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Non-alcoholic fatty liver disease across the lifespan:
The effects of early-life nutrition on adult disease

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Non-alcoholic fatty liver disease across the lifespan:
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ABSTRACT

Non-alcoholic fatty liver disease across the lifespan:
The effects of early-life nutrition on adult disease

By Ahlia Sekkarie

The prevalence of non-alcoholic fatty liver disease (NAFLD) has been increasing in children, hinting that it may have early-life origins. Supporting this hypothesis, animal studies have shown that maternal diets are associated with hepatic fat in offspring. The association between prenatal and early childhood nutrition and later NAFLD in humans, as well as the natural history of NAFLD throughout childhood, have not been well characterized.

In this dissertation, I examine the association between early-life nutrition and adult NAFLD using secondary data from two longitudinal birth cohorts. In aim 1, I examine the effect of improved protein-energy nutrition from conception to two-years on NAFLD prevalence in mid-adulthood in the Guatemalan Institute of Nutrition of Central America and Panama (INCAP) cohort. In aim 2, I utilize the UK-based Avon Longitudinal Study of Parents and Children (ALSPAC) cohort to examine the effect of (a) maternal free sugar intake and nutritional status and (b) early childhood high free sugar and sugary beverage intake on hepatic steatosis at 24 years. In aim 3, I use the ALSPAC cohort to determine whether hepatic enzymes throughout childhood and adolescence are associated with hepatic steatosis in young adulthood.

There was a high prevalence of NAFLD in mid-adulthood in the INCAP cohort. Early-life protein-energy supplementation was not significantly associated with NAFLD. In the ALSPAC cohort, maternal diabetes, overweight, obesity, and excess gestational weight gain, were positively associated with hepatic steatosis in adult offspring, although the relationship was mediated by body mass index at 24 years. Free sugar and sugary beverage intake at three years were positively but weakly associated with adult hepatic steatosis. Higher alanine aminotransferase concentration in adolescence, but not prior to puberty, was positively associated with hepatic steatosis at 24 years.

While I did not find that early-life diet and nutrition had strong independent effects on adult NAFLD, the work in this dissertation makes an important contribution to the limited body of research on the dietary and nutritional predictors of NAFLD. There is a need for further longitudinal studies on the causes of NAFLD throughout the life course.

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Chapter 1: Introduction

The global prevalence of non-alcoholic fatty liver disease (NAFLD), which is marked by increased hepatic steatosis, is currently estimated to be 24%.[1] Nationally representative U.S. data shows a significant increase in NAFLD in children, from 4% in the late 1980s to 11% in 2010.[2] The prevalence among obese children and adolescents is even higher, at about 34%.[3] The etiology of NAFLD and subsequent natural history in children is not fully understood, although risk factors such as genetics, Hispanic ethnicity, male sex, older age, and high-sugar diets are all thought to play a role.[4] Additionally, there is growing evidence that exposure to an unfavorable environment in the womb and in early childhood (collectively referred to as "early life" in this dissertation) may lead to hepatic steatosis through direct programming effects on the liver or indirectly through adiposity and metabolic dysfunction.[5]

Both undernutrition and overnutrition in early life can lead to hepatic fat storage[6]. In the context of undernutrition, maternal restriction of calories and nutritional components such as protein have been associated with increased hepatic fat in offspring in animal models.[7] The only human studies have used birthweight and child growth as markers for nutrition, and have found that low birthweight and catch-up growth are risk factors for NAFLD in adulthood.[8] In the context of overnutrition, infants born to mothers with obesity and insulin-resistance have been shown to have relatively more hepatic fat present at birth.[9] No human studies have specifically looked at maternal high-energy diets and NAFLD in offspring, although one study of 585 U.S. mothers and their children showed that a diet high in saturated fat and sugar intake during pregnancy is associated with an increased risk of obesity in offspring.[10]

Additionally, there is a sparsity of information on the natural history of NAFLD, especially prior to its diagnosis; therefore, the ideal age for screening to identify those at greatest risk of NAFLD is not known.[4]

In the context of these knowledge gaps, the overall goal of this dissertation was to further understand the developmental early-life origins of non-alcoholic fatty liver disease from an under- and overnutrition perspective. To meet these goals, I aimed to answer the following questions:

Aim 1: In an undernourished population, is exposure to a protein-energy supplement in early development associated with lower prevalence of NAFLD in adulthood?

Aim 2A: Are maternal diet and nutritional status factors (as indicated by pre-pregnancy weight, pregnancy free sugar intake, diabetes status, and gestational weight gain) associated with offspring hepatic steatosis in young adulthood?

Aim 2B: Is high intake of free sugars and sugary beverages in early childhood associated with hepatic steatosis in young adulthood?

Aim 3: Do liver enzyme concentration trends from childhood to young adulthood differ among those with severe vs low hepatic steatosis at 24 years?

To meet these aims, this dissertation includes four original research studies using data from two longitudinal birth cohorts. A review of the relevant literature and knowledge gaps will be outlined in chapter two along with a summary of the aims and hypotheses of this dissertation. Chapter three describes the methodology used to meet the research aims of this dissertation. Each of the four original research studies will be presented in chapters four through seven. Finally, in chapter eight the overall conclusions, implications, and future directions of this research will be discussed.

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Chapter 2: Background

This chapter will provide a review of the literature that sets the background for this dissertation. First, I will present the definition, prevalence, outcomes, and risk factors for non-alcoholic fatty liver disease (NAFLD) that indicate why it is of significant clinical and public health concern throughout the life course. Next, I will cover some of the risk factors for NAFLD in detail including prenatal and early childhood diet and nutrition. The next section will review the literature on early identification of NAFLD and those at risk for developing it. Finally, I will conclude with a summary of the aims and hypotheses of this dissertation.

2.1 Definition and Epidemiology of NAFLD

Non-alcoholic fatty liver disease is a chronic liver disease defined by accumulation of fat in the liver in the absence of alcohol or other secondary causes of steatosis.[1] NAFLD is considered the hepatic manifestation of metabolic syndrome, and it has recently been suggested that NAFLD be renamed to metabolic-dysfunction associated fatty liver disease (MAFLD) because of its strong relationship to type II diabetes, dyslipidemia, and insulin resistance.[1,2] NAFLD is histologically categorized into nonalcoholic fatty liver (NAFL), defined as high hepatic steatosis (fat) without hepatocellular injury, and nonalcoholic steatohepatitis (NASH) which is defined as hepatic fat plus hepatocellular injury with or without fibrosis.[1] In a healthy person, almost no fat is stored in the liver, despite the fact that the liver is a major site of metabolism for dietary fat, cholesterol, triglycerides, and free fatty acids. NAFLD develops when the balance of fat entering and exiting the liver becomes dysregulated in the setting of insulin resistance. A cutoff of 5% hepatic fat is a traditionally accepted boundary between normal and abnormal hepatic fat, although the percentage varies by measurement method.[3-5]

The prevalence of NAFLD has been increasing globally in both adults and children. The global prevalence of NAFLD is currently estimated to be 24%.[6] As obesity rates increase throughout the world,

especially in places that were previously considered malnourished, the prevalence of NAFLD is expected to further increase. In adolescents 12 to 19 years of age, using nationally representative U.S. National Health and Nutrition Examination Survey (NHANES) data, there has been a significant increase in NAFLD prevalence (estimated using alanine aminotransferase (ALT) levels and overweight status as surrogate markers of NAFLD) from 4% in the late 1980s to 11% in 2010.[7] The prevalence of obesity among children and adolescents from 1 to 19 years of age is even higher at about 34%.[8]

NAFLD can lead to life-long health problems. It is the leading cause of liver disease for both adults and children in the U.S.[7,9] It is strongly associated with obesity and metabolic syndrome.[10-16] NAFLD increases risk of type II diabetes, cardiovascular disease (CVD) and the metabolic syndrome.[17-22] For example, a person with NAFLD and fibrosis has 2.5 to 3.5 times the risk of cardiovascular disease death and increased risk of type II diabetes compared to a similarly overweight person without NAFLD.[23] An estimated one-third of adults with NASH, the more severe form of NAFLD, will go on to develop cirrhosis and liver cancer and it is the most rapidly increasing reason for liver transplants in adults.[24,25] NAFLD in children is of particular concern as they may have a more progressive form of the disease compared to adults.[26] Liver transplants are also increasing among younger adults partly because of youth-onset NASH.[27]

A mix of genetic and environmental factors, that can begin even prior to birth, contribute to the development of NAFLD[28]. Known risk factors for NAFLD include genetics, Hispanic ethnicity, advanced age, high-sugar diets, and obesity[28]. Furthermore, adiposity amplifies the risk of NAFLD in those with genes with an adipogenic effect such as PNPLA3. The PNPLA3 allele is most common in Hispanics (frequency =0.49), the group most susceptible to NAFLD.[29]

2.2 Developmental Origins of NAFLD

The development of NAFLD may begin as early as in utero through exposure to an unfavorable enviornment.[30] The “developmental origins of health and disease” (DOHAD) paradigm links environmental factors, including nutrition, during the early-life stage (both the fetal and early childhood

periods) to the risk of non-communicable diseases such as obesity, diabetes, and metabolic syndrome.[31] This theory was first proposed by Barker et al. in England; that infants with low birthweights had increased risk for cardiovascular disease and type II diabetes in adulthood.[32] The clinical discovery of NAFLD in children and the presence of steatosis at birth in some newborns suggests that its origins also may lie in exposures earlier in life.[33-36] The role of the postnatal events, including the "second hit" of poor diet, on hepatic steatosis is also yet to be elucidated.[37]

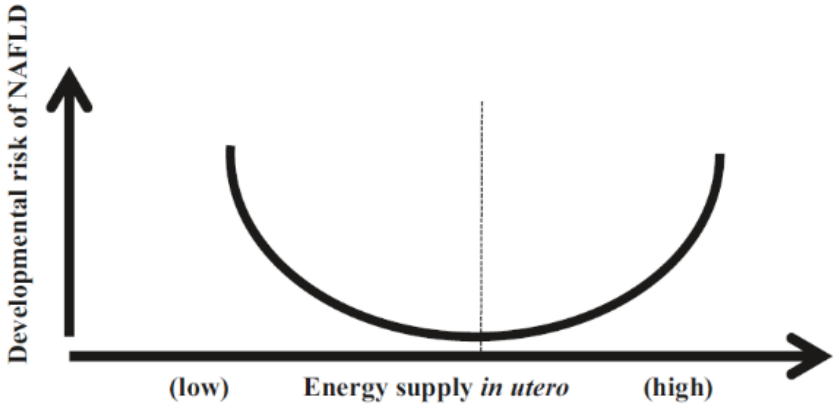

Figure 2-1. *Conceptual model of the impact of under- and overnutrition in utero on the risk of NAFLD. Itoh & Kanayama, 2018.*

Both undernutrition and overnutrition in utero and beyond can lead to hepatic fat storage as shown in the conceptual framework in ***Error! Reference source not found.***.[38] Fetuses may be " programmed" through an under- or oversupply of nutrients at sensitive periods of development leading to long-term effects on the structure or function of specific organs in offspring predisposing them to metabolic and cardiovascular diseases. The development of the fetal liver begins at four weeks gestation and is susceptible to fundamental changes to its metabolic pathways through epigenetic changes and mitochondrial dysfunction caused by inflammation resulting from environmental insults.[37]

2.2.1 Undernutrition

Undernutrition during early life increases the risk of developing features of the metabolic syndrome later in life.[39-47] There is also evidence in support of the impact of undernutrition specifically on NAFLD. Protein malnutrition was one of the first described causes of fatty liver in children in the 1960s.[48] In animal models maternal restriction of total calories, and protein specifically, have been

associated with increased hepatic fat in offspring. In one rat study maternal restriction of protein during pregnancy and lactation was associated with hepatic steatosis in adult offspring, but not with general adiposity.[44] The rats were assigned to a normal-protein diet (19%) or a low-protein diet (5%). At weaning, offspring were fed a standard chow or a high-fat diet forming four groups. The high-fat diet in the offspring intensified the effects of perinatal protein restriction on systolic blood pressure and hepatocyte number (P=0.05; two-way ANOVA). In sheep, which have more similar organogenesis of the liver to humans, a high-fat diet in offspring only led to increased hepatic fat when it was preceded by a period of fetal nutrient restriction to ~50% of total energy requirements.[43] In humans, several studies have found that infants born small for gestational age or with low birth weight are predisposed to NAFLD later in life, although another study showed that the strongest risk factor for NAFLD in adulthood is actually the catch-up growth in the first three months of life rather than small birth weight.[39,41] No human studies have directly looked at the association of early-life diet (specifically protein) in undernourished populations with offspring NAFLD.

2.2.2 Overnutrition

Maternal overnutrition also increases the risk of offspring developing NAFLD.[34,35,47,49-64] Studies in various animal models including rodents, sheep, and nonhuman primates have reported that maternal high-fat diets, Western-style diets, and maternal obesity and diabetes during pregnancy, predispose the developing offspring to NASH and insulin resistance. For example, Thorn et al, conducted a study in non-human primates that were fed a high-fat or control diet during pregnancy.[65] Only the offspring of mothers that became insulin resistant had increased liver triglyceride content and upregulated pathways for de novo lipogenesis, regardless of the offspring's diet. These offspring had the NAFLD phenotype despite the absence of postnatal obesity, insulin resistance, or inflammation indicating that the mechanism driving excess hepatic fat storage may be different from that underlying adipose tissue expansion. Many studies in rodent models have also described that high-fat diets can program hepatic steatosis or even NASH in

the offspring.[49,57,60,61,63,64] Fewer studies have looked at the association between high-sugar diets, which have been shown to be strongly associated with increased dyslipidemia, insulin resistance, and obesity, and the risk of offspring NAFLD.[52,53,66] Two such studies gave rats chocolate and sucrose-sweetened soft drinks to more realistically mimic human diets. Kjaergaard et al. created a dietary situation comparable to humans by giving pregnant rats ad libitum access and the possibility to choose between regular chow and the high-sugar chocolate and soft drinks. The high-fat and sugar diet constituted about 20% of their total dietary caloric intake.[52] The high-fat/high-sugar diet did not induce obesity or diabetes in dams during gestation. However, at 29 weeks of age their offspring had hepatic steatosis and altered lipid gene expression profiles.

The evidence from animal studies on the impact of maternal nutrition on offspring NAFLD is compelling, however, however these models cannot be considered predictive for what occurs in humans. Animal models have several limitations that may lead to stronger or different effects than what may happen in humans including 1) variations in dose levels, 2) highly controlled settings and 3) different pathophysiology. First, animal studies may provide very high or controlled nutrient levels in order to trigger a biologic response that do not be represent actual nutrient consumption in humans. Additionally, animal studies can be highly controlled which is useful for understanding the mechanistic process through which disease may occur and for generating hypothesis – but ultimately, these hypothesis must be tested in humans where real-life settings are not controlled and can include many interacting factors that lead to different outcomes. Finally, the pathophysiology of a disease may differ in humans compared to animal models. For example, liver development begins early in gestation in humans but , but in rodents it occurs later in gestation.

While human studies of NAFLD have been difficult to investigate due to the invasive nature of a definitive diagnosis with liver biopsy as well as the difficulty of following individuals over long periods of time, several studies have contributed to the evidence that gestational overnutrition is associated with fetal and infant hepatic steatosis (**Table *5-5***).[33-35,67] One retrospective autopsy study found that 78.8% of

stillborn fetuses of diabetic mothers had hepatic steatosis compared to 16.6% of offspring of non-diabetic mothers ($p<0.0001$) regardless of maternal body mass index (BMI).[33] Three studies have examined the associations of maternal pre-pregnancy BMI and gestational diabetes (GDM) with infant offspring intrahepatocellular lipid (IHCL) content using MRI.[34,35,68] Modi et al, found an increase of 8.6% (95% CI 1.1, 16.8) in IHCL 1-2 weeks after birth per maternal BMI unit increase (n=105).[34] Brumbaugh, et al. compared infants born to women with normal weight (n=13) and women with both obesity and GDM (n=12).[35] Infants born to women with obesity and GDM had a mean IHCL that was 68% higher compared with infants born to mothers with normal weight. In both studies, the infant's IHCL, but not subcutaneous adiposity, correlated with maternal pre-pregnancy BMI, indicating that maternal nutrition factors may have had direct "programming" effects on the fetal liver. A third study based in the UK of 86 infants found no association between maternal GDM and infant IHCL.[68] However, in this study the GDM mothers had good glycemic control (55% received metformin and/or insulin treatment resulting in a mean(SD) 5.3% (0.3) HbA1c) and little obesity (median BMI=24.2, IQR (21.7, 30.3) whereas glycemic status was not known in the other studies.

Four studies have looked at the prospective associations of maternal BMI, diabetes, and weight gain on offspring NAFLD in children and adolescents.[69-73] In the Generation R Study, higher maternal BMI (but not excess gestational weight gain) was associated with hepatic steatosis in offspring at ten years, regardless of BMI status.[69] In the RAINE cohort, which included 1170 adolescents of European descent in Australia, NAFLD was associated with maternal obesity and gestational weight gain but not with maternal diabetes, and associations were stronger in females.[71] In the EPOCH cohort based in the U.S., adolescent hepatic steatosis quantified by MRI was strongly associated with maternal pre-pregnancy obesity and was largely mediated by offspring adiposity at time of outcome.[72] Maternal diabetes was not significantly associated with later NAFLD. Finally, in the UK-based ALSPAC cohort, Patel et al, found a strong association between maternal diabetes and offspring hepatic steatosis at 17 years that was not mediated by child adiposity or BMI at 17 years.[73] One of the primary limitations in all of these studies is

that they may not have been powered to look at maternal diabetes as an outcome, and the types of diabetes were not looked at separately.

2.2.3 Biological mechanism

In a context of undernutrition, the liver is one of the organs most impacted by fetal growth restriction. Growth restricted offspring exhibit fewer but larger hepatic lobules and enzymatic alterations, which, along with a decreased pancreatic b-cell mass, can lead to insulin-resistance.[74,75]

In a context of overnutrition, the developmental programming of NAFLD may occur indirectly through the development of adiposity or by direct programming effects on the liver. The fetal liver begins

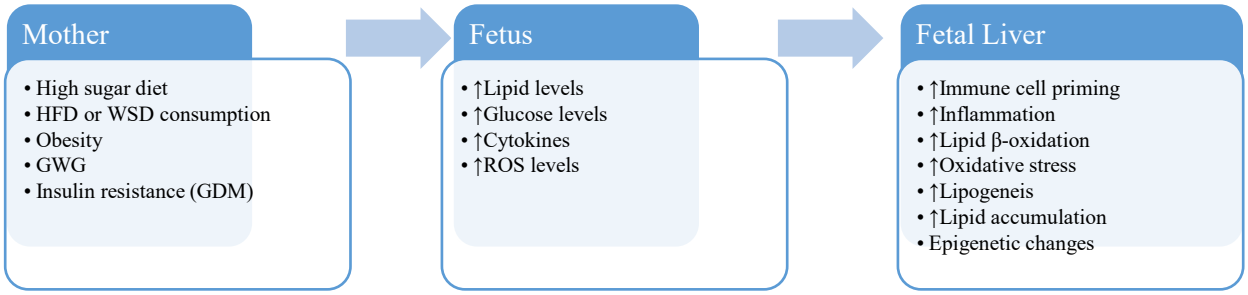

Figure 2-2. Priming for NAFLD during gestation modified from Wesolowski et al, 2018.

Abbreviations: HFD= high fat diet, WSD = western style diet, GWG, gestational weight gain, GDM = gestational diabetes mellitus, ROS=reactive oxygen species

development at four weeks of gestation and throughout most of its development is susceptible to its environment. An unfavorable environment can lead to fundamental changes to the liver's metabolic pathways which may persist into adult life and increase susceptibility to metabolic disease as summarized in ***Error! Reference source not found.***.[76] Since the fetal liver develops in a low-oxygen environment, it h as fewer mitochondria and very little gluconeogenesis, making it more susceptible to oxidative stress.[77] Maternal pregnancy diabetes, obesity, and high-fat/sugar diets are characterized by increased delivery of fuels such as glucose, free fatty acids, and amino acids to the developing fetus.[78] This could result in priming of hepatic macrophages and hepatocytes leading to greater adiposity and a more adverse cardiometabolic health profile, including NAFLD, later in life.[37] In the environment of maternal

overnutrition, the fetal liver develops under conditions of both excess nutrients and inflammation. Prior to the third trimester, the fetus does not have subcutaneous adipose tissue storage compartments or mature hepatic lipid oxidation pathways necessary to buffer against excess nutrient. Therefore, the fetal liver may be used as an ectopic site of excess fat deposition promoting metabolic and cellular stress and inflammation.[76] This also leads to mitochondrial dysfunction and epigenetic changes which result in whole-body insulin resistance and susceptibility to fatty liver throughout life. Only after approximately 28 weeks gestation, are there exponentially increasing rates of subcutaneous fat storage that continue through the third trimester. Therefore, neonatal overnutrition could prime hepatic lipid synthesis pathways both developmentally and biochemically that are associated with the onset and long-term risk for NAFLD.

2.3 High-sugar diets and sugary beverages

The majority of animal studies have primarily looked at the relationship between high-fat diets and NAFLD, but high-sugar diets are also of concern in humans because of their high prevalence and metabolic effects.[79] Added sugars include refined sugars and syrups that are added to foods or beverages during processing or preparation[80]. They do not include naturally occurring sugars such as those found in milk (lactose) and fruits (fructose). Free sugars also include sugars that are naturally present in honey, syrups, fruit juices and fruit juice concentrates. Sugar sweetened beverages (SSBs) include all beverages with added sugars, and sugary beverages (SBs), additionally include fruit juices, are the main source of sugar intake in the total daily energy intake of children.[81]

Added and free sugars, particularly the fructose component, are the primary sugars of public health concern because of their high prevalence in human diets and their metabolic effects[80]. The World Health Organization (WHO) dietary guidelines strongly recommend limiting free sugar intake to less than 10% of daily energy intake, and further suggest a further reduction to below 5%.[80] In the U.S. and the UK, all age groups have free sugar intakes that exceed the 10% recommended limit .[82,83] Free sugar percent of total energy in children 1 to 3 years of age is about 11% in both the UK and the US and

increases as they get older, peaking in adolescence.[83,84] From 2003 to 2012, the average percent of total daily calories from added sugars was approximately 15% for U.S. women of childbearing age.[82]

The WHO also considers SSBs a "probable contributor" to the obesity epidemic. Sugar sweetened beverages are of particular concern because of their high fructose content, satiety, and high levels of consumption. Liquids are less satiating causing more postprandial hunger, therefore leading to increased energy intake.[85] In the U.S., from 2009-2012 sugar sweetened beverages made up 39% of all added sugar intake in ages two and older.[86] In the UK, according to the national diet and nutrition survey years 2014-2016, children 1.5 to 3 years old consume an average of 32.6g of sugar a day, comprising 11.3% of their total energy intake, and sugary beverages contributed 21% of free sugar consumption.[83]

Due to their metabolic effects, high-sugar diets are considered one of the primary predictors of metabolic conditions, including NAFLD. Added sugar is usually in the form of sucrose, composed of glucose and fructose. A high intake of dietary fructose is associated with NAFLD, and it has been shown that children with NAFLD absorb and metabolize fructose more effectively than normal-weight children (obese children without NAFLD had an intermediate response).[87,88] Sugars, in particular fructose, are metabolized through the liver. The liver converts fructose into fat via de novo lipogenesis. Fructose metabolism skips the rate-limiting enzyme for glucose metabolism (phosphofructokinase) and is metabolized by fructokinase, which has no negative feedback system. This leads to an increased production of triglycerides which are loaded onto very low-density lipoproteins (VLDL).[89]

Epidemiologic studies have demonstrated that there is an association between high sugar intake and adiposity across the life-course, including prenatally and early childhood.[90-93] High sugar and sugary beverage intake is also associated with hepatic steatosis.[94] In a prospective study, higher sugar sweetened beverage intake at one year old was associated with higher odds of hepatic steatosis in mid-childhood independent of BMI at time of outcome (OR: 1.34, 95% CI: 0.97, 1.83).[95] In the same study, children with overweight or obesity had stronger associations between sugary beverage intake at one year and mid-childhood steatosis compared to children with normal weight. Children susceptible to and with

NAFLD have up-regulated de novo lipogenesis compared to non-NAFLD children, leading to higher VLDL.[96] Several studies have shown that children and adults with NAFLD have a higher mean fructose intake, mainly resulting from a higher consumption of soft drinks and fruit juices, as compared with individuals of the same age without NAFLD.[97] Due to the association between high sugar intake and NAFLD and the high prevalence of sugar intake in children, the strongest recommendation for the prevention and treatment of NAFLD is to reduce sugar sweetened beverage consumption.[28]

Given the overall high prevalence of sugar intake, its association with hepatic steatosis, and since it is a modifiable behavior, I chose free sugars and sugary beverages as the dietary exposure of interest in the ALSPAC cohort studies.

2.4 Screening for NAFLD in children

If NAFLD is identified early, outcomes can be improved primarily by improving diet quality (e.g., reducing sugar) and increasing physical activity.[28,98] Thus far, medications have had limited effectiveness in treating NAFLD, highlighting the importance of lifestyle change as both prevention and treatment[28]. The ability to detect NAFLD in its earliest stages is crucial to mitigating the consequences of childhood NAFLD. Most children with NAFLD typically present clinically between 10 to 13 years old.[99] Identification of NAFLD in children is largely dependent on screening since they are usually asymptomatic.[28] Screening for NAFLD is commonly done using serum liver enzymes, alanine aminotransferase (ALT) and/or evidence of liver fat on ultrasound among overweight and obese children. The gender-specific upper limit cut-offs for ALT in children are 22 mg/dl for girls and 26 mg/dl for boys[100]. The other two primary liver enzymes, aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) are not used as screening markers for NAFLD in children, but they are associated with a worse histology of the disease when they are elevated along with ALT.[28,101] Serum ALT is a widely available, minimally invasive, and inexpensive test with acceptable sensitivity. For example the use of two times the gender-specific ALT in overweight and obese children age over 10 years of age has a

sensitivity of 88% and a specificity of 26% for diagnosing NAFLD.[101] However, some children with NAFLD may present ALT values in the normal range. Additionally, elevated ALT levels can indicate several other liver diseases, although other tests can exclude these.[28]

Screening recommendations for NAFLD in children continue to be refined and the optimal age for screening is not yet clear. In 2007 an Expert Committee on childhood obesity recommended that children that are overweight or obesity be screened for NAFLD.[102] Prior to this, less than one-third of obese children were screened for NAFLD.[103] A study of a large health care system in the U.S. from 2009 to 2018 found that 54.0% of obese and 24.0% of overweight children were screened for NAFLD. Of the children screened that had elevated ALT (>30U/L), only 12.3% received further workup for NAFLD.[104] In 2017, the North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) recommended that screening for NAFLD in children that are overweight, obese or with other risk factors begin between ages 9–11 years, however this recommendation is based on limited evidence due to the sparsity of studies on the natural history of NAFLD especially prior to puberty.[28] One cross-sectional study of 742 children 2 to 19 years of age found the largest increase in prevalence of histologically diagnosed NAFLD in children between 5 to 9 years (3.3%) and 10 to 14 years (11.3%).[105]

2.5 Summary, specific aims, and hypotheses

The majority of studies on the association between early life nutrition exposures and consequent NAFLD have been conducted in animals. The few studies in humans have not looked directly at dietary factors. The only human studies on maternal undernutrition and later NAFLD have used birthweight and growth as markers for nutrition.[41] In the context of overnutrition, no human studies have specifically looked at maternal high-sugar diets and NAFLD in offspring. Studies examining early life nutritional status factors, such as pre-pregnancy obesity or gestational weight gain, have not looked at offspring NAFLD outcomes beyond adolescence. These studies have also had mixed results on whether this association is mediated through offspring adiposity. Finally, there is a sparsity of information on the natural history of pediatric NAFLD, especially prior to its diagnosis.

To understand some of these gaps in knowledge, I aimed to answer four questions in this dissertation using data from two longitudinal birth cohorts (***Error! Reference source not found.***). The f ollowing are the specific aims and hypotheses of this dissertation:

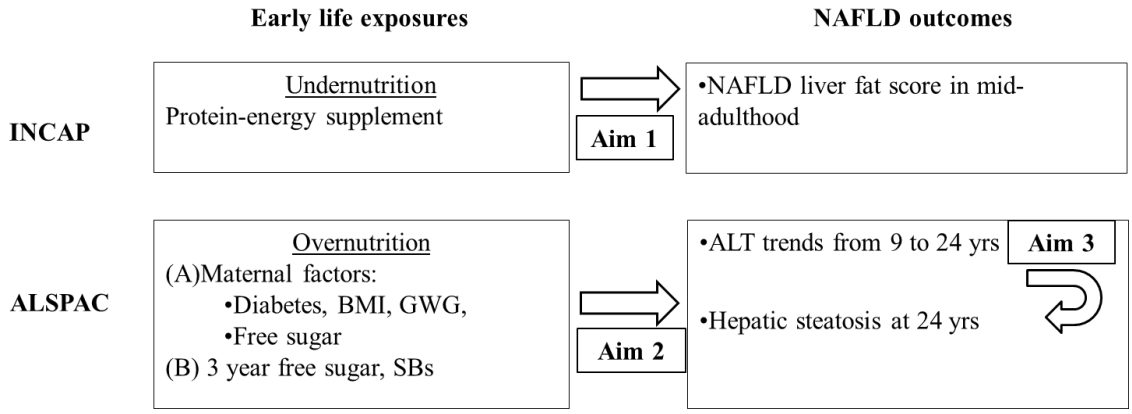

Figure 2-3. *Dissertation aims*

Abbreviations: NAFLD = Non-alcoholic fatty liver disease, INCAP = Institute of Nutrition of Central American and Panama cohort, ALSPAC = Avon Longitudinal Study of Parents and Children cohort; BMI = Body mass index, GWG = Gestational weight gain, SBs = Sugary beverages, ALT = Alanine amino transferase, yrs=years.

Aim 1: In an undernourished population, is exposure to a protein-energy supplement in early development associated with lower prevalence of NAFLD in adulthood?

Hypothesis 1: Improved protein-energy nutrition from conception to two years in an undernourished population will be associated with a lower prevalence of NAFLD in adulthood.

Aim 2A: Are maternal diet and nutritional status factors (as indicated by pre-pregnancy weight, pregnancy free sugar intake, diabetes status, and gestational weight gain) associated with offspring hepatic steatosis in young adulthood?

Hypothesis 2A: Women with pre-pregnancy overweight and obesity, diabetes, excess gestational weight gain, and high free sugar intake during pregnancy will have offspring with more prevalent hepatic steatosis in young adulthood.

Aim 2B: Is high intake of free sugars and sugary beverages in early childhood associated with hepatic steatosis in young adulthood?

Hypothesis 2B: Higher consumption of free sugars and sugary beverages in early childhood will be associated with more prevalent hepatic steatosis in young adulthood.

Aim 3: Do liver enzyme concentration trends from childhood to young adulthood differ among those with severe vs low hepatic steatosis at 24 years?

Hypothesis 3: Young adults with severe vs. low hepatic steatosis will have had higher serum ALT concentrations beginning in puberty.

The use of two longitudinal cohorts, a Guatemalan cohort from an Institute of Nutrition of Central America and Panama (INCAP) study and the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort will allow us to look at these associations from gestation into adulthood. I will use the INCAP cohort for aim 1 and the ALSPAC cohort for aims 2 and 3. The INCAP randomized nutritional intervention from 1969 to 1977 is a well-studied and rich source of information about the importance of nutrition for growth and development of children in developing countries and has had over six follow-up studies and hundreds of publications. The ALSPAC recruited pregnant women between 1991 and 1992 in Avon, UK and has followed their children for over two decades to understand how genetic and environmental characteristics influence health in parents and children. Both cohorts measured early life diet, NAFLD outcomes in adulthood, as well as a number of potential confounders.

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Chapter 3: Extended Methods

This chapter describes the two parent cohort studies, INCAP and ALSPAC, used in aim 1 and aims 2 and 3, respectively. The rationale and methods for the nutrition assessments, NAFLD outcome assessments, and statistical analyses used in the three aims of this dissertation are also described in detail. Additional information regarding the methods that are specific to each aim are presented in chapters 4-7.

3.1 Overview of Parent Studies

3.1.1 INCAP

In the 1960s and 70s, the Institute of Nutrition of Central America and Panama (INCAP) conducted a nutrition supplementation trial in four villages in southeastern Guatemala to assess the effect of improved nutrition on child growth and development. Malnutrition was prevalent in the villages where the trial was conducted: 45% of children under three years of age had severe stunting (< -3 standard deviations below the mean), and an even greater proportion had any stunting (< -2 standard deviations below the mean). At 24 months, 86% were stunted.[1]

The complete methodology of this study has been thoroughly described previously.[2-4] In summary, the INCAP randomized two pairs of size-matched villages to receive either *Atole* (an improved protein and energy-dense supplement) or *Fresco* (a no-protein, low-calorie supplement) from January 1, 1969 and February 28, 1977. The four villages were chosen from over 300 communities screened for and matched on size, diet, nutritional status, access to healthcare, demographic characteristics, and other factors. The attendance and consumption of the supplement was recorded for all breastfeeding and pregnant women and children seven years of age and younger.

The intervention was successful in improving the diets and outcomes of the sample population. Schroeder et al, published a comprehensive characterization of the quantity of the supplement consumed

with participation rates ranging from 65% to 85%.[5] The total dietary intakes of young children less than three years from *Atole* villages were greater by nine grams of protein, 100 kcal/day, and in micronutrients when compared to diets of children from *Fresco* villages.[3,6] As further evidence of the impact of the intervention, the groups exposed to *Atole* vs *Fresco* had significant reduction in the prevalence of severe stunting in children below two years of age and increased length (3 cm in the first three years of life).[3]

Cardiometabolic disease risk from 1,139 of the original 2,392 participants that were not lost to follow-up was measured between ages 37 to 54, the age range when increased risk for cardiometabolic disease is more readily detectable. Liver enzymes used to assess NAFLD, ALT and AST, were also measured and used to calculate the NAFLD liver fat score as described below. This cohort was used in aim 1 of this dissertation to look at the association between improved protein-energy nutrition in early life and later NAFLD.

3.1.2 ALSPAC

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a population-based birth cohort study based at the University of Bristol (Bristol, UK). The study was designed to investigate gene-environment interactions and their effect on health, behavior, and development of children from gestation through childhood and into adulthood. It was the first European longitudinal cohort study to begin in and measure diet in pregnancy. The study design of the ALSPAC has previously been described in detail.[7-9] Briefly, ALSPAC enrolled 14,541 pregnant women in the greater Bristol, UK area with expected delivery dates between 1st April 1991 and 31st December 1992. In 1998 to 1999 when participants would have been seven years of age, attempts were made to boost the sample by recruiting from offspring that would have been eligible to enroll in the original study, resulting in a total of 15,454 pregnancies and 14,901 children alive at the age of one year. Clinical, dietary, and demographic information were collected from the mothers starting in pregnancy. Other waves of fieldwork occurred when the participants were ages seven through 24 years. When the offspring were 24 years of age, 10,018 participants were invited to a

clinic visit known as Focus@24, which included the collection of biological samples and anthropometric measures including hepatic fat. Data from the 24-year clinic visit were collected and managed using REDCap electronic data capture tools hosted at the University of Bristol.[10,11] The study website contains details of all the data that are available through a fully searchable data dictionary and variable search tool.[12]

In aim 2 of this dissertation I use dietary FFQ data, clinical, and maternal self-reported information to assess nutrition exposures. Aims 2 and 3 utilize the FibroScan® measurements of hepatic fat as a primary outcome.

3.2 Nutrition Assessment Methods:

3.2.1 Improved protein-energy nutrition

In the INCAP study utilized for aim 1, improved protein-energy nutrition came from *Atole*, a nutritional supplement made from dry skimmed milk, sugar, and Incaparina (a vegetable protein mixture developed by INCAP [6.4g protein, 0.4g fat, and 90 kcal per 100 mL]). All calories in the control supplement, *fresco,* came from sugar (33 kcal per 100 mL) and it was fortified to match the micronutrient content of *Atole*. The supplements were available to everyone in each village at a central location twice every day via a pre-filled 180 mL cup. More was given if desired and any leftovers were subtracted from the amounts given to the nearest 10 mL.[2]

In aim 1, I and co-authors defined early exposure to improved protein-energy nutrition based on birth village (assigned as *atole* or *fresco*) from conception to age two years (based on birth date). While in the original study exposure was recorded up until seven years (within the study dates), we are only interested in the period through two years of age since it is a time of rapid development and can significantly impact later health. In the main analysis, the dichotomous exposure variable considered individuals who were exposed for the entire period from conception to age two years to be fully exposed and compared them to individuals that were partially exposed or had no exposure. In a sensitivity analysis, we created a three-category exposure variable: those who were fully exposed, those who were

partially exposed, and those without any exposure from conception to age two years. It also would have been ideal to look at exposure during pregnancy and early childhood separately. However, due to the limited sample size we were only able to look at them combined; 3.7% (n=40) of the original cohort were exposed to *Atole* only in pregnancy.

3.2.2 Food frequency questionnaire

In the Avon cohort, maternal diet was assessed via an unquantified food frequency questionnaire at 32 weeks gestation. The questionnaire covered 43 types of foods and drinks typically consumed in the UK in the 1990s, but was not validated.[13] Similar FFQs in the full sample were used throughout childhood from three to thirteen years of age, with adaptations for foods often consumed by children and changes in types of foods consumed over time. The three-year FFQs were completed by the parents. The questionnaires asked about weekly intake of each food consumed "nowadays" as: never or rarely (assumed weekly frequency=0), once in 2 weeks (=0.5), 1 to 3 times per week (=2), 4 to 7 times per week (=5.5), or more than once day (=10). More detailed questions were asked about specific foods including soft drinks and sugar. Since there were no questions about portion sizes, standard portion sizes were assumed for the nutrient estimation.[14,15] Nutrient information for foods were primarily obtained from the 5th edition of McCance and Widdowson's food tables.[16] Intakes for energy and non-milk extrinsic sugars (as well as other macro- and micronutrients) were calculated by multiplying the weekly frequency of consumption of each food by the nutrient content of a portion and summing this for all consumed foods. This was then divided by seven to get the daily intake. Approximately three percent of participants were excluded due to high or low energy intakes based on inspection of histograms.[13,17] Nutrient intakes were available for 11,923 women and were mostly adequate. When compared to a representative national sample of non-pregnant women, the nutrient intake estimates were similar, although higher sugar intake was reported in the Avon cohort.[13] At three years, dietary data was available from 10,137 (response rate 69.7%) participants[17].

3.2.3 Non-milk extrinsic sugars

Non-milk extrinsic sugars (NMES) were calculated by deducting sugars from milk and fruits and vegetables (contained within cellular walls) from total sugars.[17] This is equivalent to the definition of free sugars, which includes isolated sugars added during food preparation and manufacturing (added sugars) as well as sugars present in unsweetened fruit juices, fruit concentrates, or honey and other syrups.[18] The terms free sugars and NMES will be used interchangeably throughout this dissertation.

The primary exposure in aim 2 is free sugar nutrient density (free sugars as a percent of total energy intake (TEI)). Individuals make changes primarily by altering dietary composition rather than absolute amount of nutrients since, generally, for an individual, total energy intake is fixed within a narrow range.[19] Advantages of the nutrient density model include 1) it can be calculated directly for an individual without the use of any statistical models, 2) it is familiar to nutritionists, and 3) it is used in dietary guidelines. A disadvantage of using percent free sugars is that TEI is likely associated with NAFLD, so free sugar intake will be confounded by TEI and dividing by TEI may induce confounding in the opposite direction. This can be addressed by controlling for TEI in the models. It is also important to adjust for TEI to remove extraneous variation due to physical activity, metabolic efficiency, and body size.[19] Using the multivariate nutrition density model, we interpret the results as the increase in the odds of hepatic steatosis associated with an increase in the percent of energy from free sugar while total energy is kept constant. To test the hypothesis that energy intake is a mediator between free sugar intake and hepatic steatosis we can compare the models with and without total energy intake.

After calculating the percent NMES variable, I categorized it based on the distribution of the variable in an attempt to have a meaningful reference group that fell within the 10% recommended cut-off for free sugar intake by the WHO and had an appropriately powered sample size.[18] In mothers the categorization was categorized into tertiles (1.3 to 10.4% (reference), to 14.3%, to 42.2%) and in children three years of age it was categorized into quintiles (0.14 to 11.5% (reference), to 13.5%, to 15.3%, to 17.7%, to 36.5%). In this group, I further looked at the association with hepatic steatosis across increasing

quintiles (i.e. linear trend). Categorizing an exposure variable has additional advantages that include: 1) avoiding misspecification of the model because an underlying exposure-disease relationship is not assumed, 2) additional insights into the nature of the exposure-disease relationship, and 3) it decreases the influence of outliers.[19] Since I used categorized dietary variables, the nutrient density approach to energy adjustment is preferred over the standard multivariate approach.[19]

I also considered keeping the variable as a continuous variable. While using a continuous variable gives the best power, the NMES percent variable was highly skewed. Log transformation of the variable to normalize the distribution makes it difficult to meaningfully interpret results, and thus was not done.

3.2.4 Sugary beverages

Sugary beverages (SB) intake per day was quantified from responses to weekly intake of pure fruit juice, tinned juice, fruit drinks, Ribena™, squash, non-diet colas, and other fizzy drink questions from the 3-year FFQ. While the impact of 100% fruit juices on child adiposity has been mixed, their consumption exceeds recommended amounts in children and they are a large source of free sugars in young children's diets[20]. I did not include tea, coffee, and alcohol intake because the majority of young children did not consume them.[21] Tea and coffee intake was negligible: Of the 30% that consumed tea at least once a day, only 10% added sugar. Only 7% reported at least one coffee a day, and of those 7.3% reported adding sugar. I included the sugary beverage exposure variable as a continuous term (number of sugary beverages consumed per day) and categorized it as <1/day (reference group), 1 to 2/day, and >2/day allowing us to compare our results to previous literature on sugary beverage intake in young children.[22] When sugary beverages are the primary exposure, I compare models with and without total energy adjustment. The association between sugary beverage consumption and adiposity is likely mediated in part by overall energy intake, therefore adjusting for energy will tend to underestimate the effect of these beverages on adiposity, including hepatic steatosis.[23,24]

I did not look at sugary beverages as an exposure during pregnancy. While the pregnancy 32-week gestation FFQ asked about the intake of some sugary beverages, there were missing drink categories

including fizzy drinks, bottled drinks, and squashes, which made the interpretation of a sugary beverage intake variable unclear.

Additionally, in the maternal surveys, questions were asked specifically about "cola" intake per week in each of the first, second, and third trimesters that would have allowed us to test the most sensitive period of gestation to excess sugars. However, I decided not to utilize the cola questions to look at sugary beverage intake in each trimester for the following reasons: First, cola drinking is possibly not representative of overall sugary beverage intake. Second, it is not clear what "cola" meant and whether it was interpreted by respondents to include all soft drinks and "fizzy" drinks. Third, the cola drinks could include diet sodas. A follow-up question about "diet soft drinks" was only asked in the third trimester. About 30% of the mothers always chose a low calorie or diet soft drink and 37% sometimes chose a low calorie or diet soft drink. Diet or low-calorie soft drinks have artificial sweeteners that are a concern on their own as they can also have negative (but possibly similar to regular sugar) metabolic effects. Finally, approximately three-quarters of mothers reported no cola intake, making it statistically possible to only look at a dichotomous exposure of no vs any cola intake which may limit ability to detect any biological association. I hypothesized that to see an impact of a sugary beverage like cola on offspring metabolic outcomes, I would need to look at those consuming excessive amounts of the beverage.

3.2.5 Maternal nutritional status

To assess overall maternal nutritional status for aim 2, I used the following proxies: Pre-pregnancy weight, maternal diabetes, and gestational weight gain.

Maternal pre-pregnancy BMI was calculated using self-reported maternal height and pre-pregnancy weight, and categorized as underweight (<18.5 kg/m^2), normal (18.5-<25 kg/m^2), overweight (25-<30 kg/m^2), or obese (≥30 kg/m^2)[25].

Data on maternal diabetes status included existing diabetes, gestational diabetes, and glycosuria. Pre-existing diabetes was self-reported at time of enrollment and there was no distinction between type I

and type II diabetes. Gestational diabetes and glycosuria were assessed by standard research protocols from antenatal and postnatal clinical records. At the time of recruitment there was no universal screening for gestational diabetes by fasting glucose or an oral glucose tolerance test. Only women with established risk factors for diabetes such as obesity, family history, or south Asian ethnicity were offered gestational diabetes tests. All women were offered glycosuria urine tests at each antenatal clinic visit. Glycosuria during pregnancy was defined as having a measurement >250 mg/100 ml on at least two occasions during the pregnancy. I and co-authors categorized maternal diabetes dichotomously as none or any diabetes/glycosuria (pre-pregnancy diabetes, gestational diabetes, or glycosuria combined due to limited sample size), similar to others.[26]

Every instance of gestational weight and corresponding gestational age and date was abstracted by trained research mid-wives from obstetric medical records. There was a median of 10 measures per woman (IQR: 8 to 11). ALSPAC derived two variables for absolute weight gain in pregnancy: one using actual weight measurements from prior to 18 weeks and 28 weeks gestation and the second using predictions from linear spline models for gestational weight gain.[27,28] This was calculated by subtracting the predicted pre-pregnancy weight at 0 weeks gestational age from the predicted weight at time of delivery. The spline model was only fitted for women with term pregnancies. I chose to use the variable using the predicted weights because of its more accurate time points as well as larger sample size. In sensitivity analysis, using the model with actual weight changes did not significantly change results. Women were then categorized into three categories according to Institute of Medicine (IoM) recommendations: adequate, less than, and more than recommended GWG. Recommended weight gain is 12.5–18 kg for underweight; 11.5–16 kg for normal weight; 7–11.5 kg for overweight; and 5–9 kg for obese women.[29]

3.3 NAFLD Assessment Methods

A diagnosis of NAFLD is suspected based on the association of fatty liver combined with risk factors (mainly obesity), after the exclusion of other causes of liver disease. The gold standard for the

diagnosis of NAFLD is a liver biopsy, and the best measure of hepatic fat levels is by magnetic resonance spectroscopy. Other surrogate markers of the disease in children include imaging by ultrasonography, liver function tests, and serum markers of liver fibrosis. A substantial problem limiting both care of patients with liver disease and research on NAFLD is the lack of a non-invasive measure of fat in the liver. Liver biopsy has been considered the best diagnostic tool for confirming NAFLD, particularly fibrosis stage, as well as the most sensitive and specific means of providing important prognostic information[30]. Liver biopsy, however, is limited by invasiveness, cost, and the potential for sampling error. Currently, accurate assessment of NAFLD remains dependent on liver biopsies for detection and severity monitoring, but a better method is needed.

The gold standard for measuring hepatic fat is magnetic resonance imaging (MRI). MRI methods including MR spectroscopy and MR proton density fat fraction and MR volumetric fat fraction are all highly sensitive and specific.[31,32] Other benefits of MRI include precision, reproducibility, assessment of a larger area of the liver, and a high level of safety.[33] High hepatic fat >5% (HHF) is often used for magnetic resonance spectroscopy based findings (but is not considered a diagnosis on its own). Currently, MRI is the most appropriate choice for non-invasive evaluation of hepatic fat.[34-37] However, these methods are expensive, involve special technology, and thus are not widely adopted in clinical practice. Additional barriers include the requirement for sedation in young children, proprietary measurement protocols, and the fact that there is not a uniform standard for classifying fatty liver by MRI.[33]

The most commonly used screening methods for NAFLD include ultrasound imaging, computed tomography (CT), and liver function tests (e.g. ALT, AST, GGT), however, these have limited sensitivity. Ultrasound and CT can only identify fat if it is >33% and neither can quantify the fat.[38] Ultrasound is also dependent on the machine and its operator and CT exposes patients to ionizing radiation. Alanine aminotransferase (ALT), the most commonly used screening biomarker, is often elevated with NASH in children; however, it is often normal in NAFL. In adults with NAFLD, the usual biochemical pattern is increased ALT relative to AST (AST/ALT $\leq$ 1.0). In contrast, an AST/ALT ratio of 2.0 or higher

or ALT level exceeding 300 U/L may be indicative of alcoholic liver disease.[39] There are also several scores, such as the liver fat score (used in aim 1) and the fibrosis score, that can be used to estimate hepatic fat and fibrosis levels in adults, respectively.

A novel non-invasive test for steatosis in the liver is transient elastography (FibroScan®, TE) with controlled attenuation parameter (CAP). TE uses ultrasound shear wave velocity to measure liver stiffness measurement (LSM) and uses a measure of the attenuation of ultrasound waves in the liver at 3.5 MHz to assess steatosis (CAP). Both measures are provided simultaneously. Advantages of the method include the following: painless, fast, reliable and reproducible, with good intra- and inter-observer levels of agreement. It is machine and operator independent. These features make CAP useful for population-wide screening of NAFLD and disease follow-up.[40] This method was used to measure hepatic fat in the ALSPAC cohort at 24 years.

3.3.1 The NAFLD Liver Fat Score and Percent

For aim 1, I calculated the NAFLD Liver Fat Score and Percent from available variables in the INCAP cohort 2015-2017 wave of data collection. Scores like the NAFLD liver fat score are useful as non-invasive predictors of the disease.[41,42] The NAFLD liver fat score equations can be used to predict the presence of (1) hepatic steatosis and (2) its quantity. Five variables (presence of metabolic syndrome, type II diabetes mellitus, fasting insulin, fasting AST, and the AST/ALT ratio) which were associated independently with NAFLD as diagnosed by proton MRS are used in the equations (*Figure 3-1*).[42] The PNPLA3 polymorphism was also a predictor of hepatic fat content, but its addition did not improve the performance of the NAFLD liver fat score. Only those with alcohol consumption <20g/day were included. Excessive alcohol consumption has also been defined as >21 drinks/week in

(1) NAFLD liver fat score = −2.89 + 1.18 * metabolic syndrome (yes = 1/no = 0) + 0.45 * type 2 diabetes (yes = 2/no = 0) + 0.15 * fS-insulin (mU/L) + 0.04 * fS-AST (U/L) − 0.94 * AST/ALT

(2) Liver fat (%) = $10^{(-0.805 + 0.282 * \text{metabolic syndrome (yes = 1/no = 0)} + 0.078 * \text{type 2 diabetes (yes = 2/no = 0)} + 0.525 * \text{LOG (fs-insulin [mU/L])} + 0.521 * \text{LOG (fS-AST [U/L])} - 0.454 * \text{LOG (AST/ALT)})}$

Figure 3-1. *The NAFLD liver fat score.*

men and >14 drinks/week in women.[43] A liver fat score above −0.640 predicted the presence of steatosis with 86% sensitivity and 71% specificity compared with MRS measured liver fat. The score has an area under the receiver operating characteristic curve of 0.87 in the estimation and 0.86 in the validation group.

3.3.2 Transient elastography

Transient elastography of the liver by FibroScan® (CAP and TE scores) are available in the ALSPAC cohort at 24 years and were utilized to define the primary outcome of hepatic fibrosis in aims 2 and 3 of this dissertation. FibroScan® CAP is a novel ultrasound-based method to measure liver steatosis. The TE score describes fibrosis. In adults, FibroScan® CAP has good accuracy in quantifying the levels of liver steatosis and fibrosis in patients with NAFLD.[40,44]

Individuals with an active implantable medical device such as a pacemaker, liver ascites, or who were pregnant were excluded from the liver scan. Participants were asked to fast overnight or for at least six hours prior to transient elastography.[45] Manufacturer and machine indications were used to conduct the scan. Different probes ("S", "M", "XL") allow for the measurement of patients with varying thoracic measurements, including obese patients using the "XL" probe. The "M" probe is usually used for children. The "S" probe is for children with a thoracic perimeter less than 75cm, however, as of 2019 only liver stiffness measurements (LSM), and not CAP for steatosis, are available using "S" probe. The initial inability of the procedure to accurately determine fibrosis and steatosis in obese patients has been addressed with the development of the obese-specific "XL" probe. Cho et al. found no significant difference in LSM values between the "S" and "M" probes in children aged 1 to 16 years.[46] They used the "M" probe for all reported measures and had a 93.9% success rate. 6.1% of the failures were due to excessive thickness of the subcutaneous adipose tissue in obese children (BMI percentile mean 99.6 +- SD 2.2). Scott et al, had a 100% success rate but required the "XL" probe in 45% of patients.[47]

Ten readings were required for each patient to derive a CAP score and fibrosis result. CAP values outside the 100-400 dB/m range were considered invalid and coded as missing. Median fibrosis results greater than or equal to 15 kPa or with an interquartile range (IQR) to median ratio greater than or equal to 30% were considered invalid and coded as missing. A meta-analysis by Karlas et al. of studies containing histology verified CAP data, established CAP cut-offs for distinguishing steatosis grades (S0-S3). Optimal cut-offs for above steatosis grade S1, S2, and S3 were 248 (11% steatosis), 268 (33% steatosis), and 279 (66% steatosis) respectively. In a validation study of Fibroscan® using liver biopsy as the comparison, CAP measurements distinguished between patients with and without steatosis, however, when classifying steatosis degree as none, mild/moderate, or marked, there was a statistically significant difference between each degree vs none, but no difference between mild/moderate and marked steatosis.[48]

For aim 2, to be consistent with previous similar studies I categorized participants into two categories of steatosis based on CAP score cut-off values derived from a meta-analysis by Karlas, et al[44]: low (<248 dB/m, <11% steatosis) vs mild to severe steatosis (248-400 dB/m, ≥11% steatosis). In sensitivity analysis, I also categorized steatosis as low to moderate steatosis (< 279 dB/m, < 66% steatosis) vs severe steatosis (279-400 dB/m, ≥ 66% steatosis). I categorized fibrosis into two groups. The first group included those with no fibrosis or portal fibrosis without septa (F0-F1, <7.9 kPA) and the second group included those with any fibrosis: portal fibrosis, septa, or cirrhosis (F2-F4, >7.9 kPA).[49]

In aim 3, to understand the differences in ALT trajectories while also maintaining adequate sample size in each group, I categorized participants into three categories of hepatic steatosis low (<248 dB/m, <11% steatosis), mild to moderate, and severe (279-400 dB/m, ≥ 66% steatosis). The same fibrosis categories as in aim 2 were utilized.

3.3.3 Hepatic enzymes

Screening for NAFLD usually occurs by elevated hepatic enzymes and/or evidence of liver fat on ultrasound among overweight and obese children. Serum ALT is a widely available, minimally invasive, and inexpensive test for the screening and initial evaluation of NAFLD. Its sensitivity is acceptable;

however, a number of adult and pediatric patients may present ALT values in the normal range. Additionally, NAFLD is considered a disease of exclusion and elevated ALT levels can indicate several other liver diseases although other tests can exclude these.[50] In the United States, sex-specific biologically based cutoffs based on the 95th percentile of ALT in normal weight children (22 mg/dL for girls and 26 mg/dL for boys) from nationally representative data have been validated in a diverse cohort. For the diagnosis of NAFLD, the use of 2 times the sex-specific ALT in overweight and obese children age 10 years or older has a sensitivity of 88% and a specificity of 26%. Aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) have not been independently tested as screening tools for NAFLD in children. If ALT is normal, but AST or GGT are elevated, this may represent a condition other than NAFLD.[50] Fatty liver due to alcohol intake can also lead to elevated ALT; however, this is less of a concern in children where alcohol intakes are low. In obese children and adolescents existing prediction scores and the tested novel biomarkers have insufficient diagnostic accuracy for diagnosing or excluding NAFLD.[51]

For aim 3, I explored whether trajectories of hepatic enzymes, ALT, AST, and GGT throughout childhood and adolescence were associated with hepatic steatosis at 24 years. I utilized the hepatic enzymes from any age they were available in the ALSPAC cohort. Fasting serum ALT, AST, and GGT liver enzyme concentrations were obtained through standard clinical chemistry assays at ages 9 (non-fasting), 15, 17 and 24 years as previously described.[52] Participants were asked to fast overnight or for at least six hours prior to phlebotomy and transient elastography. Blood samples were immediately centrifuged and frozen at −80 °C. At 24 years, elevated ALT was defined as >19 U/L in women and >30 U/L in men.[45]

3.4 Statistical Analyses

This section will cover the various statistical analyses methods used in this dissertation including dealing with missing data and the various models used in the three aims including difference-in-difference, mixed multilevel models, and logistic models.

3.4.1 Missing data

In an epidemiologic or clinical study one of the primary limitations is missing data. If missing data is ignored or not dealt with properly, it can lead to biased estimates of the regression parameters, wrong standard errors or invalid statistical inference.[53] Missing data can be in the form of missing observation (e.g. participants lost to follow-up) or missing values for certain variables. Missing data can also be missing completely at random (MCAR), missing at random (MAR), or not missing at random (NMAR). In MCAR missingness is not related to the values of any variables; in MAR missingness may be related to the values of other variables, but not to its value; in NMAR the probability that the value is missing depends on its value.[53]

Most commonly, missing data is dealt with by excluding them from analysis (i.e. complete case analysis) as a regression necessitates complete data for an individual to be included, so if they are missing a value for one of the variables they are excluded. Other methods to deal with missing values include single imputation where a missing value is replaced with some other value (usually via mean imputation) or conditional imputation where missing values are replaced with the mean from cases that are similar to the case with the missing values. Both single and conditional imputation produce values that are determined without error and do not reflect uncertainty about the predicted values, thus resulting estimated variances of the parameter are biased towards 0. The parameter estimates are overly precise (narrower CI) resulting in higher type 1 error (more false positive significant tests).

It is recommended to use multiple imputation (MI) to deal with missing data when MAR criteria, normality of data, and enough data is available for imputation. Multiple imputation fills in each missing value with a set of plausible values that reflect the uncertainty about the correct missing value. MI represents a random sample of missing values resulting in valid statistical inference that properly reflects the uncertainty due to missing values.

After looking at missingness patterns and determining whether MI is necessary and valid, the following are the steps I took to conduct MI in SAS. First, narrow the sample to eligible participants. Second, log transform any other non-normal variables as MI requires continuous variables to be normally

distributed. Third, using PROC MI, conduct multiple imputation for all covariates with missing data and predictors in wide format dataset. Many researchers believe it is inappropriate to use imputed values of the dependent variable in the analysis model, especially if the variables used in the imputation model are the same as the variables used in the analysis model. Imputed values likely add no information since they were generated using the model that is being analyzed. However, the dependent variable should be included in the imputation model. The MI model should include all variables to be imputed, the dependent variable, and any other variables that could help predict missing values. If there are both categorical and continuous variables to impute, use the fully conditional specification (FCS) method. In SAS, Select "Regpmm" for continuous variables, "Logistic" for ordinal and binary variables, and "Discrim" for nominal variables. Impute at least five datasets and more is preferred. Commonly, analysts use 20 imputations. Fourth, for mixed models transform each of the datasets to long format. Fifth, run models using the imputation datasets. Finally, pool results from each of the separate regressions using PROC MIANALYZE.[54]

In aim 1 utilizing data from the INCAP cohort, I used multiple imputation to deal with missing data for maternal height (20.5% missing), maternal years of schooling (3.5% missing), and maternal age (1.6% missing). I used the fully conditional specification method for five imputations. To impute missing values, I used predictive mean matching for continuous variables and the logistic regression method for ordinal variables. I included all predictor and outcome variables that were not linear effects of each other as predictors in the imputation model.[55] I used PROC MI and PROC MIANALYZE in SAS v 9.4.[54]

There were also missing values in the ALSPAC cohort – however, for a variety of reasons I chose not to use multiple imputation in the primary analysis of aims 2 and 3. Below, I outline some of the challenges with multiple imputation and rationale for not using them in these aims.

1) I was not confident that the MAR assumption was met for all variables. MAR is an assumption that cannot be verified statistically, but researchers can rely on what is known about the

associations in their variables of interest. For example, it is possible that women missing dietary intake had different dietary patterns that were associated with lower or higher free sugar intake.

2) After exclusions, with a few exceptions, only a small percentage of data were missing. In the trend analysis (aim 3), less than 1% of participants were missing BMI at 24 years. About 34% (n=590) of those that were eligible for the study were missing all or three of the four ALT values measured over time, decreasing the analytic sample size from 1746 to 1156. In a sensitivity analysis including everyone with at least one ALT value (only excluding those with no ALT at any time point) there was no major differences in results. For the aim 2 analysis focused on pregnancy, the following variable and % were missing: maternal highest education (1.9%), pregnancy physical activity (7.5%), pregnancy smoking (0.3%), pregnancy alcohol intake (0.3%), maternal pre-pregnancy BMI (7.6%), maternal diabetes (2.6%), gestational weight gain (9.6%), dietary information (3.7%), birthweight (1.3%), breastfeeding (6.9%), 24 year BMI (0.9%), AUDIT-C (2.2%), fibrosis (5.8%). In fully adjusted complete-case analysis, our sample size decreased from 3353 to 2668.

3) In a sensitivity analysis using MI, there were no meaningful changes in estimates. They were slightly closer to the null, but all remained in the same direction. In the aim 3 MI, there was also an increase in significance and narrower confidence limits. However, this increase in significance is possibly due to the methods of the MI. When I pooled the least square mean differences from the imputed datasets, I was not able to do an adjustment for multiple comparisons. This makes it difficult to do an apples-to-apples comparison of the p-values and confidence limits for my estimate from the multiple imputation vs complete case analysis, therefore I did not report the results from this sensitivity analysis MI.

Another missing data issue specific to cohort studies is the loss-to-follow up of participants. Differential loss-to-follow up, whereas participants with certain characteristics may be less likely to participate in future waves of a study, can lead to biased estimates. One way to understand this type of

loss is non-response analysis. In our studies we compare sample characteristics at of the complete sample at baseline with the analytic sample at end-point to understand if there was differential loss-to-follow-up.

3.4.2 Difference in Difference models

In aim 1, the double-difference (D-D) modeling strategy was used to model the exposure variable, capturing a two-layered comparison in both the type (*atole* vs. *fresco*) and timing (full vs. partial exposure from conception to 2 years of age) of supplementation. It is important to note that the original trial followed cluster randomization at the village level, and the analysis will be conducted at individual level. By using the D-D model, we "broke" the initial randomization because we introduced the timing of exposure factor. This necessitates the consideration of clusters both at village level and at household level, because certain characteristics were not randomized.

The following generalised regression model was used to estimate the difference-in-difference intent-to-treat effect of *Atole* vs *Fresco* from conception to age two years, net of the differences attributable to village or birth year effects. Generalized models allow for adjustment of standard errors for within family correlations since most participants had at least one sibling in the trial. Depending on the outcome, linear or logistic forms of the model was be used.

$$\boldsymbol{NAFLD\ outcome = \alpha + \beta_1 X_i + \beta_2 Z_j + \delta X_i \times Z_j + (\gamma_1 V_1 + \ldots + \gamma_m V_m) + \varepsilon}$$

- ***NAFLD outcome*** **Yij**= liver fat percent, ALT (continouous outcomes); ***Logit P(X)***= dichotomous yes/no NAFLD using the NAFLD liver fat score.
- ***Xi*** (1=*atole*, 0=*fresco*); ***Zj*** (1=complete exposure duration, 0=partial exposure duration).
- **Xi×Zj** (interaction term which estimates the double-difference effect of *atole* vs. *fresco* for a given duration of exposure).
- $\boldsymbol{V_1}$ ***to*** $\boldsymbol{V_m}$ covariates.
- $\boldsymbol{\varepsilon}$ is the error term that captures residual variances.

The double difference estimate subtracts the average outcome for those completely exposed to *atole* minus fresco from the average outcome for those not completely exposed to atole minus *fresco* from conception to age 2 years. The use of the double-difference modeling allows us to control for within-village constants. We controlled for village-level fixed effects by using dummy variables for the birth village. Including birth year controls for the age range of the sample. We also controlled for maternal height (as an indicator of chronic maternal nutrition status), maternal grades of schooling, childhood SES, adulthood SES status in 2015-2017, participant completed grades of schooling, and participant rural vs urban (Guatemala City) residence (*Figure 3-2*). This helps mitigate the effects of lifestyle factors (particularly among those with better socioeconomic status) on the progression of cardiometabolic diseases and get closer to a true association. Additionally, in randomized trials, biases may emerge after randomization because of differences between treatment groups. Previously published studies using this data looking for similar associations have controlled for similar variables.[56] *Sex* will be considered an effect modifier. It will be controlled for using sex-specific models and adjusted for in pooled analysis.

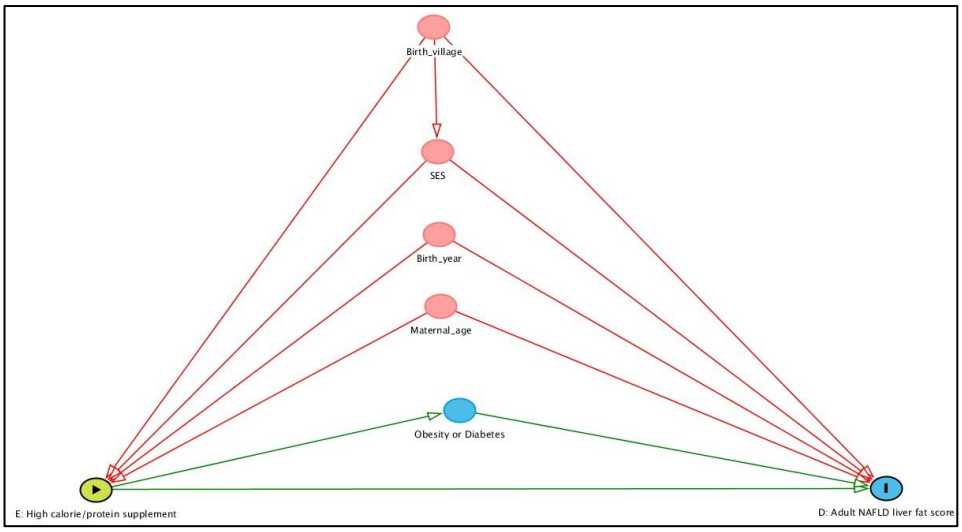

Figure 3-2. *Aim 1 directed acyclic graph.*

We excluded individuals with high alcohol intake (>21 drinks/week in men and >14 drinks/week in women).[43] We expected alcohol consumption to bias the NAFLD liver fat score estimate to be higher

than expected, since alcohol consumption can lead to similar outcomes. However, it is not expected to bias the association between diet and NAFLD if alcohol consumption is non-differential with respect to the intevention.

3.4.3 Logistic models

We used the following general logistic multivariable regression to model our binary dependent hepatic steatosis variable in aim 2:

$$LogitP(Y) = \alpha + \beta 1Xi + (\gamma 1V1 + \ldots + \gamma mVm) + \varepsilon.$$

- ***Logit P(Y)=*** Hepatic steatosis at 24 years
- ***Xi:*** **Nutrition and diet exposures** in pregnancy (aim 2A) and early childhood (aim 2B).
    - 2A: Maternal pre-pregnancy BMI, gestational weight gain category, maternal diabetes, and tertile of free sugar intake as percent of total energy intake.
    - 2: Quintiles of three year free sugar intake as percent of total energy intake.
- V_1 ***to*** V_m: covariates (see *Figure 5-1* for aim 2a and *Figure 3-3* for aim 2b).
- ε is the error term that captures residual variances

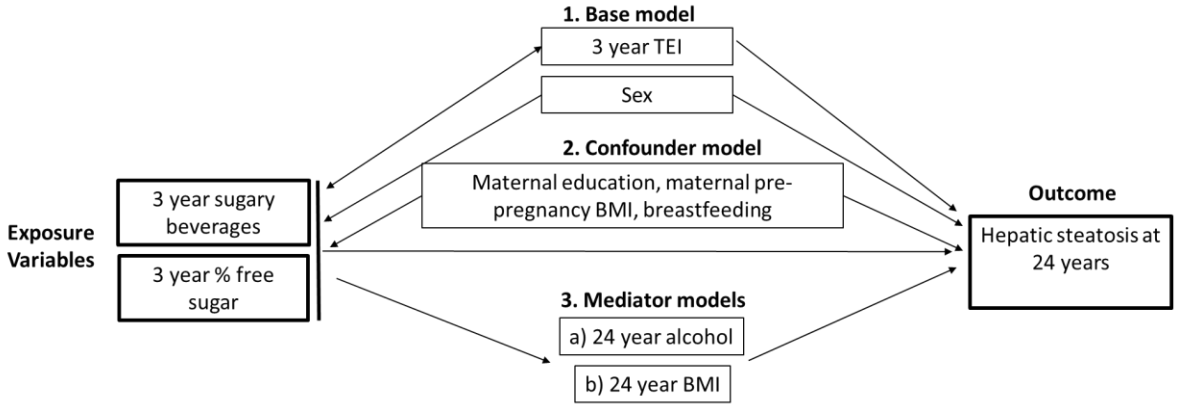

Figure 3-3. *Conceptual model for aim 2b.*

Relates 3-year % free sugar of total energy and sugary beverage intake exposures to hepatic steatosis outcome at 24 years. Abbreviations: TEI = total energy intake, BMI= body mass index.

3.4.4 Mixed models

In aim 3, we used multi-level linear mixed models to assess the trend differences of log-transformed ALT, AST, and GGT levels from ages 9 to 24 years by hepatic steatosis level at 24 years. We also modeled differences of log-transformed ALT by fibrosis. We included the fixed effects for categorical age, random effects for the intercept, and an unstructured error covariance structure. To back-transform the log-transformed means from the models, we exponentiated them to geometric means. We then calculated geometric mean ratios (GMR) to assess the differences between steatosis levels at each age. We used PROC MIXED using the REPEATED statement in SAS to conduct this analysis. The REPEATED statement controls the covariance structure imposed upon the residuals or errors. In SAS the REPEATED and RANDOM statements give the same results if we specify "intercept" as the only random component in the RANDOM statement.[57] We allow for different intercepts, but not slopes.

Since we had repeated measures of biomarkers (e.g. ALT) throughout childhood in association with NAFLD (a categorical outcome), we chose to use mixed multilevel models to account for the non-independence (i.e. clustering) of repeated measurements within individuals, change in variance of measures over time, and differences in the number of measurements between individuals.[58]

The problem of how to deal with time-varying exposure data in a way that accounts for its longitudinal features is different than most regression settings where the independent variable and dependent variable are either both cross-sectional, both longitudinal, or the outcome is longitudinal. Commonly, the methods used to analyze longitudinal data include multiple cross-sectional models which can lead to the problem of multiple hypothesis testing or to include exposure measures at each time point simultaneously in a multivariate logistic regression model which is problematic because of the correlated exposure measures leading to inflated standard error estimates and biased odds ratio estimates.[59] Despite not being temporally logical, a mixed model approach to contrast trajectories allows us to depict trends of

exposure, and is acceptable if the focus is not to establish causality or quantify risk.[59] This method treats X (ALT) as a longitudinal dependent variable and Y (NAFLD) as a time-invariant independent binary variable.

A multi-level model is comprised of two sub-models. The level-1 sub-model for individual change describes how each person changes over time. The leve-2 sub-model for inter-individual heterogeneity in change describes how these changes differ across subjects.

We chose to include age in the model as a categorical term (as opposed to a linear term) since the trends were not linear and it allowed us to make comparisons at each age point.

In multilevel models all individuals with at least one observation can contribute to the model under the assumption that data are missing at random, that is, the probability of an observation being missing is related to other observed variables for that individual, but does not depend on the true value of the missing observation.[52]

Mixed multi-level models also allow for exploration of different covariance structures.[60] They allow for the modeling of the variance-covariance matrix directly from the observed data. Assumptions about homoscedasticity (constant variances), compound symmetry (constant covariances) aren't necessary. We tested several different structures including heterogeneous first-order autoregressive (ARH(1)), unstructured (UN), and compound symmetry (CS) by comparing AIC and BIC scores. We found that an unstructured covariance matrix gave the best fit. The measurements on a subject over time may have different variances. The length of time between pairs of measurements can also impact the correlations between the measurements. An unstructured covariance assumes that the variance at every time and the correlation between every pair of times is unique. It's important to test several different variances since over-modeling the covariance structure can reduce power and precision for fixed effects estimates and tests. For example, even though an unstructured model is the most flexible, if a simple model adequately accounts for the observed covariance this can lead to over-modeling. There are covariance structures that

occupy middle ground between the extremes of CS and UN that allow for unequal spacing,[60] but we found UN to have the best fit.

In our sample, 27% of participants had only one visit. Multilevel models are designed to handle unbalanced data sets in most circumstances, however, if many participants have just one or two waves of data (e.g. > 10 to 20%) this can cause problems such as poor estimates of some parameters.[61] Therefore, we chose to only include those with more than one visit.

In our models, we adjusted for BMI at 24 years, ethnicity, and maternal education. We considered both BMI and waist to hip ratio as potential measures of adiposity. Both were significant, but since they were collinear, we chose to include BMI since it had better model fit alone. In sensitivity analysis, we adjusted for hazardous alcohol intake at 24 years using the AUDIT-C score.

The composite model for our multilevel model for change of ALT in the Avon cohort for aim 3 is:

$$ALT_{ij} = [\gamma_{00} + \gamma_{10}AGE_{ij} + \gamma_{01}NAFLD_i + (\gamma_{02}V1 + \gamma_{0m}Vm) + \gamma_{11}AGE_{ij} * NAFLD_i + \gamma_{12-m}(V_i * AGEij)] + [\zeta_{0i} + \zeta_{1i}\,AGE_{ij} + \varepsilon_{ij}]$$

- **ALT_{ij}** for ith individual at j = 9, 15, 17, and 24 years
- **γ_{00}:** The mean ALT among children at baseline taking confounders into consideration (fixed effect).
- **AGE_{ij}**: The average change in ALT between two ages among children born to low steatosis mothers taking confounders into consideration.
- **NAFLD**: The average difference in ALT at baseline among children with high vs low hepatic steatosis controlling for confounders. We also made a comparison with moderate hepatic steatosis (not included here for simplicity).
- ***V_1 to V_m***: BMI, maternal education, and ethnicity
- **$AGE_{ij} * NAFLD_i$:** The average difference in the rate of change in ALT between children that end with high vs low hepatic steatosis taking confounders into consideration.
- **$V_i * AGEij$:** The difference in the slope relating Age and mean ALT between those in upper vs lower level of covariate taking other covariates into consideration.

- ζ_{0i} ε_{ij}**:** Random effect for the intercept and error term, respectively

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Chapter 4: Prevalence of NAFLD in Guatemala following exposure to a protein-energy nutrition intervention in early life

Aim 1

Sekkarie A, He S, Welsh JA, Ramakrishnan U, Stein AD, Vos MB. Prevalence of NAFLD in Guatemala following exposure to a protein-energy nutrition intervention in early life. *Annals of Hepatology*. 2020; 19(4)

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4.1 Abstract

Introduction and Objectives: The global prevalence of non-alcoholic fatty liver disease (NAFLD) is approximately 25%, with Hispanic populations at greatest risk. We describe the prevalence of NAFLD in a cohort of Guatemalan adults and examine whether exposure to a protein-energy supplement from conception to two years is associated with lower prevalence of NAFLD.

Materials and Methods: From 1969-1977, four villages in Guatemala were cluster-randomized to receive a protein-energy supplement (*Atole*) or a no-protein, low-energy beverage (*Fresco*). We conducted a follow-up of participants from 2015-2017. We assessed blood samples (n=1093; 61.1% women; aged 37-53 years) for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and estimated NAFLD prevalence using the liver fat score. We used generalized linear and logistic models to estimate the difference-in-difference effect of *Atole* from conception to two years on NAFLD.

Results: Median ALT and AST were 19.7 U/L (interquartile range, IQR: 14.1, 27.4) and 26.0 U/L (IQR: 21.4, 32.8), respectively. The median NAFLD liver fat score was 0.2 (IQR: -1.2, 1.6) in women and -1.2 (IQR: -2.2, 0.5) in men ($p<0.0001$). The prevalence of NAFLD was 67.4% among women and 39.5% among men ($p<0.0001$). The association between *Atole* exposure from conception to two years and NAFLD was not significant (OR: 0.90, 95% CI: 0.50-1.63).

Conclusions: NAFLD prevalence among Guatemalan adults exceeds the global average. Protein-energy supplementation in early life was not associated with later NAFLD. There is a need for further studies on the causes and onset of NAFLD throughout the life course.

4.2 Introduction

Non-alcoholic fatty liver disease (NAFLD)[1] is the hepatic manifestation of metabolic syndrome. The long- term consequences of NAFLD include increased risk of end-stage liver disease, liver cancer, and cardiovascular disease.[1] The global prevalence of NAFLD has risen to 25%, with some of the highest estimates reported in South and Central America.[2,3] In Guatemala, one study reported a prevalence of 56.5%.[4] Known risk factors for NAFLD include genetics, age, nutrition, and obesity. Individuals with adipogenic genes such as Patatin-like phospholipase domain-containing protein 3 (PNPLA3), which is common in Hispanics, have a higher risk for NAFLD.[5]

The clinical manifestation of NAFLD in children and the presence of steatosis at birth in some newborns suggest that its origins also may also lie in utero.[6-9] Maternal restriction of calories and protein have been associated with increased hepatic fat in offspring in animal models.[10] In rats, maternal restriction of protein during pregnancy and lactation is associated with hepatic steatosis in offspring.[11-15] Furthermore, a high fat diet intensified the effects of perinatal protein restriction on liver outcomes.[12,14] The combination of early life undernutrition and later exposure to an obesogenic environment, characteristic of many countries undergoing the nutrition transition, may therefore contribute to the risk of developing NAFLD.[16]

The impact of undernutrition in early life on later NAFLD has been studied in two cohorts. Exposure to the Great Chinese Famine in early life was associated with an increased risk of NAFLD in adulthood.[17,18] In a study using the Helsinki Birth Cohort (n=1587), individuals who were in the smallest body mass index (BMI) tertile in early childhood and obese as adults had the greatest risk of NAFLD.[19]

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[1] NAFLD: non-alcoholic fatty liver disease, ALT: alanine aminotransferase, AST: aspartate aminotransferase, INCAP: Institute of Nutrition of Central America and Panama, IQR: interquartile range, OR: odds ratio, BMI: body mass index, SES: socioeconomic status

Other studies have found associations between NAFLD and proxies of in utero malnutrition (such as being small for gestational age and of low-birth weight), as well as accelerated growth in the first three months in early life.[20-22] The effect of protein-energy intake in early life on later NAFLD has not been described in humans.[23]

In the 1960s and 70s, the Institute of Nutrition of Central America and Panama (INCAP) conducted a nutrition supplementation trial in four villages in southeastern Guatemala to assess the effect of improved nutrition on child growth and development. Children in the improved nutrition group had greater total intake of protein (9 g/day) and energy (100 kcal/day) compared to the control group.[24,25] The population in these villages was undernourished; about 45% of children under three years had severe stunting and 86% had any stunting at 24 months.[25,26] At follow-up in 2015-2017, there was a high prevalence of cardiometabolic diseases in the cohort, including 32% with obesity and 67% with metabolic syndrome.[27] We assessed the prevalence of NAFLD and related markers of NAFLD, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), in the cohort in mid-adulthood. Additionally, we investigated the impact of exposure to a protein-energy supplement from conception to age two years on NAFLD. This period of early life, also known as the first 1000 days, is a time of rapid development and a crucial window for influencing later health.[28] We hypothesized that exposure to increased protein and energy nutrition during early life would result in decreased risk of NAFLD later in life.

4.3 Material and methods

4.3.1 Study population

Between January 1, 1969 and February 28, 1977, two pairs of size-matched villages were randomized to receive either *Atole* (a protein and energy-containing supplement) or *Fresco* (a no-protein low-energy supplement). *Atole* was a nutritional supplement (6.4g protein, 0.4g fat, and 90 kcal per 100 mL) made from dry skimmed milk, sugar, and *Incaparina*, a vegetable protein mixture developed by

INCAP. *Fresco* contained sugar water and flavoring (33kcal per 100 mL) and had no protein. *Atole* and *Fresco* were fortified to have matching micronutrient content. The supplements were available to everyone in each village at a central location twice every day. Study staff in the original trial recorded supplement intake for pregnant and lactating women and children under 7 years. The full study design has been previously published.[29]

A follow-up was conducted in 2015 to 2017 to examine the cardiometabolic health of the cohort. Of the original 2392 participants enrolled in the nutrition-supplementation trial, 1161 were not lost to follow-up and provided informed consent. Of these participants, 1118 provided at least one plasma sample (*Figure 4-1*). Characteristics of those lost to follow-up in comparison to those who participated have previously been published.[27]

Of the 1118 individuals who provided plasma samples, for the present study we excluded six who were pregnant or lactating. Since alcohol consumption also impacts liver function and can lead to steatosis, we additionally excluded six individuals who reported alcohol consumption >21 drinks/week in men and >14 drinks/week in women.[1] Finally, we excluded 13 individuals for missing the NAFLD outcome.

This study and the informed consent process were approved by the Institutional Review Boards of Emory University (Atlanta, GA) and INCAP (Guatemala City, Guatemala).

4.3.2 Data collection and lab assays

In addition to the birth village and date of birth, the original study collected data on maternal characteristics such as age, height, and schooling years. In the 2015 to 2017 wave of data collection, we obtained data on participant characteristics such as years of schooling, socioeconomic (SES) status, residence in Guatemala City, and self-reported alcohol intake. Trained field workers and phlebotomists collected anthropometric measurements and fasting blood samples, as previously described.[27]

Plasma samples were frozen for storage at -80°C until being shipped on dry ice to a biomarker core laboratory in the United States. The samples were thawed at 4°C in batches, each containing approximately 40 plasma samples. Plasma ALT and AST values were assessed using the AU480 analyzer (Beckman Coulter Diagnostics, Fullerton CA, US) using enzymatic methods (Sekisui Diagnostics P.E.I. Inc., Canada). Insulin was assayed using immunoturbidimetric methods (Kamiya Biomedical Company, WA, US).

4.3.3 Variable specification

Exposure

We determined exposure to *Atole* or *Fresco* by birth village. We used the child's birth date and the trial start and end dates to determine the age of exposure as previously described.[27] Early exposure was defined as 1000 days from conception (assumed to be 266 days before the birth date to approximate the average length of pregnancy) to age two years. In our main analysis, we considered individuals who were exposed for the entire period from conception to age two years to be fully exposed and those who were exposed for only part of that time to not be exposed. For sensitivity analysis, we also created a three-category exposure variable: those who were fully exposed, those who were partially exposed, and those without any exposure from conception to age two years.

NAFLD and Related Outcomes

We used the NAFLD liver fat score equations developed by Kotronen et al. to determine the presence of hepatic steatosis (liver fat score) and its quantity (liver fat percent).[30] The equations include metabolic syndrome, type 2 diabetes, fasting insulin, fasting AST, and the AST/ALT ratio. A higher score indicates greater steatosis. We used the optimal cut off point of -0.640 for the NALFD liver fat score to define the presence of suspected NAFLD. This cut off was determined by Kotronen et al. using the Youden index.[30]

We defined metabolic syndrome by the presence of at least three of the following criteria: 1) central obesity (waist circumference >88 cm in women, >102 cm in men), 2) elevated fasting glucose (≥100 mg/dL) or use of diabetes medication, 3) elevated triglycerides (≥150 mg/dL) or statin use, 4) low high density lipoprotein (HDL cholesterol (<40 mg/dL in men, <50 mg/dL in women), and 5) hypertension (≥85 mm Hg diastolic) or hypertension medication use.[31] We defined type II diabetes as a fasting plasma glucose ≥126 mg/dL, a post challenge glucose of ≥200 mg/dL or the use of diabetes medication.[32] We calculated the AST/ALT ratio by dividing AST by ALT.

Covariates

We calculated body mass index (BMI) by weight in kilograms divided by height in meters squared.[33] We calculated the waist-to-height ratio by dividing the waist circumference in centimeters by height in centimeters. We categorized childhood and adulthood socioeconomic status into tertiles. We had previously derived socioeconomic status from a principal component analysis of household characteristics and consumer durable goods.[27] We characterized current residence dichotomously as Guatemala City or other to capture rural vs urban location. We included age at follow-up and completed grades of schooling for mothers and their children as continuous variables.

4.3.4 Statistical analysis

We calculated median and interquartile range (IQR) values for continuous variables and percent for categorical variables. We used Wilcoxon Rank Sum tests to compare differences in medians between men and women for continuous clinical covariates and NAFLD related outcomes. We used a Pearson chi-square test to compare NAFLD, type II diabetes, and metabolic syndrome percent in men and women.

We used multiple imputation to deal with missing data for maternal height (20.5% missing), maternal years of schooling (3.5% missing), and maternal age (1.6% missing). We used the fully

conditional specification method for five imputations. To impute missing values, we used predictive mean matching for continuous variables and the logistic regression method for ordinal variables. We included all predictor and outcome variables that were not linear effects of each other as predictors in the imputation model.[34]

We used generalised regression models to estimate the difference-in-difference intent-to-treat effect of *Atole* vs *Fresco* from conception to age two years, net of the differences attributable to village or birth year effects. We estimated the difference-in-difference effect by an interaction term between the type of exposure (*Atole* vs. *Fresco*) and the timing of exposure (during the full first 1000 days versus other). The base model included birth village in the form of three dummy variables (this also serves to account for treatment assignment of *Atole* vs *Fresco*), a dichotomous variable to account for duration of exposure (full vs other), birth year, maternal height (as an indicator of maternal nutrition status), maternal years of schooling, sex, and childhood SES. We additionally controlled for several adult sociodemographic and clinical factors including SES status, years of schooling, residence, BMI, waist-to-height ratio, and height. We considered sex as a potential effect modifier. We tested for stratum heterogeneity by examining the significance of the third-order interaction between sex, exposure type and duration.

We conducted all statistical analysis in SAS version 9.4. We used PROC GENMOD, for modeling and PROC MI and PROC MIANALYZE for multiple imputation. Statistical significance was determined by $p<0.05$ and p-values were two-sided.

4.4 Results

The final sample size was 1093 individuals (61.1% women) (*Table 4-1).* Selected characteristics of the study population are in *Table 4-1*. The median age at follow-up was 44 years (IQR 41-47). Twenty-

two percent of participants were exposed to *Atole* during the full period from conception to age two years. An additional 17.8% were exposed to *Atole* for part of the period from conception to age 2 years.

The median NAFLD liver fat score was 0.2 (IQR: -1.2, 1.6) in women and -1.2 (IQR: -2.2, 0.5) in men ($p<0.0001$). The median liver fat percent was 5.4% (IQR: 3.1, 8.0) in women and 3.0% (IQR: 2.0, 6.) in men ($p<0.0001$) (*Table 4-1; Figure 4-2*). Median ALT was 19.7 U/L (IQR: 14.0, 27.4), median AST was 26.0 U/L (IQR: 21.3, 32.8), and the median AST/ALT ratio was 1.3 (IQR 1.0, 1.8). Men had significantly higher ALT and AST values ($p<0.0001$). The prevalence of NAFLD was 56.5% and was higher in women (67.4%) than men (39.5%; $p<0.0001$).

The assocations between exposure to *Atole* in early life and NAFLD-related parameters are provided in **Table *4-2***. The estimates for ALT, AST, AST/ALT ratio, liver fat score, and liver fat percent were not statistically significant in the base model or with additional adjustment for adult factors. Similarly, the odds ratio for NAFLD was not significant in the base model (OR: 1.26, 95% CI: 0.75 to 2.11) or after accounting for adult factors (OR: 0.90, 95% CI: 0.50 to 1.63) (**Table *4-3***). There was no significant stratum heterogeneity by sex. In sensitivity analysis, we found no significant differences in the models for those with partial vs no exposure and full vs no exposure.

4.5 Discussion

We describe the prevalence of NAFLD among a cohort that participated in an improved protein-energy supplementation trial during early life in Guatemala. Their prevalence of NAFLD in mid-adulthood was 56.5% and was significantly higher in women compared to men. Exposure to the protein-energy supplement from conception to two years was not associated with NAFLD in mid-adulthood.

The NAFLD prevalence we report is consistent with the only other study on NAFLD prevalence in Guatemala, despite the use of different NAFLD criteria [4]. Rivera-Andrade used the Fatty Liver Index

and found a NAFLD prevalence of 66.5% and 50.6% in women and men over 40 years old, respectively.[4] NAFLD is usually more common in men, although this difference is less pronounced with age.[35] Women in our study had a higher prevalence of metabolic syndrome and type II diabetes, which could partially explain their higher NAFLD prevalence, especially since we used a NAFLD score that includes type 2 diabetes and metabolic syndrome in its calculation.

In a previous publication using the same population, the *Atole* supplement had mixed effects on cardiometabolic outcomes. It was associated with increased odds of obesity (OR: 1.99, 95% CI: 1.16 to 3.41), reduced odds of diabetes (OR: 0.47,95% CI: 0.22 to 0.99), and was not significantly associated with metabolic syndrome.[27] The diverging associations between early life nutrition and later cardiometabolic conditions may be due to various interlinked mechanisms, including the role of protein in directing the development of metabolically active tissues.[36]

Additionally, we may have failed to observe a significant association between early life protein-energy supplementation and adulthood NAFLD because the lifetime cumulative effect of other factors such as BMI trajectory or diet that dominate over the effect of early life exposure to *Atole*. Excluding those with obesity, which might overpower any effects from early life, did not meaningfully change our estimates.

Animal models have linked early life protein and energy restriction to later NAFLD and described potential pathways through which this occurs. In rats, maternal protein restriction results in upregulation of the transcription factors sterol regulatory element binding protein (SREBP-1c), carbohydrate-responsive element-binding protein (ChREBP) and peroxisome proliferator-activated receptor-γ (PPAR-γ). This effects downstream genes important to lipid metabolism and insulin signaling.[11,14,15] At the organ level, growth restricted offspring exhibit fewer but larger hepatic lobules and enzymatic alterations, which, along with a decreased pancreatic b-cell mass, lead to insulin resistance.[37,38]

In the liver, insulin resistance inhibits b-oxidation and leads to decreased output of lipids. This, along with the increase of free fatty acid influx into the liver due to insulin resistance, results in steatosis of the liver.

There are several strengths in our study. One strength is the sample size and use of multiple imputation to account for missing data to maximize model size. Although attrition is a concern as about 50% of the original cohort were in the 2015-2017 wave of data collection, there is no evidence that attrition has affected the validity of this study. Ford et al. found that attrition was not differential with respect to *Atole* exposure from conception to age two years. Additionally, individuals lost to follow-up were similar to those who participated in the 2015-17 study, except for sex.[27]

Another strength of this study is the use of the composite NAFLD liver fat score. ALT levels alone have poor sensitivity and specificity in adults.[39,40] Therefore, scores like the NAFLD liver fat score are useful as non-invasive predictors.[30] The original NAFLD liver fat score was developed in a large Finnish cohort in 2009 so it is possible it is not transferable to other populations. However, when compared to three other NAFLD prediction scores, the NAFLD liver fat score had the best non-invasive prediction score for NAFLD and associated mortality including in Hispanics.[41]

There are a few limitations in our study. The ideal exposure period may only be a subset of the first 1000 days such as during liver organogenesis in utero, which begins at four weeks and continues throughout gestation. In our study, it is not possible to examine exposure during pregnancy and early childhood separately due to the limited sample size; 3.7% (n=40) of the original cohort were exposed to *Atole* only in pregnancy. Additionally, sufficient data were not available in our cohort on variables that have been previously associated with NAFLD including gestational age and birthweight of which 63% and 57% were missing, respectively. Among those with data available about 10% were born pre-term (<37 weeks gestation) or with low birthweight (<2.5kg).

Conclusions

Understanding the developmental origins of NAFLD and other metabolic conditions is particularly important because of the growing prevalence of these diseases.[10] The high prevalence of NAFLD in Guatemala highlights the need for population-based studies that will help identify the underlying mechanisms of the onset and development of NAFLD throughout the life course, including and beyond early life nutritional exposure alone.

4.6 References

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4.7 Tables & Figures

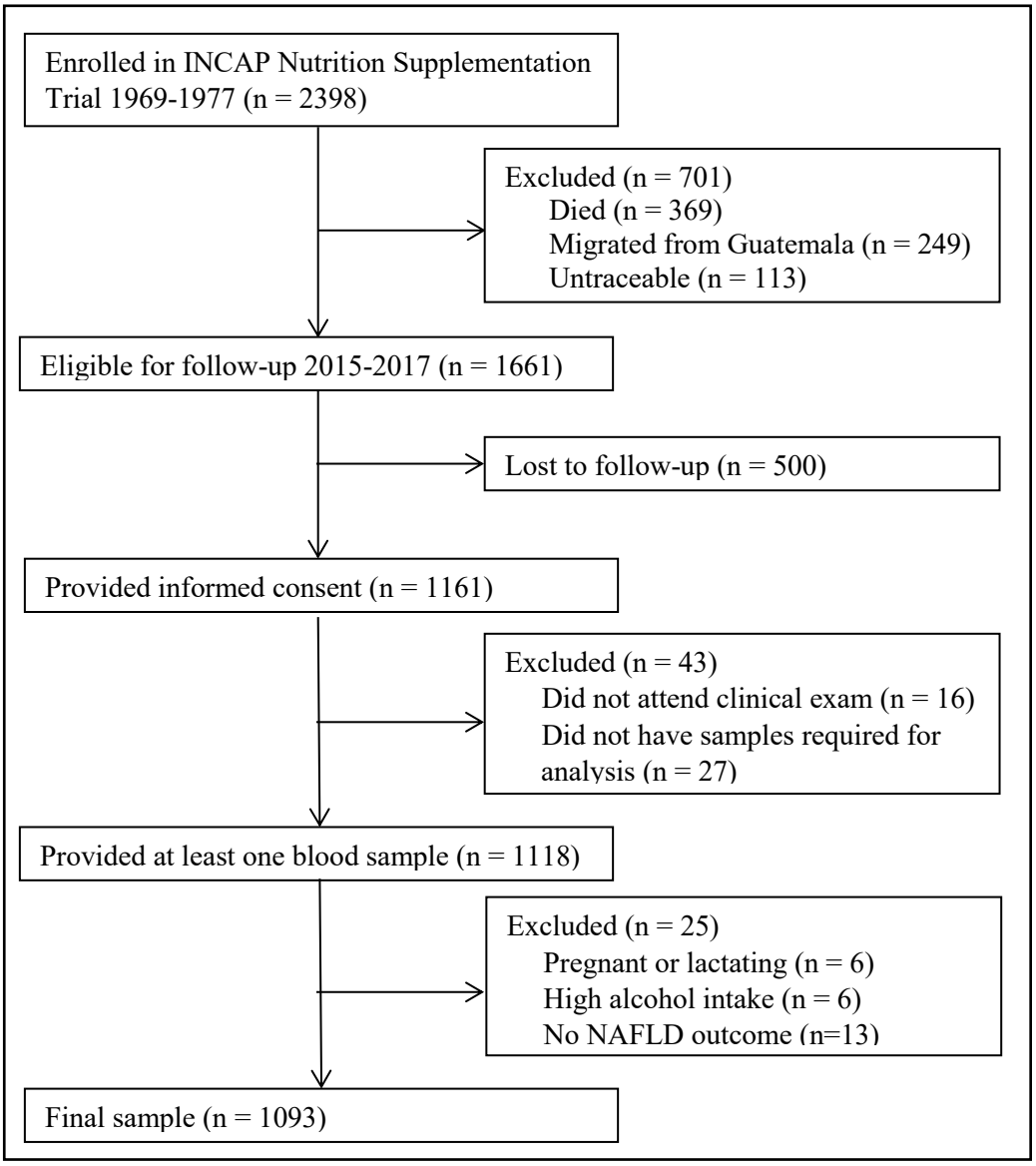

Figure 4-1. *Flow diagram*

Flow diagram of original 1969–1977 INCAP Guatemala study to 2015–2017 data collection and analysis. Of the original 2398 participants 369 had died, 249 had migrated outside Guatemala, and 113 were untraceable resulting in 1661 who were eligible for enrollment in 2015–2017. Of these, 1118 provided informed consent and provided at least one sample during the clinical exam. During analysis we excluded an additional 6 for pregnancy/lactation status, 6 for high alcohol intake, and 13 for not having data on the non-alcoholic fatty liver disease (NAFLD) outcome for a final analytic sample size of 1093.

Table 4-1. Sociodemographic and clinical characteristics (n=1093) in the INCAP cohort

	Pooled (n=1093)	Women (n=668)	Men (n=425)
	Median (IQR) or %	**Median (IQR) or %**	**Median (IQR) or %**
Maternal characteristics			
Maternal age, years[1]	26 (21, 32)	26 (21, 32)	25 (21, 32)
Maternal height, cm[1]	148.7 (145.4, 152.3)	148.7 (145.3, 152.2)	148.8 (145.7, 152.6)
Maternal schooling, years[1]	1 (0, 2)	0 (0, 2)	1 (0, 3)
Participant characteristics			
Age at follow-up, years	44 (41, 47)	44 (41, 47)	44 (40, 47)
Schooling, years	3 (2, 6)	3 (2, 6)	3 (2, 6)
Residing in Guatemala City (%)	18.9	18.4	19.5
Atole from conception to 2 yrs (%)	22.3	22.6	21.2
Clinical[3]			
Height, cm	155.3 (150.1, 162.2)	151.5 (148, 155.1)	163.9 (159.6, 167.8)
Waist circumference, cm	97.5 (90.8, 105.6)	100.3 (93.3, 108.2)	94.0 (87.5, 100.6)
Body mass index, kg/m2	27.7 (24.8, 31.1)	28.7 (25.7, 32.1)	26.4 (23.8, 29.1)
Waist-to-height ratio	0.6 (0.6, 0.7)	0.7 (0.6, 0.7)	0.57 (0.5, 0.6)
Insulin, mU/L	12.5 (7.7, 20)	14.7 (9.1, 22.3)	9.7 (6.3, 16.5)
Type II Diabetes (%)	13.5	16.4	8.7
Metabolic Syndrome (%)	56.1	69.5	35.1
ALT, U/L	19.7 (14.1, 27.4)	18.8 (13.4, 26.4)	21 (15.3, 29.7)
AST, U/L	26.0 (21.4, 32.8)	25.2 (20.7, 31.8)	27.3 (22.6, 33.5)
AST/ALT	1.3 (1, 1.8)	1.3 (1, 1.8)	1.3 (1, 1.7)
Liver Fat Score[2]	-0.3 (-1.7, 1.3)	0.2 (-1.2, 1.6)	-1.2 (-2.2, 0.5)
Liver Fat Percent[2]	4.5 (2.4, 7.4)	5.4 (3.1, 8)	3.0 (2.0, 6.0)
NAFLD (%)[2]	56.5	67.4	39.5

[1]Maternal age (n=1075), maternal height (n=869), maternal schooling (n=1054) and post-challenge glucose (n=1011) were missing values.

[2]Calculated using fasting insulin, type II diabetes, metabolic syndrome, fasting AST, and fasting AST/ALT ratio[1].

[3]$p<0.0001$ for all clinical covariates and NAFLD outcomes comparing men and women (except for AST/ALT $p=0.04$).

Abbreviations: ALT= alanine aminotransferase, AST= aspartate aminotransferase; AST/ALT=ratio between AST and ALT, NAFLD=non-alcoholic fatty liver disease, IQR=interquartile range

Table 4-2. Adjusted[1] linear difference-in-difference estimates for exposure to *Atole* from conception to age 2 years vs other, by non-alcoholic fatty liver disease related outcomes (n=1093)

	Pooled			Women			Men		
	β	95% CI	p-value	β	95% CI	p-value	β	95% CI	p-value
ALT, U/L									
Model 1	0.60	(-3.30, 4.51)	0.76	-0.79	(-5.92, 4.35)	0.76	2.87	(-3.05, 8.8)	0.34
Model 2	-0.71	(-4.54, 3.11)	0.71	-1.18	(-6.26, 3.9)	0.65	-0.25	(-5.91, 5.41)	0.93
AST, U/L									
Model 1	1.31	(-2.31, 4.92)	0.48	1.65	(-3.11, 6.4)	0.50	0.88	(-4.64, 6.41)	0.75
Model 2	0.37	(-3.23, 3.97)	0.84	1.13	(-3.59, 5.85)	0.64	-0.67	(-6.2, 4.87)	0.81
AST/ALT									
Model 1	-0.01	(-0.16, 0.14)	0.90	0.09	(-0.09, 0.28)	0.33	-0.17	(-0.43, 0.09)	0.20
Model 2	0.01	(-0.14, 0.17)	0.86	0.10	(-0.09, 0.28)	0.31	-0.09	(-0.35, 0.17)	0.51
Liver Fat Score									
Model 1	0.32	(-0.27, 0.91)	0.29	0.36	(-0.44, 1.16)	0.38	0.28	(-0.55, 1.12)	0.51
Model 2	-0.01	(-0.52, 0.51)	0.97	0.10	(-0.63, 0.83)	0.80	-0.14	(-0.78, 0.5)	0.66
Liver Fat %									
Model 1	0.34	(-0.68, 1.36)	0.51	0.17	(-1.17, 1.52)	0.80	0.60	(-0.94, 2.15)	0.44
Model 2	-0.23	(-1.15, 0.69)	0.63	-0.22	(-1.47, 1.02)	0.72	-0.17	(-1.45, 1.11)	0.79

[1]Model 1 (base model) adjusted for birth village, duration of exposure, birth year, maternal height, maternal years of schooling, sex (in pooled models), and childhood SES. Model 2 additionally adjusted for adult SES, years of schooling, residence, body mass index, waist-to-height ratio, and height.
Abbreviations: ALT= alanine aminotransferase, AST= aspartate aminotransferase; AST/ALT=ratio between AST and ALT, CI=confidence interval, SES=socioeconomic status

Table 4-3. Adjusted[1] binomial logistic difference-in-difference OR for exposure to *Atole* for full period from conception to age 2 y vs. other, for NAFLD (n=1093)

	Pooled			Women			Men		
	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
NAFLD									
Model 1	1.26	(0.75, 2.11)	0.39	1.39	(0.71, 2.74)	0.34	1.01	(0.44, 2.30)	0.98
Model 2	0.90	(0.50, 1.63)	0.73	1.10	(0.52, 2.33)	0.80	0.57	(0.18, 1.80)	0.34

[1]Model 1 (base model) adjusted for birth village, duration of exposure, birth year, maternal height, maternal years of schooling, sex (in pooled models), and childhood SES. Model 2 additionally adjusted for adult SES, years of schooling, residence, body mass index, waist-to-height ratio, and height.
Abbreviations: OR=odds ratio, CI=confidence interval, NAFLD=non-alcoholic fatty liver disease, SES=socioeconomic status

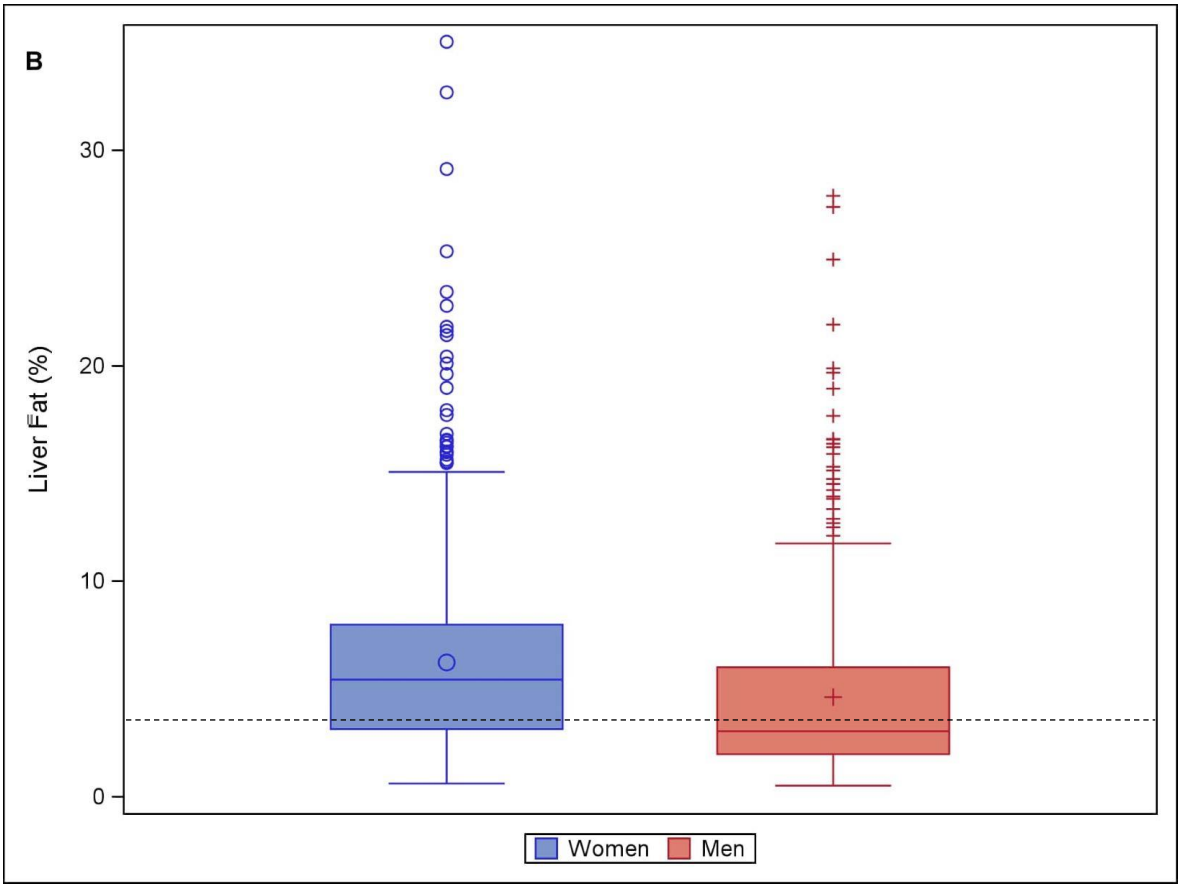

Figure 4-2. *Liver fat percent histogram by sex*

Histogram of liver fat percent by sex (blue(0) = women, red(+) = men) in the Guatemala INCAP study cohort 2015–2017 (n = 668 women, 425 men). The liver fat percent is calculated using presence of metabolic syndrome, type 2 diabetes, fasting insulin, fasting aspartate aminotransferase(AST), and fasting AST/ALT ratio. The dotted black line indicates the 5% cut off for NAFLD.

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Chapter 5: Associations of maternal diet and nutritional status with offspring hepatic steatosis in the Avon Longitudinal Study of Parents and Children

Aim 2a

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5.1 Abstract

Background & Objective Priming for cardiometabolic diseases, including non-alcoholic fatty liver disease (NAFLD), is hypothesized to begin in utero. The primary objective of this study is to determine whether there is an association between maternal nutritional status and offspring NAFLD.

Methods Data come from the Avon Longitudinal Study of Parents and Children (ALSPAC) in the UK. The analytic sample included 3353 participants who had maternal information on pre-pregnancy BMI, gestational weight gain, diabetes, and free sugar intake (percent of total energy intake) and were assessed for hepatic steatosis at 24 years by transient elastography (mild-severe; controlled attenuation parameter score ≥ 248dB/m). Multiple logistic regression was used to evaluate the association between maternal factors and offspring hepatic steatosis at 24 years.

Results In confounder-adjusted models the independent associations for each maternal factor with mild to severe vs low hepatic steatosis at 24 years were: pre-pregnancy overweight (OR: 1.84, 95%CL: 1.43-2.38) or obesity (OR: 2.73, 95%CL: 1.84-4.03), more than recommended gestational weight gain (OR: 1.30, 95%CL: 1.04-1.64), diabetes (OR: 1.39, 95%CI: 0.87, 2.21), and high free sugar intake during pregnancy (OR: 1.04, 95% CI: 0.82, 1.33). These associations were largely mediated by BMI at 24 years, but not by birthweight or breastfeeding.

Conclusions Our results suggest that maternal nutrition status is associated with the development of NAFLD in their adult offspring, although the relationship is largely mediated by offspring BMI in adulthood.

5.2 Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as having excessive hepatic steatosis in the absence of other liver disease, extreme alcohol intake, or medication-induced steatosis.[1] Established risk factors for NAFLD include increasing age, male sex, high diet sugars, and obesity. In children, the prevalence of NAFLD has increased considerably over recent decades in parallel with the rise of obesity.[2] This is concerning because pediatric NAFLD can progress to nonalcoholic steatohepatitis (NASH), which is characterized by inflammation, as well as cirrhosis and end stage liver disease in adulthood.[3] NAFLD is also associated with increased risk of diabetes and cardiovascular disease.[4,5]

The clinical manifestation of NAFLD in children and the presence of steatosis at birth in some newborns suggest that its origins may lie in utero.[6-9] The Developmental Origins of Health And Disease (DOHAD) paradigm posits that environmental factors during fetal and early life program the risk of metabolic diseases including NAFLD.[10,11] Currently, the fetal environment occurs in a context of high prevalence of maternal obesity as well as high sugar intake that continues to increase globally.[12,13] Studies in animal models have shown that maternal diets high in sugar and fat predispose offspring to developing NAFLD phenotypes.[14-19] In humans, a maternal diet high in added sugar intake during pregnancy has been associated with an increased risk of obesity in children.[20,21] While no human studies have directly looked at the association between maternal energy rich diets and offspring NAFLD explicitly, pre-pregnancy obesity and overweight, maternal diabetes, and gestational weight gain have all been associated with increased hepatic fat in infants and adolescents.[7,8,22-24] There is evidence that breastfeeding has a protective effect on hepatic steatosis in individuals that were exposed to excess nutrition in utero.[22] However, whether outcomes are dependent on child sex and the impact of childhood factors such as

adiposity in mediating the outcome have been inconsistent. It is also not known whether this association extends into adulthood.

The overall aim of this paper is to explore whether the associations between maternal nutritional status and offspring NAFLD extend into adulthood in the Avon Longitudinal Study of Parents and Children (ALSPAC), based in the UK. Secondary aims are to determine whether there is sexual dimorphism in these associations and whether the associations are mediated by birthweight, breastfeeding, or BMI at time of outcome.

5.3 Methods:

5.3.1 Study population

We used data from the Avon Longitudinal Study of Parents and Children, a population-based birth cohort study that has previously been described in detail.[25-27] In summary, ALSPAC enrolled 14,541 pregnant women in the greater Bristol, UK area with expected delivery dates between 1st April 1991 and 31st December 1992. At the age of seven, attempts were made to boost the sample, resulting in a total of 15,454 pregnancies and 14,901 children alive at the age of one year. Clinical, dietary, and demographic information was collected from the mothers starting in pregnancy. When the offspring were 24 years of age, 10,018 participants were invited to a clinic visit known as Focus@24, which included the collection of biological samples and anthropometric measures. Data from the 24-year clinic were collected and managed using REDCap electronic data capture tools hosted at the University of Bristol.[28,29] The study website contains details of all the data that are available through a fully searchable data dictionary and variable search tool.[30]

Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Informed consent for the use of collected data via questionnaires

and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time.

5.3.2 Assessment of maternal nutrition

Pre-pregnancy BMI

Maternal pre-pregnancy weight and height were self-reported via postal questionnaire and were used to calculate body mass index (BMI) as weight in kilograms divided by height in meters squared.[31] We classified BMI according to World Health Organization categories as underweight (<18.5 kg/m^2), normal (18.5 to <25 kg/m^2), overweight (25 to <30 kg/m^2), and obese (≥ 30 kg/m^2).[32]

Gestational weight gain (GWG)

Gestational weight and the corresponding gestational age and date were abstracted by trained research midwives from obstetric medical records.[33,34] As previously described, weight gain was predicted from linear spline models as the difference between predicted weight at time of delivery and weight at gestational age of 0 weeks.[33,34] Women were then categorized into three categories according to Institute of Medicine (IoM) recommendations: adequate, less than, and more than recommended GWG. Recommended weight gain is 12.5–18 kg for underweight; 11.5–16 kg for normal weight; 7–11.5 kg for overweight; and 5–9 kg for obese women.[35]

Maternal diabetes

Due to limited sample size, maternal diabetes was defined as a composite variable that included pre-existing diabetes assessed by self-reported questionnaire at time of recruitment, gestational diabetes mellitus (GDM), and glycosuria (≥ 13.9mmol/l on at least two occasions during pregnancy) abstracted from medical records, as previously described.[24]

Maternal free sugar intake

Maternal intake of 43 food groups was assessed by food frequency questionnaire at 32 weeks gestation. This information was combined with nutrient information on standardized portion sizes to calculate macronutrient intakes, as described in detail previously.[36] Non-milk extrinsic sugars (NMES) were

calculated by deducting sugars from milk and fruits and vegetables (contained within cellular walls) from total sugars.[37] This is equivalent to the definition of free sugars which includes isolated sugars added during food preparation and manufacturing (added sugars) as well as sugars present in unsweetened fruit juices, fruit concentrates, or honey and other syrups.[38] We calculated the percent of total energy consumed as free sugars by dividing each participant's estimated non-milk extrinsic sugar intake by total energy intake and categorized it into tertiles (hereafter referred to as percent free sugar).

5.3.3 Assessment of liver outcomes

At 24 years old, participants were assessed by transient elastography for non-invasive quantification of liver steatosis and fibrosis (FibroScan® 502 Touch, Echosens, Paris, France). Individuals with an active medical implant (e.g. pacemaker), liver ascites, or who were pregnant were excluded from the liver scan. Participants were asked to fast overnight or for at least six hours prior to transient elastography.[39] Transient elastography provides a controlled attenuation parameter (CAP) measure of steatosis and a measure of liver stiffness to quantify fibrosis. Manufacturer and machine indications were used to conduct the scan. Ten readings were required for each patient to derive a CAP score and fibrosis result. CAP values outside the 100-400 dB/m range were considered invalid and coded as missing. Median fibrosis results greater than or equal to 15 kPa or with an interquartile range (IQR) to median ratio greater than or equal to 30% were considered invalid and coded as missing.

We categorized participants into two categories of steatosis based on CAP score cut-off values derived from a meta-analysis by Karlas, et al: low (<248 dB/m, <11% steatosis) vs mild to severe (248-400 dB/m, ≥11% steatosis).[40] In sensitivity analysis we also categorized steatosis as low to moderate steatosis (< 279 dB/m, < 66% steatosis) vs severe steatosis (279-400 dB/m, ≥66% steatosis). We categorized fibrosis into two groups. The first group included those with no fibrosis or portal fibrosis without septa (F0-F1, <7.9 kPA) and the second group included those with any fibrosis: portal fibrosis, septa, or cirrhosis (F2-F4, >7.9 kPA).[41]

5.3.4 Covariates

Maternal age at delivery was derived from the mother's report of her date of birth and the infant's date of birth. Offspring birthweight and sex were extracted from medical records. We used highest level of maternal education reported during pregnancy as a proxy for socioeconomic status.[42] Mothers self-reported one of five categories: None/CSE (certificate of secondary education), Vocational (vocational courses after 16 years of age), O (ordinary level exams at 16 years), A (optional advanced level exams at 18 years), and University degree and above.[43] Mothers' self-reported smoking (1st and 3rd trimester) and alcohol intake (1st, 2nd, and 3rd trimester); we categorized each as none vs any at any time point in pregnancy. Physical activity was assessed via questionnaire in the first trimester and was categorized as at least once per week versus less than weekly. Infant birthweight was abstracted from medical records and we classified it as low (<2500g), normal, and high (>3999g).[44] Breastfeeding duration was assessed from maternal reports when the child was 15 months old and categorized as never, less than 3 months, 3 to 6 months, and greater than 6 months. Offspring BMI at 24 years was calculated from height and weight collected by standardized clinic protocols and categorized as described above for the mothers. We defined hazardous alcohol consumption as an Alcohol Use Disorder Identification Test for Consumption (AUDIT-C) score greater than or equal to four in women and five in men.[45,46]

5.3.5 Inclusion/Exclusion

We included all participants who attended the Focus@24 visit and had valid transient elastography measures. We excluded participants from twin pregnancies and those who were missing all four maternal exposures (pre-pregnancy BMI, gestational weight gain, diabetes, and percent free sugars). In models with percent free sugar as the primary exposure, we additionally excluded those with mothers who had pre-existing or gestational diabetes since this could alter their consumption patterns.

5.3.6 Statistical analysis

We conducted statistical analyses in SAS version 9.4 (Cary, NC). We calculated median values and IQR for continuous variables and counts and percentages for categorical variables, for the full sample and stratified by level of hepatic steatosis (low vs mild to severe) at 24 years. Wilcoxon rank sum (equal variance) and Kolmogrov-Smirnov (unequal variance) tests were used to compare differences in continuous variables across hepatic steatosis levels. Chi-squared tests were used to compare differences between categorical variables.

Figure 5-1 shows a simple conceptual model on which our modeling strategy was based. We assessed each exposure for potential effect modification by sex by including an interaction term between sex and the exposure. If this term was not significant for all exposures, sex was added as a term in the adjusted models. The primary outcome was mild to severe hepatic steatosis. Unadjusted binary logistic regression was conducted for each exposure in the model 1 series: pre-pregnancy BMI, maternal diabetes, gestational weight gain, and percent free sugar intake.

In the model 2 series we adjusted for confounders: maternal age, highest level of maternal education, maternal smoking in pregnancy, alcohol intake in pregnancy, and physical activity in pregnancy. Model 2a included pre-pregnancy BMI, maternal diabetes, and gestational weight gain. Model 2b, which focused on percent free sugar exposure, additionally adjusted for total energy intake.

If the association between exposure and steatosis remained after confounder adjustment, we considered the following potential mediators: a) birthweight, b) breastfeeding, and c) offspring BMI at 24 years.

A sensitivity analysis was performed with the primary outcome defined as severe steatosis. In addition, to understand the impact of alcohol consumption, an important predictor of hepatic steatosis, we added hazardous alcohol intake at 24 years as a covariate in the models.

5.4 Results

Of the 10,018 ALSPAC participants who were invited to attend the Focus@24+ visit, 3,877 (39% participants had FibroScan® performed. Of these, 3,766 (97%) participants had a valid CAP score. After exclusions for twin pregnancies and those missing all four maternal exposures, our sample size was 3,353 (86% of those with FibroScan®, *Figure 5-2*). Demographic characteristics of the sample overall and stratified by offspring steatosis are presented in *Table 5-1*. Approximately 20% of the offspring had hepatic steatosis at 24 years. Among the mothers, 16.7% were overweight or obese pre-pregnancy, less than 4% had diagnosed pre-existing diabetes, gestational diabetes or glycosuria, and over half had greater than recommended gestational weight gain. Those in the lowest tertile of percent free sugar intake had values ranging from 1.3% to 10.4%, middle to 14.3%, and upper tertile to 42.2%. The median maternal age was 29 years (IQR: 26.0, 32.0). Lower maternal education, smoking during pregnancy, and no breastfeeding were more prevalent in the mothers of offspring with steatosis. Most participants were female (62.2%), with male sex more strongly associated with hepatic steatosis. Over a third (37.5%) of participants were overweight or obese at age 24. Only 2.4% had hepatic fibrosis.

There was no heterogeneity by sex for any exposure-outcome associations. The results from logistic regression models are presented in *Table 5-2*. In both unadjusted and confounder-adjusted analysis, maternal pre-pregnancy overweight (aOR: 1.84, 95% CI: 1.43-2.38), obesity (aOR: 2.73, 95% CI: 1.84-4.03) and more than recommended GWG **(**aOR: 1.30, 95% CI: 1.04-1.64) were positively and independently associated with offspring hepatic steatosis. Being in the highest tertile of percent free sugar consumption was not associated with offspring hepatic steatosis as compared to offspring of mothers in the lowest tertile (aOR: 1.04, 95% CI: 0.82-1.33).

Birthweight (*Table 5-2*, *column 3a*) and breastfeeding (*Table 5-2*, *column 3b*) were not important mediators in the relationship between maternal factors and offspring steatosis. However, these associations were largely attenuated after adjusting for offspring BMI category at 24 years (*Table 5-2*, *column 3c*), indicating that they are largely mediated by offspring adiposity.

In sensitivity analysis, adjusting for hazardous alcohol intake among offspring did not significantly change the associations (

Table 5-3). The associations between maternal factors and hepatic steatosis were strengthened when we redefined the outcome to severe steatosis, although these associations were also completely mediated by offspring BMI (*Table 5-4*).

5.5 Discussion

Maternal pre-pregnancy overweight, obesity, and more than recommended gestational weight gain were positively and independently associated with offspring hepatic steatosis at 24 years in confounder-adjusted models. These associations were fully mediated by offspring BMI at 24 years.

This is the first study to look at whether the associations between maternal nutritional exposures and offspring hepatic steatosis extend into adulthood and also the first to directly explore associates with maternal free sugar intake. Several previous studies have looked at the associations of maternal BMI, diabetes, and weight gain and offspring NAFLD in children and adolescents.[22-24,47,48] (*Table 5-5*) In the RAINE cohort, which included 1170 adolescents of European descent in Australia, NAFLD was associated with maternal obesity and gestational weight gain but not with maternal diabetes, and associations were stronger in females.[23] Breastfeeding for over 6 months, especially when combined with delayed introduction of infant formula milk, had a protective associated with NAFLD in this cohort.[22]

Breastfeeding, which may influence NAFLD through the gut microbiome, has also been associated reduction in the risk of NASH in children and adolescent.[49,50] In our study, outcomes were not mediated by breastfeeding duration and we did not see differences by sex, except that males had greater prevalence of hepatic steatosis at age 24 years. It is possible we did not see a similar protective effect of breastfeeding on NAFLD because we were not able to further adjust for exclusive breastfeeding.

In the EPOCH cohort based in the US, adolescent hepatic steatosis quantified by MRI was strongly associated with maternal pre-pregnancy obesity and was largely mediated by offspring adiposity at time of outcome.[48] Maternal diabetes was not significantly associated with later NAFLD. In contrast to the other cohorts and our study in the same ALSPAC cohort, Patel et al, found a strong association between maternal diabetes and offspring hepatic steatosis at 17 years that was not mediated by offspring adiposity or BMI.[24] This difference is likely not due to differences in methodology. In both our study and the Patel study, maternal diabetes was defined consistently. While hepatic steatosis was measured differently, by ultrasound at 17 years and controlled attenuation parameter at 24 years, these methods have similar sensitivity and specific in detecting hepatic steatosis so we would not expect such a large increase in prevalence of hepatic steatosis due to assessment method alone.[51] Our statistical models were also similar. However, at 17 years a smaller subset of the cohort (n=1,215 of 4,253 that attended the 17-year clinic) was screened for hepatic steatosis, so selection bias is possible. Another possibility for this difference in outcomes may be related to the large increase in prevalence of hepatic steatosis as the cohort reached early adulthood. At 17 years, only 2.1% of a sub-sample of participants had hepatic steatosis, and by 24 years, 20% had hepatic steatosis. It is possible that early-onset steatosis has a different etiology/pathophysiology and the associations are attenuated when overall 24-year prevalence of hepatic steatosis is considered. Adulthood hepatic steatosis may be more associated with adiposity. For example, if the development of NAFLD requires obesity (particularly as individuals age), then the attenuation of estimates by adjusting for BMI is what we would expect to find, and the true association is what we derive from models not adjusting for BMI or adiposity. Future studies should explore the changes in

hepatic steatosis that occur from birth to adulthood, and specifically in the ALSPAC cohort from adolescence to adulthood.

Several studies have found associations between maternal obesity/GDM and fetal and infant hepatic steatosis (*Table 5-5*).[6-8,52,53] Modi et al, found intrahepatocellular lipid (IHCL) content in infants increased with maternal BMI.[7] They did not look at the effect of gestational diabetes due to the small number of affected women in the study sample. Brumbaugh et al., found that infants born to obese mothers with GDM had increased IHCL compared with infants born to normal weight mothers.[8] In both studies, IHCL correlated with maternal pre-pregnancy BMI but not with infant subcutaneous adiposity. In the Feeding Study cohort from Italy, mothers of 1 year old children with bright livers, a sign of hepatic steatosis, had greater gestational weight gain compared to mothers of those without bright livers.[53] Logan, et al., found no association of maternal GDM, with infant IHCL.[52] In that study, the GDM mothers had good glycemic control (55% received metformin and/or insulin treatment resulting in a mean(SD) 5.3% (0.3) HbA1c) and little obesity (median BMI=24.2, IQR (21.7, 30.3), whereas glycemic status was not known in the other studies. Finally, in a retrospective autopsy study, stillborn fetuses of diabetic mothers had 78.8% hepatic steatosis compared to 16.6% in non-diabetic mothers ($p<0.0001$) regardless of maternal BMI. Further studies of mothers with more prevalent and different types of diabetes are needed to determine whether the association of maternal diabetes with offspring NAFLD is independent of maternal obesity. Additionally, hepatic fat measures in longitudinal birth cohorts ranging from infancy and throughout childhood could clarify the question of whether elevated hepatic fat seen in infancy remains throughout childhood and their relation to overall adiposity.

We found no association between high maternal percent free sugar intake in the third trimester and offspring NAFLD. Women in the lowest tertile had intakes less than 10.4% of total energy and those in the highest tertile had intakes ranging from 14.3% to 42%. The WHO recommended cut-off for intake of free sugars is 10% of total daily energy intake.[38] While some studies have found associations between gestational diet and overall offspring adiposity, none have looked specifically at free sugars in relation to

a NAFLD outcome. Animal studies have found strong associations between maternal high fat and sugar diets and offspring hepatic steatosis. [14,15,54] The complicated nature of human diet makes it difficult to delineate interactions among nutrients. Another complicating factor is that the most sensitive period of fetal development for hepatic steatosis may be prior to the third trimester. The development of the fetal liver begins at four weeks gestation and is susceptible to fundamental changes to its metabolic pathways through epigenetic changes and mitochondrial dysfunction caused by inflammation due to excess nutrients. Maternal diabetes, obesity, and high fat/sugar diets are all characterized by increased delivery of fuels such as free fatty acids to the developing fetus.[55] Specifically, the liver may be utilized as a site for excess lipid storage, especially prior to 28 weeks gestation when subcutaneous fat storage exponentially increases.[56] Future studies should be designed to accurately measure maternal diet and biomarkers throughout pregnancy to elucidate the exact mechanism through which maternal obesity can prime offspring for later metabolic dysfunction.

Strengths and Limitations

This study contains the largest sample size to date of maternal factors and offspring NAFLD and is from a population-based longitudinal cohort study followed from pregnancy to young adulthood. Many potential confounding variables were available in the dataset. We also used a validated and accurate tool for hepatic steatosis measurement in adults (CAP score based on transient elastography).[40] We also were able to account for alcohol intake among participants. It has previously been reported that no participants had medical conditions or were taking medications that could influence hepatic function leading to greater confidence that our measure of hepatic steatosis is primarily capturing those with NAFLD.[24] While hepatic fibrosis was also measured in this study, we were underpowered to look at this as an outcome due to the small number of individuals with fibrosis.

The results of this study may not be generalizable to other populations due to the homogenous nature of the cohort population. Additionally, there was differential loss to follow-up within the cohort which could lead to selection bias. Females and participants with mothers with higher education were more

likely to participate in follow-up visits.[57] Many characteristics, including maternal diet and maternal pre-pregnancy weight, were self-reported and are subject to recall bias and social desirability bias. However, it has been shown in this cohort that maternal self-reported weight had a high correlation (r=0.95) with the weight measurement obtained at the first antenatal visit.[24] Maternal diet was collected by an FFQ that was not validated nor designed specifically to measure sugar intake. Measurement of maternal diet throughout pregnancy and with a validated FFQ may have led to different results. Because of the small sample sizes, we were not able to distinguish between types of maternal diabetes. Finally, residual confounding, particularly by lifestyle, is possible.

Conclusions

Maternal nutritional factors were positively and independently associated with offspring hepatic steatosis at 24 years, however these associations were completely mediated by offspring BMI at 24 years. Importantly, these are all modifiable risk factors and recommendations can be tailored to high-risk women to improve outcomes. Due to the high prevalence of metabolic conditions in pregnant women and the potential for transgenerational amplification of these diseases, further prospective study is needed to better understand the developmental origins of NAFLD and other metabolic conditions.[13,58]

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5.7 Tables & Figures

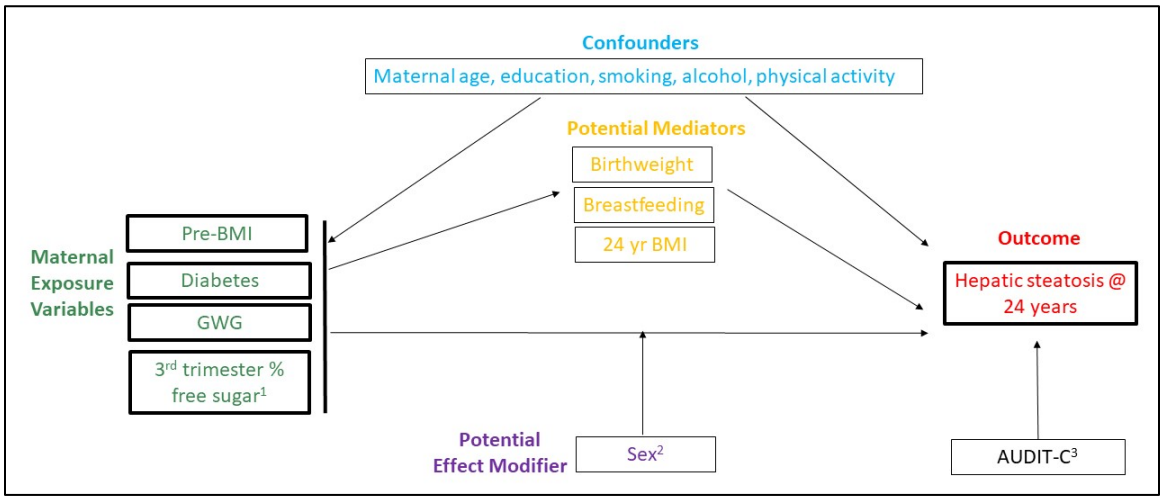

Figure 5-1. *Conceptual model relating maternal exposures to hepatic steatosis at 24 years in the ALSPAC cohort.*

[1]In models with % free sugars as the primary exposure, those with pre-existing or gestational diabetes were excluded. Total energy intake was also added as a confounder in these models.
[2]Sex was considered a confounder if the interaction term was not significant at $p<0.05$.
[3]Hazardous alcohol intake, quantified by AUDIT-C score, was adjusted for in sensitivity analysis.
Abbreviations: BMI= body mass index, GWG = gestational weight gain, BMI= body mass index, AUDIT-C = alcohol use disorders identification test consumption.

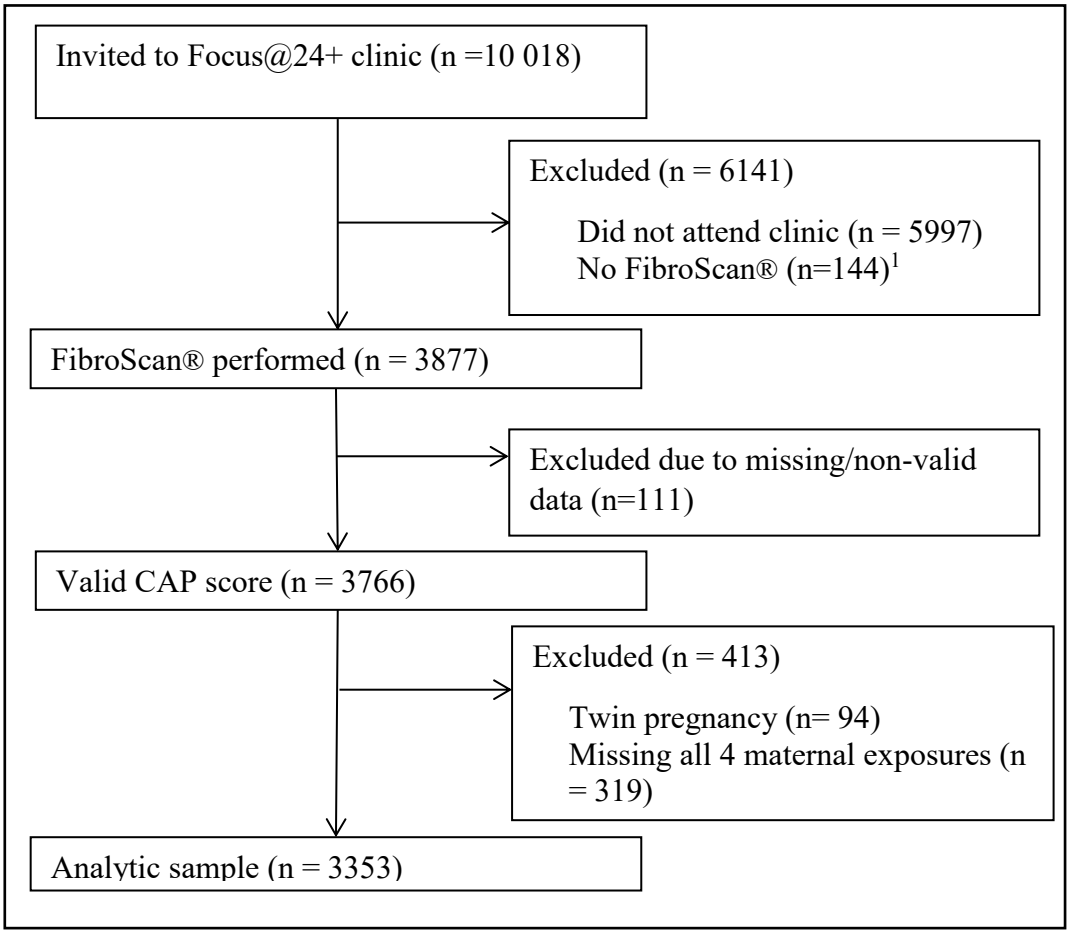

Figure 5-2. *Data flow chart for ALSPAC cohort analysis*

Of the 10,018 ALSPAC participants were invited to attend the Focus@24+ visit, 3,877 had Fibroscan for hepatic steatosis measure performed. Of these, 3,766 had a valid CAP score. After exclusions for twin pregnancies and those missing all four maternal exposures, the final sample size was 3,353. [1]No consent for liver scan, not eligible, or excluded due to active implant, liver ascites, or pregnancy.
Abbreviations: CAP= controlled attenuation parameter.

Table 5-1. Demographic and clinical factors by hepatic steatosis[1] at 24 years in the ALSPAC cohort (n=3353)

Median (IQR) or n (%)	Total (n=3353)	Low (n=2662;79.4%)	Mild-Severe (n=691; 20.6%)	P-Value[4]
Maternal & Infant Factors				
Maternal age	29.0 (26.0, 32.0)	29.0 (27.0, 32.0)	29.0 (26.0, 32.0)	**<0.001**
Maternal Highest Education				**<0.001**
Missing	65 (1.9%)	60 (2.3%)	5 (0.7%)	
CSE/None	313 (9.3%)	236 (8.9%)	77 (11.1%)	
Vocational	246 (7.3%)	178 (6.7%)	68 (9.8%)	
O-level	1113 (33.2%)	861 (32.3%)	252 (36.5%)	
A-level	939 (28.0%)	766 (28.8%)	173 (25.0%)	
Degree	677 (20.2%)	561 (21.1%)	116 (16.8%)	
Pregnancy physical activity				0.828
Missing	253 (7.5%)	201 (7.6%)	52 (7.5%)	
No	2098 (62.6%)	1672 (62.8%)	426 (61.6%)	
Yes	1002 (29.9%)	789 (29.6%)	213 (30.8%)	
Pregnancy smoking				**0.027**
Missing	11 (0.3%)	9 (0.3%)	<5[3]	
No	2799 (83.5%)	2241 (84.2%)	558 (80.8%)	
Yes	543 (16.2%)	412 (15.5%)	131 (19.0%)	
Pregnancy alcohol intake				
Missing	10 (0.3%)	9 (0.3%)	<5[3]	**0.002**
No	1823 (54.4%)	1410 (53.0%)	413 (59.8%)	
Yes	1520 (45.3%)	1243 (46.7%)	277 (40.1%)	
Maternal pre-pregnancy BMI	22.0 (20.5, 24.1)	21.8 (20.4, 23.7)	22.7 (20.9, 25.7)	**<0.001**
Missing	255 (7.6%)	208 (7.8%)	47 (6.8%)	
Underweight	127 (3.8%)	109 (4.1%)	18 (2.6%)	
Normal	2411 (71.9%)	1968 (73.9%)	443 (64.1%)	
Overweight	420 (12.5%)	294 (11.0%)	126 (18.2%)	
Obese	140 (4.2%)	83 (3.1%)	57 (8.2%)	
Maternal diabetes				0.314
Missing	86 (2.6%)	70 (2.6%)	16 (2.3%)	
None	3140 (93.6%)	2500 (93.9%)	640 (92.6%)	
Pre-existing	11 (0.3%)	7 (0.3%)	<5[3]	
Gestational	13 (0.4%)	10 (0.4%)	<5[3]	
Glycosuria	103 (3.1%)	75 (2.8%)	28 (4.1%)	
Gestational weight gain				**0.002**
Missing	321 (9.6%)	262 (9.8%)	59 (8.5%)	
Recommended	900 (26.8%)	746 (28.0%)	154 (22.3%)	
< Recommended	370 (11.0%)	299 (11.2%)	71 (10.3%)	
> Recommended	1762 (52.5%)	1355 (50.9%)	407 (58.9%)	
Total energy intake (kJ/day)[2]	7127 (6000, 8361)	7162 (6050, 8371)	6977 (5813, 8237)	**0.009**
Free sugars (g/day)[2]	51.7 (36.8, 70.8)	52.3 (36.9, 71.3)	50.1 (36.7, 69.5)	0.189
% Free sugars[2]	0.12 (0.10, 0.16)	0.12 (0.10, 0.16)	0.12 (0.09, 0.16)	0.871
Sex (% male)	1276 (38.1%)	946 (35.5%)	330 (47.8%)	<0.001
Birthweight (g)	3440 (3130, 3760)	3423 (3120, 3750)	3483 (3180, 3805)	0.014

Median (IQR) or n (%)	Total (n=3353)	Low (n=2662;79.4%)	Mild-Severe (n=691; 20.6%)	P-Value[4]
Missing	43 (1.3%)	32 (1.2%)	11 (1.6%)	0.409
Low	106 (3.2%)	84 (3.2%)	22 (3.2%)	
Normal	2783 (83.0%)	2223 (83.5%)	560 (81.0%)	
High	421 (12.56%)	323 (12.1%)	98 (14.2%)	
Breastfeeding				**0.011**
Missing	230 (6.9%)	185 (6.9%)	45 (6.5%)	
Never	509 (15.2%)	381 (14.3%)	128 (18.5%)	
<3 months	640 (19.1%)	509 (19.1%)	131 (19.0%)	
3-5 months	543 (16.2%)	418 (15.7%)	125 (18.1%)	
≥6 months	1431 (42.7%)	1169 (43.9%)	262 (37.9%)	
Adult characteristics				
BMI (kg/m^2)	23.8 (21.5, 26.9)	23.0 (21.0, 25.4)	28.6 (25.2, 33.1)	**<0.001**
Missing	31 (0.9%)	23 (0.9%)	8 (1.2%)	**<0.001**
Underweight	103 (3.1%)	99 (3.7%)	<5[3]	
Normal	1961 (58.5%)	1807 (67.9%)	154 (22.3%)	
Overweight	842 (25.1%)	592 (22.2%)	250 (36.2%)	
Obese	416 (12.4%)	141 (5.3%)	275 (39.8%)	
AUDIT-C score	5 (4, 7)	5 (4, 7)	5 (3, 7)	**0.011**
Missing	73 (2.2%)	58 (2.2%)	15 (2.2%)	**0.019**
High alcohol	1798 (53.6%)	1460 (54.8%)	338 (48.9%)	
Liver steatosis (CAP value; dB/m)	203 (172, 238)	191 (166, 214)	278 (261, 304)	**<0.001**
Fibrosis (kPA)	4.5 (3.8, 5.4)	4.5 (3.8, 5.4)	4.6 (3.8, 5.5)	
Missing	194 (5.8%)	169 (5.6%)	25 (7.5%)	0.38
None	3080 (91.9%)	2454 (92.2%)	626 (90.6%)	
Any	79 (2.4%)	61 (2.3%)	18 (2.6%)	

[1]Hepatic steatosis based on CAP score cut-off values: low (<248 dB/m, <11% steatosis) vs mild to severe (248-400 dB/m, ≥11% steatosis).[1]

[2]n=125 were missing free sugar and total energy intake values.

[3]Groups with less than five participants are expressed as $n < 5$ in line with the Avon Longitudinal Study of Parents and Children (ALSPAC) confidentiality policy.

[4]Wilcoxon rank sum (equal variance) and Kolmogrov-Smirnov (unequal variance) tests were used for continuous variables. Chi-squared tests were used for categorical variables. Significant p-values are bolded.

Abbreviations: IQR=Interquartile Range, CSE=certificate of secondary education, BMI=Body mass index, AUDIT-C= Alcohol use disorders identification test consumption, CAP=controlled attenuation parameter.

Table 5-2. Associations between maternal factors and offspring mild to severe hepatic steatosis at 24 years in the ALSPAC cohort

	1) unadjusted			2) + confounders[4]			3a) + birthweight			3b) + breastfeeding			3c) + 24-year BMI		
Hepatic Steatosis[1]	**OR**	**95% CL**		**OR**	**95% CL**		**OR**	**95% CL**		**OR**	**95% CL**		**OR**	**95% CL**	
Diabetes[2]															
No	Ref			Ref			Ref			Ref			Ref		
Yes	1.49	1.00	2.22	1.39	0.87	2.21	1.36	0.85	2.19	1.34	0.83	2.16	1.12	0.65	1.92
Pre-pregnancy BMI															
Underweight	0.73	0.44	1.22	0.67	0.37	1.20	0.68	0.38	1.23	0.67	0.37	1.23	1.12	0.59	2.11
Normal	Ref			Ref			Ref			Ref			Ref		
Overweight	1.90	1.51	2.40	1.84	1.43	2.38	1.85	1.43	2.40	1.82	1.40	2.36	1.23	0.91	1.65
Obese	3.05	2.14	4.34	2.73	1.84	4.03	2.71	1.82	4.03	2.55	1.69	3.85	0.95	0.59	1.51
Gestational weight gain															
< Rec.	1.15	0.84	1.57	1.11	0.79	1.55	1.11	0.79	1.56	1.06	0.75	1.52	1.25	0.86	1.84
Rec.	Ref			Ref			Ref			Ref			Ref		
> Rec.	1.46	1.18	1.79	1.30	1.04	1.64	1.33	1.06	1.68	1.35	1.07	1.71	1.15	0.89	1.48
Free sugar[3] tertiles															
1.3-10.4%	Ref			Ref											
10.4-14.3%	1.01	0.82	1.25	1.12	0.88	1.42									
14.3-42.2%	1.02	0.83	1.26	1.04	0.82	1.33									

[1]Hepatic steatosis based on CAP score cut-off values: low (<248 dB/m, <11% steatosis) vs mild to severe (248-400 dB/m, ≥11% steatosis).[1] Sample sizes for each model were 1.Diabetes = 3,267; 1.Pre-pregnancy BMI=3,098; 1.GWG=3,032, 1.Free sugar=3,204; 2.Diabetes, BMI, and GWG =2668; 2.Free sugar=2,646; 3a = 2,639; 3b=2,522; 3c=2645.

[2]Diabetes is defined as maternal existing diabetes, gestational diabetes, or glycosuria during pregnancy.

[3]Free sugars are presented as percent of total energy intake.

[4]Confounders include maternal age, highest level of maternal education, maternal smoking in pregnancy, alcohol intake in pregnancy, physical activity in pregnancy, and sex. The maternal exposures (pre-pregnancy BMI, maternal diabetes, and gestational weight gain) were also included as covariates in model 2. The model focused on free sugar exposure, additionally adjusted for total energy intake and did not adjusted for maternal diabetes since those individuals were excluded.

Abbreviations: OR=odds ratio, CL=confidence limits, BMI=body mass index, Rec=recommended., GWG=gestational weight gain.

Table 5-3. Adjusted[1] associations between maternal factors and 24-year mild to severe hepatic steatosis, also adjusting for hazardous alcohol intake (n=2,609)

Hepatic Steatosis[2]	**OR**	**95% CL**	
Diabetes[3]			
No	Ref		
Yes	1.27	0.79	2.05
Pre-pregnancy BMI			
Underweight	0.69	0.38	1.24
Normal	Ref		
Overweight	1.87	1.44	2.42
Obese	2.78	1.88	4.12
Gestational weight gain			
< Recommended	1.15	0.82	1.62
Recommended	Ref		
> Recommended	1.31	1.04	1.65

[1]Adjusted for maternal age, highest level of maternal education, maternal smoking in pregnancy, alcohol intake in pregnancy, physical activity in pregnancy, and sex. The maternal exposures (pre-pregnancy BMI, maternal diabetes, and gestational weight gain) were also included as covariates.
[2]Hepatic steatosis based on CAP score cut-off values: low (<248 dB/m, <11% steatosis) vs mild to severe (248-400 dB/m, ≥11% steatosis).[1]
[3]Diabetes is defined as existing, gestational, and glycosuria.
Abbreviations: BMI = body mass index, OR=odds ratio, CL=confidence limits, Ref=reference group.

Table 5-4. Associations between maternal factors and offspring *severe* hepatic steatosis at 24 years in the ALSPAC cohort

	1) unadjusted			2) +confounders[4]			3a) +birthweight			3b) +breastfeeding			3c) +24-year BMI		
Severe Steatosis[1]	**OR**	**95% CL**		**OR**	**95% CL**		**OR**	**95% CL**		**OR**	**95% CL**		**OR**	**95% CL**	
Diabetes[2]															
No	Ref			Ref			Ref			Ref			Ref		
Yes	1.74	1.06	2.84	1.32	0.72	2.41	1.39	0.76	2.56	1.13	0.59	2.16	1.02	0.51	2.01
Pre-pregnancy BMI															
UW	0.73	0.35	1.51	0.68	0.29	1.58	0.69	0.29	1.61	0.72	0.31	1.68	1.27	0.50	3.24
N	Ref			Ref			Ref			Ref			Ref		
OW	1.84	1.35	2.50	1.78	1.27	2.49	1.83	1.31	2.57	1.69	1.20	2.39	1.11	0.76	1.63
OB	3.89	2.60	5.81	3.63	2.32	5.69	3.74	2.36	5.92	3.36	2.09	5.41	1.16	0.68	1.98
Gestational weight gain															
< Rec.	1.05	0.67	1.63	1.11	0.69	1.80	1.09	0.67	1.76	1.20	0.74	1.96	1.25	0.73	2.14
Rec.	Ref			Ref			Ref			Ref			Ref		
> Rec.	1.59	1.20	2.11	1.42	1.03	1.96	1.46	1.06	2.02	1.48	1.07	2.06	1.20	0.84	1.71
Free sugar[3] tertiles															
1.3%-10.4%	Ref			Ref											
10.4%-14.3%	0.97	0.73	1.28	1.04	0.76	1.42									
14.3%-42.2%	0.91	0.68	1.20	0.87	0.62	1.21									

[1]Severe steatosis=279-400 dB/m, ≥66% steatosis. Sample sizes for each model were 1.Diabetes = 3,267; 1.Pre-pregnancy BMI=3,098; 1.GWG=3,032, 1.Free sugar=3,204; 2.Diabetes, BMI, and GWG =2668; 2.Free sugar=2,646; 3a = 2,639; 3b=2,522; 3c=2645.

[2]Diabetes is defined as existing, gestational, and glycosuria.

[3]Free sugars are presented in as percent of total energy intake.

[4]Confounders include maternal age, highest level of maternal education, maternal smoking in pregnancy, alcohol intake in pregnancy, physical activity in pregnancy, and sex. The maternal exposures (pre-pregnancy BMI, maternal diabetes, and gestational weight gain) were also included as covariates in model 2. The model focused on free sugar exposure, additionally adjusted for total energy intake and did not adjusted for maternal diabetes since those individuals were excluded.

Abbreviations: BMI = body mass index, UW=underweight, N=Normal, OW=overweight, OB=obese, OR=odds ratio, CL=confidence limits, Ref=reference group, Rec=Recommended.

Table 5-5. Summary of literature associating maternal nutrition with offspring hepatic steatosis.

Citation	Sample	n	Age	Method	Prevalence[1]	Maternal Exposure	Association (95% CI)
Patel, 2014	US Hospital	81	Fetal	Autopsy	na		
						Diabetes	78.8% v 16.7% in controls, p<0.0001
						BMI	No association
Modi, 2011	UK Hospital	105	Infants	MRI	na		
						Obesity	β 8.6% (1.1, 16.8)
						Diabetes	No association
Brumbaugh, 2013	US Hospital	25	Infants	MRI	na		
						Obesity/GDM	+68%
Logan, 2016	UK Hospital	86	Infants	MRI	na		
						GDM	+3.5% (-35.4, 65.6)
Bedogni, 2019	Feeding Study, Italy	389	1 year	Ultrasound	4.0%		
						GWG	+2kg GWG
						Fatty acids[2]	No difference
Santos, 2019	Generation R, Netherlands	2354	10 yrs	MRI	2.0% LFF[3]		
						BMI	+0.15 (0.11, 0.19) SDS
						GWG	No association
Ayonrinde, 2018	RAINE cohort, Aus European descent,	1170	17 yrs	Ultrasound	15.2%		
						Obesity[4]	OR 3.46 (1.49, 8.50)
						GWG[4]	OR 1.10 (1.04, 1.15)
						GDM	No association
Bellatorre, 2018	EPOCH cohort Mixed race/eth, US	254	16 yrs	MRI	5.9%		
						Obesity	β 1.59 (0.66, 2.52)
						Overweight	β 0.39 (-0.44, 1.23)
						GDM	β -0.46 (-1.37, 0.45)
Patel, 2016	ALSPAC White, UK	1215	17 yrs	Ultrasound	2.1%		
						Obesity	OR 2.72 (1.20, 6.15)
						Diabetes[5]	OR 6.74 (2.47, 18.40)
Sekkarie	ALSPAC White, UK	3354	24 yrs	CAP	20.0%		
						Overweight	OR 1.84 (1.42, 2.39)
						Obesity	OR 2.76 (1.85, 4.12)
						GWG	OR 1.27 (1.01, 1.61)
						Diabetes[4]	OR 1.40 (0.88, 2.40)
						% free sugar	OR 1.02 (0.80, 1.29)

[1]Prevalence of hepatic steatosis. In infants, intrahepatocellular lipid content (IHCL) content was measured.
[2]Short chain, monounsaturated, polyunsaturated, omega-3, and omega 6 as absolute and % of total fatty acids.
[3] Median liver fat fraction percent was 2.0% (95% range: 1.2-5.2) in the overall group. Did not present prevalence of hepatic steatosis.
[4]Only in females, GWG was ≥ 6 kilograms in the first trimester. Breastfeeding was independently associated with hepatic steatosis.
[5]Includes pre-existing diabetes, gestational diabetes mellitus, or glycosuria during pregnancy.
Abbreviations: UK=United Kingdom, US=United States, Aus=Australia, yrs=years, MRI=magnetic resonance imaging, CAP=controlled attenuation parameter, GDM=gestational diabetes mellitus, GWG=gestational weight gain, OR=odds ratio, SDS =standard deviation score, LFF = median liver fat fraction, na=not applicable.

Chapter 6: Associations between free sugar and sugary beverage intake in early childhood and adult NAFLD in a population-based UK cohort

Aim 2b

In preparation for submission to Children.

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6.1 Abstract

Background High sugar intake is prevalent among children and is associated with non-alcoholic fatty liver disease (NAFLD) in children and adults.

Objectives To determine whether high intake of free sugars and sugary beverages (SB) in early childhood is associated with NAFLD in early adulthood.

Methods The sample included 3095 participants who were assessed for severe hepatic steatosis at 24 years (controlled attenuation parameter >280 dB/m) and had dietary data collected via food frequency questionnaire at age 3 years. A series of multiple logistic regression models were undertaken to control for total energy intake, confounders (sex, maternal education breastfeeding duration, maternal pre-pregnancy BMI), and mediator (offspring BMI at 24 years).

Results Increasing quintiles of free sugar intake was associated with severe hepatic steatosis at 24 years, after adjusting for total energy the odds ratio (OR) per quintile increase was 1.07 (95% CL: 0.99, 1.17). The OR after full confounder and mediator adjustment was 1.07 (95% CL: 0.96, 1.19). Comparing lowest vs highest free sugar consumers, the association was OR:1.28 (95% CL: 0.88, 1.85) and 1.14 (0.72,1.82) after full adjustment. The OR for high SB consumption (>2/day) compared to <1/day was 1.23 (95% CL: 0.82, 1.84) and was OR: 0.98 (95% CL: 0.60, 1.60) after adjusting for confounders and BMI at 24 years.

Conclusions High free sugar and sugary beverage intake at 3 years was positively but weakly associated with severe hepatic steatosis at 24 years. These associations were completely attenuated after adjusting for confounders and 24-year BMI.

6.2 Introduction

The prevalence of non-alcoholic fatty liver disease (NAFLD) in children has increased considerably over recent decades, in parallel with the rise of obesity.[2] This is concerning because pediatric NAFLD can progress to nonalcoholic steatohepatitis (NASH), which is characterized by inflammation, as well as cirrhosis and end stage liver disease in adulthood.[3] NAFLD is also associated with increased risk of diabetes and cardiovascular disease.[4,5] Established risk factors for NAFLD include age, male sex, obesity, and high sugar diets.[6]

Added sugars are sugars that are added to foods during processing.[7] Free sugars, which also include sugars that are naturally present in honey, syrups, fruit juices and fruit juice concentrate, are the primary sugars of public health concern because of their high prevalence in human diets. Sugar sweetened beverages (SSBs) which include all beverages with added sugars, and sugary beverages (SBs), which also include fruit juices, are the largest source of free sugars in children[8]. The World Health Organization (WHO) dietary guidelines for children and adults recommend limiting free sugar intake to less than 10% of daily energy intake to reduce risk of overweight and obesity, and a further reduction to below 5% for additional health benefits.[7] The WHO also considers SSBs a "probable contributor" to the obesity epidemic.[9] Liquids are less satiating causing more postprandial hunger, therefore leading to increased energy intake.[10] SBs are also high in fructose, which directly contributes to the development of hepatic fat. In the United Kingdom (UK), the recommendation by the Scientific Advisory Committee on Nutrition (SACN) is for no more than 5% of total energy intake to come from free sugars.[11] In the UK, according to the national diet and nutrition survey, years 2014-2016, children 1.5 to 3 years old consume an average of 32.6g of sugar a day, comprising 11.3% of their total energy intake.[12] Only 13% had intakes below or equal to 5% of total energy.[12] In the ALSPAC cohort, the largest increase in intake of free sugars occurred during the preschool period when children increased their intake of free sugars from 12.3% at age 1.5 years to 16.4% of total energy at age 3.5 years.[13] In the NDNS, sugary beverages contributed 21% of free sugar consumption in children aged 1.5 to 3 years.[12]

A high intake of dietary fructose is associated with NAFLD.[14,15] Several studies have shown that children and adults with NAFLD have a higher mean fructose intake, mainly resulting from a higher consumption of soft drinks and fruit juices, as compared with individuals of the same age without NAFLD.[16] Reducing free sugar intake also can lead to a decrease in hepatic fat.[17] Due to the association between high sugar intake and NAFLD and the high prevalence of sugar intake in children, the strongest recommendation for treatment of NAFLD is to reduce sugar sweetened beverage consumption[6]. Since dietary behavior, including sugar intake, can be modified and dietary patterns in early childhood can track into adulthood, more evidence regarding recommendations on a public health and clinical level is important.[18]

The primary aim of this study is to describe intake of free sugar and sugary beverage intake in three-year-old children in a UK based cohort and determine whether this intake is associated with hepatic steatosis in early adulthood (24 years of age).

6.3 Methods

6.3.1 Study population

We used data from the Avon Longitudinal Study of Parents and Children, a population-based birth cohort study that has previously been described in detail.[19-21] In summary, ALSPAC enrolled 14,541 pregnant women in the greater Bristol, UK area with expected delivery dates between 1st April 1991 and 31st December 1992. When the oldest children were 7 years of age, attempts were made to increase the initial sample by recruiting from offspring that would have been eligible to enroll in the original study but did not join at the time, resulting in a new total of 15,454 pregnancies and 14,901 children. Clinical, dietary, and demographic information was collected throughout infancy and childhood with the first food frequency questionnaire (FFQ) conducted at three years. When the offspring were 24 years of age, 10,018 participants were invited to a clinic visit known as Focus@24, which included the collection of biological samples and anthropometric measures. Data from the 24-year clinic were collected and managed using

REDCap electronic data capture tools hosted at the University of Bristol.[22,23] The study website contains details of all the data that are available through a fully searchable data dictionary and variable search tool.[24]

Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the data from the 24 Year Clinic was approved by the National Research Ethics Service Committee South West—Frenchay: 14/SW/1173 ALSPAC Focus at 24 + (24 February 2015, confirmed 20 March 2015).

6.3.2 Assessment of free sugar intake at three years of age

Non-milk extrinsic sugars (NMES) as a percentage of total energy was calculated from responses to a non-quantitative FFQ, which aimed to cover all the primary foods consumed in Britain at the time and was completed by the mother. It included questions on weekly consumption frequency (never or rarely, once in two weeks, one to three times per week, four to seven times per week, and more than once a day) of 52 food groups. Since portion sizes were not collected as part of the FFQ, standard portion sizes for children were assumed for nutrient estimates.[25] Nutrient intakes were calculated by multiplying the frequency of each food with the nutrient content of a portion of food.[26] NMES were calculated by deducting the sugars from milk, fruits, and vegetables from total sugars.[13] We then categorized the NMES percent into quintiles, with the lowest quintile as the reference.

Sugary beverage intake per day was quantified from mothers' responses about their child's weekly intake sugary beverages. The number of sugary beverages consumed per day was included as a continuous measure and categorized [<1/day (reference group), 1 to 2/day, and >2/day].[27,28] SBs included pure fruit juice, tinned juice, fruit drinks, Ribena™, squash, non-diet colas, and other "fizzy drink" questions. We did not include tea, coffee, and alcohol intake. Tea and coffee intake were negligible: Of 30% that consumed tea at least once a day, only 10% added sugar. Only 7% reported at least one coffee a day, and of those 7.3% reported adding sugar.

6.3.3 Assessment of liver outcomes

At 24 years of age, participants were assessed by transient elastography for non-invasive quantification of liver steatosis and fibrosis (FibroScan® 502 Touch, Echosens, Paris, France). Individuals with an active medical implant such as a pacemaker, liver ascites, or who were pregnant were excluded from the liver scan (n=144). Participants were asked to fast overnight or for at least six hours prior to transient elastography.[29] Transient elastography provides a controlled attenuation parameter (CAP) measure of steatosis and a measure of liver stiffness to quantify fibrosis. Manufacturer and machine indications were used to determine whether the M or XL probe would be used to conduct the scan. Ten readings were required for each patient to derive a CAP score and fibrosis result. CAP values outside the 100-400 dB/m range were considered invalid and coded as missing. Median fibrosis results greater than or equal to 15 kPa or with an IQR to median ratio greater than or equal to 30% were considered invalid and coded as missing.

We categorized participants into two categories of steatosis based on CAP score cut-off values derived from a meta-analysis by Karlas, et al: low to moderate (<280 dB/m, <66% steatosis) vs severe steatosis (≥280 dB/m, ≥66% steatosis).[1] For sensitivity analysis, we categorized CAP scores as low (<248 dB/m, <10% steatosis) vs mild to severe hepatic steatosis.

6.3.4 Covariates

Offspring sex was extracted from medical records. We used highest level of maternal education reported during pregnancy as a proxy for socioeconomic status.[30] Mothers self-reported one of five categories: None/CSE (certificate of secondary education), Vocational (vocational courses after 16 years of age), O (ordinary level exams at 16 years), A (advanced level exams at 18 years), and University degree and above.[31] Breastfeeding duration was assessed from maternal reports when their children were 15 months (never, <3 months, 3-6 months, >6 months). BMI at 24 years was calculated from weight in kilograms divided by height in meters squared.[32] We classified BMI at 24 years as underweight (<18.5 kg/m^2), normal weight (18.5 to <25 kg/m^2), overweight (25 to <30 kg/m^2), and obese (≥ 30 kg/m^2).[33] We adjusted

for hazardous alcohol consumption using the Alcohol Use Disorder Identification Test for Consumption (AUDIT-C) score ≥4 in women and ≥5 in men.[34,35]

6.3.5 Inclusion/exclusion

We excluded individuals missing dietary information at three years and those missing a hepatic steatosis measure at 24 years.

6.3.6 Statistical Analysis

We conducted statistical analyses in SAS version 9.4 (Cary, NC). We calculated median and interquartile ranges (IQR) values for continuous variables and counts and percentages for categorical variables for the full sample and stratified by sugar intake quintile. F-tests were used to compare differences in continuous variables and chi-squared tests were used to compare differences for categorical variables.

We used multiple logistic regression to model our associations between each exposure (free sugar percent quintiles and sugary beverage intake) and hepatic steatosis at 24 years. Free sugar percent quintiles were considered categorically to assess pairwise comparisons between those with lowest intake (Q1) and highest intake (Q5). In separate models, free sugar percent quintile was considered as a continuous term to assess the overall trend of increasing free sugar intake. The sugary beverage exposure was considered continuously and categorically as described above. In the base model we adjusted for total energy intake. The second model adjusted for confounders including offspring sex, maternal education, maternal pre-pregnancy BMI, and duration of breastfeeding. The third model adjusted for BMI category at 24 years as a potential mediator. In a fourth model we adjusted for AUDIT-C score since alcohol intake is strongly associated with hepatic steatosis. Finally, in a sensitivity analysis, to further understand the role of total energy intake as a possible mediator in the association between a diet high in free sugars and hepatic steatosis we compared models with and without total energy adjustment.

6.4 Results

Of the 10,018 active ALSPAC participants who were invited to participate in the Focus@24+ clinic, 4,021 attended the clinic, and 3,877 participants had FibroScan® performed. Of these participants, 3,766 had a valid CAP score. After exclusions for those missing dietary intake data at three years, our sample size was 3,095 (*Figure 6-1*).

Table 6-1 presents sample characteristics by percent free sugar quintiles. Those in the lowest quintile (Q1) had intakes ranging from 0.14% to 11.5% (median = 28.5g/day) and those in the highest quintile (Q5) had intakes ranging from 17.7% to 36.5% (median = 64.9g/day). Less than 1% met the level of free sugar intake recommended by the UK SACN (<5% of total energy). The median number of sugary beverages consumed per day was 1.6 (IQR: 1.3, 2.0) (*Figure 6-2*). Approximately 17% consumed SBs less than once per day and 21% consumed SBs more than twice a day. There was no association across quintiles of percent free sugar intake with child sex. Higher percent free sugar intake was associated with lower maternal education, shorter breastfeeding duration, higher total energy intake at three years, higher sugary beverage intake at three years, higher sugar intake at 13 years, and hazardous alcohol use at 24 years.

There was a positive but small association between increasing free sugar intake at three years and severe hepatic steatosis at 24 years (

Table **6-2***, Figure 6-3*). Pairwise associations comparing those in the lowest free sugar intake group with higher levels of sugar intake were generally positive, but these associations had wide confidence intervals (

Table **6-2***, Figure 6-3*). Adjusting for confounders and mediators did not meaningfully change the estimates.

We found no strong associations between continuous SB intake at three years and severe hepatic steatosis at 24 years (*Table 6-3* When SB intake was categorized, those consuming more SBs had higher odds of hepatic steatosis at 24 years compared to those consuming SBs less than once a day (>2 SB/day OR: 1.23, 95% CL: 0.82, 1.84) (*Table 6-4*). This association was attenuated after adjusting for confounders and BMI at 24 years (OR: 0.98, 95% CL: 0.60, 1.60). Changing the primary outcome to

mild-severe steatosis (as opposed to just severe steatosis) attenuated estimates (*Table 6-5, Table 6-6*). When comparing models that did and did not adjust for total energy intake, estimates did not substantively change (*Table 6-7*, *Table 6-8*). When we considered only sugar sweetened beverages (i.e. not including pure fruit juice) as the primary exposure, effect estimates were stronger but confidence intervals were wider making it difficult to draw definitive conclusions (*Table 6-9*).

6.5 Discussion

Increased intake of free sugars at three years of age was positively but weakly associated with severe hepatic steatosis at 24 years. There was also a weak positive association between high sugary beverage intake at three years of age and severe hepatic steatosis, however this association was completely attenuated after controlling for confounders and offspring BMI category at time of outcome. A previous ALSPAC study also looked at dietary intake at three years with hepatic steatosis at 17 years of age in a sub-sample of the overall study, although they did not specifically look at free sugars.[36] In that study, every 100 kcal increase in energy intake at three years of age (calculated from multi-level models that incorporated FFQ and food diary data), was associated with greater hepatic steatosis in adolescents (OR: 1.79, CL: 1.14-2.79). The food dietary data was available on only 10% of the sample. This association was mediated by fat mass in adolescence. There were no strong associations with any macronutrient intakes, including total sugar intake.[36]

In other ALSPAC studies that looked specifically at sugary beverage intake, the primary outcome was adiposity, not hepatic steatosis. A previous analysis of sugar-sweetened beverage consumption in five- and seven-year old children in the ALSPAC cohort found no evidence of an association with adiposity at age nine years.[37] However, that study did find a positive association between consumption of low-energy beverages in five- and seven-year old children with adiposity at age nine years. The authors suggested that those at risk of obesity may be modifying their diets in an unsuccessful attempt to prevent obesity.[37] A separate ALSPAC study focused on central adiposity, found that higher consumption of SSBs from 10 to 13 years of age was associated with a larger waist circumference at 13 years independent

of total adiposity.[30] The results of these two studies are not necessarily in contrast since it has been shown that fructose, which is found in high amounts in many sugary beverages, specifically increases visceral adiposity.[38,39] The liver directly converts fructose to fat via de novo lipogenesis.[40] Fructose metabolism skips the rate-limiting enzyme for glucose metabolism (phosphofructokinase) and is metabolized by fructokinase, which has no negative feedback system. This leads to an increased production of triglycerides.[40]

Other studies have looked at the association between sugary beverages and hepatic fat but have not extended the outcome beyond childhood. In several cross-sectional studies, high fructose and sucrose intake have been reported in children with existing NAFLD.[41,42] In the Generation R cohort, more than two sugary beverages per day compared to less than one per day at one year of age was associated with higher odds of MRI measured hepatic steatosis at 10 years old, independent of BMI at time of outcome (OR: 1.34, 95% CL: 0.97, 1.83).[27] We found similar associations, but in our study, they were mediated by BMI at time of outcome. If the development of NAFLD requires obesity (particularly as individuals age), then the attenuation of estimates by adjusting for BMI is what we would expect to find, and the true association is what we derive from models not adjusting for BMI or adiposity.

The Generation R Cohort study also found that compared to children with normal weight, children with overweight and obesity had stronger associations between SB intake at one year and mid-childhood steatosis. It has been shown that overweight and obese children with NAFLD absorb and metabolize fructose more effectively than normal-weight children.[14,15] Children susceptible to and with NAFLD have up-regulated de novo lipogenesis compared to non-NAFLD children.[43]

The association between free sugar and sugary beverage consumption and hepatic steatosis is likely mediated in part by overall energy intake, therefore adjusting for energy will tend to underestimate the effect of these exposures on hepatic steatosis.[44,45] We saw no differences between models with and without energy adjustment indicating that any associations we found were not primarily mediated by total energy intake.

Strengths and Limitations

The largest strength of our study is that it utilizes a large, population-based longitudinal cohort from childhood to young adulthood. We also used a validated and accurate measure of hepatic steatosis, the CAP score based on transient elastography.[1]

While we did not exclude participants with other liver conditions, a previous report from this population reported that no participants had viral hepatitis or were taking nucleos(t)ide analogues or direct-acting antivirals and very few were taking medications for autoimmune hepatitis.[46] Additionally, adjusting for hazardous alcohol intake did not change the estimates, therefore, we are confident that NAFLD is the primary cause of hepatic steatosis in our population.

One of the possible reasons that we did not see strong associations was that most participants had intake of free sugar above recommended levels. Less than 1% of our sample had intakes within the recommended levels and those in the lowest quintile had free sugar intakes up to 11.5% of total energy. Additionally, there are limitations to the FFQ that was used to measure dietary intake. Portion sizes were not ascertained so calculated intakes may be inaccurate. Furthermore, there were other factors that contribute to hepatic steatosis that we were not able to adjust for including genetics and lifestyle variables that extend from childhood to adulthood.

A limitation of this study was the relatively homogenous nature of the sample, who are primarily of white ethnicity, and results may not be generalizable to other populations. We expect that the associations we found would differ in samples with a larger proportion of higher-risk individuals, such as Hispanics.[47] Individuals with adipogenic genes such as Patatin-like phospholipase domain-containing protein 3 (PNPLA3), which is more common in Hispanics, are more susceptible to NAFLD.[48] Additionally, there was differential loss to follow-up within the cohort, whereby females and participants with mothers with higher education were more likely to be followed-up.

Conclusions

Free sugar intake in three-year old children, as measured in our study, was positively, but weakly associated with hepatic steatosis in young adulthood. The positive association between high sugary beverage intake at three years and hepatic steatosis in young adulthood, was mediated by BMI at time of outcome. Children should continue to limit intake of free sugars and sugary beverages. Further longitudinal studies with validated measures of sugar intake and hepatic steatosis throughout childhood are important.

6.6 References

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6.7 Tables & Figures

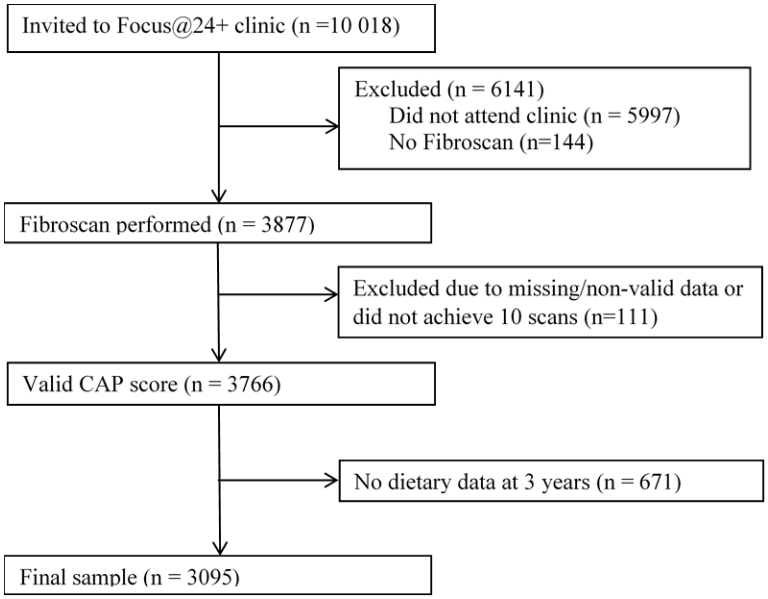

Figure 6-1. *Flowchart of participants included in final analysis.*

Of the 10,108 participants invited to the 24-year clinic, we excluded those that did not attend the clinic or did not get a liver scan because they were ineligible or excluded due to active implant, liver ascites, or pregnancy. We also excluded participants with missing or non-valid CAP scores or had missing dietary data from the three-year FFQ. Our final sample size was 3095.

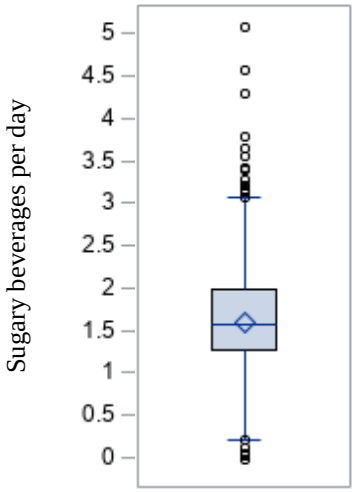

Figure 6-2. *Box plot of children's sugary beverage intake per day at 3 years.*

Table 6-1. Sample characteristics by percent free sugar of total energy intake quintile presented as n (%) or median (IQR) in the ALSPAC cohort

Variable	Overall N=3095	Q1 (0.14-11.5)	Q2 (11.5-13.5)	Q3 (13.5-15.3)	Q4 (15.3-17.7)	Q5 (17.7-36.5)	P-Value
Free sugars (g) at 3 years	43.5 (33.6, 54.7)	28.5 (23.2, 33.6)	38.4 (32.5, 44.5)	43.6 (37.3, 50.3)	50.9 (43.9, 59.0)	64.9 (52.9, 78.3)	**<.001**
TEI (kJ) at 3 years	5072 (4346, 5858)	4849 (4074, 5515)	5095 (4377, 5842)	5049(4342, 5800)	5187(4486, 6011)	5287 (4430, 6297)	**<.001**
SB per day	1.6 (1.3, 2.0)	1.4 (0.8, 1.6)	1.5 (1.1, 1.7)	1.6 (1.4, 1.9)	1.7 (1.4, 2.2)	1.9 (1.5, 2.4)	**<.001**
Male sex	1200 (38.8%)	234 (37.8%)	241 (38.9%)	243 (39.3%)	250 (40.4%)	232 (37.5%)	0.839
Maternal education							
CSE/None	257 (8.3%)	46 (7.4%)	40 (6.5%)	47 (7.6%)	49 (7.9%)	75 (12.1%)	
Vocational	206 (6.7%)	43 (6.9%)	34 (5.5%)	33 (5.3%)	39 (6.3%)	57 (9.2%)	
O-level	1042 (33.7%)	180 (29.1%)	197 (31.8%)	193 (31.2%)	226 (36.5%)	246 (39.7%)	**<.001**
A-level	895 (28.9%)	202 (32.6%)	194 (31.3%)	186 (30.0%)	170 (27.5%)	143 (23.1%)	
Degree	657 (21.2%)	140 (22.6%)	144 (23.3%)	153 (24.7%)	131 (21.2%)	89 (14.4%)	
Maternal BMI							
Underweight	117 (3.8%)	27 (4.4%)	32 (5.2%)	20 (3.2%)	18 (2.9%)	20 (3.2%)	0.09
Normal	2239 (72.3%)	442 (71.4%)	419 (67.7%)	474 (76.6%)	458 (74.0%)	446 (72.1%)	
Overweight	392 (12.7%)	68 (11.0%)	98 (15.8%)	65 (10.5%)	83 (13.4%)	78 (12.6%)	
Obese	122 (3.9%)	29 (4.7%)	26 (4.2%)	20 (3.2%)	21 (3.4%)	26 (4.2%)	
Breastfeeding duration							
Never	468 (15.1%)	84 (13.6%)	74 (12.0%)	80 (12.9%)	89 (14.4%)	141 (22.8%)	
< 3 m	613 (19.8%)	113 (18.3%)	118 (19.1%)	121 (19.5%)	130 (21.0%)	131 (21.2%)	**<.001**
3 – 5 m	512 (16.5%)	107 (17.3%)	105 (17.0%)	110 (17.8%)	116 (18.7%)	74 (12.0%)	
> 6 m	1378 (44.5%)	284 (45.9%)	303 (48.9%)	288 (46.5%)	262 (42.3%)	241 (38.9%)	
AUDIT-C at 24 yrs	5 (4, 7)	5 (3, 7)	5 (4, 7)	5 (4, 7)	6 (4, 7)	5 (3, 7)	**0.024**
BMI at 24 yrs							
Underweight	90 (2.9%)	16 (2.6%)	19 (3.1%)	19 (3.1%)	18 (3.0%)	18 (2.9%)	
Normal	1846 (60.2%)	373 (61.3%)	359 (58.5%)	386 (62.6%)	365 (59.5%)	363 (59.2%)	0.7780

Overweight	762 (24.9%)	147 (24.4%)	156 (25.4%)	147 (23.8%)	159 (25.9%)	153 (25.0%)	
Obese	369 (12.0%)	73 (12.0%)	80 (13.0%)	65 (10.5%)	72 (11.7%)	79 (12.9%)	
Severe hepatic steatosis[1]	304 (9.8%)	56 (9.0%)	52 (8.4%)	61 (9.9%)	63 (10.2%)	72 (11.6%)	0.378

[1]Steatosis is defined from controlled attenuation parameter scores: severe (>279 dB/m).
The following variables had missing values: sugary beverage intake (n=7, 0.2%), maternal education (n=38, 1.2%), maternal BMI (n=225, 7.3%), breastfeeding (n=124, 4.0%), BMI at 24 years (n=28, 0.9%), AUDIT-C score (n=63, 2.0%). F-tests were used to compare differences in continuous variables and chi-squared tests were used to compare differences for categorical variables.
Abbreviations: IQR= interquartile range, CSE =certificate of secondary education, TEI= total energy intake, yrs= years, AUDIT-C = Alcohol use disorder identification test – concise, BMI = body mass index, m=months.

Table 6-2. Percent free sugar of total energy intake quintiles at 3 years and severe hepatic steatosis at 24 years in the ALSPAC cohort

		Q1 (0.14-11.5)	Q2 (11.5-13.5)			Q3 (13.5-15.3)			Q4 (15.3-17.7)			Q5 (17.7-36.6)			Per quintile			p-trend
Model	**n**	**REF**	**OR**	**95% CL**		**OR**	**95% CL**		**OR**	**95% CL**		**OR**	**95% CL**		**OR**	**95% CL**		
1.TEI	3095	1.00	0.90	0.61	1.34	1.08	0.74	1.58	1.11	0.76	1.62	1.28	0.88	1.85	1.07	0.99	1.17	0.103
2.1+Confounders	2742	1.00	0.81	0.53	1.23	0.98	0.65	1.47	1.01	0.67	1.50	1.09	0.73	1.62	1.04	0.95	1.14	0.394
3.2+24y BMI	2715	1.00	0.67	0.41	1.08	1.04	0.65	1.66	0.99	0.62	1.58	1.14	0.72	1.82	1.07	0.96	1.19	0.204
4.2+24 y Alc	2685	1.00	0.77	0.50	1.18	0.99	0.66	1.49	0.99	0.66	1.49	1.09	0.73	1.63	1.05	0.95	1.15	0.355

Model 1: adjusts for total energy intake.
Model 2: model 1 + sex, maternal education, maternal pre-pregnancy body mass index, and breastfeeding duration.
Model 3: model 2 + body mass index category at 24 years.
Model 4: model 2 + AUDIT-C (Alcohol Use Disorder Identification Test – Concise) score at 24 years.

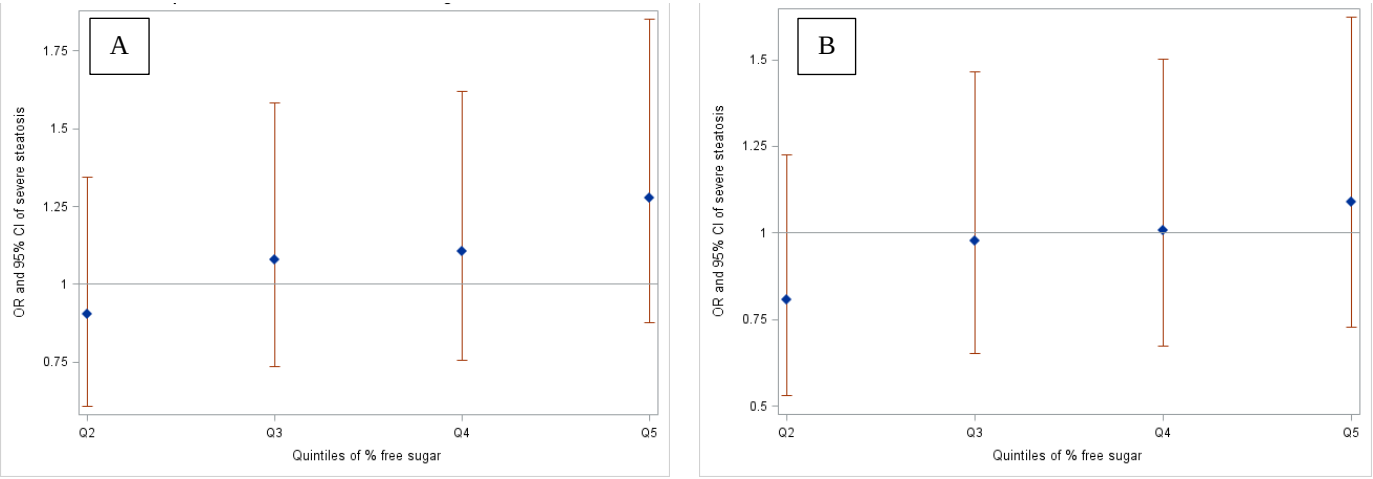

Figure 6-3. (A) Base and (B) adjusted associations between percent free sugar of total energy intake quintiles (Ref=Q1) at 3 years and severe hepatic steatosis at 24 years in the ALSPAC cohort

Table 6-3. Sugary beverage intake per day at 3 years and severe hepatic steatosis at 24 years in the ALSPAC cohort

Model	n	OR	95% CL	
1	3088	1.04	0.87	1.24
2	2739	1.04	0.86	1.25
3	2715	0.92	0.73	1.15
4	2682	1.03	0.85	1.25

Model 1: adjusts for total energy intake.
Model 2: model 1 + sex, maternal education, maternal pre-pregnancy body mass index, and breastfeeding duration.
Model 3: model 2 + body mass index category at 24 years.
Model 4: model 2 + AUDIT-C (Alcohol Use Disorder Identification Test – Concise) score at 24 years.

Table 6-4. Sugary beverage (SB) intake per day category at 3 years and severe hepatic steatosis at 24 years in the ALSPAC cohort

		<1 SB/day	1-2 SB/day			>2 SB/day		
Model	n	REF	OR	95% CL		OR	95% CL	
1	3088	1.00	1.25	0.89	1.77	1.23	0.82	1.84
2	2739	1.00	1.18	0.81	1.70	1.19	0.77	1.83
3	2715	1.00	1.03	0.68	1.57	0.98	0.60	1.60
4	2682	1.00	1.19	0.82	1.73	1.20	0.78	1.86

Model 1: adjusts for total energy intake.
Model 2: model 1 + sex, maternal education, maternal pre-pregnancy body mass index, and breastfeeding duration.
Model 3: model 2 + body mass index category at 24 years.
Model 4: model 2 + AUDIT-C (Alcohol Use Disorder Identification Test – Concise) score at 24 years.

Table 6-5. Sugary beverage intake per day at 3 years and mild to severe hepatic steatosis at 24 years in the ALSPAC cohort

Model	n	OR	95% CL	
1	3088	1.02	0.89	1.16
2	2739	1.05	0.91	1.21
3	2715	0.99	0.83	1.17
4	2682	1.05	0.91	1.22

Model 1: adjusts for total energy intake.
Model 2: model 1 + sex, maternal education, maternal pre-pregnancy body mass index, and breastfeeding duration.
Model 3: model 2 + body mass index category at 24 years.
Model 4: model 2 + AUDIT-C (Alcohol Use Disorder Identification Test – Concise) score at 24 years.

Table 6-6. Sugary beverage (SB) intake per day category at 3 years and mild to severe hepatic steatosis at 24 years in the ALSPAC cohort

		<1 SB/day	1-2 SB/day			>2 SB/day		
Model	**n**	**REF**	**OR**	**95% CL**		**OR**	**95% CL**	
1	3088	1.00	0.99	0.78	1.26	0.95	0.71	1.26
2	2739	1.00	0.96	0.74	1.24	0.96	0.70	1.31
3	2715	1.00	0.84	0.62	1.13	0.81	0.57	1.16
4	2682	1.00	0.98	0.75	1.27	0.97	0.71	1.33

Model 1: adjusts for total energy intake.
Model 2: model 1 + sex, maternal education, maternal pre-pregnancy body mass index, and breastfeeding duration.
Model 3: model 2 + body mass index category at 24 years.
Model 4: model 2 + AUDIT-C (Alcohol Use Disorder Identification Test – Concise) score at 24 years.

Table 6-7. Adjusted[1] associations between free sugar percent quintiles at 3 years and severe hepatic steatosis at 24 years with and without total energy intake (TEI) in the ALSPAC cohort

		Q1 (0.14-11.5)	Q2 (11.5-13.5)			Q3 (13.5-15.3)			Q4 (15.3-17.7)			Q5 (17.7-36.6)			Per quintile			p-trend
	n	REF	OR	95% CL		OR	95% CL		OR	95% CL		OR	95% CL		OR	95% CL		
NO TEI adjustment	2742	1.00	0.82	0.54	1.24	0.99	0.66	1.48	1.02	0.69	1.52	1.11	0.75	1.65	1.05	0.95	1.14	0.35
TEI adjustment	2742	1.00	0.81	0.53	1.23	0.98	0.65	1.47	1.01	0.67	1.50	1.09	0.73	1.62	1.04	0.95	1.14	0.39

[1]Both models are also adjusted for sex, maternal education, maternal pre-pregnancy body mass index, and breastfeeding duration.

Table 6-8. Adjusted[1] associations between sugary beverage intake at 3 years and severe hepatic steatosis at 24 years with and without total energy intake (TEI) in the ALSPAC cohort

		continuous SB/day			<1/day	1-2/day			> 2/ day		
	n	OR	95% CL		REF	OR	95% CL		OR	95% CL	
NO TEI adjustment	2739	1.05	0.87	1.26	1.00	1.18	0.82	1.71	1.21	0.79	1.85
TEI adjustment	2739	1.04	0.86	1.25	1.00	1.18	0.81	1.70	1.19	0.77	1.83

[1]Both models are also adjusted for sex, maternal education, maternal pre-pregnancy body mass index, and breastfeeding duration.

Table 6-9. Sugary sweetened beverage (not including pure fruit juice) intake per day at 3 years and severe hepatic steatosis at 24 years in the ALSPAC cohort

		continuous SB/day			<1/day	1-2/day			> 2/ day		
Model	n	OR	95% CL		REF	OR	95% CL		OR	95% CL	
1	3088	1.11	0.91	1.34	1.00	1.21	0.93	1.57	1.37	0.82	2.30
2	2739	1.03	0.83	1.27	1.00	1.13	0.86	1.49	1.22	0.70	2.12
3	2715	0.90	0.70	1.15	1.00	0.97	0.70	1.34	1.18	0.62	2.25
4	2682	1.02	0.83	1.27	1,00	1.12	0.85	1.49	1.24	0.71	2.16

Model 1: adjusts for total energy intake.
Model 2: model 1 + sex, maternal education, maternal pre-pregnancy body mass index, and breastfeeding duration.
Model 3: model 2 + body mass index category at 24 years.
Model 4: model 2 + AUDIT-C (Alcohol Use Disorder Identification Test – Concise) score at 24 years.

Chapter 7: ALT trends through childhood and adolescence associated with hepatic steatosis at 24 Years: A population-based UK cohort study

Aim 3

Sekkarie A, Welsh JA, Northstone K, Cioffi CE, Stein AD, Figueroa J, Ramakrishnan U, Vos MB. ALT Trends through Childhood and Adolescence Associated with Hepatic Steatosis at 24 Years: A Population-Based UK Cohort Study. *Children*. 2020 Sep;7(9):117.

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7.1 Abstract

Background: Alanine aminotransferase (ALT) is used to screen for non-alcoholic fatty liver disease (NAFLD) in children; however, the optimal age to commence screening is not determined. Our objective was to describe whether ALT trends from 9–24 years were associated with hepatic steatosis at 24 years in a population-based UK cohort. Methods: The sample included 1156 participants who were assessed for hepatic steatosis at 24 years and had at least two ALT measurements at 9, 15, 17, and/or 24 years. Controlled attenuation parameter scores were used to assess steatosis (low (<248 dB/m), mild/moderate (248–279 dB/m), severe (>279 dB/m)). Sex-stratified mixed-effects models were constructed to assess the liver enzyme trends by steatosis level. Results: The final sample was 41.4% male and 10.4% had severe steatosis. In both sexes, ALT trends from 9 to 24 years differed in those with low vs. severe steatosis at 24 years ($p < 0.001$). There was no evidence of differences prior to puberty. At 17 years, the low vs. severe geometric mean ratio (GMR) was 0.69, 95% CI: 0.57–0.85 in males and (0.81, 0.65–1.01) females. At 24 years, the GMR was (0.53, 0.42–0.66) in males and (0.67, 0.54–0.84) females. Conclusions: Higher ALT concentration in adolescence was associated with hepatic steatosis at 24 years. The increased screening of adolescents could strengthen NAFLD prevention and treatment efforts.

7.2 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome. [1] NAFLD is defined as having steatosis involving greater than 5% of hepatocytes, typically assessed by liver biopsy or imaging, in the absence of other causes of hepatic steatosis including heavy alcohol intake.[2] Established risk factors for NAFLD include age, male sex, and obesity. The prevalence of NAFLD has increased considerably over recent decades in youth in parallel with the rise of obesity.[3] This is concerning because pediatric NAFLD can progress to nonalcoholic steatohepatitis (NASH), which is characterized by inflammation, as well as cirrhosis and end stage liver disease in adulthood.[4] NAFLD is also associated with increased risk of diabetes, cardiovascular disease and pregnancy complications.[5,6]

The onset and subsequent natural history of NAFLD in childhood and adolescence is not well characterized.[2] Despite reports of infants with hepatic steatosis[7-10], little is known about the disease in pre-pubertal children. Most children with NAFLD typically present clinically between 10 to 13 years old.[11] If the disease is caught early, outcomes can be improved through drugs or lifestyle changes, primarily by improving diet quality (e.g., reducing sugar) and increasing physical activity.[2,12] Therefore, the ability to detect NAFLD in its earliest stages is crucial to mitigating the consequences of childhood NAFLD.

Currently**,** the recommended screening method for NAFLD in children includes elevated alanine aminotransferase (ALT) serum concentration, concurrent with obesity and other risk factors for metabolic disease such as family history of diabetes.[2] The two other primary liver enzymes, aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) are not used as screening markers for NAFLD in children, however when they are elevated in addition to ALT, they are associated with worse histology of the disease.[2,13]

Due to the sparsity of studies on the incidence and natural history of pediatric NAFLD, especially prior to its diagnosis, the ideal age for screening for NAFLD is not known.[2] No studies using serial measurements have been published on the trajectory of liver enzymes from childhood to adulthood. Additionally, the association between liver enzyme trends from childhood into adulthood and risk of NAFLD has not been previously explored. In this paper we describe trends in ALT and other liver enzyme concentrations in the UK population-based Avon Longitudinal Study of Parents and Children (ALSPAC) birth cohort from 9 to 24 years and determine whether these trends differ by category of hepatic steatosis at 24 years.

7.3 Materials & Methods

7.3.1 Study design and population

We used data from a population-based birth cohort study (ALSPAC) based at the University of Bristol (Bristol, UK) that has previously been described in detail.[14-16] Briefly, ALSPAC enrolled 15,454 pregnant women in the greater Bristol area with expected delivery dates between April 1, 1991 and December 31, 1992. Of their children, 14,901 were alive at one year of age. When the offspring were 24 years of age, 10,018 participants were invited to a clinic visit (that wave was known locally as Focus@24) between June 5, 2015 and October 31, 2017, which included the collection of biological samples and anthropometric measures, and a total of 4,021 attended. Other waves of field work occurred when participants were ages 7 through 17 years. Data from the 24 year clinic were collected and managed using REDCap electronic data capture tools hosted at the University of Bristol.[17,18] The study website contains details of all the data that are available through a fully searchable data dictionary and variable search tool.[19]

Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Informed consent for the use of collected data via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee at the time.

7.3.2 Assessment of liver outcomes

Participants were asked to fast overnight or for at least six hours prior to phlebotomy and transient elastography. Blood samples were immediately centrifuged and frozen at -80°C. Fasting serum ALT, AST, and GGT liver enzyme concentrations were obtained through standard clinical chemistry assays at ages 9 (non-fasting), 15, 17 and 24 years as previously described.[20] At 24 years, elevated ALT was defined as >19 U/L in women and >30 U/L in men.[21]

At 24 years old, participants were assessed by transient elastography for non-invasive quantification of liver steatosis and fibrosis (FibroScan® 502 Touch, Echosens, Paris, France). Individuals with an active implant, with liver ascites, or who were pregnant were excluded from the liver scan. Transient elastography provides a controlled attenuation parameter (CAP) measure of steatosis and a measure of liver stiffness to quantify fibrosis. Manufacturer and machine indications were used to determine whether the M or XL probe would be used to conduct the scan. Ten readings were required for each patient to derive a CAP score and fibrosis result. CAP values outside the 100-400 dB/m range were considered invalid and coded as missing. Additionally, median fibrosis results greater than or equal to15 kPa or with an IQR to median ratio greater than or equal to 30% were considered invalid and coded as missing.

We categorized participants by level of steatosis at 24 years based on CAP scores and using cut-off values derived from a meta-analysis by Karlas, et al.[22] Low steatosis (<10%) was defined as <248 dB/m, mild/moderate steatosis (10-66%) was defined as 248-279 dB/m, and severe steatosis (>66%) was defined as >279 dB/m. We categorized fibrosis values into two groups. The first group included those with no fibrosis or portal fibrosis without Septa (F0-F1, <7.9 kPA) and the second group included those with any fibrosis: portal fibrosis, septa, or cirrhosis (F2-F4, >7.9 kPA).[23]

7.3.3 Covariates

We calculated body mass index (BMI) as weight in kilograms divided by height in meters squared.[24] We classified BMI at 24 years as underweight (<18.5 kg/m^2), normal weight (18.5 to <25 kg/m^2), overweight (25 to <30 kg/m^2), and obese (≥ 30 kg/m^2). We used highest level of maternal education reported during pregnancy as a proxy for socioeconomic status.[25] Mothers self-reported one of five categories: None/CSE (certificate of secondary education), Vocational (vocational courses after 16 years of age), O (ordinary level exams at 16 years), A (advanced level exams at 18 years), and University degree and above.[26] Ethnicity was based on maternal ethnicity and categorized as White or Other.

7.3.4 Inclusion/Exclusion

We included all participants that had liver enzyme measures (ALT, AST, and GGT serum concentrations) available at two of the four available time points (9, 15, 17, and 24 years) and that had valid transient elastography measures at 24 years. We excluded women who self-reported pregnancy at 17 or 24 years. We excluded respondents with hazardous alcohol consumption defined by an Alcohol Use Disorder Identification Test for Consumption (AUDIT-C) score greater than or equal to 4 (women) and five (men).[27] [28]

7.3.5 Statistical Analysis

We calculated median and interquartile ranges (IQR) values for continuous variables and counts and percentages for categorical variables for the full sample and stratified by level of hepatic steatosis (low, mild/moderate, or severe) at 24 years. Kruskal-Wallis tests were used to compare differences in continuous variables across steatosis levels. Chi-squared tests were used to compare differences between categorical variables. For cell counts less than five, Fisher's exact tests were used. We reported sex-stratified ALT, AST, and GGT medians and inter-quartile ranges by age and hepatic steatosis level at 24 years.

Due to non-normal distributions assessed by the Shapiro-Wilk test for normality, we analyzed ALT, AST, and GGT as log-transformed outcomes in all regression analyses. We used repeated-measures linear mixed models to assess trend differences of log-transformed ALT, AST, and GGT levels from ages 9 to 24 years by hepatic steatosis level at 24 years. We included fixed effects for categorical age, random effects for the intercept, and an unstructured error covariance structure. We also modeled differences of log-transformed ALT by fibrosis. We decided to conduct sex-stratified analysis a priori. Covariates were adjusted for in a stepwise manner, whereby model 1 was unadjusted, model 2 included BMI at age 24 years as a covariate, and model 3 included maternal education and ethnicity as covariates. We calculated geometric mean ratios to assess differences between steatosis levels at each age. Comparisons were made using Tukey-adjusted pairwise tests. We checked all model residuals for normality.

A sensitivity analysis was performed to compare characteristics of the original cohort to those in our analytic sample. In addition, to understand the impact of alcohol consumption, we (1) analyzed the association between high AUDIT-C score and hepatic steatosis, and (2) ran the models with AUDIT-C score as a covariate instead of an exclusion criterion.

We conducted statistical analyses in SAS version 9.4 (Cary, NC).

7.4 Results

Of the 10,018 active ALSPAC participants who were invited to participate in the Focus@24 + clinic, 3877 participants had FibroScan® performed (*Figure 7-1*). Of these, 3766 participants had a valid CAP score and 3600 participants had a valid fibrosis score. After exclusions for having ALT measures obtained on no or only one occasion (n = 590), pregnancy (n = 7), and high AUDIT-C score (n = 2013), our analytic sample size was 1156.

Selected demographic and clinical characteristics of the study sample are presented in

Table **7-1**. Most of the participants were female (58.6%) and reported being of White ethnicity (97.4%). The majority (77.8%) had low hepatic steatosis, but 11.9% had mild to moderate and 10.4% had severe hepatic steatosis (

Table **7-1**). There was a positive association between liver enzymes and hepatic steatosis at 24 years ($p < 0.001$). All the clinical biomarkers measured at 24 years had a positive association with hepatic steatosis, except for high-density lipoprotein (HDL) which had a negative association ($p < 0.001$). In sensitivity analysis, there was no association between high alcohol intake and hepatic steatosis level (Chi-square = 2.4, $p = 0.30$). Compared to those enrolled in the original cohort, participants in our sample were more likely to be female and have mothers with a higher education status (both $p < 0.001$).

The trends of ALT, AST, and GGT over time differed across levels of steatosis for both sexes (

Table **7-2**). *Figure 7-2* shows the geometric means and 95% CIs of each liver enzyme plotted over time and stratified by steatosis level at 24 years for each sex from model 1. ALT values increased with age in both sexes, with strong evidence for higher ALT values in those with severe vs. low hepatic steatosis starting at 17 years in males and 24 years in females (*Figure 7-2A,B, Table 3*). AST levels declined in both sexes, until 17 years when they started to increase. In both sexes, strong evidence for differences in AST between those with severe vs. low hepatic steatosis at 24 years were only apparent at 24 years and not in childhood and adolescence (*Figure 7-2C,D, Table 7-3*). In both sexes, GGT values were higher throughout childhood and into adulthood in those with severe vs. low steatosis at 24 years (*Figure 7-2E,F*). Adjusting for BMI at 24 years attenuated the estimates of differences in liver enzymes towards the null, although strong differences between those with severe vs. low hepatic steatosis levels remained at 24 years (*Table 7-3*). Additionally, adjusting for ethnicity and maternal education did not meaningfully change differences. Differences between all levels of hepatic steatosis and for models 1–3 are presented in *Table 7-4*. In males, ALT levels did not differ at any age between those with any vs. no fibrosis at 24 years (*Figure 7-3A*). In females, ALT levels were higher at 17 years, which was maintained at 24 years, in those with any vs. no fibrosis at 24 years (*Figure 7-3B*).

In the sensitivity analysis, controlling for alcohol intake using the AUDIT-C score, instead of excluding those with hazardous alcohol intake, did not meaningfully change estimates (*Table 7-5*).

7.5 Discussion

We found that young adults with severe hepatic steatosis as measured by CAP had a steeper ALT trend from 9 to 24 years compared to those with low hepatic steatosis, with the largest increase occurring from the late teen to young adult years. In males, the differentiation of ALT trends appears to coincide with the beginning of puberty. The association between puberty and increases in steatosis and ALT has been described in previous studies.[29,30] In girls, differences in trend first appeared in late adolescence and with stronger differences occurring in young adulthood. Interestingly, girls with mild/moderate and low steatosis had nearly identical trajectories for ALT, perhaps indicating that ALT is not as sensitive of an indicator in girls, unless they have severe steatosis. NAFLD is known to be a sexually dimorphic disease. Evidence specific to young women indicates that they are better able to partition fatty acids towards ketone body production rather than very low-density lipoprotein (VLDL)-triacylglycerol packaging compared to young men, leading to protection from dysmetabolic conditions such as NAFLD.[31,32]

Concentrations of GGT were consistently higher in those with severe vs low steatosis over time. However, we found that most associations, particularly for GGT, were attenuated after controlling for BMI at 24 years. The strong association of BMI with ALT and GGT has been previously shown.[30,33] This finding supports the current recommendation that screening for NAFLD be done using elevated ALT in at-risk populations, including overweight obese children and adolescents.[2] ALT is an inexpensive, widely available, minimally invasive blood test with acceptable sensitivity, making it a useful screening tool in at-risk pediatric populations.[2] Additionally, these results indicate that more research should be conducted to see if GGT should play a larger role for NAFLD screening in childhood and adolescence.

At 24 years, 10.4% of the ALSPAC sample had severe and 11.9% had mild or moderate hepatic steatosis. Abeysekera, et al. using the same cohort also found a similar 20.7% prevalence of steatosis.[34] This represents a large increase from a NAFLD prevalence of 2.5% (defined as moderate or severe steatosis) in the cohort as measured by ultrasound at age 17 to 18 years.[34,35] This likely partially reflects a

true increase in the prevalence, however, other explanations including selection bias and poorer diagnostic ability of the ultrasound technology compared to transient elastography and CAP measures for assessment of hepatic steatosis are also possible. Estimates of the prevalence of NAFLD in adolescents in the U.S. and Australia defined by a variety of methods including autopsy, ultrasound, and ALT range from 10% to 17%.[3,29,36]

Heavy alcohol consumption is of concern in young adults, particularly in the UK.[37] Approximately half of the sample reported hazardous alcohol consumption at 24 years, a known risk factor for hepatic steatosis. In this cohort at age 24 years, 42.2% reported hazardous alcohol consumption, and 12.7% reported harmful alcohol consumption.[34] To limit potential confounding by alcohol consumption, we excluded all participants with an elevated AUDIT-C score, an instrument used to identify hazardous drinkers. Our criteria led to the exclusion of almost half the sample, therefore, we conducted a sensitivity analysis including participants with any level of drinking and controlling for the AUDIT-C score. We found no meaningful changes to our results. Similar to Abeysekera, et al, we also found no evidence of an association between hazardous alcohol consumption and hepatic steatosis.[34] Future studies should focus on the impact of high alcohol consumption in this population on hepatic steatosis, fibrosis, and other health outcomes.

Strengths and Limitations

The largest strength of our study was the use of serial measurements in a large, population-based longitudinal cohort study from childhood to young adulthood. We also used the CAP score based on transient elastography to define hepatic steatosis levels at 24 years, which is a validated and accurate marker of hepatic steatosis in adults.[22] An additional strength is the use of the AUDIT-C score to exclude individuals with hazardous alcohol consumption, increasing our confidence that participants in our analysis with severe hepatic steatosis had non-alcoholic fatty liver disease. The AUDIT-C has been shown to perform well among adolescents with good internal consistency and accuracy.[38] The AUDIT-C

has also been shown to be more useful than the AST/ALT ratio, an indicator of alcoholic fatty liver disease (vs NAFLD), for predicting hazardous drinking.[39]

While we did not exclude participants with other liver conditions. A previous report from this population reported that no participants had viral hepatitis or were taking nucleos(t)ide analogues or direct-acting antivirals and very few were taking medications for autoimmune hepatitis.[34] Therefore, we are confident that there was little, if any, confounding by other liver disease.

A limitation of this study was the relatively homogenous nature of the sample, of primarily white ethnicity, and results may not be generalizable to other populations. Additionally, there was differential loss to follow-up within the cohort, whereby females and participants with mothers with higher education were more likely to be followed-up. We expect that the associations we found would differ in samples with a larger proportion of higher-risk individuals, such as Hispanics.[40] Individuals with adipogenic genes such as Patatin-like phospholipase domain-containing protein 3 (PNPLA3), which is more common in Hispanics, are more susceptible to NAFLD.[41]

Conclusions

This is the first description of liver enzyme trends extending from childhood through to adulthood and their relation with later hepatic steatosis. ALT trends were associated with hepatic steatosis level in young adulthood. Higher ALT and GGT levels in adolescence were associated with severe hepatic steatosis at 24 years, whereas, prior to puberty, liver enzymes may not be a useful indicator of future risk. Increased testing of liver enzymes in adolescents could strengthen early NAFLD prevention and treatment efforts. There is a need for further quality longitudinal data on the natural history of pediatric NAFLD.

7.6 References

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7.7 Tables & Figures

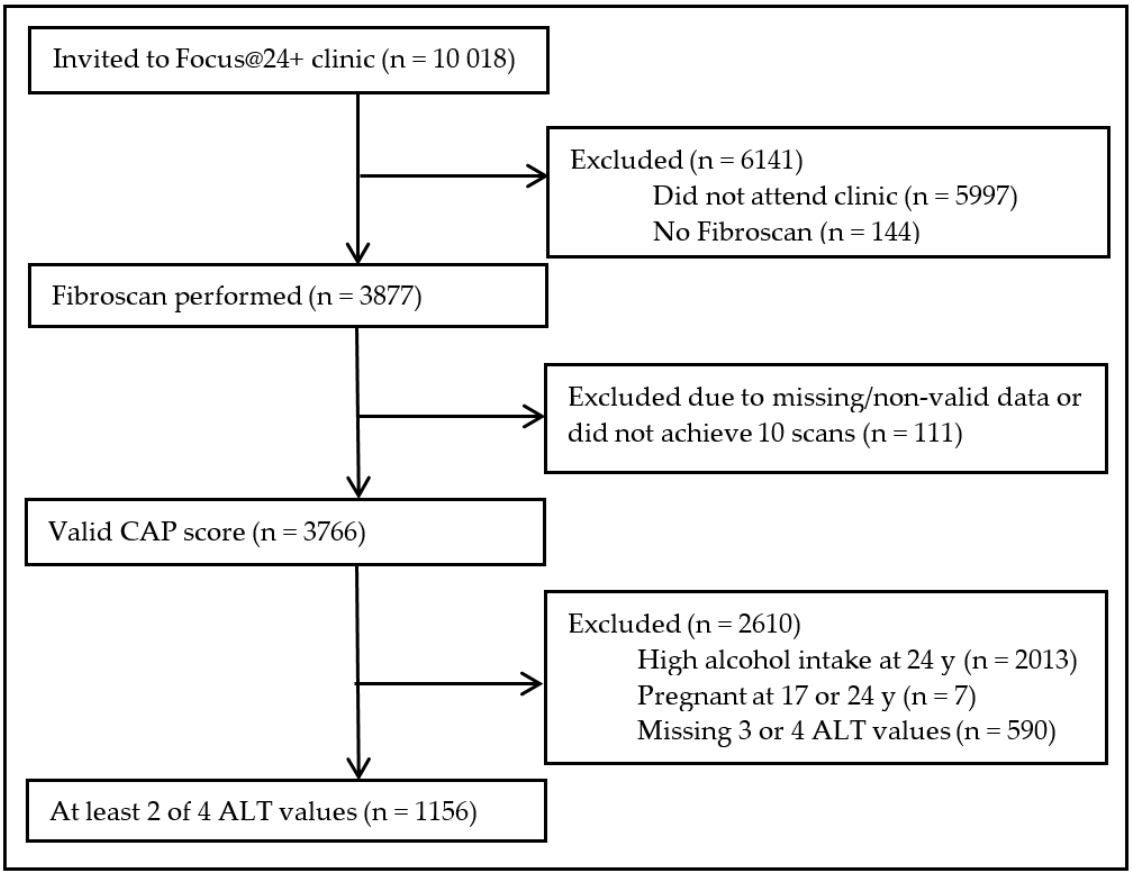

Figure 7-1. *Flow diagram*

Flowchart of the participants included in final analysis. Of the 10,108 participants invited to Focus@24-year clinic, we excluded those that did not attend the clinic or did not get a liver scan because they were ineligible or excluded due to an active implant, liver ascites, or pregnancy. We also excluded participants with missing or non-valid controlled attenuation parameter (CAP) scores, who had a high alcohol intake, were pregnant at the 17- or 24-year clinic, and had less than 2 alanine aminotransferase (ALT) values measured at 9, 15, 17, and/or 24 years. Our final sample size was 1156.

Table 7-1. Demographic and clinical factors at 24 years according to hepatic steatosis level (*n* = 1156)[1].

	Total (*n* = 1156)	**Low (*n* = 899;77.8%)**	**Mild/Moderate (*n* = 137; 11.9%)**	**Severe (*n* = 120; 10.4%)**	***p*-Value**
Age	24 (23, 35)	24 (23, 25)	24 (24, 24)	24 (23, 25)	0.751
Sex					
Male	479 (41.4)	348 (38.7)	65 (47.5)	66 (55.0)	
Female	677 (58.6)	551 (61.3)	72 (52.6)	54 (45.0)	<0.001
Ethnic Group					
White	1041 (97.4)	812 (97.8)	124 (96.1)	105 (95.5)	
Other	28 (2.6)	18 (2.2)	5 (3.9)	5 (4.6)	0.086
Mother's education					
CSE/None	111 (10.4)	83 (10.0)	17 (13.3)	11 (11.5)	
Vocational	70 (6.5)	55 (6.6)	10 (7.8)	5 (4.5)	
O-level	372 (34.8)	280 (33.7)	48 (37.5)	44 (39.6)	
A-level	327 (30.6)	266 (32.0)	32 (25.0)	29 (26.1)	
Degree	190 (17.8)	147 (17.7)	21 (16.4)	22 (19.8)	
BMI, kg/m^2	23.6 (21.2, 27.0)	22.8 (20.7, 25.3)	27.4 (24.5, 29.9)	32.1 (28.5, 35.9)	0.580
BMI category					
Underweight or Normal	659 (62.4)	608 (73.2)	42 (33.6)	9 (9.0)	<0.001
Overweight	260 (24.6)	178 (21.4)	53 (42.4)	29 (29.0)	
Obese	137 (13.0)	45 (5.4)	30 (24.0)	62 (62.0)	
CAP, dB/m	204 (175, 242)	193 (166, 213)	261 (255, 270)	313 (292.5, 343)	
Fibrosis, kPA	4.6 (3.9, 5.5)	4.6 (3.9, 5.4)	4.5 (3.8, 5.4)	5 (4, 6)	<0.001
Any Fibrosis [3]	29 (2.6)	21 (2.4)	<5 [2]	6 (5.3)	
ALT, U/L	20.7 (15.5, 30.0)	19.6 (15.0, 27.9)	20.8 (16.5, 30.8)	35.2 (23.3, 64.0)	<0.001
Elevated ALT	437 (41.0)	308 (36.8)	52 (41.3)	77 (75.5)	0.015
AST, U/L	23.9 (20.5, 29.3)	23.4 (20.3, 28.6)	24.4 (20.0, 28.5)	29.3 (23.9, 36.7)	0.175
GGT, U/L	15.0 (12.0, 21.0)	15.0 (12.0, 19.0)	17.0 (13.0, 22.0)	23.5 (16.0, 35.0)	<0.001
Cholesterol, mmol/L	4.3 (3.8, 4.9)	4.3 (3.8, 4.9)	4.2 (3.9, 4.9)	4.6 (4.1, 5.2)	<0.001
Triglycerides, mmol/L	0.8 (0.6, 1.1)	0.8 (0.6, 1.1)	0.9 (0.7, 1.2)	1.1 (0.8, 1.9)	<0.001
HDL, mmol/L	1.5 (1.2, 1.7)	1.5 (1.3, 1.8)	1.4 (1.1, 1.6)	1.1 (1, 1.4)	<0.001
LDL, mmol/L	2.4 (1.9, 2.9)	2.4 (1.9, 2.9)	2.4 (2.2, 3.2)	2.8 (2.3, 3.2)	0.004

	Total (*n* = 1156)	Low (*n* = 899;77.8%)	Mild/Moderate (*n* = 137; 11.9%)	Severe (*n* = 120; 10.4%)	*p*-Value
VLDL, mmol/L	0.4 (0.3, 0.5)	0.4 (0.3, 0.5)	0.4 (0.3, 0.5)	0.5 (0.4, 0.8)	<0.001
Insulin, mu/L	7.7 (5.4, 11.3)	7 (5, 9.7)	10.8 (7.2, 15.2)	16.6 (10.8, 25.1)	<0.001
Glucose, mmol/L	5.3 (5.0, 5.6)	5.3 (5, 5.6)	5.4 (5.1, 5.7)	5.5 (5.3, 5.8)	<0.001

[1] Values represent the median (IQR) or number of participants (%). Chi-squared tests were used to compare the differences between categorical variables. For cell counts <5, Fisher's exact tests were used. Kruskal–Wallis tests were used to compare the differences across steatosis levels for continuous variables. Some variables had missing values: ethnic group (*n* = 87), mother's highest education level (*n* = 86), BMI (*n* = 9), fibrosis (*n* = 56), and biomarker values (*n* = 91). [2] Groups with less than five participants are expressed as *n* < 5 in line with the Avon Longitudinal Study of Parents and Children (ALSPAC) confidentiality policy. [3] Any fibrosis includes those with portal fibrosis, septa, or cirrhosis (F2–F4, >7.9 kPA). Abbreviations: CSE = certificate of secondary education, BMI = body mass index, CAP = controlled attenuation parameter, ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma-glutamyl transferase, HDL = high-density lipoprotein, LDL = low-density lipoprotein, VLDL = very low-density lipoprotein.

Table 7-2. ALT, AST, and GGT by age and hepatic steatosis at 24 years (n = 479 males, 677 females)[1].

	9 Years	15 Years	17 Years	24 Years	*p*-Value
Hepatic Steatosis			**ALT, U/L**		
Males					<0.001
Low	11.8 (9.0, 15.3)	16.9 (13.5, 20.6)	15.0 (12.3, 20.6)	24.4 (18.2, 32.8)	
Mild/Moderate	11.8 (8.5, 16.0)	17.5 (14.1, 22.7)	17.7 (13.0, 21.2)	28.2 (20.6, 39.7)	
Severe	11.9 (8.8, 14.0)	17.9 (14.2, 23.5)	22.6 (15.3, 30.4)	46.8 (34.6, 73.3)	
Females					<0.001
Low	11.7 (9.2, 14.8)	13.5 (10.6, 17.0)	13.7 (11.1, 17.2)	17.2 (13.4, 23.0)	
Mild/Moderate	11.2 (8.8, 13.9)	13.8 (10.9, 16.1)	14.5 (11.3, 20.1)	16.9 (14.7, 23.6)	
Severe	12.0 (8.6, 15.0)	13.1 (9.9, 19.2)	15.9 (12.3, 27.2)	25.4 (17.9, 32.9)	
			AST, U/L		
Males					<0.001
Low	32.1 (28.4, 35.6)	22.9 (19.6, 26.9)	20.2 (17.2, 23.4)	26.1 (21.7, 31.2)	
Mild/Moderate	30.8 (28.0, 34.2)	23.1 (20.8, 29.4)	20.5 (17.5, 27.1)	26.5 (24.1, 31.6)	
Severe	32.0 (28.2, 35.3)	22.9 (19.9, 27.4)	21.7 (18.2, 25.6)	33.8 (25.9, 38.7)	
Females					<0.001
Low	30.5 (27.1, 34.7)	19.5 (17.2, 22.0)	18.6 (16.2, 22.0)	22.2 (19.5, 26.0)	
Mild/Moderate	29.4 (27.1, 33.3)	18.5 (16.4, 21.7)	17.8(15.5, 22.2)	21.9 (18.9, 26.0)	
Severe	29.1 (25.8, 32.2)	19.2 (15.7, 22.3)	17.6 (16.6, 21.9)	25.5 (22.2, 31.3)	
			GGT, U/L		

	9 Years	15 Years	17 Years	24 Years	*p*-Value
Males					<0.001
Low	15.0 (14.0, 18.0)	16.0 (14.0, 18.0)	17.0 (14.0, 21.0)	16.0 (14.0, 21.0)	
Mild/Moderate	17.0 (14.0, 22.0)	17.0 (15.0, 23.0)	21.0 (16.0, 25.0)	20.5 (15.0, 30.5)	
Severe	17.0 (14.0, 22.0)	19.0 (16.0, 23.0)	21.0 (16.0, 32.0)	27.0 (19.0, 38.0)	
Females					0.002
Low	15.0 (13.0, 18.0)	14.0 (11.0, 16.0)	14.0 (12.0, 18.0)	14.0 (11.0, 18.0)	
Mild/Moderate	16.0 (14.0, 20.0)	14.0 (12.0, 19.0)	17.0 (13.0, 22.0)	14.0 (12.0, 18.0)	
Severe	18.0 (14.0, 22.0)	14.0 (12.0, 19.0)	17.0 (13.0, 20.0)	19.0 (14.0, 31.0)	

[1]Median (IQR). *p*-value from a type 3 test of fixed effects interaction between age and hepatic steatosis level in model 1. Steatosis is defined from the controlled attenuation parameter scores: low (< 248 dB/m), mild/moderate (248–279 dB/m), severe (>279 dB/m). Abbreviations: ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma-glutamyl transferase.

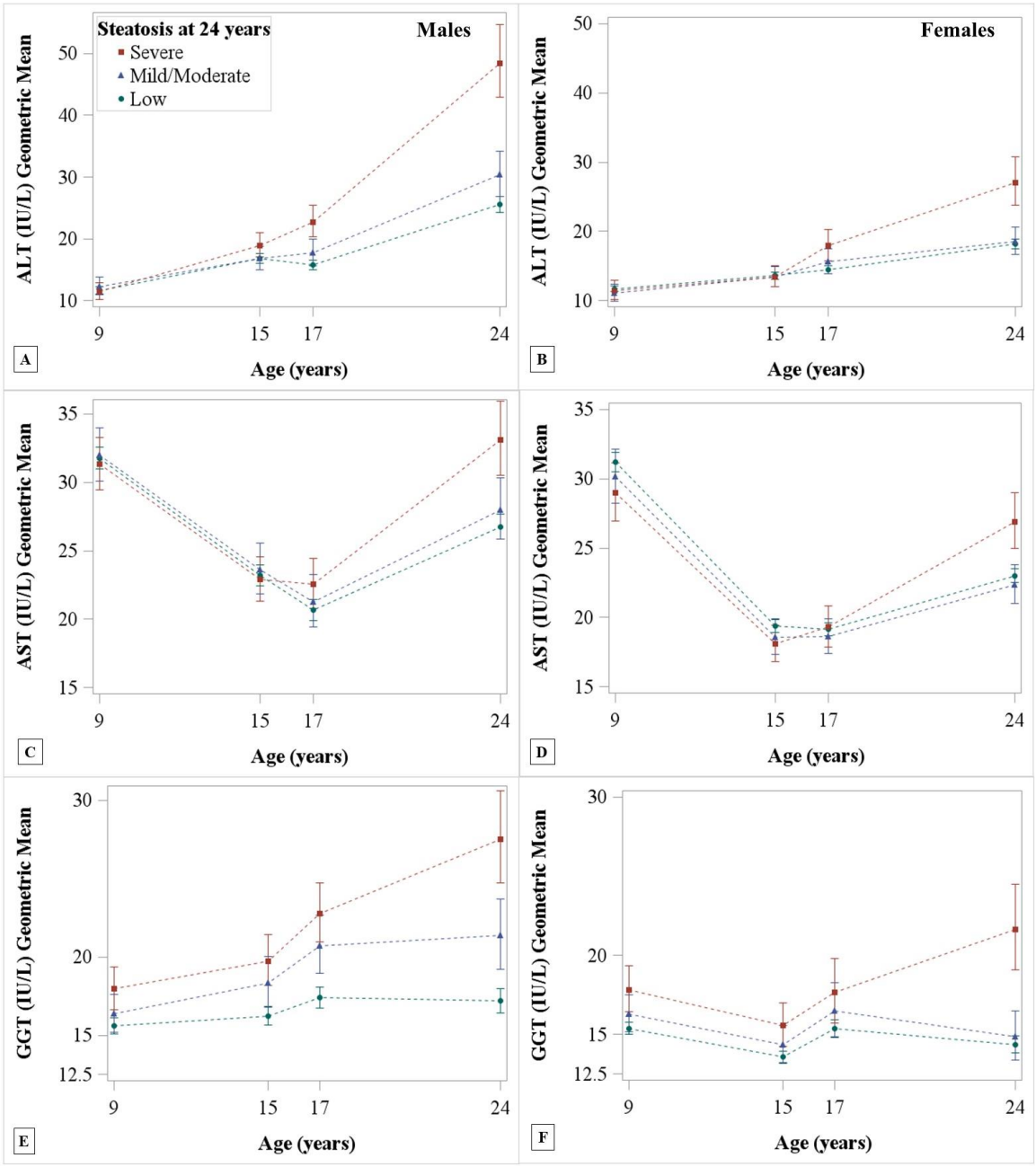

Figure 7-2. *ALT, AST, GGT geometric mean and 95% CI trends by hepatic steatosis level and sex.*

*Sample size was 479 males (**A**,**C**,**E**) and 677 females (**B**,**D**,**F**). Steatosis is defined from controlled attenuation parameter scores: low (<248 dB/m), mild/moderate (248–279 dB/m), severe (>279 dB/m). Low is marked by filled green circles, mild/moderate by blue filled triangles, and severe by filled red squares.*

Table 7-3. Geometric mean ratios and 95% CIs of liver enzymes for low vs. severe hepatic steatosis level at each age (years) and by sex [1].

	Males (*n* = 479)		Females (*n* = 677)	
ALT	**Unadjusted**	**Fully Adjusted [2]**	**Unadjusted**	**Fully Adjusted [2]**
9 years	1.02 (0.82, 1.27)	1.15 (0.90, 1.48)	1.02 (0.82, 1.26)	1.13 (0.89, 1.44)
15 years	0.89 (0.74, 1.07)	1.01 (0.81, 1.26)	1.01 (0.83, 1.24)	1.15 (0.91, 1.45)
17 years	0.69 (0.57, 0.85)	0.8 (0.64, 1.01)	0.81 (0.65, 1.01)	1.01 (0.79, 1.29)
24 years	0.53 (0.42, 0.66)	0.63 (0.5, 0.81)	0.67 (0.54, 0.84)	0.75 (0.59, 0.96)
AST				
9 years	1.01 (0.91, 1.13)	1.03 (0.91, 1.18)	1.08 (0.95, 1.22)	1.08 (0.93, 1.24)
15 years	1.01 (0.89, 1.15)	1.02 (0.88, 1.19)	1.07 (0.94, 1.22)	1.02 (0.88, 1.18)
17 years	0.92 (0.79, 1.06)	0.93 (0.79, 1.10)	0.99 (0.87, 1.13)	1.02 (0.88, 1.18)
24 years	0.81 (0.70, 0.94)	0.86 (0.72, 1.01)	0.86 (0.75, 0.97)	0.84 (0.73, 0.97)
GGT				
9 years	0.87 (0.76, 1.00)	1.01 (0.86, 1.18)	0.86 (0.75, 0.99)	1.00 (0.85, 1.18)
15 years	0.82 (0.71, 0.96)	0.99 (0.85, 1.16)	0.87 (0.75, 1.02)	0.99 (0.82, 1.19)
17 years	0.76 (0.66, 0.89)	0.90 (0.75, 1.07)	0.87 (0.71, 1.07)	1.03 (0.82, 1.30)
24 years	0.62 (0.51, 0.76)	0.73 (0.59, 0.89)	0.66 (0.53, 0.83)	0.77 (0.61, 0.98)

[1] Steatosis is defined from controlled attenuation parameter scores: low (<248 dB/m), mild/moderate (248–279 dB/m), severe (>279 dB/m). [2] Adjusted for BMI at 24 years, maternal ethnicity and education. Abbreviations: ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma-glutamyl transferase.

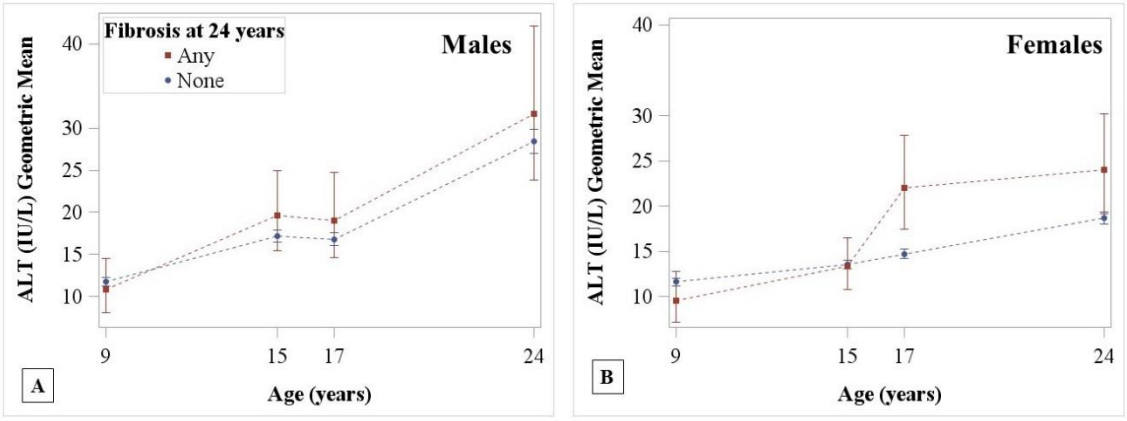

Figure 7-3. *ALT geometric mean trends by fibrosis category and sex.*

(A) *In males, and (**B**) in females. Any fibrosis (red square) includes those with portal fibrosis, septa, or cirrhosis (F2–F4, >7.9 kPA*

Table 7-4. Geometric mean ratios and 95% CIs of liver enzymes for hepatic steatosis levels at each age stratified by sex

		Males (n=479)			Females (n=677)		
Age	**Steatosis**	**Model 1**	**Model 2**	**Model 3**	**Model 1**	**Model 2**	**Model 3**
ALT							
9	Low v Med	0.95 (0.77, 1.18)	1.05 (0.84, 1.31)	1.02 (0.81, 1.27)	1.06 (0.87, 1.29)	1.10 (0.90, 1.34)	1.10 (0.89, 1.36)
9	Low v Severe	1.02 (0.82, 1.27)	1.23 (0.96, 1.56)	1.15 (0.9, 1.48)	1.02 (0.82, 1.26)	1.13 (0.90, 1.43)	1.13 (0.89, 1.44)
9	Med v Severe	1.07 (0.81, 1.42)	1.17 (0.87, 1.56)	1.13 (0.85, 1.52)	0.96 (0.73, 1.27)	1.03 (0.78, 1.37)	1.03 (0.76, 1.38)
15	Low v Med	1.00 (0.82, 1.23)	1.10 (0.89, 1.35)	1.12 (0.91, 1.38)	1.02 (0.84, 1.23)	1.07 (0.88, 1.30)	1.09 (0.88, 1.34)
15	Low v Severe	0.89 (0.74, 1.07)	1.06 (0.86, 1.31)	1.01 (0.81, 1.26)	1.01 (0.83, 1.24)	1.15 (0.92, 1.43)	1.15 (0.91, 1.45)
15	Med v Severe	0.88 (0.69, 1.14)	0.97 (0.75, 1.26)	0.90 (0.69, 1.18)	1.00 (0.77, 1.30)	1.07 (0.82, 1.41)	1.06 (0.80, 1.41)
17	Low v Med	0.89 (0.71, 1.11)	0.97 (0.78, 1.21)	0.97 (0.78, 1.21)	0.93 (0.76, 1.14)	0.98 (0.80, 1.20)	1.00 (0.81, 1.23)
17	Low v Severe	**0.69 (0.57, 0.85)**	0.84 (0.67, 1.04)	0.8 (0.64, 1.01)	0.81 (0.65, 1.01)	0.92 (0.72, 1.17)	1.01 (0.79, 1.29)
17	Med v Severe	0.78 (0.59, 1.03)	0.86 (0.66, 1.13)	0.83 (0.63, 1.09)	0.87 (0.66, 1.16)	0.94 (0.70, 1.25)	1.01 (0.75, 1.36)
24	Low v Med	0.84 (0.68, 1.05)	0.92 (0.74, 1.14)	0.92 (0.74, 1.14)	0.98 (0.81, 1.19)	1.03 (0.85, 1.24)	1.04 (0.86, 1.27)
24	Low v Severe	**0.53 (0.42, 0.66)**	**0.63 (0.50, 0.80)**	**0.63 (0.50, 0.81)**	**0.67 (0.54, 0.84)**	**0.74 (0.58, 0.95)**	**0.75 (0.59, 0.96)**
24	Med v Severe	**0.63 (0.47, 0.83)**	**0.69 (0.52, 0.91)**	**0.69 (0.52, 0.92)**	**0.69 (0.52, 0.91)**	**0.73 (0.55, 0.97)**	**0.72 (0.54, 0.96)**
AST							
9	Low v Med	0.99 (0.89, 1.11)	1.01 (0.90, 1.14)	1 (0.89, 1.13)	1.03 (0.92, 1.16)	1.03 (0.91, 1.16)	1.03 (0.91, 1.16)
9	Low v Severe	1.01 (0.91, 1.13)	1.05 (0.92, 1.19)	1.03 (0.91, 1.18)	1.08 (0.95, 1.22)	1.08 (0.94, 1.24)	1.08 (0.93, 1.24)
9	Med v Severe	1.02 (0.88, 1.18)	1.04 (0.89, 1.20)	1.03 (0.88, 1.20)	1.04 (0.89, 1.22)	1.05 (0.88, 1.24)	1.05 (0.88, 1.25)
15	Low v Med	0.98 (0.85, 1.13)	1.00 (0.86, 1.15)	1.00 (0.86, 1.16)	1.04 (0.92, 1.18)	1.04 (0.91, 1.18)	1.05 (0.92, 1.20)
15	Low v Severe	1.01 (0.89, 1.15)	1.05 (0.91, 1.21)	1.02 (0.88, 1.19)	1.07 (0.94, 1.22)	1.06 (0.92, 1.22)	1.02 (0.88, 1.18)
15	Med v Severe	1.03 (0.86, 1.23)	1.05 (0.88, 1.26)	1.02 (0.85, 1.24)	1.03 (0.87, 1.22)	1.02 (0.86, 1.22)	0.97 (0.81, 1.16)
17	Low v Med	0.97 (0.83, 1.14)	0.99 (0.84, 1.16)	0.99 (0.84, 1.17)	1.03 (0.91, 1.16)	1.02 (0.9, 1.16)	1.04 (0.92, 1.18)
17	Low v Severe	0.92 (0.79, 1.06)	0.95 (0.81, 1.11)	0.93 (0.79, 1.10)	0.99 (0.87, 1.13)	0.99 (0.85, 1.14)	1.02 (0.88, 1.18)
17	Med v Severe	0.94 (0.77, 1.15)	0.96 (0.79, 1.18)	0.94 (0.76, 1.16)	0.96 (0.81, 1.14)	0.97 (0.81, 1.15)	0.98 (0.83, 1.17)
24	Low v Med	0.96 (0.83, 1.11)	0.97 (0.84, 1.13)	0.97 (0.84, 1.12)	1.03 (0.92, 1.15)	1.02 (0.91, 1.15)	1.03 (0.92, 1.16)
24	Low v Severe	**0.81 (0.70, 0.94)**	**0.84 (0.71, 0.98)**	0.86 (0.72, 1.01)	**0.86 (0.75, 0.97)**	**0.84 (0.73, 0.97)**	**0.84 (0.73, 0.97)**

		Males (n=479)			Females (n=677)		
Age	**Steatosis**	**Model 1**	**Model 2**	**Model 3**	**Model 1**	**Model 2**	**Model 3**
24	Med v Severe	0.85 (0.70, 1.02)	0.86 (0.71, 1.04)	0.88 (0.73, 1.08)	**0.83 (0.71, 0.98)**	**0.82 (0.69, 0.97)**	**0.81 (0.69, 0.97)**
GGT							
9	Low v Med	0.95 (0.83, 1.09)	1.03 (0.90, 1.19)	1.02 (0.89, 1.18)	0.94 (0.83, 1.07)	0.99 (0.87, 1.12)	0.99 (0.87, 1.13)
9	Low v Severe	**0.87 (0.76, 1.00)**	1.01 (0.86, 1.18)	1.01 (0.86, 1.18)	0.86 (0.75, 0.99)	0.97 (0.82, 1.13)	1.00 (0.85, 1.18)
9	Med v Severe	0.91 (0.76, 1.09)	0.97 (0.81, 1.17)	0.98 (0.82, 1.18)	0.91 (0.76, 1.09)	0.98 (0.81, 1.17)	1.01 (0.84, 1.22)
15	Low v Med	0.89 (0.75, 1.04)	0.95 (0.81, 1.12)	0.97 (0.84, 1.13)	0.95 (0.82, 1.09)	1 (0.86, 1.16)	1.01 (0.86, 1.19)
15	Low v Severe	**0.82 (0.71, 0.96)**	0.95 (0.81, 1.13)	0.99 (0.85, 1.16)	0.87 (0.75, 1.02)	0.98 (0.82, 1.17)	0.99 (0.82, 1.19)
15	Med v Severe	0.93 (0.76, 1.14)	1.00 (0.82, 1.23)	1.02 (0.85, 1.23)	0.92 (0.75, 1.13)	0.98 (0.8, 1.21)	0.98 (0.78, 1.22)
17	Low v Med	**0.84 (0.71, 0.99)**	0.90 (0.77, 1.06)	0.91 (0.77, 1.07)	0.93 (0.78, 1.12)	0.98 (0.82, 1.18)	1.03 (0.85, 1.26)
17	Low v Severe	**0.76 (0.66, 0.89)**	0.89 (0.75, 1.05)	0.90 (0.75, 1.07)	0.87 (0.71, 1.07)	0.99 (0.8, 1.23)	1.03 (0.82, 1.30)
17	Med v Severe	0.91 (0.74, 1.12)	0.98 (0.80, 1.20)	0.99 (0.80, 1.22)	0.93 (0.72, 1.21)	1.01 (0.77, 1.31)	1.00 (0.75, 1.32)
24	Low v Med	**0.80 (0.66, 0.97)**	0.86 (0.72, 1.04)	0.87 (0.72, 1.04)	0.97 (0.80, 1.16)	1.02 (0.84, 1.23)	1.05 (0.86, 1.28)
24	Low v Severe	**0.62 (0.51, 0.76)**	**0.72 (0.59, 0.88)**	**0.73 (0.59, 0.89)**	**0.66 (0.53, 0.83)**	**0.76 (0.60, 0.95)**	**0.77 (0.61, 0.98)**
24	Med v Severe	**0.78 (0.60, 1.00)**	0.84 (0.66, 1.07)	0.84 (0.66, 1.07)	**0.69 (0.52, 0.90)**	**0.74 (0.56, 0.98)**	**0.74 (0.55, 0.98)**

Note: Medium indicates mild or moderate steatosis. Steatosis is defined from controlled attenuation parameter scores: low (<248 dB/m), mild/moderate (248-279 dB/m), severe (>279 dB/m). Model 1 is unadjusted, model 2 is adjusted for BMI at 24 years, and model 3 is additionally adjusted for maternal ethnicity and education. Ratios that do not cross 1.0 are bolded.

Table 7-5. Geometric mean ratios and 95% CIs of liver enzymes for hepatic steatosis levels at each age stratified by sex including participants regardless of AUDIT-C score.

		Males (n=1038)			Females (n=1513)		
Age	**Steatosis**	**GMR**	**95% CI**		**GMR**	**95% CI**	
ALT							
9	Low v Med	1.00	0.86	1.17	1.03	0.90	1.18
9	Low v Severe	0.93	0.79	1.09	0.92	0.79	1.06
9	Med v Severe	0.93	0.75	1.14	0.89	0.74	1.08
15	Low v Med	0.99	0.85	1.16	0.99	0.87	1.12
15	Low v Severe	0.86	0.74	0.99	0.97	0.84	1.11
15	Med v Severe	0.87	0.71	1.05	0.98	0.82	1.17
17	Low v Med	0.93	0.80	1.09	0.97	0.84	1.11
17	Low v Severe	0.77	0.66	0.89	0.89	0.76	1.03
17	Med v Severe	0.82	0.67	1.00	0.92	0.75	1.12
24	Low v Med	0.90	0.78	1.04	0.96	0.83	1.10
24	Low v Severe	0.60	0.52	0.70	0.70	0.60	0.82
24	Med v Severe	0.67	0.55	0.81	0.73	0.60	0.89
AST							
9	Low v Med	1.01	0.93	1.09	1.04	0.96	1.12
9	Low v Severe	1.00	0.91	1.09	1.03	0.95	1.12
9	Med v Severe	0.99	0.89	1.11	0.99	0.89	1.10
15	Low v Med	0.99	0.89	1.10	1.02	0.94	1.11
15	Low v Severe	0.98	0.88	1.08	1.01	0.92	1.11
15	Med v Severe	0.99	0.86	1.13	0.99	0.88	1.11
17	Low v Med	1.00	0.90	1.11	1.04	0.95	1.14
17	Low v Severe	0.96	0.87	1.06	1.01	0.92	1.11
17	Med v Severe	0.96	0.84	1.10	0.97	0.86	1.09
24	Low v Med	1.02	0.92	1.13	1.05	0.96	1.16
24	Low v Severe	0.86	0.77	0.96	0.89	0.80	0.99
24	Med v Severe	0.84	0.73	0.97	0.85	0.74	0.97
GGT							
9	Low v Med	0.97	0.88	1.06	0.94	0.86	1.03
9	Low v Severe	0.90	0.81	0.99	0.89	0.80	0.98
9	Med v Severe	0.93	0.82	1.05	0.94	0.83	1.07
15	Low v Med	0.94	0.84	1.04	1.01	0.91	1.11
15	Low v Severe	0.82	0.74	0.91	0.85	0.77	0.95
15	Med v Severe	0.88	0.76	1.01	0.85	0.74	0.97

		Males (n=1038)			Females (n=1513)		
Age	**Steatosis**	**GMR**	**95% CI**		**GMR**	**95% CI**	
17	Low v Med	0.90	0.80	1.02	0.94	0.83	1.06
17	Low v Severe	0.77	0.68	0.86	0.88	0.77	1.00
17	Med v Severe	0.85	0.73	0.99	0.94	0.79	1.11
24	Low v Med	0.82	0.72	0.95	0.91	0.79	1.04
24	Low v Severe	0.65	0.57	0.75	0.72	0.62	0.84
24	Med v Severe	0.79	0.66	0.95	0.80	0.66	0.96

Note: Medium indicates mild or moderate steatosis. Steatosis is defined from controlled attenuation parameter scores: low (<248 dB/m), mild/moderate (248-279 dB/m), severe (>279 dB/m). Model controls for AUDIT-C score.

Chapter 8: Conclusions

8.1 Key conclusions & discussion

My overall goal in this dissertation was to contribute to the evidence base on dietary and nutritional risk factors in utero and in early childhood that predict NAFLD in adulthood. I also examined the natural history of childhood hepatic enzyme trend associations with adult NAFLD. In this final chapter, I will start by summarizing the key conclusions and discussion points for each of the aims, followed by highlighting the greatest strengths and weaknesses of my original studies. I will conclude this chapter with sections on the synthesized overall public health implications and future directions for this work.

8.1.1 Protein-energy supplementation in an undernourished population

The first aim of this dissertation was focused on the effects of a protein-energy supplement in utero and in early childhood on NAFLD in mid-adulthood in an undernourished population in Guatemala. We found a high prevalence of overall NAFLD (56.5%), particularly in women.[1] We did not find that exposure to the protein-energy supplement *atole* from conception to age two years was significantly associated with NAFLD in adulthood.

Data on prevalence NAFLD in Central and South America is sparse, despite a known higher risk of NAFLD in Hispanics[2,3]. We add to the known information about the prevalence of NAFLD in Guatemala – showing that it may be higher than previous reports in the region. However, our measure of NAFLD was from a liver fat score so it may not be as accurate as measures from gold-standard methods that quantify hepatic fat directly. Additionally, the reported prevalence is only for one sub-population (INCAP study participants that were followed-up) and thus may not represent the overall prevalence in Guatemala.

Further prevalence studies should use nationally representative samples and validated methods, such as transient elastography, to measure liver fat and fibrosis.

One possible reason that we did not see a significant association between *atole* and later NAFLD may be that the lifetime cumulative effect of other factors such as BMI trajectory or diet may overcome the effect of early life exposure to *atole*. For example, the intake of dietary sugars, which are a known risk factor for NAFLD, were very high in this cohort.[4] Using food frequency questionnaire data collected between 2002 and 2004, dietary carbohydrates contributed 66-72% of total energy intake in this cohort.[5] Added sugar consumption, which was approximately 17% of energy intake, was almost double the 10% limit for free sugars recommended by the World Health Organization to prevent chronic disease.[5,6]

NAFLD is strongly associated with both diabetes and obesity and it is regarded as the hepatic manifestation of metabolic syndrome. The differences in the prevalence of cardiometabolic conditions and biomarkers of insulin resistance in the INCAP cohort have previously been published.[7,8] Early protein-energy supplementation through *atole* was associated with reduced odds of diabetes, increased odds of obesity, lower fasting glucose concentration, and had no association with metabolic syndrome or NAFLD.[7] These differences in association could be due to interlinked mechanisms, including the role of protein in directing the development of metabolically active tissues. For instance, myogenesis and adipogenesis sometimes compete against each other.[9] Future research could focus on the diverging associations between early life nutrition and later cardiometabolic conditions.

Another hypothesis for the diverging associations with cardiometabolic conditions in this population is that the impact of the *atole* exposure on improving socioeconomic status, human capital, and economic productivity led to changes in lifestyle that contribute to obesity such as less physical activity and more calorie-dense diets.[10,11] The combined effect of the *atole* intervention along with socioeconomic factors on cardiometabolic outcomes is a next step for research in this cohort.

8.1.2 Prenatal and early childhood overnutrition

The second aim of this dissertation was focused on the effects of overnutrition in early life on hepatic steatosis in young adulthood in a UK population-based cohort. In aim 2a, we find that maternal nutritional factors particularly overweight, obesity, excess gestational weight gain were positively associated with hepatic steatosis at 24 years, however, this association was completely mediated by adiposity. We did not find a positive association between high free sugar intake in the third trimester of pregnancy and later offspring hepatic steatosis. In aim 2b, higher free sugar intake in early childhood was positively but weakly associated with hepatic steatosis in young adulthood. The consumption of one or more sugary beverages per day was associated with higher odds of hepatic steatosis compared to those consuming less than one sugary beverage per day. This association was attenuated after adjustments for confounders, and was then further attenuated after adjustment for BMI at time of outcome.

The developmental programming of NAFLD has been hypothesized to occur indirectly through the development of adiposity or by direct programming effects on the liver. In our original studies as well as other previous cohort studies, the association between early life nutrition and later NAFLD is mediated by adiposity. The mediation of the association between fetal exposures and adolescent and adult NAFLD by offspring adiposity in our study and others indicates that the programming effect is mediated by adiposity, and not directly through the liver.[12,13] However, other studies that have measured hepatic fat in infants, as well as an ALSPAC study that measured hepatic fat at 17 years, showed that increased hepatic fat was independent of adiposity.[14-16] These studies, along with evidence from many animal studies, indicate that there can be direct programming on the liver.[17,18] One explanation for this divergence in outcomes is that any direct liver early life "programming" effects may be overcome by lifestyle factors later in life. Alternatively, if the development of NAFLD occurs in parallel with obesity (i.e. obesity is in the pathway for the development of NAFLD) than the attenuation of estimates by controlling for adiposity is exactly what we would expect to find, and the true association is what we derive from models not adjusting for BMI or adiposity. Hepatic fat measures in longitudinal birth cohorts starting in infancy and throughout childhood could clarify the question of whether elevated hepatic fat seen in infancy

remains throughout childhood and its relation to overall adiposity. Very few cohorts have measured hepatic fat longitudinally and most commonly it is only obtained at one time point.

In a seminal study in non-human primates, it was demonstrated that a maternal high fat diet led to increased de novo lipogenesis and hepatic fat in offspring regardless of the offspring's diet; however, this association was only apparent in mothers that developed insulin resistance.[19] Human studies have had mixed results on the impact of insulin resistance and diabetes on offspring hepatic steatosis. This is likely due to limitations of the data, as diabetes prevalence has been low in the studied populations and the type of diabetes has not been specified. Disentangling the effects of obesity and insulin resistance in pregnancy is a further avenue of research. More precision is needed in studies to evaluate the gradations of insulin resistance and their interaction with nutrition on NAFLD outcomes.

8.1.3 Pediatric hepatic enzyme levels association with adult NALD

The purpose of the final aim was to describe for the first time whether hepatic enzyme trends from childhood to adulthood are associated with the level of hepatic steatosis at 24 years (low, mild-moderate, or severe). In this study, we found the overall trend in ALT was steeper in those that had severe hepatic steatosis in young adulthood, although it was only after puberty that ALT concentration levels were associated with later severe hepatic steatosis. GGT concentrations were consistently higher in those that ended up with severe vs low steatosis as early as nine years of age; however, these associations were attenuated after controlling for BMI at 24 years. The strong association of BMI with ALT and GGT has been previously shown.[20,21] This finding supports the current recommendation that screening for NAFLD be performed using elevated ALT in at-risk populations including overweight and obese children and adolescents.[22] ALT is an inexpensive, widely available, minimally invasive blood test with acceptable sensitivity, making it a useful screening tool in at-risk pediatric populations.[22] Additionally, we provide evidence of the importance of the timing for screening: screening too early may miss children with later disease. Additional research, including measures of hepatic enzymes at more time points throughout childhood is needed to determine the optimal screening window. In this population, there is also a large

increase in the prevalence of hepatic steatosis from adolescents to young adulthood (~20%). Increased testing of liver enzymes in adolescents could strengthen NAFLD prevention and treatment efforts.

8.1.4 Limitations & Strengths

The greatest strength of this dissertation is that it utilizes longitudinal data from two different, large birth cohorts that have been followed into adulthood. Both the INCAP and ALSPAC cohorts measured early life diet, liver outcomes in adulthood, as well as potential confounders. Liver indices have often been left out of cardiometabolic focused cohorts, therefore the inclusion of measures of hepatic enzymes, steatosis, and fibrosis are important in these studies to further understand longitudinal metabolic health.

We were able to get a good assessment of NAFLD in both cohorts. The ALSPAC used a validated and accurate measure of hepatic steatosis, the CAP score based on transient elastography.[23] Hepatic steatosis was not measured directly in the INCAP cohort, but we were able to calculate a NAFLD liver fat score which has the best non-invasive prediction score for NAFLD, including in Hispanics, and has higher sensitivity and specificity than ALT concentration levels alone.[24,25] Hepatic steatosis can be caused by high alcohol intake, but we were able to adjust for alcohol intake in both cohorts.

While the ultimate goal of research is often to determine causality, in our observational studies, we were only able to describe associations between exposures and outcomes. Many lifestyle, genetic, social, and environmental factors may confound these associations. While we were able to adjust for many potential confounders, residual confounding, particularly by lifestyle, remains a possibility (as in any observational study). Additionally, selection bias (through differential loss to follow-up and missing data) is of concern.

Nutritional epidemiology has been criticized for its reliance of self-report data which is subject to measurement error due to recall bias, social desirability bias, underreporting bias (especially among those who are overweight or obese), etc. While, it is true that this measurement error occurs, self-reported dietary data provides useful information on food intake, behaviors, and patterns[26]. Self-reported dietary

data can be successfully used to inform guidelines and policy through appropriate study design, improvements in dietary assessment instruments, and statistical methods.

Specific to this dissertation, there were several limitations of the dietary sugar variables. First, there are limitations to the FFQs that were used to measure dietary intake. The FFQs are self-reported and thus subject to recall and social desirability bias. Portion sizes were not ascertained, so calculated intakes may be inaccurate. The FFQs utilized by the ALSPAC cohort study were not designed to measure sugar intake. Additionally, at three-years of age, most participants had intake of free sugar above recommended levels for prevention of poor health outcomes. Less than one percent of the three-year old children had intakes within the recommended levels, and those in the lowest quintile had free sugar intakes up to 11.5% of total energy. Parental report of the three-year diet may not be accurate as they may not be the only caregivers of the children. Other caregivers may provide the children with unreported food quantities and types. Finally, maternal sugar intake was only assessed in the third trimester, and this may not reflect dietary intake throughout pregnancy. Despite these limitations, this data is useful in understanding patterns of dietary intake. Reported intake levels fall within ranges of what we would expect to see based on internal comparisons using more rigorous dietary assessment methods such as dietary records and external comparisons with national dietary intake levels.

8.2 Public health implications

This dissertation adds to the evidence-base on the hypothesized relationship between early-life nutrition and later NAFLD. We are the first to look at maternal dietary factors, including protein-energy supplementation and added sugars, as exposures for later offspring NAFLD. Our studies are also the first to extend the associations between early life nutritional factors with NAFLD outcomes into adulthood. Using a life-course approach to understand diseases, particularly cardiometabolic conditions for which risk builds throughout life is important in developing strategies to minimize their health effects. The World Health Organization has declared a “life-course approach” to be essential for achieving the United

Nations 2030 Agenda for Sustainable Development Goals for population health.[27] The first 1000 days of life spanning from conception to two years of age is one of the most vulnerable life stages.[28,29] There is increasing evidence that exposures during this time-period are associated with future metabolic disease risk including hepatic steatosis.[30]

As obesity increases worldwide, there have recently been calls for more research on maternal nutrition and its role in improving health outcomes for women and children.[31] Maternal diets are the most direct method that can alter structure, function, and metabolism during fetal development.[31] Diet is also a modifiable behavior, yet, very little is known about how different maternal diets can change the perinatal environment and can lead to lasting changes in the offspring metabolic profile. There is also evidence that dietary patterns in early childhood track into adulthood, and that tastes for specific foods may even start as early as in utero.[32,33] Therefore, identifying the most important modifiable factors in early life is an important area of research to improve outcomes for mothers and children. Moreover, understanding the developmental origins of NAFLD and other metabolic conditions is particularly important because of the potential for transgenerational amplification of these diseases.[34] In 2017 to 2018 approximately 40% of U.S. women 20-39 years were obese and the prevalence of obesity continues to rise worldwide, compounding the amplification of metabolic diseases through generations. [35,36,37]

Finally, we show that in a UK population, ALT concentration begins tracking higher after puberty in those that end up with severe steatosis, and GGT concentration is elevated as early as nine years, although this is mediated by BMI. This highlights the importance of finding the right window for screening for NAFLD in children. Studies on the prevention and treatment of NAFLD, including through diet, will benefit from understanding if mild increases in hepatic enzymes throughout childhood are markers of later NAFLD.

8.3 Future directions for research

While we did not find any strong independent associations between early life nutrition and adulthood NAFLD, our findings highlight the need for further studies on the causes, underlying mechanisms, and development of NAFLD throughout the life course. Understanding these risk factors could lead to targeted recommendations to prevent and treat NAFLD starting in the earliest phases of life.

The most sensitive period of fetal development for hepatic steatosis may be prior to the third trimester. The development of the fetal liver begins at four weeks gestation and is susceptible to fundamental changes to its metabolic pathways through epigenetic changes and mitochondrial dysfunction. The liver may be utilized as a site for excess lipid storage, especially prior to 28 weeks gestation when subcutaneous fat storage exponentially increases.[38] Future studies should be designed to accurately measure maternal diet and biomarkers throughout pregnancy and to elucidate the exact mechanism through which gestational nutrition can prime offspring for later metabolic dysfunction.

While increased hepatic fat has been seen in infancy and most children are diagnosed with NAFLD at the onset of puberty, very little is known about the period prior to puberty.[22] This is a potentially sensitive period in which NAFLD could be prevented prior to the onset of puberty associated insulin resistance and adiposity. Ideal studies should utilize longitudinal birth cohorts with validated measures of nutrition starting in pregnancy as well as measure of hepatic steatosis throughout the life-course.

There is a strong need for further longitudinal studies in non-European and Caucasian populations. Hispanic populations have one of the largest burdens of NAFLD, as a combination of higher risk behaviors (e.g. sugar intake) and genetic predisposition. Individuals with adipogenic genes such as Patatin-like phospholipase domain-containing protein 3 (PNPLA3), which is more common in Hispanics, are more susceptible to NAFLD.[39] We expect that the associations we found would differ in samples with a larger proportion of higher-risk individuals, such as Hispanics.[40]

While it is known that high sugar diets and sugary sweetened beverages have detrimental effects on cardiometabolic health, there is a still a very high prevalence of sugar intake, particularly in children.[41] Reducing free sugar intake to less than 3% of total energy leads to improvements in hepatic fat in children with NAFLD, but there have been no studies on the prevention of NAFLD by sugar reduction. There is a need for research on the best strategies to promote the reduction of sugar intake in children and in pregnancy.

In this dissertation we only explored the influence of a few specific dietary factors including energy-protein supplementation in an undernourished population, high free sugars, and high sugary beverage intake. Future research could focus on the independent and combined effect of other early life dietary factors on NAFLD. For example, in most animal studies, high-energy maternal diets achieved through a combination of high fat and high sugar are utilized to induce hepatic steatosis in offspring.[18,42-44] Beyond macronutrient distributions, exploring the effect of dietary patterns is important because they reflect the way we eat in real-life and take into account the synergistic effects of different foods and beverages on health outcomes.

8.4 References

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