# *Immunoglobulin Classes and Acute Phase Proteins In The Breast Milk and Plasma of Nigerian HIV-Infected Lactating Mothers*

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***ABSTRACT***

*Relationship between infections and human breast milk still requires more research especially, in developing countries. This study aims to evaluate the effects of HIV infection on immunologic factors in the breast milk and plasma of HIV infected lactating mothers. Plasma and breast milk concentrations of immunoglobulins G, A, M and E, caeruloplasmin (CLP), complement factor IIIc (C3c), a1-antitrypsin (A1AT), and transferring (TRF) were determined in 20 asymptomatic HIV-infected lactating mothers (HIM) and 30 age-matched HIV-free (seronegative) lactating mothers (HFM) using Enzyme Linked Immunosorbent Assay and Single Radial Immunodiffusion technique. The mean plasma level of IgM was significantly elevated in HIM compared with HFM while the mean plasma level of IgE was significantly low in HIM compared with HFM. The mean breast milk levels of IgA, IgM, CLP, C3c and TRF were significantly elevated in HIM compared with HFM. Significant positive correlation existed between IgE in the plasma and breast milk of HIM and plasma IgG had significant positive correlation with the milk IgG in HFM. Severe hypergammaglobulinaemia may not be a feature of Nigerian HIM and increased levels of CLP and TRF indicate low levels of Cu and Fe respectively in the breast milk of HIM.*

***Key words**: Acute phase protein, breast milk, HIV, immunoglobulin*

## Nijerya'da HIV'le Enfekte Emziren Kadınların Plazma ve Sütünde İmmunoglobulin Sınıfları ve Akut Faz Proteinleri

***ÖZET***

*İnsan anne sütü ve enfeksiyon arasındaki ilişki özellikle gelişmekte olan ülkelerde daha fazla araştırmaya ihtiyaç duymaktadır. Bu çalışma HIV enfeksiyonunun HIV enfekte emziren annelerin plazma ve anne sütündeki immünolojik faktörler üzerine etkisini araştırmayı amaçlamıştır. Immünoglobülin G,A,M ve E, ceruloplasmin (CLP), kompleman faktör IIIc (C3c), a1-antitripsin (A1AT), ve transfer faktör (TRF) 20 asemptomatik HIV enfekte emziren kadın (HIA) ve 30 yaş-uyumlu HIV (-)(seronegatif) emziren anne (HFA)'de Enzyme Linked Immunosorbent Assay ve Single Radial Immunodifüzyon teknikleri kullanılarak değerlendirildi. IgM'nin Ortalama plazma düzeyi HIA'da HFA ile karşılaştırıldığında belirgin olarak yükselmiş, ortalama plazma IgE düzeyi belirgin olarak düşük olarak tespit edildi. IgA, IgM, CLP, C3c ve TRF'nin ortalama anne sütü değerleri HIA'lerde HFA annelere kıyasla belirgin olarak artmıştı. HIA'lerin plazma ve anne sütündeki IgE arasında belirgin pozitif korelasyon ortaya çıkmıştı ve plazma IgG HFA'lerin süt IgG'si ile belirgin pozitif korelasyona sahipti. Şiddetli hipergamaglobulinemi Nijeryalı HIA'lerin bir bulgusu olmayabilir ve artmış CLP ve TRF düzeyleri sırasıyla HIA'lerin sütünde düşük Cu ve Fe düzeylerini göstermektedir.*

***Anahtar kelimeler**: Akut faz proteini, anne sütü, HIV, immünoglobulin*

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## INTRODUCTION

Human Immunodeficiency Virus (HIV) is a retrovirus that primarily infects vital organs and cells of the human immune system thereby culminating in low cellular immunity (1,2). Previous studies have shown that the levels of IgG, IgM and IgA are elevated in the sera of HIV-infected individuals (3,4). Semba et al (5) and Arinola et al (4) reported that no significant differences exist between the plasma levels of some acute phase proteins (transferrin, complement factor IIIc, α1-antitrypsin among others) in HIV-infected individuals compared with the non-infected people. Various immunologic factors have been discovered to be present in human breast milk. These factors provide a passive immunologic support system from the mother to her infant in the first days to months after birth, awaiting endogenous maturation of the baby's own immunologic system (6,7). Human milk contains all classes of immunoglobulin (IgG, IgA, IgM, IgD and IgE) but secretory IgA is the most abundant one (8). They function as opsonin, agglutinin and complement activator among others. Shugars et al (9) reported that secretory IgA antibody could neutralize some strains of HIV. α1-antitrypsin, complement factor III, caeruloplasmin and transferrin are humoral immune factors involved in trypsin inhibition (10), opsonization (11), transport of Cu and Fe transport in the systemic circulation respectively. The interactions between HIV and humoral factors in the blood have been well studied (2). However, the relationship between HIV infection and humoral immune factors in human breast milk is yet to be explored as it is often encountered in other infections. This thus, necessitated this study.

## MATERIALS AND METHODS

### *Subjects*

The subjects were 20 asymptomatic HIV-infected lactating mothers (HIM) (28 ± 6.29 years of age) and 30 age-matched HIV-free lactating mothers (HFM) (26.1 ± 4.11 years of age). They were recruited from the Sexually Transmitted Infections (STI) and Immunization Clinics of Adeoyo Maternity Teaching Hospital, Yemetu, Ibadan, Nigeria after obtaining an informed consent from each patient. Ethical approval was also obtained from the Adeoyo Hospital Management (AMH/OG/1208). Five milliliters (5 ml) of venous blood and mature breast milk (15 days - 2 months post birth) were collected from each participant on the same day. Those on special medication (aside ARV), history of recent blood transfusion, hepatitis or mastitis infections, those infected post-partum and those with pre-term delivery were excluded from the study. The blood samples were collected into heparinized bottles to obtain plasma while the milk samples were collected into trace metal-free plastic tubes. The breast milk samples were spun at 8000 X g for 5 minutes and the fat layer was carefully removed to obtain fat-free milk plasma (12). A rapid assay using agglutination procedure (Capillus HIV-1 and 2) was used for the screening of suspected subjects and controls. Those tested positive to HIV-antibodies were confirmed with purified multiple recombinant antigens (Hexagon HIV-1 and 2).

### *Determination of IgE*

Enzyme linked Immunosorbent Assay (MICRO-ELISA, Leinco Technologies, USA) was used in determining the levels of IgE in the plasma and breast milk (13). The assay system utilizes two unique antibodies (a mouse monoclonal and a goat polyclonal) directed against distinct antigenic determinants on the IgE molecule. Plastic wells were coated with anti-IgE (mouse monoclonal) then test samples/controls containing IgE were added to the wells to form immune complexes. Anti-IgE (goat polyclonal) enzyme-labeled with horseradish peroxidase was added to each well and incubated for 45 minutes at room temperature, the IgE molecule is sandwiched between the solid phase and enzyme-labeled antibodies. The samples were decanted and washed severally to remove unbound-labeled antibody. An enzyme chromogen was added to the wells and incubated for 15 minutes at room temperature resulting in the development of a blue colour. A stopper was added to each well to stop the reactions and this was confirmed by the change of the blue colour to yellow. The intensity of the yellow colour is directly proportional to the concentration of IgE in the sample. Determination of immunoglobulin classes (IgG, IgM and IgA), caeruloplasmin, transferrin, complement factor IIIc and alpha-1-antitrypsin. The above mentioned parameters were determined using single radial immunodiffusion (14). The diameter of precipitin ring formed after antigen-antibody reaction in a buffered agar gel is proportional to the concentration of each parameter present in either the plasma or breast milk. A volume of diluted monospecific antiserum, specific for each parameter, was properly mixed with noble agar and poured on glass plate. Wells of equal diameter were made in the antibody/agar gel

***Table 1**. Levels (mean ± SD) of the immunoglobulin classes, TRF, CLP, A1AT and C3c in the plasma of HIV-infected mothers (HIM) and HIV-free mothers (HFM).*

| Parameter | HIM (n: 20) | HFM (n: 30) | t-values | p-values |
|---|---|---|---|---|
| IgG (g/L) | 5.062±1.50 | 4.886±1.66 | 0.375 | 0.709 |
| IgA (g/L) | 3.132±2.57 | 3.519±2.32 | 0.545 | 0.589 |
| IgM (g/L) | 8.154±7.00 | 3.166±3.29 | 3.371 | *0.000 |
| IgE (µg/L) | 20.632±4.19 | 44.035±4.40 | 18.489 | *0.000 |
| TRF (g/L) | 2.676±0.96 | 2.657±1.04 | 0.064 | 0.949 |
| CLP (g/L) | 1.085±0.90 | 0.903±0.83 | 0.725 | 0.448 |
| A1AT (g/L) | 1.115±0.38 | 1.047±0.28 | 0.716 | 0.478 |
| C3c (g/L) | 0.995±0.44 | 0.893±0.43 | 0.812 | 0.421 |

**p is significant at p< 0.05 value (2-tailed)*

and filled with standard plasma or test. The plates were incubated for 4 hours (IgG and TRF) and 18 hours (IgA, IgM, CLP, C3c and A1AT) at room temperature and the diameters of precipitin rings were measured using an illuminated Hyland viewer with a micrometer eyepiece.

***Statistical analysis***

Student t-test (pooled variance) was used to compare the differences between the mean ± SD of the parameters. Pearson's correlation coefficient was used to test the correlation between blood plasma and milk plasma using SPSS version 15.0. P < 0.05 value was considered significant.

**RESULTS**

As shown in Table 1, the mean plasma level of IgM increased significantly in HIM (8.154±7.00g/L) compared with HFM (3.166±3.29 g/L). In contrast, the mean plasma level of IgE was significantly low in HIM (20.632±4.19µg/L) compared with HFM (44.035±4.40µg/L). Also in Table 1, the mean plasma levels of IgG (5.062±1.50g/L), IgA (3.132±2.57g/L), CLP (1.085±0.90g/L), C3c (0.995±0.44g/L), A1AT (1.115±0.38g/L) and TRF (2.676±0.96g/L) were not significantly different in HIM compared with HFM (IgG: 4.886±1.66g/L, IgA: 3.519±2.32g/L, CLP: 0.903±0.83g/L, C3c: 0.893±0.43g/L, A1AT: 1.047±0.28g/L and TRF: 2.657±1.04g/L). In Table 2, the mean breast milk levels of IgA (3.114±0.30g/L), IgM (2.655±5.24g/L), CLP (0.013±0.02g/L), C3c (0.526±0.57g/L) and TRF (0.211± .21g/L) significantly increased in HIM compared with HFM (IgA : 0.308±0.29g/L, IgM: 0.039±0.14g/L, CLP: 0.000±0.00g/L, C3c: 0.056±0.14g/L and TRF: 0.000±0.00g/L). No significant differences were observed in the mean breast milk levels of IgG (0.860±0.00g/L), IgE (15.395±2.06µg/L) and A1AT (0.020±0.06g/L) in HIM compared with HFM (IgG: 0.867±0.38g/L, IgE: 15.552±2.64µg/L and A1AT: 0.059±0.09g/L) as also shown in Table 2. IgE in the breast milk of HIM showed significant positive correlation with IgE in the plasma. Similarly, IgG in the breast milk of HFM showed significant positive correlation with IgG in the plasma.

***Table 2**. Levels (mean ± SD) of the immunoglobulin classes, TRF, CLP, A1AT and C3c in the breast milk of HIV-infected mothers (HIM) and HIV-free mothers (HFM).*

| Parameter | HIM (n: 20) | HFM (n: 30) | t-values | p-values |
|---|---|---|---|---|
| IgG (g/L) | 0.860±0.00 | 0.867±0.38 | 0.793 | 0.432 |
| IgA (g/L) | 3.114±0.30 | 0.308±0.29 | 32.246 | *0.000 |
| IgM (g/L) | 2.655±5.24 | 0.039±0.14 | 2.752 | *0.008 |
| IgE (µg/L) | 15.395±2.06 | 15.552±2.64 | 0.219 | 0.828 |
| TRF (g/L) | 0.211±0.21 | 0.000±0.00 | 0.000 | *0.000 |
| CLP (g/L) | 0.013±0.02 | 0.000±0.00 | 2.987 | *0.004 |
| A1AT (g/L) | 0.020±0.06 | 0.059±0.09 | 1.744 | 0.088 |
| C3c (g/L) | 0.526±0.57 | 0.056±0.14 | 4.3524 | *0.000 |

**p is significant at p < 0.05 value (2-tailed)*

***Table 3.** Correlation analysis between plasma and breast milk parameters*

| | HIM (n: 20) | | HFM (n: 30) | |
|---|---|---|---|---|
| Parameter | r-values | p-values | r-values | p-values |
| IgG (g/L) | _ | _ | 0.417 | 0.022* |
| IgA (g/L) | -0.224 | 0.356 | -0.129 | 0.498 |
| IgM (g/L) | -0.092 | 0.707 | -0.114 | 0.550 |
| IgE (µg/L) | 0.543 | 0.016* | 0.112 | 0.562 |
| TRF (g/L) | -0.015 | 0.950 | _ | _ |
| CLP (g/L) | -0.173 | 0.479 | _ | _ |
| A1AT (g/L) | -0.152 | 0.535 | 0.055 | 0.771 |
| C3c (g/L) | 0.032 | 0.897 | -0.199 | 0.291 |

*p is significant at P < 0.05 value (2-tailed) (HIM: HIV infected mothers, HFM: HIV-free mothers)

## DISCUSSION

Breast milk is a complex fluid rich in nutrients with anti-infective properties for the healthy physical and mental development of babies in the first four to six months of life (15-17). It was reported that bacterial and viral infections of the intestinal tract and respiratory system in breast milk-fed infants are significantly lower when compared with the non-breast-fed infants (18). This is due to the presence of secretory antibodies, against bacteria and virus found in human milk (19). As shown in Table 1, IgM plasma level was found to be significantly higher in HIM compared with HFM. This was in concordance with the observations of the previous reports (3,4, 20,21). These studies also reported significant elevation in the serum levels of IgA and IgG, however we did not observe such changes in our study. Immune-modulating effects of pregnancy could be responsible for the observed differences since their subjects were not recruited post-partum. The elevated level of IgM observed in this study could be due to preferential production of IgM required to protect the HIV-infected mother from opportunistic infections at a time when the growing infant needs her optimal attention since IgM is necessary for both complement fixation and agglutination which are vital in control of viral infections. The mean plasma level of IgE in HIM was significantly low compared with HFM. This is in contrary to the reports of Arinola and Igbi (20) who reported that the viral envelope protein (gp 41) induces polyclonal B-cell activation resulting in excessive production of immunoglobulin classes. The low level of IgE could be a consequence of Nevirapine that was used to prevent mother-to-child transmission of HIV which might have immune modulating effect(s). This could be useful so as to prevent mass extrusion of IgE into the mother's breast milk which could mediate type-1 hypersensitivity in the infant, resulting in a fatal condition. The mean plasma levels of TRF, A1AT, CLP and C3c in HIM were not statistically significantly different compared with the mean levels in HFM as shown in Table 2. The similar levels of TRF observed in HIM and HFM are in agreement with the reports of Arinola et al. (22,4). This could be due to a balance between reduction of transferrin (as a result of its short half-life) and elevation in transferrin level (as a result of iron deficiency anaemia). Iron deficiency anaemia is a common feature in HIV-seropositive individuals due to chronic diarrhoea, mal-absorption and frequent desquamation of the skin and malnutrition commonly found in HIV-infection (22). The observed non-significant levels of A1AT and C3c corroborated the reports of Arinola et al (4) who reported similar serum levels of some acute phase proteins (alpha-1-fetoprotein and alpha-1-glycoprotein) in Nigerian HIV infected patients. However, the non-significant plasma level of CLP contradicts the report of Arinola et al (4). They claimed that raised plasma level of CLP in their subjects could either be due to Cu deficiency (a common finding in HIV subjects) or reduced phagocytic activity which will cause non rapid consumption of CLP as its production. These reasons might not hold in our subjects because they might be on supplement during pregnancy and more so, they were all on ARV therapy which could confer on them a near-normal immune response. In the breast milk however (Table 2), significant elevations in the levels of IgA, IgM, TRF, C3c and CLP were observed in the breast milk of the HIM compared with HIF. No significant differences were observed in the breast milk levels of IgG, IgE and A1AT between the two groups. The significant elevation in the levels of IgA, IgM, transferrin and caeruloplasmin, and presence of IgG, A1AT could either be due to increased mobilization from the blood into the breast milk as a consequence of HIV infection (to protect the suckling infant) or due to

Nevirapine (used to prevent mother-to-child transfer), which might have immune modulating activities as stated earlier. Wilson et al (23) reported that CCL28 controls the IgA plasma cell accumulation in the lactating mammary gland and that CCL28 has anti-microbial activities (24). It is possible that there is an upregulation of CCL28 (mucosae-associated epithelial chemokine) activities. These functions of CCL28 may be responsible for the high level of IgA observed in the breast milk of HIM. In the same vein, a similar upregulation in activities of control mechanisms of IgM, transferrin and caeruloplasmin could also occur. The elevation of IgA level in HIM breast milk could also be due to increased levels of certain cytokines as observed by Musumeci et al (25) in the breast milk of HIV-infected mothers. Interleukin-6 (IL-6) has been shown to affect the synthesis of IgA (26) and IgM (27). Elevated level of C3c observed in the breast milk of HIM compared with HFM is a direct consequence of high breast milk level of IgM (involved in complement fixation) observed in this group. This high complement activation may protect the mother's breast or as a result of complement-mediated cell lysis commonly found in viral infections so as to reduce HIV viral load in the breast milk. Transferrin and caeruloplasmin were observed only in the breast milk of HIM but not detected at all in the breast milk of HFM. Goldman (28) reported that an analogue of transferrin known, as lactoferrin is the Fe transporting protein found in human milk. Although the absence of caeruloplasmin in the breast milk samples of HFM was in contrary with the reports of Nabukhotnyi et al (29) and Puchkova et al (30), it could be that single radial immunodiffusion technique is not sensitive enough to detect low concentrations of CLP since CLP level decreases post-delivery in apparently healthy lactating mothers. The presence of transferrin and CLP in the breast milk of HIM may indicate Fe and Cu deficiencies respectively in the breast milk of the HIV mothers. It might also be due to redistribution from the plasma into the breast tissue as a result of the infection thereby culminating in increased extrusion of the elevated parameters. The relationship between the presence of transferrin and/or caeruloplasmin in breast milk and HIV infection could have been substantiated if the CD4+ count of the patients was determined. Therefore, more researches are still required to establish the relationship between breast milk transferrin/caeruloplasmin and stage of HIV infection. Significant positive correlation (Table 3) existed between IgE in the plasma and breast milk of HIM. Similarly, significant positive correlation existed between IgG in the plasma and breast milk of HFM. This could be due to increased level of IgG synthesis at the third trimester of pregnancy. It may be concluded from this study that certain humoral immunologic factors are not deficient in the breast milk of HIV-infected mothers. It could also be concluded from this study that severe hypergammaglobulinaemia may not be a feature of HIV-infected lactating mothers on ARV therapy. The increased levels of IgA, IgM and C3c in the breast milk of HIV-infected lactating mothers may be to protect the mothers and their infants from HIV-infection or even to neutralize some strains of HIV. However, increased levels of CLP and TRF may indicate low levels of Cu and Fe respectively in the breast milk of HIM.

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