The immune response to sub-clinical mastitis is impaired in HIV-infected women
Roxane Schaub, Stéphanie Badiou, Johannes Viljoen, Pierre Dujols, Karine Bolloré, Philippe van de Perre, Marie-Louise Newell, Ruth Bland, Nicolas Nagot, Edouard Tuaillon

To cite this version:
Roxane Schaub, Stéphanie Badiou, Johannes Viljoen, Pierre Dujols, Karine Bolloré, et al.. The immune response to sub-clinical mastitis is impaired in HIV-infected women. Journal of Translational Medicine, BioMed Central, 2018, 16 (1), pp.296. 10.1186/s12967-018-1667-4. hal-01906492

HAL Id: hal-01906492
https://hal.archives-ouvertes.fr/hal-01906492
Submitted on 3 Feb 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
The immune response to sub-clinical mastitis is impaired in HIV-infected women

Roxane Schaub1,2,10*, Stéphanie Badiou3, Johannes Viljoen5, Pierre Dujols1,2, Karine Bolloré1, Philippe Van de Perre1,6, Marie-Louise Newell7,8, Ruth Bland4,8,9, Nicolas Nagot1,2 and Edouard Tuaillon1,6

Abstract

Background: Subclinical mastitis (SCM) is relatively common in lactating women and may be associated with HIV shedding in breast milk. The potential association between HIV infection and breast milk immunologic factors and immune response to SCM needs to be addressed.

Methods: In this cross-sectional study, SCM (Na/K ratio > 1) was tested in 165 mature breast milk samples collected from 40 HIV-infected women who didn’t transmit HIV to their child by breastfeeding and 43 HIV-uninfected women enrolled in an interventional cohort in South-Africa (Vertical Transmission Study). The level of 33 immune markers related to Th1/Th2 related response, inflammation and bacterial exposure were compared in ART-naive HIV-infected versus HIV-uninfected women. The associations between HIV infection and SCM on the concentration of immune factors were tested separately by Wilcoxon rank-sum test and corrected for false discovery rate. To control for potential confounder effects and take into account the clustering of breast milk samples from a single woman, multivariate mixed linear models adjusted on child age at the time of sampling were performed for each immune factor.

Results: Subclinical mastitis was detected in 15 (37.5%) HIV-infected women and 10 (23.3%) HIV-uninfected women. In the absence of SCM, the breast milk levels of IP-10 and MIG were higher and IL1-RA lower in HIV-infected women than in HIV-uninfected women (respectively p < 0.001, p = 0.001, p = 0.045). In HIV-uninfected women, SCM was characterized by a robust immune response with higher concentrations of a broad panel of Th1 and inflammatory related immune markers than in samples without SCM. By contrast, in HIV-infected women a limited number of immune markers were increased and lower increases were observed in samples with SCM than without SCM.

Conclusion: HIV infection in ART-naive women was associated with elevated breast milk levels of IP-10 and MIG, which are Th1-related cytokines induced by IFN-γ. During SCM, a lower and narrower immune response was observed in HIV-infected than HIV-uninfected women, suggesting that HIV infection affects the capacity of the mammary gland to respond to SCM.

Keywords: HIV, Breast milk, Subclinical mastitis, Cytokines

*Correspondence: roxane.schaub@gmail.com; roxane.schaub@ch-cayenne.fr

10 Present Address: CIC AG/Inserm 1424, Centre Hospitalier de Cayenne, Av. des flamboyants, BP 6006, 97 306 Cayenne CEDEX, French Guiana, France

© The Author(s) 2018. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background
Mother-to-child HIV transmission (MTCT), can occur during pregnancy, delivery or breastfeeding and remains a public health concern especially in sub-Saharan Africa. Despite an overall 48% decrease of newly infected children between 2009 and 2014 due to the implementation of prophylaxis interventions and antiretroviral therapy (ART), there were still 160,000 newly infected children (<15 years old) in 2016 [1]. Postnatal HIV transmission through breastfeeding in the presence of ART contributes to a significant part of this residual transmission with a pooled risk estimate of 2.93% at 12 months [2].

World Health Organization (WHO) guidelines recommend lifelong ART for mothers living with HIV [3]; exclusive breastfeeding for the first 6 months of life; appropriate complementary foods thereafter and to continue breastfeeding for at least 12 months [4].

Breast milk, the main and optimal nutrient source of the infant, contains numerous active immune factors [5, 6]. These immune factors protect the infant from infections [7], participate in the maturation of the infant’s immune system [6], down-regulate gut inflammation and promote gut adaptation after birth when the newborn is confronted with the antigens of colonizing commensal bacteria [8]. Breast milk soluble immune factors consist of a wide array of bioactive agents like cytokines, chemokines, growth factors and acute phase proteins [6, 9]. These immune factors are involved in multiple immune functions including Th1 response, antimicrobial, anti-inflammatory, pro-inflammatory and immunomodulatory properties [5, 6, 9, 10] and influence the immune response by attracting, activating or down-regulating different cell effectors [9, 10]. During lactation, inflammatory processes of the mammary gland like mastitis [11–15] and subclinical mastitis (SCM) [16–19] induce considerable changes in the breast milk immune factors. In asymptomatic lactating women, SCM is generally defined by an increased breast milk sodium/potassium ratio (Na/K > 1) [17, 18, 20, 21]. Whereas clinical mastitis occurs in less than 10% of lactating mothers [22–24], SCM (Na/K > 1) is more frequent, especially at start of breastfeeding in the first few days after delivery and again at the time of weaning, with a prevalence ranging from 9 to 45% in mature milk in HIV-uninfected mothers [17, 18]. Studies have suggested that SCM may be associated with HIV shedding in breast milk and HIV mother-to-child transmission [25–34].

Human immunodeficiency virus is known to induce immune activation in blood [35], gut [36] and female genital tract [37, 38] but its impact on milk immune factors remains only partially defined. Few studies have compared breast milk components in ART-naïve HIV-1-infected versus HIV-uninfected women, and a limited number of parameters were assessed [39–41]. We hypothesized that HIV infection induces modifications of the composition of immune factors in breast milk, and impairs immune response to SCM in breast milk.

In a first step, our study assessed the influence of HIV infection (without ART exposure) on the pattern of breast milk immune factors. We further sought to explore the association between HIV infection and immune response to SCM.

Materials and methods
Study population and clinical features
This study was part of the ANRS 1271 project exploring factors associated with HIV-1 transmission from mother to child through breastfeeding. The study was nested in a South-African non-randomized prospective intervention cohort, the Vertical Transmission Study (VTS), examining the effect of feeding practices on infant HIV infection and survival rates, in a community where HIV prevalence in pregnant women was 23.7% [42]. A total of 2722 HIV-infected and uninfected pregnant women attending antenatal clinics in KwaZulu-Natal were enrolled after providing informed consent between October 2001 and April 2005 [43]. Participants were naïve to antiretroviral therapy except for single-dose nevirapine provided to all HIV-infected women and their newborns during delivery as per national guidelines at the time. The intervention included a personalized antenatal counseling session on infant feeding choices, and a postnatal home-based breastfeeding counseling intervention for women who chose to breastfeed. Formula feeding women were supported at their clinic visits by study nurses. Daily infant feeding practices and maternal breast health problems or breastfeeding difficulties were recorded. Maternal health and infant growth and morbidity were regularly recorded in monthly scheduled clinic visits until 9 months and every 3 months afterwards until 2 years of age. Mother’s blood samples were taken before delivery and at 6 months post-delivery to measure HIV viral load and CD4 count and child’s HIV status was checked at each study clinic visit on dried blood spots. Breast milk samples were taken separately from right and left breast at each clinic visit if the mother was still breastfeeding [44]. The VTS study and breast milk analyses were approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and women gave informed consent.

Forty HIV-infected women who did not transmit HIV to their infant during follow-up and 43 HIV-uninfected women were enrolled for this study. Subjects were selected based on availability of mature breast milk samples (breast milk collected >15 days after delivery) and in a random draw from the VTS cohort. Breast milk
samples from HIV-infected women who transmitted HIV to their child were excluded in our study to focus on the impact of HIV infection on breast milk immune factors and on response to SCM. A total of 156 breast milk samples were tested. Paired left and right breast samples were available for 73 (88.0%) women.

SCM was defined by a Na/K ratio in breast milk > 1, as described elsewhere [17, 18, 45, 46]. Maternal breast health problems (such as mastitis, engorgement, cracked/bleeding nipple, blocked duct, breast thrush, breast/nipple oozing pus and abscess [24]) were considered if they occurred during the week before the sample collection date because mastitis markers are normalized within 1 week after symptom resolution [13]. Breastfeeding practices were defined as in the VTS cohort [43], but were only included on the 15 days before the sample collection date, in order to detect a recent breastfeeding practice change that may be indicative of a breast health problem [47].

We explored four groups of breast milk samples: samples without SCM from HIV-uninfected women, samples with SCM from HIV-uninfected women, samples without SCM from HIV-infected women and samples with SCM from HIV-infected women.

**Immune factors and biochemical assays in breast milk**

Whole breast milk samples were stored at −80 °C until processing at the Montpellier University Teaching Hospital, France. All parameters were measured in lactoserum after centrifugation at 1200g for 15 min. Lactoserum sodium and potassium concentrations were measured with ion selective electrode (AU640 analyzer, Beckman Coulter, Fullerton, CA). β2microglobuline (B2M) and C-reactive protein (CRP) were determined by immunoturbidimetric methods (AU640, Beckman Coulter, Fullerton, CA). Lactoserum erythropoietin (EPO) (IMMULITE2000 EPO assay, Diagnostic Products Corporation, Los Angeles, CA), α-defensin (Hycult Biotech, Uden, The Netherlands), lactoferrin (Calbiochem, Dramstadt, Germany), secretory leukocyte peptidase inhibitor (SLPI) (R&D Systems, Minneapolis, MN), lipopolysaccharide-binding protein (LBP) (Hycult Biotech), soluble CD14 (sCD14) (Hycult Biotech) and S100A9 protein (PS100A9) (CycLex, Nagano, Japan) were quantified by commercial enzyme immunoassays as recommended by the manufacturer. Interleukins (IL) 1β, 2, 4, 5, 6, 7, 8 (IL-8 or CXCL8), 10, 13, 15 and 17, interleukin 12p40/70, receptor antagonist of interleukin 1β (IL-1RA), interleukin 2 receptor (IL-2R), granulocyte and macrophage growth factor (GM-CSF), tumor necrosis factor-α (TNF-α), γ interferon (IFN-γ), α interferon (IFN-α), macrophage inflammatory protein 1α and 1β (MIP-1α and MIP-1β), inflammatory protein 10 (IP-10 or CXCL10), monokine induced by gamma interferon (MIG or CXCL9), eotaxin, regulated upon activation normal T-cell expressed and secreted (RANTES or CCL5), and monocyte chemoattractant protein-1 (MCP-1 or CCL2) were quantified using a multiplex microbeads assay (Invitrogen Human Cytokine 25-Plex Panel, Marne-La-Vallée, France) and a Luminex 100 apparatus (Luminex, Oosterhout, The Netherlands) following the manufacturer’s instructions.

Concentrations below the lower limit of quantification were assigned half the value of the lower limit, as described elsewhere [39, 48]. Conversely, concentrations above the upper limit of quantification were assigned the value of the upper limit [17]. Immunologic factors for which 50% or more of the samples in a group were below the lower limit of quantification of the test weren’t quantitatively analyzed.

**Statistical analyses**

In the first step of the study, breast milk samples were tested for SCM based on Na/K ratio. Then, the association between ART-naïve HIV infection and breast milk environment in the absence of SCM were analyzed by comparing concentrations of immune markers in samples from ART-naïve HIV-infected versus HIV-uninfected women. Next, changes induced by SCM were explored by comparing levels of immune factors in SCM samples to breast milk samples without SCM in ART-naïve HIV-1 infected and uninfected women.

Demographic and clinical characteristics of women, immunologic factors detection rates and concentrations were compared between HIV positive and negative women using the Chi-square or Fisher exact test for qualitative variables and the Student or Wilcoxon rank-sum test for quantitative variables according to the variable’s distribution. The associations between HIV infection and SCM on immune factor’s concentration were tested separately by Wilcoxon rank-sum test. Spearman’s non-parametric test was used to assess correlation between immunologic factors by group. When immunologic factors were compared in sets of bivariate analyses, p-values were corrected for false discovery rate (FDR; \( p < 0.05 \)) to correct for multiple testing [49].

To control for potential confounder effects, multivariate mixed linear models were performed for each immunologic factor. Models were done with the SAS PROC MIXED procedure, adjusted on child age at the time of sampling [48, 50–52] and taking into account the clustering of left and right breast milk samples of a woman by introducing a random effect. All statistical analyses were conducted using SAS statistical software version 9.2 (SAS Institute, Cary, NC).
Results

Characteristics of women and breast milk samples

Demographic, obstetrical and clinical characteristics did not differ significantly between the 40 HIV-infected women and the 43 HIV-uninfected women (Table 1). None of the women reported any breast health problem in the week preceding the sample collection. A subclinical mastitis (Na/K ratio > 1) in at least one breast milk sample was detected in 15/40 (37.5%) HIV-infected women and 10/43 (23.3%) HIV-uninfected women. SCM was bilateral (in samples of both left and right breast at the same time) in 4/15 (26.7%) HIV-infected women and in 5/10 (50.0%) HIV-uninfected women.

Subclinical mastitis was detected in 34/156 breast milk samples (21.8%): 19/74 (25.7%) samples from HIV-infected women and 15/82 (18.3%) from HIV-uninfected women. In the breast milk of HIV-infected women, HIV RNA was detected in 6/69 samples (8.7%; ranging from 615 to 22,428 copies/mL).

The number of samples analyzed by group included 67 samples without SCM from HIV-uninfected women, 15 samples with SCM from HIV-uninfected women, 55 samples without SCM from HIV-infected women and 19 samples with SCM from HIV-infected women.

Seven breast milk immune factors out of 34 were detected over the lower limit of quantification in all samples (IL-8, IP-10, EPO, MCP-1, LBP, sCD14 and B2M) and 13 were quantified in at least half of the samples (IL-2 receptor, IL-12p40/70, IL-15, MIG, IL-7, Lactoferrin, MIP-1α, MIP-1β, SLPI, RANTES, CRP, PS100A9, IL-1RA) (see Additional file 1: Table S1 for immune factors detection rates by group). Fourteen immune factors were not further considered because their concentrations were below the lower limit of quantification in the majority of the samples.

Association between HIV infection and breast milk immune factors in the absence of SCM

The association between maternal HIV status and breast milk immune environment was explored by comparing concentrations of immune factors in samples without SCM (Fig. 1). The concentrations of immune factors were similar in the HIV-infected and uninfected women, except for MIG, IP-10 and CRP which were higher in HIV-infected women (respectively median [IQR]: MIG = 215 pg/mL [62–668] vs 79 pg/mL [10–153], corrected p = 0.002; median IP-10 = 932 pg/mL [320–1976] vs 337 pg/mL [165–519], corrected p = 0.001 and median CRP = 0.2 μg/L [0.1–0.3] vs 0.1 μg/L [0.1–0.1], corrected p = 0.001). In contrast, the level of IL-1RA was significantly lower in HIV-infected women (326 pg/mL [131–731] vs 531 pg/mL [255–905], corrected p = 0.045). A trend was observed towards higher B2M and lower sCD14 concentration in HIV-infected women (corrected p = 0.087, for each).

Table 1 Maternal demographic, obstetrical and clinical characteristics

| Characteristics                                      | HIV+ women (n = 40) | HIV− women (n = 43) |
|------------------------------------------------------|---------------------|---------------------|
| Maternal age at delivery (years)                     | Median [range] or N (%) | Median [range] or N (%) |
| Previous pregnancies (at least one)                  | 26 [17–39]          | 23 [17–46]          |
| Mode of delivery                                     | 30 (75.0%)          | 25 (58.1%)          |
| Vaginal                                              | 36 (90.0%)          | 38 (88.4%)          |
| Caesarean                                            | 4 (10.0%)           | 5 (11.6%)           |
| Infant age at breast milk sampling (days)            | 176 [38–494]        | 162 [31–493]        |
| Breastfeeding type until sampling                    |                     |                     |
| EBFa                                                 | 20 (50.0%)          | 17 (39.5%)          |
| MBFb                                                 | 12 (30.0%)          | 18 (41.9%)          |
| Unknown                                              | 8 (20.0%)           | 8 (18.6%)           |
| Breastfeeding type during the past 15 days before sampling |                 |                     |
| EBFa                                                 | 24 (60.0%)          | 25 (58.1%)          |
| MBFb                                                 | 16 (40.0%)          | 18 (41.9%)          |
| Sub-clinical mastitis in at least 1 sample (Na/K > 1) | 15 (37.5%)          | 10 (23.3%)          |
| Postnatal CD4-cell count (per μL)c                   | 595 [78–2473]       | –                   |
| Postnatal plasma HIV viral load (copies/mL)c         | 5300 [25–110,000]   | –                   |

a EBF: exclusive breastfeeding (breast milk only)
b MBF: mixed breastfeeding (MBF is defined as breast milk plus 1 day of solid food and/or breast milk plus 3 days of fluids other than breastmilk)

c Approximately 6 months after delivery
Multivariate mixed models, taking into account the clustering of left and right breast milk samples of a woman and adjusted on infant age at sampling, confirmed the association between HIV infection and MIG, IP-10, IL-1RA and CRP, as well as B2M and sCD14 (see Additional file 2: Table S2 for multivariate mixed models assessing the effect of HIV infection on breast milk immune factors, on samples without subclinical mastitis).

Correlations between the factors were analyzed to explore the immune network pattern in SCM-negative samples (Fig. 2). Significant and strong correlations were found between Th1-related cytokines in breast milk samples collected from HIV-infected women, especially between CXC chemokines secreted in response to IFN-γ: MIG and IP-10 (ρ = 0.86, p < 0.001), IP-10 and IL-12p40/70 (ρ = 0.84, p < 0.001) and between Th1-related cytokines and Th1-related CC chemokines, such as RANTES with both IL-12p40/70 (ρ = 0.82, p < 0.001) and IP-10 (ρ = 0.82, p < 0.001). The pattern of correlations was similar in samples of HIV-infected women with significant and strong correlations between IP-10 and both MIG (ρ = 0.87, p < 0.001) and IL-12p40/70 (ρ = 0.81, p < 0.001), MIG and IL-12p40/70 (ρ = 0.87, p < 0.001), between RANTES and IL-12p40/70 (ρ = 0.91, p < 0.001) and between IL-15 and MIP-1β (ρ = 0.85, p < 0.001).

We found only a few weak correlations (maximum |ρ| = 0.44) between breast milk immune factors and plasma HIV viral load, CD4 count or breast milk HIV.
RNA (see Additional file 3: Table S3 for correlations between breast milk immune factor concentration, HIV plasma parameters and breast milk HIV RNA).

Association between HIV infection and breast milk immune factors in the presence of SCM

The concentrations of immune factors in samples with SCM were similar in the samples of HIV-infected and uninfected women (see Additional file 4: Table S4 for breast milk immune factor comparisons between samples with sub-clinical mastitis from HIV-uninfected and HIV-infected women).

In HIV-uninfected women, the concentrations of 14 out of 20 immune factors in breast milk were significantly higher in samples with SCM compared to samples without SCM. Differences remained significant after correcting p-values for multiple testing. Hence, Th1-related cytokines (IL-2 receptor, IL-12p40/70, IL-15, MIG and IP-10), factors secreted in response to bacterial exposure (MIP-1α, MIP-1β, MCP-1, SLPI) and inflammatory markers (RANTES, B2M, PS100A9 and IL-8) were
significantly elevated in SCM-positive samples compared to SCM-negative samples. Among anti-inflammatory markers, only IL-1RA was increased. A trend was observed for higher LBP and sCD14 concentrations (Table 2).

The multivariate mixed models, taking into account the clustering of left and right breast milk samples of a woman and adjusted on infant age at sampling, confirmed the significant association between SCM and higher concentrations of breast milk IL-12p40/70, MIG, IP-10, IL-1RA, MCP-1, SLPI, B2M, and IL-8 in HIV-uninfected women (see Additional file 5: Table S5 for multivariate mixed models assessing the effect of SCM on breast milk immune factors, by HIV group).

In HIV-infected women, breast milk concentrations of five immune factors were significantly higher in SCM-positive samples than in SCM-negative samples: IL-12p40/70, MIP-1α, LBP, RANTES and IL-8 and a trend was observed for IL-7, B2M and PS100A9 (see Table 2). Interpretable multivariate mixed models confirmed the significant effect of SCM on higher concentrations of IL-12p40/70, MIP-1α, LBP, RANTES and IL-8, as well as IL-1RA and MIP-1β (see Additional file 5: Table S5).

**Discussion**

We provided the first comparative analysis of a large number of soluble immune factors in the breast milk of ART-naive HIV-infected women and uninfected women and highlighted the association between HIV infection and immune response to SCM. Our study was performed on samples collected years ago, before the era of universal, life-long maternal ART, offering a unique opportunity to better understand the interactions between HIV infection and inflammation in the mammary gland without interference of therapy.

Although pregnant and lactating women have today

| Immune factor concentration | HIV+ samples | HIV- samples |
|----------------------------|-------------|-------------|
|                            | No SCM      | SCM         | Corrected p-value* | No SCM      | SCM         | Corrected p-value* |
| Th 1                       |             |             |                  |             |             |                  |
| IL-2R                      | Median [q25–q75] | Median [q25–q75] |                  | Median [q25–q75] | Median [q25–q75] |                  |
| IL-12p40/70                | 65 [20–105] | 82 [66–109] | 0.251            | 66 [20–101] | 166 [66–536] | 0.008            |
| IL-15                      | 47 [32–66] | 77 [49–126] | 0.035            | 30 [13–71] | 100 [27–262] | 0.021            |
| MIG                        | 215 [62–668] | 442 [207–1077] | 0.021          | 79 [10–153] | 1185 [314–1328] | < 0.001          |
| IP-10                      | 932 [320–1976] | 1913 [557–2653] | 0.021          | 337 [165–519] | 1806 [502–3379] | < 0.001          |
| IL-7                       | 27 [13–58] | 62 [26–109] | 0.035            | 35 [13–177] | 179 [13–279] | 0.131            |
| Anti infl.                 |             |             |                  |             |             |                  |
| EPO                        | 23 [10–31] | 17 [13–33] | 0.970            | 31 [16–40] | 25 [18–33] | 0.920            |
| Lactoferrin                | 3.8 [2.8–6.6] | 4.5 [2.8–13.8] | 0.059           | 4.8 [2.2–8.7] | 18.8 [2.8–20.2] | 0.136            |
| IL-1RA                     | 326 [131–731] | 430 [252–1203] | 0.183          | 531 [255–905] | 1718 [470–3980] | 0.012            |
| Anti bact. response        |             |             |                  |             |             |                  |
| MIP-1α                     | 23 [8–34] | 34 [24–137] | 0.030            | 20 [8–45] | 145 [20–808] | 0.007            |
| MIP-1β                     | 17 [5–31] | 32 [11–141] | 0.030            | 16 [5–34] | 163 [24–801] | 0.003            |
| MCP-1                      | 400 [228–1281] | 690 [338–1713] | 0.024          | 524 [240–1221] | 6460 [1994–7200] | 0.003            |
| LBP                        | 63 [43–138] | 248 [97–394] | 0.036           | 84 [54–164] | 357 [85–550] | 0.064            |
| sCD14                      | 4828 [2479–10,231] | 6573 [2560–9186] | 0.084          | 9059 [5941–18,212] | 27,778 [10,650–57,686] | 0.064            |
| SLPI                       | 20 [13–60] | 40 [32–82] | 0.206            | 35 [14–174] | 102 [55–562] | 0.011            |
| Infl. markers              |             |             |                  |             |             |                  |
| RANTES                     | 72 [45–122] | 151 [88–268] | 0.030           | 87 [51–147] | 238 [110–304] | 0.006            |
| CRP                        | 0.20 [0.10–0.7] | 0.20 [0.10–0.70] | 0.591         | 0.10 [0.10–0.10] | 0.10 [0.10–0.20] | 0.203            |
| B2M                        | 8.5 [7.6–11.0] | 10.1 [8.8–13.0] | 0.088         | 8.1 [7.2–9.3] | 13.6 [10.3–26.8] | < 0.001          |
| PS100A9                    | 2875 [1405–17,183] | 18,403 [4166–23,934] | 0.088       | 3480 [2235–9930] | 23,129 [8035–25,854] | 0.021            |
| IL-8                       | 365 [233–1188] | 1275 [503–2803] | 0.030          | 460 [240–844] | 3243 [414–10,160] | 0.007            |

Sub-clinical mastitis is defined as a Na/K ratio > 1 in breast milk

[q25–q75] interquartile range

◊: pro inflammatory marker; Infl. markers: inflammatory markers; Anti infl.: anti inflammatory markers; Antibact. response: anti bacterial response

All concentrations are in pg/mL except SLPI, B2M, CRP (μg/L), lactoferrin (g/L), EPO (mIU/mL), LBP, sCD14 (ng/mL)

*p-values are for the test of the difference between samples with and without SCM, separately for each HIV group; Italic values indicate significance of p-value (<0.05) after FDR correction
increasing access to ART, HIV replication during lactation still occurs in cases of virological failure or poor adherence, and in HIV-infected women unaware of their status. Furthermore, data collected before the era of ART are necessary to further explore the immune response to SCM in HIV-infected women undetectable for HIV on ART, in whom SCM may still occur, especially during weaning.

Our results showed that HIV infection moderately alters the soluble immune factor environment in breast milk samples without SCM or any other breast health problem. Hence, in the absence of SCM, the concentrations of immune factors in breast milk appeared different between HIV-uninfected women and HIV-infected women who didn’t transmit the infection by breastfeeding, regarding MIG and IP-10 that were significantly higher in the breast milk of HIV-infected women. These two CXC-chemokines are induced by IFN-γ and belong to a Th1 response and a cascade that is critical in antiviral defense. In addition, the level of the anti-inflammatory IL-1RA cytokine was significantly lower. Furthermore, the network of soluble immune factors appeared only slightly impaired, with correlations between the Th1 cytokines themselves and with the inflammatory markers that were slightly higher in the samples of HIV-positive women.

Three other studies who did not exclude women having transmitted HIV to their infants have assessed the impact of HIV infection on breast milk immune profile regardless of the presence of a SCM. Bosire et al. who compared MIP-1α, MIP-1β, RANTES and Stromal cell-Derived Factor-1α (SDF-1α) in the breast milk of Kenyan women several times after childbirth, found that MIP-1β was significantly higher at 10 days and RANTES at 1 month among HIV-infected versus HIV-uninfected women [39]. Shapiro et al. found significantly higher levels of total IgM, IgG, IgA and SLPI in HIV-infected women compared to HIV-uninfected women in Botswana [40]. Henrick et al. found significantly higher levels of soluble toll-like receptor 2 (sTLR2) in HIV-infected women [41].

SCM can be viewed as an initial stage of infection and inflammation that carries a risk of subsequent progression to a more severe mastitis [19]. Regarding the low frequency of mastitis, the immune response in the mammary gland is most of the time able to prevent the adverse evolution of SCM into symptomatic mastitis. Hence, only a few women reported clinical mastitis (1% in HIV-infected and 0.5% in HIV-uninfected women) or other breast health problems in the VTS cohort [24, 53]. In our study, approximately a quarter of HIV-uninfected women and a third of HIV-infected women had SCM, which is consistent with other studies conducted in both HIV-infected and uninfected women [16, 17, 19, 54].

Previous studies have explored breast milk immune factors during SCM (Na/K ratio > 1). Increased IL-8, lactoferrin, SLPI and RANTES were observed in HIV-uninfected women with SCM [16, 17, 54]. We recently explored breast milk environment during SCM in HIV-uninfected mothers from the VTS cohort. Our findings indicated that SCM is associated with higher levels of B2M, PS100A9, TNF-α, IL-6, IL-8, IL-17, RANTES, IL-2R, IL-12p40/70, IFN-α, IFN-γ, MIG and IP-10 [19]. All these results highlighted a robust, prompt and predominant Th1 and pro-inflammatory response to SCM in HIV-uninfected women. Our data confirmed that, in the presence of SCM, breast milk immune environment of HIV-uninfected women was characterized by a robust immune response involving a broad panel of Th1 and inflammatory related immune factors, as well as anti-bacterial response. By comparison, the breast milk immune factor environment appeared severely impaired during SCM in HIV-infected women who didn’t transmit HIV to their child by breastfeeding, with only five immune factors that were significantly increased compared to fourteen parameters in HIV-uninfected women. Furthermore, the magnitude of immune factors concentration differences in the presence of SCM was slower in HIV-infected women compared to HIV-uninfected women.

These findings suggest that HIV infection is associated with a chronic stimulation of the Th1-related cytokines cascade in breast milk and an impaired ability to respond to SCM. The local inflammation of the mammary gland during SCM modulates both breast milk cell-free and cell-associated HIV levels and was found to increase HIV transmission through breastfeeding in several studies [25–30]. SCM is probably involved in mechanisms fueling local viral replication and traffic of infected cells in the mammary gland from the vascular compartment [31, 32]. Cytomegalovirus and Epstein–Barr virus that are part of the normal environment of the mammary gland can also facilitate breast milk HIV shedding and were found associated with HIV-1 transmission by breastfeeding in the same cohort [33]. Furthermore, in a recent study we reported that impaired capacity to secret IL-8 in breast milk during SCM was associated with detection of Epstein–Barr virus in breast milk from HIV-infected Zambian women which may in turn fuel HIV shedding [34].

As a cross-sectional study the absence of follow-up is one of the limitations of our study. Infant age at sampling varied, but all specimens were mature breast milk and multivariate analysis took into account this possible confounding factor known to influence immunologic environment in the mammary gland. In addition, the relative homogeneity of our study population was a strength to address the issue of the multiple environmental factors
that influence the immune composition of breast milk [55]. Exclusion of mother having transmitted HIV by breastfeeding may be viewed as a selection bias regarding the global population of HIV infected women. Based on the Vertical Transmission Study, the overall risk of postnatal HIV infection has been estimated at 3.9% among children breastfed for less than 6 months, each additional month of breastfeeding beyond 6 months of age being associated with a 1% risk of acquisition of HIV [56]. To be representative of the global population of HIV-infected mothers at risk of postnatal transmission, our study should have include an estimated number of two to three mothers having transmitted HIV infection by breastfeeding. However, we did not include women who transmitted HIV to their infant postnatally, assuming that the soluble immunologic pattern in breast milk from HIV-transmitters could form a peculiar type of pattern, as mentioned by several authors [46, 57–59].

**Conclusion**

HIV infection is associated with a raise of breast milk IP-10 and MIG concentrations, which are cytokines induced by IFN-γ and belong to antiviral defense. Results of this study including only women who didn't transmit HIV by breastfeeding suggest that during SCM, the breast milk environment is characterized by a lower and narrower immune response associated with maternal HIV infection. Further studies including women having transmitted HIV in the postnatal period are needed to confirm that the capacity of the mammary gland to face SCM is impaired in HIV-infected women and may contribute to facilitate HIV transmission by breastfeeding.

**Additional files**

**Additional file 1: Table S1.** Detection rates of immune factors in samples with and without sub-clinical mastitis, by HIV group. This table compares the detection rates of immune factors measured in mature breast milk samples with and without sub-clinical mastitis, separately for samples from HIV-infected and HIV-uninfected women. There were no detection rate differences between samples with and without SCM in samples from HIV-infected women. In comparison, in samples from HIV-uninfected women, interferon-α, interferon-γ, interleukin-6, and cytokines were more frequently detected in samples with SCM compared to samples without SCM.

**Additional file 2: Table S2.** Multivariate models assessing the effect of HIV on immunologic factor concentration in samples without SCM. This table indicates the adjusted regression coefficients and associated p-values of multivariate mixed linear models assessing the effect of sub-clinical mastitis on each breast milk soluble factor concentration, adjusted on child age at the time of sampling, separately for samples from HIV-infected and HIV-uninfected women. Sub-clinical mastitis was associated with and increase of 9/13 immune factors analyzed in samples from HIV-uninfected women compared to 7/17 immune factors analyzed in samples from HIV-infected women.

**Additional file 3: Table S3.** Correlations between breast milk immune factor concentration, HIV plasma parameters and breast milk HIV RNA. This table indicates the Spearman’s non-parametric correlations and associated p-values between breast milk soluble immunologic factor concentration and plasma HIV viral load (approximately 6 months after delivery), plasma CD4 count (approximately 6 months after delivery) and breast milk HIV RNA (at the time of breast milk sampling), in samples from HIV-infected women only. There are only a few weak correlations (maximum |ρ| = 0.44).

**Additional file 4: Table S4.** Breast milk immune factor comparisons between samples with sub-clinical mastitis from HIV-uninfected and HIV-infected women. This table compares the concentration of immune factors measured in mature breast milk samples with sub-clinical mastitis, between samples from HIV-infected and HIV-uninfected women. There were no statistically significant concentration differences in samples with sub-clinical mastitis between HIV-infected and HIV-uninfected women.

**Additional file 5: Table S5.** Multivariate models assessing the effect of SCM on immunologic factor concentration, by HIV group. This table indicates the adjusted regression coefficients and associated p-values of multivariate mixed linear models assessing the effect of sub-clinical mastitis on each breast milk soluble factor concentration, adjusted on child age at the time of sampling, separately for samples from HIV-infected and HIV-uninfected women. Sub-clinical mastitis was associated with and increase of 9/13 immune factors analyzed in samples from HIV-uninfected women compared to 7/17 immune factors analyzed in samples from HIV-infected women.

**Abbreviations**

ANRS: Agence Nationale de Recherche sur le SIDA; ART: antiretroviral therapy; B2M: β2-microglobulin; CD4: cluster of differentiation 4; CRP: C-reactive protein; EBF: exclusive breastfeeding; EPO: erythropoietin; FDR: false discovery rate; GM-CSF: granulocyte and macrophage growth factor; HIV: human immunodeficiency virus; IFN: interferon; Ig: immunoglobulin; IL: interleukin; IL-1RA: receptor antagonist of interleukin 1β; IL-2R: interleukin 2 receptor; IP: inflammatory protein; LBP: lipopolysaccharide-binding protein; MBF: mixed breastfeeding; MIP: monocyte chemotactic protein; MCP: monokine induced by gamma interferon; MIP: macrophage inflammatory protein; MTCT: mother-to-child HIV transmission; PS100A9: S100A9 protein; RANTES: regulated upon activation normal T-cell expressed and secreted; RNA: ribonucleic acid; sCD14: soluble CD14; SCM: sub-clinical mastitis; SDF-1α: stromal cell-derived factor-1α; SLPI: secretory leukocyte peptide inhibitor; S100L2: soluble toll-like receptor 2; TH: T helper; TNF-α: tumor necrosis factor-α; VTS: vertical transmission study; WHO: World Health Organization.

**Authors’ contributions**

MLN and RB contributed to the design, implementation and analysis of the VTS. PvdP, NIN, Jv and ET conceived and designed the ANRS 1271 study. SB and KB carried out laboratory measures. RS and PD analyzed the data. RS and ET interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

**Author details**

1. Pathogenesis and Control of Chronic Infections, INSERM, EFS, Université Montpellier 1, Montpellier, France. 2. Département d’Information Médicale, CHU de Montpellier, Montpellier, France. 3. Département de Biochimie, CHU de Montpellier, Montpellier, France. 4. Africa Centre for Health and Population Studies, University of KwaZulu-Natal, Durban, South Africa. 5. Department of Medical Virology, University of Pretoria and NHLS, Pretoria, South Africa. 6. Département de Bactériologie-Virologie, CHU de Montpellier, Montpellier, France. 7. Institute for Developmental Science, Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, UK. 8. School of Public Health, Faculty of Health Sciences, University of Witwatersrand, Johannesburg, South Africa. 9. Royal Hospital for Children, Institute of Health and Wellbeing, University of Glasgow, Glasgow, UK. 10. Present Address: CIC AG/Inserm 1424, Centre Hospitalier de Cayenne, Av. des flamboyants, BP 6006, 97 306 Cayenne CEDEX, French Guiana, France.
Acknowledgements
We thank the participants and co-workers of the Vertical Transmission Study.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The VTS study and breast milk analyses were approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and all women enrolled gave informed consent.

Funding
The work was supported by funding from Wellcome Trust and Agence Nationale de Recherche sur le SIDA (ANRS) (Grant Numbers VTS 063009/Z/00/Z, ANRS 1271).

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 2 May 2018   Accepted: 17 October 2018
Published online: 25 October 2018

References
1. HIV/AIDS data and statistics. Geneva: WHO; http://www.who.int/hiv/data/en/. Accessed 29 Jan 2018.
2. Bispo S, Chikhungu L, Rollins N, Siegfried N, Newell M-L. Postnatal HIV transmission in breastfed infants of HIV-infected women on ART: a systematic review and meta-analysis. J Int AIDS Soc. 2017;20:21251. https://doi.org/10.7448/ias.20.1.21251.
3. World Health Organization. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach. 2nd ed. Geneva: World Health Organization; 2016. http://www.ncbi.nlm.nih.gov/books/NBK374294/. Accessed 20 Mar 2018.
4. World Health Organization. Guidance: updates on HIV and infant feeding: the duration of breastfeeding, and support from health services to improve feeding practices among mothers living with HIV. Geneva: World Health Organization; 2016. http://www.ncbi.nlm.nih.gov/books/NBK9872/. Accessed 20 Mar 2018.
5. Goldman AS. The immune system in human milk and the developing infant. Breastfeed Med Off J Acad Breastfeed Med. 2007;2:195–204.
6. Field CJ. The immunological components of human milk and their effect on immune development in infants. J Nutr. 2005;135:1–4.
7. Bachrach VRG, Schwarz E, Bachrach LR. Breastfeeding and the risk of hospitalization for respiratory disease in infancy: a meta-analysis. Arch Pediatr Adolesc Med. 2003;157:237–43.
8. Walker A. Breast milk as the gold standard for protective nutrients. J Pediatr. 2010;156(2 Suppl):53–7.
9. Garofalo R. Cytokines in human milk. J Pediatr. 2010;156(2 Suppl):S36–40.
10. Hosea Blewett HJ, Cicalo MC, Holland CD. Field CJ. The immunological components of human milk. Adv Food Nutr Res. 2008;54:45–80.
11. Buescher ES, Hair PS. Human milk anti-inflammatory component contents during acute mastitis. Cell Immunol. 2001;210:87–95.
12. Fetherston CM, Wells J, Hartmann PE. Severity of mastitis symptoms as a predictor of C-reactive protein in milk and blood during lactation. Breastfeed Med Off J Acad Breastfeed Med. 2006;1:127–35.
13. Fetherston CM, Lai CT, Hartmann PE. Relationships between symptoms and changes in breast physiology during lactation mastitis. Breastfeed Med Off J Acad Breastfeed Med. 2006;1:136–45.
14. Hunt KM, Williams JE, Shafi F, Hunt MK, Behre R, Ting R, et al. Mastitis is associated with increased free fatty acids, somatic cell count, and interleukin-8 concentrations in human milk. Breastfeed Med Off J Acad Breastfeed Med. 2013;8:105–10.
15. Mizuno K, Hatsuno M, Aikawa K, Takeichi H, Hirti MT, Kaneko A, et al. Mastitis is associated with IL-6 levels and milk-fat globule size in breast milk. J Hum Lact Off J Int Lact Consult Assoc. 2012;28:529–34.
16. Filteau SM, Rice AL, Ball JJ, Chakraborty J, Stolzafus R, de Francisco A, et al. Breast milk immune factors in Bangladeshi women supplemented postpartum with retinol or beta-carotene. Am J Clin Nutr. 1999;69:593–8.
17. Rasmussen LBW, Hansen DT, Kaestle P, Michaelsen KF, Friis H, Larsen T. Milk enzyme activities and subclinical mastitis among women in Guinea-Bissau. Breastfeed Med Off J Acad Breastfeed Med. 2008;3:215–9.
18. Aryeetey RNO, Marquis GS, Timms L, Larrey A, Brakohiapa L. Subclinical mastitis is common among Ghanaian women lactating 3 to 4 months postpartum. J Hum Lact Off J Int Lact Consult Assoc. 2008;24:263–7.
19. Tuallion E, Vlijoen I, Dujoy P, Cambonie G, Rubbo P-A, Nagot N, et al. Subclinical mastitis occurs frequently in association with dramatic changes in inflammatory/anti-inflammatory breast milk components. Pediatr Res. 2016;81(4):556–64.
20. Filteau SM, Lietz G, Mulokogzi G, Bilotta S, Henry C, Tomkins AM. Milk cytokines and subclinical breast inflammation in Tanzanian women: effects of dietary red palm oil or sunflower oil supplementation. Immunology. 1999;97:595–600.
21. Richards AA, Darboe MK, Kelling T, Smith GD, Prentice AM, Lavor DA. Breast milk sodium content in rural Gambian women: between- and within-women variation in the first 6 months after delivery. Paediatr Perinat Epidemiol. 2010;24:235–61.
22. Foxman B, D’Arcy H, Gillespie B, Bobo JK, Schwartz K. Lactation mastitis: occurrence and medical management among 946 breastfeeding women in the United States. Am J Epidemiol. 2002;155:103–14.
23. Inch S, Von Xylander S. Mastitis: causes and management. Geneva: WHO; 2000. http://apps.who.int/iris/bitstream/10665/66230/1/WHO_FCH_CAH_00.13_eng.pdf. Accessed 29 Jan 2018.
24. Bland RM, Bequet R, Rollins NC, Coutousdouts A, Coovadia HM, Newell ML. Breast health problems are rare in both HIV-infected and HIV-uninfected women who receive counseling and support for breastfeeding in South Africa. Clin Infect Dis Off Publ Infect Dis Soc Am. 2007;45:1502–10.
25. Sembra RD, Kumwenda N, Hoover DR, Taha TE, Quinn TC, Mtumwalye L, et al. Human immunodeficiency virus load in breast milk, mastitis, and mother-to-child transmission of human immunodeficiency virus type 1. J Infect Dis. 1999;180:93–8.
26. Williamson IF, Filteau SM, Coutousdouts A, Newell M-L, Rollins NC, Coovadia HM, et al. Breast milk RNA viral load in HIV-infected South African women: effects of subclinical mastitis and infant feeding. AIDS Lond Engl. 2003;17:407–14.
27. Lunney KM, Iliff P, Mutasa K, Ntzezini R, Magder LS, Moulton LH, et al. Associations between breast milk viral load, mastitis, exclusive breastfeeding, and postnatal transmission of HIV. Clin Infect Dis Off Publ Infect Dis Soc Am. 2010;50:762–9.
28. Cantarci S, Koulinska IN, Aboud S, Fawzi WW, Villamor E. Subclinical mastitis, cell-associated HIV-1 shedding in breast milk, and breast-feeding transmission of HIV-1. J Acquir Immune Defic Syndr. 1999;20:651–4.
29. Phiri W, Kasonda L, Collin S, Makasa M, Sinkala M, Chintu C, et al. Factors influencing breast milk HIV RNA viral load among Zambian women. AIDS Res Hum Retroviruses. 2006;22:607–14.
30. Hoffman IF, Martinson FE, Steward PW, Chilongozi DA, Leu S-Y, Kazembe PN, et al. Human immunodeficiency virus type 1 RNA in breast-milk components. J Infect Dis. 2003;188:1209–12.
31. Danaviah S, de Oliveira T, Bland R, Vlijoen I, Pillay S, Tuallion E, et al. Evidence of long-lived founder virus in mother-to-child HIV transmission. PLoS ONE. 2015;10:e0120389.
32. Vlahov D, Tuallion E, Ali Tabaya Y, Rout F, Rubbo P-A, Media N, et al. CD4+ T cells spontaneously producing human immunodeficiency virus type I in breast milk from women with or without antiretroviral drugs. Retrovirology. 2011;8:34.
34. Sanosyan A, Rutagwera DG, Moulés J-P, Bollere K, Peries M, Kankasa C, et al. Increased Epstein–Barr virus in breast milk occurs with subclinical mastitis and HIV shedding. Medicine. 2016;95. https://doi.org/10.1097/md.0000000000004005.

35. Paiadurn M, Muller-Trutwin M. HIV-associated chronic immune activation. Immunol Rev. 2013;254:78–101.

36. Tinti C, Douek DC, Marchetti G. Gut barrier structure, mucosal immunology and intestinal microbiota in the pathogenesis and treatment of HIV infection. AIDS Res Ther. 2016;13:19.

37. Lajoie J, Juno J, Burgher A, Rahman S, Mogk W, Wachihi C, et al. A distinct cytokine and chemokine profile at the genital mucosa is associated with HIV-1 protection among HIV-exposed seronegative commercial sex workers. Mucosal Immunol. 2012;5:277–87.

38. Igbal SM, Ball TB, Kimani J, Kiama P, Thottingal P, Embree JE, et al. Elevated T cell counts and RANTES expression in the genital mucosa of HIV-1-resistant Kenyan commercial sex workers. J Infect Dis. 2005;192:728–38.

39. Bosire R, Guthrie BL, Lohman-Payne B, Mabuka J, Majiwa M, Wariua G, et al. Longitudinal comparison of chemokines in breastmilk early postpartum among HIV-1-infected and uninfected Kenyan women. Breastfeed Med Off J Acad Breastfeed Med. 2007;2:129–38.

40. Shapiro RL, Lockman S, Kim S, Smeaton L, Rahkola JT, Thor I, et al. Infant morbidity, mortality, and breast milk immunologic profiles among breastfeeding HIV-infected and HIV-uninfected women in Botswana. J Infect Dis. 2007;196:562–9.

41. Henrick BM, Yao X-D, Dranik AG, Abimiku A, Rosenthal KL, INFANT Study Team. Soluble toll-like receptor 2 is significantly elevated in HIV-1 infected breast milk and inhibits HIV-1 induced cellular activation, inflammation and infection. AIDS Lond Engl. 2014;28:2023–32.

42. Rice BD, Batzger-Fegenbaum J, Hosegood V, Tarser F, Hill C, Bar-nighausen T, et al. Population and antenatal-based HIV prevalence estimates in a high contracing female population in rural South Africa. BMC Public Health. 2007;7:160.

43. Coovadia HM, Rollins NC, Bland RM, Little KE, Coutsoudis A, Bennish ML, et al. Mother-to-child transmission of HIV-1 infection during exclusive breastfeeding in the first 6 months of life: an intervention cohort study. Lancet. 2007;369:107–16.

44. Bland R, Coovadia H, Coutsoudis A, Rollins N, Newell M. Cohort profile: mananengane or the Africa centre vertical transmission study. Int J Epidemiol. 2010;39:351–60.

45. Bosire R, John-Stewart GC, Mabuka JM, Wariua G, Gichuki C, Wamalwa D, et al. Breast milk alpha-defensins are associated with HIV type 1 RNA and CC chemokines in breast milk but not vertical HIV type 1 transmission. AIDS Res Hum Retroviruses. 2007;23:198–203.

46. Farquhar C, Mbora-Ngacha DA, Redman MW, Bosire RK, Lohman BL, Piantadosi AL, et al. CC and CXC chemokines in breastmilk are associated with mother-to-child HIV-1 transmission. Curr HIV Res. 2005;3:361–9.

47. Abou-Dakn M, Richardt A, Schaefer-Graf U, Wöckel A. Inflammatory breast diseases during lactation: milk stasis, puerperal mastitis, abscesses of the breast, and malignant tumors—current and evidence-based strategies for diagnosis and therapy. Breast Care. 2010;5:33–7.

48. Hawkes JS, Bryan DL, James M, Gibson RA. Cytokines (IL-1 beta, IL-6, TNF-alpha, TGF-beta1, and TGF-beta2) and prostaglandin E2 in human milk during the first three months postpartum. Pediatr Res. 1999;46:194–9.

49. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B Methodol. 1995;57:289–300.

50. Yilmaz HL, Saygili-Yilmaz ES, Gunesacar R. Interleukin-10 and -12 in human milk at 3 stages of lactation: a longitudinal study. Adv Ther. 2007;24:603–10.

51. Mekki A-RMA, Saleem TH, Al-Ghazali MH, Sayed AA. Interleukins-6, -8, -10 and tumor necrosis factor-alpha and its soluble receptor I in human milk at different periods of lactation. Nutr Res. 2003;23:845–55.

52. Kverka M, Buranová J, Lodiño-Adaniková R, Kocourková I, Cinova J, Tucková L, et al. Cytokine profiling in human colostrum and milk by protein array. Clin Chem. 2007;53:955–62.

53. Bland RM, Little KE, Coovadia HM, Coutsoudis A, Rollins NC, Newell M-L. Intervention to promote exclusive breast-feeding for the first 6 months of life in a high HIV prevalence area. AIDS Lond Engl. 2008;22:883–91.

54. Sembda RD, Kurnwendha N, Taha TE, Hoeer DR, Lan Y, Eisinger W, et al. Mastitis and immunological factors in breast milk of lactating women in Malawi. Clin Diagn Lab Immunol. 1999;6:71–4.

55. Ruiz L, Espinoza-Martos I, Garcia-Carral C, Manzano S, McGuire MK, Meehan CL, et al. What's normal? Immune profiling of human milk from healthy women living in different geographical and socioeconomic settings. Front Immunol. 2017;8:696.

56. Becquet R, Bland R, Leroy V, Rollins NC, Ekouevi DK, Coutsoudis A, et al. Duration, pattern of breastfeeding and postnatal transmission of HIV: pooled analysis of individual data from West and South African cohorts. PLoS ONE. 2009;4:e7397.

57. Arsenault JE, Webb AL, Koulimska IN, Aboud S, Fawzi WW, Villamor E. Association between breast milk erythropoietin and reduced risk of mother-to-child transmission of HIV. J Infect Dis. 2010;202:370–7.

58. Walter J, Kuhn L, Ghosh MK, Kankasa C, Semrau K, Sinkala M, et al. Low and undetectable breast milk interleukin-15 concentrations are associated with reduced risk of postnatal HIV transmission. J Acquir Immune Defic Syndr. 1999;20:6:100–7.

59. Walter J, Ghosh MK, Kuhn L, Semrau K, Sinkala M, Kankasa C, et al. High concentrations of interleukin 15 in breast milk are associated with protection against postnatal HIV transmission. J Infect Dis. 2009;200:498–502.