Sialic acid levels in breast milk from HIV-positive Tanzanian women and impact of maternal diet.

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**ABSTRACT**

***Objective*:** To quantify total sialic acid in milk from HIV-positive Tanzanian mothers and to determine the impact of maternal diet on milk sialic acid levels*.*

***Design*:** Milk samples were analyzed from 74 HIV-positive, Tanzanian women enrolled in a randomized, controlled clinical study of a dietary macronutrient supplement. Women were provided with a daily protein-calorie supplement plus a micronutrient supplement (PCS+MNS) or MNS only during the last trimester of pregnancy and up to the first six months of breastfeeding.

***Methods:*** Milk samples were collected at approximately two weeks and ≥3 months post-partum and assayed for total sialic acid. Milk sialic acid was assessed relative to maternal macronutrient intake, age, body-mass index (BMI), CD4+ cell count and infant birth weight.

***Results:*** Themean concentration of milk sialic acid was highest in the first two weeks postpartum (6.89 ± 2.79 mM) and declined rapidly by three months (2.49 ± 0.60 mM). Sialic acid content in milk was similar between both treatment arms of the study, and did not correlate with maternal macronutrient intake. No correlation was found between maternal age, BMI, CD4+ cell count or infant birth weight and total milk sialic acid concentration.

***Conclusions*:** Milk sialic acid levels in HIV-positive, Tanzanian women without malnutrition are comparable to reported values for women of European descent and show a similar temporal decline during early lactation. These findings suggest that total milk sialic acid is maintained despite macronutrient deficiencies in maternal diet and support a conserved role for milk sialic acid in neonatal development.

**Introduction**

Breast milk provides a critical component in the early defense of newborns against acquisition of infectious diseases. This is evidenced by the low incidence of HIV-1 transmission among breastfed infants born to HIV-positive mothers, even in the absence of antiretroviral therapy (ART) [1-6]. A variety of innate factors in human milk have been linked to reduced HIV-1 infectivity in vitro and lower rates of postnatal HIV-1 transmission [7-16]. Among these, sialyated human milk oligosaccharides (sHMO) may influence the risk of post-natal transmission in HIV-positive mothers and impact the survival and development of HIV-exposed infants [17-20].

Sialic acid is one of the most variable fractions in human milk. The level and diversity of sHMOs differ among women, even those at the same stage of lactation [21-27]. Recent findings demonstrate the impact of sHMOs on early development of the gut microbiota [28-32]. These findings are consistent with the recognized role of sialylated milk components in promoting infant gut maturation, immunity and brain development [33-36].

Production of sHMOs is associated with genetic determinants [37,38] and may be influenced by environmental factors such as maternal diet, ethnicity, age and geographical location. While changes in the level of sialic acid in human milk throughout lactation have been described in studies of women of European descent [21-27], less is known about temporal changes in milk sialic acid in African women, and in particular those with HIV infection, which may be associated with metabolic dysregulation [18]. Moreover, the influence of maternal diet during pregnancy and lactation on milk sialic acid content in this population is unknown.

In the present study, we quantified total sialic acid concentration in milk from HIV-positive Tanzanian women, and examined the influence of maternal diet on milk sialic acid levels. These studies were conducted in the context of a larger randomized, controlled clinical trial to test the impact of dietary intervention with a macronutrient (protein-calorie) supplement during late pregnancy and early lactation in these women [39-41].

**Methods**

**Study Design**

 A nested study was conducted within a randomized, controlled clinical trial to test the effect of a macronutrient supplement on health outcomes in HIV-positive, breastfeeding women and their infants in Dar es Salaam, Tanzania. The details of this clinical study are described elsewhere [39]. In brief, after informed consent, HIV-positive women in their last trimester of pregnancy were randomized to receive a protein-calorie supplement plus a micronutrient supplement (PCS+MNS) or a control regimen of a MNS alone, beginning in the third trimester of pregnancy and continuing for up to the first six months of breastfeeding. Specimens from all women for whom at least one milk sample was available were analyzed for this substudy. A total of 74 subjects were analyzed; 38 received the PCS+MNS and 36 received the MNS only.

 Eligibility required mothers to have a BMI ≥18.5 kg/m2 and a CD4 count ≥250 cells/uL, and have the intent to exclusively breastfeed their infant for at least three months postpartum. Short-term ART was prescribed for all women in this study. Clinical evaluation included anthropometric measurements, 24-hour dietary recall and food insecurity assessments as described [39]. The study was approved by the Institutional Review Board at the Geisel School of Medicine at Dartmouth and the Research Ethics Committee of the Muhimbili University of Health and Allied Sciences (MUHAS) in Dar Es Salaam, Tanzania.

*Nutritional supplements.*  A culturally acceptable and locally sourced dietary macronutrient supplement was developed to address identified deficiencies in protein and calories in the study subjects [40,41]. The supplement provided 1062 Kcal and 42 gm of protein per daily serving [39]. The MNS was the standard combination of vitamins and minerals provided to HIV-infected women in Tanzania [42]. All mothers also received 60 mg ferrous sulfate once daily during pregnancy.

*Maternal and infant assessments.* All subjects with live infant births had a home visit performed within one month of delivery at a time when they had commenced breastfeeding. Subjects were interviewed for dietary intake and food insecurity, and data on maternal weight, height and anthropometric measurements were recorded. All mothers were further seen at the study clinic monthly for up to six months after infant birth. At each visit, subjects were provided with the dietary supplements, and anthropometric measurements and a 24-hour dietary recall were performed. Milk samples were collected during the initial home visit at approximately two weeks postpartum and again at ≥3 months postpartum. HIV testing of infants was performed at 15 months of age as described [39]. Those infants testing positive for HIV were provided with standard of care.

*Milk Collection.* Milk was collected during home visits and again during follow-up clinic visits by manual expression into sterile tubes. Immediately after collection, samples were placed on ice, transported to the laboratory and processed within 4 hrs of collection. Whole milk was centrifuged at 500 x g for 10 minutes to pellet cells and debris, and to separate the skim and lipid components. Aliquots of skim milk were frozen on-site and batched for shipment to the Geisel School of Medicine at Dartmouth (Lebanon, NH). Samples were shipped on dry ice and stored at -80°C upon receipt.

*Quantification of milk sialic acid.* Total sialic acid content was quantified in skim milk fractions by ELISA (EnzyChrom™, Bioassay Systems, Haywood, CA) as per the manufacturer’s instructions. Samples were first tested undiluted. Those samples exceeding the upper detection limit of the assay were diluted 1:10 in sterile water and re-tested. Levels of sialic acid were quantified following acid hydrolysis of samples at 80°C for 60 minutes followed by neutralization. Total sialic acid concentration was determined relative to a standard curve ranging from 0.02 to 1 mM. Results were read at OD570nm using a BioTek Synergy 2 Multimode Microtiter plate reader.

*Data analysis.* Temporal trends of loge-transformed sialic acid values were compared between groups using linear mixed-effects regressions with child-specific random effects and quadratic time trends; the P value is from a Wald test. Correlations between sialic acid and the markers of interest were quantified by Spearman’s rank correlation coefficients. Univariate linear regressions were used to evaluate the mean differences in sialic acid per standard deviation increase of the continuous correlates. All statistical analyses were performed using Stata, version 13.0 (StataCorp LP, College Station, TX, USA). P values are from two-sided tests.

**Results**

The initial study visit occurred on average at 2.5 weeks postpartum and milk samples collected during this visit had a mean sialic acid concentration of 6.89 ± 2.79 mM. This value declined to 2.49 ± 0.60 mM during the second visit, which occurred on average at 13.2 weeks postpartum. A similar trend of declining sialic acid concentration occurred in milk collected from women receiving either the PCS+MNS or the MNS dietary intervention (Figure 1). In linear mixed-effects models, sialic acid levels did not differ significantly between the two study arms (mean percent difference for MNS+PCS group relative to MNS group, 95% CI: -2.8%, -13.3% to 8.9%; p=0.62).

Nutrient intake was assessed on the study visit corresponding most closely to the time of milk collection at the first postpartum visit. No statistically significant correlation was found between total milk sialic acid concentration and maternal age (Spearman’s rho=0.16, p=0.17), total daily protein intake (Spearman’s rho=0.14, p=0.25) or total daily caloric intake (Spearman’s rho=0.09, p=0.44). Additionally, we found no statistically significant correlation between infant birth weight and maternal milk sialic acid concentration in samples collected at the first postpartum visit (Spearman’s rho= (-0.16), p=0.17). Additionally, we found no statistically significant effect of maternal/infant variables on total milk sialic acid in mothers receiving the PCS+MNS as compared to those receiving the MNS only.

In a further combined analysis of dietary data from women enrolled in the two treatment arms of the study, we found no statistically significant correlation of total milk sialic acid and maternal consumption of total calories, total protein, animal protein, fat, carbohydrate and fiber (Table 1). Similarly, in a combined analysis, we found no statistically significant effect of maternal age, body mass index (BMI), CD4+ cell count or infant birth weight on total milk sialic acid (Table 1).

Four infants in the MNS arm of the study were confirmed to be HIV positive within 15 months of age, while no infants in the PCS+MNS arm of the study were found to be HIV infected [39]. Available milk samples collected within one month post-partum from three of the mothers with HIV-positive infants revealed that two had total milk sialic acid concentrations below the median value for all study mothers during this period of lactation (4.39, 4.63 vs 6.89 mM).

**Discussion**

Recent studies have defined a critical contribution of sialylated milk oligosaccharides in promoting infant growth and regulation of gut microbiota in the setting of infant nutritional deficiency and stunting [32]. While higher levels of sialylated components in mother’s milk were linked to improved infant outcomes and included enhanced maturation of the infant gut microbiome, factors that influence milk sialic acid concentrations, such as the stage of lactation and maternal diet remain obscure. Earlier studies have demonstrated a temporal change in total milk sialic acid content, characterized by high concentrations in the first few weeks postpartum, followed by rapidly declining levels with maturing lactation [21-27]. These studies were conducted primarily in women with no reported nutritional deficiencies, and suggest a beneficial role for milk sialic acid in the early postpartum period, consistent with temporal establishment of the infant gut microbiota. Our results support this concept and demonstrate similar temporal trends in milk sialic acid among African women with HIV infection. The nature of this trend is consistent with dynamic changes in milk composition that occur during the transition from early to mature milk, and support the concept that sialylated components in human milk adjust to meet the changing physiologic needs of the infant [33, 35, 43, 44].

We found no evidence to suggest that correcting macronutritional deficiencies in maternal diet during late pregnancy and lactation in HIV-positive Tanzanian mothers, including boosting total caloric and protein intake, had any significant effect on total milk sialic acid levels in the early postpartum period. The timing of this dietary intervention mirrors changes in sialic acid reported to occur in maternal saliva during pregnancy, which correlate with rapid accumulation of sialic acid in the fetal brain [35, 45]. Despite evidence that perinatal intake of protein, fatty acids [46] or gangliosides [47,48] can boost brain development, we found no significant difference in total milk sialic acid in women receiving protein supplementation, and no association with other dietary macronutrients including carbohydrates, fat and fiber.

Overall our findings reinforce the dynamic nature of sialic acid production in human milk and underscore the importance of timing sample collection to capture changes in milk sialic acid content throughout lactation, in particular focusing on the first two weeks post-partum. Importantly, our results suggest that total milk sialic acid is relatively refractory to maternal macronutrient deficiencies and may be maintained in the setting of HIV infection. This further supports an essential and conserved role for sHMO in shaping early events in infant development.

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**Figure Legend**

**Figure 1:** Change in total milk sialic acid concentration during lactation in HIV-positive Tanzanian mothers. Total sialic acid was measured in skim milk samples collected at approximately two weeks and ≥3 months postpartum. Temporal trends of loge-transformed sialic acid values were compared between subjects enrolled in the two intervention arms of the study, PCS+MNS or MNS.

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Table 1: Relationship of milk sialic acid at first postpartum visit to maternal baseline and dietary characteristics.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Marker | N | Mean (SD) | Mean Difference (95% CI) in Milk Sialic Acid (mM) per 1 SD Higher Marker Level | *P value* |
| Baseline Characteristics | Maternal Age, yrs | 71 | 31.3 (5.1) | 0.29 (-0.37, 0.95) | *0.38* |
| Maternal BMI, kg/m2 | 73 | 23.9 (3.9) | 0.23 (-0.42, 0.88) | *0.48* |
| Maternal CD4 Counts | 69 | 519 (288) | 0.39 (-0.28, 1.10) | *0.25* |
| Infant Birth Weight, kg | 74 | 3.1 (0.6) | -0.36 (-0.95, 0.23) | *0.28* |
| Maternal Daily Consumption | Calories | 74 | 2595 (710) | 0.25 (-0.40, 0.89) | *0.45* |
| Total Protein, g | 74 | 69.5 (26.1) | 0.28 (-0.37, 0.92) | *0.40* |
| Animal Protein, g | 74 | 32.9 (24.0) | -0.01 (-0.65, 0.64) | *0.99* |
| Fat, g | 74 | 96.8 (47.8) | -0.06 (-0.70, 0.59) | *0.86* |
| Carbohydrates, g | 74 | 379 (103) | 0.36 (-0.28, 1.00) | *0.26* |
| Fiber, g | 73 | 29.1 (11.6) | 0.18 (-0.47, 0.83) | *0.58* |



**Figure 1:** Change in total milk sialic acid concentration during lactation in HIV-positive Tanzanian mothers. Total sialic acid was measured in skim milk samples collected at approximately two weeks and ≥3 months postpartum. Temporal trends of loge-transformed sialic acid values were compared between subjects enrolled in the two intervention arms of the study, PCS+MNS or MNS.