UNIVERSITÀ DEGLI STUDI DI TRIESTE
Sede amministrativa del Dottorato di Ricerca

Universidade Federal do Pernambuco Instituto Materno Infantil do Pernambuco

Laboratório de Imunopatologia Keizo Azami

XX CICLO DEL DOTTORATO IN RICERCA IN

MEDICINA MOLECOLARE
(Settore scientifico-disciplinare MED/03 GENETICA MEDICA)

Genomic variability of host factors in AIDS: role of antimicrobial peptides in resistance to lentiviral infections

DOTTORANDO
Dr. Michele Milanese

COORDINATORE DEL COLLEGIO DEI DOCENTI
Chiar.mo Prof. Gianni Del Sal,
Università degli Studi di Trieste

SUPERVISORE
Chiar.mo Prof. Luiz Claudio Arraes,
Universidade Federal do Pernambuco

RELATORE
Chiar.mo Prof. Sergio Crovella,
Università degli Studi di Trieste

Supervisor:

Prof. Sergio Crovella
Università degli studi di Trieste
Via Dell'Istria 65/1
34100 Trieste

External supervisor:

Prof. Luiz Claudio Arraes,
Universidade Federal do Pernambuco
Rua Arnóbio Marques, 310
Santo Amaro
50100130 - Recife, PE - Brasil

Committee:

Prof. Tedesco Francesco
Università degli studi di Trieste
Piazzale Europa 1
34100 Trieste

Prof. Gustincich Stefano
Scuola Internazionale di Studi Superiori Avanzati
c/o AREA Science Park,
Ss 14, Km 163.5
34012 Basovizza (TS)

Prof. Massimo Levero
Università degli studi di Roma "La Sapienza"
Regina Elena Cancer Insitute
Via delle Messi d'Oro 156
00158 Roma

A chi, nonostante tutto,
non si ferma alla superficie

TABLE OF CONTENTS

ABSTRACT

AIDS is one of the most important pandemics currently taking place on Hearth. Unfortunately the high mutation rate of the virus is preventing immunologist to find an effective vaccine against its etiological agent: HIV. Therefore new biological targets needs to be identified to develop new approaches to prevent and treat HIV infection.
Antimicrobial peptides are important molecules involved in natural immune response characterized by both antimicrobial and antiviral activity and, given their role as a first defense barrier against pathogens, probably play a relevant function in HIV-1 transmission and infection. They probably interact on infection site to give a first defense barrier against virus ingression. Many studies reported anti-HIV-1 effects of different antimicrobial peptides; nonetheless none of those studies took into account the fact that different classes of antimicrobial peptides, being contemporarily present at the site of infection, are likely to cooperate synergistically.

In our work we were able to investigate the role of single nucleotide polymorphisms (SNPs) in a number of genes related to the innate immunity on HIV-1 infection and vertical transmission in a group of Brazilian children coming from impoverished areas of Recife and its surroundings.
The groups we studied were: healthy controls of same ethnic origin of the patients (ethnic origin has been verified by D-loop mitochondrial DNA sequencing), infected patient born from HIV-1 infected mothers and exposed uninfected children that were born from HIV-1 positive mothers but didn't contract the infection. In no case mothers underwent caesarian section or antiretroviral therapy before delivery to reduce infection risk.
We were able to demonstrate that two SNPs in the 5'UTR of the *DEFB1* gene, namely -20(G/A) and -52(G/A), are able to influence susceptibility to HIV-1 infection.

Furthermore we evidenced, with *in vitro* studies that these two SNPs together with a third SNP located in the same region -44(C/G), do possess a functional activity on gene transcription.

We were also able to show that one of two studied SNPs in the *LTF* gene, encoding for lactoferrin, is strongly correlated with protection from HIV-1 infection. The SNP: R29K does confer a stronger antimicrobial activity to lactoferrin and it also modifies the peptide sequence of the N-terminal antimicrobial portion of lactoferrin: lactoferricin.

Studies on the sequence of the *hCAP18* gene, coding for the only known human cathelicidin, LL37, lacked of significant results. We failed to evidence the presence of any coding non-synonymous polymorphism in the studied patients. We, however, were able to identify some novel mutations that, unfortunately, are too rare to possess any statistical significance in our study.

The defensin locus 8p23 is known to be subjected to high recombination rate. As a consequence many of the defensin genes are present in the human genome in a variable number of copies that could influence protein expression.

We therefore employed the MLPA technique to study the influence of copy-number polymorphism of the following genes: *DEFA4*, *DEFA5*, *DEFA6*, *DEFB1*, *DEFB4*, DEFB107B, *DEFB108, DEFA3*, *DEFA7*, *DEFB4*, *DEFB103A*, *DEFB104*, *DEFB105*, *DEFB106*, and *DEFB107B*.

We were able to find that low copy-number polymorphisms of the *DEFB104* gene significantly increase susceptibility to HIV-1 transmission.

Functional studies showed that mRNA expression is linearly dependant on the number of present copies of the *DEFB104* gene.

There was no correlation between the other studied genes and HIV-1 infection or vertical transmission.

Moreover, to strengthen our study, we performed further analysis on other innate immunity genes, other than defensins, namely *MBL2* and it's associated serine protease (*MASP2*).

We were able to evidence how MBL2 haplotypes characterized by high MBL production are protective towards HIV-1 infection. The studied SNPs were 52 Arg-Cys, 54

Glu-Asp and 57 Glu-Gly in the first exon of *MBL2* and -550(G/C) and -221(G/C) in the gene promoter.
We also genotyped our groups for SNPs in the *MASP2* gene, that is functionally complementary to MBL. We find no risk genotype or haplotype for the D105G and the R99K polymorphisms.

In conclusion we were able to demonstrate that polymorphisms in many innate immunity genes could influence HIV-1 infection and vertical transmission, thereby providing a target for designing new strategies to fight HIV-1 spreading.
It should be noted, however, that no general rule could be inferred from the obtained data, as it seems that, at least in the HIV-1 case, the innate immunity could play a dual role, as a reduced gene expression sometimes inhibit HIV-1 activity and sometimes promotes it

LIST OF PAPERS INCLUDED IN THE THESIS

Milanese M, Segat L, Pontillo A, Arraes LC, de Lima Filho JL, Crovella S. 2006. DEFB1 gene polymorphisms and increased risk for HIV-1 infection in Brazilian children. Aids. 20(12): 1673-5

Milanese M, Segat L, Crovella S.
Transcriptional effect of DEFB1 gene 5' untranslated region polymorphisms. Canc. Res. 2007 Jun 15;67(12):5997

LIST OF OTHER PAPERS NOT DIRECTLY RELEVANT TO THE THESIS

Boniotto M, Segat L, Milanese M, Crovella S. 2005. Detection of two functional polymorphisms in the promoter region of the IL-18 gene by single-tube allele specific PCR and melting temperature analysis. J Immunol Methods 304(1-2):184-8.

de Souza PR, Arraes LC, de Lima Filho JL, Bruneska D, Milanese M, Crovella S. 2005. CCR5 promoter polymorphisms and HIV-1 perinatal transmission in Brazilian children. J Reprod Immunol. 69(1):77-84

Segat L, Milanese M, Boniotto M, Crovella S, Bernardon M, et al. 2006. DEFB-1 genetic polymorphism screening in HIV-1 positive pregnant women and their children. J Matern Fetal Neonatal Med.19(1):13-6.

Grasso DL, Guerci VI, Zocconi E, Milanese M, Segat L, Crovella S.
MBL2 genetic polymorphisms in Italian children with adenotonsillar hypertrophy. Int J Pediatr Otorhinolaryngol. 2007 Jul;71(7):1013-1016.

Segat L, Milanese M, Crovella S.
Pin 1 promoter polymorphisms in hepatocellular carcinoma patients. Gastroenterology. 2007 Jun; 132(7):2618-9.

Araujo J, Brandão LAC, Guimarães RL, Santos S, Falcão EA, Milanese M, Segat L, Souza PR, de Lima-Filho JL, Crovella S.
Mannose Binding Lectin gene polymorphisms are associated with type 1 diabetes in Brazilian children and adolescents. Hum Immunol. 2007 Sep;68(9):739-43

Milanese M, Segat L, Marziliano N, Crovella S.
The expression of innate immunity genes in Italian Crohn disease patients. Eur J Histochem. 2007 Jul-Sep;51(3):199-202.

Arosio B, Segat L, Milanese M, Galimberti L, Calabresi C, Zanetti M, Trabattoni D, Annoni G, Crovella S, Vergani C.
PIN-1 promoter polymorphisms in mild cognitive impairment and susceptibility to Alzheimer's disease: a preliminary report.
Aging Clin Exp Res. 2007 Oct;19(5):406-9.

Milanese M, Segat L, De Seta F, Pirulli D, Fabris A, Morgutti M, Crovella S.
MBL2 Genetic Screening in Patients with Recurrent Vaginal Infections
Am J Reprod Immunol 59 (2008) 146–151

Segat L, Fabris A, Padovan L, Milanese M, Pirulli D, Lupo F, Salizzoni M, Amoroso A, Crovella S.
MBL2 and MASP2 gene polymorphisms in patients with hepatocellular carcinoma
J Viral Hepat (in press)

Brandão LAC, Guimarães RL, Carrera M, Milanese M,, Segat L, de Lima-Filho JL, Arraes LC, Crovella S.
MBL2 functional polymorphisms and increased risk for the development of atopic dermatitis in Brazilian children
Arch Dermatol (in press)

INTRODUCTION

The AIDS pandemic

Promising developments have been seen in recent years in global efforts to address the AIDS (acquired immunodeficiency syndrome) spreading, including increased access to effective treatment and prevention programs.

However, the number of people living with HIV-1, the etiologic agent of AIDS, continues to grow, as does the number of deaths due to AIDS.

According to the World Health Organization, about 40 million people were living with HIV-1 in 2006, meaning that, if we accept the estimate of 6.6 billions of people in the world, almost one every 160 people in the world is infected with the HIV-1 virus.

Reporting UNAIDS (*www.unaids.org*) data on HIV-1 epidemiology, Sub-Saharan Africa continues to bear the brunt of the global epidemic, with two thirds (63%) of all adults and children with HIV, globally living in sub-Saharan Africa, with its epicenter in southern Africa. One third (32%) of all people with HIV-1 live in southern Africa and 34% of all deaths due to AIDS in 2006 occurred there.

Declines in national HIV-1 prevalence are being observed in some sub-Saharan African countries, but such trends are currently neither strong nor widespread enough to reduce the epidemics' overall impact in the cited regions. Almost three quarters (72%) of all adult and child deaths due to AIDS in 2006 occurred in sub- Saharan Africa. Overall sub-Saharan Africa is home to an estimated 25 million adults and children infected by HIV-1. In the past two years, the number of people living with HIV-1 increased in every region of the world. The most striking increment did occur in East Asia, Eastern

Europe and Central Asia, where the number of people living with HIV-1 in 2006 was over one fifth (21%) higher than in 2004.

The 270000 adults and children newly infected with HIV-1 in Eastern Europe and Central Asia in 2006 showed an increase of almost 70% over the 160000 people who were estimated to have acquired HIV-1 in 2004. In South and South-East Asia, the number of new HIV-1 infections rose by 15% in 2004–2006, while in the Middle East and North Africa it grew by 12%. In Latin America, the Caribbean and North America, new infections in 2006 remained roughly the same as in 2004.

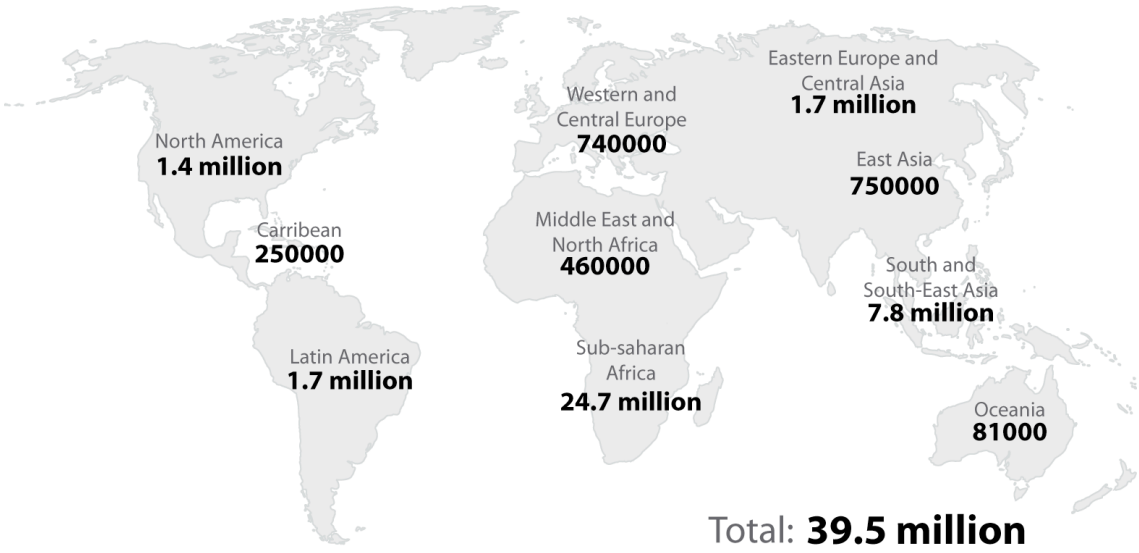

Figure 1 Number of adults and children estimated to be living with AIDS in 2006 according to UNAIDS/World Health Organization

The 270000 adults and children newly infected with HIV-1 in Eastern Europe and Central Asia in 2006 showed an increase of almost 70% over the 160000 people who acquired HIV-1 in 2004. In South and South-East Asia, the number of new HIV-1 infections rose by 15% in 2004–2006, while in the Middle East and North Africa it grew by 12%. In Latin America, the Caribbean and North America, new infections in 2006 remained roughly the same as in 2004.

Access to treatment and care has greatly increased in recent years, albeit from a very low starting level in many countries. Through the expanded provision of antiretroviral treatment, an estimated two million life years were gained since 2002 in low- and middle-income countries. In sub-Saharan Africa alone, some 790000 life-years have been gained, the vast majority of which in the past two years of antiretroviral treatment scale-up. In Latin America, where wide-scale treatment provision began earlier, some 834000 life-years have been gained since 2002. Unfortunately, while the benefits of increased diffusion of cares are clear, accession to healthcare is still difficult in many regions of the world.

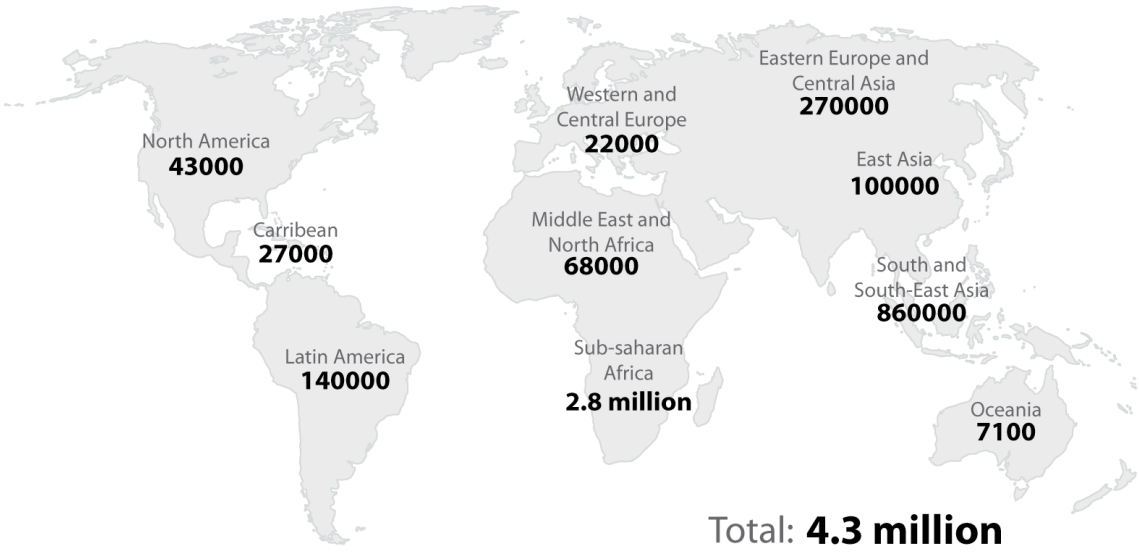

Figure 2 estimated number of adults and children newly infected with HIV during 2006 (according to UNAIDS/ World Health Organization data)

Moreover, a complication that's still present in underdeveloped countries, and in which we focused our attention in the present study, is vertical transmission of the HIV-1 virus from mother to child at the moment of delivery.

The use of highly active antiretroviral therapy leads to maximal virological suppression, thus minimizing the risk of drug resistance, but it is only available in developed countries. On the other hand, in developing countries, the use of short-course regimens is becoming more widespread.

HIV-1 infected women do present higher risk for complications during pregnancy, including ectopic pregnancy, early abortions, bacterial pneumonia, urinary tract infection,

oral and recurrent vaginal thrush, malaria, and tuberculosis. Regional anesthesia is often the treatment of choice when administering anesthesia in an HIV-infected pregnant woman. Infected children present decreased survival rates, while uninfected children born to infected mothers present a higher incidence of poor weight gain, short stature, and wasting than would be expected for the general population (Rigopoulos et al., 2007).

As said before, those problems have virtually disappeared in modern countries because of the strict control on pregnant women, the use of antiretroviral drugs that keep the viral charge low, and, perhaps most importantly, because of the routine practice of caesarian section when managing HIV-1 positive mothers.

In fact, HIV-1 is not normally able to diffuse across undamaged placental membrane and infection can only happens if the newborn is exposed to his mother's infected blood: an occurrence that's much more likely to take place during natural delivery. Moreover HIV-1 transmission can occur via breast-feeding.

Unfortunately, in poor areas it's not easy to follow HIV-infected pregnant as they often arrive at the hospital to receive medical treatment only after the delivery took place and they seldom did receive some medical advice on their disease before.

This is the case, in particular, for mothers coming from the poorest quarters of Brazilian cities, the so-called *favelas*, from which the groups we studied came from.

Notwithstanding the fact that cures are becoming more and more widespread the great problem of the AIDS pandemic is long from being solved.

Actually, despite more than 20 years passed from the identification of HIV-1 as the etiologic agent of AIDS, no great results have been achieved so far in the research of a reliable vaccine. In fact, as the knowledge on the virus is progressing, vaccinologists have learned that the biological properties of the virus hamper themselves the discovery of an effective vaccine; therefore new targets are urgently needed to control HIV-1 spreading in human populations and AIDS morbidity and mortality over the world.

New antiretroviral therapies are now available that allow to treat AIDS almost as a chronic disease, and lifespan of HIV-1 positive people has considerably extended in the last 10 years.

Unfortunately those therapies are mostly limited to patients living in developed countries, leaving the areas of the planet with the highest risk without a reliable and affordable treatment. New approaches, using autologous dendritic cells (Lu et al., 2004) did show some interesting results, but still have the drawbacks of the difficulty of the protocol and the need of a patient-tailored treatment.

Clearly the actual array of molecular targets toward HIV-1 has revealed to be insufficient to effectively fight the virus, and new targets are needed.
One of the routes that researchers worldwide took to discover new targets has been to study the genetic susceptibility to HIV-1 infection and progression, in order to pinpoint genes that can modulate the steps of viral infection and replication. In this way we are trying to explain the inter-personal variability to be infected by the HIV-1 virus.
In fact, the clinical consequences of viral exposure are variable. Some individuals can be repeatedly exposed to the virus but remain free from infection. Others can be infected but remain free from clinical disease.

Susceptibility to HIV-1 is a complex phenomenon that result from the interaction of environmental factors and the host's genetic background; however, differences between individuals in disease-free survival and resistance to infection have mostly been attributed to viral variability rather than to genetic difference (Deacon et al., 1995).

Genetic factors involved in HIV-1 susceptibility

Two main families of host's genes, namely major histocompatibility complex (MHC) (Pareja et al., 1997) and chemokine receptors (Murphy, 1996), were demonstrated to control HIV-1 transmission, thus limiting the spread of the epidemic.
Many different groups showed that a number of chemokine receptors also act as HIV-1 coreceptors and are therefore required for HIV-1 entry in cells (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996). Among these receptors, the most important is likely CCR5: a highly polymorphic gene, with several structural variants specific for different ethnic groups. Among these variants, the mutant CCR5 Δ32 allele bears a 32 base pairs deletion in its coding region, leading to the production of a truncated protein that is not expressed on cell surface. This polymorphic variant is quite frequent in Cau-

casians, where 1% of healthy individuals are homozygous for the variant (Stephens et al., 1998). Homozygous carriers of the CCR5 Δ32 variant display a strong (Samson et al., 1996), although incomplete (Theodorou et al., 1997), resistance to HIV-1 infection, since the absence of CCR5 from the cell surface impairs the virus' entrance to the cell.

It has been shown that most of the HIV-resistant individuals bear an A2/A28 human leukocyte antigen (HLA)-A and the authors suggested that enhanced presentation of HIV-1 peptides by HLA A2/A28 to CD8+ lymphocytes, that eliminate HIV-infected cells before the establishment of a productive infection (Kaul et al., 2000), is responsible for this phenomenon (MacDonald et al., 2000). Furthermore, also HLA concordance between mother and fetus has been suggested to influences mother to child transmission (MacDonald et al., 1998; Polycarpou et al., 2002).
Taken together, these data suggest that HLA molecules play a significant role in differential susceptibility to HIV-1 infection.
HLA genes have also been studied as candidate genes able to modify host's genetic background on disease progression. The rationale for these studies is that HLA genes are extremely polymorphic and that the host's HLA genotype is an important factor in determining the gravity of other infectious diseases (Hill, 1996). The molecular basis for these differences is that each allelic variant of a class I or class II HLA molecule presents a restrained and specific repertoire of peptides to CD8+ or CD4+ T lymphocytes, which in turn mount a specific response against the pathogen. In other words, antigen-presenting cells can be differently able of present a specific antigenic peptide to T lymphocytes, depending on the HLA type exhibit on their surface. Therefore, patients carrying HLA variants that more efficiently present peptides critical for the survival of the virus also mount a better immune response against the virus. This concept has usually been accepted by people working on the genetics of infectious disease, but only recently proved, in a study that demonstrated that viral sequences evolve during HIV-1 infection and that this evolution is under the control of the host's HLA genotype (Moore et al., 2002).

HLA class I molecules can be classified in two major categories, depending on the presence of isoleucine or threonine (Bw4 subtype) versus asparagine (Bw6 subtype) at position 80. Patients with isoleucine at position 80 and bearing also a KIR3DS1 natural

killer activator receptor in their genomes progressed less rapidly to clinical AIDS than the others. The most plausible explanation for this finding is that patients bearing this compound genotype use one more cell-mediated cytotoxic pathway to eliminate HIV-infected cells. Indeed, HLA isoleucine-80 molecules present on the surface of HIV-infected cells are recognized by the KIR3DS1 activator receptor, giving thus a positive signal for killing by natural killer or natural killer T cells, which usually express this receptor on cell surface. (Martin et al., 2002).

Furthermore, gene variants could directly influence disease-free survival times. Indeed, many studies showed that patients heterozygous for CCR5 Δ32 have a delayed progression towards clinical AIDS and that they develop a symptomatic primary infection less frequently than patients with two functional alleles of CCR5 (Dean et al., 1996; Meyer et al., 1997; Zimmerman et al., 1997).

Moreover, the HIV-1 viral load at early stages of disease is lower in the CCR5 Δ32 heterozygous patients. The protective genetic effect of CCR5 Δ32 is now openly recognized, as shown by a recent meta-analysis of different seroconverter cohorts assembled in different countries (Ioannidis et al., 2001). The same protective effect has been also found in pediatric cohorts (Misrahi et al., 1998).

Comparable data have been obtained for many different variants in the promoter of CCR5 that seem to influence progression of HIV-1 disease, probably through modulation of CCR5 mRNA production (Martin et al., 1998; McDermott et al., 1998).

There is also increased evidence that genetic variations in the promoter of the RANTES (regulated upon activation, normal T cell expressed and secreted) gene, one of the ligands of CCR5, could also be relevant for both susceptibility and disease progression in HIV-1 disease (An et al., 2002; Liu et al., 1999; McDermott et al., 2000). This effect is likely due to an effect on RANTES mRNA transcription efficiency that, in turn, can modulate RANTES production. Variations in the production of the RANTES protein influences the availability of CCR5 free molecules for HIV-1 binding and cell entry, since RANTES inhibits CCR5-mediated HIV-1 entry by competitive binding and down-modulation of CCR5.

Moreover, the protective effect of CCR5 Δ32 polymorphism is reported to be SDF-1 genotype's restricted (Sei et al., 2001).

SDF-1 is a molecule that belongs to the chemokine family and shows a great chemotactic activity on T- lymphocytes and macrophages. It presents two isoform (α and β) sub-

sequently to alternative splicing. And it has been shown to bind the HIV-1 coreceptor CXCR4 and block, at least in vitro, HIV-1 infection of T CD4+ cells (Bleul et al., 1996).
A polymorphism in the 3'UTR region of the gene coding for SDF-1 has been associated to HIV-1 infection and disease progression. (Brambilla et al., 2000; John et al., 2000; Mummidi et al., 1998).

Another polymorphic chemokine receptor gene that is also a minor coreceptor for HIV-1 is CX3CR1, for which three structural variants exist in Caucasians. All three have different binding affinities for the natural ligand of the receptor and patients homozygous for the I249M280 variant show a very rapid disease progression towards AIDS (Faure et al., 2000). However, the deleterious effect of the I249M280 variant was not found in several cohorts analyzed after the initial report (McDermott et al., 2000).
Of course it is possible that differences between the reports are a consequence of the selection process of the patients enrolled in these cohorts. A typical example concerns studies of disease-free survival in cohorts enrolling patients with a known date of infection (or seroconversion). The date of infection is usually defined as the midpoint between a negative and a positive HIV-1 test; however, the distance allowed between these two points to enroll a patient as a seroconverter is variable from one cohort to another (from one to several years), leading an approximation of the infection date and disease-free survival with little precision
Cytokine gene polymorphisms and particularly single nucleotide polymorphisms in the promoters of interleukin-4 (IL-4) and IL-10 have also been shown to influence HIV-1 disease progression in seroconverter cohorts. The molecular basis of this difference is thought to be modulation of mRNA production of IL-4 and IL-10, two T helper (Th) 2-type cytokines with pleiotropic effects on the immune system. However, both studies are single-cohort studies and confirmation in other cohorts is necessary to really appreciate the impact of these findings (Nakayama et al., 2000; Shin et al., 2000).
Interleukin-18 (IL-18) is a very important cytokine in both innate and acquired immunities and IL-18 production seems to be an integral part of the host's innate response to viral pathogens (Pirhonen et al., 1999; Tanaka-Kataoka et al., 1999). The role of IL-18 in HIV-1 infection is not totally clarified yet, albeit a more than fourfold increase of plasma IL-18 concentration has been found in HIV-1-infected subjects compared to

healthy individuals as well as a positive correlation between plasma IL-18 concentration and HIV-1 viral load. (Wiercinska-Drapalo et al., 2004) and similar results have been obtained by other research groups (Ahmad et al., 2002; Stylianou et al., 2003). A polymorphism (C607A) in the promoter region of the IL-18 gene has been correlated to HIV-1 infection in a pediatric Brazilian population (Segat et al., 2006).

Gene	Genotypes related to disease progression	Consistency of the findings across different studies
CCR5	CCR5 Δ32 heterozygosity delays disease progression in children and adults.	Yes (confirmed by meta-analysis)
	Promoter variants influence disease progression	Several studies without possibility for cross-validation
CCR2	CCR2 64I delays disease progression in adults	No
RANTES	Promoter variants influence disease progression	Several studies without possibility for cross-validation
SDF1	Homozygous for SDF1-3A show a delayed disease progression	No
CX3CR1	Patients homozygous for the I349 M280 haplotype progress rapidly to AIDS	No
HLA	Several alleles or allelic groups inluence disease progression	Yes
	Homozygosity for HLA A,B or C molecules influence disease progression	Single study
	Combined genotype of the Bw4 supertype with KIR haplotype influences disease progression	Single study
IL-4	Promoter variants influence disease progression	Single study
IL-10	Promoter variants influence disease progression	Single study
MBL	Structural variants influence disease progression	No

Table 1 Summary of genes that influence HIV-1 progression to AIDS

Although the presented genes are probably the ones that have the most striking effect on HIV-1 infection and progression, there is no doubt that a plethora of other genes do play

a role in modulating and modifying host's susceptibility to infection and therefore are good candidate genes to design novel approaches to develop anti-HIV-1 drugs.
Genetic variability is a very important factor to assess the susceptibility of the newborn to be infected at delivery, and we think that polymorphisms in innate immunity gene could be significant in modifying liability to be infected by HIV-1.

Innate immunity as source of targets for HIV-1 research

The human body is permanently exposed to thousands of potentially pathogenic micro-organisms, including bacteria, viruses and fungi.
Therefore it's not surprising that the human body developed, during millennia of evolution, a system to face such a bewildering array of infectious agents and to prevent damages due to infections.
The role of the immune system is far from being simple: pathogens could present themselves in a great variety of forms. Moreover, microorganisms, due to their short life cycle, evolve much more rapidly than the immune system itself. Lastly, many microorganisms behave like opportunistic pathogens, meaning that they are constantly present on the epidermis, mucosae or intestinal tract and only gave problems when the body homeostasis is impaired, while being generally harmless when normal physiology occur. Only to name a few of them we can recall Escherichia coli, useful in favoring digestion and potentially lethal if enters the blood stream, Staphyilococcus aureus, which often infects the relatively vulnerable hair follicle and glands, or Candida albicans, a normal component of the vaginal flora that causes candidiasis.

Many immunology textbooks classify the immune system in two distinct classes: the innate immunity and the acquired immunity; the first responsible for an early action of defense against pathogens and the latter accountable for a subsequent, more precise and strong response.
Advancements in immunology have clearly demonstrated that this classification is becoming more and more didactic and it is now generally accepted that innate and acquired immunity share a strong interplay. In addition, innate immunity, whose role has been neglected for almost a century, is gaining the importance it deserves in the field of immunology.

Innate immunity, in fact, forms an efficient first line of defense against infection by microorganisms, often providing an adequate protection of the body without the need of any acquired immune response. The main advantage of the innate immunity when confronted with the acquired immunity is the fact that the proteins and peptides of the former are generally already presents, formed and available at the site of infection, while maturation of the acquired immunity requires a long time.

Proteins belonging to the innate immune system have a great potential for being ideal molecular targets to study host's susceptibility to HIV-1 infection, since they are the first part of the immune system to actually get in touch with the virions. Innate immunity proteins are expressed in a wide array of tissues and especially on the most vulnerable areas of the human body, like the oral cavity or the reproductive tract.
It is then plausible to assume that inter-individual differences in HIV-1 infection and AIDS progression are partly explainable with variations in the genetic background of innate immunity.

Mannose binding lectin and its related proteases

The existence of mammalian serum lectins was first predicted in 1975 by Robinson et al. (1975), and the human protein was first isolated by Wild et al. (1983) in liver tissues. More recently, extra-hepatic transcription of MBL has been reported and this may have implications regarding its role in localized host defense (Seyfarth et al., 2006).
MBL structure has been studied extensively and consists of multimers of an identical polypeptide chain of 32 kDa. Each chain comprises four distinct regions encoded by different exons of the *MBL2* gene.
Each chain has a C-terminal, calcium-dependent carbohydrate-recognition domain (CRD); a short, α-helical, hydrophobic neck region (in the so-called coiled-coil configuration); a collagene-like region and a cysteine-rich N-terminal region. Three polypeptide chains form a triple helix within the collagenous region, stabilized by hydrophobic interactions and interchain disulphide bonds within the N-terminal cysteine-rich region. In serum, MBL consists of oligomers ranging from dimers to hexamers, and X-ray crystallographic studies demonstrated that in this form the oligomers show a bou-

quet-like structure due to an interruption in the collagenous region, giving rise to a slight bending of the triple helix structure.

Clustering of the structural subunits provides a flat platform, permitting binding of MBL to the arrays of repeating sugar groups on microbial surfaces. Although the binding affinity of each individual interaction between the carbohydrate-recognition domain and the sugar is relatively low at 10^3 M (Iobst et al., 1994), the formation of higher order oligomers provides a way for MBL to bind to the bacterial sugars with high avidity.

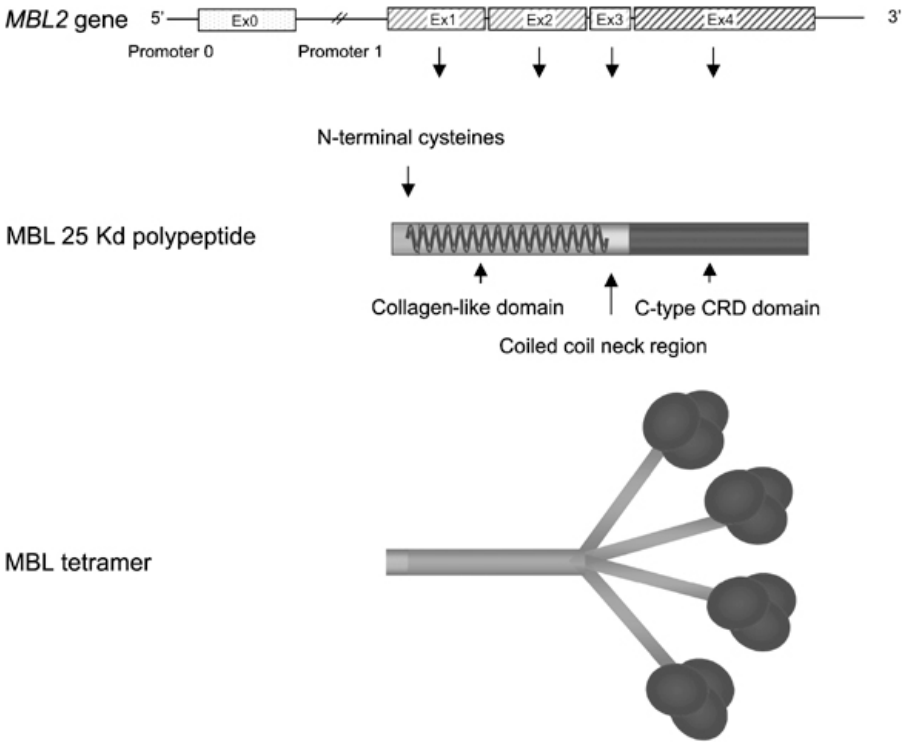

Figure 3 MBL2 gene structure, MBL polypeptide structure and MBL oligomeric structure. Upper panel shows the exon structure of the human MBL2 gene. Exon 0 (Ex0) is not translated into protein. Middle panel shows the organization of the MBL polypeptide. The MBL polypeptide consists of four domains, including an N-terminal cysteine-rich region cross linking the polypeptides, a collagen-like stalk region, an -helic neck region and a C-type carbohydrate recognition domain. The lower panel illustrates one of the predominant forms of MBL found in serum, which consists of four subunits of triple helices of MBL polypeptides

MBL is a major pattern-recognition molecule of the innate immune system that primarily recognizes specific sugar groups on the surface of a wide range of bacteria, viruses, fungi and protozoa.

The understanding of MBL function has grown rapidly over the past three decades. It is now recognized to have a role in different processes, as complement activation, promotion of complement-independent opsonophagocytosis, modulation of inflammation, recognition of altered self-structures and apoptotic cell clearance.

A role for MBL in host defense was first proposed in 1987 when Ikeda et al. observed that the protein was able to trigger the classical pathway of complement (Ikeda et al., 1987). However, it is now clear that MBL activates a novel third pathway of complement, often termed the MBL o lectin pathway, in an antibody- and C1-independent way. Similar to the C1 complex of the classical pathway, the initiating complexes of the lectin pathway comprise separate recognition and enzyme components. The recognition components, MBL and serum ficolins, bind directly to sugars or N-acetyl groups on pathogenic cells and activate three different enzymes, called MBL-associated serine proteases (MASPs-1 to -3), to activate complement (Schwaeble et al., 2002; Turner, 1996).

MASPs are homologues of C1r and C1s of the classical pathway. They all have the same modular organization consisting of two domains found in complement component Clr/Cls, Uegf and bone morphogenic protein 1 (CUB domains), separated by a Ca2+-binding epidermal growth factor (EGF)-like domain and followed by two complement control protein (CCP) modules and a C-terminal serine protease domain (Schwaeble et al., 2002) .

MASPs-1 and -3 are encoded by a common gene through alternative splicing and have identical N-terminal domains but different linker regions and serine protease domains.

By screening a liver cDNA library, Stover et al. (Stover et al., 1999) identified a cDNA encoding a shorter variant of MASP2, which they termed MAP19 (MBL-associated plasma protein of 19 kD) that is an alternatively spliced product of the MASP-2 gene (Takahashi et al., 1999).

The deduced 185-amino acid MAP19 protein retains the signal peptide, the N-terminal CUB1 domain, and the EGF-like domain of full-length MASP2, but it has a unique C-

terminal sequence (EQSL) and lacks the serine protease catalytic domain. Northern blot analysis revealed higher expression of the 1.0-kb MAP19 transcript than of the 2.6-kb transcript encoding the serine protease domain of MASP2. Immunoblot analysis indicated that un-cleaved MASP2 is expressed as a 76-kD protein, while the A chain has a molecular mass of 52 kD and the B chain has a mass of 31 kD. Stover et al. (Stover et al., 1999) proposed o role for MAP19 in modulating the activation of complement via the MBL pathway.

MASPs normally circulate as zymogens. However, when lectin–MASP complexes bind to target epitopes on pathogens, MASP-1 and -2 activate through autolysis at a single site within the short linker region between the CCP-2 module and serine protease domain. The active protease domain remains attached to the N-terminal fragment through a single disulphide bond. MASP-3 is also activated through cleavage of the linker region. However, the zymogen cannot autoactivate, so is probably activated through the action of an unidentified serum (Zundel et al., 2004).

Currently, only MASP-2 has a clearly defined role in complement activation (Thiel et al., 1997). It initially cleaves C4 to produce the peptide anaphylatoxin C4a and the C4b fragment, which attaches to the activating surface of the pathogen upon exposure of the highly reactive thioester group. C2 then binds to the immobilized C4b molecule and is also cleaved by MASP-2 to generate C2b and C2a. C2a remains attached to C4b to become the catalytic component of the C3 convertase, the enzyme that catalyse the next step in the reaction pathway (Kerr, 1980). The roles of MASP-1 and MASP- 3 are not yet known. MASP-1 cleaves C2 but not C4, so it might enhance complement activation triggered by lectin–MASP-2 complexes, but cannot initiate activation itself (Chen and Wallis, 2004). No natural substrate for MASP-3 has been identified.

In addition to its role in complement activation, in a study by Kulhman et. al (1989) it's shown how MBL is able to directly interact with cell surface receptors to promote opsonophagocytosis. Subsequently, a number of putative MBL-binding receptors have been proposed including C1qR/calreticulin (Malhotra et al., 1990), C1qRp (Tenner et al., 1995) and CR1 (Klickstein et al., 1997). However, it is still uncertain if MBL is acting as a direct opsonin or is merely enhancing other complement pathways and/or antibody-mediated phagocytosis.

The role of MBL as a modulator of inflammation appears to be complex and, accordingly, its mechanism of action remains unexplained. One possible explanation is that MBL is able to trigger proinflammatory cytokine release from monocytes (Chaka et al., 1997; Soell et al., 1995).

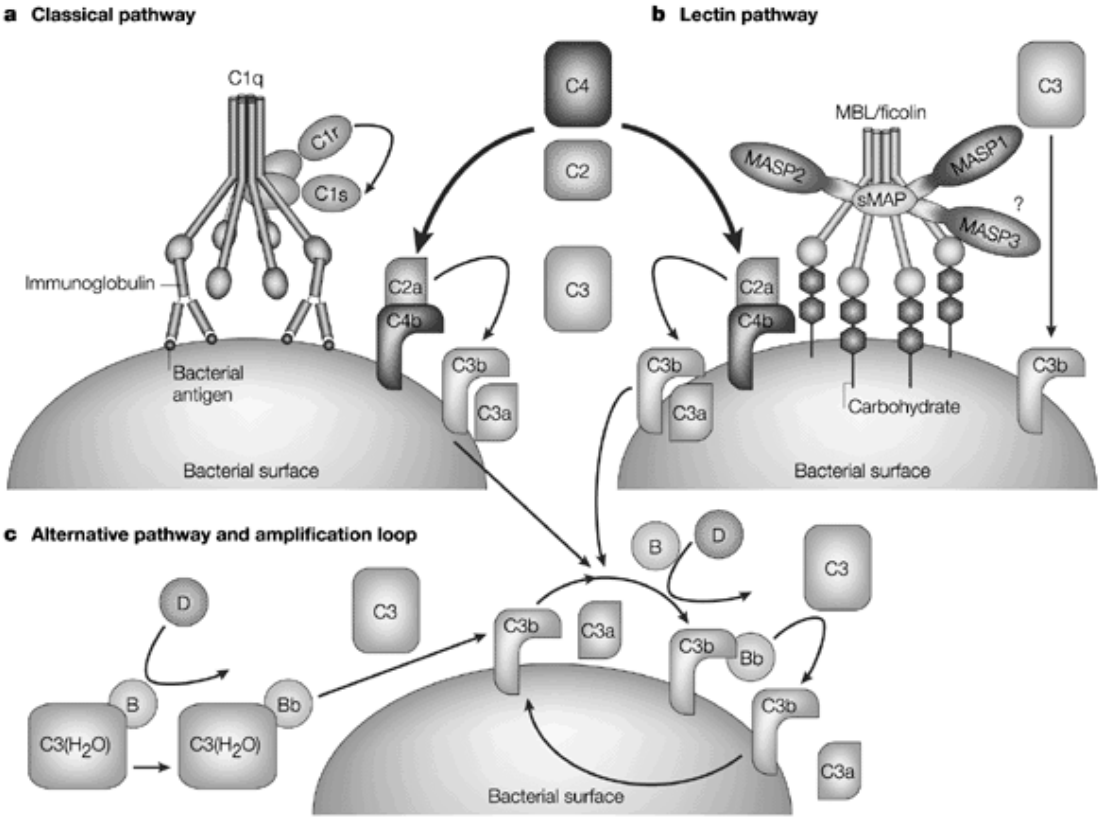

Figure 4 schematic representation of complement activation pathways (Nature Reviews Immunology)

A role for MBL in the clearance of apoptotic cells was first proposed by Ogden et al. in 2001 (Ogden et al., 2001). MBL was found to bind directly to apoptotic cells that expose terminal sugars of cytoskeletal proteins, thereby permitting their recognition and directly facilitating their phagocytosis by macrophages. Defects in the clearance of apoptotic cells have been implicated in the pathogenesis of certain autoimmune conditions, although the precise role of MBL, if any, remains elusive.

Changes in cell surface structures during oncogenic transformation appear to promote binding of MBL to cancer cells (Hakomori, 2001) where the protein can mediate cyto-

toxic effects including MBL-dependent cell mediated cytotoxicity (Ma et al., 1999; Nakagawa et al., 2003).

There are two human MBL genes, but only *MBL2* encodes for the functional protein, while *MBL1* is a pseudogene. The functional *MBL2* gene is located on chromosome 10 (q11.2-q21) and comprises four exons.

Exon 1 encodes the signal peptide, a cysteine-rich region and part of the glycine-rich collagenous region. Exon 2 encodes the remainder of the collagenous region and exon 3 encodes an α-helical coiled-coil structure. Finally, exon 4 encodes the carbohydrate-binding domain. The promoter region of the *MBL2* gene contains a number of regulatory elements, which affect transcription of the protein.

Polymorphisms in MBL2 gene and MBL deficiencies

MBL deficiency has been reported and is largely explained by three single point mutations in codons 52, 54 and 57 of exon 1 in the *MBL2* gene (Turner, 1996). These mutations are frequently referred to as variants D, B and C, respectively, with A indicating wild-type. The B variant mutation occurs in 22–28% of Eurasian populations, whereas the C variant mutation is characteristic of sub-Saharan African populations in whom it reaches frequencies of 50–60%. The D mutation reaches frequencies of 14% in European populations but can be much lower elsewhere.

The exon 1 mutations in the *MBL2* gene are believed to impair oligomerization and lead to a functional deficiency. In the case of the B and C mutations, critical axial glycines of the triple collagenous helix are replaced by dicarboxylic acids which would be expected to distort the helix (Sumiya et al., 1991) In the case of the D variant, the effect of the mutation is to replace an arginine residue by a cysteine (Madsen et al., 1994) and it has been claimed by Wallis and Cheng (1999) that the presence of this extra cysteine residue causes the formation of adventitious disulphide bonds, dramatically reducing the formation of higher order oligomers.

In addition to the above structural gene mutations, several polymorphisms have been described in the promoter region of the *MBL2* gene (Madsen et al., 1995). These are the H/L, X/Y and P/Q allele at positions –550, –221 and +4 of the *MBL2* gene.

These promoter polymorphisms combine to form haplotypes that are in strong linkage disequilibrium with the exon 1 mutations, resulting in seven common extended haplotypes, namely HYPA, LYPA, LYQA, LXPA, HYPD, LYPB and LYQC. Other rare haplotypes have also been described (Boldt and Petzl-Erler, 2002).

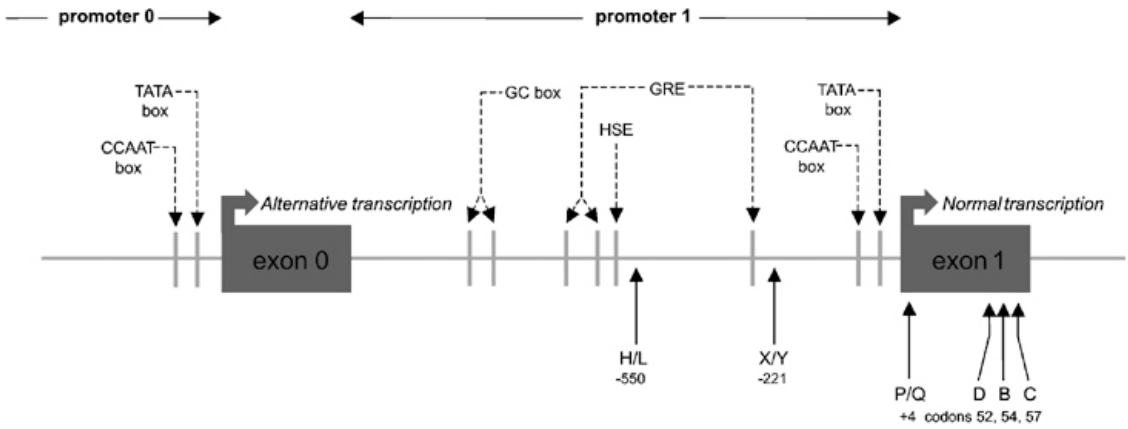

Figure 5 MBL2 polymorphisms. Two promoters, promoter 1 and promoter 0 regulate the transcription of the human *MBL2* gene. Similar to promoter 1 promoter 0 also includes a TATA box for transcription initiation, and transcription factor binding DNA sequences. Six DNA polymorphisms in the *MBL2* gene are known to be associated with variation in quantity and/or function of MBL in serum. Three variants affect the expression of the *MBL2* gene. They are localized in the promoter 1 (position -550, *H/L* variant and –221, *X/Y* variant) and in the 5'-untranslated region (position +4, *P/Q* variant) of the *MBL2* gene. Three base substitutions in exon 1 in codons 52 (*D*), 54 (*B*) and 57 (*C*) result in amino-acid changes (arginine to cysteine, glycine to aspartic acid and glycine to glutamic acid, respectively) and decreased level and function of MBL. The normal allele is named *A*.

The combination of structural gene and promoter polymorphisms results in a dramatic variation in MBL concentration in apparently healthy individuals of up to 1000-fold (Caucasian: range <20–10,000 ng/ml). In addition, Ezekowitz and colleagues (1988) presented evidence that MBL was an acute-phase reactant. The authors found that MBL messenger RNA transcripts were barely detectable in normal liver but that induction was seen in liver exposed to acute stress. Subsequent studies have shown that MBL levels can increase between 1.5 and threefold during the acute phase, but this response is variable between individuals (Thiel et al., 1992). It should also be noted that even dur-

ing an acute-phase response, individuals heterozygous or homozygous for *MBL2* mutations appear unable to achieve the protein levels of those possessing a wild-type genotype. Approximately one-third of the Caucasian population possesses genotypes conferring low levels of MBL, with approximately 5% having very low levels.

The high frequency of variant alleles observed in certain populations was initially puzzling since it suggests that functional MBL deficiency may as well be advantageous. Similarities have been proposed between the MBL genetic system and the role of the hemoglobin mutation that leads to sickle cell anemia gene in protection against malaria as occurs in carriers of the sickle cell hemoglobin allele (Allison 1954). The argument runs as follows: certain intracellular parasites use C3 opsonization and C3 receptors on monocytes/macrophages to enter their host. Therefore, any reduction in complement-activating function of the host may reduce the probability of parasitization. In support of this notion is a study on patients with visceral leishmaniosis, which revealed that such patients are more likely to have high MBL levels than uninfected controls (Santos et al., 2001). A small study of Ethiopian patients with lepromatous or borderline lepromatous leprosy also found that their MBL levels were significantly higher than those of healthy blood donors (Garred et al., 1994). An alternative explanation of the unexpectedly high frequency of low MBL phenotype individuals found in many tropical regions is that excessive complement activation can result in immunopathologically mediated host damage; therefore, any mechanism that reduces complement activation may be beneficial (Lipscombe et al., 1992).

The identification of MBL deficiency as the cause of the so-called common opsonic defect has been followed by a plethora of disease association studies aimed at defining the precise role of this protein. A number of the early studies concentrated on paediatric populations and MBL was suggested to provide substitute 'antibody-like' activity during the 'window of vulnerability' (approximately 6–24 months), when maternal immunoglobulin G (IgG) antibody levels have waned but the infant's own adaptive immune response is still immature (Super et al., 1989). Nevertheless, studies in adults suggested that there might be a role for MBL throughout life (Summerfield et al., 1995). Notwithstanding these reports, the majority of individuals possessing a variant MBL allele apparently suffers no adverse effects and remains essentially healthy. Nevertheless, MBL deficiency has been shown to be associated with increased susceptibility to many infec-

tious diseases such as bacterial diseases in neutropenic patients, meningococcal meningitis, invasive fungal infections, and viral infections (Eisen and Minchinton, 2003; Turner, 2003). Several reports suggest that MBL can also modulate the disease severity and can be used as a marker to predict the therapeutic efficacy in some disorders, e.g. the response to interferon treatment in patients with type C chronic hepatitis (Matsushita et al., 1998). Interestingly, it has been shown that individuals with mutations in the *MBL2* gene are at increased risk of having autoimmune diseases such as systemic lupus erythematosus, celiac disease and rheumatoid arthritis (Boniotto et al., 2005; Davies et al., 1997; Garred et al., 2000; Huang et al., 2003).

Polymorphisms in MASPs genes and MASP deficiencies

Inherited MASP-2 deficiency has been described as the result of a mutation causing the exchange of aspartic acid with a glycine at position 105, located in the first domain of the protein, CUB1, involved in calcium binding. This mutation abolishes MASP-2 binding to MBL and ficolins, and deprives it of functional activity. The index case suffered from recurrent severe infections and autoimmune reactions. The gene frequency of the mutation among Caucasians is 3.6% albeit it is not found in Chinese, who present a different mutation also associated with MASP-2 deficiency (Sorensen et al., 2005).

The same mutation, and more generally MASP dysfunction, is suspected to be a major modifier gene in cystic fibrosis (Olesen et al., 2006).

In their work, Thiel et al (2007) examined human populations for MASP-2 levels, MASP-2 function and for the presence of mutations in coding exons of MASP2. The MASP-2 levels differed between populations, being lowest in Africans from Zambia (median, 196 ng/ml) followed by Hong Kong Chinese (262 ng/ml), Brazilian Amerindians (290 ng/ml) and Danish Caucasians (416 ng/ml). In the Chinese population, a novel four amino-acid tandem duplication was associated with low levels of MASP-2. This new SNP was only found in Chinese (gene frequency 0.26%) while D105G was found only in Caucasians and Inuits from West-Greenland. The P126L and R99Q were present in Africans and Amerindians only, except for R99Q in one Caucasian. MASP-2 levels were reduced in individuals who presented the V377A mutation. The MASP-2 present in individuals homozygous for p.377A or p.99Q had a normal enzyme activity whereas MASP-2 in individuals homozygous for p.126L was non-functional.

To date, no MASP-1/MASP-3 deficiencies are known, however a polymorphism resulting in the amino acid substitution of a glycine with a glutamic acid residue in the MASP-1/ 3 gene, which may influence the function of the gene product, has recently been decribed. (Weiss et al., 2007).

MBL and human immunodeficiency virus

While numerous viral factors will determine the fate of an individual exposed to HIV, there are data to indicate that MBL can influence both susceptibility and severity of HIV-1 infection.

Thanks to its ability to bind to carbohydrate moieties, the likely target for HIV-1 binding is the gp120 glycoprotein, that is heavily glycosilated. While MBL can be readily demonstrated to bind to purified gp120 (Ezekowitz et al., 1989), the capacity of MBL to neutralize primary HIV-1 isolates is less convincing. Recent data indicate the MBL can opsonize HIV-1 but does not induce neutralization at the levels at which it is normally present in serum. However, binding and opsonization of HIV-1 by MBL may alter virus trafficking and viral antigen presentation during HIV-1 infection. MBL may influence uptake by dendritic cells (DC), which express a cell surface lectin called 'DC-specific intracellular adhesion molecule 3-grabbing non-integrin' (DC-SIGN). DC-SIGN has been shown to mediate a type of infection called 'trans'-infection, where DC bind HIV-1 and efficiently transfer the virus to T cells. Preincubation of HIV-1 strains with MBL prevents DC-SIGN-mediated trans-infection of T cells and indicates that at least *in vitro*, MBL may inhibit DC-SIGN-mediated uptake and spread of HIV-1 (Ying et al., 2004).

Whatever the mechanism of MBL interactions with HIV, a number of clinical studies have suggested that deficiency of MBL is a risk factor for acquiring HIV-1 infection.

MBL deficiency appears to increase the acquisition of HIV-1 infection by between three- and eightfold (Garred et al., 1997; Garred et al., 1997; Nielsen et al., 1995; Prohaszka et al., 1997). There is also an increased risk of vertical transmission from infected mothers to their offspring (Boniotto et al., 2000). However, these findings have not been replicated in all populations, with some studies failing to demonstrate a role for MBL in HIV-1 infection (Malik et al., 2003; McBride et al., 1998; Senaldi et al., 1995).

There is even less clarity with regard to the role of MBL in HIV-1 disease progression. Garred et al. (1997) demonstrated that men with MBL variant alleles had a shorter survival time following the onset of AIDS than did patients with wild-type MBL alleles. However, in a well-characterized cohort of homosexual men, variant MBL alleles had an insignificant effect on survival following the diagnosis of AIDS (Maas et al., 1998). In this latter study, there appeared to be a protective effect of MBL variant alleles, with a delay in the development of AIDS from the time of HIV-1 seroconversion. Patients with MBL variant alleles had lower CD4 counts at the time of developing AIDS, indicating that MBL deficiency may influence the onset of AIDS for any given CD4 count. Furthermore, MBL mutations appeared to protect against the development of Kaposi sarcoma (Maas et al., 1998). In another study, Prohaszka et al. (1997) found that MBL levels were lower in asymptomatic HIV-positive individuals compared with HIV-negative controls, whereas in the AIDS patients they were not; patients with high MBL levels had significantly lower numbers of CD4 cells. A possible explanation is that enhanced proinflammatory cytokine production in advanced HIV-1 disease acts to increase MBL synthesis (Arai et al., 1993), elevating levels in patients with late-stage disease. Indeed, a recent study has shown *in vitro* that MBL can enhance proinflammatory cytokine production and viral replication (Heggelund et al., 2005). In the light of studies indicating a role for MBL in inflammatory modulation, it is tempting to suggest that under some circumstances, MBL may act to promote inflammatory cells activation, thereby accelerating the rate of CD4+ T-cell depletion.

Few studies have assessed the impact of MBL in the context of effective antiviral therapy. However, one study has attempted to relate MBL status and HIV-infected long-term non-progressors (LTNPs) (Hundt et al., 2000). MBL levels were consistent with a wild-type genotype in the six LTNPs studied. Amoroso and colleagues had also suggested such an effect in a study showing that children with rapidly progressing disease were more likely to have MBL variant alleles (codon 54) than slower progressors (Amoroso et al., 1999).

Lactoferrin

Lactoferrin is an avid iron-binding glycoprotein that (with transferrin and melanoma tumor antigen p97) belongs to a family of iron-binding proteins that modulate iron me-

tabolism, hematopoiesis, and immunologic reactions that are all evolutionary products of gene duplication.

The gene transcript is assembled from 17 exons, like the bovine (bLf) and murine lactoferrin (mLf) genes, which are separated by introns ranging in size from about 300 bp to 3.3 kb. The total length of the gene is about 24.5 kb,. In situ fluorescence hybridization indicated that the human Lactoferrin gene (LTF) was mapped in the region 3p21.3 (Kim et al., 1998).

Figure 6 Localization of LTF gene

Lactoferrin is found on mucosal surfaces, within the specific granules of polymorphonuclear leukocytes, and in biological fluids, like milk, seminal fluid and saliva, suggesting a protective role in the innate immune response.

Milk is by far the most abundant source of lactoferrin with human colostrum, the early milk, containing up to 7 g/l (Masson and Heremans, 1971). There is a great variation in the concentration of lactoferrin in other human body fluids. The concentration in tears is as high as 2 mg/ml whereas that in blood is normally only as high as 1 μg/ml, although it can rise to 200 μg/ml during inflammatory processes (Masson and Heremans, 1971).

Lactoferrin is a monomeric glycoprotein with a molecular mass of about 80 kDa. The protein comprises two homologous lobes corresponding to its amino- (residues 1–333) and carboxyl- (residues 345–692) terminal halves, connected by a three-turn α-helix at residues 334–344. Each lobe is further subdivided into two domains, with a single iron-binding site situated between the inner faces of the inter-domain cleft. Each iron (Fe3+) atom is coordinated to four protein ligands, namely, 2 tyrosines, 1 aspartate, and 1 histidine, and also to a synergistic anion that, in vivo, is normally carbonate.

The two lobes have 125 amino acids in common (37% homology) and exhibit very similar tertiary structures, consistent with the hypothesis that the two lobes arose as a product of gene duplication (Metz-Boutigue et al., 1984).

Many roles have been proposed for lactoferrin, and although some of these are clearly related to its iron-binding properties, for example its ability to provide bacteria with a source of iron, others appear to be independent of iron binding.

The antimicrobial activity of lactoferrin is well established. This activity has been for a long time ascribed to the ability of lactoferrin to sequester iron, thus depriving potential pathogens of this essential nutrient. However, lactoferrin is now known to possess a second type of antimicrobial activity as a result of a direct interaction between the protein and the bacteria.

It has been well established that iron is an essential nutrient for the growth of almost all bacteria that have developed mechanisms for obtaining iron as they are often exposed to iron-limited conditions. In the normal situation, iron in the body is protein-bound, rather than "free", in order to minimize the generation of unwanted free radicals as a result of iron-catalyzed cascades. The siderophores produced by bacteria to obtain iron for their metabolism frequently have a higher affinity for iron than lactoferrin and therefore can acquire iron at the host's expense. The iron–siderophore complex is then taken up into bacteria by siderophore-specific receptors. Since this mechanism for iron intake does not require a direct interaction between the bacteria and the host's iron-binding protein, it is not host-specific.

In recent years it has become apparent that, under iron-restricted conditions, some bacteria can express membrane-bound proteins for their host's iron-containing proteins and exploit an iron-uptake mechanism that requires the direct interaction of the protein with a specific bacterial receptor. Bacterial species such as *Haemophilus influenzae*, *Actinobacillus pleuropneumonie*, and *Bordetella pertussis* are able to use transferrin- and lactoferrin-bound iron directly without the involvement of siderophores (Gonzalez et al., 1990; Herrington and Sparling, 1985).

The anti-bacterial activity of lactoferrin was initially ascribed to its ability to bind and sequester environmental iron, thereby depriving potential pathogens of this essential nutrient (Arnold et al., 1977). The ability of lactoferrin to inhibit bacterial growth in vitro was indeed one of the earliest functions described for the protein. The antimicrobial activity of lactoferrin was demonstrated against a number of bacteria (Arnold et al., 1977). Further studies have shown that lactoferrin was bactericidal only when in its iron-free state and that iron-saturated lactoferrin has a reduced antimicrobial activity (Arnold et al., 1980; Kalmar and Arnold, 1988; Yamauchi et al., 1993).

However, Arnold and co-workers demonstrated iron-independent killing by lactoferrin in 1982. Previous work in 1981, had shown that lactoferrin was bactericidal for Streptococcus mutans even when exogenous iron was added to the experimental media (Arnold et al., 1981) and it has been proposed that this iron-independent lactoferrin killing could be a result of a direct interaction of lactoferrin with the bacterial surface (Bortner et al., 1986). Lactoferrin was shown to interact with lipopolysaccharide (LPS) of the Gram-negative bacterial membrane of *E. coli* (Appelmelk et al., 1994), with the release of the LPS from the membrane. However, this release was blocked by addition of Ca2+ and Mg2+ ions. The presence of Ca2+ ions also inhibited the ability of lactoferrin to increase the susceptibility of *E. coli* to the antibiotic rifampicin (Ellison et al., 1990).

Many studies suggested that lactoferrin could bind to the isolated lipid A portion of LPS; in fact the addition of lipid A inhibited lactoferrin binding to LPS, as did the addition of polymyxin B (Appelmelk et al., 1994).

In a study by Naidu et al. lactoferrin has been demonstrated to interact reversibly with *E. coli*'s membrane porins, even if the porins may be protected from attack by the polysaccharide portion of the LPS and thus antibacterial activity may be decreased in vivo (Naidu et al., 1993). Binding of lactoferrin to moieties on the bacterial membrane surface such as LPS and its killing effects have been shown to be correlated, with the peptide concentration being crucial for cell death (Naidu et al., 1993).

Lactoferrin has been shown to modulate the activity of known antibacterial agents such as lysozyme and antibiotics, so may work synergistically (Ellison and Giehl, 1991; Ellison et al., 1988).

Polymorphisms in the LTF gene

Because of the biological functions of lactoferrin, polymorphisms in the LTF gene could be an important factor in genetic susceptibility to environmental insults and diseases.

More than 60 SNPs have been identified in the lactoferrin gene, either in the promoter region, exons or introns (Teng and Gladwell, 2006).

Recently a single-nucleotide polymorphism in the lactoferrin gene has been associated with susceptibility to diarrhea in North American travelers to Mexico, underlying the antimicrobial role of this protein against enteric pathogens (Mohamed et al., 2007). A

single-point A/G nucleotide mutation causing a non-conservative threonine/alanine substitution at position 11 (T11A) of the secreted lactoferrin protein has been associated with aggressive periodontitis (Jordan et al., 2005).

A Lys/Arg polymorphism at position 29 in the N-terminal region of human lactoferrin that results from a single nucleotide polymorphism in exon 1 of the human lactoferrin gene has been identified. When tested against different bacterial species lactoferrin containing lisine at position 29 exhibited significantly greater bactericidal activity against the gram-positive species *Streptococcus mutans* and *Streptococcus mitis* than did lactoferrin containing arginine. In addition, the lysine-containing lactoferrin stimulated bovine tracheal epithelial cells to synthesize much higher levels of tracheal antimicrobial peptide mRNA than did the arginine-containing variant. The lysine and arginine alleles show different frequencies among healthy human subjects, and patients with localized juvenile periodontitis, suggesting that these two lactoferrin variants are functionally different and that these differences may contribute to the pathogenesis of localized juvenile periodontitis (Velliyagounder et al., 2003).

Antiviral activity of lactoferrin

Besides being characterized by a broad spectrum antimicrobial activity against bacteria and fungi, lactoferrin is able to inhibit replication of a wide range of viruses. Most studies indicate that lactoferrin prevents infection of the host cell, rather than inhibiting virus replication after the target cell has become infected. Infection of the target cell is prevented by direct binding to virus particles for hepatitis C virus (HCV), herpes simplex virus (HSV), polio- and rotavirus. Studies on HCV demonstrated that human lactoferrin is able to bind to E1 and E2 envelope proteins *in vitro*, with interaction with the E2 protein also taking place *in vivo* (Yi et al., 1997).

Another mechanism by which lactoferrin exert its antiviral activity is binding to host cell molecules that the virus uses as a receptor to entry the cell such as heparin sulphate proteoglycans (HSPGs). Many viruses tend to dock on HSPGs of target cells; after this initial contact, the virus particles roll to their specific viral receptor and subsequently enter the host cell, for instance by fusing with the host cell membrane (Laquerre et al., 1998). Binding of lactoferrin to membrane's HSPGs at some extents prevents this first contact and therefore subsequent infection of the host cell.

For some viruses, it was found that Apolactoferrin (that is the iron-depleted form of lactoferrin) was more effective than the metal-saturated complexes of lactoferrin in blocking infection, although the reason is still unknown. Nevertheless, it is speculated that binding to target cell may lead to an increased uptake of metal ions like Zn^{2+}, which has been shown to be effective against poliovirus (Esposito and Obljeski, 1976). Another reason for the increased activity of apolactoferrin could be that many enzymes, including viral enzymes, require metal ions as a co-factor for their function.

Other researches demonstrated that an indirect antiviral mode of action of lactoferrin takes place through the upregulation of the antiviral response of the immune system. Administration of lactoferrin to cell cultures *in vitro*, or animals and healthy volunteers led to an upregulation of cell types that play an important role during the early phases of viral infection, such as natural killer (NK) cells, monocyte/macrophages and granulocytes (Crouch et al., 1992; Levay and Viljoen, 1995).

Lactoferrin and human immunodeficiency virus

Lactoferrin has also been reported to be active against HIV-1, (Harmsen et al., 1995) while it does not inhibit HIV-2 replication.

It has been reported that lactoferrin exerts its effect in the early phase of infection, at the level of virus adsorption and penetration

Human lactoferrin contain a cluster of negative charges between residues 210-240. This peptide sequence contributes to two beta-sheets, with a net negatively charged loop (residues 231-245). This loop is relatively easily accessible and may interact with the positively charged domains in the V3 loop of the viral envelope protein gp120. These data also confirm that lactoferrin inhibits viral replication at an early stage, probably during virus-cell fusion and binding, by binding to the gp120 molecule, thus inhibiting subsequent interaction with the CD4 receptor and possibly with the CXCR4 and CCR5 co-receptors.

The antiviral activity of lactoferrin in HIV-1 infection has been tested (Berkhout et al., 2002). Bovine lactoferrin was capable to completely impede the infection spreading at a concentration of 10 μM and considerable inhibition of virus replication was observed at concentration as low as 0.1 μM. Since it was suggested that lactoferrin, apart from its interaction with the V3 loop, could possibly inhibit virus-cell interaction through com-

petitive binding to the CXCR4 and CCR5 coreceptors, the antiviral activity of lactoferrin against HIV-1 variants with different V3 domains and co-receptor usage was tested. Bovine lactoferrin was able to inhibit various HIV-1 strains that use the CXCR4 and CCR5 co-receptor, confirming the broad activity spectrum of lactoferrin (Moriuchi and Moriuchi, 2001). This result suggests that it is possible for lactoferrin to bind to both the V3 loop of gp120 and the co-receptor CXCR-4 or CCR5.

There are also indications that lactoferrin is able to inhibit viral processes within the host cell. Lactoferrin was shown to inhibit the reverse transcriptase, protease and integrase enzymes (Ng et al., 2001; Wang et al., 2000,). Interestingly, it was also found that lactoferrin resistance coincided with a loss of viral replication capacity or fitness (Berkhout et al., 2002).

In a preliminary study, 6 months' lactoferrin administration (3 g/day, orally) was evaluated in 22 human immunodeficiency virus type 1 (HIV-1) vertically infected children, showing how this protein was effective in increasing CD4+ cell count and useful in addition to antiretroviral therapy (Zuccotti et al., 2006).

Antimicrobial peptides

A relevant part of the innate immunity is formed by peptides with direct antimicrobial activity, able to directly kill pathogens or impair their activity. Initial reports of plant peptides with antibacterial or antifungal properties date from the early 1970s (Fernandez de Caleya et al., 1972). Over the past 30 years, a hundreds of peptide antibiotics and antimicrobial peptides from animals and plants have been discovered, therefore requiring databases as *http://www.bbcm.univ.trieste.it/~tossi/amsdb.html*, to be established updated and maintained by professor A. Tossi, and *http://aps.unmc.edu/AP/main.html*, (Wang and Wang, 2004) .

Most of the progresses that have been done in studying and understanding antimicrobial peptides are due to the serious problem of resistance of bacteria and fungi to the commonly used antibiotics, a fact that generated a growing interest in the field of peptide antibiotics. The effect of this is that there's an increasing awareness that antimicrobial peptides or their synthetic analogues could be used to design and product novel therapeutic agents (Reddy et al., 2004).

Antimicrobial peptides take their name and classification from their principal characteristic: their antibacterial and antifungal activity. It should however be noted and never forgotten that many of these peptides do have a much broader range of biological properties: some of them, for example, possess antiviral as well as anticancer properties; furthermore, they can influence inflammation, proliferation, wound healing, release of cytokines, homeostasis, chemotaxis and the preservation of a balance between proteases and protease inhibitors (Bals, 2000). Those effects have only recently been discovered and clearly show how strong could be the interplay between innate and acquired immunity and how relevantly the former can influence the latter *in vivo*.

Notably, antimicrobial peptides markedly differ in their biochemical properties (amino acid composition, length and secondary structure). However, they all play essential roles in non-specific host defenses by preventing or limiting infections by their ability to selectively recognize potential pathogens.

Most peptides exert their antifungal or antibacterial effects by taking advantage of their high surface electrostatic charge to destabilize the microbial membrane, leading to cell death. This mechanism could also account for antiviral activity versus coated viruses like HIV-1, and different works have shown that defensins directly interact with the HIV-1 capside (Quinones-Mateu et al., 2003).

However, many different mechanisms of action are proposed for several peptides, including inhibiting synthesis of specific membrane proteins (Axen et al., 1997; Engstrom et al., 1984) or stress proteins (Groisman, 1996), arrest of DNA synthesis (Boman et al., 1993), breakage of single-strand DNA (Bateman et al., 1991), interaction with DNA (Park et al., 1998), and production of hydrogen peroxide (Leem et al., 1996). Antimicrobial peptides can also act by triggering apoptosis in eukaryotic cells (Velasco et al., 1997; Yoo et al., 1997) or autolysis in bacterial targets (Chitnis et al., 1993). In many cases probably more than one mechanism is employed by antimicrobial peptides to perform their task, making the comprehension of this peptides' role even more complex.

Many works published so far showed that antimicrobial peptides also exhibit a direct antiviral activity (Leikina et al., 2005; Oppenheim et al., 2003; Yang et al., 2004). While this effect is not easily explainable for uncoated viruses it's quite reasonable that antimicrobial peptides could interact with coated viruses' membranes and interfere with infection.

It has been demonstrated, for example, that the θ-defensin retrocyclin 2 (RC2) inhibited influenza virus infection by blocking membrane fusion mediated by the viral hemagglutinin. RC2 was effective even after hemagglutinin attained a fusogenic conformation or had induced membrane hemifusion. RC2, that is a multivalent lectin, prevented hemagglutinin-mediated fusion by erecting a network of crosslinked and immobilized surface glycoproteins (Leikina et al., 2005). In the same study it has been shown that this effect is not restricted to influenza virus, as RC2 also inhibited fusion mediated by Sindbis virus and baculovirus.

Human ß-defensin 3 and MBL were also able to block viral fusion by creating a protective barricade of immobilized surface proteins. The authors proposed that this general mechanism might explain the broad-spectrum antiviral activity of many multivalent lectins of the innate immune system.

Many antimicrobial peptides, in particular, have been demonstrated, beside other, to directly interact with HIV-1 viral particles (Quinones-Mateu et al., 2003) and interfere with the normal viral transmission, either by disgregation of the viral membrane or by binding to viruses' surface thus acting as an obstacle to the mechanism of infection at the point that some of them have been demonstrated to inhibit HIV-1 replication *in vitro* (Nakashima et al., 1993).

Antimicrobial peptides are able to directly bind to the viral membrane, destabilizing it and maybe destroying it, eventually generating a naked virion unable to infect CD4+ cells. Obviously, in order to take full advantages from antimicrobial peptides ability to interfere with HIV-1 the peptides should be able to interact with viral membranes and have to be present at the site of infection in such a concentration to inactivate the initial viral charge.

Genetic polymorphisms that are able to mutate the primary sequence of antimicrobial peptides can straightly determine the peptide's affinity to viral membrane, while single nucleotide polymorphisms in the promoter can modify peptide's expression in the infection site.

Defensins

Defensins represent one of the most important classes of antimicrobial peptides. They have a typical length of 30-40 amino acid residues with a molecular weight that falls in the 3-4 kD range (Ganz and Lehrer, 1994; Huttner and Bevins, 1999)

All human defensins are characterized by the presence, in their sequence, of six cysteine residues that give them the ability to form three intramolecular disulfide bridges in a peculiar pattern (Ganz, 2005; Lehrer, 2004). According to the distinctive spacing between their six cysteine residues and their disulfide bond pattern, defensins can be classified as α- and β- forms (Huttner and Bevins, 1999) that, regardless of their differences in secondary structure, share an analogous three-dimensional structure in solution (Bauer et al., 2001; Hill et al., 1991; Zimmermann et al., 1995).

Genomic organization of the defensin locus does present some interesting properties.

In fact all α- and most β-defensins genes map in a cluster in 8p23.1, a region that is known to be a frequent site of chromosomal rearrangements mediated by two olfactory repeat regions (ORRs) 5 cM apart. For example, an inversion polymorphism between these two ORRs can be found in normal population (Giglio et al., 2001; Giglio et al., 2002; Shimokawa et al., 2004; Sugawara et al., 2003). Within this cluster, two different defensins gene sub-clusters can be distinguished: one that contains the genes *DEFB1*, *DEFA6*, *DEFA4*, *DEFA1*, *DEFT1*, *DEFA3* and *DEFA5*; the other one that comprises the genes *DEFB109p*, *DEFB108*, *DEFB4*, *DEFB103*, *SPAG11*, *DEFB104*, *DEFB106*, *DEFB105*, and *DEFB107*. The latter cluster is also duplicated in reverse complementary (Taudien et al., 2004) and even a high copy number variation in this locus can occur, originating an euchromatic variant (EV) (Barber et al., 1998; Hollox et al., 2005; O'Malley and Storto, 1999).

Moreover, genes of both sub-clusters can vary inter-individually in their copy numbers. (Hollox et al., 2003; Mars et al., 1995).

α-defensins

The human α-defensins that have been discovered so far are 29-35 amino acids long and are characterized by a disulfide bridges pattern in which cysteine 1 is bound to cysteine 6, cysteine 2 to cysteine 4 and the third disulfide bond links cysteine 3 and 5, re-

sulting in peptides forming a triple-stranded b-sheet structure with a β-hairpin loop containing cationic charged molecules.

In humans, six α-defensins (HNP-1 to 6) have been discovered and are either expressed in neutrophils or present in the granules of Paneth cells of the small intestine and in the epithelial cells of the female urogenital tract (Jones and Bevins, 1993). The finding of an abundant defensin mRNA expression in human Paneth cells supports the notion that these epithelial cells may play a key role in host defense of the human bowel and strengthen the hypothesis that peptide-based host defenses are prevalent at mucosal surfaces in mammals.

HNP-1 to 4 can be found in the azurophilic granules of granulocytes. Half of the azurophilic protein content is composed of HNP-1, 2 and 3, whereas HNP-4 is present at lower concentrations (Ganz et al., 1990). Expression of HNP-1 to 3 is also detectable in B cells and natural killer cells. In neutrophils, the α-defensins play a role in the oxygen-independent killing of phagocytosed microorganisms.

The two other α-defensins, HD-5 and 6, are referred to as enteric defensins, and are found in the granules of Paneth cells, that provide host defence against microbes in the small intestine.

Paneth cells are functionally similar to neutrophils and secrete a number of antimicrobial molecules into the lumen of the crypt when exposed to bacteria or bacterial antigens, thus contributing to the maintenance of the gastrointestinal barrier (Ganz, 2000).

All the six genes of the α-defensins are found in the same region of chromosome 8 and are located in the cluster present in the band 8p23.

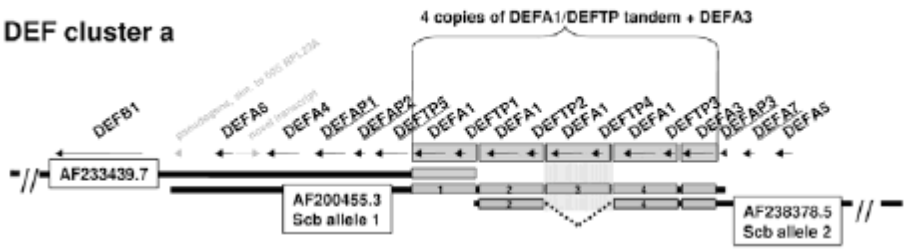

Figure 7 Schematic representation of the α-defensin cluster.

The genes DEFA1, from which both HNP-1 and HNP-3 are derived, and DEFA5, encoding the HD-5 peptide, present a copy number polymorphism, while the other α-defensin genes are present in single copy per aploid genome.

α-Defensins, which are expressed as prepropeptides with no antimicrobial activity, are activated by proteolitic cleavage that releases the C-terminal part of the peptide, responsible for the antimicrobial properties of the peptide. In the case of the enteric defensins, a single metalloproteinase is responsible for the release of the active peptide.
The three-dimensional fold of α-defensins give raise to a highly amphiphilic molecule. Therefore, microbicidal and cytotoxic properties of defensins are most likely a consequence of their ability to insert into biological membranes and to generate pores.

β- defensins

The second class of human defensins are β-defensins, that are larger than α-defensins. Although there is little primary sequence homology between α- and β- defensin families, their tertiary structures are very similar because of the presence of three disulfide bonds. In β-defensins the three disulfide bridges are between residues 1 and 5, 2 and 4, and 3 and 6, also resulting in peptides with a triple-stranded β-sheet structure and a β-hairpin loop containing cationic charged molecules (Lehrer, 2004).
The first human β-defensin (hBD-1) was isolated from hemofiltrate of patients undergoing dialysis treatment. hBD-1, a short basic peptide of 36 amino acid residues, has been found to be expressed in epithelia that are directly exposed to the environment or microbial flora (e.g. in the lung, mammary gland, salivary gland, kidney, pancreas and prostate) (Bensch et al., 1995).
A second member of the family, hBD-2, was first characterized in psoriatic skin. hBD-2 is widely expressed in epithelia (lung, gut, urogenital system, pancreas and skin), leukocytes and the bone marrow. In contrast to hBD-1, exposure of epithelial tissue to LPS or pro-inflammatory agents (TNF-a or IL-1b) up-regulates the expression of hBD-2 (Harder et al., 1997).
Another defensin (hBD-3) was isolated from human lesional psoriatic scales and cloned from keratinocytes. While hBD-1 and 2 show microbicidal activity predominantly against Gram-negative bacteria, and only low, if any, microbicidal activity against Gram-positive bacteria, hBD-3 demonstrated a salt-insensitive broad spectrum of potent antimicrobial activity against many potentially pathogenic microbes including multiresistant *S. aureus* and vancomycin-resistant *Enterococcus faecium*. Ultrastructural analy-

ses of hBD-3-treated *S. aureus* revealed signs of cell wall perforation, thus providing more evidence about defensins mode of action.

Investigation of different tissues revealed skin and tonsils to be major hBD-3 mRNA-expressing tissues. Molecular cloning and biochemical analyses of antimicrobial peptides in cell culture supernatants revealed keratinocytes and airway epithelial cells as cellular sources of hBD-3. Like hBD-2, hBD-3 is also induced by inflammatory stimuli, such as Tumor Necrosis Factor-α and contact with bacteria (Harder et al., 2001). These first three human β-defensins were discovered through the identification of antimicrobial substances in large amounts of biological material. The genes of these three β-defensins are located in a single cluster at chromosomal region 8p23.

Figure 8 Schematic representation of the β-defensins cluster.

In this gene cluster, three other β-defensin genes were discovered. The predicted peptides conserve the six-cysteine motif identical with hBD-1, and were named hBD-4, hBD-5 and hBD-6 (Garcia et al., 2001; Yamaguchi et al., 2002). Yamaguchi et al. (2002) showed that hBD-4, hBD-5 and hBD-6 are expressed in the human epididymis; hBD-4 was found to be expressed in the testis but this data have not been confirmed. The low basal expression of hBD-4 in lung epithelial cells could be upregulated by contact with bacteria or by phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C. However, expression of hBD-4 was not induced by exposure to IL-1a, IL-6, IFNc or TNF-a. hBD-4 inhibited the growth of Gram-positive and Gram-negative bacteria and of the yeast, *Saccharomyces cerevisiae* (Garcia et al., 2001,).

Yamaguchi et al. (2002) proposed the division of the β-defensins in two groups: the epididymis-specific isoforms and the other isoforms. The epididymis-specific isoforms include hBD-4, hBD-5, and hBD-6. The other isoforms include hBD-1, hBD-2 and hBD-3.

Recently, more β-defensin-like genes have been discovered with a computational search tool based on hidden Markov models, in combination with the BLAST research implementation (Schutte and McCray, 2002). Using this strategy 28 new human β-defensin genes were discovered in five syntenic chromosomal regions.

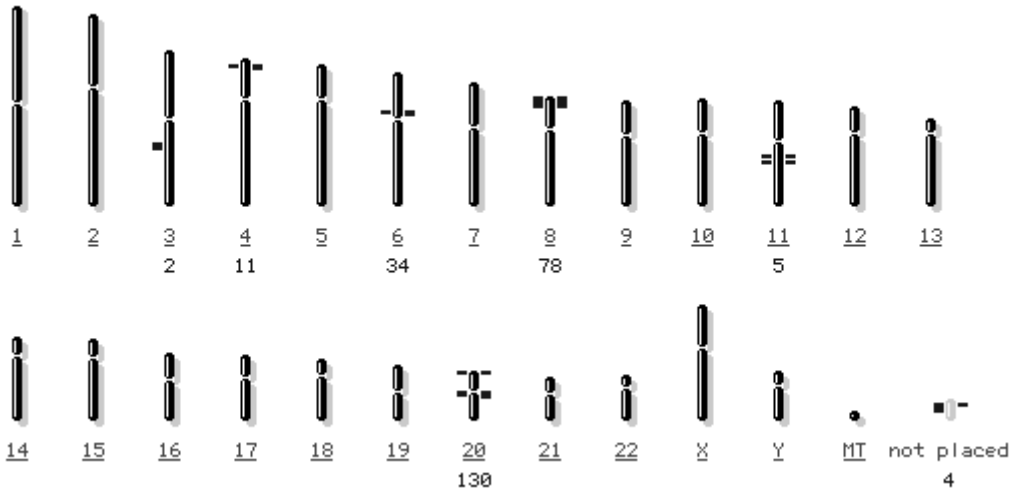

Figure 9 Schematic representation of the localization of β-defensins genes (indicated in red) on human chromosomes.

Within each syntenic cluster, the gene sequences and organization were similar, suggesting each cluster pair arose from a common ancestor and was retained because of conserved functions. Reported preliminary analysis indicates that at least 26 of the predicted genes are actually transcribed.

Human β-defensin 118 is one of these recently characterized defensin and appears to be an epididymis-specific peptide that has potent antibacterial activity that is dose, time, and structure dependent.

Incubation of *Escherichia coli* for 60 min with 10 μg/ml DEFB118 reduced bacterial survival to 20% of the control, and 25 μg/ml reduced survival to 5% of the control. DEFB118 concentrations of 50 and 100 μg/ml further reduced survival to less than 2 and 1%, respectively. It should be noted that complete reduction of disulfide bonds and alkylation of cysteines resulted in the complete loss of antibacterial activity, providing strong evidence about the importance of a correct folding disulfide bonds formation on defensins physiological effect. DEFB118 has been shown to cause rapid permeabiliza-

tion of both outer and inner membranes of *E. coli* and striking morphological alterations in the bacterial surfaces visible by scanning electron microscopy consistent with a membrane-disruptive mechanism of bacterial killing. In contrast, eukaryotic cell membranes were not permeabilized by DEFB118, as indicated by using a rat erythrocyte hemolytic assay. Again, studies on DEFB118 inhibition of macromolecular synthesis and membrane permeability in *E. coli* were consistent with a primary effect at the cell membrane level, similarly to the case of other studied defensins. DEFB118 may contribute to epididymal innate immunity and protect the sperm against attack by microorganisms in the male and female reproductive tracts (Yenugu et al., 2004).

Defensins activities

Besides their direct antimicrobial activity that likely relies on membrane disruption by generation of pores, defensins also show additional properties (Kamysz et al., 2003; Oppenheim et al., 2003).

Lichtenstein et al. (1986), for example, examined the activity of defensins against various tumor targets. The three human α-defensins, HNP-1, HNP-2, and HNP-3 were able to lyse human and murine targets when tested with chromium release and dye exclusion assays. Defensin-mediated tumor cell lysis was concentration-dependent, inhibited by serum, and dependent on temperature-sensitive events. Lysis was first detected by three hours of incubation and it reached a plateau between eight and 14 hours. Moreover, *in vitro* exposure of murine teratocarcinoma cells to HNP 1-3 abrogated their *in vivo* oncogenicity. Nonmalignant target cells were also susceptible to defensin-mediated lysis. When tumor cells were incubated with human defensins in combination with hydrogen peroxide, synergistic cytotoxicity was detected.

Defensins are also effective mitogens *in vitro* when used at the same concentration range in which they display effective antimicrobial activity in *in vitro* experiments. The concentrations that induce the mitogenic process are in the same range as those expected to be present *in vivo* during the wound healing process (Murphy et al., 1993).

Charp et al. (1988) tested the three major alpha-defensins, showing that HNP-2 appeared to be more potent than HNP-1 and HNP-3. Kinetic studies indicated that defensins inhibited protein kinase C (PKC) noncompetitively with respect to phosphatidylserine (a phospholipid cofactor), Ca2+ (an activator), ATP (a phosphoryl donor) and his-

tone H1 (a substrate protein) with Ki values ranging from 1.2 to 1.7 μM. Defensins did not inhibit the binding of (3H) phorbol 12,13-dibutyrate to PKC; however they inhibited the PKC activity stimulated by 12-O-tetradecanoylphorbol-13-acetate. Defensins had little or no effect on myosin light chain kinase (a calmodulin/Ca2+-dependent protein kinase) and the holoenzyme or catalytic subunit of cyclic AMP-dependent protein kinase, indicating a specificity of action of defensins. The authors concluded that defensins might have profound effects on functions of neutrophils and other mammalian cells, in addition to their well-recognized antimicrobial activities.

Another recognized function of defensins is their monocyte-chemotactic activity, which was found predominantly in the defensin-containing fraction of the neutrophil granules (Territo et al., 1989). Purified preparations of each of the three human defensins (HNP-1, HNP-2, HNP-3) were tested showing a significant chemotactic activity of HNP-1 at the nanomolar range. HNP-2 was less active, while HNP-3 failed to exhibit chemotactic activity. Further analysis of monocyte response to HNP-1 and HNP-2 confirmed that their activity was chemotactic rather than chemokinetic. Neutrophils demonstrated a low level of response to defensins but this reaction was primarily chemokinetic, suggesting that defensins may play a role in the recruitment of monocytes by neutrophils into inflammatory sites, strengthening the relationship between innate and acquired immunity.

Finally, it was shown that HNP-1 could stimulate cytokine and adhesion molecule expression (Chaly et al., 2000). In fact HNP-1 up-regulated the expression of TNF-α and IL-1 β in monocytes activated with *Staphylococcus aureus* or PMA, while expression of IL-10 mRNA was down-regulated while production of IL-8 was not affected. HNP-1 alone was unable to induce TNF-α or IL-1 β expression in resting monocytes. It was also demonstrated that HNP-1 could attenuate the inhibitory action of dexamethasone on TNF-α production. HNP-1 also caused about 5-fold suppression of VCAM-1 expression in TNF-α-activated human umbilical vein endothelial cells, while the ICAM-1 expression was not affected; suggesting that neutrophil defensins have the potential to modulate the inflammatory responses through regulation of cytokine production and adhesion molecule expression.

Antiviral activity of defensins

Defensins do possess a dual role in antiviral activity: the first aspect of antiviral activity involves direct interaction with viral envelopes, probably in a way comparable to their antibacterial activity, and the other involves indirect antiviral activity through interactions with possible target cells. These defensin–cell interactions are complex and possibly mediated by interacting with cell-surface glycoproteins and/or interfering with cell-signalling pathways that are required for viral replication.

Direct effect on the virion has been shown, for example, for HNP-1, that was originally reported to have a direct effect on several enveloped viruses but not on non-enveloped viruses (Daher et al., 1986). Among those enveloped viruses tested, HNP-1 has a potent direct inhibitory effect on herpes simplex virus-1 (HSV-1) and HSV-2, while his direct effect was moderate on vesicular stomatitis virus (VSV) and influenza virus, and much smaller on cytomegalovirus (CMV). The shown differential inhibitory effect of HNP-1 against different enveloped viruses could be due to variability in viral envelopes' lipid composition of different viruses, as the lipid composition of bacterial membranes has been shown to influence membrane permeabilization by rabbit neutrophil defensins (Hristova et al., 1997).

The exact mechanism of direct inactivation of the virion by defensins is not totally understood yet, and current models, including viral membrane disruption or binding to viral glycoproteins, need to be further investigated. Factors such as serum and salt are known to alter the functions of defensins *in vitro*; therefore, the different antiviral mechanisms of defensins might be operative in mucosal surfaces rather than blood, depending on the salt concentration or the presence of serum. This seems to be the case with the direct antiviral effect. Serum has been shown to diminish the direct effect of defensins on the virion (Chang et al., 2005; Daher et al., 1986).

High concentrations of defensins are known to cause cytotoxicity in the absence of serum, and this is associated with changes in cell-membrane permeability in a similar way to the antibacterial activity of the defensins. This cytotoxicity can be abolished by the presence of serum (Okrent et al., 1990; Van Wetering et al., 1997) and defensin-mediated cytotoxicity might partially account for the antiviral effect (Mackewicz et al., 2003). In addition, most defensins have potent direct antibacterial activities in conditions of low salt concentration (Lehrer et al., 1993). However, the required conditions for optimum activity vary depending on the specific function of defensins. For example,

neither a low concentration of salt nor the absence of serum is required for the chemotactic effects of defensins (Chertov et al., 1996; Yang et al., 1999).

The anti-HIV-1 activity of HNP-1, HNP-2 and HNP-3 has recently been investigated (Chang et al., 2003; Mackewicz et al., 2003; Zhang et al., 2002). HNP-1, HNP-2 and HNP-3 all have similar activities against HIV-1 primary isolates (Wu et al., 2005), in contrast to their differential chemotactic activities on monocytes, which HNP-3 does not effect (Territo et al., 1989). HNP-1, HNP-2 and HNP-3 have at least two mechanisms of anti-HIV-1 activity. They can inhibit HIV-1 replication by a direct interaction with the virus as well by affecting the target cells (Chang et al., 2003; Chang et al., 2005; Mackewicz et al., 2003). In the absence of serum, HNP-1 can directly inactivate the virus before it infects a cell (Chang et al., 2005). In the presence of serum and at non-cytotoxic concentrations (low dose), HNP-1 acts on infected cells and blocks HIV-1 infection at the steps of nuclear import and transcription. Furthermore, in primary CD4+ T cells, HNP-1 interference with PKC signalling is associated with the ability of HNP-1 to inhibit infection after HIV-1 enters the cell, although other signalling pathways might also be involved (Chang et al., 2005). For example, in macrophages, HNP-1 and HNP-2 upregulate the expression of CC-chemokines, which could contribute to inhibition of HIV-1 through competition for receptors (Guo et al., 2004). CC-chemokines can also induce the release of HNPs from neutrophils by degranulation (Jan et al., 2006). Both effects could have a role in vivo in an innate immune response to HIV. At the mucosal surface, HNPs might work to directly inactivate the virions in the absence of serum; however, in the presence of serum, their inhibitory effect would largely be on the infected cell. HNPs are positively charged, so direct binding to HIV-1 virions through charge interactions might account for some of their direct inhibition of HIV-1 virions, as well as for their sensitivity to serum through competing inter- actions with serum proteins. HNP-1, HNP-2 and HNP-3 have been reported to function as lectins, by binding to the HIV-1 envelope glycoprotein gp120 and CD4 with high affinity, although their interference with the interaction between HIV-1 gp120 and CD4 has not been well defined (Wang et al., 2004). HNP binding to gp120 is strongly attenuated by serum, therefore accounting for the loss of the direct effect on the virion in the presence of serum. Interestingly, in contrast to HNP-1, HNP-2 and HNP-3, HNP-4 acts in a lectin-independent manner and does not bind to CD4 or HIV-1 gp120 (Wang et al., 2004; Wu et al., 2005). However, HNP-4 inhibits HIV-1 replication more effectively than HNP-1,

HNP-2 and HNP-3 (Wu et al., 2005), although it is not clear whether the antiviral activity of HNP-4 is mediated through a direct effect on virions or on the infected cells. Other α-defensins, including HD5, mouse cryptdin-3 and cryptdin-4, and rhesus macaque myeloid have been tested for their ability to block HIV-1 infection (Tanabe et al., 2004). At the high concentrations associated with cytotoxicity, RMAD4 blocks HIV-1 replication, whereas cryptdin-3 enhances viral replication. The other peptides tested do not have anti-HIV-1 activity in the assay systems reported. The mechanism of enhanced HIV-1 replication by cryptdin-3 and the effect of these peptides on HIV-1 replication following viral entry are not clear. Because experiments carried out with these defensins used a transformed cell line, alternative assay systems including primary cells will help to better define the anti-HIV-1 activity of these defensins. For example, HNP-1 causes post-entry inhibition of HIV-1 in primary CD4+ T cells and macrophages but not in several transformed T-cell lines (Chang et al., 2003; Chang et al., 2005). The anti-HIV-1 activities of HBD2 and HBD3 have been shown under different conditions (Quinones-Mateu et al., 2003; Sun et al., 2005). One condition used mimics the oral mucosal environ- ment, with low salt concentrations and the absence of serum (Quinones-Mateu et al., 2003), and another condition used has high salt concentrations and the presence of serum (Sun et al., 2005). Similar to HNP-1 (Chang et al., 2005), HBD2 and HBD3 have dual anti- HIV-1 activities through direct interactions with the virus and indirectly by altering the target cell. HBD2 and HBD3 have been shown, by electron microscopy, to interact with cellular membranes as well as HIV-1 virions, although membrane disruption is not apparent. HBD2 does not affect cell–cell fusion but instead inhibits the formation of early reverse-transcribed HIV-1 DNA products (Sun et al., 2005). There are conflicting reports on the downregulation of expression of HIV-1 co-receptors by HBD1 and HBD2 did not modulate cell-surface HIV-1 co-receptor expression by primary CD4+ T cells. By contrast, Quinones-Mateu et al. (Quinones-Mateu et al., 2003) showed HBD2- and HBD3-mediated downregulation of surface CXCR4 but not CCR5 expression by peripheral-blood mono- nuclear cells (PBMCs) at high salt conditions and in the absence of serum. These conflicting reports might be due to differences in the source of the defensin and/or experimental conditions used (that is, the presence or absence of serum). Interestingly, HBD2 is constitutively expressed in healthy adult oral mucosa but the level seems to be diminished in HIV-infected individuals (Sun et al., 2005).

In response to viral infection, target cells can produce cytokines, chemokines and other antiviral factors to control viral replication. In a similar way to the cytokine induction that occurs as an early innate immune response to viral infection, HIV-1 induces mRNA expression of HBD2 and HBD3, but not HBD1, in normal human oral epithelial cells, even in the absence of HIV-1 replication (Quinones-Mateu et al., 2003). These cells lack cell-surface expression of the HIV-1 entry receptors CD4, CC-chemokine receptor 5 (CCR5) and CXC-chemokine receptor 4 (CXCR4), or galactosylceramide, so it is unclear what inter actions between the virus and the cell are responsible for this induction of expression.

Defensins could also act as chemotactic agents for T cells, monocytes and immature dendritic cells, and can induce cytokine production by monocytes and epithelial cells (Yang et al., 2004). Therefore, defensins might control viral replication by modulating the immune system, in addition to acting as direct effectors. Increasing evidence indicates that some activities of defensins are receptor mediated, resulting in activation of downstream signaling events. For example, the chemotatic activity of HBD1, HBD2 and HBD3 for memory T cells and immature dendritic cells is mediated through binding to CCR6, which is the receptor for CC-chemokine ligand 20 (CCL20; also known as MIP3) (Yang et al., 1999; Zlotnik and Yoshie, 2000). In addition, HBD2 has multiple activities on mast cells, including induction of mast-cell migration, degranulation and prostaglandin D2 production. These activities can be blocked by pertussis toxin and by phospholipase C inhibitor, indicating that Gi-protein-coupled receptors and phospholipase C signaling pathways are involved (Niyonsaba et al., 2003).

Murine β-defensin 2 can recruit bone-marrow-derived immature DCs through CCR6 and can induce DC maturation through TLR4 (Biragyn et al., 2002). Although the specific receptors responsible for the chemotactic activity of HNP-1, HNP-2 and HNP-3 have not been identified, their chemotactic activity can also be blocked by pertussis toxin, indicating the involvement of a G-protein-coupled receptor (Chertov et al., 1996; Yang et al., 2000). Several studies have implicated a role for specific receptors in other biological functions of HNPs (Higazi et al., 1996; Higazi et al., 2000; Kougias et al., 2005; Singh et al., 1988). For example, HNPs bind to low-density-lipoprotein-receptor-related proteins and interact with protein kinase C leading to decreased smooth-muscle contraction in response to phenylephrine (Nassar et al., 2002). HNPs also interact with

adrenocorticotropic hormone receptors and heparan sulphate proteoglycans (HSPGs) to modulate other bio- logical activities (Higazi et al., 1996; Higazi et al., 2000). HNP-1 has been shown to inhibit the activity of conventional PKC isoforms in a cell-free system (Charp et al., 1988). This PKC inhibitory activity seems to be important for the HNP-1-mediated inhibition of HIV-1 replication in primary CD4+ T cells (Chang et al., 2005). Taken together, these studies indicate that several biological functions involved human receptors with subsequent regulation of cell-signaling pathways. However, the role of these receptor interactions and signaling pathways in defensin-mediated antiviral activities remains to be determined.

Polymorphisms in defensins genes

Polymorphisms of human defensin genes clearly influence susceptibility to viral infection and disease progression, as has been shown for HIV-1 infection (Gonzalez et al., 2005; Gonzalez et al., 2002; Hogan and Hammer, 2001; Kaslow et al., 2005). The human α-defensins genes DEFA1 (encoding HNP-1) and DEFA3 (encoding HNP-3) have polymorphisms in both copy number and the location of 19-kilobase (kb) tandem repeats on chromosome 8p23.1 (Aldred et al., 2005; Mars et al., 1995). Gene expression of HNP-1 and HNP-3 at the RNA level in leukocytes correlates with the number of copies of the corresponding gene (Mars et al., 1995). Similarly, DEFB4 (encoding HBD2), DEFB103 (encoding HBD3) and DEFB104 (encoding HBD4), is polymorphic in copy number, with a repeat size of at least 240 kb (Hollox et al., 2003). The DEFB104 copy number correlates with the level of transcription. Although correlation between the protein levels of defensins and their gene-copy numbers has not been reported, it is tempting to speculate that variable expression levels of these defensins could lead to differential susceptibility to infection with or progression of infectious diseases. Polymorphisms in the DEFB1 gene (encoding HBD1) have been associated with susceptibility to diseases, including chronic obstructive pulmonary disease (COPD) and asthma, and are associated with the severity of cystic-fibrosis-associated pulmonary disease (Dork and Stuhrmann, 1998; Levy et al., 2005; Matsushita et al., 2002; Salvatore et al., 2002; Vankeerberghen et al., 2005). Although viral infections are one of the main triggers of exacerbations of obstructive airway diseases such as asthma and COPD (Message and Johnston, 2004), the association of polymorphisms in DEFB1 with susceptibility to vi-

ral respiratory infections is not known. Interestingly, a single-nucleotide polymorphism in the 5 reported to be associated with perinatal transmission of HIV-1 in a cohort of Italian children (Braida et al., 2004). However, the significance of this mutation in the control of HIV-1 infection remains to be explored.

Cathelicidins: LL37

Cathelicidins are a family of antimicrobial peptides derived from proteolitic cleavage of bigger polypeptides, that contain a very well conserved signal sequence and a pro-region that is highly homologus to cathelin, a cathepsin L inhibitor. On the other hand, the cathelicidin C-terminal domain shows substantial heterogeneity (Hancock and Diamond, 2000).

So far only one cathelicidin has been characterized in humans: LL37. This peptide is derived by proteolysis from the C-terminal end of the CAP18 protein (hCAP18)

Larrick et al. (1996) cloned the human CAP18 gene from a human genomic phage library. Sequence analysis revealed that, like several other genes expressed late in polymorphonuclear leukocyte development, the hCAP18 gene does not contain typical TATA box or CCAAT sequences. The gene is located on chromosome band 3p21.3, it is 1963 base pairs long, and consist in 4 exons: exons 1-3 encode for a signal sequence and the cathelin region while exon 4 contains the information for the mature antibacterial peptide. Potential binding sites for acute-phase-response factors were identified in the promoter and in intron 2. It is mainly expressed in leukocytes such as neutrophils, monocytes, NK cells, T cells and B cells, and in epithelial cells of the testis, skin, and the gastrointestinal and respiratory tracts (Agerberth et al., 2000; Bals et al., 1998; Cowland et al., 1995; Frohm et al., 1997).

Figure 10 Localization of the LL37 gene.

LL37 is induced by inflammatory or infectious stimuli (Frohm et al., 1997) and has been shown to have antimicrobial activity against both Gram-positive and Gram-

negative bacteria (Turner et al., 1998). Besides its antimicrobial activity, the peptide binds and neutralizes LPS and protects against endotoxic shock in a murine model of septicemia (Bals et al., 1999). Furthermore, it is chemotactic for neutrophils, monocytes, mast cells and T cells, induces degranulation of mast cells, alters transcriptional responses in macrophages, stimulates wound vascularization and re-epithelialization of healing skin (Zanetti, 2004), and has antitumor activity (Okumura et al., 2004). The LL37 peptide is 37 amino acid residues long, and, contrarily to defensins, has a linear structure because it does not contain any cysteine residue that permits the formation of disulfide bonds. The peptide adopts a largely random coil conformation in a hydrophilic environment, and an α-helical structure in a hydrophobic environment (Turner et al., 1998). That accounts for the antimicrobial effect of LL37; in fact the amphipathic α -helical conformation is adopted when LL37 interacts with biological membranes. Besides, LL37 is believed to multimerize, generating channels in the membrane, from which endoplasm can easily flow through (Henzler Wildman et al., 2003). LL37 is also able to trigger the p38 kinase pathway in monocytes and it's also chemotactic for T cells (Bowdish et al., 2004).

Antiviral effects of LL37

Antiviral activity of LL37 has been evidenced by several studies. LL37, for example, shown a direct antiviral activity against orhopox and has been described to inhibit vaccinia virus replication in a concentration-independent manner (Howell et al., 2004). Vaccinia virus treated with LL37 showed altered morphology, suggesting that LL37 might have a direct effect on the virion. Importantly, the physiological role of cathelicidin has been shown with a strain of mice that were know-out for the murine homologue of LL37: CRAMP. These mice have enhanced morbidity or mortality following exposure to vaccinia virus compared with control wild-type mice.

Notably, LL37, similar to defensins, has chemotactic activity and other activities that are mediated through alterations in receptor-mediated cells signaling. Moreover it is expressed also in testis (Agerberth et al., 1995) and could protect semen from pathogenic microorganisms.

So far no studies have been performed on the relationship between LL37 and HIV-1 infection and susceptibility in humans but the cathelicidin LL37 has been shown to spe-

cifically inhibit lentiviral vector infectivity (Steinstraesser et al., 2005). The strongest inhibition was seen if the lentiviral vectors were preincubated with LL37, suggesting a direct interaction with the vector particles. Since, in the same study, lentiviral vectors and retroviral vectors were inhibited to a similar degree even if not sharing any viral protein, the authors suggest that the target for LL37 on the particles is probably cell-derived. This could either be the lipid membrane derived from the cell, which surrounds the vector particles or cellular membrane proteins that are frequently incorporated in lentiviral and retroviral particles during budding (Gould et al., 2003). LL37 inhibited HIV-1 to lesser degree then the lentiviral vectors *in vitro*, and due an IC50 of 88 µg/ml against wild type HIV-1, the authors debated whether LL37 concentrations are sufficiently high at mucosal membranes to play a role in host defense against HIV-1. In their recent work, Bergman et al. (Bergman et al., 2007) have evaluated the capacity of LL37 to inhibit HIV-1 infection in vitro. They demonstrated that LL37 inhibits HIV-1 replication in PBMC, including primary CD4+ T cells. This inhibition was readily reproduced using various HIV-1 isolates without detectable changes in the target cell expression of HIV-1 chemokine receptors. Given the epithelial expression of LL37, it may contribute to the local protection against HIV-1 infection.

Lactoferricin

The presence of stable antimicrobial peptides resulting from proteolytic cleavage of lactoferrin was demonstrated by experiments that shown that limited acid proteolysis of bovine lactoferrin yielded a hydrolysate that had greater antibacterial activity than lactoferrin (Saito et al., 1991).

Bellamy and co-workers described the generation, after pepsin hydrolysis, of fragments from human and bovine lactoferrins that showed enhanced antimicrobial activity but lacked any iron-binding capacity. The fragments were characterized and named human (H) and bovine (B) lactoferricin. Both peptides are derived from the N-terminal region of the N-lobe and have greater antibacterial activity than their parent proteins (Bellamy et al., 1992; Bellamy et al., 1992). Lactoferricin H corresponds to amino acid residues 1–47 from the N-terminal region of the lactoferrin protein and includes an 18-residue loop formed by an internal disulphide bridge. Residues 1–11 constitute a separate fragment, which remains bound to the main loop by a disulfide bridge.

Lactoferricin H is similar to lactoferricin B in that it has a loop region held together by a disulphide bridge. It has been suggested that antimicrobial activity was independent of the presence of this bridge (Bellamy et al., 1992; Bellamy et al., 1992), but other studies pointed toward the importance of the disulfide bridge in antimicrobial activity by conferring rigidity and stability to the peptide as observed with cyclic peptides. Notwithstanding the fact that the total and net positive charges carried by the lactoferricin H are higher than those carried by lactoferricin B, the proportion of basic amino acid residues is greater in the bovine peptide. The lower cationicity within the 18-residue loop might be at the heart of the difference in antimicrobial activity exhibited by human lactoferrin-derived peptides.

To date little is known about the mechanism by which lactoferricins exerts their activity, even if it has been shown that they causes damage by generating blisters on the outer membrane of Gram-negative bacteria (Yamauchi et al., 1993), and that regions within lactoferricin bind to the LPS (Elass-Rochard et al., 1995). Interaction between LPS and both lactoferricins have been investigated, using a synthetic octadecapeptide corresponding to residues 20–37 in lactoferricin H, concluding that the 28–34 loop region of the peptide were found to be involved in LPS binding (Elass-Rochard et al., 1995).

There is no clear evidence of specific receptors linked to the killing mechanism. The only direct interaction experiments have been against *Candida albicans*, which is killed by lactoferricin B, where ^{14}C-labelled lactoferricin B bound to *C. albicans* and the rate of binding appeared to be consistent with the rate of killing induced by the peptide. Again, as with gram-negative bacteria, cells exhibited profound ultrastructural damage, which appeared to reflect the induction of an autolytic response (Bellamy et al., 1993).

Activity studies against *E. coli* in the pH range 5.5 and 7.5, showed that the peptide was most effective in slightly alkaline conditions, and that bacterial effectiveness was reduced in the presence of Na^+, K^+, Mg^{2+} or Ca^{2+} ions.

The sequence for human lactoferricin forms a surface-exposed α-helix with a hydrophobic tail within the native proteins (Odell et al., 1996). However, the peptides may adopt a different conformation when released from the parent protein, and/or when in contact with their target. The 3D structure of synthetic bovine lactoferricin has been solved by NMR spectroscopy and it was shown to adopt an anti-parallel β-sheet in wa-

ter, with a hydrophobic core surrounded by hydrophilic and charged amino acids (Hwang et al., 1998; Schibli et al., 1999).

Lactoferricins also exhibit moderate antiviral activity, and bovine lactoferricin does peresent antiviral activity at 100 μM (Berkhout et al., 1997). However, this peptide shows little anti-HIV-1 activity when compared to intact lactoferrin, indicating that other parts of the lactoferrin molecule are important. To date there are no studies on the relation between human lactoferricin and HIV-1 infection.

AIMS OF THE STUDY

With our study we wanted to clarify the effect that antimicrobial peptides exert, both alone or synergistically, in HIV infection and in AIDS progression in humans. At present, in fact, no studies have been performed on this subject that, in virtue of its potential effect on public health, deserves great attention.

In particular there is a lack of knowledge on the synergistical effect that different types of antimicrobial peptides could elicit in the site of infection.

Since genetic association studies proved to be very effective in identifying polymorphic proteins associated to resistance to host pathogens or differential progression of infection and thus it will be the primary tool used in the research.

Since recent scientific studies and interesting experiments demonstrated the importance of several peptides in preventing HIV infection by blocking virus–host cells interactions, we focusedthis project on the classes of peptides that were already described in the first part of this thesis, namely alpha and beta defensins, cathelicidin (LL-37/hCAP-18) and lactoferrin.

We believe that the results of our work will be beneficial to a better understanding both of antimicrobial peptides' and HIV-1's physiology and could be a starting point to develop better treatments for AIDS.

MATHERIALS AND METHODS

Enrollment of patients

Two distinct groups of subjects have been enrolled for this study.

The first DNA bank was collected in 2002 and incuded genomic DNA extracted from 303 Brazilian newborns, further subdivided in three groups: 128 infected children, 60 exposed-uninfected children and 115 healthy controls.

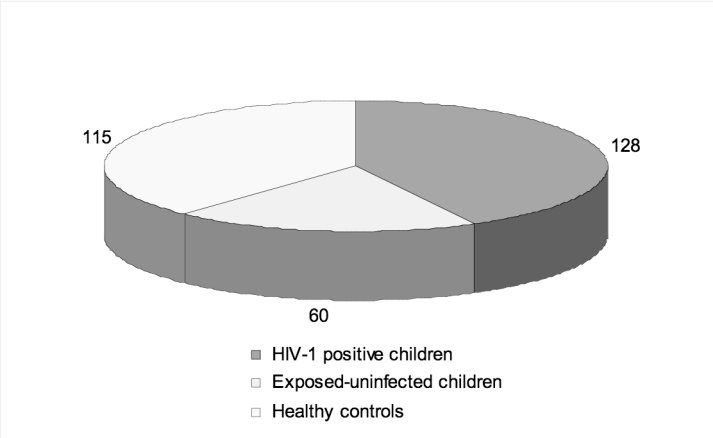

Figure 11 First DNA collection (2002)

The infected group consisted of samples of DNA extracted from children born from HIV-1 positive mothers and that contracted the infection during delivery.

The exposed-uninfected group was the most valuable and comprised children that, despite being born from HIV-1 infected mothers, weren't infected during delivery.

The healthy controls group was formed by samples extracted from newborns of HIV-1 negative mothers.

All mothers came from the impoverished areas of Recife and were admitted at the gynecological service of the IMIP. In no case the HIV-1 positive mothers did underwent antiretroviral therapy prior to delivery and parturition was not aided by caesarian section in order to prevent mother's blood contact to the children.
The aims of the study were explained and written consent was obtained.

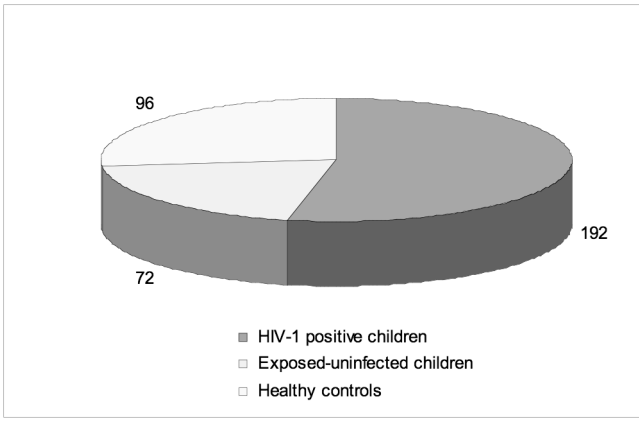

Figure 12 Figure 14: Second DNA collection (2006)

In 2006 a new sample collection took place at the IMIP. The new DNA bank counted 360 DNA samples and, compared to the previous DNA bank, was enriched in HIV-1 positive children, and, more importantly, in exposed uninfected children. Globally, in the new DNA bank we had 96 healthy controls, 192 HIV-1 infected children and 72 exposed-uninfected children. For the collection of the samples we continued to enroll the same kind of patients that were admitted at the gynecological service of the IMIP.
Written informed consent was obtained and the study has been approved by IMIP's ethical committee.

DNA extraction

Blood samples collected in 2002 were frozen to -20°C after collection in EDTA containing tubes and was extracted with phenol/chloroform procedure as follows.
Sample was thawed and 0.8 ml of SSC buffer (3M sodium cloride, 0.3M sodium citrate, pH 7.0) were added to 1 ml of blood. The sample was then microcentrifuged for 1 minute at 12000 rpm

One ml of the supernatant was then discarded and 1ml of SSC buffer was added before another centrifugation for 1 minute at 12000 rpm. Then all the supernatant was discarded and 375μl of a 0.2M solution of sodium acetate was added to the pellet and vortexed.

Then 25μl of 10% SDS and 5μl of proteinase K solution (20mg/ml) were added and the mix was incubated for 1 hour at 55°C.

After the incubation 120 μl of phenol/chlorophorm/isoamyl alcohol were added and the sample was vortexed for 30 second and then centrifuged for 2 minutes at 12000 rpm. The aqueous supernatant was then put in a new 1.5ml tube and 1 ml of 100% ethanol, stored in ice, was added. The mix was then incubated at -20°C for 15 minutes. After a centrifugation for 2 minutes at 12000 rpm the supernatant was decanted and drained. Then 180μl of 10:1 TE buffer were added and put at 55°C for 10 minutes before adding 20μl of a 2M sodium acetate solution. Then 500μl of cold 100% ethanol were added and centrifuged at maximum speed for one minute.

The resulting pellet was washed with 1 ml of 80% ethanol and centrifuged again before being dried to the air.

The DNA pellet was resuspended with 100μl of TE buffer overnight.

The samples collected in 2006 were characterized by a higher quality of the DNA, which was extracted with the Wizard Genomic DNA Purification Kit from Promega according to manufacturer's instructions.

Mitochondrial D-loop sequencing

Direct sequencing of the hypervariable D-loop sequence has been performed on the DNA collected in 2006 as previously described (Alves-Silva et al., 2000) in order to assess genetic homogeneity among the three different groups.

PCR amplification of the mtDNA control region (nucleotide position 16060-16362) was performed in a 25μl reaction volume using primer forward 5'-TCAAAGCTTACACCAGTCTTGTAAAAC-3' and primer reverse 5'-CTGAAGTAGGAACCAGATG-3', both at a 0.8μM concentration and 1X PCR Master Mix (Applied Biosystems).

Thermal cycler conditions where 10 minutes of preincubation at 95°C, then 30 cycles of 95°C for 60 seconds, 55°C for 30 seconds and 72°C for 60 seconds. A final extension step was done holding at 72°C for 4 minutes.

PCR products obtained this way usually contain excess primers and dNTP that are not compatible with the direct sequencing procedure. Samples were then digested with the two enzymes exonuclease I (Exo), that destroys every single-stranded template or primer, and Shrimp Alcaline Phosphatase (Sap), that degrades nucleotides.

The digestion reaction consisted of 1 unit of Sap (United States Biochemical, Harvard) and 5 units of Exo (United States Biochemical, Harvard) in a total volume of 7μl with a DNA quantity ranging from 60 to 80ng as estimated by agarose gel electrophoresis. The mixture was kept for 15 minutes at 37°C and then the enzymes were inactivated by 15 minutes at 85°C.

DNA sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit 2.0 (Applied Biosystems, Foster City, CA): that briefly consist in a labeling reaction of the PCR product with fluorescent nucleotides to allow detection of the fragments by the automatic sequencer. 1.88μl of water, 0.8μl of BigDye terminator kit and 0.32μl of 10pM primer were added to the digested PCR products and the mix were then put in a thermal-cycler with the following thermal profile: 1 minute at 96°C followed by 26 cycles composed by10 seconds at 96°C, 5 seconds at 50°C and 4 minutes at 60°C.

After a DNA precipitation and purification step, the labeled mixture was then added with 20μl of formammide to proceed with automatic sequencing in an ABI 3730XL sequencer (Applied Biosystems) with a 36 cm capillary filled with POP-6 polymer. Sequences were handled using SeqScape 1.0 Software.

Analysis of polymorphism in the *DEFB1* gene

SNP genotyping

We analyzed three SNPs located in the 5'UTR of the DEFB1 gene: -52(G/A), -44(C/G) and -20(G/A) (NCBI SNPs database id: rs1799946, rs1800972 and rs11362, respectively).

The studied SNPs have been firstly described by Dork and Stuhrmann (1998) and successively have been associated with increased susceptibility to vertical transmission in an Italian cohort by Braida et al. (2004).

Analysis of the frequencies of the three 5' UTR polymorphisms have been performed with direct sequencing using primers (5'-GAAGTTCACCCTTGACTGTGGC-3' and 5'-CATCCGAGACTCACATCAGCC-3'.) designed using the software Primer Express 2.0 (Applied Biosystems, Foster City, CA) according to the human sequences available in GenBank. Reactions took place in a final volume of 25μl and were conducted with 1X PCR Buffer II (Applied Biosystems), 2 mM $MgCl_2$, 50 µM each dNTPs, 0.2 µM forward primer, 0.2μM reverse primers, 0.5 U of AmpliTaq Gold Polymerase (Applied Biosystems).

Thermal-cycler conditions consisted of a pre-cycling period of 10 minutes at 95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 57°C and an elongation step at 72°C for 30 seconds. PCR reaction was terminated by a final elongation step at 72°C for 4 minutes.

PCR products were then processed in the same way as for the mitochondrial D-loop region sequencing.

Functional characterization of DEFB1 SNPs

Further analysis involved investigation over the functional effect of the studied polymorphisms.

With the aim of verifying an eventual transcriptional functional effect of the SNPs in the 5'UTR region of DEFB1 gene we employed a Dual Luciferase assay.

Starting from the wild type DEFB1 5'UTR region we generated three mutated inserts using primers designed to introduce the desired mutation into the wild type sequence. The four different fragments (wild type, -52A, -44G and -20A) have been cloned into pGL3 promoter vector (Promega) and Caco2 cells were transfected in 24-well plates (Costar, Corning NY) while at 70-80% confluence. Caco2 cells were transfected for 48 h with 1μg of the reporter plasmids and 20 ng of control renilla luciferase expression plasmid (phRG-TK, Promega) using 5μl of GenePORTER transfection reagent (Gene Therapy Systems) according to manufacturers instruction. After transfection cells were harvested with 100μl of Passive Lysis Buffer (Promega) and firefly luciferase activity

from hBD-1-pGL3 reporter vector and *Renilla* luciferase activity from the control vector were measured using the Dual Luciferase assay system (Promega) on a TD-20/20 luminometer (Turner Design, CA). Promoter activity was calculated as the ratio between firefly and *Renilla* luciferase activities measured in each sample.

Analysis of polymorphisms in the lactoferrin gene

In our study we focused our attention on polymorphisms located in the second and fourth exon of the *LTF* gene. This way, with only 2 direct sequences, we were able to genotype a total of 7 coding non-synonymous single nucleotide polymorphisms out of the 11 reported by the NCBI database, namely rs1126477, rs17855464, rs17855496 and rs1126478 (in exon 2) and rs17851346, rs17851991 and rs1126479 (in exon 4). Primers used to amplify exon 2 where 5'- AGCAAGATGCCTCCACTTGT-3' and 5'-TCTCCCTTCCATTCAGCTTG-3', while to amplify exon 4 the primers used where 5'-TCAATTCTGTCTGCCCCTTT-3' and 5'-CACTATCCCCCAGCCATCT-3'.

Successively we focused on the two most promising SNPs, namely rs1126477 and rs1126478, both located in exon 2 and we developed two allele specific PCRs in order to genotype a larger number of subjects with a faster and cheaper technology.

To genotype rs1126477 polymorphism, also reported in the literature as T11A, we employed the two allele specific primers 5'-CTCGGGTTGGGATACGGC-3' and 5'-CTCGGGTTGGGATACGGT-3' while using 5'-TTGTGGAGAATGGCTGGACA-3' as a common reverse primer.

Reactions, in a final volume of 25μl, were conducted with 1X PCR Buffer II (Applied Biosystems), 2 mM $MgCl_2$, 50 µM each dNTPs, 0.2 µM of C or T allele-specific and reverse primers, 0.5 U of AmpliTaq Gold Polymerase (Applied Biosystems) and 1X SYBR Green I (Molecular Probes). Thermal-cycler conditions were as follows: a pre-cycling period of 10 minutes at 95°C followed by 40 cycles composed by 30 seconds at 95°C, 30 seconds at 57°C and an elongation step at 72°C for 30 seconds.

Genotyping of the rs1126478 has been performed by using 5'- CAATGGCAAAG-GAATATGAGAAA-3' and 5'- CAATGGCAAAGGAATATGAGAAG-3' as forward, allele-specific primers and 5'-AGCACAGTTCCCTGTGAGAGA-3' as reverse primer.

Reactions, similarly to the T11A case, were conducted at 25μl total volume using 1X PCR Buffer II (Applied Biosystems), 2 mM $MgCl_2$, 50 µM each dNTPs, 0.2 µM of A or

G allele-specific and reverse primers, 0.5 U of AmpliTaq Gold Polymerase (Applied Biosystems) and 1X SYBR Green I (Molecular Probes). Thermal-cycling conditions were as follows: initial hold at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds.

Runs were conducted on an ABI 7900 real-time instrument and genotyping was performed on the evaluating the C_t values of the two allele-specific reactions.

Analysis of polymorphism in the *MBL2* gene

Three single nucleotide polymorphisms: 52 Arg-Cys, 54 Glu-Asp and 57 Glu-Gly, (rs5030737, rs1800450, and rs1800451, respectively) in the first exon of the MBL2 gene, encoding for the MBL protein, are linked to decreased protein expression levels and correlated with impaired immune response in a number of different diseases (Eisen and Minchinton, 2003; Turner and Hamvas, 2000).

As suggested by Garred et al. (1997) the three variant alleles found in the first exon of the MBL2 gene were grouped in the same category of allele 0, since they have a similar functional effect on MBL, impeding a correct formation of oligomers, as indicated by Larsen et al (2004).

PCR reactions were performed in a total volume of 25μl containing 1X PCR Buffer II (Applied Biosystems, Foster City, CA), 2 mM MgCl2, 50 μM each dNTPs, 0.5 μM of forward primer (5'-GGCTTCCCAGGCAAAGATG-3'), 0.5μM of reverse primer (5'-AGCCCAACACGTACCTGGTT-3'), 1X SYBR green (Molecular Bioprobes, Eugene, OR) and 0.5 U of AmpliTaq Gold Polymerase (Applied Biosystems). Thermal-cycling conditions were as follows: initial hold at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. After the amplification step a melting temperature analysis (MTA) was performed, from 60°C to 95°C in an ABI 7900 instrument. The first derivative of the raw data curves was automatically calculated by the instrument's software and the resulting curves were visually analyzed to perform the genotyping.

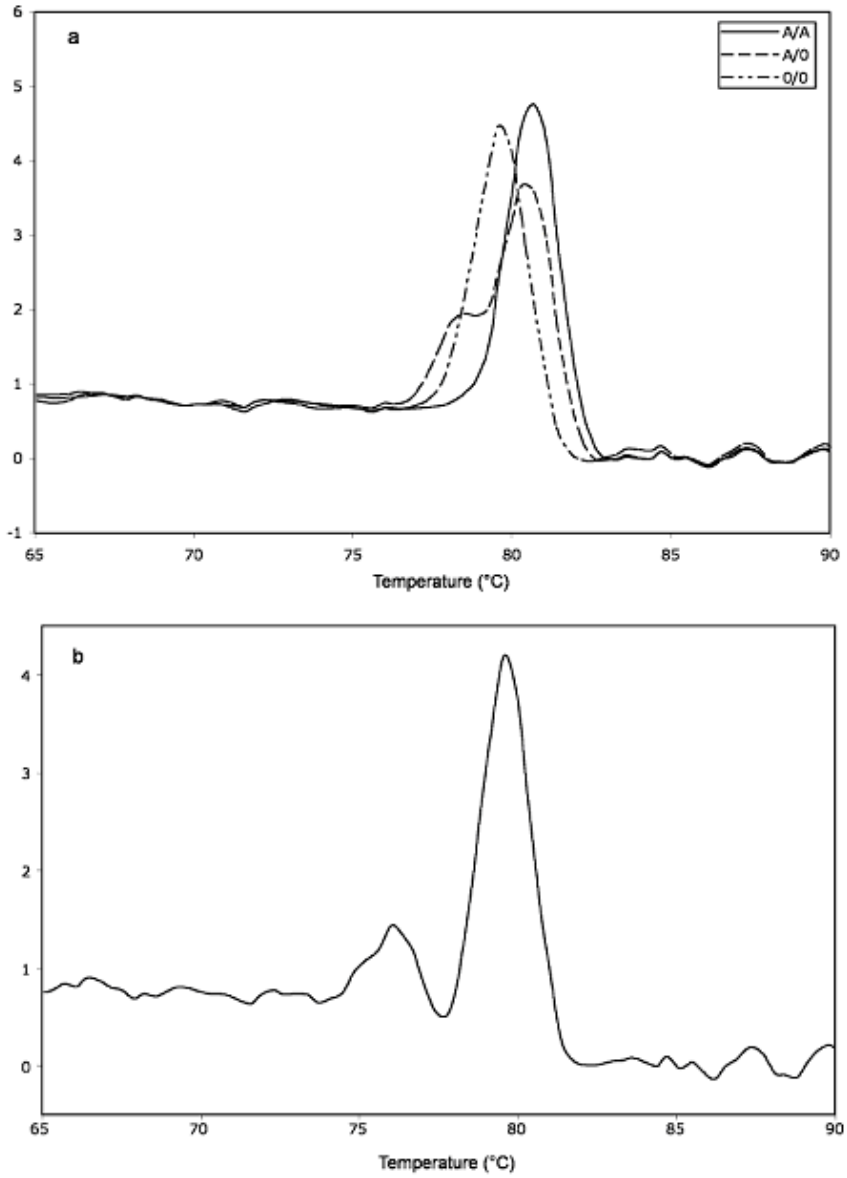

Figure 13 a) Example MTA profiles obtained for different genotypes with our technique

b) Example curve of a double heterozygous sample with the characteristic two peaks

Genotypes discrimination was performed on the basis of samples' melting profile, since the shape of the resulting curves allowed to discern samples with different genotypes: the wild-type genotype gave a peak at 80.4ºC±0.2ºC while the heterozygous samples gave a peak that has a maximum at about the same temperature as the wild-type (80.0ºC±0.4ºC) but presents a markedly different shape in the left part of the curve that

permits to clearly discriminate between samples with different genotypes. The mutated samples gave a sharp peak at 79.5ºC±0.3ºC and thus could be distinguished from the wild-type samples simply observing the experimental Tm (see figure 13a).

We also genotyped composite heterozygous samples (successively considered as 0/0 subject in the statistical analysis and whose experimental MTA curve is reported on figure 13b) that had one allele mutated at the codon 52 and one allele mutated at the codon 54. The resultant MTA profile was quite peculiar, with two peaks at 76.1ºC±0.3ºC and 79.6ºC±0.2ºC; the resulting profile makes the determination of the genotype very easy.

Moreover we developed two allele-specific PCRs for the two most important promoter polymorphisms: G-550C (rs11003125, known as H/L variant) and G-221C (rs7096206, known as X/Y variant).

To genotype the H/L polymorphic variant we used 5'-TGCTTCCCCTTGGTGTTTTTAC-3' and 5'-TGCTTCCCCTTGGTGTTTTTAG-3' as the two allele-specific reverse primers and 5'-GCCAGGGCCAACGTAGTAAG-3' as a common forward primer.

For the X/Y polymorphisms the reverse allele-specific primer used were 5'-CTGGAAGACTATAAACATGCTTTC-3' and 5'-CTGGAAGACTATAAACATGCTTTG-3', and 5'-CCGAAGAGGACATGGAGAGA-3', as a common forward primer

Both genotyping reactions took advantage of the same experimental parameters: reactions were performed in a final volume of 12.5μl, were conducted with 1X PCR Buffer II (Applied Biosystems), 2 mM $MgCl_2$, 50 µM each dNTPs, 0.2 µM of allele-specific and reverse primers, 0.5 U of AmpliTaq Gold Polymerase (Applied Biosystems) and 1X SYBR Green I (Molecular Probes). Thermal-cycler conditions were as follows: a pre-cycling period of 10 minutes at 95°C followed by 40 cycles composed by 30 seconds at 95°C, 30 seconds at 57°C and an elongation step at 72°C for 30 seconds.

Analysis of polymorphism in the *MASP2* gene

The genotyping of MASP rs12711521 SNP was performed by using TAQman pre-designed SNPs Genotyping Assays (assay id. C__22271950_20; Applied Biosystems) according to manufacturer's instructions. This assay is based on the allelic discrimination using VIC and FAM fluorophores for each SNP-specific labeled probe. The PCR

was carried out in a final volume of 10 µl, in a 96–well PCR plate and the amplified results was analyzed at the end point by using the ABI 7900HT Sequence Detection System (Applied Biosystems).

MASP D105G genotyping was performed by direct sequencing by using primers (Primer Reverse 5'-CCCAAGGAGTAGCCAGGGTT-3' Primer Forward 5'-TGTGGATGATGTCAGGCCAG-3') designed using the software Primer Express 2.0 (Applied Biosystems, Foster City, CA) according to the human sequences available in GenBank.

PCR reactions were carried out in a GeneAmp 9700 Thermal cycler (Applied Biosystems, Foster City, CA) using PCR buffer 1X, 1 unit of Taq Gold, 0.2mM dNTPs and MgCl2 2 mM). The cycling was performed with an initial denaturation for 10 minutes at 95°C, followed by 40 cycles at 95°C for 20 seconds, at the annealing temperature of 55°C for 30 seconds and 72°C for 30 seconds with a final extension to 72°C for 7 minutes.

DNA sequencing of PCR products was performed using the BigDye Terminator as previously described.

Analysis of the sequence of the LL37 gene

Since no coding non-synonymous polymorphisms are described in the NCBI database so far we analyzed the entire gene coding sequence to look for novel polymorphisms.

Analysis of the coding sequence of the hCAMP-18 gene was performed by direct sequencing of 3 distinct amplicons that, together, spanned all the 4 exons that compose the gene.

The first exon was amplified using the following primers: 5'-CTAGAGGGAGGCAGACATGG-3' and 5'-AGCAGAATGTCCCCCAAAG-3'. The second and the third exons were amplified together with the following couple of primers: 5'-CCCCATTTCCTCCTCTGACT-3' and 5'-ACACTCTATGCCCCCATCAG-3' while the fourth exon was amplified with the primers 5'-CCTGTTTCTTCCTGTACACAACC-3' and 5'-GAGGCAAAACAAGTGAGACTGA-3'.

All reactions took place in a final volume of 25μl and were performed with 1X PCR Buffer II (Applied Biosystems), 2 mM $MgCl_2$, 50 µM each dNTPs, 0.2 µM forward

primer, 0.2μM reverse primers, 0.5 U of AmpliTaq Gold Polymerase (Applied Biosystems). Thermal-cycler conditions were a pre-cycling period of 10 minutes at 95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 58°C while the elongation step was set at 72°C for 30 seconds. PCR reaction was terminated by a final elongation step at 72°C for 4 minutes.

We then analyzed the sequence of the 5'UTR and promoter region of the hCAMP-18 gene.

Do to this two couples of primers were designed on the NM_004345 reference sequence whose amplicons overlapped covering the entire coding sequence.

The first couple of primers was 5'-TGGGTCCAGAGAGCATGTGGTAT-3' and 5'-GGAAGAGAGCCAGGTGCCTAT-3' while the second couple was 5'-GAACTCCTTATCTCAGGTGATCC-3' and 5'-TCTTCATGGTCCCCATGTCTG-3'.

Reactions took place in a final volume of 25μl and were conducted with 1X PCR Buffer II (Applied Biosystems), 2 mM $MgCl_2$, 50 µM each dNTPs, 0.2 µM forward primer, 0.2μM reverse primers, 0.5 U of AmpliTaq Gold Polymerase (Applied Biosystems).

Thermal-cycler conditions consisted of a pre-cycling period of 10 minutes at 95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 57°C and an elongation step at 72°C for 30 seconds. PCR reaction was terminated by a final elongation step at 72°C for 4 minutes. PCR products were then processed in the same way as for the mitochondrial D-loop region sequencing.

Analysis of defensins gene copy number polymorphism

Multiple Ligation-dependent Probe Amplification

Multiple Ligation-dependent Probe Amplification (MLPA) relies on the use of progressively longer oligonucleotide probes in order to generate locus-specific amplicons of increasing size that can be resolved electrophoretically. In the original description of the method (Schouten et al., 2002,), these long oligonucleotides were generated using a series of proprietary M13-based vectors, since their size is beyond that attainable by standard chemical synthesis.

MLPA protocol consists of five steps: hybridization of the probes, a ligation reaction, a PCR reaction, followed by separation of amplification products by electrophoresis and data analysis.

The probes designed to be used in MLPA reactions consists of two different oligonucleotides, each of which containing one of the common PCR primer sequences. It is only when these two probe parts are both hybridised to their target sequence that they can be ligated to each other. Only ligated probes will be amplified exponentially in a PCR reaction. The number of probe ligation products depends on the number of target sequences in the sample.

Multiplexing is made possible by stuffer sequences that are inserted in the probes in order to make them all different in size. This way up to 45 unique MLPA probes can be multiplexed in a single reaction. The Salsa defensin kit p139 that we used to perform our experiments contained 19 MLPA probes specific for 13 different defensin genes, as well as 2 MLPA probes specific for the defensin-like gene SPAG11. In addition, 13 probes for other genes present at the same locus of the defensin genes, at 8p23, are present. To ensure a proper copy number quantification, 9 MLPA probes specific for genes located on other chromosomes were included as controls.

In the first step, hybridization of the specific probes, specific probes are allowed to hybridize overnight at 60°C after a rapid denaturation of the DNA sample at 95°C.

After hybridisation with the target, the two components of the probe will be ligated by a specific ligase enzyme, but only when they are both hybridised at adjacent sites. It must be noted that the ligation reaction is specific enough to allow the possibility to distinguish sequences differing in a single nucleotide.

The resulting ligation product contains both PCR primer sequences in one fragment, and hence will be amplified exponentially during the PCR reaction. In contrast, probe oligonucleotides that are not ligated will contain only a single primer sequence. As a consequence, non-ligated probe sequences will not be amplified exponentially and will not generate a signal. The removal of unbound probes is thus not necessary in MLPA.

The amplified amplicons were then separated with an ABI 3730XL automatic sequencer (Applied Biosystems). Following the PCR reaction 1 to 3 µl of the PCR reaction were mixed with 0.3 µl internal size standard and 9 µl of HiDi formamide (ABI nr.4311320). The mix was then incubated for 2 minutes at 94°C, and hold at 4°C for 5 min.

Sequencer parameters were as follows: internal standard: 600LIZ filter set 500ROX (ABI nr. 401734) filter set D; Capillaries were 36 cm long, filled with POP-7 polyemer. Temperature of run was set at 60°C while capillary fill volume was 184 steps; Pre run voltage was set at 15 kV with a pre run time of 3 minutes. Voltage ad injection was 3.0 kV; injection time was: 30 sec; run voltage was 15 kV.

Data analysis was performed with the Coffalyser software, downloadable at the *www.mlpa.com* web site following manufacturer's instructions and suggestions.

Generation of MLPA control

Due to the difficulties in obtaining a "standard" sample that contained all the genes studied with MLPA in two copies per diploid genome (four copies for the genes that are normally duplicated) we decided to create artificially a standard control that had certain amounts of each gene. Therefore we created 44 different plasmids, each containing one kind of target sequence. To do so we employed specially designed primers, with whom we were able to amplify, for each target sequence, a fragment of about 150 base pairs, flanked on each side by an *Xho*I restriction site. We then inserted this fragment in a common pGL3 and selected the plasmids with only one inserted target sequence. Insertion and ligation were performed with standard molecular biology methods as already described in the third edition of "Molecular Cloning" (Sambrook and Russel, 2001)

Sequence determination has been done with an ABI 3130XL automatic sequencer as described above.

An *E. Coli* strain has been transfected with the different plasmids and, after one night of growth at 37°C, amplified plasmid has been purified with a Millipore Plasmid Miniprep and the resulting DNA has been spectrophotometrically quantified.

Then, equals amounts of DNA for each different plasmid have been quantified with a NanoDrop spectrphotometer and then adequate amounts of each plasmid has been added to a pool to generate the standard DNA mix.

In silico analysis of the pGL3 plasmid sequence, performed with BLAST, showed that none of the target sequences were present before recombination.

Statistical analysis

Data analysis has been performed using the most relevant statistical test.

Allele and genotype frequencies were calculated by direct gene counting; Hardy-Weinberg equilibrium was calculated by expected genotype frequencies.

Assessment for differences in genotypic and allelic frequencies to do association studies have been done using Chi-square test or exact Fisher test.

Choice of one of the two methods was not relevant in most cases: in fact the Chi-square test is just a simplification of the exact Fisher test and gives similar results if the number of samples is large enough. The use of Chi-square test in spite of Fisher test is generally accepted when none of the tested subgroups is composed by less than five units.

The employed test is clearly specified in the text in a case-by-case manner.

Haplotypes and linkage disequilibrium have been estimated with the Arlequin software 3.01 (*http://cmpg.unibe.ch/software/arlequin3/*) .

To test for differences in the copy number distributions of MLPA experiments the use of a parametrical test was not suitable and therefore we opted for the Wilcoxon-Mann non-parametric test.

When relevant we executed correction of p-values for multiple observation. This technique is greatly useful when computing a large number of p-values during an experiment in order to reduce the probability to obtain false-positive results.

To do multiple comparison correction we take advantage of two different methods: the classical one proposed by Bonferroni and another proposed by Benjamini and Hochberg (1995). Choice of one method in the different cases has been done on the basis of the achieved results and taking into account the different peculiarities of the different methods. The Bonferroni method, in fact, is way more restrictive than the Benjamini and Hochberg one, the latter being especially designed to minimize false negative errors in biological experiments. We have generally preferred to use the Benjamini and Hochberg during association studies for polymorphisms and the Bonferroni method in the MLPA experiments in order to avoid the higher risk of false positive that's implied in the analysis when computing a large number of p-values. When referring to a corrected p-value the method that was used to obtain it is clearly stated, however, for all experiments p-values have been calculated with both methods.

Statistical analyses have been performed with the open-source R package, available at the *www.r-project.org site*.

RESULTS AND DISCUSSION

General Remarks

Samples

Both the 2002 and the 2006 sample collects took place at the Instituto Materno Infantil do Pernambuco (IMIP) with the collaboration of the Laboratorio de Imunopatologia Keizo Azami (LIKA).

It should be noted that Brazilian health system exists on two different levels: public assistance that's free for everyone and private assistance that's only available to a smaller part of population, characterized by medium or high social level, scholarity and economical conditions. Mothers that are cured in private hospitals belong to the smaller part of population mentioned above, are more informed and undergo a proper follow-up, with all relevant clinical analysis.

The IMIP is a hospital that's really peculiar in the Brazilian scene. In fact it's neither a public nor a private hospital; it was founded and constructed with money coming from donations, without government's founding, with the aim of give Recife's poor people free medical assistance. It's located in the center of one of Recife's biggest *favela* in order to be near to the people it wants to help.

Since getting money is a great problem all hospital's doctors work for free and the entirety of the funding are employed to ameliorate the center and to pay for drugs.

Because of the lack of a monetary fee, most doctors that work at the IMIP are volunteers that offer their free time or doctors that came from abroad thanks to co-operational project. As a consequence all personnel is greatly motivated and was happy to participate to the sample collection, despite the extra work required for this.

All the mothers whose children had their DNA extracted were selected to come from poor areas of the Recife metropolitan region. To assure maximum homogeneity, none of the enrolled subjects underwent antiretroviral therapy or caesarian section before delivery, due to the discussed limitations.

During the 2002 collection a problem arose that persisted also in the 2006 subsequent collection. In fact it has been difficult to obtain mothers' biological samples, a fact that impaired the efforts to perform statistical analysis on mothers' genetic background and forced us to perform our analysis only on children's DNA.

As a matter of fact this problem is a direct consequence of the choice of reducing our cohort only to children born from impoverished mothers, since we had to face the difficult situation of Brazilian health system. It appeared clear that due to our study finalities the sample collection must take place among the poor people, but it was equally clear that public hospitals didn't had the means, both in terms of monetary founds and available personnel, to provide an adequate way to systematically collect samples.

Still we had to face the problems correlated with the population we studied. Medical conditions in the *favelas* are dramatic, with an elevated HIV-1 incidence and little or no medical advice on AIDS prevention and treatment. Mothers often came to the hospital after the parturition or in such advanced labor conditions that caesarian section was impossible. A rightly done medical follow up is usually impossible because mothers fail to return for clinical tests.

The lack of data on mothers should be seen as the major drawback of the entire thesis work. In fact, it's plausible that vertical transmission could be also influenced by mother's genetical background: a genetic defect that induces high viral load in the mother could, for example, increase the risk of mother to son infection.

We paid the unique opportunity to work on large cohorts with the fact that we could not get the entire picture of the phenomenon and we are forced to obtain our result solely on the basis of the analysis we can do on children's DNA.

First sample collection

In order to evaluate the role of antimicrobial peptides in HIV-1 vertical transmission we have started to study the DNA bank that was already present at the Burlo Garofolo

Children Hospital and that has been collected in 2002 at the Instituto Materno Infantil do Pernambuco (IMIP).

The DNA bank consisted of DNA extracted from 303 Brazilian newborns and classified as healthy controls, HIV-1 positive subjects and exposed non-infected subjects. Healthy controls subjects were children born from uninfected mothers and that therefore didn't incur in any exposition to HIV-1 virus, while both the HIV-1 infected children and the exposed uninfected subjects were born form HIV-1 positive mothers and were differenced on the presence of the absence of HIV-1 transmission during delivery.

These DNA samples were used to assess the role of polymorphisms in the 5'UTR of *DEFB1* gene and defensin gene copy number polymorphisms on HIV-1 vertical transmission.

Second sample collection

A second collection of samples was performed in 2006 with similar procedures to the ones employed for the first round of sample accumulation. The second DNA bank, that was gathered, as in the previous case, at the IMIP, had some qualitative advantages over the first DNA bank, though.

In fact we were able to take advantages of the new facilities of the virology service at the LIKA. The virology department has been renewed in 2004 and now is more closely bound to IMIP's clinical service. As a consequence of this we were able to detect viral presence by Real-time PCR methods that weren't available during the first collection, making the diagnosis much more precise.

Furthermore we changed the methodology used to extract DNA, abandoning the phenol-chloroform method for a more modern and rapid method based on the Wizard Genomic DNA Purification Kit from Promega. This way we could ensure maximum quality constancy for the extracted DNA.

The new DNA bank we were able to gather was composed of 360 DNA samples and was enriched in HIV-1 positive children whose percentage rose by more than 10 percentages point compared to the previous bank, and, more importantly, in exposed uninfected children, probably the most valuable group, whose percentage was taken to 20%, compared to 16.67% in the previous group. The new DNA bank comprised globally 192 HIV-1 infected children, 72 exposed-uninfected children and 96 healthy controls. For

samples collection we continued to enroll patients that were admitted at the gynecological service of the IMIP, as for the previous collection.

Due to the higher quality of DNA, and to the fact that many samples belonging to the first DNA bank were no longer available, subsequent experiments have been performed solely on the second DNA bank. Thus analysis on Lactoferrin, *MBL2* and LL37 genes has been performed on these samples collected in 2006.

Mitochondrial D-loop sequencing.

Brazilians form one of the most heterogeneous populations in the world, as a result of colonization and immigration waves from Europe and 5 centuries of interethnic crosses between peoples from three continents: the European colonizers (represented mainly by the Portugueses), African slaves and the autochthonous Amerindians. (Alves-Silva et al., 2000)

It's widely recognised that allelic frequency can vary significantly among ethnicity. Since the effect of colonization and immigration have had a differential impact in different regions of Brazil and the different ethnic groups of origin can actually contribute to the gene pool of present-day Brazilians in different percentages, polymorphisms frequencies could vary greatly among populations coming from different areas of the country. Moreover, due to historical reasons, a quite tight correlation between ethnical origin and actual social condition can be evidenced also within the same geographical region.

Thus it is possible, at least theoretically, that differences of allelic and genotypic frequencies are not indicative of disease association but are the results of stratification of the studied population.

In addiction to enroll samples that all come from poor areas of the Recife metropolitan region, to further ensure genetical homogeneity among the three different groups in which was subdivided the DNA bank we decided to perform an analysis of the hypervariable D-loop mitochondrial sequence.

The D-loop region is important for phylogeographic studies. Because the region does not code for any genes, it is free to vary with only a few selective limitations on size and heavy/light strand factors. The mutation rate is among the fastest of anywhere in either the nuclear or mitochondrial genomes in animals. Mutations in the D-loop can

effectively track recent and rapid evolutionary changes such as within species and among very closely related species. The characteristics of the D-loop region have been exploited to measure the genetic contributions of African, European and Native American pools to the genetic background of today's Brazilian population.

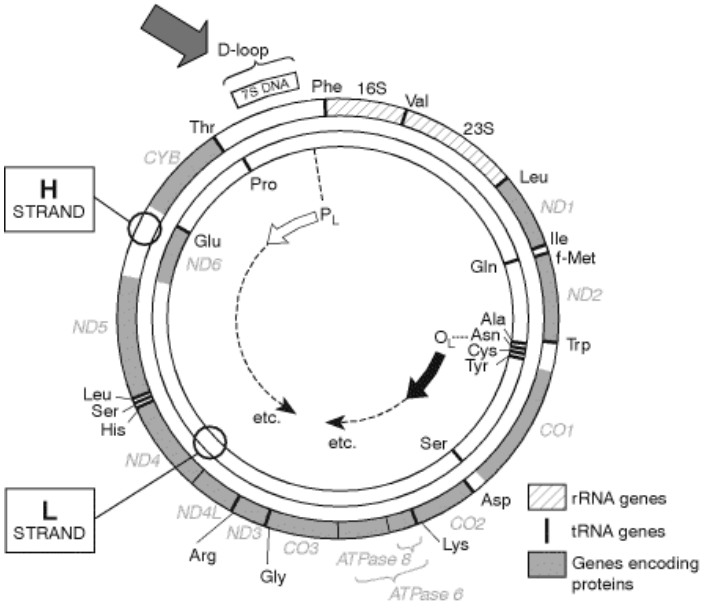

Figure 14 Organization of mitochondrial genome. A red arrow indicates the D-loop.

Alves-Silva et al. (2000) employed a restriction fragment length polymorphism (RFLP) approach to mitochondrial DNA to identify DNA ancestry in different continents. They analyzed 247 samples from different areas of Brazil and demonstrated that the total sample showed nearly equal amounts of Native American, African, and European matrilineal genetic contribution but with regional differences within Brazil. They concluded that mitochondrial DNA pool of present-day Brazilians evidently reflects the imprints of the early Portuguese colonization process involving directional mating, as well as the recent immigrant waves from Europe of the last century.

In particular the Recife's population's genetic background was derived from an admixture of African, Caucasian and native American populations with a proportional weight estimated, respectively, at 44, 34 and 22%.

Starting from this available data we decided to perform the same test of Alves-Silva (2000) in order to observe if there was any difference in the mtDNA pool among our studied groups.Results are summarized in table 1

Table 2 genetic contributions of African, European and Native American pools to the genetic background of Brazilian HIV-1 positive children, exposed-uninfected children and healthy controls.

	Healthy Controls	Exposed-Uninfected	HIV Positive
Continental fraction			
Native American	22.45%	20,19%	23,86%
African	46.15%	47,12%	45,33%
European	31.40%	32.69%	30.81%

The three groups do not significantly differ one from another; nevertheless we found a slightly higher African contribution than reported (Alves-Silva et al., 2000). This fact could be due to the reason that in the study by Alves-Silva et al. there was probably a bias due to selection of the sample, that was especially drawn among people working or studying at university, therefore belonging to the upper-high class that's historically more prone to receive European genetic contribution

DEFB1 polymorphisms in HIV-1 vertical transmission and infection

Literature on the influence of antimicrobial peptides on vertical transmission is scarce, but, on the contrary, there is more than enough literature on the anti-HIV-1 activity of many of those peptides to let us to be confident on a role for these molecules in influencing susceptibility to be infected at the moment of the delivery.

Given the peculiar genetic background of Brazilians, we decided to begin our work by trying to replicate in this population the data that were previously obtained in our lab about the influence of three SNPs of the *DEFB1* gene on vertical transmission in Italian children (Braida et al., 2004).

The 5' UTR of the *DEFB1* gene has been then sequenced in our cohort of 303 Brazilian subjects in order to study the influence of the -52 (G/A) -44 (C/G) and -20 (G/A) polymorphisms (NCBI accession numbers, respectively: rs1799946, rs1800972, rs11362) on HIV-1 infection and vertical transmission.

Results are reported on table 3.

Table 3 Alleles and genotypes count (and frequency) of polymorphisms in the 5'UTR of the DEFB1 gene in HIV-1 positive children, exposed-uninfected children and healthy controls.

	Healthy Controls	Exposed-Uninfected	HIV Positive
SNP -52 (G/A)			
G	125 (0.54)	65 (0.54)	171 (0.67)
A	105 (0.46)	55 (0.46)	85 (0.33)
G/G	34 (0.30)	60 (0.32)	53 (0.41)
G/A	57 (0.50)	27 (0.45)	65 (0.51)
A/A	24 (0.21)	14 (0.23)	10 (0.08)
SNP -44 (C/G)			
C	197 (0.86)	104 (0.87)	237 (0.93)
G	33 (0.14)	16 (0.13)	19 (0.07)
C/C	84 (0.73)	44 (0.73)	109 (0.85)
C/G	29 (0.25)	16 (0.27)	19 (0.15)
G/G	2 (0.02)	0 (0.00)	0 (0.00)
SNP -20 (G/A)			
G	145 (0.63)	70 (0.58)	124 (0.48)
A	85 (0.37)	50 (0.42)	132 (0.52)
G/G	42 (0.36)	20 (0.33)	15 (0.12)
G/A	61 (0.53)	30 (0.50)	94 (0.73)
A/A	12 (0.10)	10 (0.17)	19 (0.15)

The genotype frequencies obtained are in accordance with the Hardy-Weinberg equilibrium for all the SNPs studied in the three populations with the exception of the polymorphism -20 (G/A) in the HIV-1 infected children. ($p<0.001$)

Escaping from the Hardy Weinberg equilibrium by the -20 (G/A) polymorphism deserve a particular consideration: in fact, while the allelic frequencies in the healthy controls group do correspond to the frequencies reported in the NCBI public database

(*http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=11362*), in the remaining two groups, and in particular in the infected children group we observed a markedly increased frequency of the -20 A allele that drove the genotype frequencies out of the equilibrium.

We can exclude the hypothesis of experimental errors since the technique we used; direct sequencing is, so far, the most reliable and precise SNP genotyping technique. Moreover all genotypes have been double checked in this group by repeating sequences.

We thus believe that the detected anomaly in the Hardy-Weinberg equilibrium could be explained taking into account the fact that the infected children group could be enriched in subjects coming from a poorer environment, and thus the Caucasoid pool of genes could be under-represented in this group. Another hypothesis we can alternatively propose is that the -20A allele has a detrimental effect on protection from HIV-1 infection even when present in heterozygosis.

It's actually not possible, with the data we have and without performing deeper population studies, to discriminate from the two proposed possible causes and it's neither possible to exclude that the real rationale could be substantially different.

In both the proposed cases departure from Hardy-Weinberg equilibrium would be caused by endo-stratification of the group and therefore was not possible to eliminate this bias factor. Nevertheless the fact that all the groups were in Hardy Weinberg equilibrium for all the other polymorphisms make us quite confident about the correctness of patients selection.

When confronting the genotype and allelic frequencies of the different groups we found that the -44C allele was over-represented in the HIV-1 infected group, with a frequency of 93%, versus 86% in the exposed non-infected group and 87% in the healthy control group thus pointing toward a protective effect for the -44G allele both in vertical transmission and in infection by HIV-1 virus.

These differences in allelic frequencies resulted in an increased frequency of the -44C/C genotype at the expenses of the -44C/G genotype as well. The -44G/G is very rare and it has been found only in two healthy control subjects.

This correlation for the -44(C/G) *DEFB1* polymorphism appears quite weak and, indeed, when genotype frequencies are tested with a chi-square test the detected difference reaches statistical significance, but it is not able to retain the above mentioned significance after multiple test correction with the Bonferroni method (HIV-1 infected vs. healthy controls gave a corrected p-value=0.052).

As said before, allelic frequencies of the –44G allele were similar between healthy controls and HIV-1 exposed uninfected children while a lower frequency of the -44G allele has been found in HIV-1 infected children. In this case the difference didn't reach statistical significance.

Notably the frequency of this polymorphism was very low in the Brazilian population we studied when compared to the reported frequencies in other populations (Jurevic et al., 2002); the fact that genotype frequencies are so much more skewed toward the presence of the -44C allele and the G allele is so under-represented could account for the difficulties we have to reach statistical significance for this polymorphism.

On the other hand, we were able to demonstrate that the *DEFB1* -52A/A genotype was significantly less frequent in HIV-1 perinatally infected children when compared to healthy controls and exposed uninfected children and that this variation, contrarily to the -44(C/G) case, is statistically significant even after Bonferroni correction for multiple testing (corrected p-values are equal to 0.023 and 0.025 respectively when confronting the HIV-1 infected group with the healthy controls and the exposed non-infected children), while frequencies didn't vary significantly between HIV-1 exposed uninfected children and healthy controls.

Allelic frequencies of the -52(G/A) SNP in infected children varied significantly when compared to those of healthy controls (corrected p-value equal to 0.030), with a 13% increase in the -52G allele in the HIV-1 infected patients. The variation wasn't statistically significant when infected children were compared to exposed uninfected children (corrected p-value equal to 0.055). This is probably due to the fact that the HIV-1 exposed uninfected children group is much smaller than the healthy control group; nevertheless we were able to show that a trend exists even in this case and that the -52G allele gives increased susceptibility to vertical transmission and infection by HIV-1 virus (odd ratios 1.688, 95% confidence limits 1.151-2.482).

Finally, the stronger and more striking association has been observed considering the -20(G/A) polymorphism, where the frequency of -20G/G genotype was significantly lower in HIV-1 positive children (12%) than in exposed uninfected children (33%) and healthy control (36%) (corrected p-values were both less than 0.005).

Allelic frequencies of the -20(G/A) SNP were significantly different between HIV-1 positive children and healthy controls (corrected p-value=0.015) while the significance

wasn't achieved between HIV-1 infected and exposed uninfected children due to multiple test correction, similarly to the -44(C/G) case.
The results obtained show that carrying the -20G allele infers protection from vertical transmission of HIV-1, while a greater susceptibility was observed in patients carrying the -20A allele (odd ratios 1.814, 95% confidence limits 1.243-2.655).
As we did not find any significant difference between the exposed uninfected children and the healthy controls, we decided to improve our statistical analysis by pooling the two groups and performing single polymorphism association tests. We were basically able to confirm the conclusions obtained with the chi-square test on the three different groups, with -52G and -20A alleles increasing susceptibility to infection and vertical transmission.

We then estimated haplotype frequency and Linkage Disequilibrium. Despite the physical proximity of the three studied polymorphisms, that stretch in less than 30 base pairs of the *DEFB1* 5'UTR, and regardless of a co-segregation rate of 93% between the -20(G/A) and -44(C/G) polymorphisms, we didn't find evidence of the presence of any haploblock that gives particular inclination to HIV-1 infection or vertical transmission of the virus.
We then performed a multiple regression analysis of our data with the R software (*www.r-project.org*); the analysis was designed to highlight any potential interaction between the studied polymorphisms, but we were not able to show any significant synergistic effect of the different mutations.
The data collected in this study demonstrate that significant correlation exists between polymorphisms in the 5' UTR of the *DEFB1* gene and the risk of being perinatally infected with the HIV-1 virus in Brazilian children.
Moreover our study presented novel data when confronted to the work of Braida et al. (2004) that initially inspired our work. In fact, differently from the work of Braida et al., in which only infected children and healthy controls were considered, the inclusion in the study of a group of children that where perinatally exposed to HIV-1 but didn't contract the infection gave us the opportunity to assess the role of the analyzed SNPs in mother to child transmission of the virus. Genotypical frequencies between infected and exposed uninfected children were significantly different in the case of the -52(G/A) and -20(G/A) polymorphisms, suggesting a role for these SNPs in vertical transmission, in

particular with a disadvantageous effect of the -52 G and -20 A alleles (odd ratios respectively equal to 1.688 and 1.814, with 95% confidence intervals equal to 1.151-2.482 and 1.243-2.655).

Notwithstanding the fact that our results are statistically significant, we can't be totally sure about a direct effect of *DEFB1* 5' UTR polymorphisms on vertical transmission.

In fact, the group of HIV-1-positive children studied here were likely infected during delivery by contact with HIV-1-infected maternal blood or mucus in the birth canal; if women carrying the polymorphisms in the 5' UTR of the *DEFB1* gene are more susceptible to HIV-1 infection or more likely to have higher viral loads during infection, thus their children may be expected to have a higher frequency of this polymorphism beside being possibly exposed to higher viral levels during delivery, even if there is not a direct effect on vertical transmission of the polymorphism itself. Unfortunately, as said before, biological samples from the mothers of HIV-1-infected children or information on their viral load at the time of delivery were not available and a direct confrontation was not possible at all.

Nonetheless, evidencing a correlation between *DEFB1* 5' UTR polymorphisms and susceptibility to HIV-1 infection and vertical transmission led us to conclude that those polymorphisms could be used as biological markers to determine how prone to viral infection a subject is.

Transcriptional effect of DEFB1 gene 5' UTR polymorphisms

Although we demonstrated that the polymorphisms located in the 5' untranslated region of the *DEFB1* gene could be employed as marker of risk in vertical transmission and infection of HIV-1, there wasn't a recognized biological rationale for this fact and the role of these polymorphisms was unknown.

Since the studied polymorphism fall out of the coding sequence and the peptide sequence is not altered, they clearly can't have any implication in differential antiviral activity.

Nevertheless we hypothesized that the previously studied polymorphisms could have a functional effect on hBD-1 expression; in particular, given that they lie in the 5' UTR of the gene, they could be related to a reduced translation of the mRNA transcript and

therefore to a diminished expression of hBD-1 leading to an impaired response of the innate immunity system to the pathogen.

The most straightforward way to assess if a functional effect existed was to measure hBD-1 levels from protein samples extracted from patients biological specimens and already genotyped for the *DEFB1* 5' UTR polymorphisms and trying to correlate genotypes and hBD-1 levels. Protein samples have been obtained with the BioRad's ReadyPrep Protein Extraction Kit following manufacturer's instruction from vaginal biopsies coming from the gynecological service of the IRCCS Burlo Garofolo.

Unfortunately a great problem mined our tentative in this direction: the almost complete lack of recombinant antibodies targeted against hBD-1. In fact, while it's possible to obtain anti-hBD1 antibodies from a number of suppliers, most of them are targeted toward the same epitope, making them useless to design a sandwich ELISA (Enzyme Linked ImmunoSorbant Assay).

The only supplier we found that had anti-hBD1 antibodies targeted toward different epitopes of the peptide was Immunodiagnostik AG Bensheim, that give us three monoclonal antibodies, targeted respectively to amino acid residues 1-5, 18-26 and 28-34. We tried to develop an ELISA with any of the three antibodies linked to the plate and any of the other two for detection without good results. In fact we had no detectable signal when adsorbing the 18-24 antibody to the plate, while the best results where obtained by adsorbing the antibody targeted toward residues 1-5 to the plate and using the antibody against residues 28-34 for detection. With this setup we were able to correctly draw a standard curve with the reduced reference peptide given by Immunodiagnostik AG Bensheim. Unluckily this setup wasn't able to recognize hBD1 when present in the native form with the presence of three disulfide bonds and we obtained no signal when testing our samples.

We then tried to reduce the disulfide bonds adding Tris-(carboxyethyl) phosphine (TCEP) to a final concentration of 25mM and repeating the experiment, but results were inconclusive and we could not draw a standard curve with serial dilutions of protein extracts. We then concluded that the ELISA technique didn't suit well our needs to quantify hBD1 in complex protein mixtures, as we hypothesized that some interfering compound was present in our samples.

Repeating the experiments with protein extracted with a simple "boil-and-go" procedure, thereby avoiding to include any external compound in the protein extract, did show the same inconclusive results.

The difficulty to correctly set an ELISA assay for antimicrobial peptides is confirmed by the fact that there's no commercial kit available for any defensin and the only reported developed ELISA test in literature is for hBD2 and it has been used only once from its first introduction in 2000 (Ortega et al., 2000).

Thus we decided to perform an *in vitro* assays, more precisely a Dual-Luciferase assay, to study protein expression.

The Dual-Luciferase Reporter (DLR) Assay System by Promega has been designed to provide an efficient means of performing two reporter assays. In the DLR Assay, the activities of firefly (Photinus pyralis) and Renilla (Renilla reniformis or sea pansy) luciferases are measured sequentially in a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a luminescent signal lasting at least one minute. After quantifying the firefly luminescence, this reaction is quenched, and the Renilla luciferase reaction is initiated simultaneously by adding the so-called Stop & Glo Reagent to the same sample. Both assays can be completed in about 4 seconds using a luminometer with reagent auto-injectors. In the DLR Assay System, both reporters yield linear assays with attomole (<10–18) sensitivities and no endogenous activity in the experimental host cells. Furthermore, the integrated format of the DLR Assay provides rapid quantification of both reporters either in transfected cells or in cell-free transcription/translation reactions.

The pGL4 and phRL series of synthetic Renilla Luciferase Reporter Vectors are designed for use with the DLR Assay Systems. A Renilla luciferase vector with constitutive expression may be used in combination with any experimental firefly luciferase vector to co-transfect mammalian cells.

This technology, while suffering from all the limitations of an *in vitro* test, offered us the opportunity to rapidly and cheaply compare differences in protein expression due to 5'UTR polymorphisms and therefore we decided to use it before investing on an *in vivo* assay, considering the difficulties that mined our efforts to develop an ELISA test.

After generation of plasmids as described in the material and methods section, we moved on testing the functional influence of the studied SNPs on the activity of the

DEFB1 gene 5'UTR when cloned upstream the Firefly luciferase, used as a reporter gene.

The cell strain we used to perform the assay was a Caco2 cell line and the decision was driven by the fact that those cells are a carcinoma-derived line and therefore come from a tissue, the skin, which is naturally able to express defensin genes. By using this approach we wanted to be sure that the cell line used was able to process the 5'UTR in the most similar way to a normal epithelial cell.

Essentially, starting from a wild-type *DEFB1* 5'UTR, as assessed by direct sequencing, fragments have been cloned in which a site-specific mutagenesis have been performed to obtain fragments with mutations corresponding to the -52 (G/A) -44 (C/G) and -20 (G/A) polymorphisms.

The PCR-obtained fragments have been then digested with restriction enzymes and then cloned into the pGL3 promoter vector by Promega. In this way we cloned the wild type *DEFB1* 5'UTR with its three major variants just upstream the firefly luciferase gene, fundamentally creating a 5'UTR for this gene.

The aim was to detect functional variations in gene expression due to mutations in the 5'UTR by measuring luciferase activity.

To avoid biases due to differences in transfection, normalization of data was performed by cotransfecting the cells with a plasmid containing the Renilla luciferase gene. To ensure the reproducibility of the results all tests have been performed in quadruplicate and repeated at a later time to confirm the obtained results. Inter-assay variability showed to be 4%, while intra-assay variability was around 10%.

Our results show that all the studied polymorphisms have a functional effect on the expression of the downstream firefly luciferase gene. In particular, expression levels of firefly luciferase were sensibly reduced in all cases, as indicated by the subsequent reduction of the ratio between the activity of firefly luciferase and the activity of *Renilla* control luciferase.

Statistical analysis has been performed using standard Student t test: a choice justified by the fact that the distributions of the observed ratios appeared to be normal after testing with Shapiro-Wilk normality test.

Notwithstanding the fact that all polymorphisms were diminishing the firefly luciferase expression, only the -44 G allele was able to reduce the expression of the reporter gene to statistically significant levels, with a calculated p-value of 0.003. The latter p-value

was significant enough to retain his significance (p= 0.009) even after an appropriate adjustment for multiple observations with the Bonferroni method.

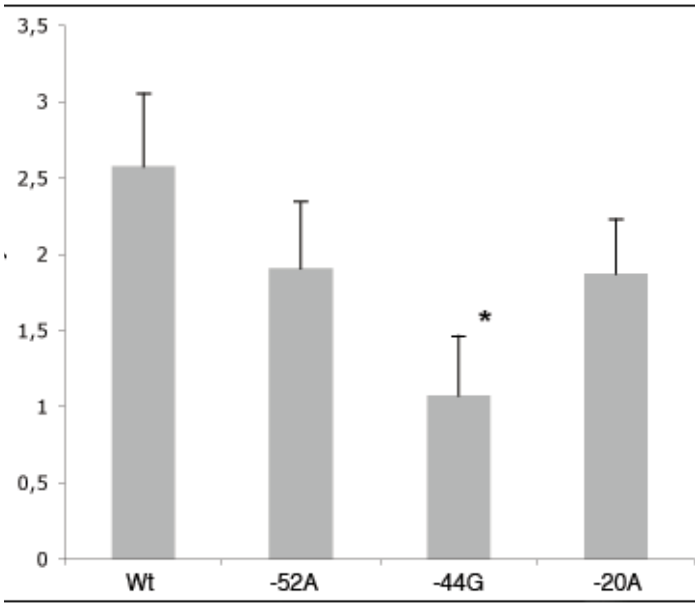

Figure 15 Expression levels of firefly luciferase (expressed as Renilla/firefly ratio) for different 5'UTR- DEFB1 gene polymorphisms

Therefore we can state that this 5' UTR polymorphism has a sensible functional effect one expression of the downstream gene in our model, suggesting that a similar effect could be also true *in vivo*.

The effect of -52A and -20A polymorphisms has proved to be lower in the magnitude of action when compared to the effect of the -44 G allele and they didn't reduce the luciferase expression to an extent at which it could be statistically significant; nevertheless the p-values calculated for these alleles suggest a functional role for these polymorphisms too, being equal to 0.086 and 0.060 for the -52A allele and for the -20A allele, respectively.

Our *in vitro* results suggest that all the studied polymorphisms can be able to hamper an effective production of hBD-1 *in vivo* and, therefore, they can be related to a lack of proper response from the innate immune system, thus indicating that they should be studied in inflammatory pathologies to seek for linkage disequilibrium with the illness.

The fact that, although not significantly, the -20 A allele is associated to a decreased transcriptional activity of *DEFB1* gene, fits quite well with the results we obtain on pa-

tients genotype. The -20A allele, indeed, was over-represented in HIV positive subject and associated to an increased risk of infection; these finding, taken together, let us suggest that reduction in the protein expression rate can be a risk factor for HIV infection.

Conversely, the results concerning -52 (G/A) -44 (C/G) SNPs are not so easy to be interpreted. In fact we were expected to find that the alleles we reported to be associated to HIV infection susceptibility (namely – 52G and -44C) correspond to lower transcriptional levels and not to increased ones, as we actually described.

A possible explanation for this can be found considering that hBD-1 could also act as chemotactic agent for T cells, monocytes and immature dendritic cells. (Yang et al., 2004). Given the fact that HIV-1 virus specifically infects T cells and monocytes, having an increased production of hBD-1 protein (associated for example with the presence of – 52G or -44C alleles) can be detrimental, since its action in recruiting cells that are prone to be infected would therefore facilitate virus spreading.

Our results aren't in accordance to the results published by Sun et al. (2006) and obtained with the same dual-luciferase report assay.

In the above mentioned study two polymorphisms have been studied: a polymorphisms in the *DEFB1* gene promoter, located 688 bases upstream to the ATG start codon and the -44 (C/G) polymorphism in the 5' UTR.

While they concluded that the mutant allele in position -688 was able to cut the luciferase expression to a half, they reported that the -44G allele had the capacity to enhance luciferase expression by a factor of 2.3.

The data presented by Sun et al. are quite strange, as the significant decrease in luciferase expression due to the -688C allele was measured in a construct that also contained the -44G mutant allele. Therefore one should assume that the -688C allele has a dramatic influence on expression, able to overwhelm the presumed positive effect of the -44G. While this is clearly possible the enhancement of expression due to the -44G allele doesn't fit either with our previous data showing a detrimental role of all three 5' UTR polymorphisms on susceptibility to HIV-1 infection, or with the data previously published by Braida et al. (2004).

Moreover the enhancement observed by Sun et al. and reportedly due to the -44G allele is based on experiments on a construct that, by admission of the authors, also contained the -20G mutation (Petros, 2007) and was therefore confronted with constructs that only contained the -20G mutation.

Since we showed a functional effect also for the -20(G/A) polymorphisms we think that the experimental protocol of Sun et al. takes into no account the bias that such mutation could have introduced in the experiment.

Another parameter that could also account for the observed differences is the fact that different cell lines were used, with our laboratory using Caco2 cells, while Sun used TSU-Pr1 and DU-145 cell lines.

Despite the fact that all three cell lines are derived from carcinoma tissue, since we dealt with highly mutated tumor cells, their inherent differences could lead to variations in dual luciferase assay results.

Our study and the one by Sun et al. are the only ones available on functional effects of *DEFB1* gene polymorphisms and the experimental differences could account for the discrepancies in observed results.

Therefore we should conclude that, while the studied polymorphisms have always been correlated to modifications in gene expression no conclusive results have been obtained so far and a more comprehensive study is required, possibly *in vivo*, to elucidate a topic that, in face of its potential clinical importance, deserves a deeper knowledge.

Defensins gene copy-number polymorphisms in vertical transmission and infection

Many defensins map in the genome in a gene cluster in position 8p23.1. Most notably genes as *DEFA3*, *DEFA5*, *DEFA7*, *DEFB4*, *DEFB103A*, *DEFB104*, *DEFB105*, *DEFB106*, *DEFB107B*, and *DEFB108* does present a copy-number polymorphism that impairs a correct genotyping of single nucleotide polymorphisms. In fact the presence of multiple copies of a gene makes difficult the discovery of new single nucleotide polymorphisms by direct sequencing in this gene, since the base mutation in one copy could be masked by the correct sequences of the other alleles. Besides, presence of an unknown number of alleles deeply compromises the assessment of polymorphisms' allelic distribution in various ethnic populations for disease association studies.

As a matter of fact, most individuals have a number of copies per diploid genome that ranges from 2 to 7, whereas carriers of the euchromatic variant show even greater variability, with 9 to 12 present copies (Hollox et al., 2003).

One interesting thing is that copy-number polymorphisms have been shown, in certain cases, to influence the total amount of expressed protein: Hollox et al. (2003) demonstrated a direct correlation between number of copies of the *DEFB104* gene and its transcript mRNA levels in a line of lymphoblastoid cells, while Linzmeier and Ganz (Linzmeier and Ganz, 2006) used the western blot technology to correlate between the total amount of HNP-1, HNP-2 and HNP-3 with the total number of *DEFA1* and *DEFB3* genes.

The published evidences are obviously not enough to affirm that copy-number polymorphisms always influence protein expression, but are sufficient to sketch a picture in which variations in the 8p23.1 cluster are able to modify the host's innate immunity environment.

This, added to the previous results that show an influence of defensins on HIV-1 infection and vertical transmission, drove us to look for a feasible way to study copy-number polymorphisms in a number of defensin genes.

In fact deletions and amplifications of a gene will usually not be detected by sequence analysis of PCR amplified gene fragments, as a normal copy is still present. An approach based on southern blot analysis could reveal many aberrations but will not always detect small deletions and is not a simple to perform routine technique, especially in our case, in which we wanted to analyze a considerable number of gene polymorphisms.

Well-characterized deletions and amplifications can be detected by specially designed PCR. However, the exact breakpoint sites of most deletions of the defensin cluster have not been determined yet. Furthermore the number of possible different deletions in this region is becoming prohibitively big.

Chen et al. (2006) have proposed an alternative approach that employed quantitative real-time PCR to detect copy number polymorphisms of human b-defensin genes.

To avoid the possibility that single nucleotide polymorphism within the primer region may affect PCR amplification, primers were designed in region that show complete sequence identity between published sequences of the same target gene.

The method was also based on the Second Derivative Maximum Method; in fact, minor deviations in the PCR amplification and the fluorescence detection within the logarithmic phase can lead to significant variation in the Cp value, that, in turn, may lead to gross misinterpretation of the gene copy number. On the contrary, with the Second De-

rivative Maximum Method, Cp determination is only defined by curve shape, not by fluorescence background or a manually defined threshold value, which avoids manual arbitrary setting in each experiment and makes the results more precise and reproducible.

We were able to test the method proposed by Chen et al. in a small subset of our cohort using the primers reported in his work. Since we were using an Applied Biosystems 7900 real-time apparatus instead of the LightCycler machinery used in the original article we had to arrange the experimental conditions to obtain consistent and reproducible amplification.

The method proved to be quite strong, with copy-number determination bound to manifold reproduction of experimental data and robust mathematical analysis. Unfortunately, due to constitutive variations in PCR amplification, to obtain consistent results all samples needed to be performed at least in triplicate, otherwise, if doing the assay in duplicate or without sample replication, we observed that copy number determination was not accurate and that intra-assay variability was largely increased. We measured the intra-assay variability in the different conditions and we obtained that when doing the procedure with triplicate samples the mean variability value 2.67% and was comparable to the one reported by Chen et al. This value grows to 6.23% when doing the assay in duplicate and to 15.1% when we weren't using sample replication in the same plate. Due to that and to the extensive number of required internal controls, only few samples could be genotyped contemporarily on a 96 wells plate and only for 3 different defensin genes: *DEFB4*, *DEFB3* and *DEFB104*.

We concluded that this methodology, while technically trustworthy, was not suitable to perform a quite large case-control association study; therefore we didn't proceed in designing the probes for the other defensin genes present at multiple copies in the 8p23.1 cluster. We still retained the assay as an internal control for the subsequent analysis.

We found that a viable and suitable alternative we could use to perform case-control association with a large number of multiple copy genes was Multiple Ligation-dependent Probe Amplification (MLPA): a technology developed by the Dutch company NRC-Holland.

MLPA is a method to establish the copy number of up to 45 nucleic acid sequences in one single reaction. Its versatility makes it applicable on genomic DNA as well as on mRNA.

With this technique it is possible to perform multiplex PCR reactions in which up to 45 specific sequences are simultaneously quantified. Amplification products are then separated by capillary electrophoresis in an automatic sequencer, using the same protocols employed in microsatellite separation. As only one pair of PCR primers is used, MLPA reactions result in a very reproducible gel pattern with fragments ranging from 130 to 490 bp. Comparison of this gel pattern to that obtained with a control sample indicates which sequences show the presence of gene copy number.

MLPA probes are able to discriminate between sequences that differ in only one nucleotide. Moreover, MLPA reactions don't require a large amount of DNA to give consistent results, with the minimum constraint, declared by MRC-Holland of 20ng of human DNA per reaction. In contrast to FISH and BAC-arrays, for example, the sequences detected are much smaller, typically being around 60 nucleotides long, thus providing an effective way to reduce the effect of eventual mutations. Moreover, one of the advantages of the MLPA technique is that the required equipment, a thermal cycler with heated lid and sequence type electrophoresis equipment, was already present in our laboratories.

To perform a case-control study on multiple defensin genes we employed the SALSA P139 Defensin kit developed by MRC-Holland. This kit contained 44 different probes for defensins present in multiple copy number, defensins present in single copy per aploid genome, other genes present at the 8p23 locus and control probes.

The genes we were interested in studying were particularly those that are present in multiple copy number, namely: *DEFA3*, *DEFA5*, *DEFA7*, *DEFB103A*, *DEFB104*, *DEFB105*, *DEFB106*, *DEFB107B*, and *DEFB108*. In the cases of *DEFA7*, *DEFB103A* and *DEFB104* each gene was targeted by two different probes designed to hybridize on different exons; this approach increased sensibility of the method for this genes and improved overall copy-number assessment.

By choosing the MLPA technique we had to face a difficulty related to the design of the experiment. In fact MLPA is especially suitable to study gross deletions or insertions like deletions in tumor tissues or trisomy of the chromosome 21. In those cases the technique takes advantage from the fact that a normal control to make a direct comparison is always available without significant aberrations. Therefore the data analysis software is able to easily discriminate between a normal sample, that is comparable to the control, and an affected individual in which the electrophoretic profile is signifi-

cantly different from the control one. Moreover in most cases the quantitative differences between the healthy subject and the affected patient are in the 50% range, meaning a loss of heterozygosity or a gene duplication, while discriminating, for example, between a sample with 9 copies of a gene and one with 10 copies means that the technique should be able to discriminate a 10% difference in normalized peak height.

The case of the defensin cluster is dramatically different since that a "normal individual" does not exist and that copy number polymorphisms are so polymorphic that grouping different samples from the same group leads to exaggerated deviations form the average value.

As a matter of fact, since the Coffalyzer software we used to perform analysis of experimental data (available for free download at the *www.mlpa.com site*) requires a control group and a case group to complete its analysis, our first approach was to perform pairwise comparisons using the three groups our cohort was divided into.

Unfortunately such approach soon showed its statistical limits: elevated deviations from the mean value made all statistical approaches virtually fruitless.

One direct solution would have be to find a subject that had only two copies per diploid genome of most of the studied genes and four copies per diploid genome of those genes that are usually duplicated. (Several defensin genes probes gave an unusually high signal when tested by MRC-Holland. Therefore, in order to have similar peak height for most of the probes when analyzing a mixture of DNA samples from healthy controls, they deliberately reduced the probe signal for some of these defensin probes by addiction of competitor oligomers). In this case this subject's DNA would have served as the control group and all others subjects would have been compared to it.

While such a subject, although rare, could exist –and we effectively found one of them later in the study- the great difficulty was to find it without actually having the possibility to check it with MLPA, since no analysis could be done on the Coffalyzer software without a control group of at least five runs and that final copy-number estimation largely depends on the quality of the control group itself.

Direct visual analysis of the electropherogram profile, as suggested by the kit producer, was even not an option. In fact this method could effectively work in the case of large deletions or duplications, but was unsuitable to discriminate such a fine copy-number polymorphisms as the ones we were interested to. Besides, automatic data analysis with the Coffalyzer software gave the opportunity to normalize the peak heights in order to

minimize the bias due to the fact that shorter fragments are preferentially amplified in a multiplex reaction. Moreover it gave the opportunity to do a strict quality check based on the control probes and the ligation-indipendent probes included in the assay.

The only viable solution was to literally create a normal DNA similar to the one detailed above. Our idea has been to create one recombinant plasmide for each probe present in the SALSA P-139 Defensin kit. A precious help in this direction come directly from the MRC-Holland support that supplied us the complete sequences that are recognized by the probes for each gene included in the kit.

As a matter of fact inserting only the ligation site was enough for the probes to detect the gene, undergo ligation and be amplified in the following PCR step.

Our first idea has been to introduce all the sequences in a single vector, but such an approach would have revealed largely impractical in a lab, like ours, that doesn't have much experience in creating recombinant organisms.

Therefore the way we followed has been to create 44 different plasmids, each containing one specific target sequence. To do so we employed specially designed primers, with whom we were able to amplify, for each target sequence, a fragment of about 150 base pairs, flanked on each side by an *Xho*I restriction site. We then inserted this fragment in a common pGL3 vector and selected the plasmids with only one inserted target sequence. Sequence determination has been done with an ABI 3130XL automatic sequencer.

Plasmids were selected to match the probes for genes that are located in single copy outside of chromosome 8 and used as controls (*IL4*, *FBN1*, *PRKCE*, *CDKN2A*, *RB1*, *FANCL*, *RTN4*, *LMO2* and *RENT2),* genes located in the 8p23 region and not belonging to the defensin family (*MFHAS1,CSMD1*, *ANGPT2*, *BLK*, *GATA4*, *MSRA*, *TNKS*, *PPP1R3B* and four different probes for MSR) defensins genes that occur in two copies per diploid genome: (*DEFA4*, *DEFA6*, *DEFB1*, and *SPAG11* each with two probes directed to different exons, and *DEFB4* with only one probe) and finally defensin genes that normally present copy-number polymorphism (one plasmid for each of the two DEFB103A, DEFFB104 and DEFA7 probes, and one plasmid for each of the probes targeted to the *DEFA3*, *DEFA5*, *DEFB105*, *DEFB106*, *DEFB107* and *DEFB108* genes.

An *E. Coli* strain has been transfected with the different plasmids and, after one night of growth at 37°C, amplified plasmid has been purified with a Millipore Plasmid Miniprep and the resulting DNA has been spectrophotometrically quantified.

Then, equals amounts of DNA for each different plasmid have been pooled to generate our standard DNA mix.

In silico analysis of the pGL3 plasmid sequence showed that none of the target sequences were present before recombination.

To confirm the functionality of our synthetic control we performed a standard MLPA reaction on each selected recombinant plasmid, on the resulting pool and on the native pGL3 plasmid and visually examined the resulting chromatograms. As expected, each plasmid gave a single peak at different positions, while the mix with pooled plasmids contained all the characteristic peaks and the native pGL3 only showed the ligation-indipendent peaks usually used as an internal control during the Coffalyzer data analysis.

Notwithstanding the fact that our synthetic mixture proved to be quite reliable, as soon as we found an individual that had only two copies of each defensin gene we turned to use this individual as the standard control and we repeated the analysis that had been done with the synthetic control DNA.

Estimation of copy-number for each gene has been performed by rounding the ratio calculated by the Coffalyzer software to the nearest integer. Data obtained this way were then employed to perform comparison of the copy number distributions among the different studied groups.

To do so we could not rely on standard t-test, since statistical distributions of copy-number polymorphisms are inherently non-normal. Indeed, due to the fact that lower values were limited to 0, distributions were, at best, skewed to the right and, in the worst cases, not complying any common distribution descriptions.

That was the case, for example of the distributions of the copy numbers of the DEFA3 that was lacking, as described (Linzmeier et al., 1999, Gene, 233, 205-11), in about 10% of the population.

We therefore employed a non-parametrical approach to the problem, using Wilcoxon rank-sum test. The Wilcoxon rank-sum test is a non-parametric test for assessing whether two samples of observations come from the same distribution. The null hypothesis is that the two samples are drawn from a single population, and therefore that their probability distributions are equal. It requires the two samples to be independent, and the observations to be ordinal or continuous measurements. More generally, the Wilcoxon rank-sum test may be thought of as testing the null hypothesis that the prob-

ability of an observation from one population exceeding an observation from the second population is equal to 0.05.

Following this approach we have been able to contemporarily analyze the number of genes present for diploid genome of the following defensin genes: *DEFA3*, *DEFA4*, *DEFA5*, *DEFA6*, *DEFA7*, *DEFB1*, *DEFB4*, *DEFB103A*, *DEFB104*, *DEFB105*, *DEFB106*, *DEFB107B, DEFB108 and SPAG11*, in order to investigate the correlation between gene copy number and HIV vertical transmission.

In our results the genes *DEFA4*, *DEFA5*, *DEFA6*, *DEFB1*, *DEFB4*, DEFB107B, *DEFB108 and SPAG11* are always present in two copies per diploid genome in healthy control, HIV-1 positive subjects and exposed uninfected children.

This subset of the collected data confirms the evidence drawn so far about the fact that those genes are present in single copy per haploid genome in the human race and therefore have not being further analyzed in this experiment.

The other studied genes, namely *DEFA3*, *DEFA7*, *DEFB1*, *DEFB4*, *DEFB103A*, *DEFB104*, *DEFB105*, *DEFB106*, and *DEFB107B*, on the contrary, were found to be present in multiple copy number in the three examined groups and the number of copies varied significantly among different individuals, thus justifying our hypothesis that differences on the number of copies of the defensin genes present in the genome could be responsible for differential response to HIV-1 infection and vertical transmission.

Data on the average number of copies present and the range of copies at which each gene appear are reported on table 4.

We were able to show that the average number of copies of the *DEFB104* gene, coding for the protein hBD-4, was significantly lower in HIV positive children when compared with exposed uninfected children (corrected p-value 0.006), with an average number of copies of 3.54 and 4.49 per diploid genome, respectively, with comparable range. Confronting HIV-1 positive children with healthy controls evidenced a similar trend (3.54 vs. 4.28 copies per diploid genome) that however didn't reach statistical significance after Bonferroni correction.

No significant differences could be evidenced among the studied groups for any of the other studied genes.

Copy-number assessment for the *DEFB104* gene was then repeated with a method based on quantitative real-time PCR, as reported by Chen et al. (Chen et al., 2006) and

we found that results were comparable to the ones obtained with MLPA (data not shown), confirming the reported differences.

Table 4 Average (and range) of gene copy number of HIV positive children, exposed-uninfected children and healthy controls

	Healthy Controls	Exposed-Uninfected	HIV Positive
DEFA3	2.44±2.38 (0-8)	3.3±2.77 (0-9)	3.94±3.67 (0-10)
DEFA7	3.78±1.25 (2-6)	3.67±1.13 (3-5)	3.58±1.20 (2-6)
DEFB4	3.78±1.1 (2-6)	4.87±1.56 (2-7)	4.14±2.13 (1-9)
DEFB103A	4.62±1.04 (3-7)	4.48±1.07 (4-8)	4.55±1.57 (2-9)
DEFB104	4.28±0.71 (2-5)	4.49±0.72 (4-7)	3.54±1.07 (2-7)
DEFB105	6.05±1.52 (3-9)	5.78±1.29 (2-4)	5.44±1.53 (2-9)
DEFB106	3.78±0.79 (2-6)	3.67±0.56 (3-5)	3.58±1.13 (2-6)

Our results showed that the *DEFB104* gene is present with reduced number of copies in children that were infected during delivery, when confronted with healthy controls and children that were exposed to the virus but didn't contract infection. While the role of some β-defensin (like hBD1, 2 and 3) in HIV infection has been quite widely investigated (Klotman and Chang, 2006), only few data concerning hBD-4 are available.
Interestingly, *DEFB104* expression has already been reported in the female reproductive tract (King et al., 2003); we can hypothesize that hBD-4 could directly interact with viral membranes destabilizing them in a way similar to that reported for hBD-2 and hBD-3 (Quinones-Mateu et al., 2003), and that the expression of hBD-4 directly correlates to the number of gene repetition as reported for *DEFA1* and *DEFA3* (Linzmeier and Ganz, 2005) and *DEFB4* (Hollox et al., 2003); our results, that the copy number of the *DEFB104* gene are lower in HIV positive children when compared with exposed uninfected children and healthy controls, suggest that this gene is involved in protecting from HIV infection and that concentration of hBD4 in the female reproductive tract may influence HIV-1 susceptibility to infection, possibly by direct interaction with HIV-1.

In our studied model, of course, genotype of the mothers, that is partly reflected on the child genotype that we studied, will be the real marker of susceptibility.
Unfortunately, clinical follow-up of the mothers couldn't be performed as most of the patients didn't return to the hospital after the first consult for medical treatment. Therefore we lack mothers' biological sample to perform a comparative study.
Although we weren't able to evidence any correlation between HIV-1 infection and vertical transmission with any other studied gene, and despite the limited number of analyzed samples, we were able to evidence that copy-number polymorphisms of defensin genes at the 8p23 locus can influence susceptibility in mother to child transmission of HIV-1.

Lactoferrin gene (LTF) polymorphisms in HIV-1 vertical transmission and infection

Because of the biological functions of lactoferrin, polymorphisms in the lactoferrin gene could be an important factor in genetic susceptibility to environmental insults and diseases.
More than 60 SNPs have been identified in the lactoferrin gene, either in the promoter region, exons or introns (Teng and Gladwell, 2006).
A single-point A/G nucleotide variation causing a non-conservative threonine/alanine substitution at position 11 (T11A) of the secreted lactoferrin protein has been associated with aggressive periodontitis (Jordan et al., 2005).
A Lysine/Arginine polymorphism at position 29 (R29K) in the N-terminal region of human lactoferrin that results from a single nucleotide polymorphism in exon 2 of the human lactoferrin gene has been associated with juvenile periodontitis, suggesting that lactoferrin R29K allelic variants are functionally different and that these differences may contribute to the pathogenesis of localized juvenile periodontitis (Velliyagounder et al., 2003).
Thus we decided to genotype T11A and R29K SNPs in our HIV samples, to asses if they may play a role in susceptibility to HIV infection or not. Genotyping results are reported in table 5; all groups were in Hardy-Weinberg equilibrium for both the investigated SNPs. Our results show no significant differences in allelic or genotype frequency for the T11A polymorphism.

Conversely, our results show that the frequencies of the R29K polymorphism are significantly different when comparing the HIV-1 infected children group with the healthy controls group.

Table 5 Alleles and genotypes count (and frequency) of the T11A and R29K polymorphisms in the LTF gene in HIV-1 positive children, exposed-uninfected children and healthy controls.

	Healthy Controls	Exposed-Uninfected	HIV Positive
SNP T11A			
A	110 (0.57)	90 (0.63)	251 (0.65)
G	82 (0.43)	54 (0.37)	133 (0.35)
A/A	30 (0.31)	27 (0.37)	84 (0.44)
A/G	50 (0.52)	36 (0.50)	83 (0.43)
G/G	16 (0.17)	9 (0.13)	25 (0.13)
SNP R29K			
G	120 (0.63)	92 (0.64)	289 (0.75)
A	72 (0.37)	52 (0.36)	95 (0.25)
G/G	36 (0.37)	25 (0.35)	105 (0.55)
G/A	48 (0.50)	42 (0.58)	79 (0.41)
A/A	12 (0.13)	5 (0.07)	8 (0.04)

The R29K GG genotype was significantly more frequent in HIV positive subjects (55%) when compared to healthy controls (37%) with a p-value, corrected for multiple comparisons with the Bonferroni method, equal to 0.0216.

Allelic frequencies were also significantly different (corrected p-value equal to 0.0108) being the G allele present in 75% of HIV infected children and only in 63% of healthy controls. The presence of the G allele is associated to an increased susceptibility to HIV infection (O.R. = 1.8233, with a 95% confidence interval of 1.23-2.69.

Differences between HIV-1 positive children and exposed uninfected children, albeit not significant, are nevertheless evident, with the frequencies of the latter group more resembling the ones of the healthy control group.

We then performed haplotype analysis, employing the Arlequin software; haplotypes frequencies are reported on table 6.

Table 6 Haplotypes frequency of the T11A/ R29K polymorphisms in HIV-1 positive children, exposed-uninfected children and healthy controls.

	Healthy Controls	Exposed-Uninfected	HIV Positive
Haplotype			
AG	0.393	0.501	0.589
GG	0.232	0.138	0.164
AA	0.180	0.124	0.065
GA	0.195	0.237	0.182
L.D.	0.16	0.44	0.59

Our results evidence that the AG haplotype is strongly associated to increased susceptibility to HIV-1 infection, being significantly more frequent in HIV positive children (59%) than in healthy controls (39%) (corrected p-value 0.0048).

This fact, coupled to the evidence that the linkage disequilibrium value was greater for the HIV-1 positive group, indicates that the two SNPs probably cooperate in determining mature lactoferrin influence on susceptibility to HIV-1 infection.

Lactoferrin has been demonstrated to have a direct antiviral effect and it has also been reported to be active against HIV-1 (Harmsen et al., 1995). Moreover interactions between lactoferrin and HIV-1 particles have been reported, with regions of the protein containing a cluster of negative charges between residues 210-240 that have been shown to interact with the positively charged domains in the V3 loop of the viral envelope protein gp12 (Swart et al., 1998).

Thus, lactoferrin seems to exert its effect in the early phase of infection, inhibiting viral replication at the level of virus adsorption and penetration, probably during virus-cell fusion.

The antiviral activity of lactoferrin in HIV infection has already been tested (Berkhout et al., 2002): bovine lactoferrin was able to completely impede infection spreading at a concentration of 10 μM and a considerable inhibition of virus replication was observed at concentration as low as 0.1 μM. Since it was suggested that lactoferrin, apart from its interaction with the V3 loop, could possibly inhibit virus-cell interaction through competitive binding to the CXCR4 and CCR5 coreceptors, the antiviral activity of lactoferrin against HIV-1 variants with different V3 domains and co-receptor usage was tested. Bovine lactoferrin was able to inhibit various HIV-1 strains that use the CXCR4 and CCR5 co-receptor, confirming the broad activity spectrum of lactoferrin (Moriuchi and Moriuchi, 2001). Therefore is plausible to suppose that polymorphisms that can influence both protein expression and activity could influence susceptibility to HIV-1 infection.

Moreover, Lactoferrin has been known for a long time to be expressed in the female reproductive tract; thus, its antiviral activity against HIV can be exerted at this level as well, suggesting that lactoferrin can be implicated in mother-to-child transmission of infection.

In our work we have evidenced that the G allele (and GG genotype) of the R29K polymorphism is more frequent in HIV positive children than healthy controls, suggesting that the presence of the G allele increases the susceptibility to be infected by the HIV-1 virus during delivery. This association between R29K G allele and augmented susceptibility to HIV-1 infection was also confirmed by Haplotype analysis.

The A allele of the R29K polymorphism (corresponding to the Lysine) has been previously described to increase lactoferrin's activity against a number of Gram-positive and Gram-negative microorganisms (Velliyagounder et al., 2003). Furthermore, in the same study, the A allele has been observed to be transcribed more efficiently than the G allele, leading to increased protein expression.

In our case, differences in both protein expression and functional activity as consequence of the presence of different allelic variants, could modify the way in which the lactoferrin protein is able to contrast HIV-1 infection and thus promote or demote host infection. More in details, the increased frequency of the G allele in HIV positive sub-

ject can account for a less effective lactoferrin in facing HIV infection thus explaning why carrying this SNP is associated to an increased risk of being infected.
Concluding, in this work we presented, for the first time, evidences that functional polymorphisms of the *LTF* gene are able to influence the receptiveness to HIV-1 infection.

hCAP-18 gene analysis

On the second cohort we performed a screening on the entire *hCAP-18* gene, coding for the LL37 human cathelicidin.
The hCAP-18 is located on chromosome 3 and is composed of 4 exons that span a region of about 2 kb. We amplified all the four exons with specific PCR reactions and then search for mutations in *hCAP-18* gene by direct sequencing with the ABI3130XL automatic sequencer.
Our approach was based on the fact that, so far, no polymorphisms have been described in the *hCAP-18* coding sequence, therefore impeding us to use more direct techniques. We genotyped 96 random samples and we found no mutations, except for a single synonymous mutation in one of the healthy controls sample: a G to C transversion in position 78 of the translated sequence. However, due to the rarity of the mutation we didn't investigate it further.
To improve the study on the *hCAP-18* sequence we further designed two pairs of primers to detect mutations in the promoter and 5'UTR region of the gene. That allowed us to study 700 base pairs of promoter region and the full 140 base pairs of 5' UTR region. The two resulting overlapping amplicons were screened by direct sequencing in 96 random samples extracted from our second collection.
We were able to identify two undescribed mutations: a T to A transversion in position -491 and a G to A transition in position -350.
The -491(T/A) mutation was detected in a sample drawn from the HIV-1 positive group, while the -350(G/A) mutation was observed in a sample belonging to the healthy controls group.
In both cases the frequency of the mutation was so low that further studied would have been statistically irrelevant unless a much larger number of samples could be studied. Therefore we decided not to go on with the investigation on the cathelicidin gene.

Such a strict conservation that we observed both in the coding sequence and in the promoter region, impaired all our efforts to perform a case-control study based on polymorphisms but nonetheless suggest an important role for LL37 in humans.

It should be noted that our negative results don't necessarily imply that variations of LL37 expression are not involved in the event of HIV-1 infection and vertical transmission as a direct effect on lentiviral infection has already been proved (Steinstraesser et al., 2005).

MBL2 and MASP-2 polymorphisms in HIV-1 vertical transmission and infection

In order to further increase our knowledge about the role of innate immunity on HIV-1 infection we decided to genotype functional polymorphisms of the *MBL2* gene.

MBL deficiency, largely explained by three single point mutations in codons 52, 54 and 57 of exon 1 in the *MBL2* gene (Turner, 1996) could, in fact, greatly impair the activity of innate immunity.

Since these three polymorphisms share a common functional effect, therefore we employed the melting temperature assay technique to contemporarily genotype them. Our methodology has the drawback of not permitting to discern which mutation is actually present in a sample, giving us only the possibility to know if there is one present. However the methodology is cheap and fast and is able to correctly pinpoint any functional impairment of MBL. On the basis of the melting temperature assay results we were able to tell if a sample was wild-type for the three polymorphisms of the first exon (A/A genotype), if there were any mutation in heterozygous form (A/0 genotype) or if there was any of the three mutation in homozygous form (0/0 genotype).

Composite heterozygous samples, presenting two alleles with distinct polymorphisms, could also be detected and, due to the functional effect of this genotype, were treated as 0/0 samples in the analysis.

Furthermore, three polymorphisms in the promoter region of *MBL2* are also responsible for reduced expression of the MBL protein: the H/L, X/Y and P/Q allele at positions –550, –221 and +4 of the *MBL* gene.

These promoter polymorphisms combine to form haplotypes that are in strong linkage disequilibrium with the exon 1 mutations, resulting in seven common extended haplotypes, namely HYPA, LYPA, LYQA, LXPA, HYPD, LYPB and LYQC.

Table 7 Alleles and genotypes count (and frequency) of polymorphisms in the first exon and in the promoter of the MBL2 gene in HIV-1 positive children, exposed-uninfected children and healthy controls.

	Healthy Controls	Exposed-Uninfected	HIV Positive
Exon-1 A/0 SNP			
A	155 (0.81)	118 (0.82)	305 (0.79)
0	37 (0.19)	26 (0.18)	79 (0.21)
A/A	62 (0.65)	48 (0.67)	120 (0.62)
A/0	31 (0.32)	23 (0.32)	65 (0.34)
0/0	3 (0.03)	1 (0.01)	7 (0.04)
Prom. X/Y SNP			
X	62 (0.32)	36 (0.25)	107 (0.28)
Y	130 (0.68)	108 (0.75)	277 (0.72)
X/X	14 (0.15)	3 (0.04)	14 (0.07)
X/Y	34 (0.35)	31 (0.43)	79 (0.41)
Y/Y	48 (0.50)	38 (0.53)	99 (0.52)
Prom. H/L SNP			
H	54 (0.28)	46 (0.32)	116 (0.30)
L	138 (0.72)	98 (0.68)	268 (0.70)
H/H	9 (0.09)	3 (0.04)	19 (0.10)
H/L	36 (0.38)	40 (0.56)	78 (0.41)
L/L	51 (0.53)	39 (0.40)	95 (0.49)

We studied the H/L and the X/Y variants by the means of real-time detected allele specific PCR, while we decided not to study the P/Q variant due to the scarce functional effect of this polymorphism. Results of *MBL2* genotyping are summarized in table 7.

No statistical differences could be evidenced by the means of Fisher exact test when confronting genomic and allelic frequencies in the different groups, thus essentially confirming negative results already reported in literature and pointing on the absence of correlation between MBL polymorphisms and HIV-1 infection (Malik et al., 2003; McBride et al., 1998; Senaldi et al., 1995).

Nevertheless there's a small but noticeable trend pointing that exposed-uninfected children are characterized by lower frequencies of the X/X and L/L genotypes, both marked by reduced expression of MBL. This effect could be biased by the fact that the exposed-uninfected group is not very large, but notwithstanding this it could suggest that impaired levels of plasma MBL favors HIV-1 infection.

It should however be noted that the negative results of Malik (2003) and McBride (McBride et al., 1998) were obtaind on groups that are considerably smaller than the one we studied, besides being performed on populations characterized by distinct genetic background from the Brazilian population we studied.

Therefore we couldn't exclude that a study on larger populations or a meta-analysis of data could confirm the negative effect of the L and X alleles on HIV-1 infection. However this putative effect, that was already described (Boniotto et al., 2000), would clearly be too faint to cover any clinical relevance.

Furthermore, none of the previous studies took into consideration the fact that MBL, even in high plasma concentrations, could not be totally functional if mutations on its associated serine proteases are present.

We therefore genotyped two functional polymorphisms in one of the most important Mannose Associated Serine Proteases: *MASP2*.

The D105G mutation wasn't detected in any but three samples and was no further considerated.

Rs12711521 (D371Y) SNP genotyping results are reported in table 8.

Once again we weren't able to detect any significant difference among the groups and we only found a weak trend (uncorrected p-value equal to 0.062) when confronting the allele frequencies of HIV-1 patients whit exposed-uninfected children, suggesting a feeble protective effect for the C allele.

Table 8 Alleles and genotypes count (and frequency) of D371Y polymorphisms in the MASP2 gene in HIV-1 positive children, exposed-uninfected children and healthy controls.

	Healthy Controls	HIV-1 positive	Exposed-Uninfected
D371Y SNP			
A	103 (0.54)	227 (0.59)	72 (0.50)
C	89 (0.46)	157 (0.41)	72 (0.50)
A/A	26 (0.27)	69 (0.36)	18 (0.25)
A/C	51 (0.53)	89 (0.46)	36 (0.50)
C/C	19 (0.20)	34 (0.18)	18 (0.25)

We then moved to perform haplotype analysis of the polymorphisms we found in the *MBL2* genes

We found that only 5 out of 8 possible haplotypes were significantly managed by the Arlequin software: LYA, HYA, LXA, LY0 and HY0; haplotypic frequencies for the various group are evidenced in figure 18; haplotype distributions are quite dissimilar between the analyzed groups.

If we group together the LYA and HYA haplotypes, associated to higher production of MBL and the LXA, LY0 and HY0 haplotypes, associated to low or medium-low protein expression we could find, via a 2X2 contingency table treated with Fisher test that the haplotypes with high expression are significantly more present in the exposed uninfected children when compared to the healthy controls and HIV-1 infected childred (Bonferroni corrected p-value <0.001 in both cases), while the percentages of high producers and low producers are similar between healthy controls and HIV-1 positive children.

This data virtually confirms the trends we observed in genotype frequencies about the H/L and the X/Y variants and points toward a role for high levels of plasma MBL in protecting from HIV-1 vertical transmission in Brazilian children.

Moreover, in order to better clarify the role played by polymorphisms in the MBL gene, and to have a general picture of the phenomenon we then computed MBL combined genotypes considering both exon-1 and promoter variants.

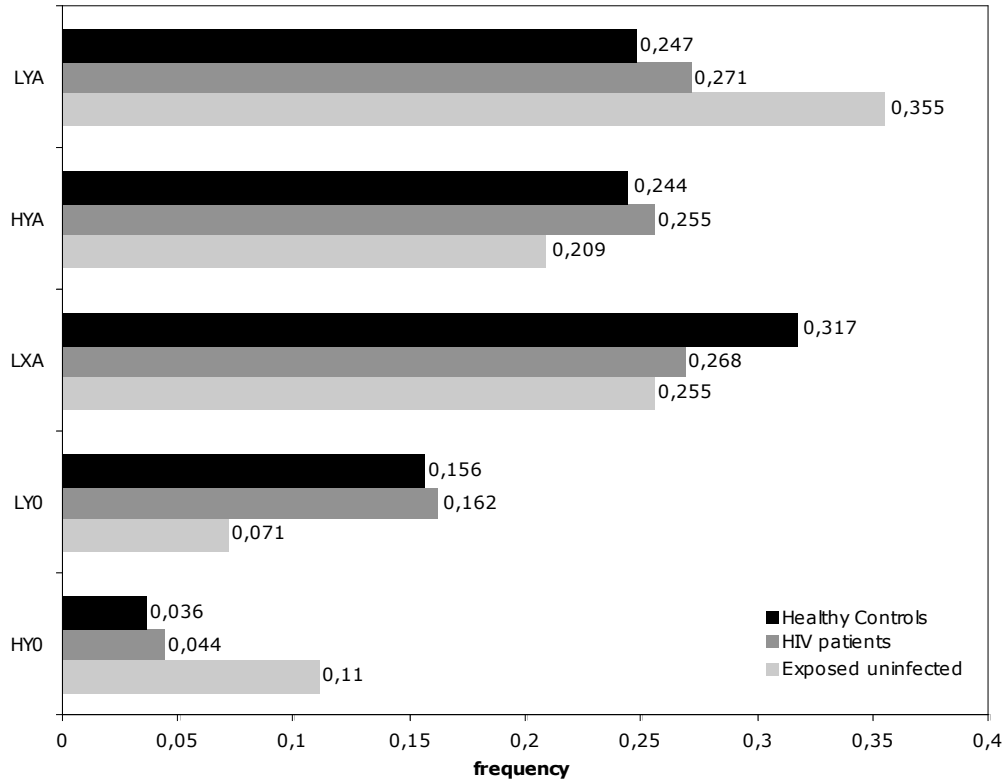

Figure 16 Haplotype frequency of MBL2 gene promoter and exon-1 polymorphisms in HIV-1 positive children, exposed-uninfected children and healthy controls.

Resulting genotypes were than ranked in three classes according to MBL production as High (HP), Low (LP) and Deficient (DP).

MBL HP genotype was significantly more frequent in Exposed uninfected children than in healthy controls and HIV-1 patients (p.value <0.001)

HIV positive children present the higher frequency of Deficient producer genotypes (18,5% vs. 10% and 14% in healthy controls and exposed-uninfected children respectively.)

Table 9 Frequency of MBL2 promoter and exon-1 combined genotype ranked according to MBL production in HIV-1 positive children, exposed-uninfected children and healthy controls.

			Healthy Controls	Exposed-Uninfected	HIV Positive
MBL2 promoter and exon- 1 combined genotypes					
	High producer (HP)	HYA/HYA HYA/LYA HYA/LXA	0.49	0.63	0.555
	Low producer (LP)	LXA/LXA HYA/0	0.41	0.23	0.26
defective	Deficient producer (DP)	LYA/0 LXA/0	0.10	0.14	0.185

Our data shows that, even if single polymorphisms in the *MBL2* gene don't affect directly HIV infection, acting synergistically they can influence protein expression levels and account for HIV susceptibility. Higher levels of the protein (as expected as a result of the presence of high producer combined genotypes) are associated to protection against HIV infections, while low levels confer susceptibility.

CONCLUSIONS

This study has been performed in order to characterize genetic factors implicated in the susceptibility to HIV infection and vertical transmission.

Different genes involved in the innate immune system have been analyzed in Brazilian pediatric population constituted by three groups: HIV infected children, children who didn't contract the infection but were exposed to the same risk conditions of the infected ones, and healthy controls that were nor infected nor exposed.

For several of the investigated genes, an involvement in disease susceptibility has been found.

The first gene of the innate immunity found to be associated to HIV infection was *DEFB1*, a gene encoding for hBD-1, a human ß-defensin constitutively expressed by many epithelia.

We identified three polymorphism in the 5'UTR of the gene at position -52, -44 and -20, that display different frequencies within the three groups analyzed.

The -44 C allele and CC genotype were over-represented in the HIV-1 infected group, thus pointing toward a protective effect for the -44 G allele both in vertical transmission and in infection by HIV-1 virus. However this correlation for the -44(C/G) DEFB1 polymorphism appeared quite weak.

On the other hand, we were able to demonstrate that the *DEFB1* -52A/A genotype was significantly less frequent in HIV-1 perinatally infected children when compared to healthy controls and exposed uninfected children.

Finally, the stronger and more striking association has been observed considering the -20 (G/A) polymorphism, where allelic and genotype frequency were significantly different between HIV-1 positive children, healthy controls and exposed uninfected children. The results obtained show that carrying the -20 G allele infers protection from vertical transmission of HIV-1, while a greater susceptibility was observed in patients carrying the -20 A allele.

Our results thus evidenced how *DEFB1* gene could be employed as a marker of risk in infection and vertical transmission of HIV-1.

Since the functional role of these polymorphisms was unknown, we performed a dual luciferase assay with the aim of to verifying if different alleles may have distinctive translational effects.

Our results showed that all the studied polymorphisms do have a functional effect. Expression levels of the reporter gene were sensibly reduced in all cases where a SNP was present.

Notwithstanding the fact that all polymorphisms were diminishing the luciferase expression, only the -44 G alleles was able to reduce the expression of the reporter gene to statistically significant levels.

Our *in vitro* results suggest that all the studied polymorphisms can be able to hamper an effective production of hBD-1 *in vivo* and, therefore, they can be related to a lack of proper response from the innate immunity, thus pinpointing the important role of this defense system in HIV infection.

In addition to their role in this particular viral infection, our results indicate that these SNPs should be analyzed in other infectious and inflammatory pathologies as well to seek for linkage disequilibrium with the illness.

In our work we also investigated for the first time if gene copy number polymorphisms of defensins genes could be related to HIV infection.

Interestingly, we were able to show that the average number of copies of the *DEFB104* gene, coding for the protein hBD-4, was significantly lower in HIV positive children when compared with exposed uninfected subjects. Confronting HIV-1 positive children with healthy controls we evidenced a similar trend.

Hypothesizing that hBD-4 could directly interact with viral membranes similarly to hBD-2 and hBD-3 and that its expression correlates to the number of gene repetition as reported for *DEFA1*, *DEFA3* and *DEFB4*, our results suggest that *DEFB104* gene copy

number polymorphisms are involved in protecting from HIV infection and that concentration of hBD4 in the female reproductive tract may influence HIV-1 susceptibility to infection.

Lactoferrin is a molecule that has already been demonstrated to have a direct antiviral effect and to be active against HIV. When analyzing its coding gene, we found a polymorphism (namely R29K) that reveals a possible involvement in HIV infection. In fact we evidenced that the G allele (and GG genotype) of the R29K polymorphism is more frequent in HIV positive children than healthy controls, suggesting that the presence of the G allele increases the susceptibility to be infected by the HIV virus during delivery. This association between R29K G allele and augmented susceptibility to HIV infection was also confirmed by Haplotype analysis. Since the presence of this SNP can influence both protein expression and functionality and since lactoferrin has been known for a long time to be expressed in the female reproductive tract, we hypothesize that lactoferrin can be implicated in mother-to-child transmission of infection.

Analyzing all the coding region and the promoter of the gene coding for LL37, allowed us to recognize strong nucleotide conservation. Indeed only a couple of SNPs were identified in a very restricted number of samples, thus precluding their suitability for association studies. The strong conservation we observed both in the coding sequence and in the promoter region suggest an important role for LL37 in host defense.

MBL is an important molecule of the innate immune system; the understanding of its function has grown rapidly over the past three decades. It is now recognized to have a role in different processes, as complement activation, promotion of complement-independent opsonophagocytosis, modulation of inflammation, recognition of altered self-structures and apoptotic cell clearance.

We analyzed polymorphisms in the promoter region and in the first exon of the gene that are known to affect protein expression levels and that has already been associated to many diseases. While we found no significant data considering the single polymorphisms, when we compute combined genotype and ranked them in three classes according to MBL resulting production as High (HP), Low (LP) and Deficient (DP), we found that MBL HP genotypes were significantly more frequent in exposed uninfected children than in healthy controls and HIV-1 patients, whereas HIV positive children present the greatest frequency of DP genotypes.

Our data show that, even if single polymorphisms in the MBL2 gene don't affect directly HIV infection, acting synergistically they can influence protein expression levels and account for HIV susceptibility. Higher levels of the protein (as expected as a result of the presence of high producer combined genotypes) are associated to protection against HIV infections, while low levels confer susceptibility.

Identifying the genetic factors involved in such a complex disease as HIV infection is quite a troublesome challenge and association studies have some well known limitations. One of the major drawback of this entire thesis work has been, of course, the lack of mothers biological samples and, as a consequence, their genetic data. In fact, it's plausible that vertical transmission could be also influenced by mother's genetical background.

Despite all, with our work we have been able to highlight the role of innate immunity and many host defense genes in conferring susceptibility or protection towards HIV infection, thus stressing up, once again, the pivotal function of this branch of the immune system.

ACKNOWLEDGMENTS

Fare un dottorato non è una cosa che si può fare da soli. E non lo si può fare a cuor leggero. I tre anni appena trascorsi sono stati molto intensi, sia dal punto di vista scientifico che, soprattutto, dal punto di vista umano.
E' pertanto inevitabile che i miei ringraziamenti vadano a tutti quelli che in questo periodo mi sono stati vicino e che han capito quando era meglio non farlo.
Innanzitutto ringrazio Sergio, che mi ha insegnato molto più di quello che era tenuto ad insegnarmi.
Poi Valentina, Ludovica e Camilla che, chi meglio e chi peggio, son riuscite a sopportarmi nei miei alterni umori. E che se non hanno capito hanno almeno fatto finta di stare ad ascoltarmi.
Marco, perché lui sì che ha capito tutto, e Üalter per non averci neppure provato; entrambi sono molto più che impagabili compagni di bevute.
Non sarò mai troppo grato al Brasile per le persone che mi ha fatto incontrare e per avermi fatto assaporare la loro visione del mondo: Lula, Zé Luis, Lucas e Raphael, tutti gli abitanti della Pousada Alquimia ed il cameriere taciturno della San Francisco.
Loris per i giri in moto, e tutti gli amici motociclisti lasciati a Trieste mentre la Eva dorme in garage.
Grazie anche alla mia famiglia, per esserci sempre: grazie ai miei genitori, ai miei zii, a mio cugino e alla sua famiglia.

Avervi vicino ha reso questi anni un vero spettacolo.

REFERENCES

Agerberth, B, et al. (1995), 'FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis.', *Proc Natl Acad Sci U S A*, 92 (1), 195-99.

Agerberth, B, et al. (2000), 'The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations.', *Blood*, 96 (9), 3086-93.

Ahmad, R, et al. (2002), 'Elevated levels of circulating interleukin-18 in human immunodeficiency virus-infected individuals: role of peripheral blood mononuclear cells and implications for AIDS pathogenesis.', *J Virol*, 76 (24), 12448-56.

Aldred, PM, EJ Hollox, and JA Armour (2005), 'Copy number polymorphism and expression level variation of the human alpha-defensin genes DEFA1 and DEFA3.', *Hum Mol Genet*, 14 (14), 2045-52.

Alkhatib, G, et al. (1996), 'CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1.', *Science*, 272 (5270), 1955-58.

Alison, AC (1954), 'Protection afforded by sickle-cell trait against subtertian malareal infection.', *Br Med J*, 1 (4857), 290-94.

Alves-Silva, J, et al. (2000), 'The ancestry of Brazilian mtDNA lineages.', *Am J Hum Genet*, 67 (2), 444-61.

Amoroso, A, et al. (1999), 'Polymorphism at codon 54 of mannose-binding protein gene influences AIDS progression but not HIV infection in exposed children.', *AIDS*, 13 (7), 863-64.

An, P, et al. (2002), 'Modulating influence on HIV/AIDS by interacting RANTES gene variants.', *Proc Natl Acad Sci U S A*, 99 (15), 10002-07.

Appelmelk, BJ, et al. (1994), 'Lactoferrin is a lipid A-binding protein.', *Infect Immun*, 62 (6), 2628-32.

Arai, T, P Tabona, and JA Summerfield (1993), 'Human mannose-binding protein gene is regulated by interleukins, dexamethasone and heat shock.', *Q J Med*, 86 (9), 575-82.

Arnold, RR, MF Cole, and JR McGhee (1977), 'A bactericidal effect for human lactoferrin.', *Science*, 197 (4300), 263-65.

Arnold, RR, M Brewer, and JJ Gauthier (1980), 'Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms.', *Infect Immun*, 28 (3), 893-98.

Arnold, RR, et al. (1981), 'Bactericidal activity of human lactoferrin: influence of physical conditions and metabolic state of the target microorganism.', *Infect Immun*, 32 (2), 655-60.

Axen, A, et al. (1997), 'Gloverin, an antibacterial protein from the immune hemolymph of Hyalophora pupae.', *Eur J Biochem*, 247 (2), 614-19.

Bals, R, et al. (1998), 'The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface.', *Proc Natl Acad Sci U S A*, 95 (16), 9541-46.

Bals, R, et al. (1999), 'Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide.', *Infect Immun*, 67 (11), 6084-89.

Bals, R (2000), 'Epithelial antimicrobial peptides in host defense against infection.', *Respir Res*, 1 (3), 141-50.

Barber, JC, et al. (1998), 'Duplication of 8p23.1: a cytogenetic anomaly with no established clinical significance.', *J Med Genet*, 35 (6), 491-96.

Bateman, A, et al. (1991), 'The effect of HP-1 and related neutrophil granule peptides on DNA synthesis in HL60 cells.', *Regul Pept*, 35 (2), 135-43.

Bauer, F, et al. (2001), 'Structure determination of human and murine beta-defensins reveals structural conservation in the absence of significant sequence similarity.', *Protein Sci*, 10 (12), 2470-79.

Bellamy, W, et al. (1992), 'Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin.', *J Appl Bacteriol*, 73 (6), 472-79.

Bellamy, W, et al. (1992), 'Identification of the bactericidal domain of lactoferrin.', *Biochim Biophys Acta*, 1121 (1-2), 130-36.

Bellamy, WR, et al. (1993), 'Role of cell-binding in the antibacterial mechanism of lactoferricin B.', *J Appl Bacteriol*, 75 (5), 478-84.

Benjamini, Y and Y Hochberg (1995), 'Controlling the false discovery rate: a practical and powerful approach to multiple testing', *J R Stat Soc*, 57 289-300.

Bensch, KW, et al. (1995), 'hBD-1: a novel beta-defensin from human plasma.', *FEBS Lett*, 368 (2), 331-35.

Bergman, P, et al. (2007), 'The antimicrobial peptide LL-37 inhibits HIV-1 replication.', *Curr HIV Res*, 5 (4), 410-15.

Berkhout, B, et al. (1997), 'Structural and functional analysis of negatively charged milk proteins with anti-HIV activity.', *AIDS Res Hum Retroviruses*, 13 (13), 1101-07.

Berkhout, B, et al. (2002), 'Characterization of the anti-HIV effects of native lactoferrin and other milk proteins and protein-derived peptides.', *Antiviral Res*, 55 (2), 341-55.

Biragyn, A, et al. (2002), 'Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2.', *Science*, 298 (5595), 1025-29.

Bleul, CC, et al. (1996), 'The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry.', *Nature*, 382 (6594), 829-33.

Boldt, AB and ML Petzl-Erler (2002), 'A new strategy for mannose-binding lectin gene haplotyping.', *Hum Mutat*, 19 (3), 296-306.

Boman, HG, B Agerberth, and A Boman (1993), 'Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine.', *Infect Immun*, 61 (7), 2978-84.

Boniotto, M, et al. (2000), 'Polymorphisms in the MBL2 promoter correlated with risk of HIV-1 vertical transmission and AIDS progression.', *Genes Immun*, 1 (5), 346-48.

Boniotto, M, et al. (2005), 'Evidence of a correlation between mannose binding lectin and celiac disease: a model for other autoimmune diseases.', *J Mol Med*, 83 (4), 308-15.

Bortner, CA, RD Miller, and RR Arnold (1986), 'Bactericidal effect of lactoferrin on Legionella pneumophila.', *Infect Immun*, 51 (2), 373-77.

Bowdish, DM, et al. (2004), 'The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes.', *J Immunol*, 172 (6), 3758-65.

Braida, L, et al. (2004), 'A single-nucleotide polymorphism in the human beta-defensin 1 gene is associated with HIV-1 infection in Italian children.', *AIDS*, 18 (11), 1598-600.

Brambilla, A, et al. (2000), 'Shorter survival of SDF1-3'A/3'A homozygotes linked to CD4+ T cell decrease in advanced human immunodeficiency virus type 1 infection.', *J Infect Dis*, 182 (1), 311-15.

Chaka, W, et al. (1997), 'Induction of TNF-alpha in human peripheral blood mononuclear cells by the mannoprotein of Cryptococcus neoformans involves human mannose binding protein.', *J Immunol*, 159 (6), 2979-85.

Chaly, YV, et al. (2000), 'Neutrophil alpha-defensin human neutrophil peptide modulates cytokine production in human monocytes and adhesion molecule expression in endothelial cells.', *Eur Cytokine Netw*, 11 (2), 257-66.

Chang, TL, et al. (2003), 'CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from alpha-defensin-1 HIV inhibition.', *J Virol*, 77 (12), 6777-84.

Chang, TL, et al. (2005), 'Dual role of alpha-defensin-1 in anti-HIV-1 innate immunity.', *J Clin Invest*, 115 (3), 765-73.

Charp, PA, et al. (1988), 'Inhibition of protein kinase C by defensins, antibiotic peptides from human neutrophils.', *Biochem Pharmacol*, 37 (5), 951-56.

Chen, CB and R Wallis (2004), 'Two mechanisms for mannose-binding protein modulation of the activity of its associated serine proteases.', *J Biol Chem*, 279 (25), 26058-65.

Chen, Q, et al. (2006), 'Screening of copy number polymorphisms in human beta-defensin genes using modified real-time quantitative PCR.', *J Immunol Methods*, 308 (1-2), 231-40.

Chertov, O, et al. (1996), 'Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils.', *J Biol Chem*, 271 (6), 2935-40.

Chitnis, SN, DB Kameswari, and KS Prasad (1993), 'Induction of autolysis in starved Escherichia coli cells by seminalplasmin, an antimicrobial protein from bovine seminal plasma.', *FEMS Microbiol Lett*, 112 (2), 147-50.

Cowland, JB, AH Johnsen, and N Borregaard (1995), 'hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules.', *FEBS Lett*, 368 (1), 173-76.

Crouch, SP, KJ Slater, and J Fletcher (1992), 'Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin.', *Blood*, 80 (1), 235-40.

Daher, KA, ME Selsted, and RI Lehrer (1986), 'Direct inactivation of viruses by human granulocyte defensins.', *J Virol*, 60 (3), 1068-74.

Davies, EJ, et al. (1997), 'A dysfunctional allele of the mannose binding protein gene associates with systemic lupus erythematosus in a Spanish population.', *J Rheumatol*, 24 (3), 485-88.

Deacon, NJ, et al. (1995), 'Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients.', *Science*, 270 (5238), 988-91.

Dean, M, et al. (1996), 'Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study.', *Science*, 273 (5283), 1856-62.

Deng, H, et al. (1996), 'Identification of a major co-receptor for primary isolates of HIV-1.', *Nature*, 381 (6584), 661-66.

Dork, T and M Stuhrmann (1998), 'Polymorphisms of the human beta-defensin-1 gene.', *Mol Cell Probes*, 12 (3), 171-73.

Dragic, T, et al. (1996), 'HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5.', *Nature*, 381 (6584), 667-73.

Eisen, DP and RM Minchinton (2003), 'Impact of mannose-binding lectin on susceptibility to infectious diseases.', *Clin Infect Dis*, 37 (11), 1496-505.

Elass-Rochard, E, et al. (1995), 'Lactoferrin-lipopolysaccharide interaction: involvement of the 28-34 loop region of human lactoferrin in the high-affinity binding to Escherichia coli 055B5 lipopolysaccharide.', *Biochem J*, 312 (Pt 3), 839-45.

Ellison, RT 3rd, TJ Giehl, and FM LaForce (1988), 'Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin.', *Infect Immun*, 56 (11), 2774-81.

Ellison, RT 3rd, et al. (1990), 'Lactoferrin and transferrin damage of the gram-negative outer membrane is modulated by Ca2+ and Mg2+.', *J Gen Microbiol*, 136 (7), 1437-46.

Ellison, RT 3rd and TJ Giehl (1991), 'Killing of gram-negative bacteria by lactoferrin and lysozyme.', *J Clin Invest*, 88 (4), 1080-91.

Engstrom, P, et al. (1984), 'The antibacterial effect of attacins from the silk moth Hyalophora cecropia is directed against the outer membrane of Escherichia coli.', *EMBO J*, 3 (13), 3347-51.

Esposito, JJ and JF Obljeski (1976), 'Enterovirus type 70 virion and intracellular proteins.', *J Virol*, 18 (3), 1160-62.

Ezekowitz, RA, LE Day, and GA Herman (1988), 'A human mannose-binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins.', *J Exp Med*, 167 (3), 1034-46.

Ezekowitz, RA, et al. (1989), 'A human serum mannose-binding protein inhibits in vitro infection by the human immunodeficiency virus.', *J Exp Med*, 169 (1), 185-96.

Faure, S, et al. (2000), 'Rapid progression to AIDS in HIV+ individuals with a structural variant of the chemokine receptor CX3CR1.', *Science*, 287 (5461), 2274-77.

Fernandez de Caleya, R, et al. (1972), 'Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro.', *Appl Microbiol*, 23 (5), 998-1000.

Frohm, M, et al. (1997), 'The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders.', *J Biol Chem*, 272 (24), 15258-63.

Ganz, T, ME Selsted, and RI Lehrer (1990), 'Defensins.', *Eur J Haematol*, 44 (1), 1-8.

Ganz, T and RI Lehrer (1994), 'Defensins.', *Curr Opin Immunol*, 6 (4), 584-89.

Ganz, T (2000), 'Paneth cells--guardians of the gut cell hatchery.', *Nat Immunol*, 1 (2), 99-100.

Ganz, T.(2005), 'Defensins and other antimicrobial peptides: a historical perspective and an update.', *Comb Chem High Throughput Screen*, 8 (3), 209-17.

Garcia, JR, et al. (2001), 'Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity.', *FASEB J*, 15 (10), 1819-21.

Garred, P, et al. (1994), 'Dual role of mannan-binding protein in infections: another case of heterosis?', *Eur J Immunogenet*, 21 (2), 125-31.

Garred, P, et al. (1997), 'Mannan-binding lectin in the sub-Saharan HIV and tuberculosis epidemics.', *Scand J Immunol*, 46 (2), 204-08.

Garred, P, et al. (1997), 'Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin.', *Lancet*, 349 (9047), 236-40.

Garred, P, et al. (2000), 'Two edged role of mannose binding lectin in rheumatoid arthritis: a cross sectional study.', *J Rheumatol*, 27 (1), 26-34.

Giglio, S, et al. (2001), 'Olfactory receptor-gene clusters, genomic-inversion polymorphisms, and common chromosome rearrangements.', *Am J Hum Genet*, 68 (4), 874-83.

Giglio, S, et al. (2002), 'Heterozygous submicroscopic inversions involving olfactory receptor-gene clusters mediate the recurrent t(4;8)(p16;p23) translocation.', *Am J Hum Genet*, 71 (2), 276-85.

Gonzalez, E, et al. (2002), 'HIV-1 infection and AIDS dementia are influenced by a mutant MCP-1 allele linked to increased monocyte infiltration of tissues and MCP-1 levels.', *Proc Natl Acad Sci U S A*, 99 (21), 13795-800.

Gonzalez, E, et al. (2005), 'The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility.', *Science*, 307 (5714), 1434-40.

Gonzalez, GC, DL Caamano, and AB Schryvers (1990), 'Identification and characterization of a porcine-specific transferrin receptor in Actinobacillus pleuropneumoniae.', *Mol Microbiol*, 4 (7), 1173-79.

Gould, SJ, AM Booth, and JE Hildreth (2003), 'The Trojan exosome hypothesis.', *Proc Natl Acad Sci U S A*, 100 (19), 10592-97.

Groisman, EA (1996), 'Bacterial responses to host-defense peptides.', *Trends Microbiol*, 4 (4), 127-8; discussion 128-9.

Guo, CJ, et al. (2004), 'Alpha-defensins inhibit HIV infection of macrophages through upregulation of CC-chemokines.', *AIDS*, 18 (8), 1217-18.

Hakomori, S (2001), 'Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines.', *Adv Exp Med Biol*, 491 369-402.

Hancock, RE and G Diamond (2000), 'The role of cationic antimicrobial peptides in innate host defences.', *Trends Microbiol*, 8 (9), 402-10.

Harder, J, et al. (1997), 'A peptide antibiotic from human skin.', *Nature*, 387(6636) (6636), 861.

Harder, J, et al. (2001), 'Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic.', *J Biol Chem*, 276 (8), 5707-13.

Harmsen, MC, et al. (1995), 'Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro.', *J Infect Dis*, 172 (2), 380-88.

Heggelund, L, et al. (2005), 'Modulatory effect of mannose-binding lectin on cytokine responses: possible roles in HIV infection.', *Eur J Clin Invest*, 35 (12), 765-70.

Henzler Wildman, KA, DK Lee, and A Ramamoorthy (2003), 'Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37.', *Biochemistry*, 42 (21), 6545-58.

Herrington, DA and PF Sparling (1985), 'Haemophilus influenzae can use human transferrin as a sole source for required iron.', *Infect Immun*, 48 (1), 248-51.

Higazi, AA, et al. (1996), 'Defensin modulates tissue-type plasminogen activator and plasminogen binding to fibrin and endothelial cells.', *J Biol Chem*, 271 (30), 17650-55.

Higazi, AA, et al. (2000), 'The alpha-defensins stimulate proteoglycan-dependent catabolism of low-density lipoprotein by vascular cells: a new class of inflammatory apolipoprotein and a possible contributor to atherogenesis.', *Blood*, 96 (4), 1393-98.

Hill, AV (1996), 'Genetic susceptibility to malaria and other infectious diseases: from the MHC to the whole genome.', *Parasitology*, 112 Suppl S75-84.

Hill, CP, et al. (1991), 'Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization.', *Science*, 251 (5000), 1481-85.

Hogan, CM and SM Hammer (2001), 'Host determinants in HIV infection and disease. Part 2: genetic factors and implications for antiretroviral therapeutics.', *Ann Intern Med*, 134 (10), 978-96.

Hollox, EJ, JA Armour, and JC Barber (2003), 'Extensive normal copy number variation of a beta-defensin antimicrobial-gene cluster.', *Am J Hum Genet*, 73 (3), 591-600.

Hollox, EJ, et al. (2005), 'Beta-defensin genomic copy number is not a modifier locus for cystic fibrosis.', *J Negat Results Biomed*, 4 9.

Howell, MD, et al. (2004), 'Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum.', *J Immunol*, 172 (3), 1763-67.

Hristova, K, ME Selsted, and SH White (1997), 'Critical role of lipid composition in membrane permeabilization by rabbit neutrophil defensins.', *J Biol Chem*, 272 (39), 24224-33.

Huang, YF, et al. (2003), 'Increased frequency of the mannose-binding lectin LX haplotype in Chinese systemic lupus erythematosus patients.', *Eur J Immunogenet*, 30 (2), 121-24.

Hundt, M, H Heiken, and RE Schmidt (2000), 'Low mannose-binding lectin serum concentrations in HIV long-term nonprogressors?', *AIDS Res Hum Retroviruses*, 16(17) (17), 1927.

Huttner, KM and CL Bevins (1999), 'Antimicrobial peptides as mediators of epithelial host defense.', *Pediatr Res*, 45 (6), 785-94.

Hwang, PM, et al. (1998), 'Three-dimensional solution structure of lactoferricin B, an antimicrobial peptide derived from bovine lactoferrin.', *Biochemistry*, 37 (12), 4288-98.

Ikeda, K, et al. (1987), 'Serum lectin with known structure activates complement through the classical pathway.', *J Biol Chem*, 262 (16), 7451-54.

Ioannidis, JP, et al. (2001), 'Effects of CCR5-Delta32, CCR2-64I, and SDF-1 3'A alleles on HIV-1 disease progression: An international meta-analysis of individual-patient data.', *Ann Intern Med*, 135 (9), 782-95.

Iobst, ST, et al. (1994), 'Binding of sugar ligands to Ca(2+)-dependent animal lectins. I. Analysis of mannose binding by site-directed mutagenesis and NMR.', *J Biol Chem*, 269 (22), 15505-11.

Jan, MS, et al. (2006), 'CC chemokines induce neutrophils to chemotaxis, degranulation, and alpha-defensin release.', *J Acquir Immune Defic Syndr*, 41 (1), 6-16.

John, GC, et al. (2000), 'Maternal SDF1 3'A polymorphism is associated with increased perinatal human immunodeficiency virus type 1 transmission.', *J Virol*, 74 (12), 5736-39.

Jones, DE and CL Bevins (1993), 'Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel.', *FEBS Lett*, 315 (2), 187-92.

Jordan, WJ, et al. (2005), 'A non-conservative, coding single-nucleotide polymorphism in the N-terminal region of lactoferrin is associated with aggressive periodontitis

in an African-American, but not a Caucasian population.', *Genes Immun*, 6 (7), 632-35.

Jurevic, RJ, et al. (2002), 'Single-nucleotide polymorphisms and haplotype analysis in beta-defensin genes in different ethnic populations.', *Genet Test*, 6 (4), 261-69.

Kalmar, JR and RR Arnold (1988), 'Killing of Actinobacillus actinomycetemcomitans by human lactoferrin.', *Infect Immun*, 56 (10), 2552-57.

Kamysz, W, M Okroj, and J Lukasiak (2003), 'Novel properties of antimicrobial peptides.', *Acta Biochim Pol*, 50 (2), 461-69.

Kaslow, RA, T Dorak, and JJ Tang (2005), 'Influence of host genetic variation on susceptibility to HIV type 1 infection.', *J Infect Dis*, 191 Suppl 1 S68-77.

Kaul, R, et al. (2000), 'HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi.', *J Immunol*, 164 (3), 1602-11.

Kerr, MA (1980), 'The human complement system: assembly of the classical pathway C3 convertase.', *Biochem J*, 189 (1), 173-81.

Kim, SJ, et al. (1998), 'Structure of the human lactoferrin gene and its chromosomal localization.', *Mol Cells*, 8 (6), 663-68.

King, AE, et al. (2003), 'Differential expression of the natural antimicrobials, beta-defensins 3 and 4, in human endometrium.', *J Reprod Immunol*, 59 (1), 1-16.

Klickstein, LB, et al. (1997), 'Complement receptor type 1 (CR1, CD35) is a receptor for C1q.', *Immunity*, 7 (3), 345-55.

Klotman, ME and TL Chang (2006), 'Defensins in innate antiviral immunity.', *Nat Rev Immunol*, 6 (6), 447-56.

Kougias, P, et al. (2005), 'Defensins and cathelicidins: neutrophil peptides with roles in inflammation, hyperlipidemia and atherosclerosis.', *J Cell Mol Med*, 9 (1), 3-10.

Kuhlman, M, K Joiner, and RA Ezekowitz (1989), 'The human mannose-binding protein functions as an opsonin.', *J Exp Med*, 169 (5), 1733-45.

Laquerre, S, et al. (1998), 'Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread.', *J Virol*, 72 (7), 6119-30.

Larrick, JW, et al. (1996), 'Structural, functional analysis and localization of the human CAP18 gene.', *FEBS Lett*, 398 (1), 74-80.

Larsen, F, et al. (2004), 'Disease-associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein.', *J Biol Chem*, 279 (20), 21302-11.

Leem, JY, et al. (1996), 'Purification and characterization of N-beta-alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine, a novel antibacterial substance of Sarcophaga peregrina (flesh fly).', *J Biol Chem*, 271 (23), 13573-77.

Lehrer, RI, AK Lichtenstein, and T Ganz (1993), 'Defensins: antimicrobial and cytotoxic peptides of mammalian cells.', *Annu Rev Immunol*, 11 105-28.

Lehrer, RI (2004), 'Primate defensins.', *Nat Rev Microbiol*, 2 (9), 727-38.

Leikina, E, et al. (2005), 'Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins.', *Nat Immunol*, 6 (10), 995-1001.

Levay, PF and M Viljoen (1995), 'Lactoferrin: a general review.', *Haematologica*, 80 (3), 252-67.

Levy, H, et al. (2005), 'Association of defensin beta-1 gene polymorphisms with asthma.', *J Allergy Clin Immunol*, 115 (2), 252-58.

Lichtenstein, A, et al. (1986), 'In vitro tumor cell cytolysis mediated by peptide defensins of human and rabbit granulocytes.', *Blood*, 68 (6), 1407-10.

Linzmeier, R, et al. (1999), 'A 450-kb contig of defensin genes on human chromosome 8p23.', *Gene*, 233 (1-2), 205-11.

Linzmeier, RM and T Ganz (2005), 'Human defensin gene copy number polymorphisms: comprehensive analysis of independent variation in alpha- and beta-defensin regions at 8p22-p23.', *Genomics*, 86 (4), 423-30.

Linzmeier, RM and T Ganz (2006), 'Copy number polymorphisms are not a common feature of innate immune genes.', *Genomics*, 88 (1), 122-26.

Lipscombe, RJ, et al. (1992), 'High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene.', *Hum Mol Genet*, 1 (9), 709-15.

Liu, H, et al. (1999), 'Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression.', *Proc Natl Acad Sci U S A*, 96 (8), 4581-85.

Lu, W, et al. (2004), 'Therapeutic dendritic-cell vaccine for chronic HIV-1 infection.', *Nat Med*, 10 (12), 1359-65.

Ma, Y, et al. (1999), 'Antitumor activity of mannan-binding protein in vivo as revealed by a virus expression system: mannan-binding proteindependent cell-mediated cytotoxicity.', *Proc Natl Acad Sci U S A*, 96 (2), 371-75.

Maas, J, et al. (1998), 'Presence of the variant mannose-binding lectin alleles associated with slower progression to AIDS. Amsterdam Cohort Study.', *AIDS*, 12 (17), 2275-80.

MacDonald, KS, et al. (1998), 'Mother-child class I HLA concordance increases perinatal human immunodeficiency virus type 1 transmission.', *J Infect Dis*, 177 (3), 551-56.

MacDonald, KS, et al. (2000), 'Influence of HLA supertypes on susceptibility and resistance to human immunodeficiency virus type 1 infection.', *J Infect Dis*, 181 (5), 1581-89.

Mackewicz, CE, et al. (2003), 'alpha-Defensins can have anti-HIV activity but are not CD8 cell anti-HIV factors.', *AIDS*, 17 (14), F23-32.

Madsen, HO, et al. (1994), 'A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein.', *Immunogenetics*, 40 (1), 37-44.

Madsen, HO, et al. (1995), 'Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein.', *J Immunol*, 155 (6), 3013-20.

Malhotra, R, et al. (1990), 'Human leukocyte C1q receptor binds other soluble proteins with collagen domains.', *J Exp Med*, 172 (3), 955-59.

Malik, S, et al. (2003), 'Absence of association between mannose-binding lectin gene polymorphisms and HIV-1 infection in a Colombian population.', *Immunogenetics*, 55 (1), 49-52.

Mars, WM, et al. (1995), 'Inheritance of unequal numbers of the genes encoding the human neutrophil defensins HP-1 and HP-3.', *J Biol Chem*, 270 (51), 30371-76.

Martin, MP, et al. (1998), 'Genetic acceleration of AIDS progression by a promoter variant of CCR5.', *Science*, 282 (5395), 1907-11.

Martin, MP, et al. (2002), 'Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS.', *Nat Genet*, 31 (4), 429-34.

Masson, PL and JF Heremans (1971), 'Lactoferrin in milk from different species.', *Comp Biochem Physiol B*, 39 (1), 119-29.

Matsushita, I, et al. (2002), 'Genetic variants of human beta-defensin-1 and chronic obstructive pulmonary disease.', *Biochem Biophys Res Commun*, 291 (1), 17-22.

Matsushita, M, et al. (1998), 'Hepatitis C virus infection and mutations of mannose-binding lectin gene MBL.', *Arch Virol*, 143 (4), 645-51.

McBride, MO, et al. (1998), 'Mannose-binding protein in HIV-seropositive patients does not contribute to disease progression or bacterial infections.', *Int J STD AIDS*, 9 (11), 683-88.

McDermott, DH, et al. (1998), 'CCR5 promoter polymorphism and HIV-1 disease progression. Multicenter AIDS Cohort Study (MACS).', *Lancet*, 352 (9131), 866-70.

McDermott, DH, et al. (2000), 'Chemokine RANTES promoter polymorphism affects risk of both HIV infection and disease progression in the Multicenter AIDS Cohort Study.', *AIDS*, 14 (17), 2671-78.

McDermott, DH, et al. (2000), 'Genetic polymorphism in CX3CR1 and risk of HIV disease.', *Science*, 290 (5499), 2031.

Message, SD and SL Johnston (2004), 'Host defense function of the airway epithelium in health and disease: clinical background.', *J Leukoc Biol*, 75 (1), 5-17.

Metz-Boutigue, MH, et al. (1984), 'Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins.', *Eur J Biochem*, 145 (3), 659-76.

Meyer, L, et al. (1997), 'Early protective effect of CCR-5 delta 32 heterozygosity on HIV-1 disease progression: relationship with viral load. The SEROCO Study Group.', *AIDS*, 11 (11), F73-8.

Misrahi, M, et al. (1998), 'CCR5 chemokine receptor variant in HIV-1 mother-to-child transmission and disease progression in children. French Pediatric HIV Infection Study Group.', *JAMA*, 279 (4), 277-80.

Mohamed, JA, et al. (2007), 'A novel single-nucleotide polymorphism in the lactoferrin gene is associated with susceptibility to diarrhea in North American travelers to Mexico.', *Clin Infect Dis*, 44 (7), 945-52.

Moore, CB, et al. (2002), 'Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level.', *Science*, 296 (5572), 1439-43.

Moriuchi, M and H Moriuchi (2001), 'A milk protein lactoferrin enhances human T cell leukemia virus type I and suppresses HIV-1 infection.', *J Immunol*, 166 (6), 4231-36.

Mummidi, S, et al. (1998), 'Genealogy of the CCR5 locus and chemokine system gene variants associated with altered rates of HIV-1 disease progression.', *Nat Med*, 4 (7), 786-93.

Murphy, CJ, et al. (1993), 'Defensins are mitogenic for epithelial cells and fibroblasts.', *J Cell Physiol*, 155 (2), 408-13.

Murphy, PM (1996), 'Chemokine receptors: structure, function and role in microbial pathogenesis.', *Cytokine Growth Factor Rev*, 7 (1), 47-64.

Naidu, SS, et al. (1993), 'Relationship between antibacterial activity and porin binding of lactoferrin in Escherichia coli and Salmonella typhimurium.', *Antimicrob Agents Chemother*, 37 (2), 240-45.

Nakagawa, T, et al. (2003), 'Antitumor activity of mannan-binding protein.', *Methods Enzymol*, 363 26-33.

Nakashima, H, et al. (1993), 'Defensins inhibit HIV replication in vitro.', *AIDS*, 7(8) (8), 1129.

Nakayama, EE, et al. (2000), 'Polymorphism in the interleukin-4 promoter affects acquisition of human immunodeficiency virus type 1 syncytium-inducing phenotype.', *J Virol*, 74 (12), 5452-59.

Nakayama, EE, et al. (2002), 'Protective effect of interleukin-4 -589T polymorphism on human immunodeficiency virus type 1 disease progression: relationship with virus load.', *J Infect Dis*, 185 (8), 1183-86.

Nassar, T, et al. (2002), 'Human alpha-defensin regulates smooth muscle cell contraction: a role for low-density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor.', *Blood*, 100 (12), 4026-32.

Ng, TB, et al. (2001), 'Inhibition of human immunodeficiency virus type 1 reverse transcriptase, protease and integrase by bovine milk proteins.', *Life Sci*, 69 (19), 2217-23.

Nielsen, SL, et al. (1995), 'The level of the serum opsonin, mannan-binding protein in HIV-1 antibody-positive patients.', *Clin Exp Immunol*, 100 (2), 219-22.

Niyonsaba, F, et al. (2003), 'Epithelial cell-derived antibacterial peptides human beta-defensins and cathelicidin: multifunctional activities on mast cells.', *Curr Drug Targets Inflamm Allergy*, 2 (3), 224-31.

O'Malley, DP and PD Storto (1999), 'Confirmation of the chromosome 8p23.1 euchromatic duplication as a variant with no clinical manifestations.', *Prenat Diagn*, 19 (2), 183-84.

Odell, EW, et al. (1996), 'Antibacterial activity of peptides homologous to a loop region in human lactoferrin.', *FEBS Lett*, 382 (1-2), 175-78.

Ogden, CA, et al. (2001), 'C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells.', *J Exp Med*, 194 (6), 781-95.

Okrent, DG, AK Lichtenstein, and T Ganz (1990), 'Direct cytotoxicity of polymorphonuclear leukocyte granule proteins to human lung-derived cells and endothelial cells.', *Am Rev Respir Dis*, 141 (1), 179-85.

Okumura, K, et al. (2004), 'C-terminal domain of human CAP18 antimicrobial peptide induces apoptosis in oral squamous cell carcinoma SAS-H1 cells.', *Cancer Lett*, 212 (2), 185-94.

Olesen, HV, et al. (2006), 'The mannan-binding lectin pathway and lung disease in cystic fibrosis--disfunction of mannan-binding lectin-associated serine protease 2 (MASP-2) may be a major modifier.', *Clin Immunol*, 121 (3), 324-31.

Oppenheim, JJ, et al. (2003), 'Roles of antimicrobial peptides such as defensins in innate and adaptive immunity.', *Ann Rheum Dis*, 62 Suppl 2 ii17-21.

Ortega, MR, T Ganz, and SM Milner (2000), 'Human beta defensin is absent in burn blister fluid.', *Burns*, 26 (8), 724-26.

Pareja, E, et al. (1997), 'The tetramer model: a new view of class II MHC molecules in antigenic presentation to T cells.', *Tissue Antigens*, 50 (5), 421-28.

Park, CB, HS Kim, and SC Kim (1998), 'Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions.', *Biochem Biophys Res Commun*, 244 (1), 253-57.

Petros, J (2007), 'Transcriptional Effect of DEFB1 Gene 5' Untranslated Region Polymorphisms.', *Cancer Res*, 67 (12), 5997.

Pirhonen, J, et al. (1999), 'Virus infection activates IL-1 beta and IL-18 production in human macrophages by a caspase-1-dependent pathway.', *J Immunol*, 162 (12), 7322-29.

Polycarpou, A, et al. (2002), 'Association between maternal and infant class I and II HLA alleles and of their concordance with the risk of perinatal HIV type 1 transmission.', *AIDS Res Hum Retroviruses*, 18 (11), 741-46.

Prohaszka, Z, et al. (1997), 'Mannan-binding lectin serum concentrations in HIV-infected patients are influenced by the stage of disease.', *Immunol Lett*, 58 (3), 171-75.

Quinones-Mateu, ME, et al. (2003), 'Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication.', *AIDS*, 17 (16), F39-48.

Reddy, KV, RD Yedery, and C Aranha (2004), 'Antimicrobial peptides: premises and promises.', *Int J Antimicrob Agents*, 24 (6), 536-47.

Rigopoulos, D, et al. (2007), 'AIDS in pregnancy, part II: Treatment in the era of highly active antiretroviral therapy and management of obstetric, anesthetic, and pediatric issues.', *Skinmed*, 6 (2), 79-84.

Robinson, D, NC Phillips, and B Winchester (1975), 'Affinity chromatography of human liver alpha-D-mannosidase.', *FEBS Lett*, 53 (1), 110-12.

Saito, H, et al. (1991), 'Potent bactericidal activity of bovine lactoferrin hydrolysate produced by heat treatment at acidic pH.', *J Dairy Sci*, 74 (11), 3724-30.

Salvatore, F, O Scudiero, and G Castaldo (2002), 'Genotype-phenotype correlation in cystic fibrosis: the role of modifier genes.', *Am J Med Genet*, 111 (1), 88-95.

Samson, M, et al. (1996), 'Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene.', *Nature*, 382 (6593), 722-25.

Santos, IK, et al. (2001), 'Mannan-binding lectin enhances susceptibility to visceral leishmaniasis.', *Infect Immun*, 69 (8), 5212-15.

Schibli, DJ, PM Hwang, and HJ Vogel (1999), 'The structure of the antimicrobial active center of lactoferricin B bound to sodium dodecyl sulfate micelles.', *FEBS Lett*, 446 (2-3), 213-17.

Schouten, JP, et al. (2002), 'Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification.', *Nucleic Acids Res*, 30 (12), e57.

Schutte, BC and PB Jr McCray (2002), '(beta)-defensins in lung host defense.', *Annu Rev Physiol*, 64 709-48.

Schwaeble, W, et al. (2002), 'The mannan-binding lectin-associated serine proteases (MASPs) and MAp19: four components of the lectin pathway activation complex encoded by two genes.', *Immunobiology*, 205 (4-5), 455-66.

Segat, L, et al. (2006), 'IL-18 gene promoter polymorphism is involved in HIV-1 infection in a Brazilian pediatric population.', *Immunogenetics*, 58 (5-6), 471-73.

Sei, S, et al. (2001), 'Protective effect of CCR5 delta 32 heterozygosity is restricted by SDF-1 genotype in children with HIV-1 infection.', *AIDS*, 15 (11), 1343-52.

Senaldi, G, et al. (1995), 'Circulating levels of mannose binding protein in human immunodeficiency virus infection.', *J Infect*, 31 (2), 145-48.

Seyfarth, J, P Garred, and HO Madsen (2006), 'Extra-hepatic transcription of the human mannose-binding lectin gene (mbl2) and the MBL-associated serine protease 1-3 genes.', *Mol Immunol*, 43 (7), 962-71.

Shimokawa, O, et al. (2004), 'Molecular characterization of inv dup del(8p): analysis of five cases.', *Am J Med Genet A*, 128 (2), 133-37.

Shin, HD, et al. (2000), 'Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10.', *Proc Natl Acad Sci U S A*, 97 (26), 14467-72.

Singh, A, et al. (1988), 'Structure of a novel human granulocyte peptide with anti-ACTH activity.', *Biochem Biophys Res Commun*, 155 (1), 524-29.

Soell, M, et al. (1995), 'Activation of human monocytes by streptococcal rhamnose glucose polymers is mediated by CD14 antigen, and mannan binding protein inhibits TNF-alpha release.', *J Immunol*, 154 (2), 851-60.

Sorensen, R, S Thiel, and JC Jensenius (2005), 'Mannan-binding-lectin-associated serine proteases, characteristics and disease associations.', *Springer Semin Immunopathol*, 27 (3), 299-319.

Steinstraesser, L, et al. (2005), 'Inhibition of early steps in the lentiviral replication cycle by cathelicidin host defense peptides.', *Retrovirology*, 2 2.

Stephens, JC, et al. (1998), 'Dating the origin of the CCR5-Delta32 AIDS-resistance allele by the coalescence of haplotypes.', *Am J Hum Genet*, 62 (6), 1507-15.

Stover, CM, et al. (1999), 'Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene.', *J Immunol*, 162 (6), 3481-90.

Stylianou, E, et al. (2003), 'Raised serum levels of interleukin-18 is associated with disease progression and may contribute to virological treatment failure in HIV-1-infected patients.', *Clin Exp Immunol*, 132 (3), 462-66.

Sugawara, H, et al. (2003), 'Complex low-copy repeats associated with a common polymorphic inversion at human chromosome 8p23.', *Genomics*, 82 (2), 238-44.

Sumiya, M, et al. (1991), 'Molecular basis of opsonic defect in immunodeficient children.', *Lancet*, 337 (8757), 1569-70.

Summerfield, JA, et al. (1995), 'Mannose binding protein gene mutations associated with unusual and severe infections in adults.', *Lancet*, 345 (8954), 886-89.

Sun, CQ, et al. (2006), 'Human {beta}-Defensin-1, a Potential Chromosome 8p Tumor Suppressor: Control of Transcription and Induction of Apoptosis in Renal Cell Carcinoma.', *Cancer Res*, 66 (17), 8542-49.

Sun, L, et al. (2005), 'Human beta-defensins suppress human immunodeficiency virus infection: potential role in mucosal protection.', *J Virol*, 79 (22), 14318-29.

Super, M, et al. (1989), 'Association of low levels of mannan-binding protein with a common defect of opsonisation.', *Lancet*, 2 (8674), 1236-39.

Swart, PJ, et al. (1998), 'Lactoferrin. Antiviral activity of lactoferrin.', *Adv Exp Med Biol*, 443 205-13.

Takahashi, M, et al. (1999), 'A truncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alternative polyadenylation is a component of the lectin complement pathway.', *Int Immunol*, 11 (5), 859-63.

Tanabe, H, et al. (2004), 'Differential effects on human immunodeficiency virus type 1 replication by alpha-defensins with comparable bactericidal activities.', *J Virol*, 78 (21), 11622-31.

Tanaka-Kataoka, M, et al. (1999), 'In vivo antiviral effect of interleukin 18 in a mouse model of vaccinia virus infection.', *Cytokine*, 11 (8), 593-99.

Taudien, S, et al. (2004), 'Polymorphic segmental duplications at 8p23.1 challenge the determination of individual defensin gene repertoires and the assembly of a contiguous human reference sequence.', *BMC Genomics*, 5 (1), 92.

Teng, CT and W Gladwell (2006), 'Single nucleotide polymorphisms (SNPs) in human lactoferrin gene.', *Biochem Cell Biol*, 84 (3), 381-84.

Tenner, AJ, SL Robinson, and RA Ezekowitz (1995), 'Mannose binding protein (MBP) enhances mononuclear phagocyte function via a receptor that contains the 126,000 M(r) component of the C1q receptor.', *Immunity*, 3 (4), 485-93.

Territo, MC, et al. (1989), 'Monocyte-chemotactic activity of defensins from human neutrophils.', *J Clin Invest*, 84 (6), 2017-20.

Theodorou, I, et al. (1997), 'HIV-1 infection in an individual homozygous for CCR5 delta 32. Seroco Study Group.', *Lancet*, 349(9060) (9060), 1219-20.

Thiel, S, et al. (1992), 'The concentration of the C-type lectin, mannan-binding protein, in human plasma increases during an acute phase response.', *Clin Exp Immunol*, 90 (1), 31-35.

Thiel, S, et al. (1997), 'A second serine protease associated with mannan-binding lectin that activates complement.', *Nature*, 386 (6624), 506-10.

Thiel, S, et al. (2007), 'Deficiency of mannan-binding lectin associated serine protease-2 due to missense polymorphisms.', *Genes Immun*, 8 (2), 154-63.

Turner, J, et al. (1998), 'Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils.', *Antimicrob Agents Chemother*, 42 (9), 2206-14.

Turner, MW (1996), 'Mannose-binding lectin: the pluripotent molecule of the innate immune system.', *Immunol Today*, 17 (11), 532-40.

Turner, MW and RM Hamvas (2000), 'Mannose-binding lectin: structure, function, genetics and disease associations.', *Rev Immunogenet*, 2 (3), 305-22.

Turner, MW (2003), 'The role of mannose-binding lectin in health and disease.', *Mol Immunol*, 40 (7), 423-29.

Van Wetering, S, et al. (1997), 'Effect of neutrophil serine proteinases and defensins on lung epithelial cells: modulation of cytotoxicity and IL-8 production.', *J Leukoc Biol*, 62 (2), 217-26.

Vankeerberghen, A, et al. (2005), 'Distribution of human beta-defensin polymorphisms in various control and cystic fibrosis populations.', *Genomics*, 85 (5), 574-81.

Velasco, M, et al. (1997), 'Macrophage triggering with cecropin A and melittin-derived peptides induces type II nitric oxide synthase expression.', *J Immunol*, 158 (9), 4437-43.

Velliyagounder, K, et al. (2003), 'One of two human lactoferrin variants exhibits increased antibacterial and transcriptional activation activities and is associated with localized juvenile periodontitis.', *Infect Immun*, 71 (11), 6141-47.

Wallis, R and JY Cheng (1999), 'Molecular defects in variant forms of mannose-binding protein associated with immunodeficiency.', *J Immunol*, 163 (9), 4953-59.

Wang, H, X Ye, and TB Ng (2000), 'First demonstration of an inhibitory activity of milk proteins against human immunodeficiency virus-1 reverse transcriptase and the effect of succinylation.', *Life Sci*, 67 (22), 2745-52.

Wang, W, et al. (2004), 'Activity of alpha- and theta-defensins against primary isolates of HIV-1.', *J Immunol*, 173 (1), 515-20.

Wang, Z and G Wang (2004), 'APD: the Antimicrobial Peptide Database.', *Nucleic Acids Res*, 32 (Database issue), D590-2.

Weiss, G, HO Madsen, and P Garred (2007), 'A novel mannose-binding lectin-associated serine protease 1/3 gene variant.', *Scand J Immunol*, 65 (5), 430-34.

Wiercinska-Drapalo, A, et al. (2004), 'Plasma interleukin-18 is associated with viral load and disease progression in HIV-1-infected patients.', *Microbes Infect*, 6 (14), 1273-77.

Wild, J, D Robinson, and B Winchester (1983), 'Isolation of mannose-binding proteins from human and rat liver.', *Biochem J*, 210 (1), 167-74.

Williams, A, CA Peh, and T Elliott (2002), 'The cell biology of MHC class I antigen presentation.', *Tissue Antigens*, 59 (1), 3-17.

Wu, Z, et al. (2005), 'Human neutrophil alpha-defensin 4 inhibits HIV-1 infection in vitro.', *FEBS Lett*, 579 (1), 162-66.

Yamaguchi, Y, et al. (2002), 'Identification of multiple novel epididymis-specific beta-defensin isoforms in humans and mice.', *J Immunol*, 169 (5), 2516-23.

Yamauchi, K, et al. (1993), 'Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment.', *Infect Immun*, 61 (2), 719-28.

Yang, D, et al. (1999), 'Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6.', *Science*, 286 (5439), 525-28.

Yang, D, et al. (2000), 'Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells.', *J Leukoc Biol*, 68 (1), 9-14.

Yang, D, et al. (2004), 'Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense.', *Annu Rev Immunol*, 22 181-215.

Yenugu, S, et al. (2004), 'The androgen-regulated epididymal sperm-binding protein, human beta-defensin 118 (DEFB118) (formerly ESC42), is an antimicrobial beta-defensin.', *Endocrinology*, 145 (7), 3165-73.

Yi, M, et al. (1997), 'Hepatitis C virus envelope proteins bind lactoferrin.', *J Virol*, 71 (8), 5997-6002.

Ying, H, et al. (2004), 'Interaction of mannose-binding lectin with HIV type 1 is sufficient for virus opsonization but not neutralization.', *AIDS Res Hum Retroviruses*, 20 (3), 327-35.

Yoo, YC, et al. (1997), 'Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk protein-derived peptide: involvement of reactive oxygen species.', *Biochem Biophys Res Commun*, 237 (3), 624-28.

Zanetti, M (2004), 'Cathelicidins, multifunctional peptides of the innate immunity.', *J Leukoc Biol*, 75 (1), 39-48.

Zhang, L, et al. (2002), 'Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor.', *Science*, 298 (5595), 995-1000.

Zimmerman, PA, et al. (1997), 'Inherited resistance to HIV-1 conferred by an inactivating mutation in CC chemokine receptor 5: studies in populations with contrasting clinical phenotypes, defined racial background, and quantified risk.', *Mol Med*, 3 (1), 23-36.

Zimmermann, GR, et al. (1995), 'Solution structure of bovine neutrophil beta-defensin-12: the peptide fold of the beta-defensins is identical to that of the classical defensins.', *Biochemistry*, 34 (41), 13663-71.

Zlotnik, A and O Yoshie (2000), 'Chemokines: a new classification system and their role in immunity.', *Immunity*, 12 (2), 121-27.

Zuccotti, GV, et al. (2006), 'Oral lactoferrin in HIV-1 vertically infected children: an observational follow-up of plasma viral load and immune parameters.', *J Int Med Res*, 34 (1), 88-94.

Zundel, S, et al. (2004), 'Characterization of recombinant mannan-binding lectin-associated serine protease (MASP)-3 suggests an activation mechanism different from that of MASP-1 and MASP-2.', *J Immunol*, 172 (7), 4342-50.

REPRINTS OF PAPERS INCLUDES IN THE THESIS

serum creatinine alone [8], serum potassium and phosphate levels, and a urinalysis to detect proteinuria and glucosuria [6,7]. Assessment for increased urinary frequency may be helpful in detecting treated patients who may be at risk. The associated consequences of tenofovir-related nephrotoxicity and hypokalemia may be profound and life threatening.

Acknowledgements

The authors would like to thank Kendall Marcus, MD, from the Center for Drug Evaluation and Research, Food and Drug Administration, for her assistance in providing the individual safety report numbers for hypokalemia among individuals taking tenofovir.

The views expressed in this article are those of the authors and do not necessarily represent the views of the Department of Veterans Affairs.

Infectious Diseases Section, Veterans Affairs Medical Center, Washington, DC, USA.

Received: 20 April 2006; accepted: 19 May 2006.

References

1. Viread (tenofovir disoproxil fumarate) package insert. Foster City, CA: Gilead Sciences; March 2006.
2. Fine DM. **Editorial comment: tenofovir nephrotoxicity – vigilance required.** *AIDS Reader* 2005; **15**:362–363.
3. Nelson M, Cooper D, Schooley R, Katlama C, Montaner J, Curtis S, et al. The safety of tenofovir DF for the treatment of HIV infection: the first 4 years. In: Abstracts of the 13th Conference on Retroviruses and Opportunistic Infections. Denver, CO, 5–9 February 2006 [A781].
4. Zimmerman AE, Pizzoferrato T, Bedford J, Morris A, Hoffman R, Braden G. **Tenofovir-associated acute and chronic kidney disease: a case of multiple drug interactions.** *Clin Infect Dis* 2006; **42**:283–290.
5. Food and Drug Administration. Viread: NDA 21-346/S-006. Rockville, MD: Food and Drug Administration; 2003.
6. Peyriere H, Reynes J, Rouanet I, Daniel N, deBoever CM, Mauboussin JM, *et al*. **Renal tubular dysfunction associated with tenofovir therapy: report of 7 cases.** *J Acquir Immune Defic Syndr* 2004; **35**:269–273.
7. Gaspar G, Monereo A, Garcia-Reyne A, de Guzman M. **Fanconi syndrome and acute renal failure in a patient treated with tenofovir: a call for caution.** *AIDS* 2004; **18**:351–352.
8. Galland JE, Parish MA, Keruly JC, Moore RD. **Changes in renal function associated with tenofovir disoproxil fumarate treatment, compared with nucleoside reverse-transcriptase inhibitor treatment.** *Clin Infect Dis* 2005; **40**:1194–1198.

DEFB1 gene polymorphisms and increased risk of HIV-1 infection in Brazilian children

Michele Milanese[a], Ludovica Segat[a], Alessandra Pontillo[a], Luiz Claudio Arraes[b,c], José Luiz de Lima Filho[c] and Sergio Crovella[a,c]

In our study we analysed three single nucleotide polymorphisms (SNPs) in the 5′ untranslated region (UTR) of the DEFB1 gene, namely −52(G/A) −44(C/G) and −20(G/A), in three groups of north-eastern Brazilian children in order to assess their role in HIV-1 infection. Our results allowed us to hypothesize that the SNPs located in the 5′ UTR of the DEFB1 gene can be employed as a marker of risk for HIV-1 infection.

Beta defensins are small secreted antimicrobial peptides that are components of innate immunity [1]. A role for beta defensins in contrasting infection from HIV-1 has been reported by Quiñones-Mateu *et al*. [2], who suggested that human beta defensin (hBD) 2 and hBD-3 are able to protect GHOST X4/R5 cells from being infected, by directly binding to the viral envelope. A similar effect, in which beta defensins interact with surface carbohydrates, has been shown by Leikina *et al*. [3]. Moreover Sun *et al*. [4] hypothesized an involvement of beta-defensins in HIV-1 oral transmission, emphasizing their protective role in the oral mucosa.

Despite the fact that Quiñones-Mateu *et al*. [2] were not able to prove a role for hBD-1 in protecting cells from infection by HIV-1, Braida *et al*. [5] recently reported an association between the −44(C/G) single nucleotide polymorphism (SNP) in the 5′ untranslated region (UTR) of the DEFB1 gene, encoding for the hBD-1 peptide, and HIV-1 infection in Italian children.

In our study we analysed the same SNPs in the 5′ UTR of the DEFB1 gene, −52(G/A), −44(C/G) and −20(G/A) (rs1799946, rs1800972, and rs11362, respectively), as reported by Braida *et al*. [5] in three groups of north-eastern Brazilian children in order to assess the role of these polymorphisms in HIV-1 infection when looking at ethnic groups other that the Caucasians studied by Braida *et al*. [5].

We enrolled 128 HIV-1 perinatally infected children (average age 4 years, range 0–13) and 60 exposed uninfected children (average age 7 years, range 0–15) born to HIV-1-positive mothers who did not receive antiretroviral therapy during gestation, and did not undergo caesarean section to prevent vertical transmission. We also recruited 115 uninfected and unexposed children (average age 8 years, range 0–19) from the same population as the healthy controls. All children came from the Instituto Materno Infantil do Pernambuco (Recife, Brazil), and informed consent was obtained from their parents.

DNA extraction and DEFB1 SNP genotyping were performed according to Braida *et al*. [5].

The significance of differences in allelic and genotype frequencies was calculated by chi-square test. Yates' continuity correction and Fisher's exact test were employed when appropriate. The correction of *P* values for multiple

Table 1. Frequencies of DEFB1 polymorphisms in children perinatally exposed to HIV-1 (infected and uninfected) and healthy controls.

SNP	HIV-1-infected children ($n = 128$)	HIV-1-exposed uninfected children ($n = 60$)	Healthy controls ($n = 115$)
−52 (G/A)			
G	0.67 (171/256)	0.54 (65/120)	0.54 (125/230)
A	0.33 (85/256)	0.46 (55/120)	0.46 (105/230)
G/G	0.41 (53/128)	0.32 (19/60)	0.30 (34/115)
G/A	0.51 (65/128)	0.45 (27/60)	0.50 (57/115)
A/A	0.08 (10/128)	0.23 (14/60)	0.21 (24/115)
−44 (C/G)			
C	0.93 (237/256)	0.87 (104/120)	0.86 (197/230)
G	0.07 (19/256)	0.13 (16/120)	0.14 (33/230)
C/C	0.85 (109/128)	0.73 (44/60)	0.73 (84/115)
C/G	0.15 (19/128)	0.27 (16/60)	0.25 (29/115)
G/G	0.00 (0/128)	0.00 (0/60)	0.02 (2/115)
−20 (G/A)			
G	0.48 (124/256)	0.58 (70/120)	0.63 (145/230)
A	0.52 (132/256)	0.42 (50/120)	0.37 (85/230)
G/G	0.12 (15/128)	0.33 (20/60)	0.36 (42/115)
G/A	0.73 (94/128)	0.50 (30/60)	0.53 (61/115)
A/A	0.15 (19/128)	0.17 (10/60)	0.10 (12/115)

SNP, Single nucleotide polymorphism.

tests was performed automatically using the R software (www.r-project.org) according to Benjamini and Hochberg [6] in order to keep the false discovery rate under 5%.

The allelic and genotype frequencies for 5′ UTR DEFB1 SNPs in the three groups studied are shown in Table 1. The genotype frequencies follow the Hardy–Weinberg equilibrium in the three populations, with the exception of the polymorphism −20(G/A) in HIV-1-infected children.

DEFB1 genotype −52 A/A was significantly less frequent in HIV-1 perinatally infected children when compared with healthy controls and exposed uninfected children (corrected *P* values are 0.023 and 0.025, respectively), whereas frequencies did not vary between HIV-1-exposed uninfected children and healthy controls. The allelic frequencies of the −52(G/A) SNP in infected children varied significantly when compared with those of healthy controls (corrected *P* value 0.030), but when compared with exposed uninfected children were not statistically significant (corrected *P* value 0.055). Nevertheless, the odd ratios calculated comparing HIV-positive children with exposed uninfected children and healthy controls were not statistically different when tested using bootstrapping techniques, suggesting that the −52 A allele has a similar protective effect in the two groups.

A weaker correlation was found for the −44(C/G) polymorphism in which the *P* values for genotype frequencies did not remain statistically significant after multiple test correction. Allelic frequencies of the −44 G allele were similar between healthy controls and HIV-1-exposed uninfected children, and although a lower frequency of the −44 G allele was found in HIV-1-infected children, the difference was not statistically significant. A stronger association was observed in the −20(G/A) SNP, in which the frequency of the −20 G/G genotype was significantly lower in HIV-1-positive children than in exposed uninfected children and healthy controls (corrected *P* values both < 0.005). Allelic frequencies of the −20 G/A SNP were significantly different between HIV-1-positive children and healthy controls (corrected *P* value 0.015), whereas the significance was not achieved between HIV-1-infected and exposed uninfected children as a result of multiple test correction.

As we did not find any significant difference between the exposed uninfected children and the healthy controls, we pooled the two groups to perform a more powerful analysis using the Haploview software [7], built to perform single SNP and haplotype association tests. We confirmed the results found with the chi-square test, but despite the proximity of the three SNPs and a co-segregation rate of 93% between the −20(G/A) and −44(C/G) SNPs, we did not find evidence of the presence of any haploblock. Multiple regression analysis performed with the R software on the new dataset did not show any significant interaction between the different SNPs.

We have demonstrated that significant correlation exists between SNPs in the 5′ UTR of the DEFB1 gene and the risk of being infected with HIV-1 in Brazilian children. We found a significant increase of the −52 A/A and −20 G/G genotypes among HIV-1-infected children, when compared with healthy controls, and the odd ratios of carrying the −52 A or −20 G allele were, respectively, 1.688 (95% confidence limits 1.151–2.482) and 1.814 (95% confidence limits 1.243–2.655), suggesting a role for these polymorphisms in increasing the susceptibility to infection. We also found a sensible, even if not significant, reduction of the frequency of the −44 G allele: the frequency of this polymorphism was very low in the

Brazilian population when compared with other populations [8], and this fact could account for the lack of statistical significance.

Finally, a particular consideration is deserved for the departure of the −20 G/A allele from the Hardy–Weinberg equilibrium. The excess of G/A heterozygous subjects in the exposed-infected children could possibly be explained by hypothesizing a dominant effect for the A allele, able to increase the susceptibility to HIV-1 infection even if present in a single dose.

In conclusion, our results obtained on Brazilian children confirm that the SNPs located in the 5′ UTR of the DEFB1 gene can be employed as a marker of risk for HIV-1 infection.

[a]*Department of Developmental and Reproductive Sciences, University of Trieste, Trieste, Italy;* [b]*Instituto Materno Infantil de Pernambuco, Recife, PE, Brazil; and* [c]*Laboratorio de Imunopatologia 'Keizo Azami', Universidade Federal de Pernambuco, Recife, PE, Brazil.*

Received: 29 March 2006; accepted: 18 May 2006.

References

1. Kagan BL, Ganz T, Lehrer RI. **Defensins: a family of antimicrobial and cytotoxic peptides.** *Toxicology* 1994; **87**:131–149.
2. Quiñones-Mateu ME, Lederman MM, Feng Z, Chakraborty B, Weber J, Rangel HR, *et al*. **Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication.** *AIDS* 2003; **17**:F39–F48.
3. Leikina E, Delanoe-Ayari H, Melikov K, Cho MS, Chen A, Waring AJ, *et al*. **Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins.** *Nat Immunol* 2005; **6**:995–1001.
4. Sun L, Finnegan CM, Kish-Catalone T, Blumenthal R, Garzino-Demo P, La Terra Maggiore GM, *et al*. **Human beta-defensins suppress human immunodeficiency virus infection: potential role in mucosal protection.** *J Virol* 2005; **79**:14318–14329.
5. Braida L, Boniotto M, Pontillo A, Tovo PA, Amoroso A, Crovella S. **A single-nucleotide polymorphism in the human beta-defensin 1 gene is associated with HIV-1 infection in Italian children.** *AIDS* 2004; **18**:1598–1600.
6. Benjamini Y, Hochberg Y. **Controlling the false discovery rate: a practical and powerful approach to multiple testing.** *J R Stat Soc* 1995; **57**:289–300.
7. Barrett JC, Fry B, Maller J, Daly MJ. **Haploview: analysis and visualization of LD and haplotype maps.** *Bioinformatics* 2005; **21**:263–265.
8. Jurevic RJ, Chrisman P, Mancl L, Livingston R, Dale BA. **Single-nucleotide polymorphisms and haplotype analysis in beta-defensin genes in different ethnic populations.** *Genet Test* 2002; **6**:261–269.

Ezetimibe effectively decreases LDL-cholesterol in HIV-infected patients

Blai Coll[a,b], Gerard Aragonés[a], Sandra Parra[b], Carlos Alonso-Villaverde[b] and Lluis Masana[b]

We tested the security and efficacy of ezetimibe in the treatment of HIV-associated dyslipemia. Twenty HIV-infected patients were randomly assigned to receive ezetimibe 10 mg/day or fluvastatin 80 mg/day. Patients receiving ezetimibe experienced a statistically significant ($P = 0.003$) 20% reduction in the concentration of LDL-cholesterol, similar to that observed with fluvastatin (24%, *P* between groups 0.70). We concluded that ezetimibe monotherapy effectively decreases LDL-cholesterol in HIV-infected patients.

The control of cardiovascular risk factors in HIV-infected patients is relevant because the incidence of myocardial infarction [1] and other atherosclerosis-related events [2] are increasing. Lipid abnormalities are commonly present in this clinical setting [3], but the majority of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, with the exception of pravastatin and fluvastatin, are metabolized by the cytochrome P450 3A4, which in turn is also the metabolic pathway of many of the antiretroviral agents, especially the protease inhibitors (PI) [4]. Recent evidence supports the use of ezetimibe in combination with statins to add a powerful lipid-lowering effect [5]. However, the metabolism of ezetimibe, which is P450 independent, and the low incidence of drug interactions and side-effects, make this drug suitable to be tested in monotherapy in HIV-infected patients.

Patients were enrolled during the 3 months of the inclusion period if they fulfilled the eligibility criteria: more than 6 months on stable HAART, more than 18 years of age, and a fasting LDL-cholesterol concentration of 3.30 mmol/l or greater. Classic cardiovascular risk factors were recorded, and fasting total cholesterol, HDL-cholesterol, triglycerides, and glucose were determined. LDL-cholesterol values were obtained using the Friedewald formula. CD4 and CD8 lymphocyte counts and HIV-1 viral load were determined using standard techniques.

Endothelial function was analysed using peripheral arterial tonometry [6]. Briefly, this system (Itamar Medical Ltd., Caesarea, Israel) utilizes a finger probe to assess digital volume changes accompanying pulse waves. The peripheral arterial tonometry data were analysed by a computer in an operator-independent manner. A ratio of less than 1.6 was considered to be a marker for endothelial dysfunction [6].

Patients were then randomly assigned (according to the HAART regime: boosted PI or non-nucleoside analogues) to receive ezetimibe 10 mg/day or fluvastatin extended release 80 mg/day in a 1 : 1 ratio. Patients were evaluated 2–3 weeks after the initiation of lipid-lowering agents to assess tolerability and adherence, and at 6 weeks, the baseline protocol was re-applied.

Results are expressed as mean (SEM) or in percentages. Univariate analyses, using non-parametric tests, were

Transcriptional Effect of DEFB1 Gene 5′ Untranslated Region Polymorphisms

To the Editor:

Human β-defensin-1 (hBD-1) has been shown to be a candidate tumor suppressor gene by Sun et al. (1) who reported that single nucleotide polymorphisms (SNP) in the *DEFB1* gene are able to modify the transcriptional activity of hBD-1 promoter. In particular, the *−44C/G* SNP was described to enhance transcription up to 2.3 times more than the wild-type sequence in DU145 or TSU-Pr1 cell lines.

Recently, we showed that significant correlation exists between SNPs in the 5′ untranslated region (UTR) of the *DEFB1* gene and the risk of being infected with HIV-1 in Italian and Brazilian children (2, 3).

With the aim of verifying an eventual transcriptional functional effect of the SNPs in the 5′UTR region of *DEFB1* gene, we employed a dual luciferase assay, the same technique used by Sun et al. (1).

Starting from the wild-type *DEFB1* 5′UTR region, we generated three mutated inserts using primers designed to introduce the desired mutation into the wild-type sequence. The four different fragments (wild-type, *−52A*, *−44G*, and *−20A*) have been cloned into pGL3 promoter vector (Promega), and Caco2 cells were transfected for 48 h with 1 μg of the reporter plasmids and 20 ng of control *Renilla* luciferase expression plasmid (phRG-TK, Promega) using 5 μL of GenePORTER transfection reagent; a dual luciferase assay (Promega) was done according to the manufacturer's instruction. To ensure the reproducibility of the results, all tests have been done in quadruplicate and repeated at a later time to confirm the obtained results.

Our results show that the three SNPs do possess a functional activity, leading to an impaired production of the gene downstream to the mutated promoter.[1] Both the *−52A* and the *−20A* alleles cause a 25% mean reduction of expression. Although quantitatively relevant, this reduction was not statistically significant. The *−44G* mutation, contrary to the data presented by Sun et al. (1), drag to a markedly reduced expression of about 53% in our experimental setup.

Based on our results (2, 3) and because all the studied SNPs could hamper an effective production of hBD-1 *in vivo*, we hypothesized that they could be related to reduced response from the innate immune system.

Despite the fact that DU145, TSU-Pr1, and Caco2 are all carcinoma-derived cell lines, their inherent differences could lead to variations in dual luciferase assay results: we believe that the functional effect of the *DEFB1* 5′UTR SNPs is still controversial and deserves a more comprehensive study.

Michele Milanese
Ludovica Segat
Sergio Crovella
Genetic Service,
Istituto di Ricovero e Cura a Carattere Scientifico
Burlo Garofolo, Trieste, Italy

References

1. Sun CQ, Arnold R, Fernandez-Golarz C, et al. Human β-defensin-1, a potential chromosome 8p tumor suppressor: control of transcription and induction of apoptosis in renal cell carcinoma. Cancer Res 2006;66:8542–9.
2. Braida L, Boniotto M, Pontillo A, Tovo PA, Amoroso A, Crovella S. A single-nucleotide polymorphism in the human β-defensin 1 gene is associated with HIV-1 infection in Italian children. AIDS 2004;18:1598–600.
3. Milanese M, Segat L, Pontillo A, Arraes LC, de Lima Filho JL, Crovella S. DEFB1 gene polymorphisms and increased risk of HIV-1 infection in Brazilian children. AIDS 2006;20:1573–5.

[1] More detailed information is available at http://www.bbcm.univ.trieste.it/~bbcm/defb1func1/.

doi:10.1158/0008-5472.CAN-06-3544