Lay Summary

Infant nutrition and maternal obesity prospectively influence the risk of nonalcoholic fatty liver disease in adolescents

Non-alcoholic fatty liver disease (NAFLD) is a disorder in which there is excessive fat deposition in the liver that is commonly associated with obesity, in the absence of excessive alcohol intake. NAFLD is now the most common liver disorder in most populations and can occur at any stage from early childhood through to old age. Concerns regarding NAFLD arise from it becoming more commonly diagnosed, associated with increased risk of diabetes, obesity, cardiovascular disease, liver cirrhosis and liver cancer in some cases.

There is currently no approved treatment for NAFLD, with lifestyle changes involving dietary care and exercise recommended. We have previously shown links between NAFLD and a Western dietary pattern, high fructose intake and trajectories of weight gain from early childhood. In this study we have examined whether there is an association between early infant feeding and maternal factors on NAFLD in well-characterised adolescents from the Western Australian Pregnancy (Raine) Cohort.

We have shown that there is an increased risk of NAFLD in offspring of mothers who are obese at the start of pregnancy. Obesity in adolescents also increases the risk of NAFLD. However, breastfeeding without starting infant formula milk for the first 6 months of life reduces the risk of NAFLD, even after taking into account maternal and adolescent obesity and an unhealthy Western dietary pattern. Adolescents diagnosed with NAFLD but who were breastfed and with delayed initiation of formula milk for at least 6 months, were less obese and had less severe risk factors for cardiovascular disease and type II diabetes.

Based on our findings we consider that reducing the risk of NAFLD needs to start before birth, aiming for a normal pre-pregnancy body mass index. Breastfeeding for at least 6 months and delaying formula milk feeding for that duration should be encouraged. Finally, child and adolescent obesity should be avoided.

Infant nutrition and maternal obesity prospectively influence the risk of nonalcoholic fatty liver disease in adolescents

Short Title: Maternal obesity and duration of breastfeeding are associated with NAFLD

Oyekoya T Ayonrinde[1-3], Wendy H Oddy[4,5], Leon A Adams[1,6], Trevor A Mori[1], Lawrence J Beilin[1], Nicholas de Klerk[4], John K Olynyk[2,3,7]

[1]School of Medicine and Pharmacology, The University of Western Australia, Perth, WA, Australia, [2]Department of Gastroenterology and Hepatology, Fiona Stanley Hospital, Murdoch, WA, Australia, [3]Faculty of Health Sciences, Curtin University, Bentley, WA, Australia, [4]Telethon Kids Institute, The University of Western Australia, Perth, WA, Australia [5]Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania, Australia, [6]Department of Hepatology, Sir Charles Gairdner Hospital, Nedlands WA, Australia, [7]Edith Cowan University, Joondalup, WA, Australia

Corresponding author

Dr Oyekoya T. Ayonrinde

Department of Gastroenterology and Hepatology

Fiona Stanley Hospital

11 Robin Warren Drive

Murdoch 6150

Australia

Tel: +61861522827

E-mail oyekoya.ayonrinde@health.wa.gov.au

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Abbreviations

NAFLD, Nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment for insulin resistance; IQR, interquartile range; CI, confidence interval; OR, odds ratio; ≥, equal to or greater than; <, less than.

Infant nutrition and maternal obesity influence the risk of nonalcoholic fatty liver disease in adolescents

Abstract

Background and aims

The pathway to nonalcoholic fatty liver disease (NAFLD) in adolescents may have its origins in adiposity gains, nutrition and sedentary lifestyle established during childhood. There is inadequate knowledge regarding associations between infant nutrition and subsequent NAFLD. We examined the association of maternal factors and infant nutrition, with the subsequent diagnosis of NAFLD in adolescents.

Methods

Adolescents aged 17 years in the Western Australian Pregnancy (Raine) Cohort study had fatty liver assessment using liver ultrasound. Prospectively recorded data on maternal pregnancy and infant feeding were examined against a NAFLD outcome during late adolescence.

Results

NAFLD was diagnosed in 15.2% of the 1170 adolescents examined. Ninety-four percent had been breastfed as infants. The duration of breastfeeding before starting supplementary milk was ≥4 months in 54.4% and ≥6 months in 40.6%. Breastfeeding without supplementary milk ≥6 months (adjusted OR 0.64, 95% CI 0.43-0.94, p=0.02), maternal pre-pregnancy obesity (adjusted OR 2.29, 95% CI 1.21-4.32, P=0.01) and adolescent obesity (adjusted OR 9.08, 95% CI 6.26-13.17, <0.001) were associated with NAFLD independent of a Western dietary pattern at age 17 years. Adolescents with NAFLD who had been breastfed for ≥6 months had a less adverse metabolic profile compared with adolescents breastfed for <6 months.

Supplementary milk intake starting before 6 months was associated with a higher prevalence and ultrasound severity of NAFLD compared with intake starting after 6 months (17.7% vs. 11.2%, p=0.003 and 7.8% vs. 3.4%, p=0.005 respectively).

Conclusion

Though NAFLD is generally mediated through adiposity gains, breastfeeding for at least 6 months, avoidance of early supplementary formula milk feeding, and normal maternal pre-pregnancy BMI may reduce the odds of a NAFLD diagnosis during adolescence.

Lay summary

Non-alcoholic fatty liver disease (NAFLD) is a common liver disorder in which there is too much fat in the liver in people who do not consume excessive amounts of alcohol.

In this large study we found that infants who consumed breast milk for less than 6 months before starting infant formula milk, infants who were obese as teenagers or had mothers who were obese at the start of pregnancy, were much more likely to have NAFLD at age 17 years.

Based on our findings we consider that reducing the risk of NAFLD in teenagers needs to start before birth, by encouraging normal body mass index before pregnancy, as well as breastfeeding without infant formula milk consumption for the first 6 months of life.

Non-alcoholic fatty liver disease (NAFLD) is a complex disorder in which there is excessive fat deposition in the liver that is commonly associated with obesity and insulin resistance in the absence of excessive alcohol intake. NAFLD is now the most common liver disorder in humans (1), with a general population prevalence of 2.6% in children (2), 15.2% in adolescents (3) and 19-25% in adults (4-6). Population data from the National Health and Nutrition Examination Survey (NHANES) in the USA showed a doubling of the prevalence of suspected NAFLD in adolescents over a 20 year period up to 2010 (7). Severe hepatic steatosis diagnosed using ultrasound has been shown to be independently associated with increased liver disease morbidity and mortality (8). Further, the histologic spectrum of NAFLD, comprising plain steatosis, non-alcoholic steatohepatitis (NASH) and NASH-associated cirrhosis can occur from childhood through to adulthood (9). However, despite increasing evidence that adiposity gain during childhood and adolescence is a significant risk factor for NAFLD in adolescence (10,11) and in adulthood (12), the role of early life nutrition, including breastfeeding has not been adequately elucidated.

Prior to 2001, the World Health Organisation (WHO) recommended that infants be exclusively breastfed for 4 to 6 months before introducing complementary foods, however, recommendations now suggest exclusive breastfeeding for the first 6 months of life (13). Benefits of breastfeeding extend beyond nutritional value to include potential reduction in rates of childhood infection, obesity and allergies later in life (14). There are, however, inconsistent reports about the influence of breastfeeding on later obesity (15), with some studies describing a possible protective effect on obesity (16,17) considered more likely with a threshold of 6 months of breastfeeding (18-21) and other studies unable to demonstrate this (22-24). The contradictions may reflect varied breastfeeding definitions (22), mixed feeding patterns including infant formula milk or complementary feeding, recall bias, maternal and individual factors including prevalent dietary habits and sedentary lifestyle. Maternal obesity

has been associated with shorter durations of breastfeeding, early introduction of supplementary formula milk and complementary food, possibly unhealthy food preferences in childhood and later obesity (25). There are, however, few studies examining whether maternal obesity, infant nutrition and early feeding habits prospectively influence the development of NAFLD. In particular, there is no current evidence that breastfeeding causally reduces NAFLD risk in humans. In the only published observational study examining the effect of breastfeeding on the development of NASH in humans, Nobili et al found longer duration of breastfeeding was associated with a reduction in the risk of NASH in children and adolescents (26).

The aim of this study was to examine associations between duration of breastfeeding and age at introduction of complementary milk or solid food, maternal pre-pregnancy obesity and adolescent obesity, on the diagnosis of NAFLD in adolescents from the Western Australian Pregnancy Cohort (Raine Cohort) at 17 years of age.

Materials and methods

The Raine study is a longitudinal cohort study with prospectively collected maternal, birth, child and adolescent data, including detailed nutritional data in the early years of life and serial follow up every 2-3 years. The Raine Study was initiated as a pregnancy and birth cohort comprising 2,868 live-born children from 2,900 pregnancies recruited mainly from the antenatal clinics of King Edward Memorial Hospital for Women in Perth, Western Australia between 1989 and 1992. The background and serial assessments of the Raine cohort has been detailed previously (21). The following terms are explained: antenatal refers to the period during pregnancy, neonate refers to the newborn and infant is the child under 1 year. Antenatal data on mothers was prospectively collected, incorporating socio-demographic characteristics, history of gestational diabetes, hypertension during pregnancy, weight and

height and calculated body mass index (BMI). Neonatal data included mode of delivery, birth anthropometry, early feeding pattern and age when discharged home. Each subsequent child assessment involved detailed questionnaires on lifestyle, health, medications, and physical assessments including anthropometry and cardiovascular assessments. Lists of medications given to the infant, including antibiotics, were documented by the mother or care-giver at the 1-year assessment. Aspects of infant nutrition examined were the duration of breast milk feeding, age at introduction of non-breast milk and solid feeding and types of milk consumed, as reported by the parents or primary caregiver of the child during the first 3 years of life. Mothers recorded the age at which breast-milk feeding stopped in a diary and this was clarified by direct interview during the ages 1,2 and 3-year surveys. Exclusive breastfeeding is defined as per the World Health Organisation as breastfeeding with no supplementary milk or complementary food intake (13). For the purpose of this study the terms breastfeeding and breast milk feeding are used interchangeably while consumption of supplementary milk or infant formula milk are considered the same. The age at which individuals stop breastfeeding and age of starting infant formula milk may have different metabolic effects. For example, in the Raine Study infants breastfed for >4 months but introduced to other milk at ≤4 months (mixed feeding) had the highest increase in BMI at age 14 years (17). Therefore, we considered the duration of breastfeeding and age at introduction of supplementary milk and complementary food separately, given the potential mixed patterns of feeding. Breastfeeding with supplementary milk intake and breastfeeding with no supplementary milk are used to describe feeding patterns regardless of any other complementary food intake.

The 17-year cross-sectional assessment of the cohort was conducted between July 2006 and June 2009, at which time the participating cohort was representative of the broader Western Australian population (3). At age 17 liver ultrasound was performed to assess for fatty liver. Other data collected at the time were derived from detailed questionnaires, anthropometric,

clinical and biochemical measurements as previously described (3). Laboratory assessments were performed with venous blood samples taken from an antecubital vein after an overnight fast. Serum glucose, insulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), ferritin, transferrin saturation, high sensitivity C-reactive protein (hsCRP), adiponectin, and leptin levels were assayed. We did not test for hepatitis B or C virus infections because notification rates for hepatitis B and C virus infections were on average less than 24/100,000 and 23/100,000, respectively, for Western Australian teenagers between the ages of 15 and 19 years over the study period (3). Anthropometric measurements [weight, height, waist circumference, hip circumference, and skinfold thickness (SFT)] and cardiovascular assessments [resting pulse rate, systolic blood pressure (SBP), and diastolic blood pressure (DBP)] were conducted by trained examiners. BMI was derived from weight (kg)/ height^2 (m^2). Central obesity in the adolescents was defined by waist circumference ≥80 cm in females and ≥94 cm in males, consistent with age and gender-specific metabolic syndrome criteria of the International Diabetes Federation (27). We defined adolescent obesity by waist circumference since we previously identified a higher proportion of adolescents with central obesity using waist circumference than using BMI (3). The homeostasis model assessment for insulin resistance (HOMA-IR) score was calculated as follows: HOMA-IR score = [Fasting insulin (μU/ml) x Fasting glucose (mmol/L)]/ 22.5.

Previously published reports describe the liver ultrasound methodology (3) and protocol (28). The diagnosis of hepatic steatosis (fatty liver) by ultrasound required a total fatty liver score of at least 2, including a liver echotexture score of at least 1. The ultrasound score was computed from liver echotexture (bright liver and hepato-renal echo contrast) 0-3, deep attenuation (diaphragm visibility) 0-2, and vessel blurring (intrahepatic vessel visibility) 0-1.

NAFLD steatosis severity was derived from the total fatty liver score as 0 to 1 (no fatty liver), 2 to 3 (mild fatty liver), or 4 to 6 (moderate to severe fatty liver). We used an alcohol intake threshold of < 140 grams per week for females and < 210 grams per week in males, consistent with recent NAFLD diagnosis and management guidelines (29), to refine the ultrasound diagnosis of fatty liver to a clinical diagnosis of NAFLD. At 14 years of age, the adolescent, the parent or care-giver completed a semi-quantitative food frequency questionnaire (FFQ) developed by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) (30,31). From the FFQ data two dietary patterns, described as the Healthy pattern or Western pattern were defined and the extent of intake of these during the preceding 12 months was estimated. A z-score was assigned for each dietary pattern, indicating how closely the reported intake corresponded with the 2 patterns (31). We have previously described associations between dietary patterns and NAFLD in adolescents in the Raine cohort (31). Institutional ethics committee approval was obtained from the Princess Margaret Hospital for Children Human Research Ethics Committee. Signed informed parental consent and adolescent assent at 17 years were obtained.

Statistical analysis

Variables were summarized by the mean and standard deviation for symmetrical distributions and median and interquartile range (IQR) for asymmetric distributions. Differences in normally distributed data were analysed using Student's t-test or Analysis Of Variance (ANOVA), while non-normally distributed data were analysed using the Mann-Whitney U test. Chi-square or Fisher's exact test, as appropriate, were used to compare proportions. Multivariable logistic regression analysis was used to identify predictors of NAFLD from maternal data, adolescent obesity, adolescent dietary patterns and infant feeding data. All statistical tests were two-sided and based on a significance level of 5%. Data were analyzed using IBM SPSS statistics for Windows (version 20.0; Armonk, NY: IBM Corp.). Because of

the change in WHO recommendations for exclusive breastfeeding from 4 months to 6 months (13) we paid particular attention to differences between effects of introducing other milk after 4 months or after 6 months.

Results

NAFLD in the Raine Cohort

The cohort comprised 1170 community-based 17-year-old adolescents. Median alcohol intake was 10 grams per week (IQR 0-90 grams per week) during the preceding 12 months. 3 adolescents were excluded from analysis due to excessive alcohol intake. Consequently, NAFLD was diagnosed in 177/1167 (15.2%), while 236/1156 (21.1%) with documented waist circumference had central obesity, comprising 32.7% female and 9.9% male ($P < 0.001$). NAFLD was more prevalent in females than in males (19.6% vs 10.8%, $P < 0.001$), consistent with the female predominance of central obesity. However, amongst the centrally obese, 63/180 (35%) of females and 34/56 (60.7%) of males had NAFLD. Comparisons of adolescents in the cohort with or without NAFLD are shown in Table 1.

Breastfeeding in the Raine Cohort

The duration of breastfeeding was documented for 1153 study participants. The median (interquartile range [IQR]) duration of breastfeeding was 7.0 (2.0-12.0) months. There was no difference in the duration of breastfeeding between males and females (7[2-12] vs. 6[2-11], p=0.84 respectively). The duration of breastfeeding was ≥4 months in 66.5%, ≥6 months in 56.3% and ≥12 months in 26.7%. True exclusive breastfeeding continued ≥4 months in 42.9% and ≥6 months in 7.4%. Breastfeeding without supplementary formula milk occurred for ≥4 months in 54.4% and for ≥6 months in 40.6%. Further, breastfeeding for ≥6 months with supplementary milk introduced at ≥4 months was seen in 47.2%.

Maternal characteristics associated with the duration of exclusive breastfeeding

After adjusting for maternal pre-pregnancy BMI, mothers who exclusively breast-fed their infant for <4 months compared with those who exclusively breastfed for ≥4 months tended to be younger, more likely to smoke during pregnancy, had lower family income and were less likely to be in a married or defacto relationship or to have completed secondary school education. A similar pattern was seen with early introduction of supplementary milk prior to 4 months and 6 months in breastfeeding mothers (Table 2). By contrast, independent predictors of breastfeeding for ≥6 months were maternal age over 30 years (OR 2.35, 95% CI 1.80-3.09, p<0.001), maternal non-smoking during pregnancy (OR 2.49, 95% CI 1.81-3.43, p<0.001), normal pre-pregnancy BMI (1.70, 95% CI 1.20-2.38, p=0.003) and annual family income >$36,000 at the time of delivery (OR 1.68, 95% CI 1.11-2.55, p=0.02).

Influence of initial feeding at birth on infant feeding and adolescent NAFLD

Most neonates (94%) were breastfeeding on leaving hospital. Neonates who were discharged from hospital breastfeeding had a higher likelihood of breastfeeding at 6 months when compared with neonates discharged bottle-feeding (59% vs. 3%, p<0.001). There was a lower prevalence of adolescent NAFLD in neonates discharged home breastfeeding vs. bottle-feeding (14.6% vs. 24.3%, p=0.03). However, there was no significant difference in the prevalence of NAFLD based merely on having ever been fed breast milk or not (14.5% vs. 19.8%, p=0.12). Adolescents with NAFLD had a shorter duration of breastfeeding compared with adolescents without NAFLD (Table 1).

Effect of breastfeeding for ≥ 4 months on the Prevalence of NAFLD

Adolescents who had been exclusively fed breast milk for ≥4 months, compared with those with exclusive breastfeeding <4 months, had a lower prevalence of NAFLD (12.1% vs.

17.1%, p=0.02). Exclusive breastfeeding for ≥4 months was associated with reduced odds of NAFLD after adjusting for maternal obesity (OR 0.67, 95%CI 0.48-0.96, p=0.03) but did not remain significant when adolescent obesity was added into the model (OR 0.72, 95%CI 0.49-1.07, p=0.10). Similarly, when breastfeeding for ≥4 months without supplementary formula milk but disregarding any solid food intake was considered, there were reduced odds of NAFLD after adjusting for maternal obesity (OR 0.70, 95%CI 0.50-0.98, p=0.04) but this did not remain significant when additionally adjusted for adolescent obesity (OR 0.72, 95%CI 0.50-1.04, p=0.10). However, breastfeeding for ≥4 months without supplementary formula milk was associated with a lower prevalence of NAFLD compared with breastfeeding supplemented with formula milk intake <4 months (12.9% vs. 17.6%, p=0.03)

Effect of breastfeeding for ≥ 6 months on the prevalence and severity of NAFLD

Breastfeeding without supplementary milk for ≥6 months was independently associated with reduced odds of NAFLD in adolescence after adjusting for adolescent and maternal obesity (adjusted OR 0.64, 95% CI 0.43-0.94, p=0.02) and after adjusting for healthy and Western dietary patterns during adolescence (adjusted OR 0.60, 95% CI 0.41-0.87, p=0.008). Breastfeeding without supplementary milk for ≥6 months compared to <6 months, was associated with a lower prevalence of NAFLD even if complementary solid food was consumed prior to 6 months (11.1% vs. 17.9%, p=0.002). Non-exclusive breastfeeding ≥6 months compared with <6 months was associated with a lower prevalence of NAFLD (12.6% vs. 18.3%, p=0.007). Breastfeeding without supplementary milk for ≥6 months, compared with <6 months, more than halved the prevalence of severe steatosis in adolescents with NAFLD (3.5% vs. 7.7%, p=0.005).

Association of duration of breastfeeding and age at introduction of supplementary milk on NAFLD and serum liver enzymes in adolescence

The prevalence of NAFLD was lower with longer durations of breastfeeding (Figure 1). There was a significant negative correlation between serum GGT in the adolescents and the duration of breastfeeding and age at introduction of supplementary milk (r= -0.08, p=0.01 and r= -0.09, p=0.006 respectively). There was no significant correlation between serum ALT and breastfeeding duration or age at starting supplementary milk (r= -0.03, p=0.70 and r= -0.04, p=0.31 respectively).

Association of duration of breastfeeding on metabolic characteristics of adolescents with NAFLD.

Adolescents with NAFLD who had been breastfed for ≥6 months had a less adverse metabolic profile compared with adolescents breastfed for <6 months. In particular, adolescents with NAFLD who were breastfed for ≥6 months had lower weight, BMI, waist circumference, subcutaneous fat, resting pulse rate, lower serum GGT, triglycerides, leptin, hs-CRP and HOMA-IR (Table 3).

Effect of the age at introduction of supplementary formula milk on breastfeeding and NAFLD

The median [IQR] duration of breastfeeding was shorter in infants introduced to formula milk <4 months compared with ≥4 months (2[0-4] months vs. 10[7-13] months, p<0.001. Adolescents with NAFLD had commenced supplementary formula milk intake at a significantly younger age than those without NAFLD (Table 1). The prevalence of NAFLD was lower the longer the delay in commencing supplementary milk (Figure 1). Formula milk intake earlier than 6 months was associated with a higher prevalence and severity of NAFLD than intake commenced after 6 months (17.7% vs. 11.2%, p=0.003 for NAFLD prevalence; 7.8% vs. 3.4%, p=0.005 for severe steatosis). Infants commencing supplementary formula milk earlier than 6 months had an increased risk of a NAFLD diagnosis during adolescence

(OR 1.71, 95% CI 1.02-2.43, P=0.003). This remained significant after adjusting for healthy and Western dietary patterns during adolescence (adjusted OR 1.62, 95% CI 1.11-2.37, p=0.01).

Effect of type of milk consumed at 1 year and age at introduction of solid food on Adolescent NAFLD

Types of milk consumed by the infants at 1 year were breast milk-only 18.4%, infant formula milk-only 9.3%, cow milk-only 60.3%, soy milk 5.7% and unstated or other types or combinations of milk 6.3%. There was no difference in the proportion of infants subsequently diagnosed with NAFLD in association with the most common types of milk consumed at age 1 year (breast milk 14.9%, formula milk 16.8%, cow milk 16.1%; p=0.30). Solid food feeding was introduced prior to 4 months in 18.6% and prior to 6 months in 79.0% of infants. The age at which solid food was commenced did not significantly contribute to the odds of being diagnosed with NAFLD during adolescence (OR 1.01, 95% CI 0.89-1.15, p=0.85), Figure 1.

Association of infant antibiotic use with breastfeeding duration and NAFLD

Data on antibiotic treatment during the first year of life was available for 1114 adolescents, amongst whom 16 had received antibiotics. Infants who had been treated with antibiotics had a shorter duration of breastfeeding compared with infants not treated with antibiotics (4.0 [1.3-5.8] vs. 7.0 [3.0-12.0] months, p=0.02). However, there was no difference in antibiotic use during infancy in adolescents with NAFLD compared with adolescents without NAFLD (0.6% vs. 1.7% respectively, p=0.35)

Prediction of adolescent NAFLD

Univariate associations of the odds of NAFLD from potential risk factors were computed (Table 4). Using multiple logistic regression analysis, predictors of adolescent NAFLD were

determined from clinically or statistically plausible covariates, including the duration of breastfeeding without supplementary milk (<6 months vs ≥6 months) or age of introduction of formula milk, maternal pre-pregnancy obesity, maternal age, maternal smoking during pregnancy, adolescent obesity and dietary patterns during adolescence. Breastfeeding without supplementary milk ≥6 months reduced the risk, while maternal obesity and adolescent obesity increased the risk of a NAFLD diagnosis after adjusting for covariates (Table 5). In the whole cohort, neither a Western dietary pattern nor healthy dietary pattern at age 14 years was significantly associated with NAFLD at age 17 years (OR 0.99, 95% CI 0.80-1.21, p=0.89 and OR 1.06, 95% CI 0.86-1.29, p=0.61 respectively). By contrast, in obese adolescents a Western dietary pattern at age 14 years was associated with an increased risk of NAFLD (OR 1.45, 95% CI 1.05-2.00, p=0.03) while a healthy dietary pattern was associated with a non-significant reduced risk of NAFLD (OR 0.76, 95% CI 0.54-1.07, p=0.12) in unadjusted analyses. However, the Western dietary pattern in obese adolescents was not associated with NAFLD after adjusting for duration of breastfeeding and maternal obesity (OR 1.251, 95% CI 0.877-1.786, p=0.217; OR 0.527, 95% CI 0.289-0.998, p=0.049, OR 3.651, 95% CI 1.426-9.351, p=0.007 respectively).

Discussion

In this study we report an inverse association between the duration of infant breastfeeding as well as the age at introduction of supplementary formula milk on the subsequent diagnosis of NAFLD in adolescence. This observation is independent of the adverse effect of maternal pre-pregnancy BMI and adolescent obesity. In unadjusted analysis, breastfeeding for ≥6 months reduced the odds of a later diagnosis of NAFLD in adolescence by over 40% compared with shorter durations of breastfeeding. Breastfeeding without supplementary milk for ≥6 months was also associated with a lower prevalence of severe steatosis. By contrast, early introduction of supplementary milk feeding before 6 months increased the odds of

NAFLD by at least 70%. Breastfeeding for at least 6 months without starting supplementary milk until after 6 months reduced the odds of a NAFLD diagnosis by nearly 40% after adjusting for maternal obesity and obesity during adolescence. The age at which complementary solid food was introduced was not associated with NAFLD.

A previous human study showed a reduced prevalence and severity of NAFLD/ NASH with longer durations of breastfeeding, in a drug-like cumulative dosing manner (26). We have now extended that observation with a finding that a longer duration of breastfeeding and later initiation of supplementary formula milk are associated with a reduced prevalence of NAFLD and of severe steatosis. In particular, there was a potentially protective effect of 6 or more months of breastfeeding on the expression of metabolic characteristics of adolescents with NAFLD. Amongst adolescents with NAFLD, those breastfed for at least 6 months had a more favorable metabolic profile compared with those breastfed for shorter durations. It is therefore plausible that breastfeeding for 6 or more months results in a longer-term favourable metabolic milieu in NAFLD that may protect against NASH.

The role of the duration of breastfeeding and of maternal obesity on later development of NAFLD in humans remains poorly detailed. Evidence from a mouse model suggests that maternal obesity during pregnancy, plus a post-natal obesogenic diet, program offspring to develop NAFLD (32,33). Animal models have additionally demonstrated that maternal pre-pregnancy and pregnancy-associated obesity, foetal and early postnatal exposure to high fat nutrition increase programming for increased hepatic lipogenesis, lipid oxidation and hepatic steatosis in rats (34). Despite the relationship of maternal obesity and smoking with NAFLD, we did not find an association between maternal hypertension or diabetes during pregnancy and NAFLD. The putative mechanism of breast milk protection against dysmetabolism, including NAFLD, is uncertain and may not involve nutritional value alone but also attenuation of the adverse metabolic programming resulting from maternal and child obesity

and effects on the gut microbiome. There is evidence that breast milk (35,36) and early antibiotic use (37) influence the gut microbiota in infants. Additionally, recent evidence shows that different gut microbiota patterns may reflect progression of NAFLD to NASH in children and adolescents (38). While breast milk contains hormones that regulate adiposity, contributes to the composition of the intestinal microbiota and possibly influences future food preferences (39), early antibiotic use during breastfeeding was recently shown to negatively influence the benefits of longer breastfeeding duration on longer-term metabolic health (40). We found antibiotic use in infants associated with a shorter duration of breastfeeding, but did not associate with the adolescent NAFLD outcome.

There is large variability in the composition of breast milk that is dependent on maternal factors such as breast milk fatty acid, triglyceride, leptin and insulin secretion, maternal obesity, maternal diet composition and genetic influences that could result in programming for obesity and NAFLD in offspring (25,32). Breast milk quality is also influenced by gestational age at delivery and lactation duration, and differs from formula milk in nutrient composition and presence of growth factors, cytokines, immunoglobulins, and digestive enzymes (41). High-protein formula milk is associated with higher weight gain than lower protein formula milk though both types of formula milk produce more weight gain than breast milk (42). Formula milk may also produce higher insulin secretion and high hepatic glucose output affecting hepatic lipogenesis (43) that contributes to the development of NAFLD.

Higher maternal pre-pregnancy BMI has been associated with reduced breastfeeding duration (44). We have now shown that early breast milk feeding after hospital delivery increases the likelihood of breastfeeding for ≥6 months, which is associated with reduced odds of a NAFLD diagnosis. Pre-pregnancy maternal obesity, short duration of breastfeeding, early formula milk feeding and subsequent child and adolescent obesity all contribute to NAFLD. NAFLD severity, in turn has been linked with liver-related morbidity and mortality (8). It is

therefore critical that the multiple opportunities to address these risk factors be identified and steps be implemented to (a) reduce obesity in women of child-bearing age, (b) encourage breastfeeding for the first 6 months of life, (c) discourage supplementary formula milk intake during the first 6 months of life, (d) reduce child and adolescent obesity. This would require multidisciplinary efforts by primary care physicians, obstetricians, midwives, pediatricians, community child nurses and other clinicians.

Limitations of our study include that (a) it is an observational study and cannot conclude causality, (b) use of ultrasound and not histology or MRI for diagnosing fatty liver, (c) reliance on parent recall for the record of infant nutrition, (d) possible underestimate of the role of maternal BMI which is also associated with duration of breast feeding and (e) since only 40% of the original cohort participated in the liver ultrasound assessment generalizability to the whole cohort or general community cannot be guaranteed. However, use of ultrasound to diagnose fatty liver is supported by both the American Association for the Study of Liver Disease (AASLD) and European Association for the Study of the Liver (EASL) guidelines that recommend liver ultrasound and not liver biopsy as the preferred first-line diagnostic test for screening patients for fatty liver (29,45). While liver histology is the gold standard to distinguish plain steatosis from NASH, liver biopsy increases study cost, is invasive, has a small complication risk and could not be justifiable in the large community-based cohort of asymptomatic adolescents participating in this non-interventional observational study. Consequently, we used a validated liver ultrasound protocol with high sensitivity and specificity for fatty liver (28) and did not rely on serum transaminase levels, which are often "normal" in NAFLD, as in this population (3). Since severe hepatic steatosis on ultrasound has been shown to be independently associated with increased liver disease morbidity and mortality, liver ultrasound may be a useful prognostic tool (8). Also, maternal recall of breastfeeding duration is considered to be a valid and reliable estimate of

breastfeeding initiation and duration when the detail is recalled after a period up to 3 years (46), as in this study. In this respect we are encouraged by the observation that maternal factors influencing breastfeeding and breastfeeding rates that we described are similar to those in a later Australian national survey (47).

In conclusion, though NAFLD is generally mediated through adiposity gains, breastfeeding for at least 6 months, avoidance of early supplementary formula milk feeding and attaining normal maternal pre-pregnancy BMI are recommended to reduce the odds of a NAFLD diagnosis during adolescence. Further research is required to better define the relative contributions of genetic, maternal, dietary and physical activity factors on the development of NAFLD.

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TABLES

Measurement		**NAFLD (N=176)**	**No NAFLD (N=991)**	**P value**
Adolescent				
Adiposity	Weight (kg)	80.6(21.1)	65.4(11.4)	<0.001
	Waist (cm)	89.8(16.1)	77.0(8.6)	<0.001
	Body mass index (kg/m^2)	27.4(6.0)	22.0(3.2)	<0.001
	Subcutaneous adipose thickness (mm)	30.5(14.5)	15.4(8.7)	<0.001
	Visceral adipose thickness (mm)	34.7(14.1)	32.3(9.7)	0.02
	Suprailiacskinfold thickness (mm)	24.8(9.8)	14.0(7.4)	<0.001
CVS	Systolic Blood Pressure (mm Hg)	115.4(11.6)	114.6(11.1)	0.40
	Diastolic Blood Pressure (mm Hg)	59.9(6.4)	59.4(6.6)	0.35
	Pulse (per minute)	67.2(10.5)	64.2(10.5)	0.001
Biochemistry	Alanine aminotransferase (U/L)	27.1(20.2)	23.2(10.3)	<0.001
	Aspartate aminotransferase (U/L)	25.1(11.0)	24.8(7.5)	0.66
	Gamma-glutamyl transpeptidase (U/L)	17.6(11.2)	14.3(7.2)	<0.001
	Triglycerides (mmol/L)	1.2(0.6)	1.0(0.5)	<0.001
	HDL-Cholesterol (mmol/L)	1.2(0.3)	1.3(0.3)	0.001
	LDL-Cholesterol (mmol/L)	2.5(0.8)	2.3(0.6)	0.01
	Total cholesterol (mmol/L)	4.2(0.9)	4.1(0.7)	0.03
	Glucose (mmol/L)	4.8(0.4)	4.8(0.6)	0.68
	Insulin (mU/L)	10(6.8-16.0)	7.0(4.7-10.3)	<0.001
	High sensitivity CRP (mg/L)	1.2(0.5-3.3)	0.5(0.2-1.2)	<0.001
	HOMA-IR	2.11(1.38-3.35)	1.46(0.98-2.16)	<0.001

	Leptin (µg/L)	29.8(12.8-55.7)	7.9(2.0-21.2)	<0.001
	Adiponectin (mg/L)	8.3(4.2)	10.0(6.0)	0.001
Maternal				
	Age when pregnant (years)	27.9(6.1)	28.9(5.7)	0.04
	Pre-pregnancy weight (kg)	64.3(17.0)	58.8(10.6)	<0.001
	Pre-pregnancy BMI (kg/m2)	23.8(6.0)	21.9(3.7)	<0.001
	Smoked during pregnancy	49(27.8%)	202(20.5%)	0.03
	Diabetes	5 (2.8%)	41 (4.1%)	0.53
	Gestational diabetes	2 (1.1%)	17 (1.7%)	0.76
	Hypertension during pregnancy	49 (27.8%)	247 (24.9%)	0.41
Infant				
	Birth weight (kg)	3.33(0.53)	3.34(0.58)	0.87
	Age discharged home after birth (days)	5 (4-7)	5 (4-7)	0.62
	Duration of breastfeeding (months)	5.0(1.0-10.5)	7.0(2.8-12.0)	0.01
	Age started formula milk (months)	3.0(1.0-6.0)	4.0(2.0-7.0)	0.006
	Age started complimentary food (months)	4.0(4.0-5.0)	4.0(4.0-5.0)	0.88

Table 1. Features of the cohort comparing adolescent, maternal and infant characteristics related to the presence or absence of NAFLD. Results are presented as mean (standard deviation), median (interquartile range) or percentages using Student's t-test or Mann–Whitney U-test respectively. P values <0.05 are considered statistically significant.

Footnote: BMI = body mass index, HDL = high density lipoprotein, LDL = low density lipoprotein, CRP = C-reactive protein, HOMA-IR = homeostasis model assessment for insulin resistance.

	True Exclusive Breastfeeding ≥4months (n=471)	P value	Breastfeeding Without Supplementary Milk ≥4 months (n=616)	P value	Breastfeeding Without Supplementary Milk ≥6 months (n=461)	P value
Mother age						
≥25 years	47.3%	<0.001	60.2%	<0.001	46.3%	<0.001
< 25 years	29.3%		36.0%		22.9%	
Overweight/ obese at start of pregnancy						
Yes	38.3%	0.10	47.0%	0.01	35.8%	0.09
No	44.9%		53.3%		42.6%	
Smoking during pregnancy						
Yes	30.9%	<0.001	36.6%	<0.001	23.6%	<0.001
No	46.3%		59.5%		45.5%	
Married/ defacto relationship when pregnant						
Yes	45.9%	<0.001	57.3%	<0.001	43.2%	<0.001
No	26.9%		39.2%		26.4%	
Maternal education						
≥12 years	50.5%	<0.001	65.0%	<0.001	50.6%	<0.001
<12 years)	37.4%		46.4%		33.1%	
Family income						
>$36,000	47.9%	0.02	60.8%	0.002	49.2%	<0.001
$12,000-$35,999	43.3%		54.4%		38.6 %	
<$12,000	33.8%		44.1%		30.3%	

Table 2. Maternal characteristics related to durations of breastfeeding. Results are expressed as percentages. Chi-square or Fisher's exact test was used to compare the different maternal characteristics. P values <0.05 are considered statistically significant

Footnote: ≥ = greater than or equal to; < = less than

	NAFLD			No NAFLD		
	Breastfeeding ≥6 months	**Breastfeeding <6 months**	**P value**	**Breastfeeding ≥6 months**	**Breastfeeding <6 months**	**P value**
Weight (kg)	74.2 (20.4)	83.8 (20.9)	0.006	65.7 (1.1)	65.3 (11.3)	0.59
Body mass index (kg/m^2)	25.2 (5.1)	28.5 (6.1)	0.001	21.9 (3.3)	22.1 (3.2)	0.36
Waist (cm)	84.8 (15.6)	92.2 (15.9)	0.008	77.1 (8.7)	76.9 (8.4)	0.63
Suprailiac skinfold thickness (mm)	21.4 (8.1)	26.8 (10.1)	0.002	13.6 (7.1)	14.4 (7.4)	0.04
Subcucutaneous adipose thickness (mm)	26.2 (13.7)	32.7 (14.4)	0.006	14.8 (8.3)	15.8 (8.9)	0.07
Visceral adipose thickness (mm)	32.3 (8.8)	36.0 (14.9)	0.15	31.8 (9.0)	32.8 (10.0)	0.12
Systolic blood pressure (mm Hg)	112.9 (12.1)	116.3 (11.1)	0.08	114.1 (11.4)	114.9 (10.7)	0.27
Diastolic blood pressure (mm Hg)	58.7 (6.0)	60.3 (6.4)	0.15	59 (7)	59 (6)	0.64
Pulse per minute	64 (9)	69 (11)	0.003	64 (10)	65 (10)	0.85
Alanine aminotransferase (U/L)	23.2 (19.6)	29.0 (20.7)	0.10	20.0 (10.2)	20.4 (10.3)	0.62
Aspartate aminotransferase (U/L)	24.3 (14.8)	25.3 (9.0)	0.60	25.0 (8.1)	24.3 (6.6)	0.19
Gamma-glutamyl transpeptidase (U/L)	14.8 (10.1)	18.9 (11.7)	0.04	13.7 (6.7)	14.7 (7.7)	0.06
Glucose (mmol/L)	4.7 (0.3)	4.8 (0.6)	0.36	4.8 (0.5)	4.7 (0.5)	0.15
Total cholesterol	4.06 (0.79)	4.33 (0.89)	0.08	4.12 (0.71)	4.07 (0.75)	0.33

(mmol/L)						
HDL cholesterol (mmol/L)	1.28 (0.31)	1.21 (0.25)	0.18	1.34 (0.30)	1.29 (0.29)	0.02
LDL cholesterol (mmol/L)	2.31 (0.71)	2.53 (0.76)	0.09	2.34 (0.61)	2.30 (0.66)	0.39
Triglycerides (mmol/L)	1.03 (0.49)	1.26 (0.62)	0.03	0.98 (0.39)	1.05 (0.59)	0.04
Leptin (µg/L)	26.2 (7.9-43.5)	36.9 (18.0-62.1)	0.01	7.9 (1.7-22.8)	8.1 (2.2-21.2)	0.75
Adiponectin (mg/L)	8.5 (5.0-12.2)	7.4 (5.2-9.8)	0.41	8.8 (6.4-12.8)	9.0 (6.1-11.9)	0.29
HOMA-IR	1.67 (1.26-2.73)	2.24 (1.56-4.0)	0.02	1.41 (0.92-2.14)	1.52 (1.00-2.18)	0.35
hsCRP (mg/L)	0.70 (0.31-1.99	1.44 (0.64-4.55)	0.003	0.47 (0.20-1.22)	0.49 (0.21-1.20)	0.67

Table 3. Relationship between duration of breastfeeding, metabolic characteristics and liver enzymes in adolescents with or without NAFLD. Results are expressed as mean (standard deviation) or median (interquartile range) using Student's t-test or Mann–Whitney U-test respectively. P values <0.05 are considered statistically significant.

Footnote: HDL = high density lipoprotein, LDL = low density lipoprotein, HOMA-IR = homeostasis model assessment for insulin resistance, hsCRP = high sensitivity C-reactive protein.

	Study participants (n=1153)		
Variable	Odds ratio for NAFLD	95% Confidence interval	P value
Exclusive Breastfeeding ≥4 months vs. <4 months	0.67	0.47-0.95	0.02
Supplementary milk start < 6 months	1.71	1.20-2.43	0.003
Breastfeeding with no supplementary milk months ≥6 months	0.57	0.40-0.82	0.002
Mother's pre-pregnancy obesity (BMI≥ 30kg/m^2)	3.16	1.83-5.44	<0.001
Mother's age (years)	0.97	0.95-0.999	0.04
Infant gender (female)	2.05	1.47-2.85	<0.001
Mother smoked during pregnancy	1.50	1.04-2.16	0.03
Neonate bottle-feeding on discharge from hospital	1.88	1.06-3.33	0.03
Infant consuming breast milk at 12 months	0.45	0.27-0.74	0.002
Infant consuming soy milk at 12 months	0.89	0.60-1.32	0.57
Infant consuming cow milk ≤12 months	1.06	0.57-1.95	0.86

Solid food commenced ≥6 months	0.78	0.51-1.20	0.49
Family income (≥$35,000 vs. <$35,000)	0.69	0.43-1.09	<0.001
Maternal education (≥12 years vs. <12 years)	0.89	0.65-1.23	0.49

Table 4. Risk of NAFLD associated with infant nutrition and maternal characteristics presented as unadjusted odds ratios and 95% confidence intervals using univariate logistic regression analysis. P values <0.05 are considered significant.

Footnote: ≥ = greater than or equal to; < = less than

	Breastfed study participants (n=1153)		
Variable	**Odds ratio for NAFLD**	**95% Confidence Interval**	**P value**
Breastfeeding without supplementary milk ≥6 months vs. <6 months	0.64	0.43-0.94	0.02
Maternal pre-pregnancy obesity	2.29	1.21-4.32	0.01
Adolescent obesity	9.08	6.26-13.17	<0.001

Table 5. Independent predictors of risk of NAFLD associated with infant nutrition, maternal and adolescent obesity, presented as adjusted odds ratios and 95% confidence intervals using multivariable logistic regression analysis. P values <0.05 are considered significant. Other variables adjusted for include age at which solid food intake was initiated maternal age, smoking during pregnancy, family income and dietary patterns during adolescence.

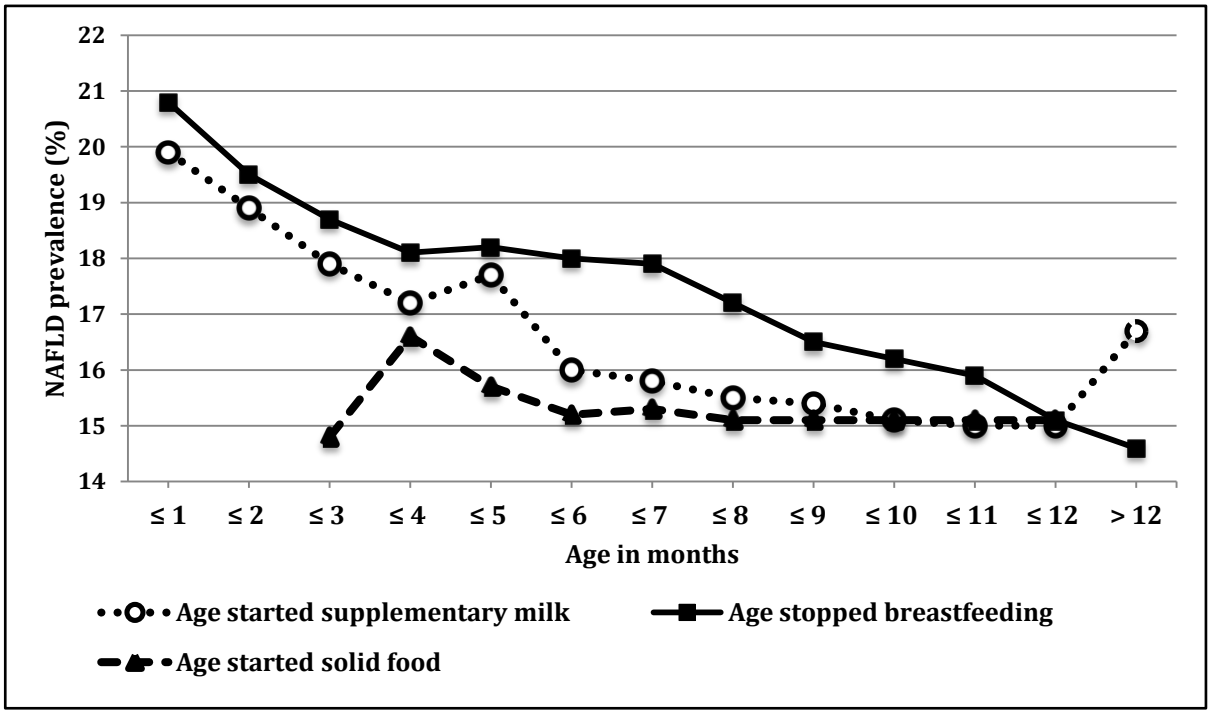
Footnote: ≥ = greater than or equal to; < = less than

Figure 1. Relationship between infant feeding patterns and NAFLD prevalence in adolescents.

The prevalence of NAFLD reduces with increasing durations of breastfeeding and delayed start of supplementary milk.

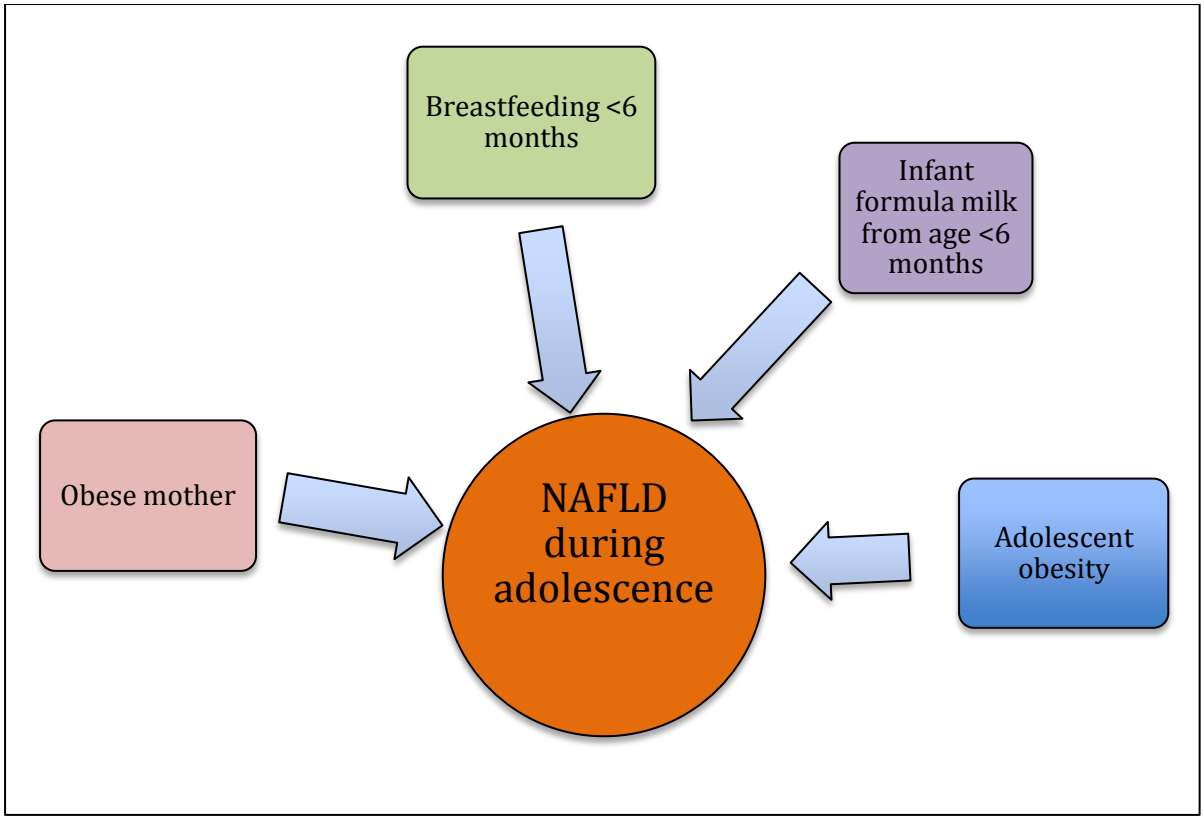
Figure

Figure 1.



Footnote: ≤ = less than or equal to; > = greater than

*Graphical Abstract



Highlights

- Maternal pre-pregnancy obesity is associated with NAFLD in adolescent offspring.
- Breastfeeding initiated at birth and continued for 6 months or longer, before commencing infant formula milk consumption, reduces the odds of NAFLD in adolescence.
- Mothers should be supported and encouraged to breastfeed infants for at least 6 months.
- Despite associations of maternal pre-pregnancy obesity, breastfeeding duration and timing of starting infant formula milk intake, obesity in the individual remains a major contributor to NAFLD in adolescents.