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Acute Moderate-Intensity Postpartum Exercise Does Not Elicit Exercise-Specific Changes in Human Milk Immune-Regulatory Proteins

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Abstract

Human milk carries immune-regulatory proteins that influence infant immune development, but whether acute maternal exercise alters this profile is unknown. Whole milk from nine women was analyzed for 75 immune-regulatory and cytokine proteins before and after time-matched sedentary and moderate-intensity exercise visits. Although nine proteins increased post-exercise, the sedentary condition induced similar effects, and no condition-by-time interactions were detected, demonstrating no exercise-specific effect on human milk immune-regulatory protein profile.

Main text

During early infancy, before a newborn's own adaptive immune system has matured, breast milk supplies much of the immune protection that the infant needs [1]. Beyond well-known components such as immunoglobulins, lactoferrin, lysozyme, and human milk oligosaccharides, breast milk also carries a range of cytokines and other immune regulatory proteins which support mucosal immune defense, help the infant gut lining mature, and shape immune development in ways that can persist well beyond infancy [2–4]. The cytokine profile of breast milk is shaped by both maternal and lactational factors, including maternal health, parity, delivery mode, stage of lactation, and diet, which have been linked to infant immunity, growth patterns, and respiratory outcomes [4–6]. Because breast milk cytokines can be influenced by everyday maternal behaviors, understanding which exposures shape them carries translational importance.

Exercise is a well-established modulator of circulating cytokine activity, and regular moderate-intensity exercise training produces a broadly anti-inflammatory effect [7]. Acutely, skeletal muscle contraction increases myokine release, including IL-6, which promotes anti-inflammatory mediators such as IL-1ra and IL-10 while suppressing TNF-α [7–9]. Leading obstetric organizations, including the American College of Obstetricians and Gynecologists and the U.S. Department of Health and Human Services, recommend that postpartum women get at least 150 minutes of moderate-intensity aerobic activity each week and have stated explicitly that this level of activity does not interfere with lactation [10,11]. What remains unclear is whether the systemic immune effects of exercise now discussed under the umbrella term "exerkines" also reach the immune compartment of human milk [12,13].

Existing studies examining maternal exercise and human milk have largely focused on milk production, macronutrient composition,[14] and selected immune factors[15]. Controlled studies suggest that acute moderate-intensity exercise does not substantially alter immunoglobulin A, lactoferrin, or lysozyme, whereas more strenuous exercise may produce transient changes in specific immune components[16,17]. However, whether a single bout of moderate-intensity exercise acutely alters the broader immune-regulatory protein profile of human milk remains unclear, particularly under controlled, time-matched conditions. Therefore, we examined whether a single bout of moderate-intensity aerobic exercise alters

cytokines and other immune-related factors in human milk compared with a time-matched sedentary condition.

To determine whether an acute bout of moderate-intensity exercise alters the immune-regulatory protein profile of human milk, nine healthy lactating women (**Supplementary Table. 1**) completed a dietary-controlled, within-participant study consisting of a sedentary control visit followed by an exercise visit on consecutive days (**Figure. 1**). Morning feeding, complete expression of both breasts, standardized breakfast intake, and pre-collection rest were time-matched across visits. During the exercise visit, participants completed 30 minutes of inclined treadmill walking at approximately 70% of VO_2max whereas during the sedentary visit they remained at rest. Serial whole-milk samples were collected before and 30 minutes after each condition and analyzed using the Olink Cytokine and Immune Surveillance panels, yielding absolute concentrations for 75 immune-regulatory proteins.

In within-condition analyses, nine proteins increased from pre- to post-exercise after multiple comparison correction, including interleukin-18 (IL-18), IL-33, IL-17C, placental growth factor (PGF), Fms-related tyrosine kinase 3 ligand (FLT3LG), keratin 18 (KRT18), amphiregulin (AREG), CD80, and oncostatin M (OSM) (log2FC, 0.17–1.17; FDR-adjusted $P < 0.05$; **Figure. 2A**). No proteins decreased significantly following exercise. Changes were also observed during the time-matched sedentary condition. IL-18 and PGF increased, whereas erythropoietin (EPO) and thymic stromal lymphopoietin (TSLP) decreased, after FDR correction (**Figure. 2B**). Moreover, each of the nine proteins that increased significantly following exercise also exhibited a positive pre-to-post change during the sedentary condition, although most did not meet the within-sedentary FDR threshold. Across the full protein panel, the pre-to-post Log2 fold-changes during the exercise and sedentary conditions were positively associated (**Figure. 2C**). For example, paired participant-level concentrations of IL-18, IL-33, and PGF, the three proteins with the largest pre-to-post increases during the exercise visit, showed generally upward trajectories during both visits (**Figure. 2D**). Consistent with these parallel temporal responses, Two-way repeated-measures ANOVA identified a nominal condition-by-time interaction for CD28 ($P = 0.036$), which did not survive FDR correction and was not supported by Sidak-adjusted pairwise comparisons. No other condition-by-time interactions were significant (all $P \geq 0.05$) (**Supplementary Table. 2**). Thus, although nine immune-regulatory proteins increased significantly within the exercise visit, the magnitudes of these changes were not statistically distinguishable from those observed during the time-matched sedentary visit. These findings therefore do not provide evidence that a single bout of moderate-intensity exercise produces an exercise-specific alteration in the measured whole-milk immune-regulatory protein profile.

We examined whether a single bout of moderate-intensity aerobic exercise acutely alters the cytokine and immune-regulatory protein profile of human milk. Although short-term changes in several proteins were observed, none demonstrated a significant condition-by-time interaction passing FDR-correction when compared with the time-matched sedentary condition. Thus, we interpret the observed within-exercise increases as a short-term temporal responses shared across visits rather than exercise-specific effects.

Importantly, the absence of condition-by-time interactions should not be interpreted as evidence that the exercise stimulus was biologically inert. In a companion analysis of the same cohort, the same acute exercise bout increased breast milk small extracellular vesicle (sEV) concentration, enriched sEV-associated proteomic, miRNA, and acylcarnitine signals, and increased mitochondrial respiratory capacity in neonatal-stage cells exposed to post-exercise sEV preparations [18]. The contrast between studies suggests compartment-specific responsiveness, where acute exercise may modify an sEV-associated signaling compartment without measurably altering the immune-regulatory proteins assayed in whole milk. The present findings also do not exclude responses later in recovery, which were not assessed.

Determining whether exercise alters human milk cytokines and growth factors is important because of their role in neonatal mucosal defense, epithelial maturation, and immune development [3,5]. IL-18, one of the strongest within-exercise signals in this study, is produced by lactating mammary epithelium and has been proposed to support neonatal host defense[19]. Studies further show that selected milk-borne proteins, including IL-8 and epidermal growth factor, can retain structural or functional integrity during neonatal gastric digestion[20]. These observations support the plausibility of local signaling in the infant gastrointestinal tract, but they cannot be generalized to every analyte measured here and do not establish systemic transfer. We did not assess digestion, uptake, bioactivity, or infant outcomes. Nevertheless, the absence of a significant exercise-specific reduction provides preliminary reassurance that moderate exercise does not acutely deplete the measured whole-milk immune-regulatory factors after exercise.

An important strength of the present study was the inclusion of a time-matched sedentary condition. Human milk is a dynamic biological fluid whose composition varies with lactation stage, maternal factors, sampling conditions, and time [4–6,21]. Previous work has also demonstrated changes in milk IgA during repeated sampling under both exercise and resting conditions[17]. A recent randomized crossover study found time-dependent changes in milk insulin despite no exercise effect[22] and identified a post-exercise increase in adiponectin compared to a sedentary condition[23]. Without the sedentary visit, the nine within-exercise changes observed here could have been misattributed to exercise. Statistical significance within one visit but not the other also does not establish a differential response; that inference depends on the condition-by-time interaction. These findings emphasize the importance of time-matched control conditions when evaluating acute exercise changes in human milk and suggest that pre-post comparisons alone may overestimate exercise-specific effects.

Our findings are consistent with previous controlled studies showing no acute effect of moderate-intensity exercise on breast milk IgA, lactoferrin, or lysozyme [16]. The stability of the human milk immune-regulatory protein profile is particularly notable given the well-established systemic response to exercise. Skeletal muscle contraction induces the release of cytokines and other exercise-responsive signaling molecules, collectively described as exerkines [7–9,12]. However, a single bout of exercise cannot determine whether repeated exercise produces longer-term adaptations. A prenatal exercise training

intervention was associated with differences in IL-8 and TNF-α in colostrum, and maternal aerobic fitness has been associated with breast milk hormone concentrations[24], but these studies do not establish the effects of exercise training initiated during lactation[25]. Randomized postpartum exercise-training studies with larger and more diverse cohorts, serial sampling, concurrent maternal plasma measurements, and compartment-specific milk analyses are warranted to relate molecular changes in breast milk to infant outcomes[26].

The within-participant design, standardized dietary intake, controlled milk collection, and individualized exercise intensity strengthened the experimental design. However, the small sample size and relatively healthy cohort limit generalizability and the ability to exclude modest effects. The study also assessed only one exercise modality and intensity over a short sampling window. Although placing the sedentary visit first reduced residual exercise effects on the subsequent visit, the fixed order prevented the conditions from being fully separated from day or order effects. The nominal condition-by-time interaction observed for CD28 warrants further investigation in larger studies with varied exercise protocols, extended sampling, and repeated training to determine whether this finding is reproducible.

In conclusion, acute moderate-intensity aerobic exercise did not elicit a distinct shift in the cytokines and immune factors of human milk. Current guidelines recommend at least 150 minutes of moderate-intensity aerobic physical activity per week during the postpartum period and support exercise during lactation [10,11]. These findings provide further reassurance regarding the efficacy of moderate-intensity exercise during breastfeeding and warrant future studies to investigate if regular exercise training produces adaptations to breast milk.

Methods

Study Design and Ethical Approval

This within-participant, time-matched study was conducted at the Mayo Clinic Clinical Research and Trials Unit in Rochester, Minnesota, USA, in accordance with the Declaration of Helsinki. All study procedures were approved by the Mayo Clinic Institutional Review Board and registered with ClinicalTrials.gov (NCT06892483), and all participants provided written informed consent.

The protocol comprised one screening visit followed within 60 days by two outpatient study visits conducted on consecutive days. The sedentary visit was completed first, followed by the exercise visit, to avoid carryover from exercise into the control condition. The same participants and study visits were included in a companion analysis of breast milk small extracellular vesicles[18]. Although eleven participants were included in our previous analyses[18], the present study examining immune-regulatory proteins in whole milk included the nine participants with whole-milk samples available at all four analyzed time points (Pre-Sed, Post-Sed, Pre-Ex, and Post-Ex).

Participants

Participants were recruited through word of mouth and flyers posted in lactation rooms across the Mayo Clinic campus. Eligible women were ≥ 18 years of age, ≥ 2 months postpartum, actively lactating following a singleton pregnancy, and were able to express ≥ 3 oz of breast milk per pumping session. Exclusion criteria were a body mass index ≥ 30 kg m^{-2}; fasting glucose ≥ 100 mg dL^{-1}; cardiovascular disease; impaired renal function (glomerular filtration rate < 60 mL min^{-1} 1.73 m^{-2}); impaired hepatic function (aspartate aminotransferase > 2× the upper limit of normal); poorly controlled thyroid disease (undetectable TSH or > 10 mIU L^{-1}); uncontrolled hypertension (resting blood pressure > 160/90 mmHg); a positive pregnancy test; current smoking or tobacco use; or alcohol abuse.

Screening Assessments

At the on-site screening visit, fasting blood samples were obtained for measurement of plasma glucose, insulin, and a comprehensive chemistry panel (complete blood count, serum creatinine, liver enzymes, thyroid-stimulating hormone [TSH], and lipid profile). A physical examination with vital signs was performed, and a urine test confirmed the absence of pregnancy. Following consultation with a research dietician, body composition was measured by dual-energy X-ray absorptiometry.

Maximal oxygen uptake was estimated using a submaximal modified Balke protocol [27]. Participants began walking at a self-selected fixed speed of 3 to 3.5 mph at 0% grade. Treadmill grade was increased by 2.5% every 2 minutes while speed remained constant. Oxygen uptake was measured by continuous indirect calorimetry (Parvo Medics, Sandy, UT), with concurrent 12-lead electrocardiography (Quinton), and manual blood pressure measurement. The test was terminated when the participant reached 90% of their age-predicted maximal heart rate. Estimated maximal oxygen uptake (VO_2max) was obtained by extrapolating the participant-specific linear relationship between heart rate and VO_2 to age-predicted maximal heart rate.

Dietary standardization

Participants consumed weighed, standardized meals during the 2 days preceding the sedentary visit. Following completion of that visit, standardized meals were provided for the remainder of the day and for breakfast before the exercise visit. Meals provided 20% of energy from protein, 50% from carbohydrate, and 30% from fat. Resting energy expenditure was estimated using the Müller Eq. 2[8]. Breakfast provided 25% of each participant's prescribed daily energy intake.

Experimental visits

On the morning of each outpatient visit, participants first breastfed their infant, fully expressed both breasts, and immediately consumed the standardized breakfast at home. Participants arrived at the

Clinical Research and Trials Unit within 1 h of breakfast and remained sedentary until the first study milk collection, which occurred exactly 2 h after breakfast.

During the sedentary visit, the initial breast milk sample was designated Pre-Sed. Participants subsequently remained sedentary for 90 min, with water permitted ad libitum but no caloric intake, after which the Post-Sed sample was collected. During the exercise visit, the initial sample was designated Pre-Ex. Participants remained sedentary for 30 min and then completed 30 min of inclined treadmill walking at a workload equivalent to approximately 70% of estimated $\dot{V}O_2$max. Treadmill speed and grade were selected on the basis of the screening exercise test. Rating of perceived exertion was recorded every 10 min using the Borg 6–20 scale. After exercise, participants remained sedentary for 30 min before collection of the Post-Ex sample. Thus, the interval between pre- and post-condition milk collections was 90 min during both visits, and the Post-Ex sample was obtained 30 min after completion of exercise. An additional sample was collected 120 min after exercise as part of the parent protocol but was not included in the present whole-milk protein analysis.

Human Breast Milk Collection and Storage

At each collection timepoint, both breasts were fully expressed. Milk from both breasts was pooled and gently mixed to reduce variability associated with differences between early- and late-expression milk. Approximately 50 mL was retained for research analysis and the remainder was returned to the participant. Whole-milk samples were divided into aliquots immediately after collection and stored at −80°C until analysis.

Whole Milk Immune-regulatory Protein Quantification

Frozen whole-milk aliquots from the Pre-Sed, Post-Sed, Pre-Ex, and Post-Ex time points were thawed and centrifuged in low bind tubes at 3,000 × g for 10 minutes at room temperature, and the skim milk fraction was collected. The skim milk fraction was then centrifuged twice at 10,000 × g for 10 minutes, and the resulting supernatant was collected after each centrifugation while avoiding any residual lipid layer. The resulting skim milk samples were analyzed using the Olink Target 48 Cytokine and Target 48 Immune Surveillance panels on the Olink Signature Q100 platform by the Mayo Clinic Targeted Biomarkers Core. These assays use proximity extension technology followed by quantitative polymerase chain reaction readout.

Absolute protein concentrations were calculated from analyte-specific calibration curvesand reported in pg mL^{-1}. All samples from an individual participant were run on the same plate with sample order randomized and laboratory personnel blinded to condition.

The two panels assayed 89 unique proteins. Proteins were retained for statistical analysis when concentrations within the quantifiable range were observed for all samples. Seventy-five proteins met

this criterion and were included in the final analysis, but data from all 89 proteins are provided in the supplementary data file.

Statistical Analysis

Protein concentrations were $\log_2$-transformed before inferential analysis. For each participant and protein, within-condition $\log_2$ fold change was calculated as $\log_2$ (post-condition concentration / pre-condition concentration). Within the exercise and sedentary visits, pre-to-post changes were evaluated using two-sided paired t tests on $\log_2$-transformed concentrations. To account for testing proteins within each of the two targeted Olink panels, P values were adjusted separately within each condition using the Benjamini–Hochberg false-discovery-rate procedure. An FDR-adjusted q value < 0.05 was considered statistically significant.

Exercise specificity was evaluated separately for each protein using two-way repeated-measures analysis of variance, with condition (sedentary vs exercise) and time (pre vs post) as within-participant factors. The condition-by-time interaction was the primary test of whether the pre-to-post response differed between conditions.

For descriptive comparison of temporal responses across the protein panel, protein-level $\log_2$ fold changes during the exercise visit were regressed against the corresponding $\log_2$ fold changes during the sedentary visit using simple linear regression. The coefficient of determination and two-sided P value for the regression slope are reported. Because individual proteins may be biologically correlated, this protein-level regression was treated as a descriptive panel-wide comparison rather than an independent participant-level test.

Analyses were performed using GraphPad Prism version 10.3.1. Statistical tests were two-sided, with P < 0.05 considered statistically significant unless adjustment for multiple testing was specified. No a priori sample-size calculation was performed because this was an exploratory analysis of available samples from the parent study.

Declarations

Competing Interests:

The authors declare no competing interests.

Author Contribution

V.D.P.: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. S.S.L.: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. K.A.K.: Investigation,

Methodology, Writing – review & editing. H.E.K.: Formal analysis, Funding acquisition, Data interpretation, Writing – review & editing. M.W.P.: Conceptualization, Methodology, Investigation, Supervision, Funding acquisition, Data interpretation, Writing – original draft, Writing – review & editing, Project administration.

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Data Availability

The data that support the findings of this study are available in the supplementary information file.

Code availability:

This study did not generate new materials or code.

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Figures

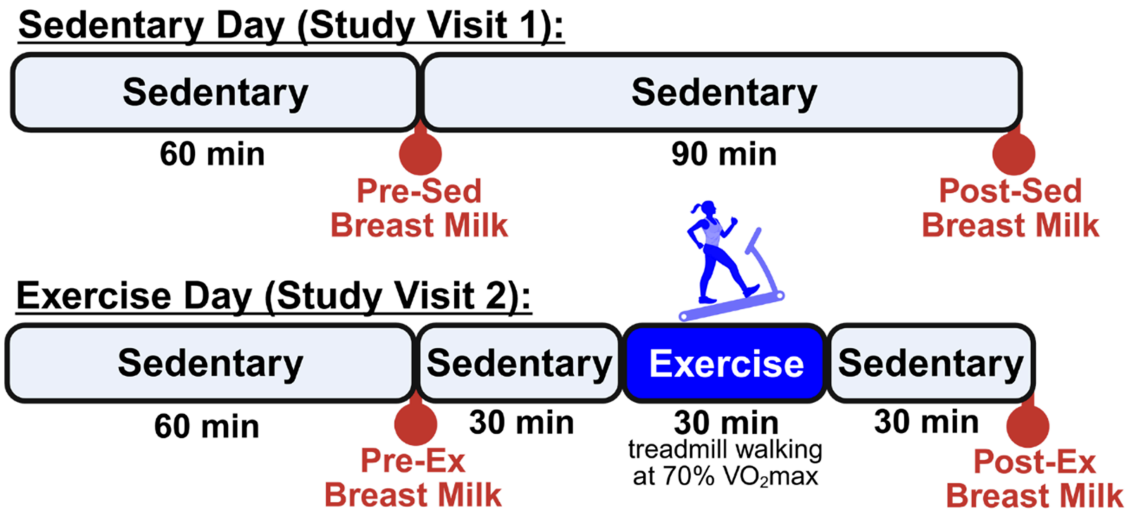


Figure 1

Study design overview. Breast milk samples were collected from nine healthy lactating women (maternal age of ≥ 18 years, ≥ 2 months postpartum) on sedentary (Sed) and exercise (Ex) study visits

(time-matched). Created in Biorender.

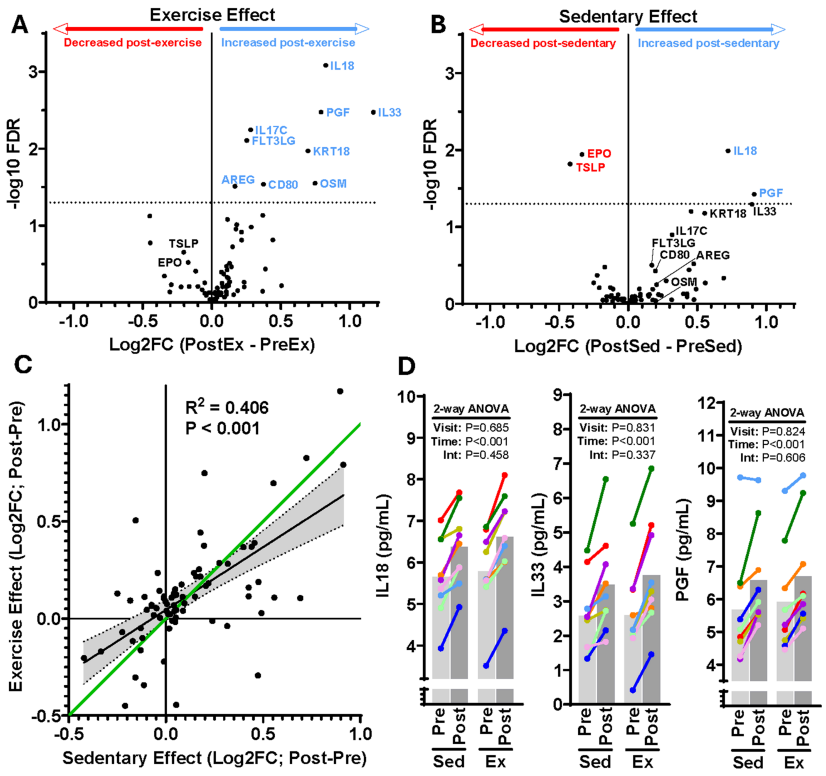


Figure 2

Changes in circulating proteins following exercise and time matched sedentary conditions.

(A and B) Volcano plots showing protein responses to exercise (A) and sedentary (B) conditions, expressed as log2 fold change (log2FC) with FDR adjusted significance. Significant proteins (FDR-adjusted $P < 0.05$) are highlighted in blue (increased) or red (decreased); dotted lines indicate the significance threshold.

(C) Association between log2 fold-changes in protein concentrations during the exercise and sedentary conditions. The black line and shaded area represent the linear regression and 95% confidence interval, respectively; the green line indicates the line of identity.

(D) Individual participant trajectories for IL-18, IL-33, and PGF during the sedentary and exercise conditions. Lines connect paired pre- and post-condition measurements; two-way ANOVA results are shown.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryDataFile.xlsx
- SupplementaryMaterials.docx