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Pregnancy lipidomic profiles and DNA methylation in newborns from the CHAMACOS cohort

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Abstract

Lipids play a role in many biological functions and the newly emerging field of lipidomics aims to characterize the varying classes of lipid molecules present in biological specimens. Animal models have shown associations between maternal dietary supplementation with fatty acids during pregnancy and epigenetic changes in their offspring, demonstrating a mechanism through which prenatal environment can affect outcomes in children; however, data on maternal lipid metabolite levels during pregnancy and newborn DNA methylation in humans are sparse. In this study, we assessed the relationship of maternal lipid metabolites measured in the blood from pregnant women with newborn DNA methylation profiles in the Center for the Health Assessment of Mothers and Children of Salinas cohort. Targeted metabolomics was performed by selected reaction monitoring liquid chromatography and triple quadrupole mass spectrometry to measure 92 metabolites in plasma samples of pregnant women at ~24 weeks gestation. DNA methylation was assessed using the Infinium HumanMethylation 450K BeadChip adjusting for cord blood cell composition. We uncovered numerous significant associations between maternal metabolite levels, particularly phospholipid and lysolipid metabolites, and newborn methylation. The majority of the observed relationships were negative, suggesting that higher lipid metabolites during pregnancy are associated with lower methylation levels at genes related to fetal development. These results further elucidate the complex relationship between early life exposures, maternal lipid metabolites, and infant epigenetic status.

Key words: DNA methylation; metabolomics; Mexican-American; epigenetics; newborns; cord blood; prenatal exposure

Introduction

Metabolomics has emerged as a useful tool to examine the profiles of small molecule metabolites in biospecimens [1]. Factors that have been associated with metabolite levels include age [2], diseases such as obesity [3], genetic variants throughout the human genome [4], and epigenetics [5, 6]. Both untargeted and targeted methodologies are commonly used in metabolomic research, with the former measuring thousands of metabolites in a biological sample and the latter focusing on a subset of metabolites, often involved in specific biological pathways [2]. For instance, lipidomics evaluates the function and distribution of lipids, which have a greater diversity of molecular species...
compared to the other classes of biological molecules in the body, such as carbohydrates and amino acids [7].

Epigenetic mechanisms regulate gene expression without changes in DNA sequence and include DNA methylation, histone modifications, and non-coding RNAs [8]. DNA methylation is the most commonly examined epigenetic mechanism and refers to the addition of a methyl group to the 5’ position of the cytosine ring in a CpG dinucleotide by DNA methyltransferases [9]. DNA methylation patterns are retained following somatic cell division [10]. In the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) cohort and as part of the Pregnancy and Childhood Epigenetics consortium, we have explored the relationship of infant DNA methylation with early life environmental exposure of mothers to chemicals during pregnancy, including endocrine disrupting chemicals [11–13] and smoking [14]. Since the epigenome undergoes remodeling and rapid cell division in the prenatal period [8], this life stage is considered particularly sensitive to environmental insults and can have implications for disease trajectories [15].

There remains a paucity of information on the relationship between metabolite profiles, in general and specific to the lipid species, and DNA methylation, particularly the link between maternal lipid metabolites during pregnancy and offspring epigenetics, which could be a mechanism through which the in utero environment exerts an effect on epigenetic mechanisms that could impact offspring health. Most of the existing research on this topic has been conducted in animal models and has focused on the relationship between maternal nutrition, including dietary supplementation with fatty acids, and offspring methylation status [16, 17]. Only one study to date has examined the association between in utero metabolic traits, including lipid metabolites, and DNA methylation profiles in newborns [5]. The authors found significant relationships between maternal fatty acid levels and newborn methylation globally and at target genes related to early growth.

Previous research in the CHAMACOS cohort examined the association between maternal prenatal phthalate urine metabolite levels and targeted metabolomic compounds present in plasma and urine, collected at 26 weeks gestation [18]. In addition, we assessed the relationship between pre-pregnancy body mass index (BMI) and maternal metabolomics profiles. We observed numerous associations of metabolomic markers involved in lipid and nucleic acid metabolism and the inflammatory response with both urinary phthalate metabolites and maternal BMI. This study aims to determine the association between maternal metabolomic profiles in pregnancy and child DNA methylation at birth, emphasizing metabolites enriched for lipid pathways that were confirmed as relevant in previous work in CHAMACOS [18]. This study will contribute to the growing body of evidence of the influence of the early life environment on epigenetic mechanisms.

**Results**

**Study Population**

Characteristics of CHAMACOS mothers and children included in the study are presented in Table 1. Additional data on CHAMACOS demographic parameters have been previously described in [19]. Most mothers were overweight or obese (BMI ≥ 25 kg/m²) prior to pregnancy. Roughly equal portions of children were boys and girls, 5% were of low birth weight (< 2500 g) and 7% born were premature (< 37 weeks).

### Table 1: characteristics of CHAMACOS children and mothers (N = 81 pairs) with newborn 450K data and assessment of maternal metabolomic profiles at 26 weeks gestation

| Characteristics                              | N (%) |
|----------------------------------------------|-------|
| Maternal age at delivery                     |       |
| 18–24                                        | 32 (39.5) |
| 25–29                                        | 36 (44.4) |
| 30–34                                        | 11 (13.6) |
| 35–45                                        | 2 (2.5) |
| Newborn sex                                  |       |
| Boy                                          | 39 (48.1) |
| Girl                                         | 42 (51.9) |
| Maternal pre-pregnancy BMI (kg/m²)           | 25.5 (4.3) |
| Newborn gestational age (weeks)              | 38.8 (1.7) |
| Newborn birth weight (grams)                 | 3413.1 (558.0) |
| Newborn white blood cell count (%)           |       |
| Granulocytes                                 | 46.4 (10.3) |
| CD4+ T                                       | 15.5 (6.7) |
| CD8+ T                                       | 13.2 (3.8) |
| B cells                                      | 9.5 (3.2) |
| nRBC                                         | 10.4 (6.5) |
| Monocytes                                    | 8.1 (1.9) |
| NK cells                                     | 0.4 (1.2) |

**Maternal Prenatal Metabolomic Profiles**

In this study, we focused on maternal metabolites previously identified to be relevant to lipid biosynthesis, arachidonate enrichment and release, and inflammatory signaling [18]. The 92 plasma metabolites in the study included fatty acids, lysolipids, phospholipids, sphingolipids, monoacylglycerols, diacylglycerols, and triacylglycerols (Table 2, Figs 1 and 2). Example distributions of specific fatty acid, lysolipid, and phospholipid metabolites are shown in Fig. 1. Median lipid metabolite counts ranged from 0.01 (C16:0e/C18:1 PSe) to 44 132.92 (C16:0/C18:1 PC) (Table 2). In Fig. 2, we plotted the median values for the metabolites within each of the four quartiles based on the ranges in the metabolite distributions. The phosphatidylcholine (PC) phospholipids had the highest median values and a broad range across subjects. All metabolites were log(1+x) transformed in analyses to reduce the influence of outliers.

**Maternal Metabolomics and Newborn DNA Methylation Analyses**

Numerous maternal prenatal metabolites were significantly associated, after correcting for multiple hypotheses testing, with DNA methylation of CHAMACOS newborns (Table 3). The metabolites that were related to newborn DNA methylation after adjusting for relevant covariates, indicated by an asterisk above the bar plots in Fig. 2, were distributed across the four quartiles based on metabolite ranges, with four of the nine significant metabolites found in the lowest quartile of ranges (Fig. 2A). After adjusting for child sex, batch, and white blood cell type estimates, four phospholipids (C16:0/C20:4 PS, C16:0e/C18:1 PS, C18:0/C20:4 alkyl PA, C18:0e/C18:1 PSe), four lysolipids (C16:0 alkyl LPA, C18:1 alkyl LPA, C18:1 LPI, C20:4 LPS), and the fatty acid C18:0 FFA were among the significant metabolites related to newborn methylation. The number of CpG sites significantly associated with a specific metabolite ranged from 1 to 6. The phospholipid C18:0/C20:4 alkyl PA, shown in Fig. 3, was one
of two phospholipid metabolites associated with methylation at six different CpG sites, shown by the data points above the red genome-wide significance threshold. Prior to adjustment of P-values for multiple hypotheses testing, this metabolite was associated with 10 338 CpG sites throughout the genome. Four of the false discovery rate (FDR) significant CpG sites associated with C18:0/C20:4 alkyl PA were also related to C18:1 LPI.

Several CpG sites had significant relationships with one phospholipid (C18:0/C20:4 alkyl PA) and/or two lysolipid (C16:0 alkyl LPA, C18:1 LPI) metabolites (lysolipids are phospholipid breakdown products). For instance, cg12106728 was negatively associated with C18:0/C20:4 alkyl PA (\(\beta = -0.104, \text{FDR } P = 0.005\)) and C18:1 LPI (\(\beta = -0.667, \text{FDR } P = 0.003\)). A CpG site that maps to CTDSP2 and MR26A2, cg17169243, was inversely related to C18:0/C20:4 alkyl PA (\(\beta = -0.454, \text{FDR } P = 0.019\)), C18:1 LPI (\(\beta = -0.807, \text{FDR } P = 0.002\)), and C16:0 alkyl LPA (\(\beta = -0.366, \text{FDR } P = 0.021\)). cg19220754, located in the body of TNPO1, was hypomethylated with increased C18:0/C20:4 alkyl PA (\(\beta = -1.843, \text{FDR } P = 0.019\)) and C18:1 LPI (\(\beta = -2.962, \text{FDR } P = 0.019\)). cg24175823 was negatively associated with C18:0/C20:4 alkyl PA (\(\beta = -1.341, \text{FDR } P = 0.002\)), C18:1 LPI (\(\beta = -2.039, \text{FDR } P = 0.018\)), and C16:0 alkyl LPA (\(\beta = -1.024, \text{FDR } P = 0.018\)). Out of the 28 total significant relationships between metabolites and CpG sites, 22 had negative regression coefficients, suggesting that increased lipidomic levels in maternal blood are generally associated with lower DNA methylation in their newborn children.

Although most of the significant associations were negative, some of the positive relationships observed between maternal metabolite levels and infant DNA methylation had the largest effect sizes. A CpG site that maps to SEPT2 and HDLBP, cg16787284, was positively related to C16:0e/C18:1 PSe (\(\beta = 25.723, \text{FDR } P = 0.026\)) and C18:0e/C18:1 PSe (\(\beta = 1.154, \text{FDR } P = 0.021\)). cg22539279, located in the body of NEK11 and ASTE1, was also hypermethylated with increased C16:0e/C18:1 PSe (\(\beta = 23.200, \text{FDR } P = 0.021\)).

In addition, seven of the FDR-significant relationships remained after using the more stringent Bonferroni adjustment. These included the associations of cg10874881 and cg16597728 with the lysolipid C18:1 LPI (\(\beta = 0.807, \text{FDR } P = 0.021\)), cg12106728 and cg17169243 with the phospholipid C16:0e/C18:1 PSe, and cg24175823 with the phospholipid C18:0/C20:4 alkyl PA, cg19220754, located in the body of TNPO1, was hypomethylated with increased C18:0/C20:4 alkyl PA (\(\beta = -1.843, \text{FDR } P = 0.019\)) and C18:1 LPI (\(\beta = -2.962, \text{FDR } P = 0.019\)). cg24175823 was negatively associated with C18:0/C20:4 alkyl PA (\(\beta = -1.341, \text{FDR } P = 0.002\)), C18:1 LPI (\(\beta = -2.039, \text{FDR } P = 0.018\)), and C16:0 alkyl LPA (\(\beta = -1.024, \text{FDR } P = 0.018\)). Out of the 28 total significant relationships between metabolites and CpG sites, 22 had negative regression coefficients, suggesting that increased lipidomic levels in maternal blood are generally associated with lower DNA methylation in their newborn children.

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In addition, seven of the FDR-significant relationships remained after using the more stringent Bonferroni adjustment. These included the associations of cg10874881 and cg16597728 with the phospholipid C16:0/C20:4 PS, cg12106728 and cg24175823 with the phospholipid C18:0/C20:4 alkyl PA, cg21883754 with the phospholipid ether C16:0e/C18:1 PSe, and cg12106728 and cg17169243 with the lysolipid C18:1 LPI.

Pathway Analysis of Gene Hits

The maternal metabolites significantly associated with newborn methylation included complex lipids and products of lipid degradation, side products generated from choline and fatty acid release for the developing fetus (Fig. 4A and B). These lipids provide a methyl source for DNA methylation and drive growth, providing lipid building blocks and energy (Fig. 4B). To determine the biological relevance of the metabolomic and methylation findings, we ran pathway analysis of the genes for which the significant CpG hits were mapped (Table 4). The most common molecular functions relevant to the genes included binding and catalytic activity. In addition, the genes were involved in a range of biological processes at the cellular and metabolic levels and participated in biological regulation, response to stimulus, and localization. The genes BDNF, PC, and SEPT2 have been related to a variety of pathways, including Huntington disease, pyruvate metabolism, and Parkinson disease, respectively.

Discussion

In this study, we characterized the lipidomic profiles of pregnant women from the CHAMACOS cohort, building upon previous research that identified associations of metabolomic data with phthalates, endocrine disrupting chemicals, and maternal pre-pregnancy BMI [18]. We focused on maternal metabolite levels assessed around 26 weeks gestation, rather than at child delivery, due to the availability of existing metabolomic data and more importantly, to better capture exposures relevant during fetal development that could relate to newborn epigenetic
Figure 2: median levels of metabolites with ranges in the (A) first (lowest), (B) second, (C) third, and (D) fourth (highest) quartiles.

*Represents metabolites that were significantly associated with DNA methylation in newborns.
Table 2: distribution of lipid metabolites in plasma of CHAMACOS mothers (N = 81)

| Metabolite Pathway | Median | IQR |
|-------------------|--------|-----|
| C16:0/C18:1 DAG | Diacylglycerol | 21.56 | [14.44, 30.06] |
| C16:0/C20:4 DAG | Diacylglycerol | 0.91 | [0.57, 1.35] |
| C18:0/C18:1 DAG | Diacylglycerol | 6.22 | [3.83, 9.53] |
| C18:0/C20:4 DAG | Diacylglycerol | 0.07 | [0.04, 0.12] |
| C12:0 AC | Fatty acid | 1.12 | [0.94, 1.41] |
| C16:0 AC | Fatty acid | 25.18 | [14.61, 40.10] |
| C16:0 FFA | Fatty acid | 1660.35 | [1282.00, 2199.70] |
| C16:0 FFA | Fatty acid | 0.91 | [0.57, 1.35] |
| C18:0/C18:1 DAG | Diacylglycerol | 6.22 | [3.83, 9.53] |
| C18:0/C20:4 DAG | Diacylglycerol | 0.07 | [0.04, 0.12] |
| C12:0 AC | Fatty acid | 1.12 | [0.94, 1.41] |
| C16:0 AC | Fatty acid | 25.18 | [14.61, 40.10] |
| C16:0 FFA | Fatty acid | 1660.35 | [1282.00, 2199.70] |
| C16:0 FFA | Fatty acid | 0.91 | [0.57, 1.35] |
| C18:0/C18:1 DAG | Diacylglycerol | 6.22 | [3.83, 9.53] |
| C18:0/C20:4 DAG | Diacylglycerol | 0.07 | [0.04, 0.12] |
profiles. The 92 lipid metabolites assessed had broad ranges of levels in the women’s plasma. We further examined the relationship between maternal metabolite counts and offspring DNA methylation. Out of the lipid metabolites assessed, we identified significant and predominantly negative associations among four phospholipids, four lysolipids, and a fatty acid with newborn methylation. In addition, several of the significant metabolites were related to multiple CpG sites. For example, the phospholipids C16:0/C20:4 PS and C18:0/C20:4 alkyl PA were both associated with 6 CpG sites. These results strengthen the evidence that maternal metabolites, particularly lysolipids and phospholipids, are related to reduced offspring DNA methylation at CpG sites in genes involved in a variety of biological processes, including catalytic and binding activities.

The wide range in lipid metabolite levels observed in CHAMACOS is similar to a study conducted in 40 mother–child dyads from a clinical birth cohort in Michigan [5]. Marchlewicz et al. examined acylcarnitine, free fatty acid, and amino acid

Table 2: (continued)

| Metabolite | Pathway | Median | IQR          |
|------------|---------|--------|--------------|
| C18:0/C20:4 alkyl PI | Phospholipid | 0.48   | [0.30, 0.89] |
| C18:0/C20:4 PA | Phospholipid | 3.23   | [2.75, 3.96] |
| C18:0/C20:4 PC | Phospholipid | 25 368.63 | [22 167.33, 28 643.58] |
| C18:0/C20:4 PI | Phospholipid | 36.05  | [21.71, 52.25] |
| C18:0/C20:4 PS | Phospholipid | 0.61   | [0.18, 0.91] |
| C18:0e/C18:1 PCE | Phospholipid | 2440.81 | [1981.18, 2796.77] |
| C18:0e/C18:1 PCE | Phospholipid | 0.7    | [0.44, 1.18] |
| C18:0e/C18:1 PGE | Phospholipid | 0.45   | [0.03, 2.34] |
| C18:0e/C20:4 PCE | Phospholipid | 4815.13 | [4010.52, 5666.14] |
| C18:0e/C20:4 PCE | Phospholipid | 0.09   | [0.03, 0.16] |
| C18:0p/C20:4 PCE | Phospholipid | 4569.48 | [3835.01, 5374.80] |
| C18:0p/C20:4 PEp | Phospholipid | 0.1    | [0.04, 0.16] |
| Cardiolipin C18:1/18:1/18:1/18:1 T2 | Sphingolipid | 1.04   | [0.80, 1.31] |
| C16:0 ceramide | Sphingolipid | 12.11  | [9.50, 13.92] |
| C18:0 ceramide | Sphingolipid | 1.79   | [1.37, 2.36] |
| C18:0 SM | Sphingolipid | 1997.51 | [1135.78, 2943.23] |
| C18:0/C16:0 ceramide-1-phosphate | Sphingolipid | 8.26   | [7.32, 10.12] |
| C18:1 SM | Sphingolipid | 2325.2 | [2009.34, 2810.72] |
| C18:1/C16:0 ceramide-1-phosphate | Sphingolipid | 92.51  | [78.65, 109.94] |
| C20:4 SM | Sphingolipid | 1400.77 | [881.66, 1765.40] |
| Lactosylceramide C18:1/C18:0 | Sphingolipid | 0.5    | [0.28, 0.77] |
| Sphinganine | Sphingolipid | 3.64   | [1.38, 11.14] |
| Sphingosine | Sphingolipid | 36.95  | [19.04, 51.62] |
| C16:0/C16:0/C16:0 TAG | Triacylglycerol | 1690.96 | [958.05, 2779.60] |
| C16:0/C18:1/C16:0 TAG | Triacylglycerol | 10 433.19 | [7397.58, 13 658.95] |
| C16:0/C20:4/C16:0 TAG | Triacylglycerol | 3969.7 | [2889.16, 5224.91] |
| C16:0/C18:0/C18:0 TAG | Triacylglycerol | 216.26 | [121.32, 356.69] |
| C18:0/C18:0/C18:0 TAG | Triacylglycerol | 868.13 | [430.36, 1782.46] |
| C18:0/C20:4/C18:0 TAG | Triacylglycerol | 2.72   | [1.61, 9.12] |

Figure 3: manhattan plot of CpG sites of CHAMACOS newborns associated with C18:0/C20:4 alkyl PA levels in the blood of their mothers during pregnancy. Red line represents genome-wide significance threshold of \(-\log_{10}(5.00E-08)\), while the blue line corresponds to the suggestive threshold of \(-\log_{10}(1.00E-05)\)
metabolite levels of mothers during the first trimester and at delivery, as well as of their children at birth. They observed wide distributions of free fatty acid levels similar to CHAMACOS, with medians ranging from 0.00 to 152.70 nmol/ml and 0.00 to 247.90 nmol/ml for the maternal first trimester and delivery metabolites, respectively. In CHAMACOS mothers, median free fatty acid metabolite counts ranged from 1.12 to 4337.56. Higher levels in the CHAMACOS cohort could be attributed to differences in study populations; since CHAMACOS participants have a high prevalence of obesity and are Mexican-

Table 3: significant results of the relationship between prenatal maternal metabolite levels in plasma and newborn cord blood DNA methylation

| CpG sites          | Chromosome | N  | β   | SE  | FDR P-value | Significant exposures | Genes | Gene location | Relation to CpG island |
|--------------------|------------|----|-----|-----|-------------|------------------------|-------|---------------|------------------------|
| cg02496111         | 16         | 81 | −3.172 | 0.467 | 0.012       | C16:0/C20:4 PS          | MYCBPAP | Body          | Island                 |
| cg03168497         | 17         | 81 | −2.118 | 0.330 | 0.021       | C18:1 alkyt LPA         | Body    | Island        | Island                 |
| cg04108939         | 1          | 80 | 1.169  | 0.187 | 0.039       | C20:4 LPS               | BEST4   | Island        | Island                 |
| cg06494167         | 2          | 80 | −1.428 | 0.214 | 0.018       | C18:1 LPI               | RMND5A  | Island        | Island                 |
| cg07370087         | 1          | 81 | 0.469  | 0.076 | 0.044       | C18:0 FFA               | Body    | Island        | Island                 |
| cg08833610         | 17         | 81 | −3.662 | 0.589 | 0.040       | C16:0/C20:4 PS          | RPAIN, NUP88 | Island       | Island                 |
| cg09931872         | 10         | 80 | −2.409 | 0.365 | 0.018       | C16:0/C20:4 PS          | WDFY4   | 5'UTR        | Island                 |
| cg10528455         | 2          | 81 | 0.492  | 0.076 | 0.019       | C18:0/C20:4 alkyt PA    | Body    | Island        | Island                 |
| cg10874881         | 7          | 80 | −2.546 | 0.346 | 0.002       | C16:0/C20:4 PS          | POUSF2  | Body          | Island                 |
| cg12106728         | 12         | 79 | −1.014 | 0.144 | 0.005       | C18:0/C20:4 alkyt PA    | Body    | Island        | Island                 |
|                   |            | 79 | −1.667 | 0.229 | 0.003       | C18:1 LPI               | Body    | Island        | Island                 |
| cg14630692         | 9          | 81 | −0.305 | 0.047 | 0.019       | C16:0 alkyt LPA         | URM1    | Body          | Island                 |
| cg14897096         | 11         | 78 | −1.911 | 0.286 | 0.018       | C16:0/C20:4 PS          | PC       | 5'UTR        | Island                 |
| cg15710245         | 11         | 79 | −0.731 | 0.114 | 0.024       | C18:0e/C18:1 PHe        | BDNF    | Island        | Island                 |
|                   |            | 79 | −0.454 | 0.070 | 0.019       | C18:0/C20:4 alkyt PA    | Body    | Island        | Island                 |
|                   |            | 79 | −0.807 | 0.105 | 0.002       | C18:1 LPI               | Body    | Island        | Island                 |
| cg16597728         | 20         | 79 | −2.063 | 0.293 | 0.005       | C16:0/C20:4 PS          | APCDD1L | Body          | Island                 |
| cg16877284         | 2          | 81 | 25.723 | 4.056 | 0.026       | C16:0e/C18:1 PSe        | SEPT2, HDLBP | Island       | Island                 |
| cg17169243         | 12         | 79 | −0.366 | 0.057 | 0.021       | C16:0 alkyt LPA         | CTDSP2, | Body          | Island                 |
|                   |            | 79 | −0.454 | 0.070 | 0.019       | C18:0/C20:4 alkyt PA    | MIR26A2 | Body          | Island                 |
|                   |            | 79 | −0.807 | 0.105 | 0.002       | C18:1 LPI               | Body    | Island        | Island                 |
| cg19220754         | 5          | 81 | −1.843 | 0.281 | 0.019       | C18:0/C20:4 alkyt PA    | TNPO1   | Body          | Island                 |
| cg21833754         | 19         | 81 | −2.962 | 0.456 | 0.019       | C18:1 LPI               | Body    | Island        | Island                 |
| cg22159279         | 3          | 81 | 23.200 | 3.607 | 0.021       | C16:0e/C18:1 PSe        | TTYH1   | TSS200       | Island                 |
| cg24175823         | 10         | 77 | −1.024 | 0.154 | 0.018       | C16:0 alkyt LPA         | NEK11, ABT3 | TSS200      | Island                 |
|                   |            | 77 | −1.341 | 0.180 | 0.002       | C18:0/C20:4 alkyt PA    | Body    | Island        | Island                 |
|                   |            | 77 | −2.039 | 0.306 | 0.018       | C18:1 LPI               | Body    | Island        | Island                 |
| cg27492749         | 7          | 76 | −1.548 | 0.235 | 0.019       | C18:0/C20:4 alkyt PA    | SGCE, PEG10 | Body, TSS1500 | Island                 |

Bolded FDR P-values indicate CpGs that were also significant based on the more conservative Bonferroni correction (uncorrected P-value < 1.36 x 10^-4). β represents regression coefficients of the relationship between log(1+x) transformed maternal prenatal metabolites and newborn DNA methylation M-values, adjusting for child sex, batch, and white blood cell composition.

Figure 4: diagram of the significant positive (+) and negative (−) associations observed between maternal metabolites during pregnancy and newborn DNA methylation (A) and the biological pathways whereby maternal lipid levels could impact DNA methylation of their children (B)
American as compared to the population in the Marchlewicz et al. study, which was mostly Caucasian; and the species of free fatty acid metabolites assessed in each cohort.

In addition to determining the distributions of lipid metabolites in maternal plasma, another critical objective of this study was to assess the relationship between maternal blood lipid levels and offspring DNA methylation at birth. Using a targeted approach, previous research in animal models has demonstrated associations between maternal fatty acid diet and increased DNA methylation globally, measured by methylation in newborns. Specifically, the relationship between maternal fatty acids measured during the first trimester were significantly and positively correlated with infant methylation at LINE-1 and LUMA, and at genes relevant for growth, including the imprinted genes H19 and IGFl. They observed that maternal fatty acids measured during the first trimester were significantly and positively correlated with infant methylation at LINE-1, IGFl, ESR1, and PPARα, and negatively correlated with H19 and LUMA. Additional correlations were found for maternal delivery and cord blood metabolite levels with newborn methylation, with similar directions of association. Although we were not able to replicate the results observed in the Marchlewicz et al. study since they used a candidate gene approach, we were also able to observe a significant relationship between maternal lipid levels and imprinted gene methylation in newborns. Specifically, the relationship between cg27492749, a CpG site within another imprinted gene (SGCE), and the lipid metabolite C18:0/C20:4 alkyl PA were all related to newborn DNA methylation.

In CHAMACOS, we observed predominantly negative relationships between maternal second trimester metabolite counts and newborn DNA methylation. Marchlewicz et al. [5] conducted a similar study in 40 predominantly white (85%) mothers and their children from Michigan. They examined whether maternal metabolomic profiles are associated with cord blood methylation globally, measured by LINE-1 and LUMA, and at genes relevant for growth, including the imprinted genes H19 and IGFl. They observed that maternal fatty acids measured during the first trimester were significantly and positively correlated with infant methylation at LINE-1, IGFl, ESR1, and PPARα, and negatively correlated with H19 and LUMA. Additional correlations were found for maternal delivery and cord blood metabolite levels with newborn methylation, with similar directions of association. Although we were not able to replicate the results observed in the Marchlewicz et al. study since they used a candidate gene approach, we were also able to observe a significant relationship between maternal lipid levels and imprinted gene methylation in newborns. Specifically, the relationship between cg27492749, a CpG site within another imprinted gene (SGCE), and the lipid metabolite C18:0/C20:4 alkyl PA were all related to newborn DNA methylation.

Several of the genes with significant CpG sites related to maternal metabolite levels in the CHAMACOS cohort are involved in cellular process or-
in biological pathways associated with early life development. For instance, the BDNF gene encodes for the growth factor, brain-derived neurotrophic factor, which is important in the development of the central and peripheral nervous systems of infants [22]. Human septins, including the protein encoded by SEPT2, can assemble into higher-order cytoskeletal structures, such as filaments and bundles, which are necessary to the growing fetus [23]. In addition, the PC gene encodes for the enzyme pyruvate carboxylase, which fuels the tricarboxylic acid cycle, the major pathway that drives the biosynthesis of amino acids [24].

The statistically significant lipids in this study can be linked with DNA methylation in several ways. First, the process of lipid degradation releases fatty acids and lysolipids, as well as choline, which is transported to the developing infant. Choline serves as a major methyl source for DNA methylation and is also necessary for the synthesis of new biological membranes for rapidly dividing cells. The observed associations between DNA methylation and lipid degradation products may be possible because the metabolic flux is relatively slow or their abundance is low. In contrast, we did not find associations for the phosphatidylcholine (PC) or triacylglycerol (TG) metabolites, which serve as major sources of either fatty acids or choline [25, 26]. The metabolic flux through PC and TG metabolism and their relative abundance is relatively high (Fig. 2), which could obscure the association. Second, fatty acids provide a major energy source which drives growth in the developing fetus. Third, ether lipids regulate cell differentiation and cellular signaling [27]. Ether lipids are an integral component of lipid membranes, provide enhanced integrity at lipid raft microdomains, and are important for membrane fusion and trafficking.

A limitation of this study includes the relatively small sample size, which could limit the power to detect significant differences based on the large number of tests performed. However, our study has more power than the most comparable study [5], which has half the number of mother–child dyads with metabolomic and DNA methylation data. Another limitation is the absence of data on child metabolomic profiles at birth. However, given that maternal lipid metabolites can be transferred to the developing fetus via the placenta and that the prenatal period is a time of epigenetic remodeling, we feel that exploring the relationship between maternal metabolite levels during pregnancy, a sensitive time for epigenetic changes, is more relevant. In addition, although DNA methylation is the most commonly studied epigenetic mechanism, maternal lipid metabolites could also be related to other epigenetic marks in children, including histone modifications.

The main strengths of this study include the use of multiple ‘omics’ data from the well-characterized CHAMACOS cohort, including the assessment of numerous classes of lipids, which expanded upon the fatty acids that have been previously assessed in relation to infant methylation data. This allowed for the discovery of novel associations between maternal metabolite levels, including phospholipids and lysolipids, and newborn DNA methylation, which could have implications on child health. Previous studies [28, 29] in pregnant women with poor birth outcomes compared to controls revealed differences in metabolomic profiles of lipid metabolites. In addition, lower levels of lysophospholipids, phospholipids, and monocytyglycerols during early pregnancy have been associated with macrosomia [30]. Epigenetics could be one mechanism whereby in utero exposure to maternal metabolites could impact health in early life. Future research should further explore the potential role of maternal metabolomic profiles on offspring DNA methylation and its role in child health.

### Methods

#### Study Population

Study participants included women and children from the CHAMACOS study, a longitudinal birth cohort originally aimed at examining the relationship of pesticide and environmental chemical exposure with the health and development of Mexican-American children from Salinas Valley, CA. A more in depth discussion of the study population has been characterized previously in Eskenazi et al. [19]. From 1999 to 2000, a total of 601 pregnant women were enrolled, resulting in the delivery of 527 liveborn singletons. Two pregnancy interviews were conducted at an average of 13.4 and 26.0 weeks gestation, with an additional visit after delivery. This study includes the 81 mother–child pairs with both metabolomic data from blood collected at the second pregnancy visit and with newborn Illumina HumanMethylation 450K BeadChip data. The University of California, Berkeley Committee for Protection of Human Subjects approved all study protocols and written informed consent was obtained from the CHAMACOS mothers.

#### Pregnancy Metabolite Measurements

Biological samples collected from 115 CHAMACOS women around 26 weeks gestation (mean = 26.4, SD = 3.2) were analysed to characterize maternal metabolomics profiles during pregnancy. Detailed descriptions of the metabolomics analyses in the CHAMACOS cohort have been previously described in Zhou et al. [18]. Briefly, blood from the women was analysed in duplicates by selected reaction monitoring (SRM) liquid chromatography and triple quadrupole mass spectrometry, a method previously validated by the Nomura research group [31]. SRM was used to identify metabolites, which were measured using the area under the curve. C12 MAGE, Pentadecanoic acid, and D3N15 Serine were utilized in the normalization of plasma non-polar positive, plasma nonpolar negative, and plasma polar metabolites, respectively. Laboratory and field blanks, as well as internal standards, were included to ensure quality, and repeat samples had good reproducibility (coefficients of variation ≤3–15%). For this study, we focused on the plasma metabolites involved in lipid pathways since lipids are involved in diverse biological activities including metabolic and structural functions, inflammation, signaling, and endocrine regulation [32].

#### Cord Blood Collection and Processing

At delivery, hospital staff collected cord blood in two types of vacutainers, one coated in heparin (green top) and the other without the anticoagulant (red top). Blood clots were aliquoted from the nonheparinized vacutainers and were stored at –80°C until use in DNA isolation.

#### DNA Preparation

DNA isolation of anticoagulant-free umbilical cord blood clot samples was performed using QIamp DNA Blood Maxi Kits (Qiagen, Valencia, CA) following the manufacturer’s protocol, with the exception of small modifications that were previously described in Holland et al. [33].

#### 450K BeadChip DNA Methylation Analysis

DNA samples of 1 μg were bisulfite converted using Zymo Bisulfite Conversion Kits (Zymo Research, Irvine, CA), whole
genome amplified, enzymatically fragmented, purified, and applied to Illumina Infinium HumanMethylation 450 BeadChips (Illumina, San Diego, CA) following the manufacturer’s instructions [34]. Samples were randomized across assay wells, chips, and plates to prevent batch bias. Robotics handled the 450K BeadChips, which were analysed using the Illumina Hi-Scan system. DNA methylation was assessed at 485 512 CpG sites.

Probe signal intensities were extracted by Illumina GenomeStudio software (version XXV2011.1, Methylation Module 1.9) methylation module and background subtracted. A variety of quality assurance and control measures were implemented and have been previously described in Yousefi et al. [35], such as determination of assay repeatability and batch effects. The All Sample Mean Normalization algorithm [35], adjusted for color channel bias, batch effects and differences in Infinium chemistry. In addition, Beta Mixture Quantile normalization was used to account for differences between the two Illumina probes [36]. Samples were retained if 95% of sites assayed had detection \( P \)-values greater than 0.01. Criteria for removal included: (i) sites with annotated probe SNPs \( n = 65 \) and with common SNPs (minor allele frequency >5%) within 50 bp of the target identified in the MXL (Mexican ancestry in Los Angeles, CA) HapMap population \( n = 49748 \); (ii) sites mapped to the \( X (n = 10708) \) and \( Y (n = 95) \) chromosomes [14]; (iii) cross-reactive probes identified by Chen et al. [57] \( n = 26950 \); and (iv) probes where 95% of samples had detection \( P \)-values greater than 0.01 \( n = 460 \). A total of 398 483 CpG sites remained for analysis. Methylation beta values across sites were log-transformed to the M-value scale to more accurately adhere to modeling assumptions [38]. Methylation observations greater or less than three times the interquartile range for a given CpG site were removed prior to regression analyses to reduce the influence of methylation outliers.

**Statistical Analysis**

To examine the distributions of individual metabolites in maternal blood during pregnancy, we computed descriptive statistics. We used Student’s \( t \)-tests to compare methylation profiles between CHAMACOS newborns with methylation data that were included \( N = 81 \) and excluded \( N = 288 \) in the current analyses, with results indicating no significant differences in methylation between the two groups. There was no difference between the subset of the participants included in this study and the rest of the CHAMACOS cohort.

To determine the relationship between maternal pregnancy metabolomics profiles as the exposure and 450K DNA methylation of their newborn children as the outcome, we fit limma linear models with empirical Bayes variance shrinkage [39]. To reduce the influence of metabolite outliers, all 92 maternal lipid metabolites were \( \log(1+x) \) transformed. We adjusted for sex and batch in each of the models. Since DNA methylation has been observed to vary by cell type [40], we also accounted for white blood cell type proportions in statistical models. To estimate cord blood proportions of seven white blood cell types, including nucleated red blood cells, we utilized a cord blood reference dataset from Johns Hopkins [41]. We adjusted \( P \)-values for multiple hypotheses testing using the Benjamini–Hochberg FDR threshold for significance of 0.05 [42]. We also identified CpGs that were significant based on the more conservative Bonferroni correction (uncorrected \( P \)-value <1.36 \( \times 10^{-6} \) to account for 36 660 436 tests). This analysis was performed using R statistical computing software (v3.5.1) [43].

**Gene Ontology Analysis**

We used the online tool PANTHER (protein annotation through evolutionary relationship) [44] to classify the function and relevant pathways of genes with FDR significant CpG sites related to lipid metabolites. Briefly, the list of genes was entered into the ‘Gene List Analysis’ tool in PANTHER and the functional classification for each gene was generated. Gene ontology related to molecular function, biological process, cellular component, protein class, and pathway was available for most of the genes identified in the regression results.

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