Lactotransferrin gene functional polymorphisms do not influence susceptibility to human immunodeficiency virus-1 mother-to-child transmission in different ethnic groups

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Lactotransferrin, also known as lactoferrin, is an iron binding glycoprotein that displays antiviral activity against many different infectious agents, including human immunodeficiency virus (HIV)-1. Lactotransferrin is present in the breast milk and in the female genitourinary mucosa and it has been hypothesised as a possible candidate to prevent mother-to-child HIV-1 transmission. To verify if two functional polymorphisms, Thr29Ala and Arg47Lys, in the lactotransferrin encoding gene (LTF) could affect HIV-1 infection and vertical transmission, a preliminary association study was performed in 238 HIV-1 positive and 99 HIV-1 negative children from Brazil, Italy, Africa and India. No statistically significant association for the Thr29Ala and Arg47Lys LTF polymorphisms and HIV-1 susceptibility in the studied populations was found. Additionally LTF polymorphisms frequencies were compared between the four different ethnic groups.

Key words: AIDS - HIV-1 - innate immunity - lactoferrin - SNPs - vertical transmission

In 2013, about 240,000 children acquired human immunodeficiency virus (HIV)-1 infection worldwide (WHO 2013). The main route of HIV-1 infection for a child is through the HIV-1 positive mother, during delivery or breastfeeding. Without antiretroviral intervention, caesarean delivery (Legardy-Williams et al. 2010) or safer alternatives to breastfeeding, the transmission rate of HIV-1 from an infected mother to her infant varies from 30-45% (Datta et al. 1994).

In this context, innate immunity effector molecules have an important role in fighting against HIV-1 infection. Among them, lactotransferrin (also known as lactoferrin) seemed to be a possible candidate to prevent mother-to-child HIV-1 transmission. Lactotransferrin is found in breast milk and at the mucosal level in the female genitourinary tract (Kazmi et al. 2006): a reduction in maternal serum lactotransferrin has been correlated with increased risk of HIV-1 MTCT (Defer et al. 1995, Semba et al. 1998). Reduced lactotransferrin serum level have been detected in HIV-1 infected individuals compared to healthy controls (Dapsanse et al. 2001) and in acquired immune deficiency syndrome (AIDS) patients compared to asymptomatic and symptomatic HIV-1 positive individuals or controls (van der Strate et al. 2001).

The LTF gene (chromosomal localisation: 3p21.3) encodes lactotransferrin; more than 60 single nucleotide polymorphisms (SNPs) have been identified within the promoter region, exons or introns. Two nonsynonymous polymorphisms, Thr29Ala and Arg47Lys (rs1126477 and rs1126478, respectively) in the first exon of the LTF gene, resulting in amino acid changes in the mature peptide and located within the strongly basic N-terminal region of the protein, that mediates its antibacterial properties (Shanbacher et al. 1992) have been described as associated with different pathologies, such as periodontitis (Velliyagounder et al. 2003, Jordan et al. 2005, Wu et al. 2009), dental caries (Azevedo et al. 2010, Fine et al. 2013), ovarian cancer (Cao et al. 2011), nasopharyngeal carcinoma (Zhou et al. 2012), dyslipidaemia (Moreno-Navarrete et al. 2008) and coronary artery stenosis (Videm et al. 2012).

The Thr29Ala amino acid change results from an A-G substitution at nucleotide 88 and the Arg47Lys change results from an A-G transition at nucleotide 140 (Teng & Gladwell 2006).

This preliminary study aims at investigating the role of these two nonsynonymous SNPs in HIV-1 positive newborns infected by vertical transmission and children not infected but born from HIV-1 positive mothers, in four different populations from Brazil, Italy, Africa and India. Frequency differences within the four ethnic groups will be also described.
SUBJECTS, MATERIALS AND METHODS

Patients - Genomic DNAs from 238 HIV-1 positive children (all within 6 and 14 years of age; 126 females - 53% and 112 males - 47%) were obtained. The sample consisted of 78 Brazilian children (40 females and 38 males) from the state of Pernambuco, Northeast Region of Brazil, collected at the Prof Fernando Figueira Integral Medicine Institute, Recife, 89 Italians (49 females and 40 males), collected at the Children Hospital Regina Margherita, Turin, 26 Zimbabwe Africans (14 females and 12 males) and 45 Kerala Indians (23 females and 22 males). The last two samples both came from a historical cohort kindly provided by Prof Trabattoni (Sacco Hospital, Milan, Italy).

The children were infected during delivery [exposed infected (EI)], Ninety-nine exposed uninfected (EU) HIV-1 negative children (all within 6 and 14 years of age; 53 females - 54% and 46 males - 46%; 35 Brazilian, 20 females and 15 males; 18 Italian, 9 females and 9 males; 15 Zimbabwe African, 9 females and 6 males; 31 Kerala Indian, 15 females and 16 males), born from HIV-1 positive mothers, were already available at the Laboratory of Immunogenetics of Scientific Institute For Research, Hospitalization and Care (IRCCS Burlo Garofolo and were used for this study. Biological material from the mothers was not available and the sole information available to us was that none of the infected mothers underwent antiretroviral therapy before delivery or caesarean section to prevent HIV-1 infection of the newborns.

DNA extraction and SNPs genotyping - DNA extraction from whole blood was performed using the DNeasy Blood and Tissue Kit (Qiagen, Germany), in accordance with the manufacturers’ manual.

The two LTF (National Center for Biotechnology Information Reference Sequence: NC_000003), functional SNPs, Thr29Ala and Arg47Lys (rs1126477 and rs126478, respectively) were genotyped by real time polymerase chain reaction (PCR) using C_6998511_10 and C_6998521_10 fluorogenic allele-specific TaqMan SNPs genotyping assay probes (Applied Biosystems-Life Technologies, USA) on the AB7900HT Real Time PCR platform (Applied Biosystems), following manufacturer instructions: an initial step for Taq polymerase activation at 95°C for 10 min followed by 40 cycles with 15 s of 95°C for denaturation and 1 min at 60°C for extension.

Allelic discrimination was done both manually and automatically with the SDS detection software (Applied Biosystems).

Real time allele specific PCRs results were double checked by direct sequencing performed on 50 randomly chosen samples and 100% of concordance was found.

Statistical analysis - LTF gene polymorphisms, allele and genotype frequencies were calculated by direct counting, while haplotype frequency were computed using the Arlequin software v.3.1 (Excoffier & Lischer 2010).

The Fisher's exact test for pairwise comparison of allele, genotype and haplotype frequencies (using contingency tables as appropriate) was performed with the open-source R v.3.1.0 (R Development Core Team 2014). Only p values < 0.01 were considered to be significant.
The Arg47Lys G allele was more represented among our Brazilian population than AMR population (57% and 47% respectively). Moreover the G/G genotype was more frequent in our Brazilian population, whereas A/G and A/A genotype were more frequent in the AMR population (Tables III, IV).

Therefore, the allele and genotype frequencies of both polymorphisms were not statistically different when comparing EI with EU children in our populations (Tables I, II).

The Thr29Ala and Arg47Lys polymorphisms were in LD ($p < 0.07$, D’statistic > 0.72; $r^2$ statistic > 0.11 for all groups) and combined to form three major haplotypes (AG, GG and GA, with frequencies > 0.01) and one minor (AA, with frequency ≤ 0.01). Comparing the haplotype frequencies between EU and EI in the four populations, no statistically significant differences were observed when using Fisher test with 4 x 2 table (Table V). The same results were obtained considering the most frequent haplotype as reference and analysing all other haplotypes vs. that reference (data not shown).

### DISCUSSION

Several biological activities of lactotransferrin against HIV-1 have been described so far, ranging from the inhibition of virus-cell interaction through CXCR4 and CCR5 binding or viral gp120 V3 loop blocking (Berkhout et al. 2002), to inhibit viral processes within the host cells (Ng et al. 2001). Recently, Wong et al. (2014) have shown the inhibitory activity of lactotransferrin fragments lactoferricin and lactoferrampin against HIV-1 reverse transcriptase and integrase, respectively.

A reduction of the serum lactotransferrin concentration has previously been associated with increased HIV-1...
infection susceptibility (Defer et al. 1995, Semba et al. 1998, Dapsanse et al. 2001) and AIDS progression (van der Strate et al. 2001). Additionally, recent works have explored the role of lactotransferrin in the oral health among HIV-1 infected subjects. Alves et al. (2014) observed higher salivary lactotransferrin levels in HIV-1 infected children during Candida sp. oral colonisation. Ferreira et al. (2014) reported no lactotransferrin level alterations in gingival crevicular fluid and saliva from HIV-1 infected subjects with chronic periodontitis. To the best of our knowledge, no studies so far investigated the possible association between LTF genetic variants and susceptibility to HIV-1 infection or with progression to AIDS and its clinical manifestations.

Two functional polymorphisms within LTF gene, named Thr29Ala and Arg47Lys, were previously associated with an increased susceptibility to periodontitis development (Velliyagounder et al. 2003, Jordan et al. 2005, Wu et al. 2009), dental caries (Azevedo et al. 2010, Fine et al. 2013), ovarian cancer (Cao et al. 2011), nasopharyngeal carcinoma (Zhou et al. 2012), dyslipidaemia (Moreno-Navarrete et al. 2008) and coronary artery stenosis (Videm et al. 2012). In our study, the Thr29Ala and Arg47Lys polymorphisms were analysed in order to investigate the possible role of these LTF genetic variants on HIV-1 MTCT.

Our association analysis, performed among children born from HIV-1 positive mothers from four different populations, indicates that the LTF Thr29Ala and Arg47Lys allele, genotype and haplotype frequencies, are similar in HIV-1 infected and HIV-1 EU children and are also comparable to those reported by the HapMap project (International HapMap Consortium 2003) and 1000 Genomes project (GPC et al. 2012); the Brazilian population does not follow the same frequency distribution.

### TABLE II

LTF Arg47Lys polymorphism allele, genotype frequencies (and counts) in human immunodeficiency virus (HIV) positive (+) and negative (-) children in the four different populations analysed

| LTF SNP | HIV- | HIV+ | HIV- vs. HIV+ |
|---------|------|------|--------------|
| Arg47Lys (rs1126478) | n = 15 | n = 26 | p (CI) OR |
| **Africa** | | | |
| G | 0.97 (29) | 0.96 (50) | Reference |
| A | 0.03 (1) | 0.04 (2) | 1.00 (0.06-70.74) 1.16 |
| G/G | 0.93 (14) | 0.96 (25) | Not calculable |
| A/G | 0.07 (1) | 0.00 (0) | Not calculable |
| A/A | 0.00 (0) | 0.04 (1) | Not calculable |
| HWE (χ²; p) | 0.02; 0.89 | 26; 3.41E-07 |
| **India** | n = 31 | n = 45 |
| G | 0.66 (41) | 0.64 (58) | Reference |
| A | 0.34 (21) | 0.36 (32) | 0.86 (0.52-2.26) 1.08 |
| G/G | 0.39 (12) | 0.38 (17) | Reference |
| A/G | 0.55 (17) | 0.53 (24) | 1.00 (0.34-2.90) 0.99 |
| A/A | 0.06 (2) | 0.09 (4) | 1.00 (0.17-17.82) 1.40 |
| HWE (χ²; p) | 1.56; 0.21 | 1.21; 0.27 |
| **Brazil** | n = 35 | n = 78 |
| G | 0.57 (40) | 0.67 (104) | Reference |
| A | 0.43 (30) | 0.33 (52) | 0.18 (0.36-1.24) 0.67 |
| G/G | 0.40 (14) | 0.49 (38) | Reference |
| A/G | 0.34 (12) | 0.36 (28) | 0.82 (0.31-2.38) 0.86 |
| A/A | 0.26 (9) | 0.15 (12) | 0.26 (0.15-1.64) 0.50 |
| HWE (χ²; p) | 3.15; 0.08 | 2.88; 0.09 |
| **Italy** | n = 18 | n = 89 |
| G | 0.36 (13) | 0.40 (71) | Reference |
| A | 0.64 (23) | 0.60 (107) | 0.71 (0.53-2.70) 1.17 |
| G/G | 0.06 (1) | 0.16 (14) | Reference |
| A/G | 0.61 (11) | 0.48 (43) | 0.79 (0.20-2.45) 0.73 |
| A/A | 0.33 (6) | 0.36 (32) | 0.66 (0.27-129.16) 2.58 |
| HWE (χ²; p) | 1.89; 0.17 | 0.005; 0.94 |

CI: confidence interval; HWE: Hardy Weinberg equilibrium; LTF: lactoferrin encoding gene; OR: odds ratio; SNP: single nucleotide polymorphism.
probably due to the ethnic heterogeneity among Brazilians, that are an admixture of African, Caucasian and native American populations (Alves-Silva et al. 2000), and considering that the AMR population that was used as a comparison is not a Brazilian one, but is an admixture of Colombian from Medellin, Mexican ancestry from Los Angeles, United States of America, Puerto Ricans, from Puerto Rico; c: an admixture of Toscans, Italy; CI: confidence interval; LTF: lactoferrin encoding gene; OR: odds ratio; SNP: single nucleotide polymorphism.

During descriptive analysis, African and Italian populations seemed to present opposite allelic frequencies. On the other hand, among the Indian and the Brazilian subjects, the frequencies of the two polymorphisms seemed to be similar.

No significant differences were found when comparing HIV-1 positive and HIV-1 negative children, suggesting that the two LTF polymorphisms could be not involved in HIV-1 vertical transmission. However, the data are preliminary and the comparison of the frequencies among the distinct human groups is quite complex to be performed: it is not excluded that the lack of association may be due in part to the small sample size of the populations investigated combined with a small effect of LTF in HIV-1 susceptibility, a multifactorial trait. Indeed, although the total number of analysed individual was 337, the classification in HIV-1 positive and negative and in the four subgroups based on ethnic origins led to a reduction of the power of the study. In fact, except for the haplotype analysis in the Brazilian population (power = 0.96), all other tests had a low power (0.07...
### TABLE IV

*LTF* Arg47Lys polymorphism allele, genotype frequencies (and counts) in human immunodeficiency virus (HIV) positive (+) plus negative (-) children in the four different populations compared with the most similar ethnic group reported by HapMap and 1000 Genomes project.

| *LTF* SNP | Total (HIV+ plus HIV-) | HapMap/1000 Genomes | Total vs. Hapmap/1000 Genomes |
|-----------|-------------------------|----------------------|------------------------------|
| **Arg47Lys (rs1126478)** | | | |
| **Africa** (n = 41) | | | |
| G          | 0.97 (79) | 0.99 (223) | Reference |
| A          | 0.03 (3)  | 0.01 (3)   | 0.19 (0.37-21.43) 2.81 |
| G/G        | 0.93 (39) | 0.97 (110) | Reference |
| A/G        | 0.07 (1)  | 0.03 (3)   | 1.00 (0.02-12.11) 0.94 |
| A/A        | 0.00 (1)  | 0.00 (0)   | Not calculable |
| **India** (n = 76) | | | |
| G          | 0.66 (99) | 0.65 (371) | Reference |
| A          | 0.34 (53) | 0.35 (201) | 1.00 (0.66-1.46) 0.99 |
| G/G        | 0.39 (29) | 0.43 (124) | Reference |
| A/G        | 0.55 (41) | 0.43 (123) | 0.22 (0.81-2.54) 1.42 |
| A/A        | 0.06 (6)  | 0.14 (39)  | 0.51 (0.21-1.78) 0.66 |
| **Brazil** (n = 113) | | | |
| G          | 0.57 (144) | 0.47 (169) | Reference |
| A          | 0.43 (82)  | 0.53 (193) | 0.000006 (0.35-0.71) 0.50 |
| G/G        | 0.40 (52)  | 0.24 (44)  | Reference |
| A/G        | 0.34 (40)  | 0.45 (81)  | 0.002 (0.23-0.75) 0.42 |
| A/A        | 0.26 (21)  | 0.31 (56)  | 0.0004 (0.16-0.63) 0.32 |
| **Italy** (n = 107) | | | |
| G          | 0.36 (84)  | 0.33 (59)  | Reference |
| A          | 0.64 (130) | 0.67 (117) | 0.25 (0.50-1.21) 0.78 |
| G/G        | 0.06 (15)  | 0.10 (9)   | Reference |
| A/G        | 0.61 (54)  | 0.47 (41)  | 0.65 (0.28-2.16) 0.79 |
| A/A        | 0.33 (38)  | 0.43 (38)  | 0.35 (0.20-1.68) 0.60 |

*a*: Yoruba, Ibadan, Nigeria; *b*: an admixture from Han-Chinese in Beijing, China, Southern Han Chinese, Japanese in Tokyo, Japan; *c*: an admixture of Colombian from Medellin, Colombia, Mexican ancestry from Los Angeles, United States of America, Puerto Ricans, from Puerto Rico; *d*: Toscans, Italy; CI: confidence interval; *LTF*: lactoferrin encoding gene; OR: odds ratio; SNP: single nucleotide polymorphism.

Previous findings suggested that lactotransferrin could play a role in HIV-1 vertical transmission, but no studies have been conducted on the ability of the two *LTF* variants to prevent HIV-1 entry to the cells or to interfere with the viral processes. Considering our negative results we could speculate that, although the two polymorphisms are not synonymous and result in two amino acidic substitutions, these variations may not modify the antiviral activity of the mature protein; the PolyPhen-2 (Adzhubei et al. 2010) software predicted the two SNPs as benign and thus not able to radically affect the protein. Another hypothesis is that if even the two polymorphisms could influence the antiviral role of the protein, perhaps an increment in gene expression, due to...
TABLE V

*LTF* haplotype frequencies (and counts) in human immunodeficiency virus (HIV) positive (+) and negative (-) children in the four different populations analysed

| LTF haplotypes (Thr29Ala-Arg47Lys) | HIV-   | HIV+   | HIV- vs. HIV+ (p) |
|-----------------------------------|--------|--------|-------------------|
| Africa                            | n = 30 | n = 52 |                   |
| AG                                | 0.77 (23) | 0.77 (40) | 1.00              |
| GG                                | 0.20 (6)  | 0.19 (10) |                |
| GA                                | 0.03 (1)  | 0.04 (2)  |                 |
| AA                                | 0.00 (0)  | 0.00 (0)  |                 |
| LD (p; D'; r²)                    | 0.07; 0.99; 0.11 | 0.01; 0.99; 0.13 | -         |
| India                             | n = 62 | n = 90 |                   |
| AG                                | 0.47 (29) | 0.39 (35) | 0.56              |
| GG                                | 0.19 (12) | 0.25 (23) |          |
| GA                                | 0.34 (21) | 0.36 (32) |              |
| AA                                | 0.00 (0)  | 0.00 (0)  |                 |
| LD (p; D'; r²)                    | < 0.01; 0.99; 0.45 | < 0.01; 0.99; 0.35 | -         |
| Brazil                            | n = 70 | n = 156 |                   |
| AG                                | 0.46 (32) | 0.51 (79) | 0.19              |
| GG                                | 0.37 (26) | 0.32 (50) |            |
| GA                                | 0.11 (8)  | 0.16 (25) |            |
| AA                                | 0.06 (4)  | 0.01 (2)  |                 |
| LD (p; D'; r²)                    | < 0.01; 0.72; 0.41 | < 0.01; 0.91; 0.45 | -         |
| Italy                             | n = 36 | n = 178 |                   |
| GA                                | 0.64 (23) | 0.60 (106) | 0.85          |
| AG                                | 0.25 (9)  | 0.30 (54)  |             |
| GG                                | 0.11 (4)  | 0.10 (17)  |             |
| AA                                | 0.00 (0)  | 0.01 (1)   |           |
| LD (p; D'; r²)                    | < 0.01; 0.99; 0.59 | < 0.01; 0.97; 0.63 | -         |

LD: linkage disequilibrium; *LTF*: lactoferrin encoding gene.

external factors other than genetic variations, may compensate the alterations within the molecule. A study conducted by Moreno-Navarrete et al. (2008) showed that the two *LTF* polymorphisms did not correlate with the plasma protein level in a Spanish population.

Unfortunately, in this study, being a retrospective one performed on historical groups of HIV-1 patients infected through vertical transmission, biological samples other than DNA were not available to perform functional studies and investigating the effective influence of *LTF* genetic variants on protein production or on protein functionality. Other limitations of the study are the lack of information about the mother’s genotypes and regarding breastfeeding occurrence.

Our results suggest that *LTF* genetic polymorphisms Thr29Ala and Arg47Lys are not associated with susceptibility to HIV-1 MTCT, but our data are preliminary, obtained on low number of individual, also lacking of functional characterisation of the analysed SNPs. Therefore, further large-scale studies and functional analysis are necessary to clarify the potential role of *LTF* gene polymorphisms in HIV-1 infection susceptibility.

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