Association of Bright Liver With the PNPLA3 I148M Gene Variant in 1-Year-Old Toddlers

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Context: Nonalcoholic fatty liver disease (NAFLD) is being increasingly diagnosed at younger ages, pointing toward an early-life origin.

Objective: To evaluate the frequency and risk factors for bright liver (BL) in 1-year-old toddlers.

Design: Secondary analysis of the 1-year follow-up of the Feeding Study. Exposures were child PNPLA3 and TM6SF2 gene variants; child anthropometry at birth and at 1 year of follow-up; child subcutaneous, visceral, and epicardial adipose tissue at 1 year of follow-up; maternal anthropometry at the start and at the end of pregnancy; and maternal red blood cell fatty-acid composition at the third trimester of pregnancy.

Setting: General population.

Participants: Among 505 mother-toddler pairs, 391 children (77%) underwent liver and abdominal ultrasonography at the 1 year follow-up.

Main Outcome: BL as diagnosed by ultrasonography.

Results: Seventeen (4%) of 391 toddlers had BL. Compared with the toddlers with the PNPLA3 CC genotype, the odds (95% CI) of BL were 3.01 (1.05 to 8.64, P < 0.05) times higher in those with the PNPLA3 CG genotype and 5.37 (1.12 to 25.77, P < 0.05) higher in those with the PNPLA3 CC genotype. We found no association between BL status and TM6SF2. Body weight, body mass index, and maternal weight gain during pregnancy were higher in BL+ than in BL− children. Visceral adipose tissue was higher but subcutaneous adipose tissue and epicardial adipose tissue were similar in BL+ and BL− children.
The frequency of nonalcoholic fatty liver disease (NAFLD) is increasing in parallel with the epidemic of obesity (1). NAFLD is being increasingly diagnosed at younger ages, pointing toward an early-life origin (2). NAFLD has long been considered the consequence of excess weight (3), even if the paradigm considering NAFLD the hepatic manifestation of the metabolic syndrome is being increasingly challenged (4).

Recent studies have shown that gene variants are involved in the pathogenesis of NAFLD (5, 6). Normal weight and metabolically healthy subjects with such gene variants may develop a disease for which the name “genetic NAFLD” has been proposed (7). The most important gene variants are the patatin-like phospholipase domain-containing protein 3 (PNPLA3 I148M) and transmembrane 6 superfamily member 2 protein (TM6SF2 E167K) mutations, which influence lipid droplet remodeling, lipid secretion from hepatocytes, and retinol metabolism (7, 8). Such mutations may protect against cardiovascular disease while increasing the risk of severe steatosis and steatohepatitis (7, 8). One in three European children carries the PNPLA3 I148M variant and one in three is overweight (7). Thus, many children are at risk for developing “mixed NAFLD” (i.e., NAFLD produced by both genetic and environmental factors) (7). It is also possible that these children may develop more rapidly nonalcoholic steatohepatitis and fibrosis (9).

The developmental origin of health and disease hypothesis postulates that in utero exposure to selected stressors contributes to the risk of later disease, including NAFLD (10). Nonhuman primates develop hepatic steatosis following in utero exposure to high-fat diets (11, 12). Both low and high birth weight children are over-represented among young NAFLD patients, suggesting the possibility of metabolic programming of NAFLD (13, 14).

The Feeding Study is a cohort study aimed at investigating the association between the in utero exposure to dietary fats and the low-grade inflammatory status and insulin resistance of the offspring at birth (15). The inclusion criteria of the mothers of the Feeding Study were: (1) age ≥18 and ≤35 years; (2) singleton pregnancy; (3) no history of smoking; (4) no history of miscarriage; (5) no history of chronic disease including diabetes; (6) no history of gestational diabetes; and (7) no history of ovulation induction or in vitro fertilization. The study was approved by the Ethical Committee of the Bambino Gesù Children’s hospital and was performed respecting national and international regulations and the Declaration of Helsinki (2000). All parents gave written informed consent. The main aim of the present secondary analysis was to estimate the frequency of BL at 1-year follow-up and its association with the PNPLA3 and TM6SF2 gene variants.

Anthropometric assessment

Weight and length at birth were measured to the nearest 5 g and 0.1 cm, respectively (17, 18). Body weight and length at 1 year of age were measured to the nearest 50 g and 0.5 cm (17). SD scores (SDS) of weight and length at birth were calculated using Italian reference data (18) and those at 1 year using the World Health Organization reference data (19).

Liver ultrasonography

Liver ultrasonography (LUS) was performed by two experienced physicians (G.D.M. and F.P.) using an ACUSON X700 ultrasound system (Siemens Healthcare, Siemens AG, Munich, Germany). VAT thickness was measured using a 1.0- to 5.0-MHz curved array transducer, SAT thickness using a 5.0- to 10.0-MHz linear transducer, and EAT thickness using a 1.0- to 4.0-MHz phased array transducer. To measure VAT and SAT, the transducers were positioned where the xiphoid line meets the waist plane. VAT was measured as the distance between the peritoneal border and the corpus of the lumbar vertebra (2, 20); SAT was measured at the same level of VAT but as the distance between the cutaneous border and the linea alba (2, 20); EAT was measured as the echo-free area extending from the epicardial surface to the parietal pericardium of the right ventricular free wall at its thickest
level using the aortic annulus and the midchordal region as landmarks for the long- and short-axis views, respectively (2, 20). Liver echogenicity was assessed using a 1.0- to 5.0-MHz curved array transducer placed on the subcostal plane along the midclavicular line. BL (0 = no; 1 = yes) was defined on the basis of the difference in echogenicity between the liver and the renal parenchyma, the degree of hepatic penetration of ultrasounds, and the degree of visualization of hepatic vessels (2, 20). Suspected cases of BL were reevaluated within 2 weeks from the first LUS by both physicians in charge of ultrasonography. A definitive diagnosis of BL was reached only when the two physicians agreed on the diagnosis of BL performed at the second LUS.

**PLNPLA3 and TMSF2 gene variants**

DNA was extracted by the cord blood samples of pregnant women using the QIAamp Blood MiniKit (Qiagen, Hilden, Germany). \textit{PNPLA3} rs738409 C$>$G and \textit{TM6SF2} rs58542926 C$>$T single nucleotide polymorphism were genotyped using the TaqMan 5'-nuclease assay (Life Technologies, Foster City, CA). The TaqMan genotyping reactions were performed in a MicroAmp Fast Optical 96 Well Reaction Plate with Barcode (Applied Biosystems, Foster City, CA) using a volume of 25 μL containing 2X TaqMan Universal PCR Master Mix No Amperase UNG (Applied Biosystems), 20X SNP Genotyping Assay, and 10 ng DNA. Reaction plates were run on a 7900 HT Fast instrument (Applied Biosystems) for 10 minutes at 95°C, then for 40 cycles of 15 seconds at 95°C and, last, at 60°C for 1 minute.

**Fatty acids**

Maternal fatty acids were measured at the third trimester of pregnancy on erythrocyte membranes as described in detail elsewhere (15, 16).

###Results

**Flow diagram**

Eight-hundred forty-seven (85%) mothers continued the study after enrollment; 505 (60%) of them underwent the 1-year follow-up visit (Fig. 1). All the mother-toddler pairs who took part in the 1-year follow-up were invited to undergo LUS; however, mostly because LUS had to be scheduled on special days on which no other examination was performed, some mothers declined the invitation. Thus, only 391 (77%) children underwent LUS at the 1-year follow-up visit.

**Comparison of children with and without LUS**

Table 1 compares the anthropometric data of the children with and without LUS at birth and at the 1-year follow-up visit. The statistically significant median differences of $-0.02$ (95% CI, $-0.04$ to $-0.001$, $P = 0.05$) years of age at follow-up and of $-1.00$ (95% CI, $-1.87$ to $-1.13$, $P < 0.05$) cm of length at follow-up between LUS+ vs LUS− children are clearly biologically irrelevant (29). The two samples are quite similar for the all features of interest.

**Comparison of children with and without BL**

Of the 391 toddlers who underwent LUS, 17 (4%) had BL. The measurements of BL− and BL+ toddlers are reported in Table 2. The median gestational age was $-1.0$ (95% CI, $-1.9$ to $-0.1$, $P < 0.05$) months

![Figure 1. Flow of the mother-newborn and mother-toddler pairs during the Feeding Study. FU, follow-up.](https://academic.oup.com/jcem/article-abstract/104/6/2163/5288763/104621636287853)
lower in BL+ than in BL− toddlers. This difference is unlikely to be clinically relevant, however. More interestingly, all toddlers had an appropriate length for their gestational age, which was expected owing to the inclusion criteria for the mothers of the Feeding Study. At the 1-year follow-up, the median weight was 1.05 (95% CI, 0.44 to 1.64; \( P = 0.001 \)) kg higher in BL+ than in BL− toddlers; the median weight SDS was 0.37 (0.32; 0.94) higher in BL+ than in BL− toddlers; and the median body mass index (BMI) was 1.14 kg/m² (95% CI, 0.18 to 2.10; \( P = 0.05 \)) higher in BL+ than in BL− toddlers. Although all of these estimates have large 95% CI because of the small number of BL+ toddlers, they agree with the available evidence showing that ponderal status and VAT are predictors of fatty liver (2, 30–32).

### Comparison of mothers of children with and without BL

The measurements of the mothers of the Feeding Study toddlers with and without BL are given in Table 3. The median maternal weight change was 2.00 (95% CI, 0.15 to 3.85; \( P = 0.05 \)) kg higher in the mothers of BL+ than in those of BL− toddlers. Not surprisingly, because of the low sample of BL+ children, this estimate has large 95% CI, but it suggests that pregnancy-related factors may be associated with fatty liver at 1 year of age.

### Association between BL and PNPLA3 and TM6SF2 variants

Compared with toddlers with the PNPLA3 CC genotype (reference group), the odds (95% CI) of BL were 3.01 (1.05 to 8.64, \( P = 0.05 \)) times higher in those with the PNPLA3 CG genotype and 5.37 (1.12 to 25.77, \( P = 0.05 \)) higher in those with the PNPLA3 CC genotype (PMLE logistic regression). Compared with the toddlers with the TM6SF2 CC genotype, the odds (95% CI) of BL were 2.08 (0.62 to 7.00) times higher in those with the TM6SF2 CT genotype and 2.08 (0.11 to 39.14) higher in those with the TM6SF2 TT genotype (PMLE logistic regression).
prompted us to perform an analysis of PNPLA3 and TM6SF2 as potential risk factors for BL in this population. The frequency of the GG, CG, and CC PNPLA3 alleles in our population is similar to that reported in the white population of the Dallas Heart Study, which showed that PNPLA3 is a risk factor for fatty liver independently from dyslipidemia and insulin resistance (6).

In line with expectations (2), BL+ children were heavier and had a higher BMI than BL− children. As far as fat distribution is concerned, VAT was higher in BL+ than in BL− toddlers, but their SAT and EAT were similar. The median difference of 1.8 mm in the VAT of BL+ and BL− children may be biologically relevant even if its 95% CIs are large (0.05 to 3.35 mm), partly because of the low number of BL+ children (n = 17). There is increasing evidence that the inability of SAT to expand may produce an early accumulation of hepatic fat (31, 33).

However, we found similar values of SAT and EAT in BL+ and BL− children at 1 year of age. Our finding that birth weight and later fatty liver are not associated agrees with the findings of a large cohort study of 17-year-old adolescents from Germany (34) and of a smaller cohort study of 18- to 24-year-old adults from Holland (35).

Interestingly, BL+ children were born to mothers who had a greater increase of body weight during pregnancy compared with those of BL− children. Few and contradictory data are available on the association between maternal weight gain and fatty liver in offspring. The Avon Longitudinal Study of Parents and Children reported that maternal overweight/obesity and gestational diabetes mellitus are associated with hepatic fat content in offspring (36). However, such association disappeared after adjustment for childhood obesity (36). The Exploring Perinatal Outcomes in Children Study found that maternal obesity before pregnancy was associated with an increased hepatic fat fraction in

### Table 2. Anthropometric and Ultrasonographic Measurements of Feeding Study Toddlers at Birth and at 1-Y Follow-Up

<table>
<thead>
<tr>
<th></th>
<th>BL− Toddlers</th>
<th>BL+ Toddlers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Median (IQR) or n (%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>374</td>
<td>179 (47.9%)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td>195 (52.1%)</td>
</tr>
<tr>
<td>Gestational age, wk</td>
<td>374</td>
<td>39 (38; 40)</td>
</tr>
<tr>
<td>Duration of breastfeeding, mo</td>
<td>374</td>
<td>5 (0; 11)</td>
</tr>
<tr>
<td>Weight at birth, kg</td>
<td>374</td>
<td>3.31 (2.99; 3.63)</td>
</tr>
<tr>
<td>Weight at birth, SDS Bertino</td>
<td>374</td>
<td>0.35 (−0.37; 0.98)</td>
</tr>
<tr>
<td>Length at birth, m</td>
<td>374</td>
<td>0.50 (0.49; 0.52)</td>
</tr>
<tr>
<td>Length at birth, SDS Bertino</td>
<td>374</td>
<td>0.00 (0.00; 0.01)</td>
</tr>
<tr>
<td>Length for gestational age, Bertino</td>
<td>374</td>
<td>374 (100.0%)</td>
</tr>
<tr>
<td>Age at follow-up, y</td>
<td>374</td>
<td>1.0 (1.0; 1.1)</td>
</tr>
<tr>
<td>Weight at follow-up, kg</td>
<td>374</td>
<td>9.95 (9.20; 10.65)</td>
</tr>
<tr>
<td>Weight at follow-up, SDS WHO</td>
<td>374</td>
<td>0.44 (−0.13; 1.08)</td>
</tr>
<tr>
<td>Length at follow-up, cm</td>
<td>374</td>
<td>76.0 (74.0; 78.5)</td>
</tr>
<tr>
<td>Length at follow-up, SDS WHO</td>
<td>374</td>
<td>0.37 (−0.29; 1.15)</td>
</tr>
<tr>
<td>BMI at follow-up, kg/m²</td>
<td>374</td>
<td>16.9 (16.0; 18.0)</td>
</tr>
<tr>
<td>BMI at follow-up, SDS WHO</td>
<td>374</td>
<td>0.27 (−0.35; 0.94)</td>
</tr>
<tr>
<td>Subcutaneous adipose tissue at follow-up, mm</td>
<td>373 c</td>
<td>3.6 (3.1; 3.9)</td>
</tr>
<tr>
<td>VAT at follow-up, mm</td>
<td>369 d</td>
<td>45.0 (43.0; 47.0)</td>
</tr>
<tr>
<td>Epicardial adipose tissue at follow-up, mm</td>
<td>369 e</td>
<td>3.4 (3.1; 3.6)</td>
</tr>
<tr>
<td>PNPLA3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>374</td>
<td>218 (58.3%)</td>
</tr>
<tr>
<td>CG</td>
<td></td>
<td>138 (36.9%)</td>
</tr>
<tr>
<td>GG</td>
<td></td>
<td>18 (4.8%)</td>
</tr>
<tr>
<td>TM6SF2</td>
<td>374</td>
<td>331 (88.5%)</td>
</tr>
<tr>
<td>CT</td>
<td></td>
<td>38 (10.2%)</td>
</tr>
<tr>
<td>TT</td>
<td></td>
<td>5 (1.3%)</td>
</tr>
</tbody>
</table>

Abbreviations: SDS Bertino, SDS per Bertino et al. (18); WHO, World Health Organization.

aP < 0.05.
bP < 0.001 (median regression).
cCould not be reproducibly measured in 1 BL− toddler.
dCould not be reproducibly measured in 5 BL− toddlers and 1 BL+ toddlers.
offspring, independent of adiposity (37). In a study of neonates born to obese mothers without gestational diabetes mellitus, intrahepatic fat content was associated with maternal BMI but not with birth weight, suggesting that maternal obesity may be an early determinant of hepatic fat content (38). In the Cardiovascular Risk in Young Finns cohort study, PNPLA3, TM6SF2, and low birth weight were predictors of NAFLD at the 31-year follow-up (39).

The current study investigated healthy infants born from healthy mothers after 38 to 42 weeks of gestation. A low number of mothers was obese before pregnancy and all children were appropriate for gestational age. In such a cohort, we cannot properly test the hypothesis that in utero development is associated with later fatty liver, because most risk factors for abnormal growth were virtually excluded by the entry criteria applied to the mothers. It is nonetheless of interest that in this healthy population of mothers, weight gain during pregnancy was also associated with BL in the offspring at 1 year. It is likely that PNPLA3 and maternal weight gain during pregnancy (and other pregnancy-related factors) do interact in determining the risk of fatty liver (39). However, the low number of toddlers with the PNPLA3 GG allele (18 BL− and 2 BL+) impeded us from formally testing this hypothesis in the current study. As other researchers have done (39), we wish to emphasize that BMI and its possible interaction with PNPLA3 are modifiable risk factors, whereas PNPLA3 is not.

Although this study reports the frequency of fatty liver in 1-year-old children from a general population, it has several limitations. First, of the 1000 mother-newborn pairs enrolled into the Feeding Study, only 505 mothers presented at the 1-year follow-up and only 391 of their children underwent LUS at 1 year; thus, we cannot claim that our data can be generalized to the whole Feeding Study population. Second, we did not evaluate insulin resistance at the 1-year follow-up, even if it is possible that BL+ children at this age are not more insulin-resistant than their BL− peers, in line with the findings of the Origin of Cardiovascular Disease Study (2). Such assessment is important because there is increasing evidence that hepatic insulin resistance may develop in response to the accumulation of triacylglycerol in the hepatocytes (33). Third, we used LUS to diagnose BL in our children. Although LUS is the only feasible option to diagnose fatty liver in epidemiological studies (30, 32), it is known to offer an acceptable assessment of fatty liver starting from an intrahepatic triglyceride content of 10% only, at least in adults (40). Thus, some cases of BL may have gone undetected in our study, and our estimate of 4% for BL frequency is to be considered conservative. Fourth, the small number of BL+ cases (n = 17, 4%) impeded us from formally evaluating the possibility of a different gene-maternal environment interaction in BL+ vs BL− children.

In conclusion, in the Feeding Study, 4% of 1-year-old toddlers had BL and PNPLA3 was a predictor of it. Larger cohorts with greater numbers of children with BL are needed to determine whether there is a gene-environment interaction between PNPLA3 and maternal and child anthropometry.
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Author Contributions: G.B. and M.M. designed the study, analyzed the data, and drafted, and revised the manuscript. M.M. coordinated the study and got funding. G.D.M., M.F., and F.P. designed and performed data collection. G.M. and F.P. run the clinical study and performed liver ultrasonography. F.S. and V.N. followed the toddlers and performed the clinical assessment. M.F., A.L., and A.C. performed laboratory and genetic tests. B.D. supervised genetic testing. All authors revised and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agree to be accountable for all the aspects of the work.

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Disclosure Summary: The authors have nothing to disclose.

References


