Association of maternal pre-pregnancy BMI and breastfeeding with NAFLD in young adults: a parental negative control study

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\textbf{ABSTRACT}

Background: The importance of the maternal-infant dyad in the genesis of nonalcoholic fatty liver disease (NAFLD) is of increasing interest. The Avon Longitudinal Study of Parents and Children (ALSPAC) showed that at age 24, 1 in 5 had NAFLD measured by transient elastography and controlled attenuation parameter (CAP). Our aim was to investigate the association between breastfeeding duration and maternal pre-pregnancy BMI on offspring NAFLD in young adulthood.

Methods: 4021 participants attended clinic for FibroScan and CAP measurement using Echosens 502 Touch\textsuperscript{R}, 440 participants with Alcohol Use Disorders were excluded. Offspring of 100 non-singleton pregnancies were excluded. 2961 valid CAP measurements for NAFLD were analysed. Exposures of interest were breastfeeding of any duration, >6 months exclusive breastfeeding, and maternal pre-pregnancy BMI. Multivariable regression models estimated the odds of NAFLD at 24 years. We performed a paternal negative control test to explore residual confounding in the analyses of pre-pregnancy BMI.

Findings: There was a modest inverse association of exclusive and non-exclusive breastfeeding >6 months having a protective effect on NAFLD in offspring (OR 0.92 [95% CI 0.86-1.0] and OR 0.90 [0.67-1.1] respectively). The odds of offspring NAFLD in overweight pre-pregnancy maternal BMI and paternal BMI was OR 0.66 [0.50-0.90], OR 0.74 [0.52-1.08], and OR 1.07 [0.60-2.22] respectively, with the ratio of effect sizes OR 0.57 [1.11-2.22]. Similarly, odds of offspring NAFLD with obese pre-pregnancy maternal BMI and paternal BMI was OR 2.66 [1.71-4.14] and OR 1.35 [0.91-2.00] respectively, with the ratio of effect sizes OR 1.90 [1.05-3.74].

Interpretation: Higher maternal pre-pregnancy BMI was associated with offspring NAFLD, having accounted for shared parental confounding. We did not replicate previous work that found a strong association between breastfeeding and NAFLD.

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Ayonrinde and colleagues demonstrated that maternal pre-pregnancy BMI is strongly associated with NAFLD specifically in male adolescent offspring [17]. The Avon Longitudinal Study of Parents and Children (ALSPAC) is a large prospective birth cohort, based in Bristol, United Kingdom (UK) [18]. ALSPAC assessed the cohort as young adults (n=4021; mean age 24 years) using transient elastography (FibroScan®) and controlled attenuated parameter (CAP) and found NAFLD prevalence to be over 20% [19].

Using existing data on maternal BMI and infant feeding practices at ALSPAC, we aim to replicate the previous Raine cohort study and test whether exposures of any breastfeeding, >6 months exclusive breastfeeding, and maternal pre-pregnancy BMI are associated with NAFLD in young adulthood. To strengthen a causal inference, we used a negative control approach by testing the association of paternal pre-pregnancy BMI and comparing this effect size to that of maternal pre-pregnancy BMI.

2. Materials and methods

2.1. Study population

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a prospective birth cohort study from southwest England [18,20]. The study website contains details of all available data through a fully searchable data dictionary and variable search tool (www.bristol.ac.uk/alspac/researchers/our-data). Briefly, ALSPAC invited pregnant women in Avon, UK with expected delivery dates between April 1, 1991 and December 31, 1992 into the cohort [21]. The initial number of pregnancies enrolled is 14,541. Of these initial pregnancies, there was a total of 14,676 foetuses, resulting in 14,062 live births and 13,988 children who were alive at 1 year of age.

When the oldest children were approximately 7 years of age, an attempt was made to bolster the initial sample with eligible cases who had failed to join the study originally. Following 3 further phases of recruitment, this resulted in an additional 913 children being enrolled. The total sample size for analyses using any data collected after the age of seven is therefore 15,454 pregnancies, resulting in 15,589 foetuses. Of these, 14,901 were alive at 1 year of age [21].

Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Consent for biological samples has been collected in accordance with the Human Tissue Act (2004). Informed consent for use of data collected when the children were aged 7 years was obtained. Consent for biological samples has been collected in accordance with the Human Tissue Act (2004). Informed consent for use of data collected when the children were aged 7 years was obtained.

Between June 2015 and October 2017, 10018 remaining “active” ALSPAC study participants were invited to the “Focus@24+” clinic (see Fig. 1). Of 10018 participants invited, 40.1% (n=4021/10018) attended; mean age 24.0 years (SD 0.8; IQR 23-25 years; 1507 males, 2510 females).

1060 participants were excluded from the final analysis. 293 non-core participants (i.e. not enrolled in the original recruitment phase of ALSPAC) did not have recorded maternal measures. 97 twins and triplets were excluded as non-singleton pregnancies are independently associated with lower breastfeeding duration [22-24]. 440 participants attending the Fibro24+ clinic had evidence of Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-V) criteria for alcohol use disorder (AUD) were also excluded from analysis [25]. 94 participants did not attend the FibroScan session. Of those who did not attend the FibroScan session. Of those who did not attend the FibroScan session. Of those who did not attend the FibroScan session. Of those who did not attend the FibroScan session. Of those who did not attend the FibroScan session. Of those who did not attend the FibroScan session. Of those who did not attend the FibroScan session. Of those who did not attend the FibroScan session.

Study data were collected and managed using REDCap electronic data capture tools hosted at University of Bristol [26]. In line with ALSPAC confidentiality policy, any analysed groups with less than...
five participants are expressed as \( n = \text{"<5"} \). This figure when expressed can include zero.

In the F@24+ clinic no one was reported to have viral hepatitis or taking nucleoside analogues/direct-acting antivirals. In the F@24+ clinic, there were less than 5 participants identified with: autoimmune hepatitis on azathioprine, or autoimmune hepatitis/primary sclerosing cholangitis overlap syndrome on prednisolone, mycophenolate mofetil, ursodeoxycholic acid, who were not removed as this was a general population study.

2.2. Outcomes

Prior to Focus@24+ clinic attendance, participants were fasted for a minimum of 6 hours or overnight before blood tests and subsequent transient elastography.

*Imaging.* In the F@24+ clinic participants were assessed with transient elastography ( FibroScan®, Echosens 502 Touch®, Echosens, Paris) and controlled attenuation parameter (CAP). These are a standardised non-invasive measure of fibrosis and quantifying steatosis in NAFLD [27,28]. CAP score cut off values for different grades of steatosis for both groups were derived from a meta-analysis on CAP technology [28]. Ten valid readings within the range of 100-400dB/m were required to derive a CAP score. NAFLD was defined as \( \geq 248 \text{dB/m} \) (S1 equivalent steatosis).

*Serology.* Fasted blood tests analysed included liver function: alanine transaminase (ALT), aspartate aminotransferase (AST) and \( \gamma \)-glutamyl transferase (GGT). Lipid profiles were taken including cholesterol, triglycerides, low-density lipoprotein-cholesterol (LDL-C), very low-density lipoprotein (VLDL-C), and high-density lipoprotein (HDL). Glucose and insulin levels were also sent and used to calculate the homeostasis model assessment for insulin resistance (HOMA-IR), using the equation HOMA-IR score = \( (\text{Fasting insulin} [\mu \text{U/ml}] \times \text{Fasting glucose} [\text{mmol/l}]) / 22.5 \) [29,30].

As a secondary analysis we sought to evaluate if there was a difference in liver enzyme levels and metabolic markers between participants with NAFLD who were breastfed for more or less than 6 months, as the previous Raine cohort study had noted some difference including lower serum GGT, triglycerides, and HOMA-IR in participants breastfed for \( \geq 6 \) months [6].

2.3. Exposures

The data used in this study were collected from ALSPAC mothers during pregnancy and over the index child’s first year of life. Data on method of infant feeding, including introduction of supplementary milk formula and complimentary feeding, were obtained from questionnaires completed by mothers when the index child was 6 and 15 months of age [31]. Two categorical variables were created. “Any breastfeeding duration” was trichotomized into never breastfed (referent), non-exclusive breastfeeding <6 months, and non-exclusive breastfeeding \( \geq 6 \) months. “Exclusive breastfeeding \( \geq 6 \) months” was compared to having never breastfed (referent), and mixed feeding (i.e. supplementary milk formula) or duration of breastfeeding <6 months combined. Exclusive breastfeeding \( \geq 6 \) months was defined as no reported supplementary milk formulas (including cow’s milk, soya, hypoallergenic and follow-on formula) in the first 6 months. A cut-off of \( \geq 6 \) months breastfeeding was specified to facilitate direct comparison with the Raine cohort analysis.

In questionnaires at around 18 weeks of gestation women reported their pre-pregnancy weight and height and this was used to calculate maternal pre-pregnancy BMI (correlations between BMI based on weight and height reported by the women and BMI based on weight and height extracted from the first antenatal clinic visit were high; Pearson correlation coefficient: 0.9).

2.4. Confounders

Confounders were defined a priori based on prior evidence of them being known or plausible causes of the exposure and outcome.
potentially causal intrauterine factors in mothers (35,36). Here we increasingly used as a negative control for maternal exposure as data development origins of disease, paternal exposure has been plausibly cause the outcome, or vice versa (32-34). In studies of the where either the exposure is changed to an exposure that does not influence the associations of pre-pregnancy BMI (in mothers and fathers), adjusted multivariable logistic regression models were used to examine the associations of pre-pregnancy BMI (in mothers and fathers), exclusive breastfeeding for more or less than 6 months, and any breastfeeding duration with NAFLD.

3.1. Paternal negative control

Negative control studies in epidemiology compare results from the hypothesised (real) association and a negative control association where either the exposure is changed to an exposure that does not plausibly cause the outcome, or vice versa [32-34]. In studies of the developmental origins of disease, paternal exposure has been increasingly used as a negative control for maternal exposure as data are often available on the same exposures in the father that are potentially causal intrauterine factors in mothers (35,36). Here we compared confounder adjusted paternal BMI categories of normal, overweight and obesity associations with offspring NAFLD to the same associations of maternal BMI categories with offspring NAFLD.

For this parental negative control comparison, we also mutually adjusted each parents’ exposure for the same exposure in the other parent as failing to do this can result in bias in the presence of assortative mating [37]. To simplify the paternal negative control test, maternal and paternal BMI were dichotomised in two models: (1) normal BMI vs overweight & obese BMI and (2) normal and overweight BMI vs obese BMI. In each model maternal and paternal BMI exposures were mutually adjusted for, following which effect size estimates were compared at the different thresholds (overweight and obese BMI). The null hypothesis was there is no difference between the maternal and paternal estimates.

3.2. Dealing with missing data

This study only included participants with complete CAP data and imputed missing exposure and confounder data, with this being the highest for maternal pre-pregnancy BMI (9.2% of participants did not have this measure). Statistical analysis was performed using Stata MP 15.1. (For further details on the imputation strategy see Supplementary Methods).

4. Results

2961 participant with valid CAP results were eligible for analysis (63.9% female). Overall, 20.7% (95%CI 19.2-22.1%) of participants attended the Focus@24+ clinic had NAFLD (≥S1 equivalent steatosis), with 10.1% having S3 equivalent steatosis CAP measurements (>66% steatosis).

Characteristics of the main exposures analysed is summarised in Table 1. Offspring NAFLD prevalence within male and female sex were interrogated (see Table 2). There did not appear to be any relationship between longer breastfeeding duration between male offspring participants with or without NAFLD (p=0.27). No relationship

| Variable                        | S0 (n=2350; 79.4%) | S1 (n=215; 7.3%) | S2 (n=99; 3.3%) | S3 (n=297; 10.6%) |
|---------------------------------|--------------------|-------------------|-----------------|-------------------|
| Sex                             | Male (n=1070)      | 789 (73.7%)       | 93 (8.7%)       | 44 (4.1%)         | 144 (13.5%)       | p=0.001*          |
|                                 | Female (n=1891)    | 1561 (82.5%)      | 122 (6.4%)      | 55 (2.9%)         | 153 (8.1%)        |                  |
| Maternal pre-pregnancy BMI      | BMI <25kg/m^2 (n=2196) | 1795 (81.7%)      | 144 (6.6%)      | 73 (3.3%)         | 184 (8.4%)        | p=0.001*          |
|                                 | Overweight (25-30kg/m^2) (n=371) | 261 (70.3%)      | 39 (10.5%)      | 14 (3.8%)         | 57 (15.4%)        |                  |
|                                 | Obesity (>30kg/m^2) | 71                | 14              | <5                | 32                |                  |
| Paternal BMI                    | BMI <25kg/m^2 (n=2196) | 972 (82.0%)       | 87 (7.3%)       | 36 (3.0%)         | 90 (7.6%)         | p=0.001*          |
|                                 | Overweight (25-30kg/m^2) (n=371) | 638 (76.7%)      | 62 (7.4%)       | 35 (4.2%)         | 97 (11.7%)        |                  |
|                                 | Obesity (>30kg/m^2) | 106               | 11              | <5                | 28                |                  |
| Breastfeeding                   | Never breastfed (n=404) | 307 (76.0%)       | 33 (8.2%)       | 15 (3.7%)         | 49 (12.1%)        | p=0.15*           |
|                                 | Mixed feeding (n=1747) | 1398 (80.0%)      | 130 (7.4%)      | 60 (3.4%)         | 159 (9.1%)        |                  |
|                                 | Exclusive breastfeeding for ≥6months (n=584) | 471 (80.6%)      | 33 (5.6%)       | 14 (2.4%)         | 66 (11.3%)        |                  |
| Maternal smoking during pregnancy No (n=2446) | 1955 (79.9%) | 175 (7.1%)       | 81 (3.3%)       | 335 (9.6%)        | p=0.40*           |
|                                 | Yes (n=407)        | 359 (76.9%)       | 35 (7.0%)       | 17 (3.4%)         | 56 (12.0%)        |                  |
| Mean (SD)                       | 29.5 (4.6)         | 28.7 (4.5)        | 28.5 (4.4)      | 28.8 (4.8)        | p=0.001*          |
| Maternal education              | Lower than O-level (n=502) | 374 (74.5%)      | 46 (9.2%)       | 19 (3.8%)         | 63 (12.5%)        | p=0.03*           |
|                                 | O-level (n=987)    | 765 (77.3%)       | 78 (7.9%)       | 38 (3.8%)         | 106 (10.7%)       |                  |
|                                 | Higher education (n=1385) | 1132 (81.7%)     | 90 (6.5%)       | 39 (2.8%)         | 124 (9.0%)        |                  |
| Social class                    | IV-V (partly skilled and unskilled occupation) (n=879) | 65 | 9 | <5 | 6 | p=0.43*           |
|                                 | III (non-manual and manual occupation) (n=530) | 686 (78.0%) | 64 (7.3%) | 33 (3.7%) | 97 (11.0%) | |
|                                 | II (managerial and technical occupation) (n=1264) | 1000 (79.1%) | 95 (7.5%) | 35 (2.8%) | 134 (10.6%) | |
| Birthweight (grams)             | Mean (SD)          | 3413.7 (513.4)    | 3475 (554.8)    | 3434 (457.0)      | 3479 (509.5)      | p=0.02*           |

Steatosis grade based on CAP score ranges from S0-S3. S0 = <248 dB/m (<10% steatosis); S1 ≥248 to <268 dB/m (10% - <33% steatosis [mild]); S2 ≥268 to <280 dB/m (33% - <66% steatosis [moderate]); and S3 ≥280 dB/m or more (≥66% steatosis [severe])

* Pearson’s Chi² test

Univariable logistic regression
was observed in female offspring participants with or without NAFLD (p=0.28). Both in male and female offspring participants, maternal pre-pregnancy BMI was strongly associated with offspring NAFLD (p=0.001 and p<0.0001 respectively). Similarly, paternal BMI was also strongly associated with offspring in both male and female sexes (p=0.01 and p<0.0001 respectively). Finally, no difference in socioeconomic status was identified between male and female offspring with and without NAFLD (p=0.47 and p=0.13 respectively).

### 4.1. Any Breastfeeding

Complete data on patterns of breastfeeding existed on 2409 participants when restricting to those with available CAP measurements. Participants were categorised ordinally into those that were not breastfed (n=342/2409), those that breastfed (with or without mixed feeding) for less than 6 months (n=1048/2409) and those that breastfed (with or without mixed feeding) for ≥6 months (n=1019/2409) as a dose response exposure. (see Table 3.) There was a weak association of breastfeeding duration ≥6 months being associated with lower odds of NAFLD, compared to <6 months or no breastfeeding (19.0% vs 20.6% vs 24.0% respectively; unadjusted OR 0.78 [0.58-1.04]; p=0.09). However, this association attenuated when adjusted for confounders including social class, maternal age, smoking in pregnancy, birthweight and the pre-pregnancy BMI exposure variable (OR 0.92 [0.67-1.26]; p=0.60). Findings were similar following the use of multiple imputation (OR 0.90 [0.67-1.21]; p=0.50).

### 4.2. Exclusive breastfeeding ≥6 months

(see Table 3.) When interrogating ≥6 months exclusive breastfeeding, participants were categorised into those who never breastfed (n=342/2409), those with mixed feeding or duration of breastfeeding <6 months combined (n=1544/2409), and those who exclusively breastfed for ≥6 months (n=523/2409). Lower prevalence of NAFLD were seen amongst participants who were exclusively breastfed for ≥6 months, compared to participants who received mixed feeding and those who never breastfed, but the confidence interval included the null (19.3% vs 20.0% vs 24.0% respectively; OR 0.78 [0.56-1.01]; p=0.14). Effect estimates were attenuated further after adjusting for confounders including social class, maternal age, smoking in pregnancy, birthweight, and the pre-pregnancy BMI exposure variable (0.89 [0.63-1.27]; p=0.53). This did not alter following imputation of a complete dataset (0.92 [0.66-1.27]; p=0.60).

### 4.3. Timing of introduction of supplementary milk formula and complementary feeding

(see Table 4.) In the first 6 months, no association was identified between the month supplementary milk formula was introduced and the presence of NAFLD in univariable analysis (OR 0.97 [0.93-1.01; p=0.13]). Whilst there was a stronger association between timing of complementary feeding being commenced in the first 6 months and lower odds of NAFLD, the confidence interval again included the null (OR 0.91 [0.82-1.01; p=0.07]).

### 4.4. Pre-pregnancy maternal BMI

(see Table 3.) 13.8% (n=371/2688) of mothers were overweight, whilst 4.5% had pre-pregnancy obesity (n=121/2688). Presence of NAFLD in offspring ranged from 18.3% amongst mother with a BMI<25 kg/m² to 41.3% amongst mother with pre-pregnancy obesity.

Complete data on maternal pre-pregnancy BMI was available for 2505 participants when interrogating NAFLD outcomes. Mothers’ BMI was categorised into normoweight (<25kg/m²; n=2051/2505), overweight (25 ≤ BMI <30kg/m²; n=346/2505), and obese (≥30kg/m²; n=108/2505). Crude associations of overweight and obese maternal pre-pregnancy BMI with offspring NAFLD outcomes were OR 2.00 [1.55-2.58]; p<0.0001 and OR 3.28 [2.20-4.88]; p<0.0001 respectively. Following adjustment for confounders including social class, maternal age and smoking in pregnancy and imputation to a complete data set overweight and obese maternal pre-pregnancy BMI with offspring NAFLD outcomes were OR 1.84 [1.44-2.37]; p<0.0001 and OR 3.06 [2.11-4.42]; p<0.0001 respectively.

### 4.5. Paternal negative control

A negative control test entails the comparison of magnitude of the maternal and paternal association estimates within a mutually adjusted model. (see Table 5.) For completeness Table 5 contains crude estimates. 2316 participants had paternal BMI data.

Prior to mutual adjustment, parental pre-pregnancy BMI was dichotomised into (1) BMI<25kg/m² vs BMI ≥25kg/m² (overweight and obese) and (2) BMI<30kg/m² vs BMI ≥30kg/m² (obese). In model (1) the odds of offspring NAFLD in overweight/obese pre-pregnancy maternal BMI and paternal BMI was OR 2.09 [95%CI 1.62-2.68; p<0.0001] and OR 1.33 [95%CI 1.07-1.65; p=0.01] respectively, with the ratio of effect sizes OR 1.57 [95%CI 1.11-2.22; p=0.01]. Similarly, in model (2) the odds of offspring NAFLD with obese pre-pregnancy BMI was OR 1.56 [95%CI 1.18-2.06; p=0.001].
Table 3
Exposures associated with presence of NAFLD (n=2961)

| Model | Independent variable | Variable | Presence of NAFLD | Crude OR [95% CI] | Adjusted (1) OR [95% CI] | Adjusted (2) OR [95% CI] |
|-------|----------------------|----------|-------------------|------------------|-------------------------|-------------------------|
| 1     | Pre-pregnancy maternal BMI | Underweight or Normal | 18.3% | Referent | Referent | Referent |
|       |                      | Overweight [imputed result] | 29.6% | 2.00 [1.55-2.58]; p=0.0001 | 1.99 [1.54-2.57]; p=0.0001 | 1.84 [1.44-2.37]; p=0.0001 |
|       |                      | Obese [imputed result] | 41.3% | 3.28 [2.20-4.88]; p=0.0001 | 3.19 [2.13-4.76]; p=0.0001 | 3.09 [2.11-4.42]; p=0.0001 |
| 2     | Paternal BMI | Number of participants with complete data available = 2505 | 18.0% | 1.39 [1.12-1.73]; p=0.003 | 1.85 [1.26-2.72]; p=0.002 | p<0.0001 |
| 3     | ≥6 months Exclusive breastfeeding | Never breastfed [imputed result] | 24.0% | 0.81 [0.62-1.07]; p=0.14 | 0.85 [0.64-1.16]; p=0.28 | 0.88 [0.66-1.18]; p=0.40 |
|       |                      | Mixed feeding [imputed result] | 20.0% | 0.83 [0.69-1.01]; p=0.18 | 0.85 [0.64-1.16]; p=0.28 | 0.88 [0.66-1.18]; p=0.40 |
|       |                      | ≥6 months exclusive breastfeeding [imputed result] | 19.3% | 0.78 [0.65-1.09]; p=0.14 | 0.83 [0.69-1.01]; p=0.18 | 0.85 [0.64-1.16]; p=0.28 |
| 4     | Any breastfeeding | Never breastfed [imputed result] | 24.0% | 0.83 [0.62-1.11]; p=0.21 | 0.84 [0.62-1.13]; p=0.24 | 0.86 [0.64-1.17]; p=0.33 |
|       |                      | <6 months [imputed result] | 20.6% | 0.84 [0.62-1.13]; p=0.24 | 0.86 [0.64-1.17]; p=0.33 | 0.89 [0.68-1.27]; p=0.40 |
|       |                      | ≥6 months [imputed result] | 19.0% | 0.78 [0.58-1.04]; p=0.09 | 0.85 [0.62-1.17]; p=0.32 | 0.92 [0.67-1.26]; p=0.60 |
|       |                      | Number of participants with complete data available = 2409 | p=0.16 | p=0.50 | p=0.70 | p=0.70 |

1 Likelihood ratio test

maternal BMI and paternal BMI was OR 2.66 [95%CI 1.71-4.14; p<0.0001] OR 1.35 [95%CI 0.91-2.00; p=0.014] respectively, with the ratio of effect sizes OR 1.98 [95%CI 1.05-3.74; p=0.04].

4.6. Association of duration of breastfeeding with serum liver enzymes, cardiometabolic markers, and young adults with NAFLD

(see Table 6.) Median ALT, AST, and GGT levels were compared between participants identified with NAFLD who had been breastfed exclusively for ≥6 months vs < 6 months breast/mixed feeding, with no difference found (p=0.21, 0.12 and 0.66 respectively). Similarly, there was no difference in mean cholesterol, LDL-C, HDL, and median triglyceride levels between groups (p=0.86, 0.22, 0.30 and 0.40 respectively). Median HOMA-IR values amongst participants with NAFLD who received exclusive breastfeeding for ≥6 months were higher compared to participants with NAFLD who breastfed for <6 months (3.5 vs 2.8, p=0.07) with both groups in the insulin resistance range. This is despite similar levels of obesity (38.4% vs 38.7% respectively) and similar sex distribution (56.2% vs 50.6% respectively) amongst the 2 groups.

Further analyses including analysing liver fibrosis (liver stiffness measurement of ≥7.9kPa equivalent to F2 fibrosis) as the outcome with the same exposures can be found in Supplementary Results.

5. Discussion

5.1. Main findings

We found evidence of a specific early life effect of overweight and obese maternal pre-pregnancy BMI with higher odds of offspring NAFLD in young adulthood, that could not be explained by the shared parental confounding structure. Whilst we found an association with maternal pre-pregnancy BMI and NAFLD outcomes in offspring, this was also the case when modelling the association of paternal BMI as a negative control exposure. Therefore, whilst there may be an early life effect, this is in addition to environmental factors in the genesis NAFLD.

We did not find evidence that longer breastfeeding duration and ≥6 months exclusive breastfeeding duration were associated with a lower odds of NAFLD in offspring by young adulthood. We found insufficient evidence supporting the hypothesis that mixed feeding is
more deleterious to offspring developing NAFLD in young adulthood compared to exclusive breastfeeding.

5.2. Strength and limitations

This study can only comment on the relationships of maternal pre-pregnancy BMI and breastfeeding duration with NAFLD outcomes in the context of singleton pregnancies as we removed twin and multiple pregnancies to limit bias, as those pregnancies are associated with lower breastfeeding duration [22-24]. By treating BMI as a categorical variable to replicate the previous Raine cohort analysis, we accepted a lower breastfeeding duration [22-24]. By treating BMI as a categorical variable to replicate the previous Raine cohort analysis, we accepted a lower breastfeeding duration [22-24].

Our diagnosis of NAFLD was based on controlled attenuation parameter diagnosis in the absence of harmful alcohol use. We acknowledge that cut-off values for CAP are validated in adults with histologically proven liver disease and these results should be interpreted in that context. However, in our large population study, gold standard liver biopsy was not ethically viable.

Whilst individuals with alcohol use disorder were removed from this analysis, ALSPAC did not collect data on grams of alcohol consumed by participants. Therefore, it is possible that participants with excess daily alcohol consumption (>30g/day in men and >20g/day in women) were included in the analysis.

We accepted that increased maternal pre-pregnancy BMI and breastfeeding cannot be considered entirely independent of each other. In addition to breastfeeding duration being lower in mothers with obesity, murine models showed in offspring of mice control diets, when breastfed by obese mice on high-fat diets, had evidence of increased hepatic tumour necrosis factor, interleukin-6 expression, and triglyceride levels—highlighting a difference in breastmilk composition between mothers with obesity and those with normal BMI [12, 38, 39]. Maternal pre-pregnancy BMI was included as a confounder in regression models evaluating breastfeeding as an exposure to replicate and compare with the previous Raine cohort analysis. Despite utilising a large birth cohort, our study remained underpowered to assess the relationship between breastfeeding duration and outcomes of NAFLD in offspring (see Supplementary Methods for power calculation). Therefore, we cannot definitively comment on a lack of protective effect of longer breastfeeding duration.

The World Health Organisation advised exclusive breastfeeding duration for 4 to 6 months during the 1990s and changed their stance in 2001 to 6 months exclusive breastfeeding [40,41]. Overall, 21.2% (n=620/2928) of our participants were exclusively breastfed for ≥6 months in the early 1990s, which is substantially higher than 1% figure quoted in the 2010 UK Infant Feeding Survey, and the Raine cohort 7-4% [6,42]. Despite this relatively high number of exclusively breastfed participants, we were unable to elucidate a strong protective effect of longer exclusive breastfeeding duration for NAFLD development.

The ALSPAC Focus@24 clinic had a large clinic non-attendance, with only 40-1% of participants attending. Therefore, the ALSPAC birth cohort may not be entirely representative of the wider population [18]. The demographic profile of the catchment area in southwest England, and the differential attrition, has created an over-representation of affluent groups and an under-representation of ethnic minorities, with ALSPAC over 97% Caucasian [18]. Thus, it is unclear how our results apply to patients of other ethnicities with NAFLD. Higher maternal educational attainment is strongly associated with only 40-1% of participants attending. Therefore, the ALSPAC birth cohort may not be entirely representative of the wider population [18].

5.3. Other evidence

To the best of our knowledge, this is one of the largest studies to interrogate the relationship between breastfeeding, maternal pre-pregnancy BMI and NAFLD development in the offspring. It remains

more deleterious to offspring developing NAFLD in young adulthood compared to exclusive breastfeeding.

5.2. Strength and limitations

This study can only comment on the relationships of maternal pre-pregnancy BMI and breastfeeding duration with NAFLD outcomes in the context of singleton pregnancies as we removed twin and multiple pregnancies to limit bias, as those pregnancies are associated with lower breastfeeding duration [22-24]. By treating BMI as a categorical variable to replicate the previous Raine cohort analysis, we accepted a lower breastfeeding duration [22-24]. By treating BMI as a categorical variable to replicate the previous Raine cohort analysis, we accepted a lower breastfeeding duration [22-24].

Our diagnosis of NAFLD was based on controlled attenuation parameter diagnosis in the absence of harmful alcohol use. We acknowledge that cut-off values for CAP are validated in adults with histologically proven liver disease and these results should be interpreted in that context. However, in our large population study, gold standard liver biopsy was not ethically viable.

Whilst individuals with alcohol use disorder were removed from this analysis, ALSPAC did not collect data on grams of alcohol consumed by participants. Therefore, it is possible that participants with excess daily alcohol consumption (>30g/day in men and >20g/day in women) were included in the analysis.

We accepted that increased maternal pre-pregnancy BMI and breastfeeding cannot be considered entirely independent of each other. In addition to breastfeeding duration being lower in mothers with obesity, murine models showed in offspring of mice control diets, when breastfed by obese mice on high-fat diets, had evidence of increased hepatic tumour necrosis factor, interleukin-6 expression, and triglyceride levels—highlighting a difference in breastmilk composition between mothers with obesity and those with normal BMI [12, 38, 39]. Maternal pre-pregnancy BMI was included as a confounder in regression models evaluating breastfeeding as an exposure to replicate and compare with the previous Raine cohort analysis. Despite utilising a large birth cohort, our study remained underpowered to assess the relationship between breastfeeding duration and outcomes of NAFLD in offspring (see Supplementary Methods for power calculation). Therefore, we cannot definitively comment on a lack of protective effect of longer breastfeeding duration.

The World Health Organisation advised exclusive breastfeeding duration for 4 to 6 months during the 1990s and changed their stance in 2001 to 6 months exclusive breastfeeding [40,41]. Overall, 21.2% (n=620/2928) of our participants were exclusively breastfed for ≥6 months in the early 1990s, which is substantially higher than 1% figure quoted in the 2010 UK Infant Feeding Survey, and the Raine cohort 7-4% [6,42]. Despite this relatively high number of exclusively breastfed participants, we were unable to elucidate a strong protective effect of longer exclusive breastfeeding duration for NAFLD development.

The ALSPAC Focus@24 clinic had a large clinic non-attendance, with only 40-1% of participants attending. Therefore, the ALSPAC birth cohort may not be entirely representative of the wider population [18]. The demographic profile of the catchment area in southwest England, and the differential attrition, has created an over-representation of affluent groups and an under-representation of ethnic minorities, with ALSPAC over 97% Caucasian [18]. Thus, it is unclear how our results apply to patients of other ethnicities with NAFLD. Higher maternal educational attainment is strongly associated with only 40-1% of participants attending. Therefore, the ALSPAC birth cohort may not be entirely representative of the wider population [18].

5.3. Other evidence

To the best of our knowledge, this is one of the largest studies to interrogate the relationship between breastfeeding, maternal pre-pregnancy BMI and NAFLD development in the offspring. It remains
the first to use a paternal negative control test to elucidate causality for maternal pre-pregnancy BMI leading to NAFLD in offspring. Whilst there was an association between increased paternal BMI and offspring NAFLD, when we mutually adjusted for maternal and paternal BMI and compared effect size estimates, there still appeared to be an increased association between overweight and obese maternal BMI and offspring NAFLD. This lends support to an early life effect increasing odds of NAFLD in offspring amongst mothers with elevated pre-pregnancy BMI, independent of environmental factors.

In a similar birth cohort, the Raine cohort (n=1170) based in Western Australia, 15.2% had NAFLD based on ultrasound assessment. Our findings evaluating the early life effects of maternal pre-pregnancy were consistent with the Raine Cohort in relation to maternal pre-pregnancy obesity and NAFLD outcomes (OR 1.29 [1.14–1.45]; p=0.01). Our paternal negative control test supported this association. In addition, a meta-analysis of current literature of maternal risk factors for paediatric NAFLD risk supports our findings regarding the relationship of elevated maternal pre-pregnancy BMI with offspring NAFLD [45]. The Raine cohort found >6 months exclusive breastfeeding to be protective vs <6 months breastfeeding without supplementary milk (OR 0.64 [0.43–0.94], p=0.02); whereas after adjustment we did not find evidence of a similar protective effect with a larger sample size. However, our assessment of a protective breastfeeding effect is limited by being underpowered.

Ayonrinde and colleagues also identified sexual dimorphism in the association of parental factors on NAFLD in offspring [17]. They found paternal obesity to be associated with male offspring NAFLD only. Female offspring NAFLD with independently associated with higher maternal pre-pregnancy BMI. Furthermore, lower parental socioeconomic status was associated with NAFLD in male offspring. We did not demonstrate such sexual dimorphism in our cohort analysis at 24 years or when our participants were 17.8 years during the first assessment for NAFLD using ultrasound [46]. This could be related to an overrepresentation of female participants in our cohort, who accounted for 63.9% of our cohort compared to 49.4% of the Raine cohort. In addition, NAFLD is far more prevalent in ALSPAC male participants compared to female participants, a reversal of the relationship seen in the previous Raine cohort analysis. This could be related to ALSPAC examining young adults as opposed to adolescents. Suzuki et al. analysed paediatric biopsy proven NAFLD identifying a relationship between increasing pubertal stage and possibly worse histological features of NAFLD, although the study was underpowered to identify sex differences [47].

Our results contrast existing epidemiological literature which largely negates the association of pre-pregnancy BMI, breastfeeding duration, and outcomes of the related outcomes of childhood adiposity and obesity. The Promotion of Breastfeeding Intervention Trial (PROBIT) randomised controlled trial involving over 17,000 offspring did not demonstrate a difference in adiposity between infants with substantially greater durations and exclusivity of breastfeeding than the control arm at aged 6.5 years or 11.5 years [46,49]. One of the largest systematic reviews and meta-analyses (including 105 studies) to interrogate the association between breastfeeding and offspring being overweight or obese found only a modest pooled OR 0.87 [0.76–0.99], involving >1500 participants [50].

Whilst we found no evidence that longer breastfeeding durations were protective for offspring developing NAFLD, other studies have suggested it may have a protective effect for mothers within a maternal-infant dyad. Using an older American cohort study, Coronary Artery Risk Development in Young Adults, Ajmara and colleagues demonstrated that mothers who breastfed for longer durations (>6 months) had >50% reduction in NAFLD outcomes compared to those who had breastfed for 0–1 months, after adjusting for age, ethnicity education level and baseline BMI [51]. The mechanism postulated for this finding was of increased maternal basal metabolic rate associated with lactation, increasing insulin sensitivity [51,52].

5.4. Implications

The wide-ranging early life benefits of breastfeeding are indisputable. Whilst our findings are insufficient to conclusively rule out an effect, we found little evidence that longer breastfeeding durations or exclusive breastfeeding > 6 months is protective for offspring developing NAFLD. With higher-income countries such as the UK having shorter breastfeeding duration, further studies are needed to clarify if a true protective benefit exists.

Similarly, we have demonstrated evidence for an early life effect associated with elevated maternal pre-pregnancy BMI, in addition to environmental factors, that contributes to offspring developing NAFLD as young adults. We must collectively address the obesogenic environment our patients live in and support them in behavioural change interventions to improve their diet and increase exercise whilst promoting policies that minimise perpetuation of unhealthy eating patterns. Ultimately this will reduce the risk of patients and their children developing advanced NAFLD fibrosis and cirrhosis.

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Authors Contributions

KW A (study design, data analysis, manuscript preparation); JGO (study design, critical review); PMD (advice on negative control study and critical review); GSF (data analysis, critical review) LZ (critical review); FHH (critical review); DAL (advice on negative control study and critical review); JH (study design, data acquisition, data analysis, critical review); MH (study design, critical review, supervised entire project).

Declaration of Interest

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