

# DHA Supplementation Attenuates Inflammation-Associated Gene Expression in the Mammary Gland of Lactating Mothers Who Deliver Preterm

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## ABSTRACT

**Background:** In a randomized trial of DHA supplementation to lactating mothers who delivered preterm, there were significant increases in DHA status in the mother and her infant.

**Objectives:** Our objective here was to characterize the mammary gland transcriptomes from the above study. We hypothesized that proinflammatory gene expression would be attenuated in the increased DHA group compared with the standard DHA group.

**Methods:** In the original trial, mothers delivering at <29 wk gestation at the University of Cincinnati Medical Center and intending to express their milk were randomly assigned to supplementation with 200 mg/d DHA (standard group: STD) or 1000 mg/d DHA (experimental group: EXP) within 7 d of delivery. Here, we conducted RNA-seq transcriptome analysis of  $n = 5$  EXP and  $n = 4$  STD extracellular mammary mRNA samples extracted from the fat layer of milk samples obtained 4 wk postenrollment. Transcripts were assessed for differential expression (false discovery rate adjusted  $P$  value <0.05) and clustering between EXP compared with STD groups. Ontological analysis of all differentially expressed genes (DEGs) was performed with Topocluster.

**Results:** There were 409 DEGs. We observed 5 main groups of biological processes that were upregulated, including those associated with improved immune regulation and management of oxidative stress; and 3 main groups of biological processes that were downregulated, including 1 associated with immune dysregulation. For example, we observed upregulation of inflammation-inhibiting genes including NF $\kappa$ B inhibitor alpha (*NFKBIA*; fold-change (FC), adjusted  $P$  value: FC = 1.70,  $P = 0.007$ ) and interleukin-18 binding protein (*IL18BP*; FC = 2.2, adjusted  $P = 0.02$ ); and downregulation of proinflammatory genes including interleukin 7 receptor (*IL7R*; FC = -1.9, adjusted  $P = 0.02$ ) and interleukin 1 receptor like 1 (*IL1RL1*; FC = -13.0, adjusted  $P = 0.02$ ).

**Conclusions:** Increased DHA supplementation during lactation can modulate the expression of inflammation-related genes within the mammary gland. This might translate to milk composition with a more optimal inflammasome profile. Future research with a larger clinical trial and greater interrogation of clinical outcomes is warranted. *J Nutr* 2022;152:1404–1414.

**Keywords:** docosahexaenoic acid, cytokine, RNA sequencing, lactation physiology, human milk, transcriptome, omega-3 fatty acids, inflammation, premature birth

## Introduction

According to the CDC, 1 in 10 births occurred prematurely in the United States in 2019 (1). Compared with term infants, preterm infants rely heavily on the nonspecific, innate immune system and consequently have a significantly higher risk of

developing infection (2). Regulatory T-cells are often less functional in preterm infants compared with full-term infants, and it is hypothesized that this sustained inflammatory state increases the infant's susceptibility to numerous inflammatory morbidities during infancy and later in life (3).

Human milk is known to contain anti-inflammatory components (4) that can reduce the risk of mortality and inflammation-associated morbidity in the preterm infant (5). One component of human milk known to possess anti-inflammatory and antioxidant properties is the long-chain omega-3 PUFA, DHA (6). DHA is an important component in the lipid raft of the phospholipid membrane, where it modulates cytokine expression and downstream signaling that influences PG production and associated metabolites (7, 8). More specifically, DHA metabolites can serve as ligands for receptors that inhibit the transcription of proinflammatory nuclear factor kappa B (*NFKB*) and activate anti-inflammatory transcription factors such as peroxisome proliferator-activated receptor  $\gamma$  (9). In preterm infants specifically, enteral DHA supplementation decreases the risk of severe retinopathy (10), and whole blood DHA concentrations are predictive of chronic lung disease and late-onset sepsis (11).

Intake of DHA from the diet or as a supplement is essential during pregnancy and lactation due to its limited ability to be adequately synthesized in the body from  $\alpha$ -linoleic acid (12). It is also important to ensure adequate DHA concentrations in the appropriate ratio with arachidonic acid (ARA) (13). In the United States, in human milk of donating mothers (14) and mothers who delivered preterm (15), the ARA:DHA ratio exceeds the recommended range of 1:1 to 2:1 (13). For this reason, it is recommended that lactating mothers who deliver preterm are prescribed supplemental DHA (16). The Institute of Medicine (17) does not set a DRI for DHA in pregnancy or lactation, but the range of intake suggested by experts varies from 200 mg/d to 1000 mg/d during pregnancy and lactation (18, 19). Maternal supplementation at the higher end of the recommendation has been shown to increase DHA concentrations in human milk, spurring further questions regarding its ability to decrease inflammation in the preterm infant (15).

In a randomized trial involving DHA supplementation in mothers who delivered preterm, investigators from our research group found that DHA supplementation strategies using 1000 mg/d compared with 200 mg/d can increase DHA concentrations in maternal milk and infant plasma (15). As part of the same randomized trial, our research group obtained extracellular mammary epithelial cell mRNA (lactocyte mRNA) of enrolled mothers. By interrogating the intersection of differentially expressed genes with known inflammation and

cytokine signaling pathways, it might be possible to understand the influence of DHA on cytokine gene expression in the lactocyte. This will help us determine whether there is a broader effect of maternal DHA supplementation on mammary gland health and breast milk quality, aside from increasing human milk DHA concentrations. We hypothesized that inflammation-associated gene expression in the lactocytes of mothers who delivered preterm would be significantly attenuated in the group randomly assigned to an increased DHA supplement (1000 mg/d) compared with the group randomly assigned to receive the standard DHA supplement amount (200 mg/d).

## Methods

### Study design and participants

We conducted transcriptome analysis of extracellular mammary mRNA that was extracted from human milk as part of a randomized controlled trial that took place between July 2013 and August 2014 (15). The original trial was conducted at the University of Cincinnati Medical Center (Cincinnati, OH), where mothers were recruited in the postpartum unit after delivery of an infant <29 completed gestational weeks of age with no congenital abnormalities that could interfere with study outcomes. All mothers recruited intended to provide their expressed milk to their infant. Mothers enrolled in the original trial were randomly assigned to begin supplementation with either 200 mg/d DHA [standard (STD) group] or 1000 mg/d DHA [experimental (EXP) group] within 7 d of delivery. As part of the original trial, mothers provided spot samples of expressed milk at 4 wk postenrollment, without controlling for the time of day or timing since the previous breast emptying. Milk DHA concentration was assayed as part of the original trial as previously described (15). Of the 18 lactating mothers who completed the original trial, 14 provided a milk sample at 4 wk postpartum at a time when a study investigator (MM) was available to process the freshly expressed sample for RNA stabilization and extraction. The details on deriving the final sample sizes used in bioinformatic analysis are described below and summarized in Figure 1. The study was approved by the University of Cincinnati Institutional Review Board.

### RNA isolation and extraction

Fresh human milk fat globules contain crescents of mammary epithelial cell cytoplasm, including mRNA, which is what allowed us to utilize freshly expressed milk samples for extracellular mammary RNA extraction, as described previously (20). Briefly, after centrifuging the fresh milk sample at 15,000 g  $\times$  10 minutes at room temperature, the milk fat layer was dispersed in TRIzol LS solution (Thermo Fisher Scientific) to stabilize the mRNA. The samples were either processed to extract mRNA immediately or stored at  $-80^{\circ}\text{C}$  until mRNA extraction using chloroform, and then isolated and purified using the PROMEGA Maxwell 16 integrated system (Promega Corp). Once isolated, the samples were screened for quality and quantity using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). Ten samples ( $n = 6$  EXP and  $n = 4$  STD) met initial quality control and were stored at  $-80^{\circ}\text{C}$  until sequencing. Just prior to sequencing, the 10 samples were sent to the Gene Expression Core at Cincinnati Children's Medical Center to confirm adequate RNA purity (rRNA 28S:18S), quality (RNA integrity number), and quantity using the Agilent 2100 Bioanalyzer. All 10 samples submitted scored in the range of 8.4 to 9.4 for RNA integrity, had an rRNA ratio (28S:18S) between 1.3 and 2.6, and RNA quantity  $>1 \mu\text{g}$ , and thus were suitable for next-generation RNA sequencing.

### RNA sequencing and processing

The 10 high-quality samples were sent to the Genomics, Epigenomics, and Sequencing Core at the University of Cincinnati for library preparation and sequencing. NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs) was used to isolate poly(A) mRNA from 1  $\mu\text{g}$  total RNA. Then, the SMARTer Apollo automated

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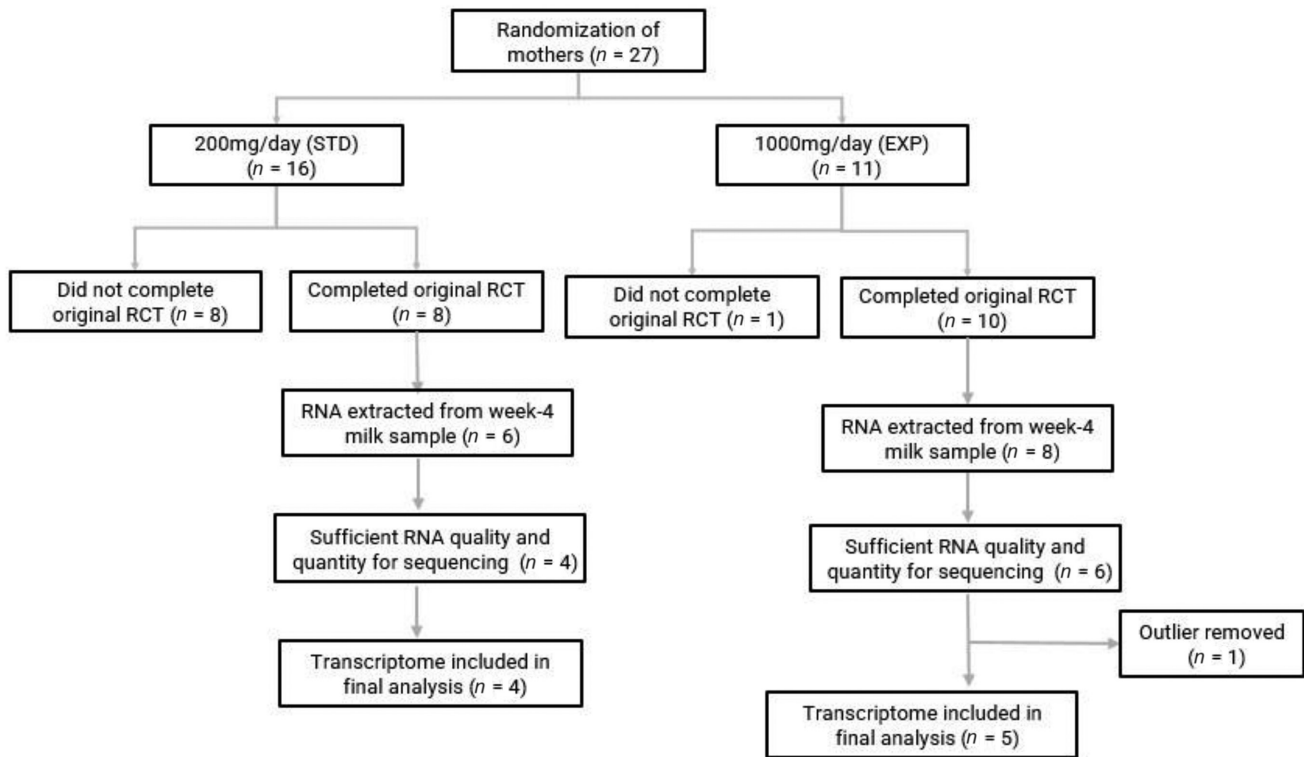
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All supplemental materials are available from the, "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/>.

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Abbreviations used: ARA, arachidonic acid; *CISH*, cytokine inducible SH2 containing protein; *CSN2*, beta casein; DEG, differentially expressed gene; EXP, experimental group; FC, fold-change; *IL1RL1*, interleukin 1 receptor like 1; *IL7R*, interleukin 7 receptor; *LALBA*, alpha lactalbumin; *LPO*, lactoperoxidase; NEC, necrotizing enterocolitis; *NFKBIA*, *NFKB* inhibitor alpha; *PDGFRA*, platelet derived growth factor receptor alpha; STD, standard group.



**FIGURE 1** Derivation of final transcriptome set from the randomized allocation of mothers to the experimental group (EXP: 1000 mg/d DHA) and standard group (STD: 200 mg/d DHA) that took place in the original randomized controlled trial (RCT) (15).

NGS library prep system (Takara Bio USA) was used to enrich the poly(A) RNA and the NEBNext Ultra II Directional RNA Library Prep kit (New England BioLabs) was used to prepare the library for sequencing through 8 cycles of PCR. Next, library quality control and quantification were performed using NEBNext Library Quant Kit (New England BioLabs) together with QuantStudio 5 Real-Time PCR Systems (Thermo Fisher). Individually indexed libraries were proportionally pooled and directional polyA RNA-seq was performed in August 2020, using the Illumina NextSeq 550 sequencer (Illumina, Inc) with the sequence setting of single read  $1 \times 85$  bp to generate  $\sim 30$  million reads per sample using standard Illumina protocols. Fastq files for downstream data analysis were automatically generated via Illumina BaseSpace Sequence Hub v2.02 (Illumina, Inc). Reads were mapped to the reference human genome GRCh38.p13 [GRCh38.p13-Genome-Assembly-NCBI (nih.gov)] using STAR (Illumina, Inc) under the first strand setting. The aligned reads were quantified and converted to relative gene expression level represented by transcripts per million using Salmon (Illumina, Inc).

### Bioinformatic analysis

The alignment result was seamlessly used as input for further analyses using the DESeq2 Bioconductor package (21). In this package, counts are modeled following a negative binomial distribution and fit by a generalized linear model for each gene. To test for differential expression in the EXP group compared with the STD group, Wald tests were applied to the fitting coefficients, and the Benjamini-Hochberg false discovery adjustment for multiple testing was applied to the  $P$  values. Significant differentially expressed genes (DEGs) were defined as those with a false discovery rate adjusted  $P$  value  $< 0.05$ .

Principal component analysis using all transcripts was performed to confirm separation by randomization group. One EXP sample was an outlier (Supplemental Figure 1A), likely a technical outlier attributable to the myriad steps involved in generating the RNA-seq transcriptome (22). It does not appear that the outlier is due to lack of compliance with the DHA supplementation protocol because milk DHA response in this outlier was centered within the range observed for the other EXP

samples (see footnote to Table 1); alternatively, outlier status could be due to the sample being from the mother with the earliest gestational age (24<sup>5/7</sup> wk), or possibly having a nonresponder phenotype as described for triacylglycerol response to DHA (23). To avoid having 1 outlier unduly distort the results in this small exploratory analysis (22), the outlier was removed and a second principal component analysis confirmed separation of samples according to randomization groups (Supplemental Figure 1B). Thus, high-quality transcripts from  $n = 5$  EXP samples and  $n = 4$  STD samples were included in further analysis of DEGs.

Ontological analysis of DEGs was performed with Topcluster (24). Genes belonging to the dominant biological processes altered by DHA supplementation (according to enrichment  $P$  value) were interrogated in the NCBI gene database (25) and GeneCards human gene database (<https://www.genecards.org/>), with a focus on inflammation. In addition, PubMed was queried for genes with previous reports in the literature pertaining to mammary gland function, inflammation, and/or neonatal morbidity. Sample and gene clusters were identified in heat maps through hierarchical clustering of normalized expression values using the average linkage rule and Pearson centered similarity measure.

## Results

### Clinical characteristics

Characteristics of study participants included in the transcriptome analysis and stratified by randomization group are shown in Table 1 and include race, mode of delivery, BMI at 4 wk postenrollment, gestational age of the infant, and milk DHA concentration at baseline and 4 wk postenrollment. There were no meaningful or statistically significant differences in clinical characteristics between the 2 groups, apart from milk DHA concentration, which increased 3-fold in the EXP group ( $P = 0.008$ ) and displayed a nonsignificant decline in

**TABLE 1** Characteristics of study participants included in transcriptome analysis<sup>1</sup>

	EXP group (1000 mg/d DHA)	STD group (200 mg/d DHA)
No. of samples	5	4
Race, <i>n</i>		
White, non-Hispanic	3	2
Black or African American	2	2
Mode of delivery, <i>n</i>		
Cesarean	4	3
Vaginal	1	1
BMI at 4 wk postpartum, median (min/max), kg/m <sup>2</sup>	27.5 (24.2/27.8)	26.4 (21.1/31.6) <sup>2</sup>
Gestational age of infant, median (min/max), wk <sup>d/7</sup>	27 <sup>2/7</sup> (25 <sup>0/7</sup> /28 <sup>6/7</sup> )	26 <sup>1/7</sup> (25 <sup>5/7</sup> /27 <sup>5/7</sup> )
Human milk DHA <sup>3</sup>		
Mean ± SD; median (min/max), baseline, mg/100 mL	8.2 ± 5.7; 6.2 (4.7/18.4)	10.1 ± 5.6; 8.1 (6.0/18.4)
Mean ± SD; median (min/max), week 4, mg/100 mL	26.9 ± 13.3; 22.7 (13.9/48.8) <sup>4</sup>	6.1 ± 2.1; 5.6 (4.1/9.2) <sup>3</sup>
Mean ± SD; median (min/max), week 4, % of baseline	363 ± 143%; 367% (224/593%)	73 ± 40%; 72% (30/117%)

<sup>1</sup>EXP, experimental; STD, standard.

<sup>2</sup>Unavailable for 2 mothers.

<sup>3</sup>For the technical outlier excluded from EXP group, human milk DHA values at baseline, 4 wk postenrollment, and week 4 as percentage of baseline, were 9.1 mg/100 mL, 27.8 mg/100 mL, and 305%, respectively; gestational week of birth was 24<sup>5/7</sup>.

<sup>4</sup>Paired *t*-test comparison of mean DHA at baseline compared with 4 wk postenrollment, EXP group, *P* = 0.008; STD group, *P* = 0.29; independent *t*-test comparison of EXP compared with STD at 4 wk postenrollment, *P* = 0.04.

the STD group (*P* = 0.29), between enrollment and 4 wk postenrollment.

### Overall gene expression and DEG results

The complete transcriptome for each sample and accompanying metadata have been uploaded to NCBI GEO database, at GSE181990. A total of 409 genes were found to be differentially expressed between the EXP and STD groups (false discovery rate adjusted *P* value <0.05) and this gene list is accessed in Supplemental File 1.

### Alignment of gene expression rank with mature lactation

Gene expression rank in each DHA group was compared with the top 20 genes with the highest expression in mature lactation in mothers who delivered at term (20). These results are shown in Table 2, and where there is significant differential expression, the log<sub>2</sub> fold-change (FC) ± SE, FC, and adjusted *P* value are also shown. Consistent with mature lactation in full-term deliveries, beta casein (*CSN2*) was the most highly expressed gene in both groups; however, it was significantly upregulated in the EXP group (FC = 2.1; adjusted *P* = 0.03). Alpha lactalbumin (*LALBA*), the second most highly expressed gene in mature-term lactation, was also upregulated in the EXP group (FC = 1.8; adjusted *P* = 0.01). Lysozyme (*LYZ*) (FC = -3.5; adjusted *P* = 0.0004) was downregulated in the EXP group along with eukaryotic translation elongation factor1 alpha 1 (*EEF1A1*) (FC = -1.5; adjusted *P* = 0.006). Generally, all the top 20 genes expressed in mature lactation were among the top 20 in preterm mothers except for thymosin beta 10 (*TMSB10*), actin beta (*ACTB*), and chordin-like 2 (*CHRD2*). However, for the 4 genes with significant differential expression there was considerable heterogeneity in gene expression within each DHA group, as evidenced by first-level hierarchical clustering not aligning with group allocation, as shown in Supplemental Figure 2E.

### Most differentially expressed genes

Supplemental Table 1 summarizes the top 20 significantly upregulated genes in the EXP group compared with the STD

group, including gene description, log<sub>2</sub> FC ± SE, FC, and adjusted *P* value. FC among the top 20 upregulated genes ranges from 110 to 7.2 and includes genes involved in positive regulation of cell growth and development (*TSHR*, *PITX1*, *VIPR2*, *FSTL1*), management of extracellular matrix deposition (*ABI3BP* and *MMP11*), support of cell–cell matrix interactions and signal transduction (*ADAM19* and *PCDHA10*), immune system development (*TMEM176B*), regulation of metabolic processes (*FAM69C*, *FZD10*, *ST8SIA4*), and regulation of transcription (*H3F3A*, *BEND4*).

Supplemental Table 2 summarizes the top 20 significantly downregulated genes in the EXP group compared with the STD group, along with their gene description, log<sub>2</sub> FC ± SE, FC, and adjusted *P* value. FC among the top 20 downregulated genes ranges from -261 to -4.0 and includes genes involved in posttranscriptional modification (*SNORA105A*), genome stability (*SLX1B-SULT1A4*), cell–cell or cell–matrix connections (*CDH12*, *LRRC4*, *LAMC2*), epithelial cell development (*MGP*), cell metabolism and intracellular transport (*ATP13A5*, *SULT1C4*, *KIF6*), proapoptotic and catabolic processes (*PMAIP1*, *SPATA18*), and proinflammatory processes (*IL1RL1*, *SERPINB2*, *NPR2*, *MUC5B*, *MILR1*, *RSAD2*). Supplemental File 4 provides literature cited in Supplemental Tables 1 and 2.

### Ontological analysis

Ontological analysis of DEGs revealed upregulation of 5 biological process themes in the EXP group, which we have characterized as: 1) improved protein handling and glycosylation, 2) improved oxidative stress response, 3) normalized immune response, 4) improved bioenergetics, and 5) improved lactocyte support. Analysis also revealed downregulation of 2 primary themes in the EXP group, which we have characterized as: 1) immune dysregulation, and 2) protein mishandling (Figure 2). In Table 3, we summarize functions of key differentially expressed genes within the 4 biological process themes most relevant to inflammation: improved oxidative stress response, normalized immune response, improved bioenergetics, and downregulation of immune dysregulation. Table 3 also includes the log<sub>2</sub> FC ± SE, FC, and adjusted *P* value for DEGs in each



**TABLE 2** Ranking of expression in EXP (1000 mg/d) and STD (200 mg/d) DHA groups compared with rank of the top 20 expressed genes in mature, term lactation (20)<sup>1</sup>

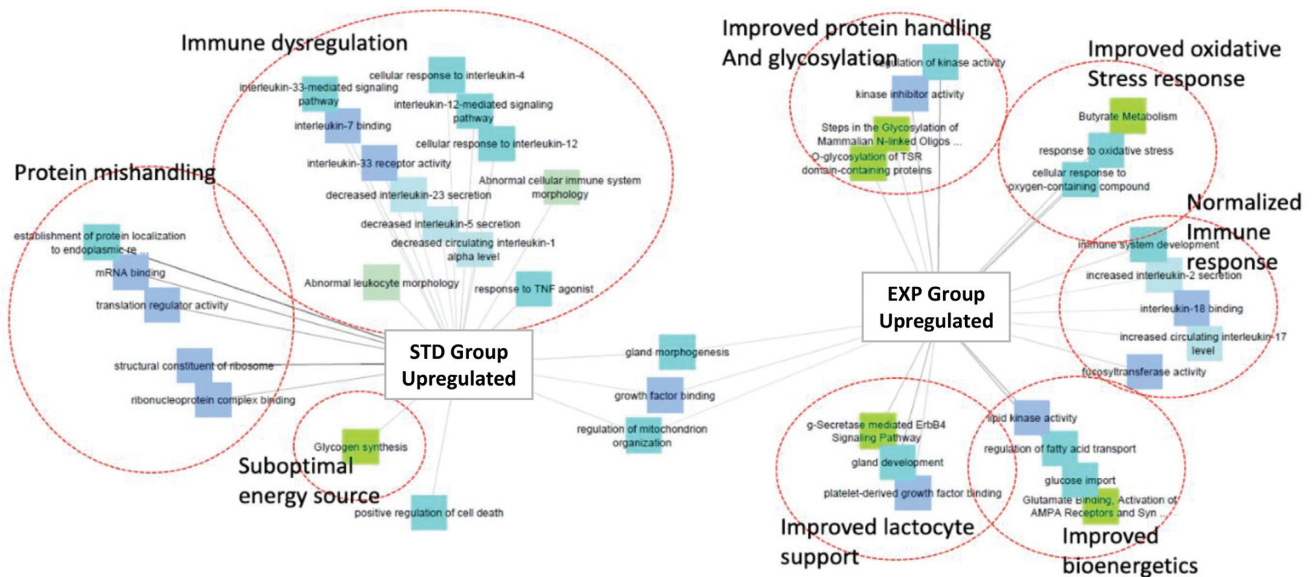
Rank in mature lactation	Gene symbol (25)	Gene name (25)	EXP rank (n = 5)	STD rank (n = 4)	Log2 FC ± SE (FC) <sup>2</sup>	Adjusted P value <sup>2</sup>
1	<i>CSN2</i>	Casein, beta		1	1.06 ± 0.31 (2.1)	0.03
2	<i>LALBA</i>	Lactalbumin, alpha	2	2	0.85 ± 0.23 (1.8)	0.01
3	<i>CSN1S1</i>	Casein, alpha s1	3	4		
4	<i>CSN3</i>	Casein, kappa	6	10		
5	<i>LTF</i>	Lactotransferrin	4	3		
6	<i>FTH1</i>	Ferritin, heavy polypeptide 1	5	5		
7	<i>CSN1S2AP</i>	Casein, alpha s2-like A	9	12		
8	<i>LYZ</i>	Lysozyme	15	7	-1.79 ± 0.36 (-3.5)	0.0004
9	<i>SPP1</i>	Secreted phosphoprotein 1	8	11		
10	<i>TMSB10</i>	Thymosin, beta 10	37	46		
11	<i>FASN</i>	Fatty acid synthase	10	8		
12	<i>TPT1</i>	Tumor protein, translationally-controlled 1	13	13		
13	<i>CEL</i>	Carboxyl ester lipase (bile salt stimulated lipase)	12	16		
14	<i>FABP3</i>	Fatty acid binding protein	20	21		
15	<i>XDH</i>	Xanthine dehydrogenase	7	9		
16	<i>ACTB</i>	Actin, beta	34	41		
17	<i>CD24</i>	CD24 molecule	14	14		
18	<i>EEF1A1</i>	Eukaryotic translation elongation factor 1, alpha 1	11	6	-0.60 ± 0.15 (-1.5)	0.006
19	<i>PIGR</i>	Polymeric immunoglobulin receptor	16	15		
20	<i>CHRD2</i>	Chordin-like 2	27	49		

<sup>1</sup>EXP, experimental group; FC, fold-change; STD, standard group.

<sup>2</sup>If adjusted P value <0.05, then log2 FC ± SE (FC) and adjusted P value are shown; positive = upregulation in EXP, negative = downregulation in EXP; significant differential expression results are based on Wald statistics with Benjamini–Hochberg false discovery adjustment to the P values.

theme. In Supplemental Figure 2A–D, we present heat maps to contrast gene expression across individual samples according to key genes from the 4 biological process themes listed above. For all 4 themes, the heat maps confirm primary-level clustering

of gene expression levels by DHA group. The complete list of significantly upregulated and downregulated gene ontologies are provided in Supplemental Files 2 and 3, respectively, with ontology category, ontology name, p-value, hit count in



**FIGURE 2** Ontological themes that are upregulated in the experimental group (EXP: 1000 mg/d DHA, n = 5) or standard group (STD: 200 mg/d DHA). Weight of line denotes strength of significance. Color indicates ontology category as follows: lime green = pathway, teal = biological process, light teal = mouse phenotype, sage green = human phenotype, lavender = molecular function. Group labels represent our description of overall ontology theme. Ontological analysis of differentially expressed genes was performed in Topocluster, which draws from >30 ontological repositories, and the figure was generated in Cytoscape.

**TABLE 3** Key differentially expressed genes within select significantly enriched ontologies<sup>1</sup>

Ontology label	Gene symbol	Gene name	Log2 FC ± SE (FC), adj. <i>P</i> value <sup>2</sup>	Function
<i>Improved oxidative stress response theme</i> Butyrate metabolism	<i>ACSM1</i>	Acyl-CoA synthetase medium chain family member 1	1.85 ± 0.54 (3.6), <i>P</i> = 0.03	Catalyzes the activation of fatty acids by CoA to produce an acyl-CoA, the first step in fatty acid metabolism; involved in the activation of lipotic acid, an essential cofactor for mitochondrial metabolism (41)
Butyrate metabolism	<i>HMGCL</i>	3-Hydroxy-3-methylglutaryl-CoA lyase	0.46 ± 0.14 (1.4), <i>P</i> = 0.03	Catalyzes a key step in ketogenesis. Terminal step in leucine catabolism. Ketone bodies are essential as an alternative source of energy to glucose, as lipid precursors, and as regulators of metabolism
Response to oxidative stress	<i>SESN1</i>	Sestrm1	0.90 ± 0.25 (1.9), <i>P</i> = 0.02	The encoded protein mediates p53 inhibition of cell growth by activating AMP-activated protein kinase, which results in the inhibition of the mammalian target of rapamycin protein; plays a critical role in antioxidant defense
Response to oxidative stress	<i>DPEP1</i>	Dipeptidase 1	2.51 ± 0.61 (5.7), <i>P</i> = 0.006	Known to regulate leukotriene activity by catalyzing the conversion of leukotriene D4 to leukotriene E4 (42)
Response to oxidative stress	<i>LPO</i>	Lactoperoxidase	1.92 ± 0.57 (3.8), <i>P</i> = 0.03	Following its secretion from salivary, mammary, and other mucosal glands, this enzyme catalyzes the generation of the antimicrobial substance hypothiocyanous acid. A milk protein with antimicrobial function; known to play host defensive roles through antimicrobial activity (43); it is responsible for most of the peroxidase activity in mature milk
Response to oxidative stress	<i>ACACA</i>	Acetyl-CoA carboxylase alpha	0.77 ± 0.18 (1.7), <i>P</i> = 0.004	Acetyl-CoA carboxylase is a biotin-containing enzyme that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid synthesis; altered expression might influence the fatty acid composition of milk
Response to oxidative stress	<i>CISH</i>	Cytokine inducible SH2 containing protein	0.87 ± 0.26 (1.8), <i>P</i> = 0.03	C/IS family members are known to be cytokine-inducible negative regulators of cytokine signaling. C/IS is involved in the negative regulation of cytokines that signal through the janus kinase-signal transducer and activator of transcription proteins pathway such as erythropoietin, prolactin, and IL-3 receptor
Response to oxidative stress	<i>COLEC12</i>	Collectin subfamily member 12	1.48 ± 0.37 (2.8), <i>P</i> = 0.007	The protein is a scavenger receptor that displays several functions associated with host defense. It can bind to carbohydrate antigens on microorganisms, facilitating their recognition and removal
Response to oxidative stress	<i>NFKBIA</i>	NFKB inhibitor alpha	0.76 ± 0.19 (1.7), <i>P</i> = 0.007	The encoded protein interacts with REL dimers to inhibit NF-κB/REL complexes, which are involved in inflammatory responses
<i>Immune dysregulation theme</i> IL12-mediated signaling	<i>SERPINB2</i>	Plasminogen activator inhibitor 2	-3.11 ± 0.82 (-8.6), <i>P</i> = 0.01	This gene is one of the top 20 downregulated genes in the EXP group compared with the STD group (Supplemental Table 2). It is induced during many inflammatory processes and infections. Dysregulated serpin B2 expression or serpin B2 polymorphisms have been associated with a number of diseases involving inflammation (44)

(Continued)

**TABLE 3** (Continued)

Ontology label	Gene symbol	Gene name	Log2 FC ± SE (FC), adj. <i>P</i> value <sup>2</sup>	Function
Abnormal cellular immune system morphology	<i>IL7R</i>	IL7 receptor	-0.91 ± 0.25 (-1.9), <i>P</i> = 0.02	The protein encoded by this gene is a receptor for IL7. Study shows that suppression of the IL7 receptor can suppress chronic inflammation by controlling antigen-specific memory T-cells (33)
Abnormal cellular immune system morphology	<i>MUC5B</i>	Mucin 5B	-2.62 ± 0.80 (-6.1), <i>P</i> = 0.04	This gene is one of the top 20 downregulated genes in the EXP group compared with the STD group (Supplemental Table 2). It encodes a member of the mucin family of proteins, which are highly glycosylated macromolecular components of mucus secretions. This gene is involved in macrophage activation processes
IL33-mediated signaling pathway	<i>IL1RL1</i>	Interleukin 1 receptor like 1	-3.70 ± 0.96 (-13.0), <i>P</i> = 0.01	This gene is one of the top 20 downregulated genes in the EXP group compared with the STD group (Supplemental Table 2). IL33 binding to IL1RL1 has been associated with a variety of disease states and inflammatory processes (34). Studies of the similar gene in mice suggest this receptor can be induced by proinflammatory stimuli and could be involved in the function of helper T-cells
Response to TNF agonist	<i>RPS3</i>	Ribosomal protein S3	-0.66 ± 0.15 (-1.6), <i>P</i> = 0.003	Plays a role in regulating transcription as part of the NF-κB/p65-p50 complex, where it binds to the RELA/p65 subunit, enhances binding of the complex to DNA, and promotes transcription of target genes; A growing body of evidence suggests that Rps3 is involved in the regulation of NF-κB activity (45)
Improved bioenergetics theme Lipid kinase activity	<i>PDGFRA</i>	Platelet derived growth factor receptor alpha	1.46 ± 0.36 (2.7), <i>P</i> = 0.007	Tyrosine kinase receptor. Required for normal development of the mucosa lining the gastrointestinal tract, and for recruitment of mesenchymal cells and normal development of intestinal villi
Regulation of fatty acid transport	<i>H2RH2</i>	Histamine receptor H2	2.01 ± 0.42 (4.0), <i>P</i> = 0.0008	The H2 subclass of histamine receptors mediates gastric acid secretion; involved in the suppressive activities of histamine; it is a potent stimulant of cAMP production, which leads to activation of protein kinase A
Regulation of fatty acid transport	<i>THBS1</i>	Thrombospondin-1	1.89 ± 0.37 (3.7), <i>P</i> = 0.0002	This protein is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions; has been shown also to have functions in the regulation of cell proliferation, migration, and apoptosis in a variety of physiological and pathological events, such as wound healing, inflammation, and inhibition of angiogenesis (46)
Glucose import	<i>ERBB4</i>	Erb-B2 receptor tyrosine kinase 4	0.81 ± 0.25 (1.8), <i>P</i> = 0.049	The protein binds to and is activated by neuregulins and other factors and induces a variety of cellular responses including mitogenesis and differentiation; required for mammary gland differentiation, induction of milk proteins, and lactation; ERBB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation
Glucose import	<i>KLF15</i>	Krüppel-like factor 15	0.80 ± 0.23 (1.7), <i>P</i> = 0.03	KLF15 plays an important role in regulation of the expression of genes for gluconeogenic and amino acid-degrading enzymes. KLF15 plays an essential role in adipogenesis through its regulation of PPAR-γ expression (47)

(Continued)

**TABLE 3** (Continued)

Ontology label	Gene symbol	Gene name	Log2 FC ± SE (FC), adj. <i>P</i> value <sup>2</sup>	Function
Glucose import	<i>PRKCA</i>	Protein Kinase C alpha	1.59 ± 0.20 (3.0), <i>P</i> = 0.0000	Is involved in positive and negative regulation of cell proliferation, apoptosis, differentiation, migration and adhesion, and inflammation. Can regulate selective LPS-induced macrophage functions involved in host defense and inflammation. But in some inflammatory responses, can negatively regulate NF-κB-induced genes, through IL1A-dependent induction of NF-κB inhibitor alpha (NFKBIA/IKBA)
<i>Normalized immune response theme</i>				
T-helper 1 type immune response	<i>IL18BP</i>	Interleukin-18 binding protein	1.13 ± 0.32 (2.2), <i>P</i> = 0.02	Functions as an inhibitor of the proinflammatory cytokine IL18
Immune system development	<i>PDGFRA</i>	Platelet derived growth factor receptor alpha	1.46 ± 0.36 (2.7), <i>P</i> = 0.007	Studies suggest that this gene plays a role in organ development and wound healing
Immune system development	<i>HOBX7</i>	Homeobox B7	2.29 ± 0.60 (4.9), <i>P</i> = 0.01	Functions as a sequence-specific transcription factor that is involved in cell proliferation and differentiation
Immune system development	<i>SAMD9L</i>	Sterile alpha motif domain containing 9 like	0.72 ± 0.22 (1.6), <i>P</i> = 0.04	Encodes a cytoplasmic protein that acts as a tumor suppressor but also plays a key role in cell proliferation and the innate immune response to viral infection
Immune system development	<i>CD101</i>	CD101 molecule	1.60 ± 0.35 (3.0), <i>P</i> = 0.001	Plays a role as inhibitor of T-cell proliferation induced by CD3. Inhibits expression of IL2RA on activated T-cells and secretion of IL2. Inhibits tyrosine kinases that are required for IL2 production and cellular proliferation
Immune system development	<i>CR2</i>	Complement C3d receptor 2	1.31 ± 0.40 (2.5), <i>P</i> = 0.04	Might be involved in the breakdown of tolerance and excessive antibody production by autoreactive B-cell clones (48). The complement system plays a pivotal role in the inflammatory response. CR2-targeted complement inhibition has been proved to be a potential therapeutic strategy for many diseases (48)
Immune system development	<i>BATF2</i>	Basic leucine zipper activating transcription factor-like transcription factor 2	0.68 ± 0.20 (1.6), <i>P</i> = 0.03	Activator protein 1 family transcription factor that controls the differentiation of lineage-specific cells in the immune system
Immune system development	<i>CSF1</i>	Colony stimulating factor 1	1.78 ± 0.49 (3.4), <i>P</i> = 0.02	The protein encoded by this gene is a cytokine that controls the production, differentiation, and function of macrophages. Plays an important role in innate immunity
<i>Major milk proteins</i>				
Major milk proteins	<i>LALBA</i>	Alpha lactalbumin	0.85 ± 0.23 (1.8), <i>P</i> = 0.01	This gene encodes α-lactalbumin, a principal protein of milk and essential enzyme in lactose synthesis
Major milk proteins	<i>CSN2</i>	Beta casein	1.06 ± 0.31 (2.1), <i>P</i> = 0.03	β-Casein is the principal protein in human milk and the primary source of essential amino acids for a suckling infant
Major milk proteins	<i>LYZ</i>	Lysozyme	-1.79 ± 0.36 (-3.5), <i>P</i> = 0.0004	Lysozyme is one of the antimicrobial agents found in human milk; it has antibacterial activity against a number of bacterial species
Major milk proteins	<i>EEF1A1</i>	Eukaryotic translation elongation factor 1, alpha 1	-0.60 ± 0.15 (-1.5), <i>P</i> = 0.006	Encodes an isoform of the α-subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome during protein biosynthesis

<sup>1</sup>Information not specifically cited is drawn from the National Center for Biotechnology Information (NCBI) gene database (25). EXP: experimental group; FC: fold-change; PPAR-γ: peroxisome proliferator-activated receptor γ; REL: v-rel avian reticuloblastosis viral oncogene homolog; STD: standard group.

<sup>2</sup>Differentially expressed gene results are based on the Wald statistic with Benjamin-Hochberg false discovery adjustment to the *P* values; EXP group, *n* = 5; STD group, *n* = 4. Shown is log2 FC ± SE (FC), adjusted *P* value; positive = upregulation in EXP; negative = downregulation in EXP.



query list, hit count in genome, and gene list in the query list.

## Discussion

Although there is evidence supporting DHA's ability to alter inflammatory pathways in human adipose tissue (8), this is the first report to our knowledge examining inflammation-associated gene expression in the mammary gland. Our findings show that increased DHA supplementation during lactation resulted in the differential expression of 409 genes, many of which are significantly enriched in key ontological functions associated with immune modulation.

In examination of the top 20 significantly upregulated genes, transcripts include inflammatory regulators such as *TMEM176B* (FC = 9.7, adjusted  $P = 0.02$ ) and *TSPAN18* (FC = 110, adjusted  $P = 0.0005$ ). *TMEM176B* is part of the significantly enriched ontology label “immune system development,” and is defined as a negative regulator of inflammasome activation (25). Research shows that *TMEM176B* inhibits proinflammatory actions of *NLRP3* (26, 27). *TSPAN18* was the most upregulated gene among the EXP group. This gene is a member of the tetraspanin superfamily and is involved in the regulation of inflammatory responses stimulated by wounds (thromboinflammation) (28). Upregulation of *TSPAN18* could prevent inflammatory signaling in endothelial cells that disrupts the endothelial barrier and allows extravasation of inflammatory cells into surrounding tissues (29). The remaining highly upregulated genes are predominantly involved in biological processes that support cell development and stability (Supplemental Table 1).

The 2 most highly downregulated genes in the EXP group were *SNORA105A* (FC = -261, adjusted  $P = 0.049$ ), which encodes a small nucleolar RNA of the H/ACA box class and is involved in posttranscriptional regulation of RNA, and *SLX1B-SULT1A4* (FC = -39, adjusted  $P = 0.0004$ ), which is involved in genome stability. More research is needed to elucidate these novel results. However, the list of the top 20 genes downregulated in the EXP group is dominated by downregulation of proinflammatory cell responses to stimuli, specifically helper T-cells, macrophages, and mast cells (25, 30, 31). Although all these cells are important to innate immunity, we could speculate that DHA prevents an exaggerated response and promotes a healthy immune response. Further, the proapoptotic gene *PMAIP1* is suppressed as well as *NPR2*, which is a gene responsible for regulating guanylyl cyclase activity and potentially development of hypotensive responses to inflammation (25). Collectively, the top differentially expressed genes listed in Supplemental Tables 1 and 2 support the role of DHA in modulating healthy immune response and promoting healthy cell development and stability.

A key gene involved in the “improved oxidative stress response” theme is NFKB inhibitor alpha (*NFKBIA*; FC = 1.7, adjusted  $P = 0.007$ ; Table 3), with uniform upregulation across all samples in the EXP group as shown in the heatmap for this theme (Supplemental Figure 2A). This gene encodes a protein that inhibits NF- $\kappa$ B, which is a transcription factor responsible for inducing the production of various cytokines and other amplifiers of the inflammatory response (32). Upregulation of *NFKBIA* could help prevent exaggerated NF- $\kappa$ B-induced activation and prevent an excessive proinflammatory cytokine response (32).

Within the “immune dysregulation” theme, interleukin 7 receptor (*IL7R*; FC = -1.9, adjusted  $P = 0.02$ ) and interleukin 1 receptor like 1 (*IL1RL1*; FC = -13.0,  $P = 0.01$ ) were significantly downregulated in the EXP group, with the latter among the top 20 most significant downregulated genes. *IL7R* is a receptor for the potent proinflammatory cytokine IL7, meaning that downregulation in *IL7R* would result in reduced activation of IL7. Recent studies conducted in nonhuman primate models show that suppression of *IL7R* can suppress chronic inflammation by controlling antigen-specific memory T-cells (33). *IL1RL1* binds IL33, which has been implicated in a variety of inflammation-associated disease states (34). Well-designed animal studies investigating *IL1RL1* have consistently displayed its upregulation in several inflammatory conditions leading to it becoming a target gene in therapeutic efforts related to inflammatory disorders (34).

Cytokine inducible SH2 containing protein (*CISH*) was significantly upregulated (FC = 1.8, adjusted  $P = 0.03$ ) and is included in the “improved response to oxidative stress” and “improved bioenergetics” themes. The product of this gene is involved in the suppression of cytokine signaling via a negative feedback system that regulates cytokine signal transduction. It negatively regulates IL3, a cytokine that has been shown to fuel cytokine storms and bolster inflammation, especially in the development of sepsis (35). Due to its ability to negatively regulate IL3, upregulation of *CISH* could provide an anti-inflammatory effect, and if biologically active in the infant, could have the potential to aid in the prevention of neonatal sepsis.

Lipids supply nearly 50% of the energy in human milk (36), and the fatty acid composition of these lipids is responsible for both the nutritional and physicochemical properties of the fat component of human milk (36). Among these fatty acids, increased concentration of the SCFA butyrate in human milk has been found to potentially reduce excessive inflammation and increase the functionality of the immature mucosal barrier in the digestive tract of premature infants (37). Within the improved oxidative stress theme, upregulation of genes involved in butyrate metabolism were seen in the EXP group. Specifically, acyl-CoA synthetase medium chain family member 1 (*ACSM1*) was upregulated in the EXP group (FC = 3.6, adjusted  $P = 0.03$ ). The product of this gene is responsible for catalyzing the production of acyl-CoA, via enzymes such as butyrate-CoA ligase, which is the first step of fatty acid metabolism. Another gene involved in butyrate metabolism, 3-hydroxy-3-methylglutaryl-CoA lyase (*HMGCL*), was also upregulated in the EXP group (FC = 1.4, adjusted  $P = 0.03$ ). Another enriched gene in the improved oxidative stress theme, acetyl-CoA carboxylase alpha (*ACACA*), was significantly upregulated in the EXP group (FC = 1.7,  $P = 0.004$ ). This gene is responsible for catalyzing the carboxylation of acetyl-CoA to malonyl-CoA, which is the rate-limiting step in fatty acid synthesis. Upregulation of genes involved in fatty acid synthesis and butyrate metabolism could result in an increase in the SCFA content of milk; however, further research is needed with a broader interrogation of milk sample composition to confirm this hypothesis. Overall, an increase in human milk metabolites, such as the SCFA butyrate, could have anti-inflammatory effects and stimulate an increase in the mucosal barrier of the infant.

Human milk contains numerous biologically active components that can aid in the prevention of infection within the immature gastrointestinal tract of premature infants (37). One of these components is lactoperoxidase, a product of the lactoperoxidase (*LPO*) gene, which plays a crucial role in

protecting the gastrointestinal tract of the infant from harmful pathogens (36). *LPO* was enriched in the improved oxidative stress cluster, because it was upregulated in the EXP group (FC = 3.8,  $P = 0.03$ ). Upregulation of *LPO* in milk could indicate increased protection against gastrointestinal infection in the infant.

Platelet derived growth factor receptor alpha (*PDGFRA*) was enriched in the improved bioenergetics cluster, because it was upregulated in the EXP group (FC = 2.7,  $P = 0.007$ ). The *PDGFRA* gene is crucial in the development of the mucosal lining of the intestines by driving the proliferation of mesenchymal cells (38). In a study conducted in rats, it was suggested that dysregulation of platelet-derived growth factor and its receptors could contribute to necrotizing enterocolitis (NEC) development during intestinal maturation. Therefore, upregulation of *PDGFRA* could potentially increase the integrity and development of the mucosal lining in the preterm infant, which could aid in protection against gastrointestinal infection and development of NEC.

*LALBA* and *CSN2* are the most highly expressed genes in the lactating mammary gland, making up ~45% of total mRNA in mature lactation; consequently, these are the most abundant proteins in human milk as well (20, 39). However, overall protein concentration in human milk is not sufficient for the preterm infant, meaning that fortification is needed to ensure optimal nutrition (40). In addition to being the top 2 genes expressed in both the STD and EXP groups, both *LALBA* and *CSN2* were significantly upregulated in the EXP group, which could translate to DHA improving protein concentration in human milk. However, these results should be interpreted with caution because upon inspection of the heat map for the primary milk protein genes (Supplemental Figure 2E), upregulation was driven primarily by 2 EXP participants, and the primary hierarchical cluster for the major milk protein heat map did not align with DHA group (in strong contrast to DHA group being the primary hierarchy for all of the heat maps generated for the key genes in each of the 4 biological process themes). Combined with the small sample sizes in this study, further research is warranted, including an in-depth examination of milk composition and volume, to confirm if DHA influences upregulation of these major milk protein genes and if this translates to higher concentrations of protein in milk.

The primary limitations of our analysis include a small sample size along with the exclusion of a sample that was likely a technical outlier, although the possibility of this sample representing a nonresponder phenotype, as described for triacylglycerol response to DHA (23), cannot be ruled out. All samples with adequate mRNA quality and quantity were included in sequencing (representing 50% of participants who completed the pilot trial). However, a larger study sample would have increased the power to detect differences in a larger number of genes, and enable detection of differential response by responder phenotype, if such a phenomenon exists in the mammary gland as seen for plasma triglyceride response in nonlactating individuals (23). Another limitation was that we were not able to corroborate the gene expression results with corresponding changes in serum biomarkers and in milk composition and volume between baseline and 4 wk postenrollment. However, as the first study to examine gene expression response to DHA supplementation, our transcriptome analysis illuminates bioactives to target in future DHA trials in lactating women.

In conclusion, our findings suggest that DHA supplementation of 1000 mg/d can modulate the expression of inflammation-associated genes within the mammary gland

and also genes involved in oxidative stress response and bioenergetics that could support improved breast milk quality and mammary gland function. The results of this study could have important clinical implications if further research shows that improving the inflammasome profile of human milk through increasing DHA supplementation translates to improved clinical outcomes. However, larger clinical trials are warranted to confirm if a 1000-mg/d DHA supplement during lactation will reduce the risk of inflammation-driven morbidity in premature infants of breastfeeding mothers, and to determine if this response is uniform across participants.

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## Data Availability

The complete transcriptome for each sample and accompanying metadata have been uploaded to NCBI GEO database, at GSE181990.

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