD liver fat score, and, in particular, incomplete adjustment for potential confounders. Indeed, although we observed a similar crude, inverse association between total fructose intake and IHL, quantified by MRI, this association was completely abrogated after additional adjustment for nutritional factors that previously have been reported to be associated with IHL.[18-21]

Further, we were able to differentiate between sources of dietary fructose in relation to IHL. For fructose from fruit, we found that the crude, inverse association with IHL was attenuated towards the null after additional adjustments including dietary fiber. It is possible that overadjustment has occurred since fruits are rich in dietary fiber. In agreement, a previous study in Chinese adults found an inverse association between fruit intake and the presence of NAFLD, but this study did not adjust for dietary fiber (and other relevant confounders).[24]

In contrast to the findings for fructose from fruit, we observed an association between fructose from fruit juice and SSB and higher IHL, even after adjustment for nutritional factors that are associated with IHL. The role of SSB in the development of NAFLD and type 2 diabetes has extensively been studied, however, with inconsistent results for NAFLD.[25,26] Of note, when this relationship was examined in a large cohort (n=2,634) and IHL was accurately assessed (by computed tomography), a positive association was observed, even after adjustment for confounders.[27] To date, only one study addressed the association of fructose from fruit juice and a surrogate marker of NAFLD in a relatively small cohort of healthy individuals and individuals with type 2 diabetes, and did not find an association.[13] Of note, a recent meta-analysis (n≈35,000) did find a positive association between fruit juice consumption and incident type 2 diabetes.[26]

The divergent associations of fructose from fruit and fructose from fruit juice and SSB with IHL may be explained by the food matrix, i.e., 'the physical domain that contains and/or interacts with specific constituents of a food (e.g., a nutrient) providing functionalities and behaviors which are different from those exhibited by the

components in isolation or a free state'.[28] For instance, the presence of fiber, vitamins, flavonoids, and antioxidants might counteract the deleterious effects of fructose.[29,30] The abrogation of the inverse association of fructose from fruit with IHL after adjustment for dietary fiber supports the concept of the food matrix. Alternatively, consumption of fruit could be a proxy of a healthy lifestyle (and vice versa for fruit juice and SSB). Although we extensively corrected for lifestyle variables, residual confounding may still be present and (partly) account for the current observations.

We generally observed stronger associations for individuals with type 2 diabetes, which warrant further investigation. One potential biological explanation could be a gene-environment interaction. Gene-environment interactions have been reported for NAFLD susceptibility genes that also predispose to type 2 diabetes.[31,32] Alternatively, the observed interactions may be methodologically flawed due to underreporting bias by specific subgroups, e.g., with higher BMI or type 2 diabetes, which is a limitation inherent to nutritional epidemiology.[33] Adjustment for total energy intake can overcome this source of bias, except when there is differential bias in the reporting of macronutrient intake.[33] We did, however, not find differences in the strength of the associations between individuals with prior diagnosed type 2 diabetes and those with newly diagnosed type 2 diabetes (i.e., who were unaware of the diagnosis), which further reduces the likelihood of underreporting bias and suggests that the associations are truly stronger in individuals with type 2 diabetes.

Previous studies have shown that advanced liver fibrosis is particularly prevalent in type 2 diabetes.[34,35] In the present study we did not find any association between fructose intake and ALT, used as a marker of hepatocyte damage. This may be explained by a lack of power (serum ALT was available in n=1,602) and/or the fairly normal ALT levels in this population. Future studies are, therefore, warranted to further investigate (different sources of) dietary fructose in relation to liver damage and fibrosis.

The present study has several strengths and limitations. We used a large population-based cohort, enriched with type 2 diabetes individuals, that was extensively phenotyped using state-of-the-art methods (e.g., 3T-Dixon-MRI of the liver and physical activity monitoring by an accelerometer). This allowed for an accurate estimation of the dependent variable and the adjustment for a wide range of potential confounders. Our study also has specific limitations. First, dietary intake was assessed by means of a FFQ, which has been validated against 24-h dietary recalls for intakes of mono- and disaccharides and fruit, but not for fruit juice and SSB.[15] Further, we could not differentiate between intakes of fresh fruit juice and packed fruit juice, which warrants further study. Second, although the self-reported intake of total fructose in our cohort was comparable to the general Dutch population[36], self-reported intakes of fructose from fruit juice and particularly from SSB were low (consistent with reduced intakes of

fruit juice and SSB with increasing age in the Dutch population[36]). Our results may, therefore, not be extrapolated to populations with high fructose consumption, such as the United States[25], although the effects of fructose restriction, if any difference, is expected to be even greater in such populations. Third, similar to a previous study[13], we only calculated intakes of fructose from homogeneous and relatively easily quantifiable food products, such as fruit, fruit juice, and SSB. We did not specifically assess the association of other dietary sources of fructose, such as vegetables and processed foods (which are more difficult to quantify), with IHL. Fourth, this is a cross-sectional study, which, by design, does not allow inference of causality. We do, however, believe that reverse causality, i.e., high IHL leads to more intake of fructose from fruit juice and SSB, is less likely. Finally, we adjusted for type 2 diabetes in the regression models because of the oversampling of type 2 diabetes in the Maastricht Study. It is likely that overadjustment has occurred since type 2 diabetes is believed to be a consequence of IHL.[3] We, therefore, performed stratified analyses and generally observed stronger associations for individuals with type 2 diabetes. The role of insulin resistance is even more complicated as it may be the consequence of both fructose intake (=exposure) and IHL (=outcome).[37] Adjustment for the Matsuda index may, therefore, have introduced collider bias and should be interpreted cautiously.[38]

In view of implications for public health, we found that individuals in the highest tertile of intake of SSB may reduce their risk of hepatic steatosis by 37% by lowering their fructose intake to the lowest tertile of intake (i.e., a reduction of ~4.5 g fructose from SSB/day). The corresponding absolute reduction of 0.3 percent point in IHL (=9%-fold change [**Table 6.2, model 5**] multiplied by the population median of 3.2 percent [**Table 6.1**]), is small, yet in line with our recently conducted double-blind randomized controlled trial showing that fructose restriction *per se* resulted in a 0.7 percent point reduction in IHL.[9] Moreover, this seemingly small reduction in IHL should be viewed in the context of the global epidemic of NAFLD. It has been estimated that a quarter of the worldwide adult population (~five billion people) is affected by NAFLD. Moreover, NAFLD is more frequently observed in type 2 diabetes and, in fact, is currently viewed as a risk factor of type 2 diabetes.[2-4] It is, therefore, expected that a relatively easily implementable change in lifestyle, i.e., reduction of fruit juice and SSB intake, will have major beneficial health effects at the population level. This finding is of particular interest since there is growing evidence that an excise tax on SSB – as already implemented in the United Kingdom and US cities including Berkeley (California) – has a beneficial, reducing effect on SSB consumption.[39,40] Of note, fruit juice (without added sugar) is currently exempted from all these levies.[39,40]

In conclusion, our population-based cohort study shows that fructose from fruit juice and SSB is associated with higher IHL, independent of confounders. These cross-

sectional findings contribute to current knowledge in support of measures to reduce the intake of fructose-containing beverages as a means to prevent hepatic steatosis at the population level.

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# Supplemental material

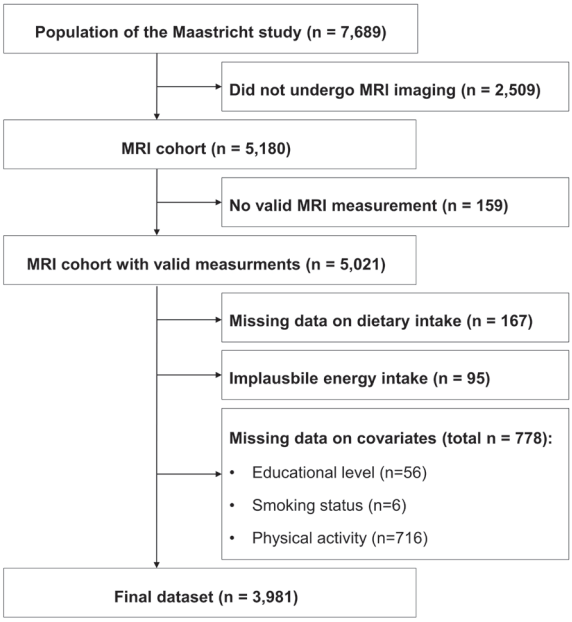


**Figure S6.1. Flowchart of the study population selection process.**
Abbreviation: MRI, magnetic resonance imaging.

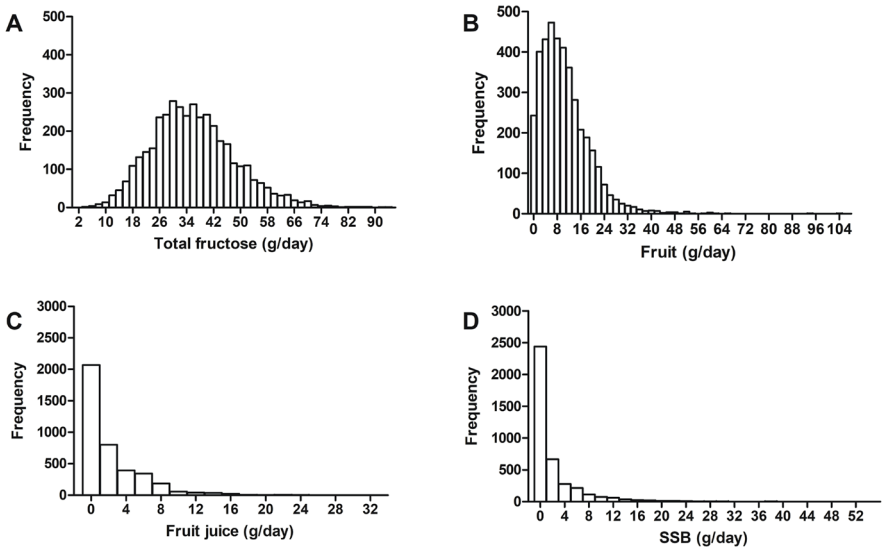


**Figure S6.2. Distribution of energy-adjusted intake of fructose (g/day): total fructose (A), fruit (B), fruit juice (C), and SSB (D).**
Abbreviation: SSB, sugar-sweetened beverages.

**Table S6.1. Multivariable-adjusted associations of energy-adjusted fructose intake and intrahepatic lipid content (IHL) stratified according to type 2 diabetes (individuals without type 2 diabetes [n=3,171] versus individuals with type 2 diabetes [n=810]).**

| | Individuals without type 2 diabetes (n=3,171) | | | | | Individuals with type 2 diabetes (n=810) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Energy-adjusted fructose intake tertiles | | | | | Energy-adjusted fructose intake tertiles | | | | |
| Total fructose* | T1 | T2 | T3 | *P* for | P for | T1 | T2 | T3 | *P* for | *P* for |
| Median g/day | 24.4 | 35.1 | 47.6 | trend | interaction | 24.4 | 35.1 | 47.6 | trend | interaction |
| Model 5 | 1 | 0.99 (0.93; 1.06) | 1.01 (0.94; 1.09) | 0.932 | 0.089 | 1 | 1.05 (0.92; 1.21) | 1.05 (0.90; 1.23) | 0.547 | 0.089 |
| Fructose from fruit* | T1 | T2 | T3 | *P* for | | T1 | T2 | T3 | *P* for | |
| Median g/day | 3.1 | 9.0 | 17.8 | trend | | 3.1 | 9.0 | 17.8 | trend | |
| Model 5 | 1 | 0.97 (0.91; 1.04) | 0.98 (0.91; 1.05) | 0.609 | 0.058 | 1 | 0.89 (0.78; 1.02) | 0.84 (0.72; 0.97) | 0.215 | 0.058 |
| Fructose from fruit juice* | T1 | T2 | T3 | *P* for | | T1 | T2 | T3 | *P* for | |
| Median g/day | 0.1 | 0.9 | 5.3 | trend | | 0.1 | 0.9 | 5.3 | trend | |
| Model 5 | 1 | 0.99 (0.93; 1.05) | 1.03 (0.96; 1.09) | 0.133 | 0.071 | 1 | 1.14 (1.00; 1.31) | 1.08 (0.94; 1.24) | 0.114 | 0.071 |
| Fructose from SSB* | T1 | T2 | T3 | *P* for | | T1 | T2 | T3 | *P* for | |
| Median g/day | 0.0 | 0.5 | 4.5 | trend | | 0.0 | 0.5 | 4.5 | trend | |
| Model 5 | 1 | 0.99 (0.93; 1.05) | 1.08 (1.01; 1.16) | 0.032 | 0.132 | 1 | 1.21 (1.06; 1.38) | 1.14 (0.99; 1.32) | 0.076 | 0.132 |

* Energy-adjusted fructose by means of the residual method.

Regression coefficients should be interpreted as the fold change in IHL that is associated with the difference between the tertile of fructose intake and the reference group (see methods section).

P for trend values were obtained from linear regression with fructose as continuous variables (see methods section).

Data are presented for the fully adjusted model (i.e., with adjustment for age, sex, educational level, smoking status, physical activity, intake of total energy, and energy-adjusted intakes of alcohol, saturated fat, protein, vitamin E, and dietary fiber).

Abbreviation: SSB, sugar-sweetened beverages; T, tertile

**Table S6.2. Multivariable-adjusted (including Matsuda index) associations of energy-adjusted fructose intake and intrahepatic lipid content (IHL) (n=1,415).**

| | Energy-adjusted fructose intake tertiles | | | |
|---|---|---|---|---|
| Total fructose* | T1 | T2 | T3 | *P* for trend |
| Median g/day | 24.4 | 35.1 | 47.6 | |
| Model 5[#] | 11 | 0.99 (0.90-1.09) | 0.97 (0.87-1.09) | 0.598 |
| Fructose from fruit* | T1 | T2 | T3 | *P* for trend |
| Median g/day | 3.1 | 9.0 | 17.8 | |
| Model 5[#] | 1 | 0.98 (0.90-1.08) | 0.96 (0.86-1.07) | 0.823 |
| Fructose from fruit juice* | T1 | T2 | T3 | *P* for trend |
| Median g/day | 0.1 | 0.9 | 5.3 | |
| Model 5[#] | 1 | 0.93 (0.85-1.02) | 1.00 (0.91-1.09) | 0.977 |
| Fructose from SSB* | T1 | T2 | T3 | *P* for trend |
| Median g/day | 0.0 | 0.5 | 4.5 | |
| Model 5[#] | 1 | 0.99 (0.90-1.09) | 1.05 (0.95-1.16) | 0.046 |

* Energy-adjusted fructose by means of the residual method.

[#] In this sensitivity analysis, the covariate type 2 diabetes was replaced by the Matsuda index.

Regression coefficients should be interpreted as the fold change in IHL that is associated with the difference between the tertile of fructose intake and the reference group (see methods section).

P for trend values were obtained from linear regression with fructose as continuous variables (see methods section).

Data are presented for the fully adjusted model (i.e., with adjustment for age, sex, the Matsuda index, educational level, smoking status, physical activity, intake of total energy, and energy-adjusted intakes of alcohol, saturated fat, protein, vitamin E, and dietary fiber).

Abbreviations: SSB, sugar-sweetened beverages; T, tertile.



**Table S6.3. Multivariable-adjusted associations of energy-adjusted fructose intake and alanine aminotransferase (n=1,602).**

| | Energy-adjusted fructose intake tertiles | | | |
|---|---|---|---|---|
| Total fructose* | T1 | T2 | T3 | *P* for trend |
| Median g/day | 24.4 | 35.1 | 47.6 | |
| Model 5 | 1 | 0.99 (0.94-1.04) | 0.99 (0.94-1.05) | 0.997 |
| Fructose from fruit* | T1 | T2 | T3 | *P* for trend |
| Median g/day | 3.1 | 9.0 | 17.8 | |
| Model 5 | 1 | 0.99 (0.95-1.04) | 0.99 (0.94-1.04) | 0.648 |
| Fructose from fruit juice* | T1 | T2 | T3 | *P* for trend |
| Median g/day | 0.1 | 0.9 | 5.3 | |
| Model 5 | 1 | 0.98 (0.94-1.03) | 0.98 (0.94-1.03) | 0.998 |
| Fructose from SSB* | T1 | T2 | T3 | *P* for trend |
| Median g/day | 0.0 | 0.5 | 4.5 | |
| Model 5 | 1 | 0.98 (0.94-1.03) | 0.99 (0.94-1.04) | 0.310 |

* Energy-adjusted fructose by means of the residual method.

Regression coefficients should be interpreted as the fold change in alanine aminotransferase that is associated with the difference between the tertile of fructose intake and the reference group (see methods section).

P for trend values were obtained from linear regression with fructose as continuous variables (see methods section).

Data are presented for the fully adjusted model (i.e., with adjustment for age, sex, type 2 diabetes, educational level, smoking status, physical activity, intake of total energy, and energy-adjusted intakes of alcohol, saturated fat, protein, vitamin E, and dietary fiber).

Abbreviations: SSB, sugar-sweetened beverages; T, tertile.

# Chapter 7

## Genetically proxied ketohexokinase function and risk of colorectal cancer: a Mendelian randomization study

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*Gut 2023;72(3):604-6*

We read with interest the findings from the longitudinal study by Hur and colleagues[1], showing that a high consumption of sugar-sweetened beverages in adulthood is associated with early-onset colorectal cancer (CRC). However attractive the association, the authors acknowledge that their finding does not establish a causal relationship since residual confounding cannot be excluded.

Biological plausibility for a causal role of fructose, one of the principal added sugars, in the pathogenesis of CRC has recently been provided by Taylor et al.[2], who demonstrated a direct link between fructose 1-phosphate (F1P) and intestinal tumor growth in mice (**Figure 7.1A**).

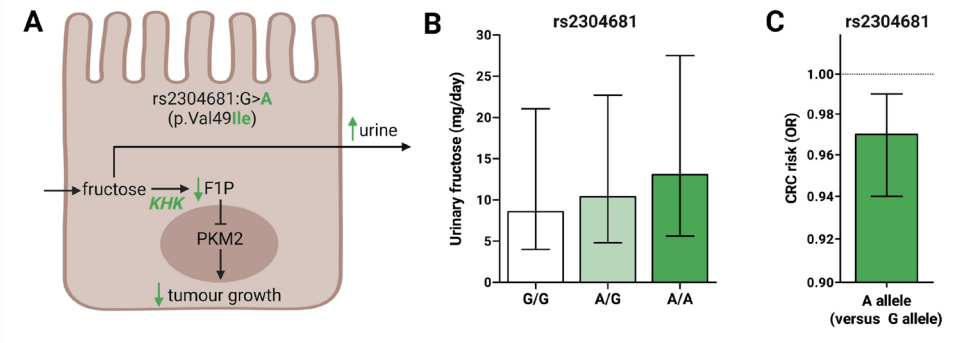


**Figure 7.1. (A)** After intestinal absorption, fructose is phosphorylated by ketohexokinase (KHK). Fructose 1-phosphate (F1P) inhibits the M2 isoform of pyruvate kinase (PKM2) by formation of inactive PKM2 monomers, which enhances hypoxic cell survival and, consequently, intestinal tumor growth.[2] It is hypothesized that a common missense variant in the *KHK* gene (rs2304681) results in impaired fructose phosphorylation and, hence, protection from colorectal carcinoma (green arrows). Furthermore, it is anticipated that the unphosphorylated fructose will escape metabolism and will subsequently be excreted via the kidneys in the urine, similar to essential fructosuria, an inborn error of metabolism.[3] **(B)** The rs2304681 A allele is associated with greater 24h urinary fructose excretion in participants of the Maastricht Study (n=1,471). **(C)** The rs2304681 A allele is associated with a lower risk of colorectal cancer (n=34,627 cases and 71,379 controls).

To translate the experimental findings by Taylor et al. to humans and infer causality from the observations by Hur et al., we performed a Mendelian Randomization (MR) analysis by studying the association between a common variant in the gene encoding ketohexokinase (*KHK*) (which catalyzes the formation of F1P, the first committed step in fructose metabolism) and CRC risk. Since genetic variants are randomly distributed among future haploid cells during meiosis, they can be used as instruments in MR to examine a potential causal relationship akin to conducting randomized controlled trial.[4]

We first assessed functionality of rs2304681, a common missense variant in *KHK* resulting in amino acid substitution Val49Ile (allele frequency: 0.37).[5] In line with our hypothesis (**Figure 7.1A**)[3], we found that the minor A allele is associated with fructosuria in 1,471 participants of the Maastricht Study, a population-based cohort study (see **Supplementary Materials** for extensive description) (regression coefficient: 0.064; 95% CI: 0.027;0.100; p=0.001 for log-transformed urinary fructose, adjusted for age, sex and oversampling of type 2 diabetes; **Figure 7.1B**). Since fructosuria reflects impaired KHK function[3], the association is consistent with rs2304681, or a highly co-related variant, being functional.

Examining the relationship between rs2304681 and CRC risk, we found the minor A allele to be protective based on analysis of 34,627 cases and 71,379 controls of European ancestry[6] (see **Supplementary Materials** for extensive description) (Odds ratio: 0.97, 95% CI: 0.94:0.99, p=0.007; **Figure 7.1C**).

Subsequent MR analysis demonstrated that genetically proxied impaired KHK function (reflected by log-transformed urinary fructose) is associated with a lower CRC risk (Wald ratio: -0.67±0.25, p=0.007; analyzed using the TwoSampleMR package, version 0.5.6, in R[7]). To explore if the association was driven by rs2304681 or another variant, we performed Bayesian co-localization analysis using genome-wide association data for both urinary fructose and CRC risk (500 kb flanking rs2304681). Findings were, however, inconclusive (posterior probabilities <0.80 for both H3 [=co-localization absent] and H4 [=co-localization present]; analyzed with R statistical software using the Coloc package, version 5.1.0[8]). A failure to demonstrate a clear relationship is likely to reflect the limited statistical power of our analysis due to the relatively weak associations.[8] Hence, we cannot exclude the possibility that the association between rs2304681 and CRC risk is explained by another variant in a neighboring gene that is in strong linkage disequilibrium.

Accepting these caveats, our findings provide additional evidence to implicate fructose in the pathogenesis of CRC. Moreover, they also suggest that pharmacological inhibition of KHK may be a viable approach to reduce CRC risk. The latter conclusion is of particular interest given that the first KHK inhibitors have recently entered phase II clinical trials as a treatment of non-alcoholic fatty liver disease.[9]

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# Supplemental material

## Association between rs2304681 and 24h urinary fructose levels

### *Study population*

The Maastricht Study is an extensively phenotyped, population-based cohort study with an oversampling of individuals with type 2 diabetes.[1] In brief, the Maastricht Study focuses on the etiology, pathophysiology, complications, and comorbidities of type 2 diabetes. All individuals between 40 and 75 years old who lived in the southern part of the Netherlands were eligible for participation. Participants were recruited through mass media campaigns and via mailings from the municipal registries and the regional Diabetes Patient Registry. In total, 7,689 participants completed the baseline measurements from November 2010 until December 2017. The Maastricht Study has been approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare and Sports of the Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

### *Twenty-four urinary fructose levels*

Levels of fructose in 24h urine collections were quantified by using a newly developed Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (UPLC-MS/MS) method[2], in the first 3,180 participants of the Maastricht Study. The UPLC-MS/MS method precisely and accurately quantifies urinary fructose in the micromolar range (intra- and inter-day assay variation ranged from 0.3 to 5.1%; accuracy of ~98%[2]). For the present study, urine samples collected for <20h or >28h were excluded from the statistical analyses.

### *Genotyping*

Genotyping was performed in those individuals with type 2 diabetes (n=1,489) who were also included in a Dutch diabetes biobank.[3] This sample was complemented with a sample of individuals without type 2 diabetes (n=1,648) who were selected mainly based on the completeness of the phenotyping data. Genotyping was done with the use of the Illumina Global Screening Array BeadChip (Infinium iSelect 24x1 HTS Custom Beadchip Kit), which includes rs2304681, at the Human Genotyping Facility of the Genetic laboratory of the department of Internal Medicine at Erasmus MC. Genotyping was successful in 95% (2,992 of 3,137) of all samples. The final dataset consists of n=1,471 individuals who have been genotyped and in whom urinary fructose levels have been measured, see flowchart (**Supplemental Figure S7.1**).

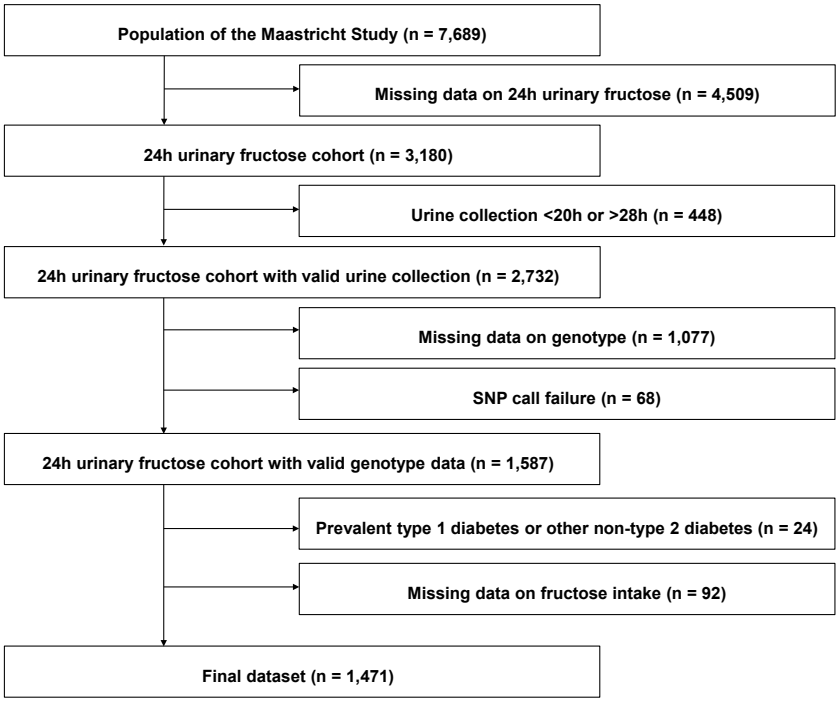


**Figure S7.1. Flow chart of the study population selection process.**



*Quality control, imputation, and association analyses*

Co-localization experiments were conducted to study whether the association signals for urinary fructose and colorectal cancer (CRC) co-localize. For this, linear regression association analyses were carried out for the genomic region of 500 kilobases around rs2304681 (250 kb on either side of the single nucleotide polymorphism [SNP]).

First, in the Maastricht Study, genotype data quality control (QC) and imputation were performed using the Rapid Imputation for COnsortias PipeLIne (RICOPILI).[4] Preliminary QC in Plink version 1.9 [5] consisted of: importing self-reported sex and checking for discrepancies between self-reported and genotype-based sex, identifying relatedness/duplicate samples through identity-by-descent estimation, dropping strandambiguous SNPs, and dropping duplicate markers. Subsequent QC consisted of multidimensional scaling (MDS) to extract ancestry principal components (PCs), and extracting European ancestry individuals based on these PCs. Furthermore, genotype data were filtered for DNA sample level missingness (--mind 0.02), SNP level missingness (--pre geno 0.05; -- geno 0.02), case-control missing rate difference (--midi

0.02), maximum number of Mendelian errors per SNP (--lmend 4), number of Mendelian errors per sample (--imend 10000), heterozygosity outliers based on F statistic (--Fhet_th 0.2), Hardy-Weinberg equilibrium violations in controls and cases, respectively (--hwe_th_co 1e-06; --hwe_th_ca 1e-10), monomorphic SNPs (--withpna 0), minimum number of chromosome X SNPs to perform sex check (--sexmin 10).
Imputation through the RICOPILI consisted of: 1) prephasing using Eagle v2.3.5 [6] and 2) imputation using Minimac3.[7]. Genotype dosage data were converted to best guess genotypes based on a minimum genotype probability of p>0.8. QC filtered out all SNPs with an INFO score <0.1 and minor allele frequency (MAF) <0.005.
Finally, linear regression association analyses were carried out using Plink version 1.9 [5] on the best guess genotypes for the genomic region of 500 kilobases around rs2304681 (250 kb on either side of the SNP), with additional filtering for SNPs with MAF > 0.01 and SNP missingness <0.01. Furthermore, related individuals were excluded at this point (IBD PIHAT threshold 0.1; n=147) by randomly selecting one family member only. Linear regression was performed using the '--linear' option in Plink. Age, sex, genotyping batch (genotyping was performed in two batches) and 5 ancestry PCs were adjusted for.

## Association between rs2304681 and colorectal cancer

*Study population*

This study population has been described in detail elsewhere.[8] In short, the study population was composed of five new CRC genome-wide association studies (GWAS) that were subsequently meta-analyzed together with 10 previously published GWAS, resulting in 31,197 CRC cases and 61,770 controls, all of European ancestry.

*Assessment of colorectal cancer*

The diagnosis of CRC was based on ICD coding (ICD-9: 153, 154; ICD-10: C18.9, C19, C20) in all populations[8], in accordance with the World Health Organization guidelines.

*Genotyping*

Genotyping was done with different arrays in the different populations, as previously described.[8] Individuals with a low single nucleotide polymorphism (SNP) call rate (<95%) were excluded. CRC risk estimates from each individual population were combined by an inverse-variance weighted fixed-effects meta-analysis.

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# Chapter 8

## Genetically proxied ketohexokinase function and risk of cardiometabolic disease: a Mendelian randomization study

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*Diabetes Care 2023;46(4):e97-e98*

# Abstract

**Background and aims**

In a recent systematic review and meta-analysis of prospective cohort studies, Lee and colleagues showed that substitution of low- and no-calorie-sweetened beverages for sugar-sweetened beverages was associated with a lower cardiometabolic risk. However, it is unknown whether these relationships are truly causal. Our aim was, therefore, to examine the association between genetically proxied impaired fructose metabolism and cardiometabolic traits.

**Methods**

We assessed the association of rs2304681 (a common variant in the ketohexokinase gene) with cardiometabolic traits obtained from publicly available databases. Subsequently, in two-sample Mendelian randomization (MR) analyses, we combined these summary-statistics for the cardiometabolic traits (=outcome) with data on genetically proxied impaired fructose metabolism, indexed by urinary fructose (=exposure; obtained from our previous study).

**Results**

We found that the rs2304681 minor A allele was associated with a lower intrahepatic lipid content (β±SE: -0.028±0.008, n=36,703). Protective effects were observed for the risk of type 2 diabetes (fixed-effects meta-analysis OR:0.985, 95%CI: 0.975;0.994, n=1,331,670), hypertension (OR:0.988, 95%CI:0.976;0.999, n=440,285), and myocardial infarction (fixed-effects meta-analysis OR:0.976, 95%CI:0.961;0.992, n=583,191). Subsequently, two-sample MR-analyses showed that genetically proxied impaired fructose metabolism protects from intrahepatic lipid accumulation (Wald-ratio: -0.63, 95% CI: -0.98;-0.28), type 2 diabetes (Wald-ratio: -0.35, 95% CI: -0.57;-0.13), hypertension (Wald-ratio: -0.28, 95% CI: -0.55;-0.01), and myocardial infarction (Wald-ratio: -0.54, 95% CI: -0.90;-0.19).

**Conclusions**

Our findings suggest that fructose *per se* has harmful cardiometabolic effects and, therefore, support and substantiate the conclusions of Lee and colleagues to use water or low- and no-calorie sweetened beverages as a health strategy to reduce the intake of sugar-sweetened beverages.

With interest we read the article in *Diabetes Care* from Lee et al.[1], who performed a systematic review and meta-analysis of prospective cohort studies and showed that substitution of low- and no-calorie-sweetened beverages for sugar-sweetened beverages was associated with a lower cardiometabolic risk. Although the authors carefully attempted to mitigate the influence of residual confounding, they deservedly conclude that they were most likely unable to exclude both unmeasured and measured residual confounding.[1]

Mendelian randomization (MR) is a powerful approach to study the lifelong effects of an exposure of interest on outcomes, independent of the disruptive effects of confounders. We recently studied a common variant in the gene encoding ketohexokinase (*KHK*).[2] KHK catalyses the phosphorylation of fructose, the main caloric constituent of sugar-sweetened beverages. Impaired KHK function results in reduced fructose metabolism and, eventually, urinary fructose excretion.[2] We showed that the rs2304681 minor A allele, a common missense variant in *KHK*, was associated with greater urinary fructose excretion and protection from colorectal cancer.[2]

To gain more insight into the causal association between dietary fructose and cardiometabolic outcomes, we studied the association between the rs2304681 minor A allele and cardiometabolic disease, by using publicly available databases.

We found that the rs2304681 minor A allele was associated with a lower intrahepatic lipid content, assessed by magnetic resonance imaging in the UK Biobank cohort[3] (β: -0.028 ± 0.008, n=36,703; **Figure 8.1**). Protective effects were also observed for the risk of type 2 diabetes in the combined Asian Genetic Epidemiology Network (AGEN) and European Diabetes and Mental Health Adaptive Notification Tracking and Evaluation (DIAMANTE) trial cohorts[4] (fixed-effects meta-analysis: OR: 0.985, 95% CI: 0.975;0.994, n=1,331,670; **Figure 8.1**), the risk of hypertension in the UK Biobank cohort[3] (OR: 0.988, 95% CI: 0.976;0.999, n=440,285; **Figure 8.1**), and the risk of myocardial infarction for the combined Coronary Artery Disease Genome-wide Replication and Meta-analysis plus the Coronary Artery Disease Genetics (CARDIoGRAMplusC4D) and UK Biobank cohorts[3,5] (fixed-effects meta-analysis: OR: 0.976, 95% CI: 0.961;0.992, n=583,191; **Figure 8.1**).

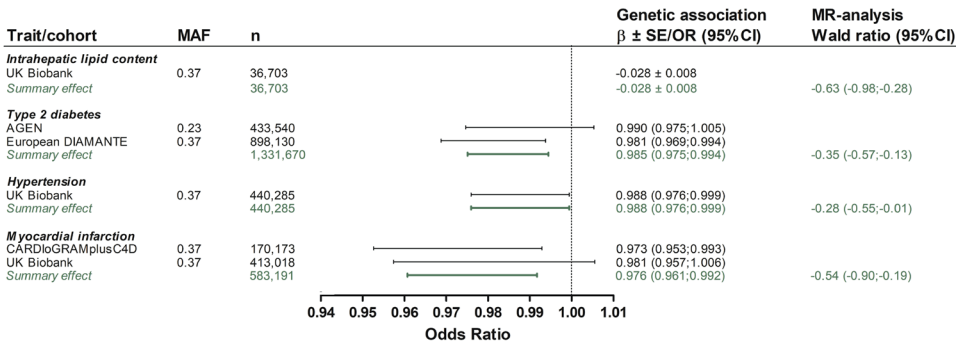


**Figure 8.1. Genetic associations between a common variant in the ketohexokinase (*KHK*) gene (rs2304681 minor A allele) and cardiometabolic traits (forest plot), and Mendelian randomization analyses for the relationship between genetically proxied impaired KHK function (indexed by urinary fructose) and cardiometabolic traits**.

Genetic associations should be interpreted as the change in intrahepatic lipid content, or the risk of type 2 diabetes, hypertension, and myocardial infarction conferred by one *KHK* risk allele.

Wald ratios should be interpreted as change in intrahepatic lipid content, risk of type 2 diabetes, hypertension, or myocardial infarction per unit increase in log urinary fructose (indicative of impaired KHK function).

Abbreviations: β, beta; CI, confidence interval; MAF, minor allele frequency; MR, Mendelian Randomization; SE, standard error; OR, Odds Ratio.

We subsequently conducted two-sample MR-analyses (TwoSampleMR package in R). The previously reported association between the rs2304681 minor A allele and ($^{10}$log) urinary fructose[2] was used as exposure and the hitherto reported associations between the rs2304681 minor A allele and cardiometabolic disease were used as outcomes. We found that genetically proxied impaired fructose metabolism protects from intrahepatic lipid accumulation (Wald-ratio: -0.63, 95% CI: -0.98;-0.28, p<0.001; **Figure 8.1**), type 2 diabetes (Wald-ratio: -0.35, 95% CI: -0.57;-0.13, p=0.002; **Figure 8.1**), hypertension (Wald-ratio: -0.28, 95% CI: -0.55;-0.01, p=0.040; **Figure 8.1**), and myocardial infarction (Wald-ratio: -0.54, 95% CI: -0.90;-0.19, p=0.003; **Figure 8.1**).

Our findings suggest that fructose *per se* has harmful cardiometabolic effects and, therefore, support and substantiate the conclusions of Lee et al.[1] on the use water or low- and no-calorie sweetened beverages as a health strategy to reduce the intake of sugar-sweetened beverages

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# Chapter 9

Summary and general discussion

Non-alcoholic fatty liver disease (NAFLD) and its cardiometabolic consequences are a major health burden worldwide.[1] Intrahepatic lipid (IHL) accumulation is the first stage of NAFLD. IHL content is, among others, driven by the conversion of simple sugars into fatty acids via hepatic *de novo* lipogenesis (DNL).[2-4] However, which simple sugar – fructose or glucose – plays a greater role in the augmentation of DNL has been the subject of much debate.[5]

The overall aim of this thesis, therefore, was to determine the role of fructose in the pathophysiology of NAFLD, and the possible underlying mechanism. We used a variety of research methodologies (each with its own strengths and limitations), including experimental studies using both mice and humans as well as nutritional and genetic epidemiology, highlighting the translational nature of this thesis.

This chapter summarizes and discusses the main findings of this thesis in the context of current scientific literature and methodological considerations. In addition, conclusions based on this thesis are reported and possible directions for future research are addressed.

# Summary and discussion

## Part I: Background – Overview of fructose metabolism and a new method to quantify fructose concentration

### Epidemic of fructose intake in relation to the pathogenesis of intrahepatic lipid accumulation

The Western society is currently witnessing an epidemic of fructose intake[6,7], as reviewed in **chapter 2**. The dramatic rise in intake of simple sugars, in particular fructose, parallels the current obesity epidemic and its sequelae such as NAFLD, dyslipidemia, type 2 diabetes (T2DM), and cardiovascular disease (CVD).[8-10] The abundancy of fructose is in particular challenging for individuals with hereditary fructose intolerance (HFI).[8,11] Patients with HFI are characterized by rare loss-of-function mutations in aldolase B (*ALDOB*; responsible for the second step in fructolysis), and do not tolerate fructose and thus need to follow a life-long fructose-free diet to avoid acute symptoms and chronic complications (**chapter 2**).[8,11] Recent advances in the study of mice and humans have provided more insight into the pathogenesis of HFI, including the paradoxical fatty liver phenotype.[12,13] Importantly, these recent studies identified a central role for hepatocellular fructose 1-phosphate (F1P) accumulation and concomitant adenosine triphosphate (ATP) and phosphate depletion in the

pathogenesis of HFI.[12,13] Moreover, treatment with a ketohexokinase inhibitor (KHK; responsible for the first step in fructolysis and thus hampering F1P accumulation) ameliorated hepatic steatosis in a mouse model with global knockout of aldolase B (ALDOB-KO mice that phenocopies HFI[12,14]). These data highlight the importance of hepatocellular F1P (and concomitant ATP and phosphate depletion) and show that increased fructolytic flux *per se* is not necessary for the pathogenetic features seen in HFI.[12,13]

Given the current epidemic of fructose intake and its parallels with the prevalence of non-communicable disease, proper fructose measurement is required for future studies investigating the (causal) role of fructose in non-communicable disease. **Chapter 3** describes how we developed and validated an Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (UPLC-MS/MS) method to precisely and accurately quantify fructose in serum and urine fractions in the micromolar range, without interference of other sugars in the sample. Furthermore, we showed in a number of exploratory experiments the utility of this UPLC-MS/MS method for the measurement of fructose (**chapter 3**).

In conclusion, the current epidemic of fructose intake in parallel with the NAFLD epidemic demonstrates the need for better understanding of fructose metabolism in relation to health. Furthermore, recent studies identified F1P as a major determinant in the pathogenesis of hepatic steatosis in HFI, yet the role of F1P in the pathogenesis of fructose-induced NAFLD remains unknown. Most importantly, these recent studies imply that blocking upstream F1P formation – by inhibition of KHK – may be a viable approach to reduce IHL content (see **Chapter 10** 'Impact paragraph').

# Part II: Fructose as a signalling molecule – Interaction between fructose and glucose metabolism: from mice to men

## Hepatic fructose metabolism – A role for fructose as signalling molecule in pathogenesis of intrahepatic lipid accumulation

We investigated possible mechanisms by which fructose participates as signalling molecule in the pathogenesis of IHL accumulation in mice, and in particular the role of F1P herein (**chapter 4**).

In the first experiment, we revisited the glucokinase regulatory protein-glucokinase (GKRP-GCK) complex (**chapter 4**). F1P is a potent disruptor of the GKRP-GCK complex, which subsequently facilitates hepatic glucose uptake, and consequently stimulates DNL.[15-17] Therefore, we crossbred ALDOB-KO mice (i.e. typified by high hepatocellular

F1P) with GKRP-KO mice (i.e. lacking hepatic GCK expression since GKRP acts as a stabilizer for GCK[18,19]), and generated ALDOB/GKRP-DKO mice to study the role of the GKRP-GCK complex in the pathogenesis of IHL accumulation in HFI. However, we found that GKRP knockout did not protect from IHL accumulation, and, therefore, conclude that GKRP-GCK does not actively contribute to hepatic steatosis in HFI.

In the second experiment, we studied the role of carbohydrate response element binding protein (ChREBP) in the pathogenesis of IHL accumulation in HFI (**chapter 4**). ALDOB-KO mice are characterized by intracellular F1P accumulation and concomitant ATP and phosphate depletion which could activate ChREBP, a lipogenic transcription factor with multiple downstream effects including stimulation of DNL.[20-27] Therefore, we treated the ALDOB-KO mice with adeno-associated viruses directed against short hairpin ChREBP to reduce hepatic ChREBP activity.[28,29] In contrast to our hypothesis, we found that hepatic ChREBP knockdown increased liver weight and tended to increase IHL content in ALDOB-KO mice. We will perform near future analyses to provide more insight into the pathways that contribute to IHL content (e.g. DNL and VLDL-secretion) and the involvement of gluconeogenetic and glycogenolytic pathways to better understand the mechanisms which are upregulated/suppressed by hepatic ChREBP knockdown. Noteworthy, as proposed by Lei and colleagues[28], the consequence of altered hepatic ChREBP activity for NAFLD development is likely dependent on the disease and/or physiological state, and (hepatic) ChREBP ablation is an effective treatment in other models.[30]

Next, we wanted to study the role of the GKRP-GCK complex in humans (**chapter 5**). Previously, Moore and colleagues elegantly showed that adding 7.5 g of fructose to a 75 g oral glucose tolerance test (OGTT) reduced plasma glucose excursions, most likely due to increased hepatic glucose uptake, in both healthy adults and individuals with T2DM.[31,32] However, it is currently not known at what threshold fructose interacts with glucose *in vivo*. Therefore, in **chapter 5**, we repeated and extended the original study by Moore and colleagues by studying the plasma glucose response during an OGTT with and without the addition of different doses of fructose, ranging from 1 gram to 15 grams. Surprisingly, we could not replicate the findings of Moore and colleagues.[31,32] Therefore, in order to explain our null findings, we measured the serum fructose response after an OGTT *without* addition of fructose by using our newly developed UPLC-MS/MS method (**chapter 3**). We found a small, statistically significant increase in serum fructose levels, which may be explained by endogenous fructose production via the polyol pathway[33], consistent with findings of a previous study.[34] Therefore, it may be that the amount of endogenously produced fructose is already sufficient to maximally dissociate GCK from GKRP, explaining why the addition of exogenous fructose did not affect plasma glucose excursions. This implies that repeating the OGTT

with lower doses of glucose – below the threshold of endogenous fructose production – might yield different results. Moreover, we believe that this study does not exclude a role for the GKRP-GCK complex since previous studies showed that individuals carrying a common variant in the gene encoding *GCKR* – resulting in dysfunctional GKRP-GCK binding – have increased DNL and IHL content as well as dyslipidemia.[35-37] Noteworthy, these OGTT experiments are presumably independent of the lipogenic factor ChREBP since its activation and action likely takes longer than the 120-minute postprandial follow-up.

In conclusion, we were unable to demonstrate the interaction between fructose and glucose metabolism in ALDOB-KO mice (**chapter 4**) and humans (**chapter 5**). Future studies are warranted to elucidate the key molecular mechanisms by which fructose participates as a signalling molecule in the pathogenesis of IHL accumulation and the role of F1P herein.

# Part III: Fructose intake and non-communicable disease at the population level

## Reduced fructose metabolism – A (dietary) modality in the protection of intrahepatic lipid accumulation

Recent studies have shown that fructose, more than glucose, augments DNL.[38] In support, Simons and colleagues conducted a double-blind randomized-controlled trial to study the effects of fructose or glucose supplementation on a background of a 6-week fructose-restricted diet.[39] The complete fructose-restricted group showed a small decrease in IHL content, suggesting that fructose *per se* has more deleterious effects on IHL deposition than glucose.[39] In agreement, in **chapter 6** of this thesis, we found that fructose from fruit juice and sugar-sweetened beverages (SSB), but not from fruit, was associated with higher IHL content (measured by magnetic resonance imaging; MRI) at the population level. These divergent associations may be explained by lifestyle: consumption of fruit could be a proxy of a healthy lifestyle (and vice versa, intake of fruit juice and SSB a proxy of an unhealthy lifestyle). Although we extensively corrected for lifestyle variables in our statistical analyses, residual confounding may still be present and (partly) account for the observations. The latter marks an important limitation inherent in traditional epidemiology. In addition, since fructose and glucose intake are intercorrelated (i.e. in the form of sucrose), we were by design unable to determine the role of fructose *per se* in this epidemiological study.

Therefore, to further explore the causal nature of this observational relationship (**chapter 6**) and determine the role of fructose *per se* we used Mendelian randomization (MR), a form of genetic epidemiology that can infer causal associations in observational data.[40] MR is based on the concept that each individual randomly receives a genetic variant during conception. Akin to a randomized-controlled trial, this random distribution of genetic variants amongst individuals allows us to study the effects of lifelong exposure to fructose on disease risk, without the disruptive effects of confounding factors. In **chapter 7**, we first showed that a common variant in the *KHK* gene (i.e. rs2304681:G>A [p.Val49Ile]; minor allele frequency: 0.37) was associated with higher urinary fructose levels quantified by our UPLC-MS/MS method (**chapter 3**). These data align with the phenotype of individuals with essential fructosuria (OMIM #229800; those who have a loss of *KHK* [EC 2.7.1.3]) who present with fructosuria, and, therefore, suggest altered functionality of the Val49Ile KHK protein (or a highly correlated variant). Next, in **chapter 8**, we showed that genetically proxied impaired fructose metabolism (reflected by higher urinary fructose levels; **chapter 7**) was associated with a lower IHL content as determined by MRI. Taken together, these studies strongly suggest a causal role for reduced fructose metabolism in the protection of IHL accumulation.

## Intestinal fructose metabolism – A role for the intestines as fructose scavenger in the protection of intrahepatic lipid accumulation

Along the liver, recent studies have identified a crucial role for the intestines (which also express KHK and ALDOB) in the pathogenesis of fructose-mediated metabolic disorders.[41] Recent mice studies have demonstrated that intestinal fructose metabolism reduced the flux of fructose via the portal circulation towards the liver, which consequently protected from fructose-induced IHL accumulation.[42,43] In support, Jang and colleagues showed that mice gavaged with fructose metabolized 90% of the absorbed fructose within the small intestine and only minute amounts reached the liver.[44] In agreement, overexpression of KHK-C promoted intestinal fructose clearance and decreased fructose-induced DNL, while knockout of intestine-specific KHK-C resulted in more fructose supply to the liver (and gut microbiota) and consequently increased fructose-induced DNL.[42] Since these data collectively show that intestinal fructose metabolism scavenges fructose away from the liver (and peripheral circulation)[42-44], we studied in **chapter 5** the serum fructose response during an OGTT with addition of different doses of fructose in healthy individuals. We observed a non-linear relationship between the fructose dose (i.e. 1, 2, 5, 7.5, 15 g) and the serum fructose response, suggesting that at lower doses less fructose escapes intestinal (and hepatic) fructose metabolism in humans. On the other hand, we did detect a

statistically significant increase in serum fructose after fructose doses even as little as 1 gram and 2 grams. This suggests that at least some fructose passes the small intestine and reaches the liver.

Furthermore, the mode of fructose exposure might exhibit distinct biological effects on intestinal fructose metabolism. Animal studies have shown that fructose or sucrose as either one large bolus or as liquid may be more deleterious for the liver than multiple small boluses or as solid.[42,45] In agreement, in **chapter 6**, we found in our cross-sectional study that fructose from fruit juice and SSB, but not from fruit, was associated with higher IHL content. These divergent associations may be explained by the 'food matrix', i.e. the presence of fibers in fruit delay intestinal fructose exposure, resulting in enhanced intestinal fructose metabolism and consequently less spillover to the liver, in contrast to fructose from SSB.[46,47] The abrogation of the inverse association of fructose from fruit with IHL content after adjustment for dietary fiber supports the concept of the food matrix (**chapter 6**).

Of interest, the importance of intestinal fructose metabolism in relation to colorectal cancer (CRC) has also recently been demonstrated: Taylor and colleagues demonstrated that F1P promotes hypoxic cell survival and the development of intestinal tumors in susceptible mice.[48] Consistently, some, but not all, epidemiological studies found an association between the intake of SSB and higher risk of colorectal adenomas and cancer.[49,50] In support, in **chapter 7**, we demonstrated that genetically proxied reduced fructose metabolism (via impaired KHK function) was associated with lower CRC risk. Although these data collectively identify fructose as culprit in the pathogenesis of CRC, further studies are needed to assess whether F1P *per se* is responsible for these findings.

In conclusion, although the hitherto mentioned considerations and methodological limitations (see 'Methodological considerations') should be taken into account, based on nutritional and genetic epidemiology collectively we conclude that fructose plays a presumptive causal role in the pathogenesis of IHL accumulation (and T2DM, hypertension, myocardial infarction, and CRC). Moreover, the findings of this thesis may have significant health consequences for clinical practice which are outlined in **Chapter 10** ('Impact paragraph').

# Methodological considerations

The studies conducted as part of this thesis further increase our understanding of the relationship between fructose and IHL accumulation. However, the variety of research methodologies used in this thesis each have notable shortcomings that should be considered when interpreting the results.

## Methodological considerations in mice studies

Although animal research is for obvious ethical considerations discouraged, a major advantage of using animals is the possibility to elucidate the underlying mechanism of fructose-induced IHL deposition. There is no good *in vitro* model to study the GKRP-GCK complex, apart from hepatocytes isolated from rats.[51] In addition, animal research allows a controlled setting (e.g. chow and drink exposure and administration of the intervention), as opposed to (uncontrolled) experiments in humans who tend to show limited compliance (particularly in the case of dietary and physical activity interventions).

However, a self-evident limitation of animal research is transferability of the findings from mice to humans, since mice are not humans and vice versa. For example, the ALDOB-KO mice have a complete aldolase B deletion (without residual aldolase A or C expression in the liver; **chapter 2**), while in humans there is residual activity in the mutant aldolase Bs[52-54] and/or compensating expression of the other aldolase isozymes[55,56], which limits generalizability of the findings between these two species. Furthermore, although the significance of intestinal fructose metabolism has recently been shown in mice[42-44,48], whether it is dominant over hepatic fructose metabolism in other species than mice is yet unclear.[57] Indeed, previous work has shown that intestinal fructose-absorbing transporter GLUT5 expression is markedly higher in rats and humans than in mice[58], which in turn may account for the higher peak portal fructose concentration in humans than in mice.[59-61] This adds another layer of difficulty to translating findings from mice to humans. Despite these limitations, as outlined in **chapter 2**, the ALDOB-KO mice phenocopies HFI (i.e. failure to thrive, liver and intestinal complications, hypoglycemia, and death after fructose intake) and, therefore, appears to be a suitable mouse-model for HFI.

Last, animal work is dependent on numerous external conditions. For example, although we used ALDOB-KO mice fed the same fructose-free chow as previous experiments with ALDOB-KO mice[12,14,38], our ALDOB-KO mice developed hepatic steatosis less severely (diminishing the possible effect size of interventions for the reduction of IHL content, which could impede the statistical power).

## Methodological considerations in human studies

A general, important limitation in human studies is variation (since the experimental setting is less controlled when compared to animal studies), which could impede the statistical power. In **chapter 3**, we used our newly developed UPLC-MS/MS method to measure 24h urinary fructose, which is the net result of renal fructose supply (i.e. determined by dietary intake, intestinal uptake/metabolism, first-pass effect in the liver, and the glomerular filtration rate) and reabsorption by the proximal tubule. All these determinants may differ among individuals and thus may exhibit different biological effects on urinary fructose levels. Nonetheless, despite this biological variation we still showed that dietary fructose intake (**chapter 3**) and a genetic variant in *KHK* (**chapter 7**) are associated with urinary fructose levels.

Although considered the gold standard to assess metabolic pathways, we did not use stable isotopes to quantify DNL, because they will not prove causality since the GKRP-GCK complex cannot be directly measured (and implementation of these expensive studies in a large number of participants is unfeasible). For this, liver biopsies are required, which has several major limitations such as poor accessibility and risk of (life-threatening) complications. As an alternative, we performed OGTT experiments in humans to provide some insight into the acute effects of oral fructose on glucose metabolism (**chapter 5**). Since OGTTs are associated with great within-subject variability[62], we aimed to reduce this by standardizing our measurements (e.g. participants received instructions regarding factors that could influence the postprandial plasma glucose response such as diet and physical activity). Furthermore, we applied a 4-day wash out to limit possible carry over effects of repeated OGTTs.[63] However, despite our efforts to standardize the procedure and minimize bias, the quality of the data from these experiments relies for a large part on the compliance with the provided guidelines. Furthermore, it must be noted that Moore and colleagues did observe effects of fructose added to an OGTT on the glucose response both in healthy adults and individuals with T2DM.[31,32] In contrast, we observed null findings while repeating these OGTT experiments in healthy humans. In addition to the aforementioned mentioned possible explanations (see 'Summary and Discussion, part II'), a statistical power issue could theoretically account for the null findings. However, we included more participants than in the studies by Moore and colleagues.[31,32] In addition, even in a meta-analysis (combining our OGTT data with that of another, similar study[64]; pooled sample size n=38) we were unable to demonstrate the acute effect of fructose on the glucose response, which further increases the likelihood of spurious findings by Moore and colleagues.[31,32]

## Methodological considerations in nutritional epidemiology

A significant strength of observational studies is the ability to explore associations in large populations and draw conclusions on the clinical relevance and health implications. However, several forms of bias can challenge the results of observational studies. The internal validity of a study refers to the accuracy of the inferences drawn for the individuals of the population of which they are part.[65] The internal validity of any epidemiological study is threatened by systematic error, which distorts the 'true' relationship between the determinant and outcome. Three forms of systematic error may occur: information bias, confounding, and selection bias. First, these three main threats to internal validity will be discussed. Second, causal inference, multiple testing adjustment, and external validity will be addressed.

### *Information bias*

Information bias is caused by erroneous information (measurement error in case of continuous data and misclassification error in case of categorical data) on the determinant, the outcome, or both.[66] The impact of such error depends on whether it is systematic or random and whether it is in the main determinant or outcome variable.[67] Systematic error in a determinant or outcome variable can cause both overestimation and underestimation of an effect size.[67] Random measurement error in the determinant variable attenuates findings towards the null (i.e. regression dilution bias), while in the outcome variable this error widens the confidence intervals (i.e. reduces estimate precision).[67] Therefore, well-designed and standardized protocols have been implemented in the Maastricht Study as a means to minimize measurement and misclassification error during data collection, handling, and analysis.

### *Information bias in the determinant variable*

There are several methods available to assess dietary intake, however there is no gold standard. Habitual food intake is commonly assessed by food-frequency questionnaires (FFQs) in large observational cohort studies, which is, like any dietary collection, subject to errors. In **chapter 6**, dietary intake was assessed by a validated 253-item semi-quantitative FFQ that was specifically designed for use in the Maastricht Study.[68] This FFQ was validated against a 24h recall for the intake of total fructose and fruit, but not for fruit juice and SSB.[68] Furthermore, FFQs are prone to recall bias (i.e. participants may incorrectly report their food intake and the portion sizes) potentially leading to under- or overestimation of nutrient intake.[69] Also, we limited our analyses to fructose from fruit, fruit juice (unable to differentiate between fresh and packed fruit juice) and

SSB while other fructose-containing food items may exhibit different associations with IHL content.

In epidemiological studies, it is usually appropriate to adjust for total energy intake to control for confounding.[70] Confounding can result if total energy intake is related to disease risk, because of associations between physical activity or body size and the probability of disease.[70] Macronutrients are correlated with total energy intake either because they contribute directly to energy intake or because individuals who consume more total energy also eat more of all specific nutrients.[70] Failure to account for total energy intake can result in spurious associations between nutrient intakes and disease risk or even reverse the direction of association.[70] Multiple methods are available to adjust for total energy intake, including the standard multivariate model, the nutrient density model, the residual method, and the energy-partition (decomposition) model.[70] As proposed by Willet and colleagues[70], in case of categorizing the determinant variable, the residual method and nutrient density methods are preferred and we, therefore, opted for using the residual method in **chapter 6**. For this, we performed regression analyses to compute residuals of nutrient intake by removing the variation caused by total energy intake (i.e. the nutrient intakes of the individuals in a study population are regressed on their total energy intakes).[70] An advantage of this method is that the nutrient residual is uncorrelated with total energy intake and this allows to directly evaluate the variation due to the nutrient composition of the diet (as opposed to the combination of dietary composition and total amount of food).[70]

Noteworthy, we generally observed stronger associations for individuals with T2DM in **chapter 6**. These observed interactions may be methodologically flawed due to underreporting bias, which is a limitation inherent to nutritional epidemiology.[71] Adjustment for total energy intake can overcome underreporting bias, except when there is differential bias in the reporting of macronutrient intake.[71] However, we did not find differences in the strength of the associations between individuals with prior diagnosed T2DM and those with newly diagnosed T2DM (i.e. who were unaware of the diagnosis at baseline), which reduces the likelihood of underreporting bias.

*Information bias in the outcome variable*

Measurement error in the outcome variable may also cause information bias, which, if random, would reduce estimate precision, and correspondingly would impede statistical significance.[65] Most previous observational studies quantified liver fat by (poor) biomarkers and/or the fatty liver index that are not based on reference methods.[72] In contrast, we used 3T Dixon MRI to non-invasively measure IHL content (**chapter 6**). This method has been validated and calibrated against proton magnetic resonance spectroscopy (1H-MRS), the gold standard to non-invasively quantify IHL.[73]

After calibration, the intraclass correlation coefficient between Dixon MRI and 1H-MRS was 0.989 (95% CI 0.979; 0.994; n=36).[73] Notably, there is inevitable biological variation (rather than methodological variation) in the outcome variable since IHL content is highly variable over time, and due to intra-liver variation and a lag time between the baseline measurements (including dietary intake assessment) and MRI of the liver (although this lag time did not significantly affect the observed relationships in **chapter 6**; data not shown in this thesis).

*Confounding, overadjustment bias, collider bias, and multicollinearity*

Confounding occurs when an association between a determinant and outcome variable can be influenced by extraneous factors (confounders). Despite our efforts to address this form of bias by including potential confounders (such as diet and lifestyle) in the regression models, we cannot fully eliminate the presence of residual confounding and, therefore, cannot infer causality in **chapter 6**.

In addition, although extensive adjustment for potential confounders is intended to reduce confounding, it may conversely cause overadjustment bias if the included variables are mediators or descendants of the outcome.[74,75] This is possibly evident in **chapter 6** when we adjusted for T2DM in the regression models because of the oversampling of individuals with T2DM in the Maastricht Study, which likely resulted in overadjustment since T2DM is believed to be a consequence of IHL[76] (see below 'selection bias').

Furthermore, collider bias may have been introduced after adjustment for insulin resistance (estimated by the Matsuda index) in the regression models in **chapter 6**[65], since insulin resistance may be the consequence of both fructose intake (= determinant) and IHL content (= outcome).[77]

Last, multicollinearity may in some cases follow from multivariable linear regression analyses. Multicollinearity is defined as a strong correlation between the determinant and one or more confounders.[78] Because strongly correlated variables provide little unique information, the regression coefficients for these corresponding variables are, consequently, based on limited information as well. Multicollinearity can result in unreliable regression estimates (i.e. affecting the strength, precision, and direction of the associations). Multicollinearity is particularly often an issue in nutritional epidemiology. Intakes of nutrients (particularly macronutrients) are positively correlated with total energy intake because the intake of nutrients contribute directly to energy intake and/or individuals who consume more total energy also consume on average more of all specific nutrients.[70] Indeed, in **chapter 6**, fructose intake was initially strongly correlated with total energy intake (variance inflation factor [VIF]: 14; data not shown in this thesis). However, adjustment of the nutrient variables for total

energy intake by means of the residual method solved the issue of multicollinearity (i.e. VIF's: <1.5), since the nutrient residual is uncorrelated with total energy.[70]

*Selection bias*

Selection bias occurs if findings are biased by the procedures used for participant selection or due to non-random factors that affect study participation.[79] It is most likely that the recruitment strategy of the Maastricht Study as well as the in- and exclusion criteria applied have led to selection bias in **chapter 6**.[80] The Maastricht Study is by study design enriched with individuals with T2DM in order to increase the statical power to identify any potential differences between individuals with and without T2DM.[80] Because of this oversampling of individuals with T2DM, we adjusted for T2DM in the regression models in **chapter 6**. It is likely that overadjustment has occurred (which might underestimate the effect size), since T2DM is believed to be a consequence of IHL.[76] Therefore, we also performed stratified analyses and generally observed stronger associations for individuals with T2DM.
Furthermore, the main analyses in **chapter 6** were performed according to the complete-case analysis principle, i.e. the participants with any missing data (determinant, outcome, or confounder) are excluded in all models.[81] An advantage of this approach is that it allows to make the comparison of consecutive regression models insightful given the same sample size in all models. However, by limiting the sample size in earlier models, it reduces the statistical power and may introduce selection bias in earlier models if participant exclusion due to missing variables is non-random.

*Causal inference*

Causality is defined by Kenneth Rothman and colleagues as 'a cause of a specific disease event as an antecedent event, condition, or characteristic that was necessary for the occurrence of the disease at the moment it occurred, given that other conditions are fixed'.[82]
The study in **chapter 6** is cross-sectional which, by design, does not allow inference of causality as residual confouding can be present. However, reverse causality, i.e. a high IHL content leading to more intake of fructose from fruit juice and SSB, seems unlikely.

*Multiple testing adjustment*

Adjustment for multiple testing is the correction of the statistical significance threshold (alpha) based on the number of statistical comparisons made.[83] We used multiple main determinants (total fructose, fructose from fruit, fructose from fruit juice, and fructose from SSB) in **chapter 6**, which theoretically would require adjustment for multiple

testing.[83] However, since the different sources of fructose intake are related, we believe it would be too rigid to adjust for multiple testing in this context.[83] In addition, adjustment for multiple testing increases the chance of missing important findings (i.e. type II error; false negative). Also, for that reason, we applied a less rigid significance threshold ($p<0.10$) for the exploratory interaction-analyses in **chapter 6**, to reduce the chance of missing important findings. We do realize this comes at the risk of detecting spurious findings.

*External validity*

External validity refers to the generalizability of the findings to individuals who are not part of the study population. Although **chapter 6** represents findings from a population-based cohort, these results do not necessarily apply to other populations. Participants of the Maastricht Study are aged between 40 and 75 years, mostly Caucasian, and more often suffer from T2DM[80], which limits generalizability of our findings to other populations of different age groups, ethnicities, and glucose metabolism status. For example, the intake of fructose from fruit juice and SSB was relatively low in the Maastricht Study suggesting that the effect may be (even) greater in populations with much higher fructose intake (such as adolescents[7]).

## Methodological considerations in genetic epidemiology

Genetic epidemiology can overcome some of the limitations inherent in epidemiological studies as outlined above. Mendelian randomization (MR) is a form of genetic epidemiology that can infer causal associations in observational data.[40] Since an individual is randomized at conception (more precisely at meiosis) to receive a gene variant that either predisposes or protects from an exposure of interest (in this case urinary fructose levels), these gene variants can be used as an instrument to test for a causal association with an outcome of interest (e.g. IHL content). Besides the advantage of assessing causality within observational study designs, findings from MR analyses are less prone to unmeasured confounding or reverse causation since inheritance of exposure-predisposing or exposure-protective alleles is independent and cannot be influenced by the outcome.[84] Also, a two-sample approach (i.e. gene-exposure data and gene-outcome data derived from different datasets) has, by design, a weak instrument bias towards the null rather than in the direction of the confounded observation, resulting in a low likelihood of a type I error (i.e. false positive).[85] Nonetheless, the MR approach has potential pitfalls, and it must meet the following assumptions prior to conducting such study:

1) The genetic variant must be (robustly) associated with the exposure trait of interest.
2) The genetic variant must not be associated with any confounder of the association between the exposure and outcome (i.e. horizontal pleiotropy).
3) The genetic variant must not be associated with the outcome of interest via other pathways than the exposure trait.[86,87]

In genetic association studies, there are three situations in which the latter two assumptions are violated, namely:

1) Pleiotropy: the genetic variant also affects other risk factors of the outcome.
2) Linkage disequilibrium: the genetic variant co-segregates with neighboring genes that affect the outcome.
3) Population stratification: the frequency of the genetic variant is different among the different subpopulations used.[86,87]

First, these three potential threats to the core assumptions of MR will be reviewed. Second, functionality of the genetic variant and statistical power will be discussed.

*Pleiotropy*

Pleiotropy refers to the phenomenon of a single genetic variant influencing multiple traits. Two forms of pleiotropy may occur.[86,87] First, vertical (mediation) pleiotropy means that the genetic variant is associated with one trait, which in turn influences another (unidirectional).[86,87] Because they are on the causal pathway, they should be considered as intermediates of the relationship between the exposure and outcome and, therefore, they do not invalidate the MR assumptions. Second, horizontal (confounding) pleiotropy refers to the genetic variant influencing the two traits through independent pathways, which makes it a confounding factor in the relationship between the exposure and outcome.[86,87]

The results from **chapter 7** and **chapter 8** should be interpreted in view of the risk of horizontal pleiotropy. The associations between impaired KHK function and non-communicable disease may be mediated by body weight since impaired KHK function and consequently reduced fructose metabolism results in caloric excretion via the urine. Although not shown in the UK Biobank[88], summary-statistics from the GIANT consortium database showed that a genetic variant in *KHK* (i.e. rs2304681:G>A [p.Val49Ile]) is associated with lower BMI (data not shown in this thesis).[89] Also, multiple observational studies have previously shown an association between adiposity and NAFLD, T2DM, hypertension, CVD, and CRC.[90-99] Therefore, body weight loss due to impaired KHK function and consequent caloric loss might, at least in part, account for the protective associations of the rs2304681 minor A allele on non-communicable

disease risk (**chapter 7** and **chapter 8**). However, we were unable to adjust for BMI in the regression models since we used summary-statistics and, therefore, cannot definitely conclude that the observed associations are truly due to fructose *per se* (which is a study limitation of the genetic studies outlined in **chapter 7** and **chapter 8**).

*Linkage disequilibrium*

Linkage disequilibrium is the non-random association of alleles at different loci in a given population.[100-106] This can exist because alleles are physically close together (i.e. since they tend to be co-inherited or for reasons of population origin in subsections of an overall population) and, therefore, demonstrate a statistical association within the overall population.[100-106] Linkage disequilibrium is influenced by many factors, including selection, the rate of genetic recombination, mutation rate, genetic drift, the system of mating, population structure, and genetic linkage.[100-106]

The results from **chapter 7** and **chapter 8** may be threatened by linkage disequilibrium since we cannot exclude the possibility that the observed associations are truly due to rs2304681 or that this genetic variant in *KHK* is in linkage disequilibrium with another gene. This is unlikely for the observed association with fructosuria since this trait is highly specific for KHK but cannot be ruled out for the observed associations with cardiometabolic outcomes. However, animal studies have shown that whole-body knockout of KHK caused both fructosuria and protection from metabolic defects.[107] In addition, we performed a Bayesian co-localization analysis in **chapter 7** using genome-wide association data for both urinary fructose (exposure) and CRC risk (outcome), to evaluate the potential shared, local genetic architecture between KHK and CRC.[108,109] Unfortunately, findings of this co-localization analysis were inconclusive. This likely is because co-localization analysis requires the presence of a genetic variant that is strongly associated with each trait in the given genetic region to conclude that there is co-localization.[110] In case of associations at a relatively weak level of statistical significance (like is the case in **chapter 7**), then the co-localization method will typically conclude that there is no causal variant for the outcome rather than evidence for the hypothesis of distinct causal variants or a shared causal variant (i.e. co-localization).[110] Such finding does not provide strong evidence either in favor of or against the validity of the MR assumptions.[110] Therefore, we cannot exclude the possibility that the association between rs2304681 and non-communicable disease risk is explained by another causal variant in a neighboring gene that is in strong linkage disequilibrium (**chapter 7**), which deserves further study.

*Population stratification*

Population stratification refers to the presence of a systematic difference in allele frequencies among the different subpopulations from a population.[111,112] The co-existence of different disease rates and allele frequencies within subpopulations lead to spurious associations between the two at the population level.[111,112] This form of confounding is a well-known issue in genetic association studies with common variants and causes an inflation of the type I error rate (i.e. false positive) and impedes statistical power.[85]

In **chapter 7** and **chapter 8** we used summary-statistics to study the associations between a genetic variant in *KHK* and cardiometabolic traits, and some[113,114], but not all[115-117], of the summary-statistics were accounted for population stratification. Of interest, Johnston and colleagues studied the prevalence and cardiometabolic associations of the Val49Ile variant among UK Biobank participants.[88] The UK Biobank is comprised of mixed ethnicities, albeit mostly white, and the Val49Ile variant is more common in white versus non-white participants.[88] Johnston and colleagues were unable to adjust for ethnicity directly (due to small sample sizes) and instead repeated the analyses exclusively among white participants only, which attenuated the association for the hypertension trait towards the null.[88] These findings further emphasize the need for future studies to properly account for population stratification in the associations between *KHK* and cardiometabolic traits.

*Functionality of the genetic variant*

Our data in **chapter 7** imply functionality of the Val49Ile variant or a highly correlated variant, since fructosuria reflects impaired KHK function. Furthermore, we additionally performed multiple *in silico* analyses to predict the functionality of rs2304681, which yielded inconclusive results (e.g. SIFT score: 0.08, where ≤0.05 is predicted to be deleterious[118]; data not shown in thesis). Therefore, a more direct functional measure like urinary fructose may be superior. Also, although measurement of urinary fructose is costly, this functional *ex vivo* measure allows easier translation compared to *in vitro* and/or animal data.

*Statistical power*

The effect size of a genetic variant is inversely related to the allele frequency and, hence, the effect sizes of common genetic variants are generally small.[119] Therefore, large study populations are required to ensure sufficient statistical power to detect a significant effect.

Although the Maastricht Study was sufficiently powered to demonstrate an association between the genetic variant in *KHK* and urinary fructose (gene-exposure data; **chapter 7**), it is probably underpowered for gene-outcome data. Therefore, we combined outcome-data from different (ultra) large-scale cohorts in order to obtain a sample size sufficiently large to detect a significant association between the common variant in *KHK* and non-communicable traits in **chapter 7** and **chapter 8**. Notably, since common gene variants generally have small effect sizes[119], this probably explains the small risks that were observed in **chapter 7** and **chapter 8**. In this context, the direction of the associations, i.e. all protective, is more relevant than the effect sizes. Based on the data from **chapter 8**, it is expected that pharmacological inhibition of KHK, which had a clinically relevant effect on IHL content[120], will also have beneficial effects on other cardiometabolic outcomes. Since fructosuria was ~2.000 times higher after pharmacological KHK inhibition (~4.5 g/day[121]) compared to the small effect of rs2304681 on fructosuria (~2.2 mg/day per risk allele) it, therefore, is anticipated that the beneficial cardiometabolic effects of pharmacological KHK inhibition will also be substantially greater.

## Overview fructose and intrahepatic lipid accumulation – What is the evidence?

In summary, the different research methodologies used in this thesis each have its strengths and caveats. Each individual study by itself does not allow casual inference but combining the results of all studies allows to do so. This concept is based on the 'triangulation' approach, i.e. the practice of strengthening causal inferences by integrating results from several different research methodologies that have different and unrelated key sources of potential bias.[122] When we apply the 'triangulation' approach to the data provided in this thesis and previously by our research group, they together strongly suggest that fructose has a causal, deleterious role in the pathogenesis of IHL accumulation (**Table 9.1**). In addition, we conclude that each study provides unique information. First, we learned from the FRUITLESS study that fructose – more than glucose – augments IHL accumulation.[39] Second, results from our observational cohort study shows that the source of fructose matters (**chapter 6**).

**Table 9.1. Overview of the current evidence of fructose and intrahepatic lipid content provided by our research group.**

| Level of evidence in the pyramid of causation (Davies, *BMJ* 2018) | Study design | Outcome | Major strengths | Major limitations |
|---|---|---|---|---|
| Higher | *Randomized controlled trial:* Six-week fructose restriction (The FRUITLESS study) | *Lower* IHL content | • Causality | • Short study duration<br>• Potential statistical power issue<br>• Small groups, resulting in unbalanced randomization<br>• External validity |
| Higher | *Genetic epidemiology:* Genetically proxied reduced fructose metabolism | *Lower* IHL content | • Strongly suggestive of causality | • Pleiotropy<br>• Population stratification<br>• Linkage disequilibrium<br>• Statistical power and effect size |
| Lower | *Observational cohort study:* Lower fructose intake at the population level | *Lower* IHL content | • (Clinical) effect size<br>• Health implications | • Information bias<br>• Confounding<br>• Selection bias<br>• External validity |

## Conclusions and future research

The impact of the findings in this thesis is elaborately discussed in the next impact paragraph (**chapter 10**). The findings presented in this thesis strongly suggest that fructose plays a causal role in the pathogenesis of IHL accumulation (and likely T2DM, hypertension, myocardial infarction, and CRC). However, we were unable to identify the underlying mechanisms by which fructose causes IHL accumulation (and could not demonstrate a role for GKRP or ChREBP).

Results of this thesis pave the way for future research. First, future studies should identify the key molecular mechanisms by which fructose (more specifically F1P) participates as a signalling molecule in the pathogenesis of IHL accumulation. Second, future studies should investigate the mechanism by which different fructose sources cause IHL accumulation in humans, and the contribution of intestinal and hepatic fructose metabolism herein.

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# Chapter 10

Impact paragraph

# Impact paragraph

Non-alcoholic fatty liver disease (NAFLD) is a major health burden worldwide (with a prevalence of 25% in the adult population).[1] NAFLD is a risk factor of other non-communicable diseases including type 2 diabetes mellitus (T2DM), chronic kidney disease, cardiovascular disease, and cancer.[2-4] Intrahepatic lipid (IHL) accumulation (the first stage of NAFLD) is, among others, driven by the conversion of simple sugars into fatty acids via hepatic *de novo* lipogenesis (DNL).[5-7] However, which simple sugar – fructose or glucose – plays the greater role in the augmentation of DNL has been the subject of much debate.[8] Therefore, the overall aim of this thesis was to determine the role of fructose in the pathophysiology of NAFLD, and the possible underlying mechanisms. This chapter describes how the findings of this thesis may impact scientific research, society, policy, and clinical practice.

## Implications for the prevention of fructose-induced non-alcoholic fatty liver disease at the population level – Societal measures

IHL accumulation is an asymptomatic disease with low awareness among patients and clinicians, despite the increasing prevalence and the associated morbidity and mortality. An understanding of disease etiology is essential to adequately treat NAFLD patients. This thesis has gained (additional) insight into the causal relationship between fructose and NAFLD and provides evidence that fructose causes IHL accumulation in humans. Furthermore, findings in this thesis imply that fructose from fruit juice and sugar-sweetened beverages (SSB) may be more prone to cause IHL accumulation than fructose from fruit. Therefore, implementation of societal measures to reduce fructose intake at the population level (in particular from fruit juice and SSB) are an important goal for the foreseeable future.

National dietary recommendations should be updated, especially since various nutritional recommendations have currently not established a place for fruit juice.[1] We showed in this thesis that fruit, fruit juice and SSB possibly have different effects on liver health and should be considered in the establishment of future nutritional recommendations.[9] Specifically, it should be advised to avoid fruit juice and SSB, rather than to completely refrain from fructose since other fructose-containing foods like fruit and vegetables contain essential nutrients (e.g. vitamin C and fibers). In addition, fruit juice is still perceived as healthy by both adults and children[10], which demonstrates the need for education to improve health literacy with a view to the adoption of a healthy diet.

Nonetheless, long-term compliance to dietary guidelines is challenging[11-14], and, thus, additional societal measures are warranted to reduce the intake of fructose at the

population level. We should redesign our living environment by disincentivizing unhealthy food products and nudging consumers towards healthy food choices (e.g. warning labels, in-store promotions of healthier beverages, fruit, and vegetables, price increases on SSB [and possibly fruit juices], and increasing the availability of low-calorie beverages[15,16]), which can to a large degree be accomplished by legislation. For example, the implementation of an excise tax on SSB has a beneficial, reducing effect on fructose intake[17,18] and, therefore, has been advocated by the World Health Organization.[19] Notably, fruit juice (without added sugar) is currently exempted from all these levies.[17,18] Furthermore, the excise tax on SSB incentivizes manufacturers of SSB to reduce the sugar content in their products.[18] Although together these changes are modest at the individual level, they can have substantial health effects at the population level (=prevention paradox).[20]

## Implications for the treatment of non-alcoholic fatty liver disease in patient groups – Pharmacological measures

Findings presented in this thesis show that reduced fructose metabolism protects from IHL accumulation (and T2DM, hypertension, myocardial infarction, and colorectal cancer). These findings provide therapeutic opportunities, namely blocking ketohexokinase (KHK) activity. Indeed, previous studies have shown that the blocking of KHK-C ameliorated fructose-induced IHL accumulation in rodents.[21,22] In addition, pharmaceutical companies have recently initiated programs aimed at developing novel and specific KHK inhibitors. For example, a potent reversible KHK inhibitor (i.e. PF-06835919; tested in a Phase 2 clinical trial), was well tolerated, resulted in pronounced fructosuria (~4.5 g/day), and reduced IHL by ~19% at the background of their normal diet in adults with NAFLD.[23] Of interest, the elevated urinary fructose levels due to pharmacological KHK inhibition appear clinically asymptomatic and benign (similar to individuals with essential fructosuria who have a loss of *KHK* [EC 2.7.1.3; OMIM #229800]).

Moreover, treatment with a pharmacological KHK inhibitor is of particular interest for patients with hereditary fructose intolerance (HFI). Patients with HFI cannot metabolize fructose due to a genetic defect (and thus accumulate hepatocellular fructose 1-phosphate [F1P]). Dietary treatment with complete fructose, sorbitol and sucrose restriction has been effective in preventing acute HFI manifestations.[24] However recent studies have shown that, despite this diet, HFI patients still suffer from hepatic steatosis[25,26], which may even evolve in non-alcoholic steatohepatitis and fibrosis.[27] These studies also identified hepatocellular F1P (or concomitant hepatocellular ATP and phosphate depletion) as culprit in the pathogenesis of HFI, which may be due to the trace amounts of fructose in the diet and/or endogenous fructose production.[22,28] This

illustrates that dietary treatment may be insufficient to prevent long-term chronic liver manifestations and that novel therapeutic approaches to HFI are an emerging need. It is expected that pharmacological KHK inhibitors – by preventing F1P accumulation – may be a complementary therapy to ameliorate clinical manifestations in HFI patients.

## Implications for future scientific studies

Results of this thesis provide starting points for future research. First, future studies should identify the key molecular mechanisms by which fructose participates as signalling molecule in the pathogenesis of IHL accumulation, and the role of F1P herein. Second, future studies should investigate the effects of different fructose sources in relation to IHL accumulation in humans, and the role of intestinal and hepatic fructose metabolism herein.

## Conclusions

Taken together, based on this thesis we can conclude that it is FRUITFUL to combat the current fructose epidemic, as it will result in a lower risk of IHL accumulation, T2DM, hypertension, myocardial infarction, and colorectal cancer. The findings of this thesis may have implications for several stakeholders, such as the general population, certain patient groups (e.g. HFI), health care professionals (including dieticians and clinicians), the pharmaceutical industry, insurance companies, policy makers, and the food industry, as they support measures to reduce fructose intake at the population level (by societal efforts such as updated dietary guidelines and an excise tax on SSB) and to reduce/impair fructose metabolism in certain patient groups (by pharmacological KHK inhibitors), in order to combat the current fructose-induced NAFLD epidemic and its sequelae.

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# Addendum

# Nederlandse samenvatting

# Bestrijden van de Fructose Epidemie

*Vruchtbaar of Vruchteloos?*

## Introductie

Onderzoek heeft aangetoond dat de inname van toegevoegde simpele suikers (glucose en fructose) bijdragen aan het ontstaan van niet-overdraagbare ziekten zoals overgewicht, diabetes type 2 en niet-alcoholische leververvetting. Niet-alcoholische leververvetting komt voor bij ~25% van de volwassenen. De aandoening bestaat uit steatose (vervetting), inflammatie (ontsteking) en/of fibrosering (verlittekening) van de lever, in afwezigheid van overmatig alcoholgebruik.
Er zijn vier verschillende mechanismen die leiden tot niet-alcoholische leververvetting:

- Verhoogde flux van vrije vetzuren richting de lever: een overmaat aan vet in de bloedbaan (afkomstig van voeding of vetcellen) kan in de lever opgeslagen worden.
- Verhoogde *de novo* lipogenese: een overmaat aan simpele suikers (glucose en fructose) uit de bloedbaan kan in de lever omgezet en opgeslagen worden als vet.
- Verlaagde beta-oxidatie: het opgeslagen vet in de lever kan onvoldoende verbrand worden als energiebron.
- Verlaagde secretie van VLDL-partikels: het opgeslagen vet in de lever kan onvoldoende getransporteerd worden naar de bloedbaan om elders in het lichaam gebruikt te worden.

Er bestaat echter nog discussie over welke simpele suiker – glucose of fructose – méér bijdraagt aan het ontstaan van leververvetting. Tevens is het exacte mechanisme dat ten grondslag ligt aan het ontstaan van leververvetting nog onbekend.
Het doel van dit proefschrift was om te onderzoeken of fructose oorzakelijk verbonden is met niet-alcoholische leververvetting en welk mechanisme hier ten grondslag aanligt. Om deze (oorzakelijke) verbanden te onderzoeken en het onderliggende mechanisme in kaart te brengen hebben wij gebruik gemaakt van verschillende onderzoeks-methoden waaronder dieronderzoek, experimenten in mensen, observationeel onderzoek en Mendeliaanse randomisatie studies.

# Belangrijkste bevindingen

## Deel I: Achtergrond – overzicht van het fructose metabolisme en een nieuwe methode voor het kwantificeren van fructose concentraties

In **hoofdstuk 2** van dit proefschrift is de huidige epidemie van fructose inname in relatie tot niet-alcoholische leververvetting beschreven. Ook is hier aandacht besteed aan patiënten met hereditaire fructose intolerantie (HFI; een stofwisselingsziekte gekenmerkt door een verminderde afbraak van fructose 1-fosfaat), hetzij als model kan worden gebruikt om zo het fructose metabolisme beter te bestuderen en begrijpen.
Vervolgens beschrijven wij in **hoofdstuk 3** de ontwikkeling en validatie van een UPLC-MS/MS methode voor het kwantificeren van fructose in urine en serum. Deze methode was cruciaal voor de uitvoering van mijn vervolgstudies in deel II en deel III van het proefschrift.

## Deel II: Fructose als een signaal molecule – interactie tussen fructose en glucose metabolisme: van muis tot mens

In het tweede deel van dit proefschrift beoogde wij te onderzoeken hoe fructose 1-fosfaat een rol speelt bij het ontstaan van leververvetting in dier en mens. In **hoofdstuk 4** hebben wij gebruik gemaakt van een muismodel dat HFI nabootst en wordt gekenmerkt door de accumulatie van fructose 1-fosfaat en levervet. In deze muisexperimenten hebben wij de rol van glucokinase regulatory protein (GKRP) en carbohydrate-responsive element-binding protein (ChREBP; een lipogene transcriptie factor) in het ontstaan van leververvetting onderzocht. Wij konden echter niet aantonen dat GKRP en ChREBP een (actieve) rol spelen bij vetstapeling in HFI.
Vervolgens hebben wij in **hoofdstuk 5** de interactie tussen fructose en glucose in mensen onderzocht. Gezonde deelnemers ontvingen 75 g orale glucosetolerantie testen (OGTTs) met toevoeging van verschillende fructose concentraties en een OGTT zonder toevoeging van fructose als referentie. Echter vonden wij geen acute effecten van oraal fructose op de plasma glucose excursie.

## Deel III: Fructose inname en niet-overdraagbare ziekten op populatieniveau

In **hoofdstuk 6** hebben wij een observationeel onderzoek uitgevoerd om de relatie tussen fructose uit verschillende voedingsbronnen en levervetting te bestuderen. Wij vonden dat fructose uit fruitsappen en frisdranken, maar niet uit fruit, geassocieerd is met méér leververvetting in een grote populatie cohort (de Maastricht Studie). De tegengestelde richting van deze relaties kan verklaard worden door de 'voedingsmatrix'

en/of leefstijl (mensen die meer fruit consumeren hebben over het algemeen een gezondere leefstijl, en vice versa mensen die meer frisdrank consumeren hebben over het algemeen een ongezondere leefstijl). De resultaten van deze observationele studie hebben belangrijke implicaties voor de publieke gezondheid en ondersteunen huidige maatregelen om de inname van fructose op populatieniveau te reduceren (bijvoorbeeld met behulp van een Btw-verhoging op suikerhoudende dranken).
Hoewel observationeel onderzoek inzicht geeft in de klinische relevantie, kan er echter geen uitspraak worden gedaan over oorzakelijke verbanden. Om te onderzoeken of er een oorzakelijk verband bestaat tussen fructose en leververvetting, hebben wij in **hoofdstuk 7** en **hoofdstuk 8** van dit proefschrift de Mendeliaanse randomisatie onderzoekstechniek toegepast. Mendeliaanse randomisatie is gebaseerd op het concept dat er binnen een populatie een grote overeenkomst is in het genetisch profiel van mensen, maar ook een klein deel dat varieert. Als gevolg van deze variatie hebben sommige mensen genen die leiden tot een verminderd functionerend fructose metabolisme terwijl andere mensen genen hebben die leiden tot een normaal functionerend fructose metabolisme. Door deze genetische variatie te bestuderen in relatie tot ziekte is het mogelijk om te onderzoeken of het hebben van een verminderd fructose metabolisme leidt tot het ontwikkelen bepaalde aandoeningen (=oorzakelijk). De resultaten van de analyses uitgezet in **hoofdstuk 7** en **hoofdstuk 8** suggereren inderdaad dat fructose een oorzakelijk verband heeft met leververvetting (alsook diabetes type 2, hypertensie, hart- en vaatziekten en colorectale kanker).

# Conclusie

Op basis van dit proefschrift concluderen wij dat fructose niet-alcoholische leververvetting veroorzaakt. Bovendien concluderen wij dat fructose uit fruitsappen en frisdranken, maar niet uit fruit, potentieel meer leververvetting veroorzaakt. De bevindingen in dit proefschrift suggereren dat het reduceren van fructose inname (met name uit fruitsappen en frisdranken) op populatieniveau een effectieve aanpak is om de epidemie van niet-alcoholische leververvetting te bestrijden. Verder blijkt dat farmaceutische remming van het fructose metabolisme een effectieve behandeling voor leververvetting kan zijn in bepaalde patiëntengroepen.
Alhoewel dit proefschrift diverse (oorzakelijke) verbanden heeft onderzocht, dienen de resultaten nader bestudeerd te worden in vervolgonderzoek. Allereerst is toekomstig onderzoek nodig om het onderliggend mechanisme van fructose-geïnduceerde levervetting te ontrafelen, en de rol van fructose 1-fosfaat hierin. Ten tweede,

toekomstige studies zijn nodig om de effecten van verschillende fructose-bronnen op levervetting in mensen te bestuderen, en de rol van de 'voedingsmatrix' hierin.
Samenvattend, op basis van dit proefschrift concluderen wij dat het 'vruchtbaar' is om de epidemie van fructose inname te bestrijden, mogelijk door het reduceren van fructose uit fruitsappen en frisdraken (= 'vruchteloos'), maar niet van fructose uit fruit (= 'vruchtbaar').

# Dankwoord

# Dankwoord

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# Scientific output

# Scientific output

## List of publications

- **Buziau AM**, Schalkwijk CG, Stehouwer CDA, Tolan DR, Brouwers MCGJ. Recent advances in the pathogenesis of hereditary fructose intolerance: implications for its treatment and the understanding of fructose-induced non-alcoholic fatty liver disease*. Cell Mol Life Sci* 2020 May;77(9):1709-1719. doi: 10.1007/s00018-019-03348-2.
- **Buziau AM**, Scheijen JLJM*, Stehouwer CDA, Simons N, Brouwers MCGJ, Schalkwijk CG. Development and validation of a UPLC-MS/MS method to quantify fructose in serum and urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 2020 Oct 15;1155:122299. doi: 10.1016/j.jchromb.2020.122299. **Equal contribution*
- **Buziau AM**, Eussen SJPM, Kooi ME, van der Kallen CJH, van Dongen MCJM, Schaper NC, Henry RMA, Schram MT, Dagnelie PC, van Greevenbroek MMJ, Wesselius A, Bekers O, Meex SJR, Schalkwijk CG, Stehouwer CDA, Brouwers MCGJ. Fructose Intake From Fruit Juice and Sugar-Sweetened Beverages Is Associated With Higher Intrahepatic Lipid Content: The Maastricht Study. *Diabetes Care* 2022 May 1;45(5):1116-1123. doi: 10.2337/dc21-2123.
- **Buziau AM**, Law PJ, Blokland GAM, Schalkwijk CG, Scheijen JLJM, Simons PIHG, van der Kallen CJH, Eussen SJPM, Dagnelie PC, van Greevenbroek MMJ, Houlston RS, Wesselius A, Went M, Stehouwer CDA, Brouwers MCGJ. Genetically proxied ketohexokinase function and risk of colorectal cancer: a Mendelian randomization study. *Gut* 2023 Mar;72(3):604-606. doi: 10.1136/gutjnl-2021-326299.
- **Buziau AM**, Blokland GAM, Schalkwijk CG, Scheijen JLJM, Simons PIHG, Eussen SJPM, Dagnelie PC, van Greevenbroek MMJ, Wesselius A, Stehouwer CDA, Brouwers MCGJ. Comment on Lee et al. Relation of Change or Substitution of Low- and No-Calorie Sweetened Beverages With Cardiometabolic Outcomes: A Systematic Review and Meta-analysis of Prospective Cohort Studies. *Diabetes Care* 2022;45:1917-1930. doi: 10.2337/dc22-1930.
- **Buziau AM**, Scheijen JLJM, Stehouwer CDA, Schalkwijk CG, Brouwers MCGJ. Effects of fructose added to an oral glucose tolerance test on plasma glucose excursions in healthy adults. *Metabol Open*. 2023 May 12;18:100245. doi: 10.1016/j.metop.2023.100245.

## Additional manuscripts (in preparation)

- **Buziau AM**, et al. Hepatic glucokinase regulatory protein and carbohydrate response element binding protein do not explain the fatty liver phenotype in aldolase B deficiency (manuscript in preparation).
- **Buziau AM**, et al. Mannose treatment does not protect from intrahepatic lipid accumulation in aldolase B deficiency (manuscript in preparation).
- **Buziau AM**, et al. Endogenous fructose production is not pathologic in the pathogenesis of intrahepatic lipid accumulation in aldolase B deficiency (manuscript in preparation).
- De Groot D, **Buziau AM**, et al. Fructose from sugar-sweetened beverages is associated with higher blood pressure: The Maastricht Study (manuscript in preparation).
- Huadong C, **Buziau AM**, et al. Fructose intake from sugar-sweetened beverages is associated with a greater risk of hyperandrogenism: UK Biobank cohort study (manuscript submitted).

## Oral presentations

- Annual Dutch Diabetes Research Meeting 2019 (Netherlands, Oosterbeek)
- Dutch Translational Metabolism Conference 2020 (online due to COVID-19)
- De Maastricht Studie publiekssymposium 2021 (online due to COVID-19)
- 57th European Association for the Study of Diabetes Annual Meeting 2021 (online due to COVID-19)
- Dutch Epidemiology Conference (WEON) 2021 (online due to COVID-19)
- EASD-NAFLD study group 2021 (online due to COVID-19)
- Annual Dutch Diabetes Research Meeting 2021-2022 (online due to COVID-1; nomination best abstract)
- Dutch Translational Metabolism Conference 2022 (Netherlands, Wageningen)
- 3rd European Fatty Liver Conference 2022 (Netherlands, Maastricht)
- Wetenschapsdag Interne Geneeskunde 2022 (Netherlands, Maastricht; winner best publication price)
- 58th European Association for the Study of Diabetes Annual Meeting 2022 (Sweden, Stockholm)

## Poster presentations

- Cardiovascular Research Institute Maastricht day 2019 (Netherlands, Maastricht)
- Dutch Endocrine Meeting 2020 (Netherlands, Noordwijkerhout)
- 38th International Symposium on Diabetes and Nutrition 2021 (online due to COVID-19)

## Grants

- September 2021: Grant (€ 15.000,-) to explore the potential mechanisms by which aldolase B deficiency leads to intrahepatic lipid accumulation provided by United for Metabolic Diseases.
- October 2022: Grant (€ 15.000,-) to explore the potential mechanisms by which aldolase B deficiency leads to intrahepatic lipid accumulation provided by United for Metabolic Diseases.
- March 2023: Grant (€ 7.350,-) to fund 1 month of salary costs and the publication costs to publish null-findings of animal experiments provided by ZonMw.
- March 2023: Grant (€ 8.625,-) to fund 1 month of salary costs and the publication costs to publish null-findings of animal experiments provided by ZonMw.

## Societal impact

- June 2021, www.mumc.nl, '*Het is hoog tijd voor meer gezonde voeding in de schappen*'
- February 2022, www.mumc.nl, '*Vruchtsuiker uit fruitsappen net zo ongezond als die uit frisdranken?*'
- February 2022, www.diabetesfonds.nl, '*Vruchten-suiker uit fruitsap mogelijk net zo ongezond als uit frisdrank*'
- February 2022, www.reuma-arnhem.nl, '*Vruchtsuiker uit fruitsappen net zo ongezond als die uit frisdranken?*'
- February 2022, www.nieuwsvoordietisten.nl, '*Meer leververvetting bij hoge inname fructose uit frisdrank én vruchtensap*'
- February 2022, www.zorgkrant.nl, '*Vruchtsuiker uit fruitsappen ongezond?*'
- March 2022, www.nu.nl, '*Suikertaks moet volgens wetenschappers ook gelden voor vruchtensap*'
- April 2022, Reformatorisch Dagblad, '*Fruitsapje bevat veel suiker*'
- May 2022, www.mumc.nl, '*Relatie tussen vruchtensuiker en darmkanker verder ontrafeld*'

- July 2022, www.nu.nl, '*Steeds meer bekend over frisdrank en het risico op darmkanker*'
- March 2023, www.hartstichting.nl, '*Onderzoek toont aan: vruchtensuiker kan hart- en vaatziekten veroorzaken*'
- March 2023, www.nieuwsvoordietisten.nl, '*Relatie tussen fructose en risico op diabetes type 2 en hart- en vaatziekten*'
- March 2023, www.limburger.nl, '*Vruchtensuikers in frisdrank verhogen kans op diabetes en hart- en vaatziekten, blijkt uit Maastrichts onderzoek*'
- July 2023, www.reuma-arnhem.nl, '*Onderzoek MUMC+ brengt leefstijlziekte leververvetting in beeld*'

Curriculum vitae

# Curriculum vitae

Amée Buziau werd geboren op 31 oktober 1990 in Haarlem en groeide op in Driebergen, Nederland. Amée is na het behalen van haar middelbareschooldiploma (De Breul, Zeist) Communicatie gaan studeren in Utrecht. Na het behalen van haar bachelor diploma is Amée Voeding- en Dieetkunde gaan studeren in Antwerpen, België (inclusief een buitenlandse stage in Auckland, Nieuw-Zeeland). Vervolgens heeft zij haar masterdiploma Voeding en Gezondheid aan Wageningen University & Research behaald (inclusief een buitenlandse stage in Brisbane, Australië).
Van augustus 2018 tot augustus 2023 deed Amée haar promotieonderzoek onder begeleiding van prof. dr. Martijn Brouwers, prof. dr. Coen Stehouwer en prof. dr. Casper Schalkwijk aan de afdeling Interne Geneeskunde, Universiteit Maastricht, binnen CARIM School for Cardiovascular Diseases (van Maastricht Universitair Medisch Centrum+). Gedurende haar promotietraject heeft Amée onderzoek gedaan naar de rol van vruchtensuiker in de pathogenese van leververvetting waarbij zij gebruik heeft gemaakt verschillende soorten onderzoeksmethoden (waaronder genetische epidemiologie, voedingsepidemiologie, humane experimenten en muisexperimenten). Daarnaast had zij verschillende onderwijstaken (tutor en assessor van essays), was zij begeleider van meerdere studenten (3 master studenten en 1 bachelor student) en vertegenwoordiger van de journal club en voortgangspresentaties op de afdeling.
Gedurende haar promotietraject heeft Amée de CARIM Postdoc Talent Fellowhip ontvangen om het eerste jaar van haar postdoc-onderzoek te financieren. Dit postdoc-onderzoek wordt uitgevoerd aan het Stamcelinstituut Leuven (België) bij de groep van prof. dr. Catherine Verfaillie.

Amée Buziau was born on October 31, 1990, in Haarlem and raised in Driebergen, the Netherlands. After graduating from secondary school (De Breul, Zeist), she enrolled the bachelor Communications in Utrecht. After graduation, she continued her studies and started the program Nutrition and Dietetics in Antwerp, Belgium (including an international placement in Auckland, New Zealand). Subsequently, she obtained her master's degree Nutrition and Health from Wageningen University & Research (including an international placement in Brisbane, Australia).
From August 2018 until August 2023, Amée conducted her PhD research under supervision of Prof. Martijn Brouwers, Prof. Coen Stehouwer, and Prof. Casper Schalkwijk at the department of Internal Medicine, Maastricht University, within CARIM School for Cardiovascular Diseases (Maastricht University Medical Center+). During her PhD-trajectory, Amée performed research regarding the role of fructose in the pathogenesis of intrahepatic lipid accumulation for which she employed multiple research methodologies (including genetic epidemiology, nutritional epidemiology,

experiments with humans, and mice experiments). In addition, she had several teaching roles (tutor and assessor of essays), supervised numerous student (3 master students and 1 bachelor student), and was representative of journal club and progress meetings at the department.
During her PhD, Amée acquired the CARIM Postdoctoral Talent Fellowship to fund the first year of her postdoctoral research. This postdoctoral research is performed at the Stem Cell Institute Leuven (Belgium) in the group of Prof. Catherine Verfaillie.