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Nutritional interventions' impacts on human milk: three trials in low-resource settings

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Human milk (HM) composition is variable to support changing infant needs and is influenced by maternal diet and health. We harmonized HM data from three randomized trials in Burkina Faso (MISAME-III), Pakistan (Mumta-LW), and Tanzania (ELICIT) within the International Milk Composition (IMiC) Consortium to test how maternal nutrition affects HM composition. Balanced energy-protein (BEP) supplements or nicotinamide given to lactating mothers increased milk B-vitamins, while macronutrients, oligosaccharides, bioactive proteins, and microbiome composition were physiologically buffered. Metabolomic and proteomic analyses revealed shifts in vitamin-related metabolites, triglyceride profiles, and intracellular protein representation. In a MISAME-III subset, maternal and infant blood showed concordant changes. Postnatal BEP improved infant growth only in the more undernourished cohort. Improved milk micronutrient quality across settings supports extending maternal supplementation into lactation.

Human milk (HM) is a complex biological system whose composition varies during feeds, over the course of lactation, between mothers, and across populations, reflecting both underlying biology and maternal environment (*1*). The postnatal period is a time of high nutrient demands, and adequate maternal nutrition is essential to support HM production and composition. HM is the sole source of nutrition for exclusively breastfed infants in the first months of life, and its composition influences infant growth, immunity, and development (*2*). Certain milk constituents (for example,

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some vitamins and fatty acids) are sensitive to maternal dietary intake (*3*), with some research finding that undernourished women produce milk with lower nutrient concentrations (*4*, *5*). In many low- and middle-income countries (LMICs), pregnant women struggle to meet the nutritional needs to sustain healthy fetal development (*6*), and prenatal malnutrition can affect HM composition (*7*). Maternal intake during pregnancy also builds fetal stores of folate, vitamin B_{12}, and iron that infants rely on in early life (*7*).

The World Health Organization (WHO) recommends exclusive breastfeeding for the first six months of life, and continued breastfeeding alongside complementary foods up to two years or beyond (*8*). In 2016, WHO antenatal care guidelines recommended universal balanced energy-protein (BEP) supplementation during pregnancy in populations with elevated rates of undernutrition, to reduce the risk of stillbirth and small-for-gestational-age (SGA) births (*9*). BEP provides macronutrients and multiple micronutrients; "moderate-certainty" evidence suggests it "probably" reduces the rate of stillbirth and SGA, though the evidence base drew from trials using diverse formulations, limiting firm conclusions on efficacy (*9*, *10*). WHO also cautioned that BEP supplementation is of "relatively high" cost compared to other antenatal interventions (*9*).

The Gates Foundation concurrently defined compositional guidelines for BEP and funded a multi-country program, including a consortium of seven intervention studies, to evaluate BEP effectiveness in pregnancy and lactation (*11*, *12*). Current WHO guidelines do not extend BEP recommendations into lactation, despite the high nutrient demands of breastfeeding and HM's dominant role in early-life nutrition (*9*, *13*), and there have been no systematic investigations into whether maternal BEP supplementation during lactation alters HM composition. Feeding the mother across pregnancy and lactation effectively "feeds the infant twice": first in utero, and again via her milk. Emerging evidence links specific HM constituents with infant growth: Higher lactose tracks with greater weight gain and higher protein with linear growth, while HM fat shows inconsistent associations (*14*). Some minerals are tightly regulated and unresponsive to short-term maternal diet (for example, iron, zinc), whereas others (for example, iodine, selenium) reflect dietary intake and health status (*15*).

In line with this, studies show that maternal docosahexaenoic acid (DHA) supplementation increases DHA in HM (*16*, *17*). By contrast, a Gambian trial giving nursing mothers an additional ~700 kcal/day for a year found no increase in milk volume or fat content, suggesting homeostatic "buffering" of some macronutrients, though those mothers were not severely undernourished (*18*). Observational cohorts also link human milk oligosaccharides (HMOs) to infant growth [for example, higher 2′-fucosyllactose associated with greater weight/length among fucosyltransferase 2 (FUT2) secretor mothers], and preclinical models show sialylated HMOs can rescue growth in settings of undernutrition via the microbiota (*19*). Much of the HM composition evidence, particularly macronutrients, bioactive proteins, and multi-omics profiles, comes from high-income populations, leaving a need for research on globally representative HM data (*20*). Beyond a clinical evaluation of any single intervention, our harmonized cross-trial design lets us distinguish milk components whose concentrations track maternal exposure from those that are homeostatically buffered.

Here we analyzed HM from three randomized trials on harmonized, HM-validated multi-omics platforms within the International Milk Composition (IMiC) Consortium, to evaluate how maternal nutritional interventions during pregnancy and lactation influence milk composition across diverse settings (*20*). MISAME-III (Micronutriments pour la Santé de la Mère et de l'Enfant-III) in Burkina Faso used a 2×2 factorial design to test prenatal and postnatal BEP supplementation relative to iron–folic acid (IFA), allowing separation of antenatal and lactational effects. Mumta-LW (Mumta Lactating Women trial) in Pakistan enrolled undernourished lactating women and randomized them to postpartum BEP, BEP plus infant azithromycin, or breastfeeding counseling alone. ELICIT (Early Life Interventions for Childhood Growth and Development in Tanzania) used a 2×2 factorial design to test postnatal maternal nicotinamide supplementation and independent infant antimicrobial interventions, with milk collected before any infant dosing.

Results

Across the three studies, 1545 milk samples from 640 mother–infant dyads were analyzed for macronutrients (protein, fat, lactose, energy), micronutrients (B-vitamins, fat-soluble vitamins, minerals, and trace elements), HMOs, bioactive proteins, targeted and untargeted metabolomic, proteomic, and microbiome profiles. Each study sampled 16 to 17% of trial participants at 2 (Mumta-LW, ELICIT) or 3 (MISAME-III) visits; child age at collection and intervention design varied by study.

We conducted four types of analyses: intervention effects on child anthropometry within the IMiC subsample, machine learning-based variable importance of classes of HM components in discriminating intervention arm membership, intervention effects on individual milk components, and pathway enrichment analyses of major metabolites and proteins. Milk components were grouped into primary (macro- and micronutrients), secondary (HMOs and bioactive proteins), tertiary (targeted metabolites), and exploratory (untargeted metabolites, proteomics, and microbiome) outcomes, with false discovery rate (FDR) correction applied within each category (see methods); FDR-adjusted *P*-values are denoted *Q*.

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An online resource provides complete analytical outputs, including baseline characteristics, HM component distributions, and sensitivity analyses (*21*).

Infant growth benefits arise primarily from prenatal, not postnatal, nutritional supplementation

In the IMiC subset of the ELICIT trial, postnatal maternal supplementation produced no differences in infant weight-for-length Z-scores (WLZ) or length-for-age Z-scores (LAZ) at 3 or 6 months (fig. S1), matching the full trial (*22*). In MISAME-III, prenatal BEP improved WLZ at 3 months; its effects on LAZ at 3 and 6 months and WLZ at 6 months were not significant (fig. S1), in line with the primary trial's finding that growth benefits come mainly from prenatal rather than postnatal supplementation (*23*). In Mumta-LW, which specifically enrolled undernourished mothers, postnatal BEP raised WLZ at 6 months in the IMiC subset (fig. S1), though this effect was not seen in the full trial (*24*).

Maternal postnatal (but not prenatal) nutritional supplementation consistently increases human milk B-vitamins and variably affects macronutrients and metabolites

Ensemble machine-learning models trained to predict the intervention arm from milk composition singled out B-vitamins across all three trials: Models fit on B-vitamins identified the treatment arm better than chance (Fig. 1A), and only the postnatal BEP and nicotinamide interventions were distinguishable. There was no signal from the antimicrobial-only arm in ELICIT (a negative control: infants received azithromycin and nitazoxanide only after milk collection) or the prenatal BEP arm in MISAME-III, and adding infant azithromycin to postnatal BEP in Mumta-LW added no signal beyond BEP alone. We therefore estimated intervention effects after combining the two nicotinamide arms in ELICIT and the postnatal BEP arms in Mumta-LW and MISAME-III (pooling the control and prenatal-only arms in MISAME-III). Stratified intervention effects are noted when reported and are available for all analyses in the online resource (*21*). The pooled interventions raised the first principal component (a measure of class-wide effect) of B-vitamins across trials and timepoints (Fig. 1B), with no effect on the first principal component of other micronutrients and an increase in macronutrients only at the 1.5-month visit in Mumta-LW. Targeted metabolites shifted at the 1.5-month visit in Mumta-LW and the 1- to 2-month visit in MISAME-III, and untargeted metabolites increased at the 3- to 4-month visit in MISAME-III.

Maternal intervention effects on HM nutrients (primary outcomes)

Complete native-unit (absolute) concentrations for all primary and secondary outcomes are tabulated in table S1, reporting per-arm sample sizes, means, standard deviations (SDs), medians, and interquartile ranges (IQRs) by trial, arm, and visit, alongside the corresponding native-unit average treatment effects, 95% confidence intervals, and FDR-adjusted *Q*-values; these complement the Z-scored effects shown for cross-component comparability in Fig. 2. Macronutrient effects were inconsistent across trials. In Mumta-LW at 1.5 months, BEP increased total energy by 11.6% (71.74 kcal/L, 95% confidence interval (CI): 29.63 to 114.00; Q = 0.003), fat by 18.0% (0.62 g/100 mL, 95% CI: 0.19 to 1.06; Q = 0.012), and protein by 11.1% (0.11 g/100 mL, 95% CI: 0.02 to 0.20; Q = 0.026). However, there were no effects at 2 months (Figs. 2 to 4), and unexpectedly in MISAME-III, postnatal BEP was associated with lower milk fat at 1 to 2 months (postnatal BEP vs. control: 12.7% reduction; −0.33 g/100 mL, 95% CI: −0.65 to −0.02; P = 0.036, Q = 0.091). Because milk fat varies substantially across the day and within a feed, and we used single spot samples rather than 24-hour collections, this result should be interpreted cautiously and does not necessarily reflect reduced infant fat intake. The difference was driven by higher fat and calories in the control arm: fat and calorie decreases were significant when the prenatal, postnatal, and prenatal+postnatal arms were each compared with control (all P < 0.05) (*21*). Nicotinamide supplementation in ELICIT did not affect macronutrients.

All three trials produced clear increases in multiple B-vitamin biomarkers (Figs. 2, 3A, and 4). In ELICIT, nicotinamide raised almost every measured B_3 metabolite, with total vitamin B_3 (expressed as nicotinamide) up 183.7% (3306 μg/L, 95% CI: 2783 to 3829; Q < 0.001), while other B-vitamins showed no significant increase after FDR correction. MISAME-III showed broad elevations of almost all B-vitamins and their metabolites at every one of the three visits (Figs. 2 and 4). The trajectory over time differed by vitamin: BEP's effect on pantothenic acid and riboflavin grew across visits, while its effect on free thiamin rose and then fell. In Mumta-LW, B-vitamins increased by a similar magnitude at 1.5 months, with smaller effects at 2 months (Figs. 2 to 4). In Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, these responses concentrated in B-vitamin pathways; nicotinate and nicotinamide metabolism was FDR-significant at every visit in all three trials (Fig. 3B and table S2).

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Beyond the B-vitamins, most milk micronutrient concentrations were unaffected across the trials, though a few time- and intervention-specific effects stood out. In Mumta-LW, BEP increased vitamin A at 1.5 months (36.1%, 0.18 mg/L; 95% CI: 0.10 to 0.26; $Q < 0.001$), and 2 months (22.3%, 0.11 mg/L; 95% CI: 0.03 to 0.20; $P = 0.011$, $Q = 0.042$), and raised α-tocopherol at 1.5 months (31.1%, 1.63 mg/L; 95% CI: 0.93 to 2.32; $Q < 0.001$). BEP in MISAME-III did not increase vitamin A but raised selenium at 1 to 2 months and 3 to 4 months, notable because selenium is present in the BEP supplement but scarce in Burkinabè diets. In MISAME-III, γ-tocopherol fell at 1 to 2 months (−16.0%, $Q = 0.020$), consistent with competition between α- and γ-tocopherol for transport into milk under vitamin E supplementation. Most other micronutrient estimates were consistent with no effect, and maternal body mass index (BMI) did not consistently modify macronutrient or micronutrient effects, as shown in Section 3 of the online resource (*21*).

To test whether BEP reduced deficiencies rather than merely raising concentrations, we classified each nutrient as deficient (below the 10th percentile of the age-specific reference from the Mothers, Infants, and Lactation Quality (MILQ) study) or not (fig. S2) (*25*). Nicotinamide in ELICIT reduced B_3 deficiency, significantly at 5 months (relative risk (RR): 0.22; 95% CI: 0.13 to 0.39; $Q < 0.001$). In Mumta-LW (1.5 months), postnatal BEP reduced pantothenic acid, B_2, and B_3 deficiencies; in MISAME-III, it reduced pantothenic acid and B_3 deficiencies at all visits and B_2 deficiency at the first visit, and lowered selenium deficiency at every visit (RR: 0.44 to 0.86). Room to reduce deficiency depended on the vitamin: in control milk, thiamin (B_1), B_6, and biotin already exceeded the MILQ median, leaving few samples deficient, whereas 24 to 38% fell below the MILQ 10th percentile for riboflavin (B_2), niacin (B_3), and B_{12}, the vitamins with the clearest intervention reductions (Fig. 4).

Maternal intervention effects on targeted non-nutrient HM components (secondary and tertiary outcomes)

Secondary outcomes were unaffected: No intervention arm in any trial significantly altered HMOs or targeted bioactive compounds at any visit (fig. S3). For tertiary outcomes, postnatal BEP consistently affected targeted metabolites, with 994 of 3911 estimates (25.4%) showing evidence of an effect and 515 (13.2%) surviving FDR correction (Fig. 5A). Most of these effects clustered in lipids, especially triglycerides, which make up 41.3% (240/581) of all targeted metabolites but 73.4% of all FDR-significant findings. They were affected at the Mumta-LW 1.5-month visit (108 hits, 72.5% of that visit's significant findings), the ELICIT 1-month visit (88, 88.0%), and all three MISAME-III visits (36, 120, and 26 hits). Triglycerides generally increased in ELICIT and Mumta-LW and at the first and third MISAME-III visits, but mostly decreased at the MISAME-III 1 to 2 month visit. After adjustment for total milk fat, most FDR-significant triglyceride effects were increases at every MISAME-III visit (fig. S4), so the apparent decrease at 1 to 2 months likely reflects the transient drop in total fat rather than a reversal of triglyceride-specific effects.

Targeted metabolite-set enrichment analyses (MSEA) showed that BEP shifted milk metabolite profiles differently across lactation. In early lactation (≤2 months), BEP broadly down-regulated amino-acid and nitrogen metabolism (glutamate, arginine/proline, methionine, glycine/serine, ammonia recycling, and glutathione), together with fatty-acid and bile-acid biosynthesis and carnitine synthesis ($Q = 6.0 \times 10^{-6}$ to 3.5×10^{-2}; Fig. 5B and table S3); up- and down-regulated pathways are reported separately in the online resource (*21*). By 3 to 4 months the pattern shifted: mitochondrial oxidative pathways were instead up-regulated (the electron transport chain, ketone-body, and phytanic-acid oxidation; $Q = 4.9 \times 10^{-2}$), while glycine/serine metabolism remained down-regulated ($Q = 4.2 \times 10^{-3}$). The milk metabolome therefore appears to respond dynamically to supplementation over time.

To connect these pathway findings to specific lipids, we broke triglyceride species down into their constituent fatty acids and tested BEP effects (Fig. 5C and table S4). In MISAME-III, BEP was consistently associated with triglycerides rich in polyunsaturated fatty acids, particularly α-linolenic and γ-linolenic acid (18:3n-3/18:3n-6), across all three visits ($P = 5.4 \times 10^{-8}$ to 6.0×10^{-3}), with stearidonic acid (18:4n-3) also elevated at 14 to 21 days ($P = 3.4 \times 10^{-3}$). Among saturated and monounsaturated fatty acids, myristic acid (14:0; Mumta-LW and ELICIT) and 9Z-nonadecenoic acid (19:1(9Z); ELICIT) were under-represented among the triglycerides that increased, including in arm-stratified analyses of Mumta-LW (*21*). Fig. 5C shows compositional enrichment rather than absolute concentrations, so these PUFA-triglyceride shifts are compatible with largely unchanged total milk fat (Fig. 2 and fig. S4).

Maternal intervention effects on the HM microbiome, untargeted metabolome and proteome (exploratory outcomes)

Microbiome

Maternal interventions left the milk microbiome largely unchanged. Observed richness, but not Shannon diversity, increased at the first visit in Mumta-LW (fig. S5), and no individual taxa reached FDR significance, as reported in Section 4 of the online resource (*21*).

Untargeted metabolomics

Among untargeted metabolites, 1347 of 353,656 average treatment effect (ATE) estimates (0.38%) reached significance at FDR < 0.05. Because untargeted features carry only putative,

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mass-based annotations, we interpret these pathway-level results as exploratory (Fig. 6A, table S5). Enrichment focused on central-carbon and nitrogen metabolism. In ELICIT, the Warburg effect was down-regulated at 1 month ($Q = 3.2 \times 10^{-4}$); in MISAME-III, bile-acid biosynthesis was down-regulated at 1 to 2 months ($Q = 1.2 \times 10^{-4}$) alongside an up-regulated glucose-alanine cycle at the same visit ($Q = 7.3 \times 10^{-4}$); and in Mumta-LW, nitrogen-handling and amino-acid pathways (purine metabolism, ammonia recycling, urea cycle, glutamate metabolism; $Q = 2.5 \times 10^{-4}$ to 3.7×10^{-4}) were up-regulated at 2 months, with the Warburg effect down-regulated earlier, at 1.5 months ($Q = 1.9 \times 10^{-4}$). Fatty-acid and eicosanoid pathways, prominent in the targeted metabolome (Fig. 5B) and in the complementary Mummichog analysis (Fig. 6B), were also FDR-significant in a subset of this untargeted enrichment (fatty acid biosynthesis, MISAME-III, 1 to 2 months, $Q = 4.6 \times 10^{-3}$; arachidonic acid metabolism, ELICIT, 1 and 5 months, $Q = 4.3 \times 10^{-2}$ and 4.1×10^{-2}, both down-regulated), corroborating the direction seen in the other two enrichment methods. Amino-acid and central-carbon pathways recurred across the targeted and untargeted metabolome, though effect directions varied by study and platform, as detailed in the online resource (*21*).

A second, independent enrichment method (Mummichog) gave a closely matching picture (Fig. 6B and table S6). In MISAME-III, BEP was again linked to FDR-significant reductions in fatty-acid and inflammatory-lipid pathways: arachidonic acid metabolism ($P = 8.4 \times 10^{-5}$, $Q = 1.0 \times 10^{-2}$; 14 to 21 days), fatty acid metabolism ($P = 8.4 \times 10^{-5}$, $Q = 5.0 \times 10^{-3}$), de novo fatty acid biosynthesis ($P = 8.4 \times 10^{-5}$ to 9.2×10^{-4}), leukotriene metabolism ($P = 3.4 \times 10^{-4}$, $Q = 1.3 \times 10^{-2}$), linoleate metabolism ($P = 3.4 \times 10^{-4}$, $Q = 3.7 \times 10^{-2}$), and fatty acid activation ($P = 7.6 \times 10^{-4}$, $Q = 3.7 \times 10^{-2}$; the last four at 3 to 4 months). These pathways build and lengthen long-chain fatty acids into inflammatory lipid mediators (interpreted further in Discussion). (Prostaglandin formation from arachidonate was also FDR-significant but in the up-regulated direction at 1 to 2 months, so it is not part of this coordinated reduction.)

Untargeted proteomics

Untargeted proteomics revealed a predominantly downward shift in intracellular protein programs following maternal BEP supplementation, with the specific pattern differing between trials (Fig. 6C and table S7). Few individual proteins reached FDR significance, and no up-regulated pathway survived FDR correction in the primary MISAME-III or Mumta-LW BEP contrasts; the pathway-level results below therefore concern the FDR-significant down-regulated enrichments. Because milk contains proteins derived from intact and exfoliated somatic cells (mammary epithelial cells, leukocytes, and milk-fat-globule membrane components) in addition to proteins actively secreted by lactocytes, these gene ontology (GO)-term enrichments should be interpreted as differences in the relative abundance of proteins from these sources between intervention and control milks, rather than as evidence of active intracellular reprogramming within secreting cells.

At the individual-protein level, five of 1320 quantified proteins reached FDR significance in MISAME-III at 1 to 2 months and 34 of 1681 in Mumta-LW at 2 months. Both proteins that increased in MISAME-III were selenoproteins: selenoprotein P (SELENOP, +0.70 SD; 95% CI: 0.35 to 1.04; $Q = 0.031$) and glutathione peroxidase 3 (GPX3, +0.69 SD; 95% CI: 0.38 to 0.99; $Q = 0.014$), concordant with the rise in milk selenium in the same trial and visit and with maternal blood, where they were the only proteins raised in both compartments (table S8). The three decreased proteins were USP21, ADH7, and thioredoxin (−0.74 to −0.91 SD). In Mumta-LW, effects were more numerous and mixed in direction: 26 proteins increased, including USP15, PALS1, ARF6, SYTL1, and SLC34A2 (+0.72 to +0.97 SD), and 8 decreased, including lactate dehydrogenase A (LDHA) and neutrophil elastase (ELANE) (−0.46 to −0.59 SD), with a weaker same-direction selenoprotein signal (GPX3 +0.57 SD, $Q = 0.17$). The milk selenoprotein effects are FDR-significant in the covariate-adjusted primary model and nominally significant without adjustment; the maternal-blood effects are FDR-significant in both.

In MISAME-III at 1 to 2 months, proteins in 27 GO pathways were depleted after FDR correction, clustering around telomere maintenance and DNA-biosynthetic processes together with chaperone-mediated protein folding and translation/ribonucleoprotein-complex biogenesis ($Q = 9.8 \times 10^{-4}$ to 4.8×10^{-2}). Several of the strongest annotations concerned telomere and DNA-biosynthetic processes; these terms, however, were largely driven by a common set of molecular chaperones: multiple chaperonin-containing TCP1 (CCT/TCP1) subunits together with HSP90AA1, HSP90AB1, and the HSP90 co-chaperone PTGES3, indicating a broad reduction in chaperone- and proteostasis-associated proteins rather than a telomere-specific effect.

In Mumta-LW at 2 months, proteins in 58 pathways were depleted after FDR correction, forming a distinct but complementary metabolic signature that encompassed glycolysis, pyruvate and carbohydrate metabolism, cellular respiration, ATP metabolism, and nucleotide and nicotinamide nucleotide metabolism ($Q = 1.6 \times 10^{-5}$ to 4.4×10^{-2}). These pathways were driven by abundant intracellular metabolic proteins, including PKM, GAPDH, ALDOA/ALDOC, PGAM1, GPI, LDHA, G6PD, IDH2, and NAMPT (table S7).

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BEP-associated changes in maternal blood, milk, and infant blood (MISAME-III)

In a MISAME-III subset with paired maternal plasma, dried-blood microsamples, and milk (see methods), we compared BEP-responsive metabolite features across compartments by direction and significance only, as intensities are not comparable across platforms. In maternal blood, chemical-class enrichment was concentrated in lipids (up-regulated oxylipins and free fatty acids, down-regulated acylcarnitines; table S9). Up-regulated features persisted across consecutive visits and new ones emerged, most markedly in infants (100% of features significant at 1 to 2 months remained so at 3 to 4 months and 87.5% from 3 to 4 to 5 to 6 months, with 5 and 10 features newly significant at those visits; table S10); these group-level counts do not show within-individual accumulation. Twenty up-regulated features were matched across at least two compartments and two (putative citrate and 4-hydroxy-2-oxoglutarate) across maternal blood, milk, and infant blood; 16 of the 20, including both, were reliably mass-matched to features detected in the BEP supplement analyzed on the same platform (table S8). Pathway analysis of these features was exploratory: no pathway met the FDR threshold, and only glyoxylate and dicarboxylate metabolism was nominally enriched (P = 0.004; pyrimidine, P = 0.063; vitamin B_6, P = 0.092; table S11). Two of the affected metabolites correspond to BEP nutrients: pyridoxal (vitamin B_6) and N1-methyl-2-pyridone-5-carboxamide (a niacin catabolite). Putative dehydroascorbate, the oxidized form of vitamin C (*26*), was supplement-matched and linked between milk (3 to 4 months) and infant blood (5 to 6 months), though not maternal blood; maternal vitamin C supplementation can raise antioxidant capacity in milk and infant urine (*27*), but we did not measure antioxidant capacity or function. SELENOP and GPX3 rose in both milk and maternal blood; where mother and infant shared a feature catalog (5 to 6 months), 16 of 18 maternally significant features agreed in direction in the infant; and a medium-chain acylcarnitine (m/z 286.202) increased in milk, maternal blood, and infant blood (Fig. 6D and table S8). These are concordant intervention-associated changes across compartments, consistent with, but not demonstrating, milk-mediated transfer. Feature identities are provisional (accurate-mass-based; annotation ongoing).

Discussion

Across randomized trials in Burkina Faso, Pakistan, and Tanzania, maternal nutritional supplementation during lactation consistently enriched milk B-vitamins, while effects on macronutrients were limited to early increases in the undernourished Pakistani cohort. High-dose nicotinamide (vitamin B_3) produced large increases in milk vitamin B_3 and associated vitamers, while BEP supplements raised riboflavin and vitamin B_6, and in Mumta-LW also vitamin A and α-tocopherol, especially where baseline maternal nutritional status, as measured by mid-upper-arm circumference (MUAC), was low. By contrast, total protein, lactose, energy, and (in most settings) fat remained stable, consistent with tight physiological buffering of macronutrients.

Maternal postnatal supplementation selectively enriched milk micronutrients while macronutrients remained buffered, a pattern consistent with prior trials showing that many water-soluble micronutrients, particularly B-vitamins, reflect maternal intake and status. Postpartum riboflavin supplementation increased milk riboflavin and relieved infant deficiency signs in The Gambia (*28*), and a South Asian trial found that providing high-dose vitamin B_{12} during pregnancy and/or lactation increased milk B_{12} and improved infant B_{12} status (*29*). A systematic review similarly concluded that maternal intake of vitamins B_1 and B_2 is generally reflected in HM concentrations, with more limited evidence for B_6 and B_{12} (*30*). In the Women First trial, maternal dietary intake of vitamin B_6 and choline (but not of other B-vitamins) tracked with their corresponding HM concentrations, although preconception or prenatal supplementation itself did not raise milk niacin (*31*). The ELICIT 6-month high-dose nicotinamide regimen, by contrast, yielded pronounced increases in milk B_3, likely reflecting differences in dose, duration, and baseline status.

Macronutrient buffering is consistent with a decade of research reporting that, unlike milk fatty acids, evidence linking maternal diet to milk carbohydrate and protein content remains limited and inconsistent (*32*). Even large changes in dietary fat rarely alter total milk fat, as the mammary gland draws on adipose stores and de novo synthesis to maintain milk output. In our pooled data, BEP caused at most a small, transient rise in milk fat in the most undernourished setting; otherwise, macronutrients remained stable. The transient drop in milk fat at one MISAME-III visit, which did not survive FDR correction, should be interpreted cautiously. One possible explanation is dilution by greater milk volume, which would lower fat concentration without changing triglyceride composition; after adjustment for total fat, the 1- to 2-month triglyceride effects resembled those at the other visits. Because volume was not measured, this study cannot distinguish dilution from other explanations, such as within-feed and diurnal variation in single spot samples, or chance. Physiologically, several vitamins, including most B-vitamins and vitamin A, have milk concentrations that track maternal intake and status because their transfer into milk depends on circulating maternal levels, whereas many minerals, folate, and macronutrients are maintained within relatively narrow ranges through mammary homeostatic regulation, including mobilization of maternal body stores to preserve milk supply (*15*).

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Improved milk micronutrient quality did not uniformly translate into early growth gains. Infant growth at 6 months was improved by BEP in Mumta-LW but unaffected by the postnatal intervention arms in the IMiC subsample of ELICIT and MISAME-III. Because infants depend on prenatal stores of nutrients like iron and B_{12}, poor pregnancy status may also constrain the benefits of postnatal HM micronutrient improvements (*7*), though MISAME-III found no evidence of a clear additive growth benefit from combining prenatal and postnatal BEP compared with prenatal supplementation alone (*23*). Lactation nevertheless remains a critical and underutilized window for addressing maternal–infant micronutrient deficiencies, even if such interventions are unlikely to alter growth trajectories except in the most malnourished populations. The absence of length and weight gains in two of the three trials should not be read as evidence of limited clinical relevance. Adequate maternal B-vitamin, vitamin A, and selenium delivery via HM plausibly supports several outcomes that anthropometry does not capture within a 6-month window. Improved milk micronutrient quality is expected to raise infant biochemical micronutrient status, for example serum thiamin, riboflavin, and B_{12}, and thereby reduce the risk of deficiency syndromes such as infantile beriberi (*33*). Early-life B-vitamin and polyunsaturated fatty acid (PUFA) exposure has also been linked to neurodevelopmental trajectories that anthropometric measures do not capture (*34*, *35*).

Beyond nutrients, supplementation was associated with systems-level shifts in the milk lipidome, metabolome, and proteome, whose functional consequences this study did not measure. The shifts in milk fatty acid composition without changes in total fat were expected, as HM fatty-acid profiles, and their PUFA fraction in particular, reflect maternal dietary fat sources (*5*, *36*). BEP formulations differed in lipid content, with the peanut-based MISAME-III supplement providing more unsaturated fats than the chickpea- and lentil-based Mumta-LW supplement, consistent with the larger triglyceride shifts observed in MISAME-III. Supplemented mothers likely had higher circulating unsaturated fatty acids that were incorporated into milk triglycerides, whereas unsupplemented mothers relied more on mobilizing stored fat or synthesizing medium-chain fatty acids. The BEP-associated enrichment of α-linolenic and stearidonic acid is relevant because these plant-derived n-3 fatty acids are precursors the infant can partially convert to longer-chain n-3 PUFAs (*37*), and milk PUFA supply has also been linked to infant allergic outcomes (*35*). In parallel, the coordinated reduction we observed in milk eicosanoid pathways (arachidonic acid and leukotriene metabolism; prostaglandin formation was the exception, rising at 1 to 2 months) is notable because these n-6-derived lipid mediators shape inflammatory signaling, and HM carries oxylipins and eicosanoids that may influence the infant gut and immune system (*38*, *39*).

Untargeted metabolomic signatures suggest that BEP supplementation alters the balance of nutrients delivered through milk in ways that may have functional consequences for infant growth and metabolic programming. The consistent reduction in long-chain (≥C20) PUFAs and their elongation pathways across settings, alongside the rise in dietary C18 precursors, points to a shift away from endogenous maternal fatty-acid synthesis and remodeling toward greater reliance on dietary PUFA availability. In a MISAME-III subset with paired blood samples, the circulating profile fit this redistribution: de novo fatty-acid biosynthesis and related eicosanoid pathways were decreased in milk but increased in maternal blood, consistent with mothers making less milk fat themselves and drawing more on dietary fat. SELENOP [the principal selenium carrier in plasma and milk, required for maternal-to-neonatal selenium transfer (*40*, *41*)] and the extracellular selenoenzyme GPX3 were the only proteins raised in both milk and maternal blood, consistent with the milk-selenium response reflecting maternal selenium supply; antioxidant activity was not measured, so this is not evidence of improved antioxidant function. A medium-chain acylcarnitine increased in both milk and infant blood, consistent with, but not demonstrating, transfer through milk.

One feature of this milk remodeling could matter for the infant in ways that length and weight would not capture, though it remains provisional and needs targeted confirmation. BEP lowered the milk pathways that supply methyl groups and simple amino acids (methionine, glycine, and serine), consistently in the targeted metabolome across trials. One-carbon metabolism supplies the methyl groups used for DNA and histone methylation and is a recognized link between early-life nutrition and epigenetic regulation (*42*), and HM is comparatively rich in one-carbon substrates such as S-adenosylmethionine (*43*). A change in milk supply of these compounds is therefore a plausible route by which maternal nutrition could influence infant developmental programming, though the strongest human evidence concerns the periconceptional window rather than lactation (*44*); for milk-borne supply this remains a hypothesis for future study. Set against this, the enrichment of carnitine and fatty-acid-oxidation pathways was among the most reproducible signals across trials and platforms. Carnitine is conditionally essential in early life, when neonates rely substantially on milk-derived carnitine (*45*, *46*) to carry fatty acids into mitochondria for β-oxidation (*47*), so this pathway shift provides a metabolic context for the medium-chain acylcarnitine that increased in both milk and infant blood. Because acylcarnitines are produced in proportion to fatty-acid-oxidation flux (*48*), the infant increase could reflect milk-borne supply, altered fatty-acid oxidation in the infant, or both, which our

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data cannot distinguish.

Across the two trials, BEP supplementation reduced proteins representing distinct domains of intracellular mammary-cell biology: chaperone-dependent protein folding, translation, and intracellular trafficking in MISAME-III, and central-carbon, respiratory, and nicotinamide adenine dinucleotide (NAD)/redox metabolism in Mumta-LW. Despite involving different pathways, both signatures reflected a lower representation of intracellular machinery in milk (*49*). HM contains viable mammary epithelial cells and extracellular vesicles carrying intracellular protein cargo of mammary epithelial origin (*50*). The reduced abundance of intracellular proteins could therefore reflect changes in mammary epithelial state, cellular turnover or shedding, extracellular-vesicle cargo, or the abundance of milk somatic cells, rather than reduced milk synthesis or direct suppression of these pathways; because increased somatic-cell shedding can accompany mammary inflammation and epithelial disruption (*51*), reduced shedding would be consistent with a less inflammatory mammary environment under BEP. The Mumta-LW signature is notable because the trial enrolled women with poorer nutritional status, and BEP simultaneously provided energy and B-vitamins central to carbohydrate and redox metabolism (B_1, B_2, B_3, B_5). The lower abundance of these proteins (PKM, LDHA, GAPDH, G6PD, NAMPT) does not necessarily indicate impaired mammary energy metabolism; one possible explanation is metabolic adaptation to improved substrate and cofactor availability, reducing reliance on endogenous glycolytic, NAD-salvage, and reductive machinery (notably NAMPT, the rate-limiting NAD-salvage enzyme, since BEP supplies its precursor niacin (*52*)). Our data do not, however, directly demonstrate altered flux or a specific feedback mechanism. Together with the metabolomic evidence for altered lipid and energy metabolism, these findings are compatible with improved maternal nutrient availability altering the metabolic demands of milk synthesis and secretion, with the strongest central-carbon response occurring in the more nutritionally constrained population.

This study's strengths include pooling harmonized data from three rigorously implemented randomized trials across diverse low-resource settings and the application of modern analytical platforms, particularly mass spectrometry, which has substantially expanded the number and diversity of HM constituents that can be quantified. Key limitations include sampling at different child ages across sites, which limits temporal comparability, and relying on single spot samples rather than 24-hour collections, without test-weigh measurement of milk volume; because milk fat and some water-soluble vitamins vary substantially within a feed and across the day, a spot sample reflects one moment, not an infant's daily intake. Supplementation could plausibly have altered milk volume and thus total nutrient delivery despite stable concentrations; MILQ-study evidence suggests concentrations are largely independent of daily volume, but short-term volume changes could still affect them, so we may underestimate BEP's full impact on delivery (*53*). In a MISAME-III subset we additionally profiled the maternal circulating metabolome and proteome and the infant circulating metabolome, showing intervention effects concordant in direction with the milk findings; these cross-compartment analyses are exploratory and limited to a single trial, with mass-based (not yet confirmed) feature identities, milk-to-blood matching limited by isobaric ambiguity, and near-detection-limit imputation that leaves the near-uniform infant increases requiring confirmation. Functional assays of antimicrobial or antioxidant activity in the milk samples were beyond the scope of the present harmonized analysis and remaining sample volumes. Targeted maternal micronutrient biomarkers, which would let us link milk alterations to maternal systemic micronutrient status, were not part of the collected data, and the cross-compartment analyses above are untargeted and limited to the MISAME-III subset (*23*, *24*). These limitations motivate future trials with longer follow-up beyond 6 months and a broader outcome set (including infant micronutrient biomarkers, neurodevelopment, and infection incidence) to capture meaningful biological and functional gains that extend beyond anthropometric outcomes, and to evaluate alternative BEP lipid formulations and integrated intervention packages combining maternal supplementation with infection-reducing or microbiome-modulating strategies.

Our findings carry several policy implications. First, postpartum supplementation can improve milk micronutrient quality, particularly B-vitamins, and may improve infant micronutrient status (not measured here) even when growth is unchanged, supporting extension of micronutrient or BEP programs beyond delivery. Growth expectations should remain realistic: supplementation alone is unlikely to reduce stunting except in severely undernourished populations with substantial baseline deficiencies, and addressing growth faltering will require integrated strategies combining maternal nutrition with infection control and nutrient-dense complementary feeding. Postpartum supplementation may also prevent maternal depletion and excessive weight loss, supporting sustained lactation and caregiving capacity, and its cost-effectiveness warrants assessment given existing investments in pregnancy nutrition.

Second, the MILQ HM reference standards (*25*) let us interpret effects against biologically meaningful population benchmarks rather than statistical significance alone. Several micronutrients showed meaningful upward shifts even when reductions in deficiency prevalence were modest: milk vitamin B_{12} shifted upward in the MILQ distribution without consistently reducing deficiency risk (illustrating the gap between distributional shifts and threshold-based metrics),

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whereas riboflavin (B_2), B_3, B_5, and B_6 responded more strongly, often rising above the MILQ median and reducing the prevalence of low values. In selenium-poor Burkina Faso, BEP raised milk selenium and cut deficiency prevalence, though at 1 to 2 and 3 to 4 months most samples stayed below the MILQ 10th percentile, likely because the dose, while effective, is too low to fully replenish local mothers (*54*), or the reference threshold is unreachable on local intake (*55*). The upward shift in concentration is still likely to be nutritionally meaningful, although without milk-volume data we cannot quantify the selenium reaching the infant.

Third, the composition of BEP supplements is intentionally designed to deliver both macronutrients and micronutrients according to defined specifications, which also include guidance on lipid quality (*11*). At the same time, BEP is often produced locally for cost and feasibility, so its lipid fraction derives from regionally available ingredients, most commonly peanuts in West Africa and chickpeas or lentils in South Asia. In our analyses, BEP-associated lipid inputs were reflected in milk triglyceride composition, more strongly in peanut-based Burkina Faso than in Pakistan, indicating that the choice of lipid sources in locally adapted formulations can shape milk lipid profiles: an opportunity to refine BEP lipid composition, within the constraints of local production, to better support infant growth and development.

In sum, maternal nutritional supplementation during lactation reshaped HM composition well beyond the nutrients supplied, with consistent multi-omic shifts across three diverse populations, strengthening the case for extending supplementation programs into the postpartum period.

Materials and methods

Study design and participants

We conducted a harmonized cross-trial analysis of individual participant human-milk data from 640 mother–infant dyads drawn from three randomized controlled trials in one low-income (Burkina Faso) and two lower-middle-income countries (Pakistan and Tanzania) participating in the International Milk Composition (IMiC) Consortium (*20*). In Pakistan, the Mumta-LW trial enrolled undernourished lactating women (MUAC <23 cm) shortly after birth and randomized them to postnatal balanced energy–protein (BEP) supplementation, BEP plus infant azithromycin prophylaxis, or breastfeeding counseling alone; BEP was provided daily for up to six months postpartum (*56*). In Burkina Faso, MISAME-III employed a 2×2 factorial design to test prenatal and/or postnatal BEP supplementation against iron–folic acid alone, with BEP administered daily during pregnancy and/or through six months postpartum (*57*). Both trials included a pre-formulated multiple micronutrient fortificant added to the BEP. In Tanzania, ELICIT used a 2×2 factorial design to evaluate maternal nicotinamide supplementation during lactation and independent infant antimicrobial interventions, with milk collected prior to any infant dosing (*58*).

MISAME-III included women aged 15 to 40 years with gestational age ≤21 weeks at randomization and excluded multifetal pregnancies and severe illness. Mumta-LW enrolled lactating women aged 13 to 49 years with recent live births, MUAC <23 cm, infant birthweight ≥1500 g, and intent to exclusively breastfeed to 6 months; women with severe maternal or neonatal illness or allergy to BEP ingredients were excluded. ELICIT enrolled infants <14 days of age with birthweight ≥1500 g and their mothers aged ≥18 years, excluding multiple gestations, major congenital anomalies, and lack of breastfeeding intent. All analyses followed each original trial's intent-to-treat population, restricted to dyads with at least one HM measurement.

The MISAME-III trial enrolled 1708 pregnant women in rural Burkina Faso and followed 1628 singleton live-born infants; 290 mother–infant dyads were included in IMiC and contributed 845 HM samples across three scheduled visits (14 to 21 days, 1 to 2 months, and 3 to 4 months postpartum): 276, 287, and 282 samples at the three visits respectively, with 25 expected samples missing across visits due to incomplete visit attendance or unobtained samples. The Mumta-LW trial recruited 957 lactating women with MUAC <23 cm in peri-urban Karachi and contributed 150 dyads with HM measured at approximately 1.5 and 2 months postpartum (300 samples; complete). ELICIT enrolled 1188 mother–infant pairs in rural Haydom, Tanzania, and 200 dyads with HM at 1 and 5 months postpartum were selected for IMiC (400 samples; complete). Across trials, the IMiC subsets thus represent approximately 16 to 17% of randomized participants per trial and yielded a combined analytic dataset of 1545 milk samples from 640 dyads. Baseline participant characteristics for the IMiC subset of each study, overall and by intervention and study arm, are available in Section 1 of the online resource (*21*).

Milk collection and processing

Milk collection protocols varied by site, but all used clean expression methods and standardized aliquoting into sterile cryovials. Samples were stored at −80°C locally and shipped on dry ice to the Manitoba Interdisciplinary Lactation Centre for centralized processing. To minimize assay batch effects, samples were randomized by intervention group and collection time before laboratory analysis. In ELICIT, HM was collected by mid-feed hand expression after washing the areola with soap and deionized water; approximately 8 mL was expressed into sterile containers and transferred to −80°C within the same day. In Mumta-LW, at least 10 mL of pre-feed milk was hand-expressed after washing the breast with warm water and soap, transported on ice, mixed, and aliquoted into four 1.5 mL cryovials before storage at −80°C.

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MISAME-III used an electric breast pump (full-breast expression from the less recently used breast); 7.2 mL of thoroughly homogenized milk was aliquoted into four 2 mL cryovials and stored in liquid nitrogen or −80°C freezers prior to shipment. Additional details, including laboratory analysis techniques, are available in the IMiC protocol paper (*20*).

Outcomes

Primary outcomes were concentrations of macronutrients and micronutrients; secondary outcomes included HMOs and targeted bioactive proteins; tertiary outcomes included targeted metabolomics; exploratory analyses considered untargeted metabolomics, proteomics, and microbiota composition. A list of all outcomes and descriptions is available in Section 2 of the online resource (*21*).

Primary HM outcomes comprised total protein, fat, and digestible carbohydrate (primarily lactose) measured in g/100 mL, and a panel of vitamins and minerals including thiamine, riboflavin, free and phosphorylated B-vitamins, pantothenic acid, niacin-related metabolites, vitamin A, vitamin E, carotenoids, calcium, iron, zinc, selenium, sodium, potassium, and magnesium. Secondary outcomes were individual HM oligosaccharides (HMOs) and selected bioactive proteins (for example, secretory IgA, calprotectin, leptin, insulin, fibroblast growth factor 21). Tertiary outcomes included targeted metabolites across aqueous and lipid classes from the MxP Quant 500 platform (for example, triglycerides, diglycerides, ceramides, free fatty acids, acylcarnitines), while exploratory outcomes included untargeted metabolomic features, untargeted proteomic features, the relative abundances and α-diversity indices of the HM microbiome, and centered log-ratio-transformed abundances of individual taxa. Milk microbiota were profiled by 16S rRNA gene sequencing of the V4 hypervariable region at the Alkek Center for Metagenomics and Microbiome Research (Baylor College of Medicine), with DNA-free-water negative controls at each processing step and the Decontam and SCruB algorithms used to identify and remove extraction- and sequencing-introduced contaminants given the low biomass of milk; full wet-laboratory and quality-control detail is in the IMiC protocol paper (*20*). Metabolomic, proteomic, and microbiome measures had below limit of detection values imputed per analysis laboratory specifications.

For presentation and modeling, HM components were expressed in native assay units, then also standardized to study×visit–specific z-scores. Intervention effects were estimated on both scales: in native assay units, which preserve interpretability and provide the absolute effect estimates reported in the text (for example, g/100 mL or μg/L), and in z-scored units, which increase comparability across HM measures with differing scales and are used throughout the figures (where effects represent mean differences in SD units).

Infant anthropometric measurements (weight, recumbent length, and head circumference) were collected by trained personnel at scheduled study visits using standardized equipment and procedures across sites, and were converted to age- and sex-standardized weight-for-age (WAZ), length-for-age (LAZ), weight-for-length (WLZ), head-circumference-for-age (HCAZ), and mid-upper-arm-circumference-for-age (MUAZ) z-scores using the WHO Child Growth Standards (*20*, *59*).

Statistical analysis

We analyzed each study separately due to differences in milk sample collection time and intervention designs. To limit dimensionality and provide complementary, biology-anchored metrics, we also evaluated pre-specified composite measures (for example, principal component analysis (PCA)–based domain scores and targeted-learning growth prediction-score summaries) alongside intervention effects on individual components. For the treatment-arm prediction analyses, SuperLearner ensemble machine learning models were used to predict multi-class treatment arm membership from all subsequent primary, secondary, and tertiary outcomes (*60*). Algorithms used within the SuperLearner library were the simple mean, generalized linear models, least absolute shrinkage and selection operator (LASSO) penalized regression (*61*), random forest, and XGBoost gradient boosting models (*62*). Prediction accuracy was assessed using the cross-validated area under the curve (CV-AUC).

We estimated ATEs on each milk component and on the first principal component of each component class using targeted maximum likelihood estimation (TMLE) (*63*), estimated using SuperLearner for outcome and propensity model, and adjusting for baseline prognostic factors where available within each trial (child sex, maternal age, maternal education, maternal height, maternal parity, season of delivery, hospital/home birth, asset-based household wealth, household food security category, number of rooms and persons in the household, improved water source, improved household flooring versus dirt, and cooking locations). Maternal BMI, MUAC, and gestational age at birth were intentionally excluded because the prenatal interventions could have affected them (post-treatment bias). Continuous covariates were median-imputed when missing and categorical covariates were assigned an explicit “missing” factor level; in both cases a missingness indicator was added to the adjustment set. For estimation, intervention arms were collapsed within each trial to a single contrast: in MISAME-III, women receiving postnatal BEP (BEP/BEP and IFA/BEP) were compared against women not receiving postnatal BEP (BEP/IFA and IFA/IFA); in ELICIT, nicotinamide and nicotinamide+azithromycin+nitazoxanide arms were pooled against the control; and in Mumta-LW, BEP and BEP+azithromycin

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arms were pooled against the control. Near–zero-variance biomarkers and covariates (R caret package nearZeroVar function defaults) were dropped before model fitting. To correct for multiple comparisons, we applied Benjamini–Hochberg FDR corrections by outcome group (primary, secondary, tertiary, and exploratory), study, and visit to calculate FDR-adjusted *P*-values, denoted *Q* throughout this paper (*64*). This applies throughout, including the metabolite-set, pathway, and GO enrichment analyses. TMLE analyses were performed using the bioTMLE package in R (*65*), using the same Super-Learner library as for the treatment-arm prediction analysis for the primary, secondary outcomes, and tertiary outcomes, and using generalized linear models alone for the higher-dimensional exploratory outcomes (untargeted metabolomics, the milk proteome, and the microbiome), for tractable computation and greater stability at small per-cell sample sizes. All analyses were conducted in R; analysis scripts are available on GitHub (*66*) and permanently archived on Zenodo (*21*).

MILQ reference standard application

To assess the adequacy of human milk (HM) nutrient concentrations, we applied age-specific reference values from the MILQ study, which provides harmonized distributions for macronutrients, minerals, and vitamins measured using HM-validated assays (*25*, *67*–*70*). For each milk sample, infant age at collection (in days) was used to assign an age band consistent with MILQ reference strata (0 to 3 days, 4 to 17 days, 18 to 31 days, 1 to 2 months, 2 to 3 months, 3 to 4 months, 4 to 5 months, and 5 to 6 months). Observed HM concentrations were harmonized to MILQ units, including conversion of B-vitamins and minerals where required, and nutrients were matched to MILQ component definitions. Deficiency was operationalized as a binary indicator defined by concentrations below the 10th percentile of the MILQ age-specific reference distribution, reflecting intake levels plausibly associated with inadequate infant supply. Components with very low prevalence of deficiency across arms within a study–visit stratum (<5 cases) were excluded from inferential analyses to avoid sparse-data bias. Intervention effects on HM deficiency were estimated separately by study, visit, and nutrient using TMLE, and P-values were FDR-corrected within each visit and outcome group.

Metabolite set enrichment analyses (MSEA)

We performed pathway-level interpretation of intervention-associated untargeted metabolomics signals using an over-representation analysis (ORA) workflow implemented locally with the MetaboAnalystR package (*71*) (run locally, so the full raw-data-to-figure pipeline is reproducible without the MetaboAnalyst web application). To enable metabolite annotation, we constructed a reference metabolome by integrating study-specific metabolite feature labels with multiple identifier sources, including MS2-verified compound identifiers, global identifiers, and MetID-based annotations, and derived a final metabolite name and corresponding Human Metabolome Database (HMDB) identifiers for each feature.

For each study and sampling time point, we generated contrast-specific metabolite lists from the intervention effect models. Foreground feature lists were defined as untargeted-metabolomics features with unadjusted $P < 0.05$ in the per-(study × timepoint × contrast × direction) ATE estimate; this permissive foreground is standard in over-representation workflows and preserves statistical power for the pathway-level test, where Benjamini–Hochberg correction is then applied to the enrichment P-values (see below). Feature lists were further stratified by direction of effect (up-regulated: estimate > 0; down-regulated: estimate < 0) and by analytical framework (combined-arm versus stratified-arm), with combined-arm analyses used for time points preceding antibiotic administration where appropriate. Study-specific metabolite feature identifiers were then mapped to matched metabolite names using the corresponding annotation keys.

ORA pathway enrichment was computed locally in MetaboAnalystR for each study, time point, and direction of effect, and annotated with relevant metadata; untargeted-metabolome enrichment (Fig. 6A) used the reference metabolome described above as the enrichment background. Results were concatenated into a unified enrichment table; for the untargeted-metabolome analysis (Fig. 6A and table S5), pathways with fewer than three annotated member metabolites were excluded from enrichment testing. For each pathway, an enrichment ratio was calculated as the observed-to-expected hit ratio (hits/expected), and a signed enrichment metric was generated by assigning negative values to down-regulated pathways to preserve directionality. Significant pathways were summarized in tabular form and visualized using volcano-style scatter plots of signed enrichment ratio versus $-\log_{10}(P)$, with points colored by study and nonsignificant results shown in gray. Pathway labels were applied to enriched pathways meeting the significance criteria indicated in the figure. For the targeted (tertiary) metabolites, over-representation was assessed against the whole measured human metabolome as the reference background (rather than the smaller targeted-assay panel) and combined the Small Molecule Pathway Database (SMPDB) small-molecule and lipid metabolite-set libraries; enrichment is therefore expressed relative to the known human metabolome, and the resulting FDR-significant pathways (Fig. 5B and table S3) are correspondingly sensitive to this reference choice. For the primary micronutrient metabolites, pathway-level effects were instead assessed with MetaboAnalystR's KEGG pathway-topology (impact) module; Fig. 3B plots each pathway's topological impact against its enrichment $-\log_{10}(P)$, with the full per-pathway statistics in table S2.

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Pathway-level interpretation of untargeted metabolomics using Mummichog

For pathway-level interpretation of untargeted metabolomics features, we performed functional enrichment analysis using Mummichog (*72*) on contrast-specific metabolite feature lists derived from the intervention effect models. Analyses were stratified by study, collection time window, and intervention contrast, and features were further separated by ionization mode and by direction of effect to define "up-regulated" (estimate > 0) and "down-regulated" (estimate < 0) feature sets.

For each subset, Mummichog was run separately for positive and negative ionization modes using a mass accuracy tolerance of 10 ppm, the human_mfn pathway/metabolite reference database, a significance threshold of 0.05, and 500 to 1000 permutations (as specified per run) to estimate empirical pathway significance. To summarize results across analyses, all outputs were concatenated and a signed enrichment ratio (pathway coverage/fold enrichment) was calculated, with negative values assigned to down-regulated pathways to preserve directionality. Significant pathways (Q < 0.05) were visualized using volcano-style plots of signed pathway coverage versus $-\log_{10}(P)$.

Compositional fatty-acid signatures of intervention-responsive triglycerides

To assess whether specific fatty acids were preferentially represented among triglycerides (TGs) significantly altered by maternal nutritional supplementation, we conducted a compositional enrichment analysis linking TG-level intervention effects to their constituent fatty acids using Biocrates MxP Quant 500 lipid annotations. Structural TG annotations were imported from the annotation metadata and parsed into lipid shorthand notation specifying the three fatty-acid side chains (sn-1, sn-2, sn-3). TG molecular species were decomposed into their constituent fatty acids, and the relative proportion of each fatty acid within a TG was calculated as the frequency of its occurrence divided by the total number of fatty-acid positions for that TG.

For each fatty acid present within a given study–timepoint–contrast stratum, we compared its relative proportion between FDR-significant TGs (Benjamini–Hochberg Q < 0.05 on the TG-level ATE estimates) and nonsignificant TGs using a Wilcoxon rank-sum test. Fatty acids were tested only when both TG groups were represented, ensuring valid statistical comparisons. Resulting P-values were adjusted for multiple testing using the Benjamini–Hochberg procedure. For each fatty acid, we additionally calculated the mean difference in proportional representation between significant and nonsignificant TGs.

This approach does not estimate absolute fatty-acid concentrations in HM. Rather, it identifies fatty acids that are compositionally enriched or depleted within TG species that respond to supplementation. In down-regulated TGs, a negative mean difference indicates fatty acids disproportionately lost among TGs reduced by the intervention, whereas a positive mean difference reflects fatty acids characteristic of TGs being reduced. By contrast, in up-regulated TGs, a positive mean difference indicates fatty acids preferentially represented among TGs increased by the intervention, and vice versa. Together, this analysis constitutes a composition-based enrichment framework linking TG-level intervention effects to underlying fatty-acid structure.

GO enrichment analysis of intervention-associated breast-milk proteomic features

The milk proteome was quantified by DIA-NN (*73*) at the protein-group (UniProt) level and analyzed for MISAME-III and Mumta-LW; ELICIT was excluded because its proteome samples came only from the control and azithromycin arms, with no nicotinamide-arm samples, so no intervention effect was estimable. For pathway-level interpretation of intervention-associated breast-milk proteomic signals, we performed GO Biological Process ORA using the clusterProfiler framework (*74*), run natively at the UniProt (protein-group) level. We first imported harmonized intervention effect model outputs and retained proteomics features (UniProt identifiers). GO Biological Process terms were assigned to each measured UniProt accession using a custom UniProt-to-GO mapping derived from org.Hs.eg.db, so that every measured protein is counted once; this avoids the annotation multiplicity that arises when a single UniProt accession is expanded into multiple Entrez genes. The background ("universe") for each test comprised all UniProt accessions measured in the corresponding study × contrast.

For each study and timepoint window, we generated contrast-specific foreground protein lists, defined as proteomic features with unadjusted P < 0.05 in the per-(study × timepoint × contrast) ATE estimate, excluding the control contrast and stratified by direction of effect (up-regulated: estimate > 0; down-regulated: estimate < 0). As with MSEA above, this permissive protein-level threshold preserves power for the pathway-level enrichment test. Over-representation of GO Biological Process terms was evaluated with clusterProfiler::enricher against the custom UniProt-to-GO term set (gene-set sizes 10 to 500), using the measured proteome (all UniProt accessions in the corresponding study × contrast) as the background so that each protein is counted once; Benjamini–Hochberg correction was applied to the returned GO-term P-values, and terms with adjusted Q < 0.05 were considered FDR-significant. Enrichment outputs were converted to tidy tables and annotated with study, timepoint, and direction metadata.

To enable directional visualization, fold enrichment values were assigned a negative sign for down-regulated results.

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We summarized enrichment results across analyses by concatenating all output tables, and visualized them using volcano-style scatterplots of signed fold enrichment versus $-\log_{10}(P)$, with point size/alpha scaled by gene count and labels applied to the most significant terms. For reporting, we exported a supplementary table containing GO identifiers, term descriptions, enrichment metrics, adjusted P-values, and the corresponding contributing protein lists (mapped back to UniProt IDs for interpretability).

Cross-compartment maternal and infant blood analyses (MISAME-III)

In a MISAME-III subset with paired samples we applied the same targeted maximum likelihood framework to maternal venous plasma, maternal prenatal and postnatal dried-blood microsamples (VAMS), infant postnatal VAMS, and maternal blood proteomics, estimating BEP-versus-control ATEs per feature, timepoint, and compartment, with false-discovery-rate control within each compartment and visit (and, for pathways, ionization mode). Because feature intensities are not comparable across platforms, cross-compartment comparisons were based only on the direction and significance of the effect. Proteins were matched across milk and blood by UniProt accession; metabolite features were matched within ionization mode by accurate mass (±25 ppm), except maternal and infant postnatal VAMS, which share one feature catalog and were matched by feature identifier. Within each compartment, we tracked whether features that were significantly up-regulated (FDR < 0.05) at one visit stayed significant at the next (retention = the fraction of the preceding visit's significant features that remained significant at the current visit). Maternal plasma and VAMS were combined as maternal blood when summarizing the number of biological compartments represented. Up-regulated features were then linked across maternal blood, milk, and infant blood by shared feature identifier, identical putative annotation (unannotated features by identical *m/z*), with concordant effect direction. To assess compatibility with the intervention, matched features were compared against untargeted metabolomics from 18 BEP-supplement replicates on the same platform (±25 ppm); the putative annotations of the linked features were analyzed locally in MetaboAnalystR (KEGG pathway module; hypergeometric over-representation with topological impact) to identify the pathways they drive (Fig. 6D). We also checked whether effects agreed in direction across all features, not just the significant ones, using rank-based methods that do not rely on a significance cutoff. Because Mummichog enrichment is direction-agnostic, we assigned a direction to each pathway with a binomial sign test: we took the features Mummichog mapped to the pathway, recorded the sign of each feature's BEP effect, and tested whether the fraction with positive effects differed from 0.5 (pathways with at least four member features; nominal *P*-values). Because matches rest on accurate mass without authentic-standard or tandem-MS confirmation, all feature identities, supplement matches, and pathway assignments are provisional (annotation ongoing).

Online resource

The online resource (*21*) includes searchable tables of all results, balance tables of trial baseline covariates, interactive visualizations of individual HM components by study arm, and results of sensitivity analyses, including unadjusted intervention effects and intervention effects on unscaled outcomes.

Ethics, oversight, and registration

Secondary analysis of de-identified samples/data was approved by the University of Manitoba Human Research Ethics Board; each contributing study obtained local ethics approval and voluntary informed consent (*56–58*). The University of Manitoba approval ID is HS23767. IMiC is registered at ClinicalTrials.gov (NCT05119166); the constituent trials are registered as NCT03533712 (MISAME-III), NCT03564652 (Mumta-LW), and NCT03268902 (ELICIT). The protocol paper provides full governance, data-flow, and laboratory/analytic standard operating procedures (*20*).

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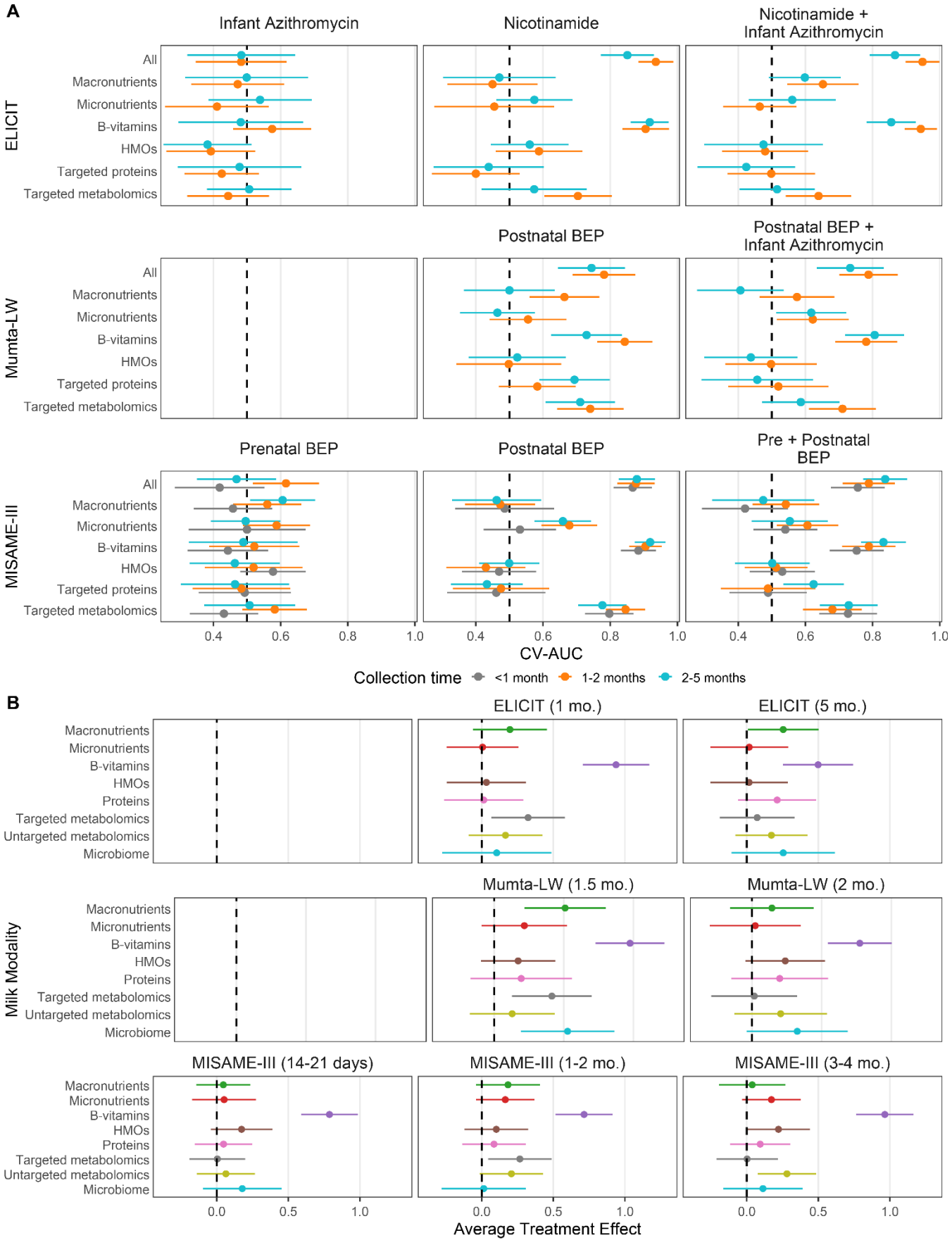
Author contributions: Conceptualization: T.D.-C., A.Me., M.B.A., L.H.A., A.H., C.L., N.A., M.D.B., F.J., and L.S. Methodology: T.D.-C., A.Me., K.F., C.-H.S., L.D., A.H., and N.A. Investigation: T.D.-C., K.F., A.J., M.B.M., L.C.T., A.Mu., A.P., L.H.A., J.-A.B., M.R.B., K.A.L., N.S., J.E.V., L.B., M.J., D.H., S.S.F., C.L., M.D.B., F.J., N.A., L.S., and J.M.M. Visualization: T.D.-C., A.Me., and M.B.A. Funding acquisition: M.B.A., C.L., M.D.B., F.J., L.C.T., and T.D.-C. Project administration: K.F. and M.B.A. Supervision: M.B.A., C.L., M.D.B., and F.J. Writing – original draft: T.D.-C. and A.Me. Writing – review and editing: K.F., C.-H.S., A.J., M.B.M., L.D., L.C.T., A.Mu., A.P., Y.S., N.I., W.K., M.I.N., M.B.A., L.H.A., J.-A.B., M.R.B., K.A.L., N.S., J.E.V., L.B., M.J., D.H., S.S.F., C.L., M.D.B., F.J., N.A., L.S., and J.M.M. **Competing interests:** M.B.A. has received speaking honoraria from nonprofit organizations that support breastfeeding (Institute for the Advancement of Breastfeeding & Lactation Education, Thai Breastfeeding Centre, UK Baby Friendly), and companies that produce human milk-related products (Prolacta Biosciences, Medela). She is a scientific advisor to TinyHealth (an infant microbiome testing company) and has consulted for DSM and AIIG (food ingredient companies). L.B. is a coinventor on patent applications related to the use of HMOs in preventing necrotizing enterocolitis (NEC) and other inflammatory diseases. N.A. is a member of the Scientific Advisory Boards of January AI, Parallel Bio and WellSim Biomedical Technologies, is a cofounder of Takeoff41 and is a paid consultant for MaraBio Systems. K.A.L. and M.J. hold equity and positions at Sapient Bioanalytics, LLC. The remaining authors declare that they have no competing interests. **Data, code, and materials availability**: Our data analysis code is deposited on GitHub at https://github.com/amertens/IMiC-intervention-effects-public and archived on Zenodo at 10.5281/zenodo.22104233 (*21*). Multi-omics human milk metabolomics (MSV000102534) and proteomics (MISAME: MSV000100366, Mumta-LW: MSV000100367, ELICIT: MSV000100365) datasets are available on MassIVE. Due to participant privacy protections and the terms of study consent across the IMiC studies, other individual-level data cannot be deposited in a public repository. De-identified datasets and accompanying metadata will be made available to qualified investigators upon reasonable request, contingent on approval by the relevant institutional review boards and execution of appropriate data-sharing agreements. No new materials were created in this study. **License information:** Copyright © 2026 the authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original US government works. https://www.science.org/content/page/science-licenses-journal-article-reuse

SUPPLEMENTARY MATERIALS

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Tables S1, S3, S6, and S7

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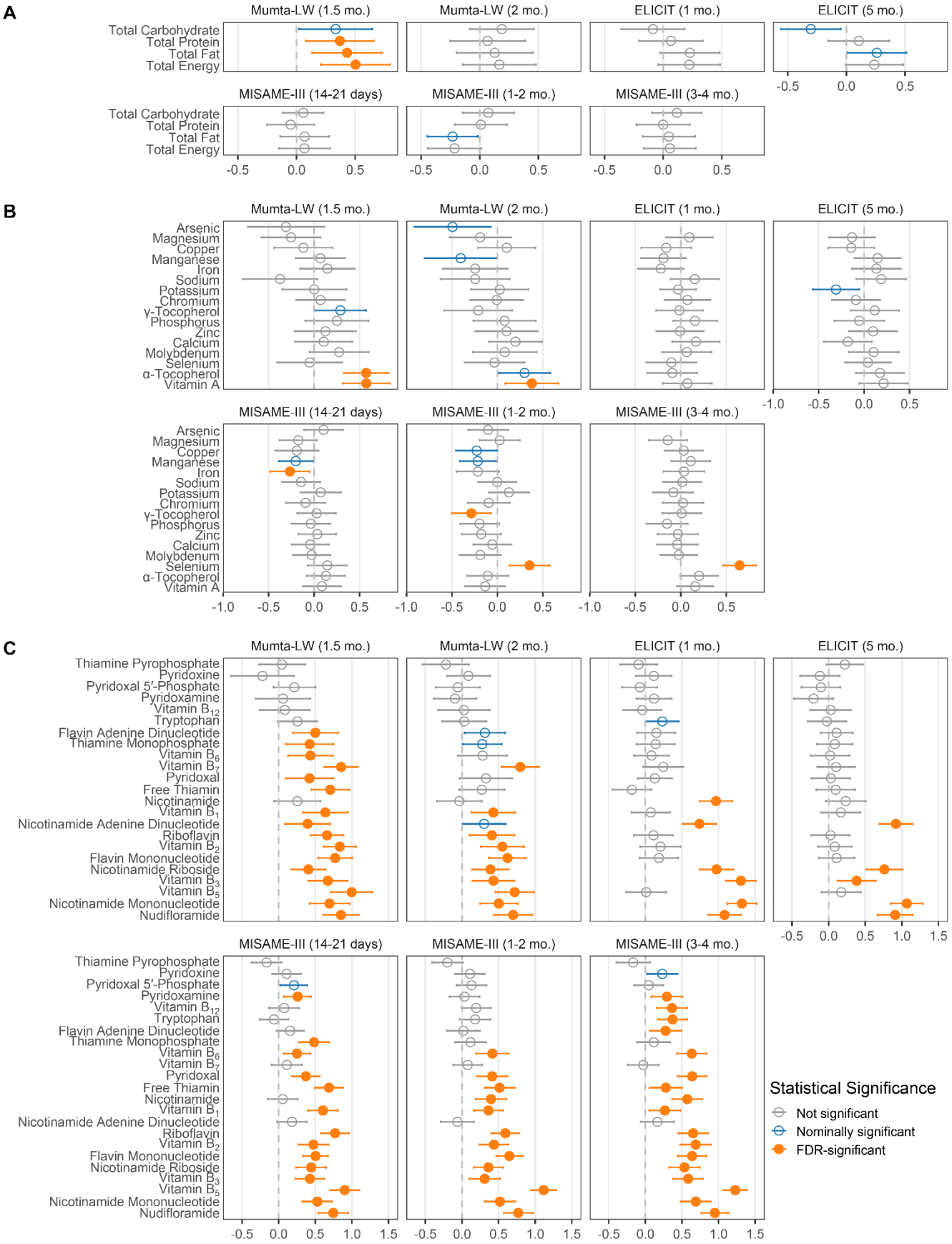
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Fig. 1. Impact of maternal BEP or nicotinamide interventions on different classes of human milk components. (**A**) The results of an ensemble machine learning analysis identifying treatment arm from class of milk components, or from all classes of components combined, with the *x* axis showing the cross-validated area under the receiver operating characteristic curve, where a CV-AUC of 0.5 is analogous to a random prediction of the treatment arm. The "micronutrient" category is all minerals and non–B vitamin micronutrients. Predictions are stratified and colored by milk collection visit. (**B**) The effects of postnatal BEP and nicotinamide interventions on the first principal component from principal component analysis of each class of milk components. Effects are scaled to always be in the positive direction for comparability. Based on results in (A), the two postnatal nicotinamide arms are pooled in ELICIT, and the two postnatal BEP arms are pooled in Mumta-LW and MISAME-III.

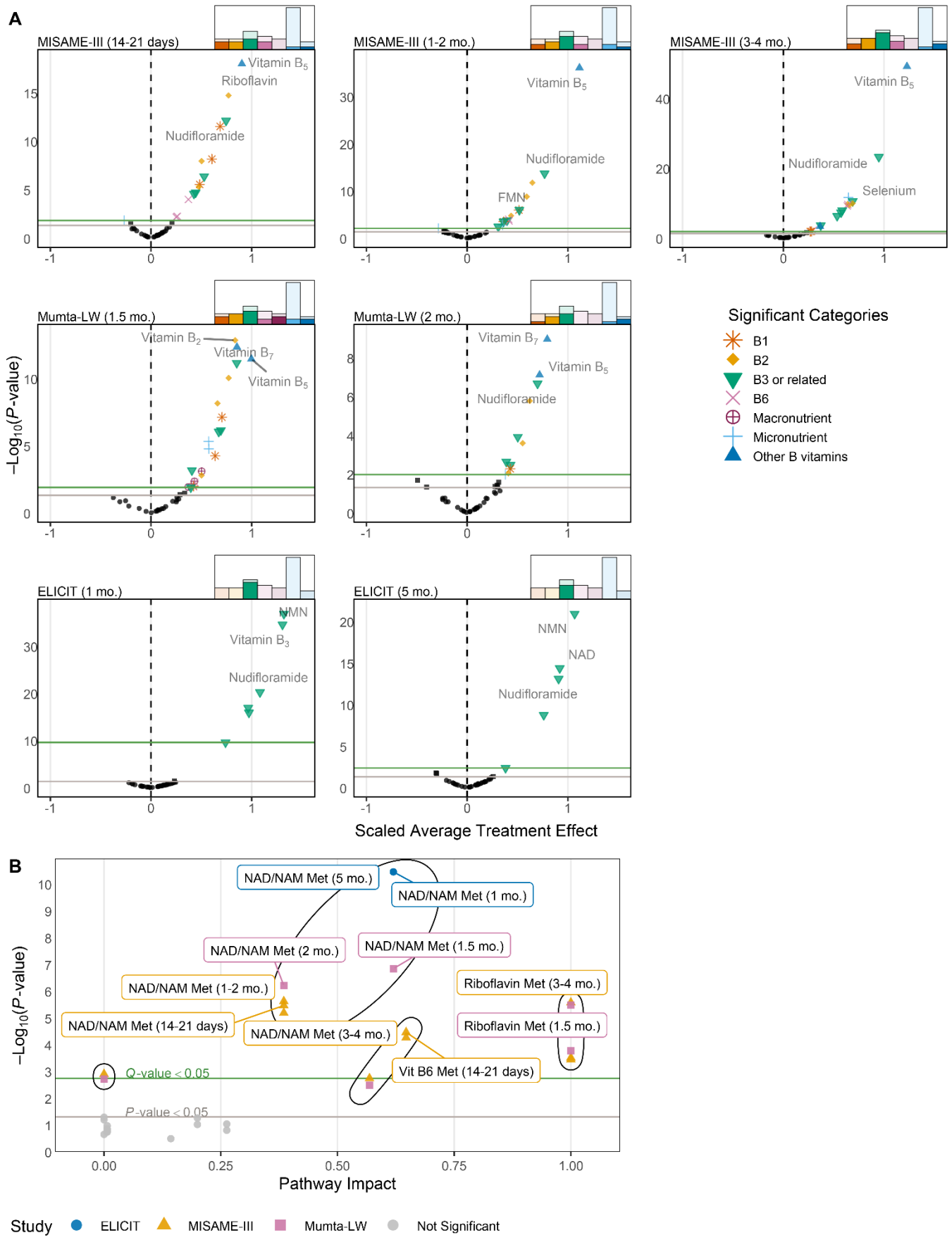
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Fig. 2. **Maternal nutritional intervention effects on human milk nutrients (primary outcomes).** Average treatment effects (ATEs) and 95% confidence intervals are plotted, with each row a separate milk component. Panels are split by component type: (**A**) macronutrients, (**B**) micronutrients and fat-soluble vitamins, and (**C**) B vitamins and metabolites. The milk components were scaled (z-scored) prior to targeted maximum likelihood estimation of the ATE by study and milk collection visit. Intervention arms were combined (detailed in methods), contrasting nicotinamide arms versus the control arm in ELICIT, BEP arms (with or without azithromycin) versus the control arm in Mumta-LW, and postnatal BEP supplementation arms versus nonpostnatal BEP supplementation arms in MISAME-III. Statistics: ATEs estimated by targeted maximum likelihood estimation (TMLE); *P*-values FDR-corrected (Benjamini–Hochberg, $Q < 0.05$) within outcome group, study, and visit.

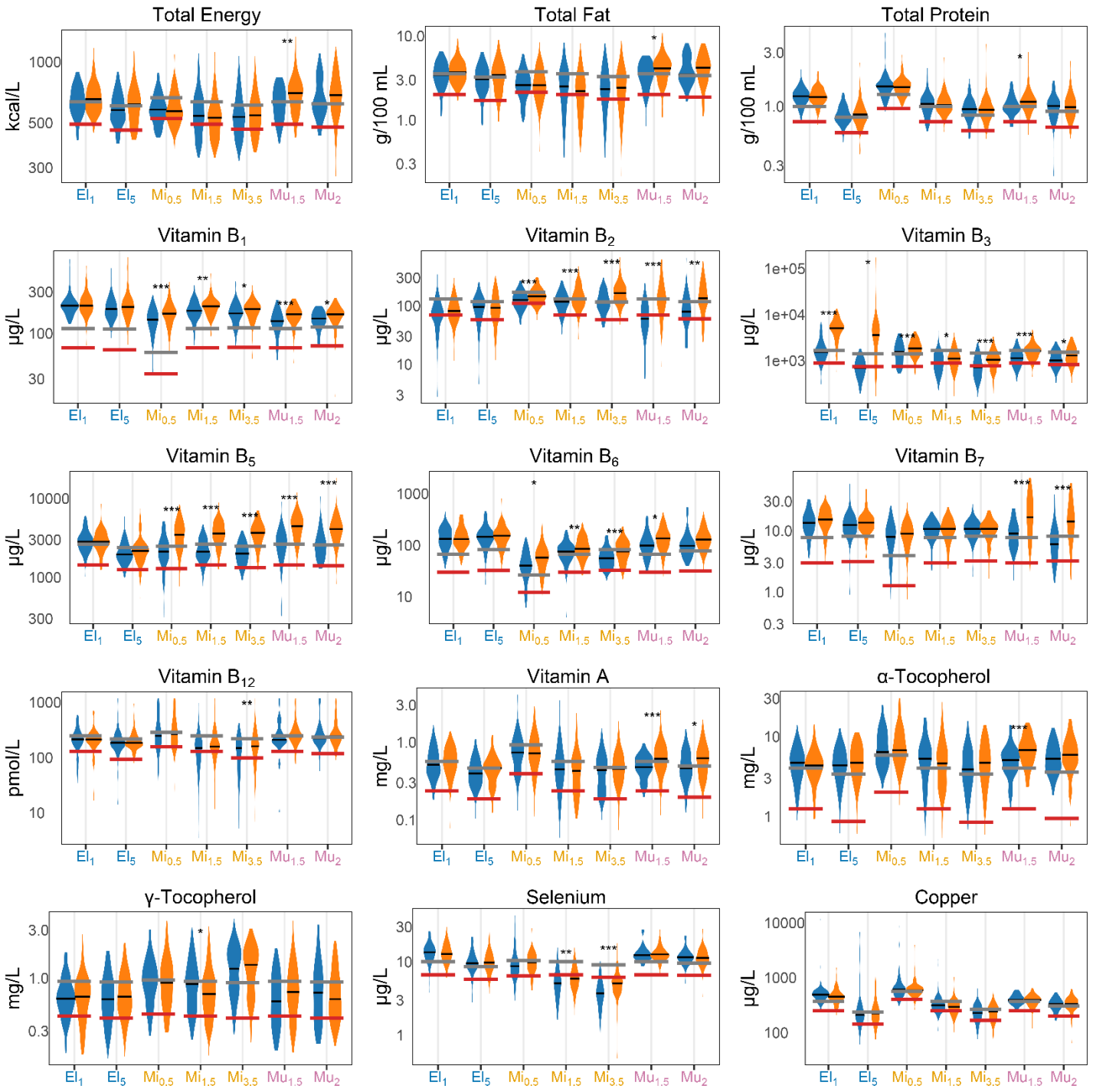
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Fig. 3. Maternal nutritional intervention effects on human milk micronutrients and macronutrients (primary outcomes). (A) Volcano plots of intervention effects on primary HM components (macronutrients, micronutrients, and vitamins). The horizontal gray line is the nominal significance cutoff (P-value < 0.05), while the green line denotes the significance cutoff after FDR adjustment for multiple comparisons. For milk components significant after FDR correction, points are colored and shaped by the category of milk components (other points are black), and the points are annotated with the labels of the three most significant milk components, with metadata in Section 5 of the online resource (*21*). Light-colored bar plots show the percent of all HM components in each category, overlaid with darker-colored bar plots showing the percent of HM components in that category significantly affected by intervention after FDR correction. (B) B vitamin–related KEGG metabolic pathways for the primary micronutrient outcomes. Each point is one pathway in one study–timepoint combination, plotting the pathway's topological impact (x) against its enrichment $-\log_{10}(P\text{-value})$ (y), colored and shaped by study; nonsignificant results are shown in gray. The horizontal gray line denotes $P < 0.05$ and the green line the false discovery rate threshold ($Q < 0.05$). Ellipses group repeated measurements of the same pathway across timepoints, and pathways nominally enriched ($P < 0.05$) in at least two study–timepoint combinations are labeled. Statistics [(A) volcano]: ATEs by targeted maximum likelihood estimation (TMLE); P-values FDR-corrected (Benjamini–Hochberg, $Q < 0.05$) within outcome group, study, and visit. Statistics (B): KEGG pathway over-representation (hypergeometric) with relative-betweenness centrality for topological impact; Benjamini–Hochberg FDR ($Q < 0.05$). Abbreviations: BEP, balanced energy–protein; IFA, iron–folic acid.

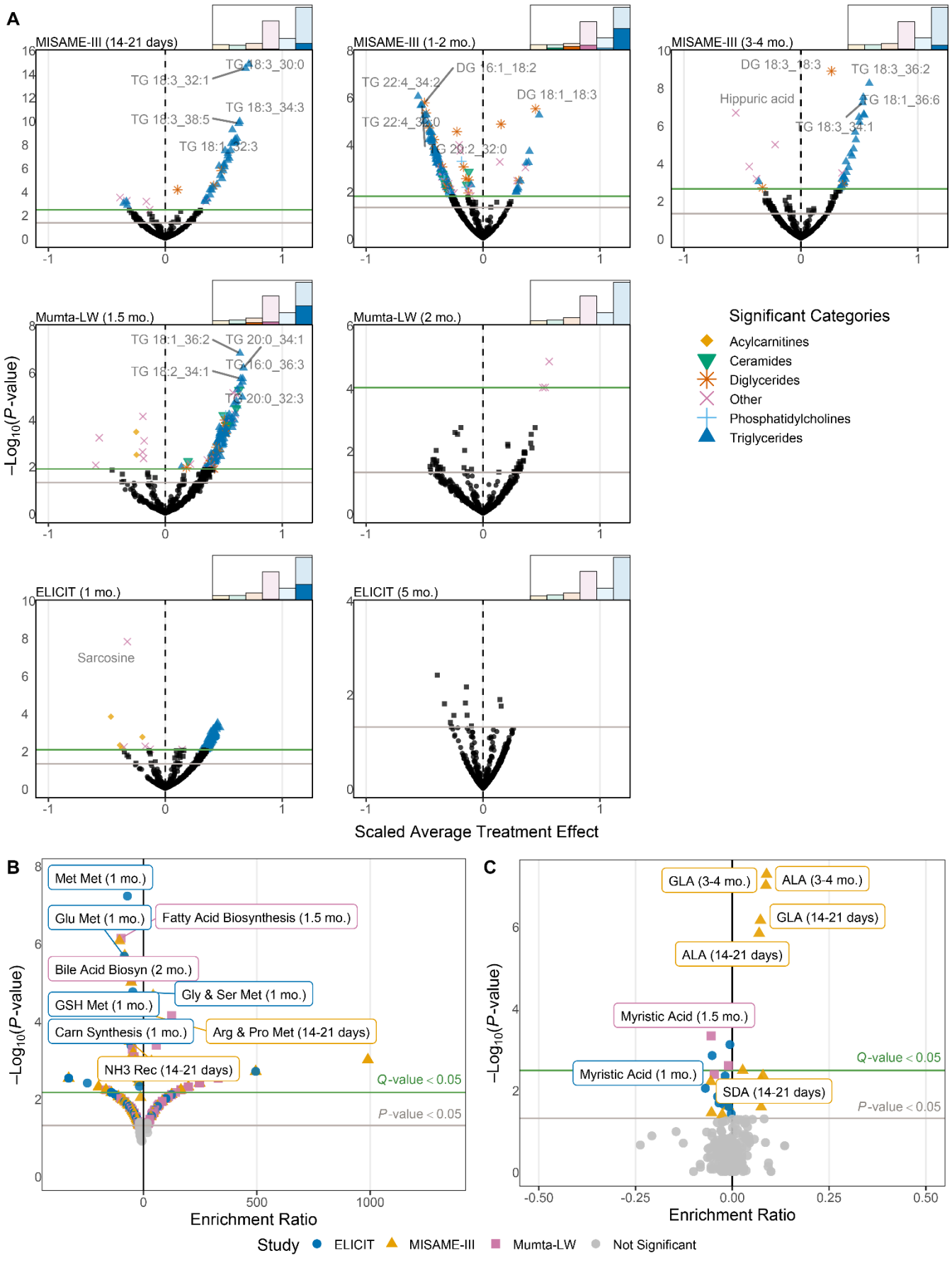
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Fig. 4. IMiC distributions of HM nutrients relative to MILQ reference standards. Each panel shows one human milk nutrient as violin plots of its concentration in native units, with the horizontal line within each violin marking the median. Horizontal gray and red lines mark the 50th and 10th percentiles of the age-specific MILQ reference distribution, respectively. Distributions are stratified by study (EI, ELICIT; Mi, MISAME-III; Mu, Mumta-LW) and visit in months, given together in the *x* axis labels, and by intervention arm, colored blue for the control arm and orange for pooled intervention arms. Asterisks mark ATEs significant after FDR correction (* = Q-value < 0.05, ** = < 0.01, *** = < 0.001). *Y* axes use a log scale.

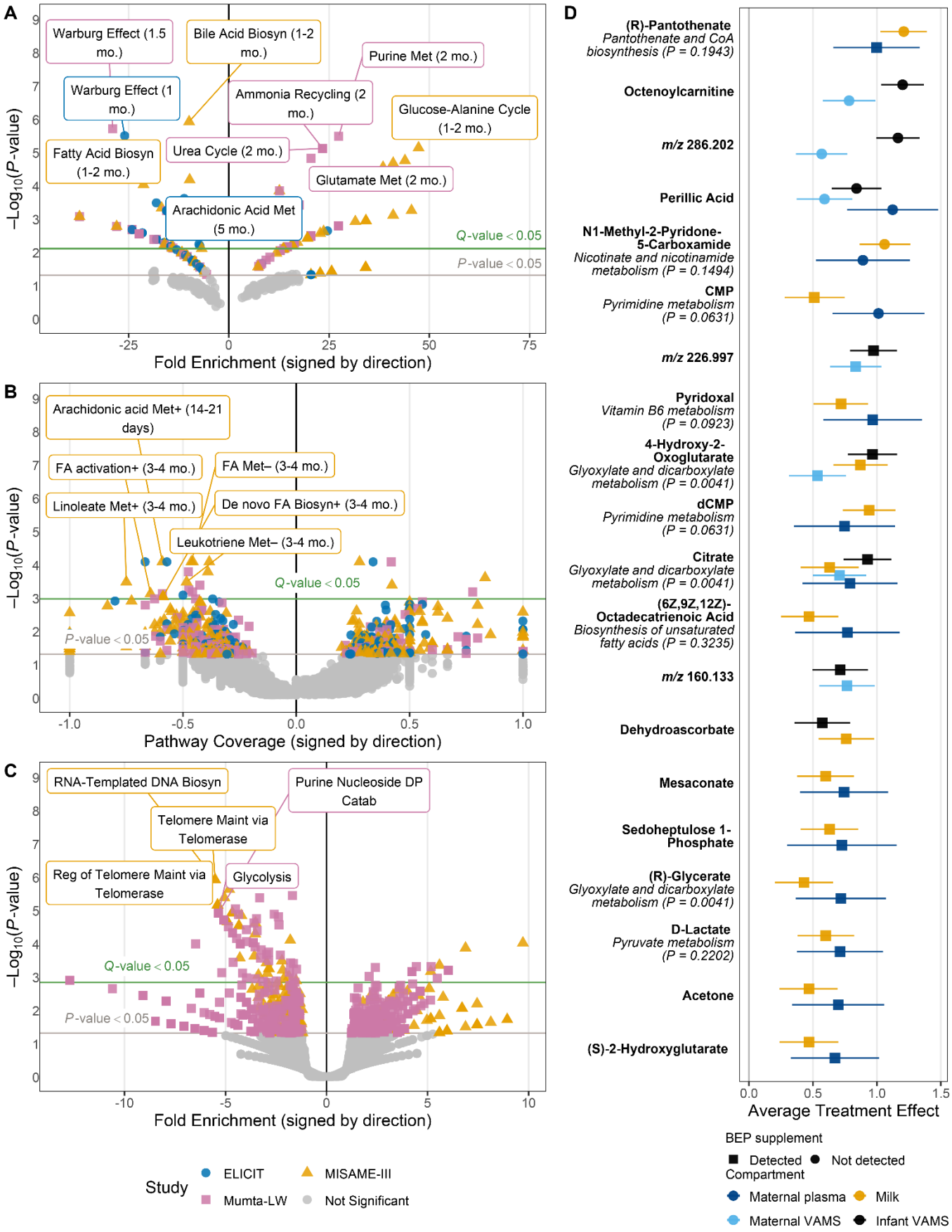
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Fig. 5. Maternal nutritional intervention effects on targeted metabolites in human milk (tertiary outcomes). (**A**) Volcano plot of intervention effects on targeted metabolites, with plot conventions as in Fig. 3A and the top 5 most significant components labeled; metadata are in Section 5 of the online resource (*21*). Bar plots show, by category, the percent of all targeted metabolites (light) versus the percent significantly affected after FDR correction (dark). (**B**) Enriched pathways identified by MSEA. Points represent pathway–timepoint–study combinations and are colored and shaped by study; nonsignificant results are shown in gray. Positive enrichment ratios reflect enrichment among up-regulated features, whereas negative values indicate enrichment among down-regulated features. The horizontal gray line denotes $P < 0.05$ and the green line indicates the FDR threshold ($Q < 0.05$). A representative subset of pathways is annotated. (**C**) Enriched fatty acids (unadjusted for total fat) derived from triglyceride–fatty acid conversion analyses. Positive enrichment ratios indicate fatty acids associated with up-regulated triglycerides, whereas negative values indicate fatty acids associated with down-regulated triglycerides. The same significance thresholds and color conventions as (B) apply. Statistics: (A) shows TMLE-derived ATEs with Benjamini–Hochberg FDR correction ($Q < 0.05$). (B) (MSEA) and (C) (triglyceride-to-fatty-acid compositional enrichment, Wilcoxon rank-sum) use Benjamini–Hochberg FDR ($Q < 0.05$). Abbreviations: ALA, α-linolenic acid; Arg, arginine; Asp, aspartate; BCFAs, branched-chain fatty acids; Carn, carnitine; GLA, γ-linolenic acid; Glu, glutamate; Gly, glycine; GSH, glutathione; Met, metabolism; NH_3, ammonia; Pro, proline; Rec, recycling; Ser, serine; Syn, synthesis; UreaC, urea cycle; VLCFAs, very-long-chain fatty acids; β-Ox, β-oxidation.

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Fig. 6. Maternal nutritional intervention effects on the untargeted metabolome and proteome of human milk (exploratory outcomes). (**A**) Enriched pathways identified by metabolite set enrichment analysis (MSEA) of untargeted metabolomics. Points represent pathway–timepoint–study combinations and are colored and shaped by study when significant; nonsignificant results are shown in gray. Positive enrichment ratios indicate enrichment among up-regulated features, whereas negative enrichment ratios indicate enrichment among down-regulated features. The horizontal gray line denotes $P < 0.05$ and the green line indicates the FDR threshold ($Q < 0.05$). The top 8 pathways by significance are annotated. (**B**) Enriched pathways identified by Mummichog analysis of untargeted metabolomics. Positive and negative annotations (±) denote pathways identified from positive or negative ionization modes, respectively. The *x* axis is the fraction of each pathway's compounds among the significant features, signed by direction. The horizontal gray line denotes $P < 0.05$ and the green line indicates the FDR threshold ($Q < 0.05$). (C) Enriched pathways identified by gene ontology over-representation analysis (GO ORA) of untargeted proteomics, plotted as signed fold enrichment against $-\log_{10}(P)$. The same point colors and symbols by study, significance thresholds, and annotation conventions as in (B) apply. (D) Cross-compartment comparison of up-regulated BEP-associated metabolite features (MISAME-III). Each row is one metabolite that was up-regulated and linked across at least two compartments (20 metabolites, 44 compartment-level estimates). Features were linked across compartments by identical putative name (unannotated features by exact *m/z*), so a feature without an annotation on one platform is not linked there; the octenoylcarnitine increases in milk and maternal plasma are therefore shown in table S8 rather than here. Points are ATEs with horizontal 95% confidence intervals, colored by compartment [maternal plasma, maternal dried-blood microsamples (VAMS), milk, infant VAMS], and shaped by whether the feature was detected in the BEP supplement analyzed on the same platform; the vertical gray line marks a null effect. Where a metabolite was significant in one compartment at more than one visit, its largest effect in that compartment is plotted, so each metabolite contributes at most one point per compartment; rows are ordered by each metabolite's largest effect. *Y* axis labels give the putative metabolite name and, for the 10 metabolites with a KEGG pathway assignment, the single most enriched pathway with its nominal over-representation *P*-value (table S11). Metabolite identities are putative (accurate-mass based). Statistics: (A) and (B) use untargeted-metabolomics pathway enrichment [MSEA in (A) Mummichog in (B); (C) uses gene ontology ORA (clusterProfiler::enricher with a custom UniProt-to-GO map)]. All pathway *P*-values in (A) to (C) are Benjamini–Hochberg FDR-corrected ($Q < 0.05$). (D) shows within-compartment standardized ATEs estimated by TMLE; because feature intensities are not comparable across analytical platforms, effects are comparable across compartments in direction and significance rather than in absolute magnitude, and the pathway *P*-values shown on its axis labels are nominal. Abbreviations. Act, activation; Ala, alanine; CENP-A, centromere protein A; Chr, chromosome; Chrom, chromatin; Deg, degradation; Diff, differentiation; Endopept, endopeptidase; ETC, electron transport chain; FA, fatty acid; FA Syn, fatty acid synthesis; Form, formation; Gly, glycine; Hydrol, hydrolase; Ile, isoleucine; Leu, leucine; Loc, localization; Met, metabolism; Mk, megakaryocyte; Myeloid, myeloid cell; Neg, negative; Nuc, nucleosome; Pept, peptidase; Pro, proline; Prot, protein; Reg, regulation; Ser, serine; Syn, synthesis; TCA, tricarboxylic acid cycle; Thr, threonine; UreaC, urea cycle; Val, valine; Warburg, Warburg metabolism.

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Nutritional interventions' impacts on human milk: Three trials in low-resource settings

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