**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**

# O PAPEL DOS POLIMORFISMOS DO GENE DA PROTEÍNA DE LIGAÇÃO À MANOSE EM PACIENTES INFECTADOS PELO VÍRUS DA IMUNODEFICIÊNCIA HUMANA

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# SUMÁRIO

## LISTA DE ABREVIATURAS

AIDS – Síndrome da Imunodeficiência Adquirida

ARV – Anti-retroviral

bp – Pares de bases

CCR5 – Receptor de quimiocina C tipo 5

CRD – Domínio de Reconhecimento de Carboidratos

DNA – Ácido desoxirribonucléico

gp120 – Glicoproteína 120

gp41 – Glicoproteína 41

HAART – terapia anti-retroviral altamente ativa

HBV – Hepatite B

HCV – Hepatite C

HIV – Vírus da Imunodeficiência Humana

HLA – Antígeno Leucocitário Humano

IL – Interleucinas

INF – Interferon

MASP – Serino Proteases Associadas à MBL

MBL – Proteína de Ligação à Manose

MHC – Complexo Principal de Histocompatibilidade

RNA – Ácido ribonucléico

SC – Sistema Complemento

SNPs – Polimorfismos de um único nucleotídeo

TNF – Fator de necrose tumoral

## RESUMO

A Proteína de Ligação à Manose (MBL) é um membro da família das colectinas humanas que atua como molécula de defesa e desempenha um papel importante na imunidade inata. A MBL se liga a carboidratos na superfície de microorganismos, desencadeando a opsonização e fagocitose. Essa ligação também resulta na ativação do sistema complemento. Já foi descrito que a MBL reconhece e se liga a proteína gp120 do Vírus da Imunodeficiência Humana-1 (HIV-1), mas o papel dessa molécula na infecção pelo HIV-1 ainda não está claro. Vários polimorfismos no gene *MBL2* foram descritos como capazes de alterar a conformação da proteína, levando a baixos níveis séricos de MBL, e susceptibilidade aumentada a infecções. Entre eles, duas variantes na região promotora (-550 H/L e -221 X/Y), e três variantes no éxon 1, Arg52Cys, Gly54Asp e Gly57Glu (coletivamente chamadas de alelo 0, enquanto a combinação dos três alelos selvagens é chamada de A) foram analisadas no presente trabalho. Nosso objetivo foi investigar o papel da MBL na infecção pelo HIV-1, através da análise dos polimorfismos localizados nas regiões promotora e do éxon 1 do gene *MBL2*. Nós investigamos a prevalência dos alelos variantes em 410 pacientes infectados pelo HIV-1 do Hospital de Clínicas de Porto Alegre e 345 indivíduos não infectados. Todos os indivíduos são da região Sul do Brasil. As variantes localizadas na região promotora foram genotipadas usando a técnica PCR-SSP e as variantes do éxon 1 foram analisadas por PCR em tempo real, usando um ensaio de temperatura de *melting* e confirmação por PCR-RFLP. As freqüências genotípicas e alélicas foram comparadas entre os dois grupos analisados, indivíduos positivos para HIV-1 e controles, usando o teste de qui-quadrado. As análises foram

realizadas subdividindo os indivíduos de acordo com a origem étnica. Entre os indivíduos Euro-descendentes, uma maior freqüência do genótipo LX/LX foi observada entre pacientes, quando comparada com controles ($p<0,001$). As análises haplotípicas também demonstraram uma maior freqüência dos haplótipos associados com baixos níveis de MBL entre os pacientes infectados pelo HIV-1 ($p=0,0001$). Entre os indivíduos Afro-descendentes, as freqüências dos genótipos LY/LY e HY/HY foram maiores entre os pacientes, quando comparadas com os controles ($p=0,009$ e $p=0,02$). Nosso trabalho encontrou uma maior freqüência dos genótipos associados com baixos níveis de MBL entre os pacientes Euro-descendentes, sugerindo um papel potencial para a MBL na susceptibilidade à infecção pelo HIV-1 entre indivíduos Euro-descendentes.

Palavras-chave: MBL, polimorfismos, imunogenética, HIV, etnia.

## ABSTRACT

Mannose-binding lectin (MBL) is a member of the collectin protein family that acts as a defence molecule and plays an important role in innate immune responses. MBL binds to carbohydrates on the surface of microorganisms, leading to opsonisation and phagocytosis. This binding also results in activation of the complement system. It was already described that MBL recognizes and binds to gp120 protein of human immunodeficiency virus-1 (HIV-1), but the role of such molecule on HIV infection is still debated. Several *MBL2* gene polymorphisms were described as capable of disrupting the MBL protein resulting in low serum levels and increased susceptibility to infections. Among them, two variants in the promoter region (-550 H/L and -221 X/Y) and three variants in exon 1, Arg52Cys, Gly54Asp and Gly57Glu (that are collectively called allele 0, while the combination of the three wild-type alleles is called A), were analyzed in the present work. Our aim was to investigate the role of MBL on HIV-1 infected subjects through the analysis of the polymorphisms located in the *MBL2* promoter and exon 1 regions. We investigated the prevalence of the variant alleles in 410 HIV-1 infected patients from the Hospital de Clínicas de Porto Alegre (HCPA), and in 345 uninfected individuals. All individuals are from Southern Brazil. The variants from the promoter were genotyped using PCR-SSP and the variants from the exon 1 were analyzed by Real-time PCR using a melting temperature assay and were confirmed by PCR-RFLP. Polymorphisms genotype and allele frequencies in the two groups analyzed, namely HIV-1 positive subjects and controls, were compared using Chi-square-test. The analyses were performed subdividing the individuals according to their ethnic

origin. Among Euro-derived individuals a higher frequency of the LX/LX genotype in patients was observed when compared to controls ($p<0.001$). The haplotypic analysis also showed a higher frequency of the haplotypes associated with lower MBL levels among HIV-1 infected patients ($p=0.0001$). Among Afro-derived individuals the frequencies of LY/LY and HY/HY genotypes were higher in patients when compared to controls ($p=0.009$ and $p=0.02$). An increased frequency of genotypes associated with low MBL levels was observed among Euro-derived patients, suggesting a potential role for MBL in the susceptibility to HIV-1 infection in Euro-derived individuals.

Key Words: MBL, polymorphisms, immunogenetics, HIV, ethnicity.

# 1. INTRODUÇÃO

## 1.1. Características da doença

A Síndrome da Imunodeficiência Adquirida (AIDS, do inglês *Acquired Immune Deficiency Syndrome*) tornou-se mundialmente conhecida a partir da década de 80, quando surgiram os primeiros sinais de uma epidemia. O Vírus da Imunodeficiência Humana, conhecido como HIV (sigla originada do inglês: *Human Immunodeficiency Virus*), é um vírus pertencente à classe dos retrovírus e causador da AIDS.

A doença aguda inicial caracteriza o período após a soroconversão. Embora alguns pacientes permaneçam assintomáticos, a infecção aguda pelo HIV geralmente apresenta sintomas semelhantes à gripe, associados a um aumento de partículas virais no sangue, ampla disseminação pelos tecidos e invasão do tecido linfóide pelas partículas virais (Mindel e Tenant-Flowers, 2001). O período médio de incubação é estimado em 3 a 6 semanas, ou seja, é esse o intervalo de tempo entre a exposição ao vírus e o surgimento de sintomas como febre, fadiga, mialgia, eritema e dor de cabeça (fase inicial). No período assintomático ocorre redução da viremia, acompanhada de replicação silenciosa do vírus nos linfonodos, lesão na arquitetura dos linfonodos, perda de linfócitos CD4+ e de células dendríticas. Esse período de latência do vírus é marcado pela forte interação entre o sistema imune e por constantes e rápidas mutações do vírus. A produção de anticorpos específicos anti-HIV é perceptível 8 a 12 semanas após a infecção (Ministério da Saúde, 2008; Chu e Selwyn, 2010).

As células do sistema imunológico mais atingidas pelo vírus são os linfócitos CD4+, cuja maquinaria enzimática é parasitada pelo HIV. Após o

período de latência clínica (que pode alcançar até 10 anos), esse compartimento celular começa a funcionar com menor eficiência e, com o tempo, a capacidade do organismo em combater doenças comuns diminui (Figura 1). Aproximadamente 50% dos indivíduos infectados, sem uso de terapia anti-HIV, vão começar a apresentar sinais da infecção, como o decréscimo de contagem das células CD4 e perda das atividades imunológicas, deixando a pessoa sujeita ao desenvolvimento de vários tipos de patologias, como infecções oportunistas (ex: tuberculose), doenças neurológicas (ex: confusão, paralisia, demência), depressão da medula óssea e cânceres. As infecções gastrintestinais contribuem para a perda de peso, além da possível ocorrência de lesão cardiovascular e renal (Abdulle *et al.*, 2008; Levy, 2009).

O paciente não tratado morre em dois anos, geralmente. No entanto, é importante mencionar que muitas das infecções que causam problemas ou que podem ser ameaçadoras para pessoas com AIDS são geralmente controladas por um sistema imune saudável (Mindel e Tenant-Flowers, 2001; Ministério da Saúde, 2008).

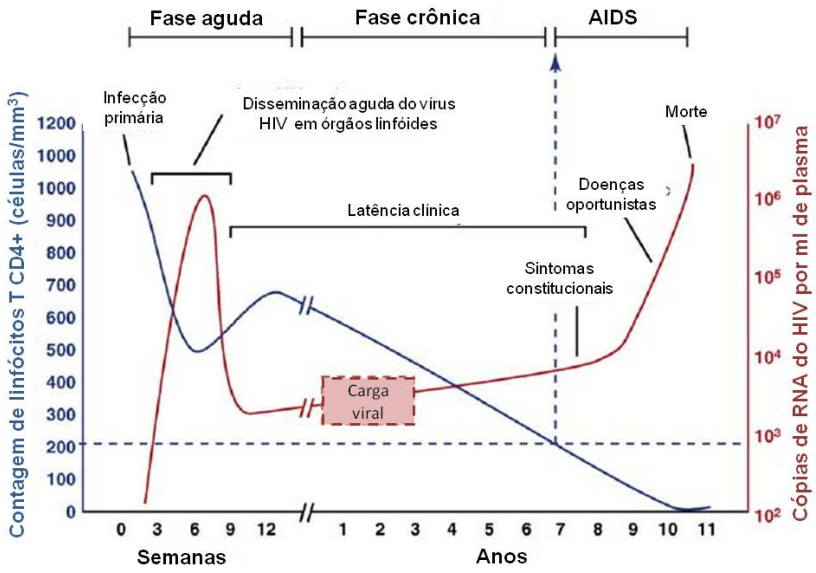


Figura 1 - Curso clínico da infecção pelo HIV (adaptado de An e Winkler, 2010).

Os seres humanos mostram notável variação na vulnerabilidade à infecção pelo HIV, especialmente nos desfechos clínicos após a infecção. Uma diferença surpreendente é o nível de vírus circulante no plasma durante a fase não sintomática que precede a progressão da doença (Fellay *et al.*, 2007). Essa variabilidade na evolução clínica permite a classificação dos pacientes infectados de acordo com a progressão da doença. Um grupo consiste dos “progressores típicos”, nos quais as funções imunes são intactas no início da infecção, mas vão perdendo suas funções conforme a progressão da doença. Outro grupo é chamado de “progressores rápidos”, que demonstram um declínio muito rápido na contagem das células CD4, normalmente de 2 a 5 anos após o contato com o vírus. A principal característica dos progressores rápidos é a carga viral elevada, que não diminui após a infecção primária. Um terceiro grupo consiste dos “sobreviventes de longo prazo” ou “não

progressores de longo prazo", que permanecem assintomáticos, com contagem de células CD4 e carga viral estáveis por mais de 5 anos, sem uso de terapia anti-retroviral. Esses pacientes têm níveis detectáveis de viremia e podem progredir para AIDS. Um quarto grupo (ou uma subclassificação do terceiro) consiste dos "controladores de elite", um grupo distinto de pacientes sem uso de terapia anti-HIV, que parece ser capaz de controlar a replicação viral. Os indivíduos têm contato com o HIV, mas permanecem saudáveis durante muitos anos, com carga viral muito baixa ou indetectável, e geralmente não apresentam sinais clínicos de progressão da doença (Levy, 2009; Blankson, 2010; Casado *et al.*, 2010).

Dois tipos diferentes de HIV, HIV-1 e HIV-2, causam infecção e doença em humanos. O HIV-1 parece ter surgido de transmissão cruzada entre espécies, de um vírus de chimpanzés para humanos, e o HIV-2 de transmissão cruzada entre espécies de um vírus do macaco *Sooty Mangabey* (Apetrei *et al.*, 2007).

Em termos mundiais, em comparação com o HIV-1, o HIV-2 é muito menos prevalente. Indivíduos infectados com HIV-2 foram primariamente encontrados no oeste da África e Índia. Além do mais, a infecção com o HIV-2 está associada com uma progressão mais lenta da deficiência imune, e o vírus parece não ser transmitido com a mesma eficiência que o HIV-1, mesmo em casos de transmissão materno-fetal. No Brasil, o HIV-1 é muito mais prevalente do que o HIV-2 (Cohen *et al.*, 2008).

A transmissão do HIV-1 depende da infectividade da pessoa que transmite o vírus e da susceptibilidade do hospedeiro. A infectividade depende da concentração de HIV-1 e da quantidade de células infectadas com vírus no

fluido corporal (sangue ou secreções do trato genital). As exigências virais e celulares para transmissão do HIV-1, no entanto, permanecem apenas parcialmente compreendidas (Cohen *et al.*, 2008).

A progressão da infecção pelo HIV é resultado de um declínio na competência imunológica que ocorre devido à replicação aumentada do HIV que estava latente. O mecanismo exato para essa ativação é pouco esclarecido (Mindel e Tenant-Flowers, 2001). A susceptibilidade à infecção pelo HIV-1 e a subseqüente progressão da doença são altamente variáveis entre diferentes indivíduos. Variações genéticas do hospedeiro têm sido apontadas como responsáveis por parte dessas diferenças (Van Manen *et al.*, 2008; An e Winkler, 2010).

## 1.2. Ciclo do HIV-1

O ciclo de replicação do HIV-1 é complexo e composto de diversas etapas, as quais dependem tanto de fatores celulares do hospedeiro quanto de fatores virais. O ciclo de vida do HIV-1 pode ser dividido em duas fases: o estágio inicial ocorre quando o vírus penetra na célula do hospedeiro e se integra no genoma e o estágio tardio é caracterizado pela replicação viral (Coiras *et al.*, 2009).

O processo de entrada do vírus na célula se dá pela fusão do envelope lipídico viral com a membrana plasmática celular. O componente viral que media a fusão é o envelope glicoprotéico, composto pela camada superficial da glicoproteína gp120 e pela camada transmembrana da glicoproteína gp41. A fusão é iniciada com a interação entre a gp120 e os receptores CD4 e subseqüente interação com os co-receptores CCR5 e/ou CXCR4, liberando o

material viral que consiste de um capsídeo contendo o RNA viral, e as enzimas transcriptase reversa e integrase, no interior do citosol da célula hospedeira (Campbell e Hope, 2008; Adamson e Freed, 2010).

A seguir, a estrutura do capsídeo é desfeita e a transcriptase reversa transcreve o RNA viral em DNA fita-dupla, que é transportado para o núcleo celular e integrado ao genoma do hospedeiro, na forma de provírus, pela integrase. O provírus pode permanecer inativo por meses ou anos, com pouca ou nenhuma produção de novas proteínas virais e dessa maneira a infecção pelo HIV pode permanecer latente (Adamson e Freed, 2010).

A ativação da transcrição é feita por citocinas e elementos reguladores do gene viral por um mecanismo pouco conhecido. Transcritos completos do RNA viral são produzidos e os genes virais são expressos como proteínas. Inicialmente são expressos os genes reguladores (*tat* e *rev*), e então os genes estruturais (*env*, *gag* e *pol*), além de várias proteínas acessórias como o fator de infectividade viral (Vif), proteína viral U (Vpu), fator negativo (Nef) e proteína viral R (Vpr) (Freed, 2001; Adamson e Freed, 2010).

Após a transcrição dos diversos genes virais, as proteínas virais são sintetizadas no citoplasma, formando a estrutura central do vírus. Esse complexo de nucleoproteínas é fechado em um envelope e o vírus é liberado da célula por brotamento (Freed, 2001; Coiras *et al.*, 2009; Adamson e Freed, 2010).

### 1.3. Epidemiologia

Em 2007, avanços na metodologia de estimativa da epidemia do HIV, aplicados a uma gama aumentada de dados de diferentes países, resultaram

em mudanças substanciais na estimativa do número de pessoas vivendo com HIV no mundo. No entanto, a interpretação qualitativa da gravidade e as implicações da pandemia pouco mudaram. Em 2008, o número estimado de pessoas vivendo com HIV no mundo era próximo a 34,4 milhões (31,1 a 35,8 milhões). De acordo com a UNAIDS/WHO (2009) 2,7 milhões de novos casos e 2 milhões de mortes ocorreram no ano de 2008.

No Brasil, desde a identificação do primeiro caso de AIDS, em 1980, até junho de 2009, foram identificados 544.846 casos da doença. Destes, 323.069 foram determinados no Sudeste (59%), 104.671 no Sul (19%), 64.706 no Nordeste (12%), 31.011 no Centro Oeste (6%) e 21.389 no Norte (4%). Segundo critérios da Organização Mundial de Saúde, o Brasil tem uma epidemia concentrada, 78% dos homens e 71% das mulheres encontram-se na faixa etária de 25 a 49 anos. A taxa de incidência de AIDS mantém-se, ainda, em patamares elevados - 18,2 casos por 100 mil habitantes - basicamente devido à persistência da tendência de crescimento entre as mulheres. A razão de sexo (homens:mulheres) diminuiu consideravelmente do início da epidemia para os dias atuais. Em 1986, a razão era de 15,1:1 e, a partir de 2002, estabilizou-se em 1,5:1 (Ministério da Saúde, 2009).

O país acumulou cerca de 217 mil óbitos devido à AIDS até junho de 2009, sendo as taxas de mortalidade crescentes até meados da década de 90, e estabilizadas em cerca de 11 mil óbitos anuais desde 1998. Após a introdução da política de acesso universal ao tratamento anti-retroviral (ARV), que combina drogas com diferentes formas de ação (HAART), observou-se uma importante queda na mortalidade. A partir do ano 2008, essa taxa se

estabilizou em cerca de 6,1 óbitos por 100 mil habitantes (Ministério da Saúde, 2009).

### 1.4. Co-infecção com hepatite B e com hepatite C

Com o progresso da doença, pessoas infectadas sofrem de infecções oportunistas, problemas na pele e boca e doenças hematológicas, muitas das quais são fáceis de tratar ou atenuar com a terapia anti-retroviral (Mindel e Tenant-Flowers, 2001). Os vírus do HIV, da hepatite B (HBV) e da hepatite C (HCV) correspondem às três infecções virais crônicas mais comuns documentadas no mundo. Esses vírus têm rotas de transmissão similares, através do sangue e seus derivados (por uso comum de agulhas para injetar drogas ou através de atividade sexual) tornando a co-infecção um evento comum. Estima-se que de 34,4 milhões de pessoas vivendo com HIV no mundo, aproximadamente 20% estejam co-infectadas com HCV. Os dados referentes à co-infecção com HBV variam de 5% em países ocidentais até 20% em regiões endêmicas da África Subsaariana e Sudeste Asiático (Soriano *et al.*, 2010). As co-infecções do HBV e HCV em pacientes HIV positivos são de extrema importância devido às conseqüências subjacentes, como problemas hepáticos associados com esses vírus, os quais têm sido relacionados com o decréscimo da expectativa de vida nos pacientes infectados com HIV (Saravanan *et al.*, 2007; Jindal *et al.*, 2008).

De acordo com algumas estimativas, há aproximadamente 400 milhões de pessoas no mundo infectadas com hepatite B, dessas, cerca de 4 milhões estão infectadas com HIV. Em pacientes infectados com HIV, a infecção do HBV aumenta a replicação do HIV e a hepatotoxicidade causada pela terapia

anti-retroviral e está correlacionada com diminuição da contagem de células CD4+ em pacientes com cirrose e hiperesplenismo. Há também evidências de que a infecção com HBV, por si, reduza a contagem de células CD4+. Em pacientes infectados com HBV, o HIV favorece a replicação do HBV, progressão a infecção com HBV crônica, e risco de carcinoma hepatocelular, e reduz os anticorpos contra a proteína nucleocapsídica do "core" da hepatite B (anti-HBe) e anticorpos contra superfície da hepatite B (anti-HBs). As co-infecções também estão associadas com eficácia reduzida da terapia anti-retroviral, incluindo risco aumentado de resistência ao medicamento lamivudina e resposta diminuída ao interferon alfa (Peters, 2007).

Entre 2 e 3% da população de países desenvolvidos apresenta infecção com o vírus da hepatite C. A co-infecção por HCV e HIV é comum devido às mesmas rotas de transmissão. O estado de ativação imune permanente proporcionado pela infecção pelo HCV pode agir deleteriamente em indivíduos HIV-positivos, favorecendo a destruição mais rápida dos linfócitos CD4+. Além disso, a recuperação imune observada após o início da terapia anti-retroviral efetiva pode ser parcialmente prejudicada em indivíduos com hepatite C, através da infecção de células imunes pelo próprio HCV (Tovo et al., 2007).

## 1.5. Terapia HAART

A terapia de combinação, que mudou o prognóstico do HIV/AIDS, é conhecida como terapia anti-retroviral altamente ativa (HAART, *highly active antiretroviral therapy*). Uma combinação de HAART típica envolve dois inibidores nucleosídicos da transcriptase reversa com um inibidor não-nucleosídico da transcriptase reversa ou um ou dois inibidores da protease.

Com o uso do esquema HAART a replicação do HIV é inibida, com redução da presença do RNA do HIV no plasma para níveis indetectáveis e prolongamento da sobrevida do paciente. Todavia, o esquema é complexo e apresenta muitos efeitos adversos; além disso, a aderência do paciente a esse tratamento é difícil, e sua administração pode ser permanente, visto que o HIV não é erradicado. O vírus permanece latente nas células T de memória, integrado no genoma do hospedeiro, constituindo uma fonte para reativação potencial se os fármacos forem interrompidos (Max e Sherer, 2000).

No entanto, com todos os três grupos de fármacos podem ocorrer interações farmacológicas indesejáveis, podendo haver variações interpessoais na absorção. Alguns fármacos penetram pouco no cérebro, podendo permitir proliferação local do vírus (Rang, 2004).

## 1.6. Base genética da susceptibilidade à infecção pelo HIV, progressão da doença e resposta ao tratamento

Durante a infecção aguda pelo HIV, o vírus replica-se amplamente atingindo níveis acima de 100 milhões de cópias/ml (Mellors *et al.*, 1996). Observações em diversos laboratórios definiram a natureza do ciclo replicativo do HIV, o qual envolve três enzimas codificadas pelo vírus: transcriptase reversa, integrase e protease, que têm se tornado alvos das terapias anti-retrovirais (Levy, 2006).

A susceptibilidade humana à infecção com o HIV não é uniforme. A resistência à infecção reflete algumas combinações de fatores genéticos, resistência inata, e resistência adquirida. Vários estudos têm demonstrado uma grande variabilidade interindividual em resposta à infecção pelo HIV. Essa

variabilidade inclui susceptibilidade à infecção pelo vírus, transmissão, progressão da doença, com fenótipos que variam desde indivíduos expostos com níveis indetectáveis de viremia, indivíduos assintomáticos até pacientes que chegam à morte em decorrência da AIDS.

Polimorfismos em receptores de quimiocinas, que servem como co-receptores de HIV têm sido associados com susceptibilidade diminuída a infecção, assim como em diferentes taxas de progressão da doença. Até hoje, a mais importante contribuição genética para a infecção pelo HIV identificada é uma mutação de deleção (alelo delta 32) no gene codificador do co-receptor CCR5, oferecendo proteção substancial contra a infecção pelo HIV. Essa foi a primeira evidência de que a transmissão das cepas do HIV usam preferencialmente o co-receptor CCR5 para a entrada na célula, e indivíduos portando essa deleção em homozigose produzem uma proteína truncada, que não é expressa na superfície celular. Esses achados levaram ao desenvolvimento de drogas que atuam nesse mecanismo (O'Brien e Nelson, 2004; Cohen *et al.*, 2008). Foi demonstrado, através de estudos de associação, que polimorfismos em genes que codificam quimiocinas ligantes do CCR5 (como CCL5 e CCRL3L1) inibem a replicação do HIV in vitro, caracterizando um papel importante desses genes na infecção e patogênese do HIV (An e Winkler, 2010).

Outra importante observação efetuada ao longo dos últimos anos é a presença de mecanismos naturais que limitam a replicação do HIV dentro das células. Esses achados oferecem novas abordagens para terapias anti-HIV. Um exemplo é a APOBEC-3G, uma citidina desaminase que tem importante atividade anti-HIV. Ela induz mutações no genoma do HIV e é bloqueada pela

proteína Vif (fator de infectividade viral), que é essencial para a replicação viral, impedindo a proteína APOBEC-3G de funcionar na célula infectada. Algumas variantes dessa proteína estão envolvidas na inibição da atividade de retrovírus, incluindo o HIV-1 (Henriet *et al.*, 2005; Wang *et al.*, 2008).

Recentemente, o gene *Trim5α* foi identificado como parte da imunidade intrínseca que protege o ser humano e outros primatas contra a infecção retroviral, provavelmente através da limitação da abertura do capsídeo do HIV dentro a célula (Diaz-Griffero *et al.*, 2007; Van Manen *et al.*, 2008).

Entre as diversas variantes genéticas estudadas, o sistema HLA (Antígeno Leucocitário Humano, do inglês *Human Leukocyte Antigen*), do Complexo Principal de Histocompatibilidade (*Major Histocompatibility Complex* - MHC) representa um foco importante das pesquisas envolvendo o a infecção pelo HIV, já que sua função é apresentar peptídeos ao sistema imune. A homozigose para os loci 1, 2 e 3 dos alelos HLA classe I foram positivamente correlacionados com progressão da doença, e indivíduos homozigotos HLA-A, -B e –C mostraram menor tempo de sobrevida após diagnóstico de AIDS. Alelos específicos do HLA estão envolvidos em reações de hipersensibilidade a algumas drogas ARV, como abacavir com *HLA-B*5701* e nevirapina com *DRB1*0101* (Mahungu *et al.*, 2009). Outros alelos HLA foram associados com a progressão da doença (*B27*, *B57* e *B35*) (Kaur e Mehra, 2009; An e Winkler, 2010).

As células *natural killer* (NK) são componentes fundamentais da imunidade inata na defesa contra infecções virais, através da ativação de citocinas e pela sua citotoxicidade. O controle da atividade das células NK é regulado por receptores com funções ativadoras e inibidoras, incluindo o

receptor KIR (*killer immunoglobulin-like receptors*), que participa da regulação das células NK através do reconhecimento de ligantes HLA nas células alvo. Alguns estudos propõem a ação combinada de variantes no gene KIR com variantes em genes HLA, na defesa contra a infecção pelo HIV-1. Já foi reportado que a combinação do alelo *KIR3DS1* com o alelo *HLA-Bw4* confere proteção contra o desenvolvimento de AIDS, e também contra infecções oportunistas nesses pacientes (Qi *et al.*, 2006).

### 1.7. Proteína de Ligação à Manose

A Proteína de Ligação à Manose também conhecida como Lectina de Ligação à Manose, é uma das proteínas mais estudadas da família das colectinas humanas [oligômeros formados por cadeias polipeptídicas caracterizados por um domínio de reconhecimento de carboidratos (CRD) ligado a uma região colagenosa] (Sorensen *et al.*, 2005). Ela desempenha um papel importante na primeira linha de defesa do organismo e teve seu papel inicialmente avaliado em 1968, em um caso de uma criança com infecção bacteriana recorrente relacionada com baixos níveis desta proteína (Worthley *et al.*, 2005; Bouwman *et al.*, 2006).

A estrutura da MBL caracteriza-se por multímeros de cadeias polipeptídicas idênticas de 32kDa, cada uma compreendendo quatro regiões distintas codificadas pelos diferentes éxons do gene *MBL2*, que será discutido posteriormente. Cada cadeia tem uma região C-terminal com um domínio de reconhecimento de carboidratos cálcio-dependente; uma pequena região hidrofóbica, denominada pescoço, em formato de hélice, uma região colagenosa contendo glicina e uma região N-terminal rica em cisteína. Três

cadeias polipeptídicas formam uma hélice tripla com a região colagenosa, estabilizada pelas interações hidrofóbicas e pontes dissulfídicas entre as cadeias com a região N-terminal (Figura 2). Essa é a forma molecular básica da MBL circulante. No soro, a MBL consiste de oligômeros, de dímeros a hexâmeros, em formato de buquê (Ezekowitz, 2003; Dommett *et al.*, 2006).

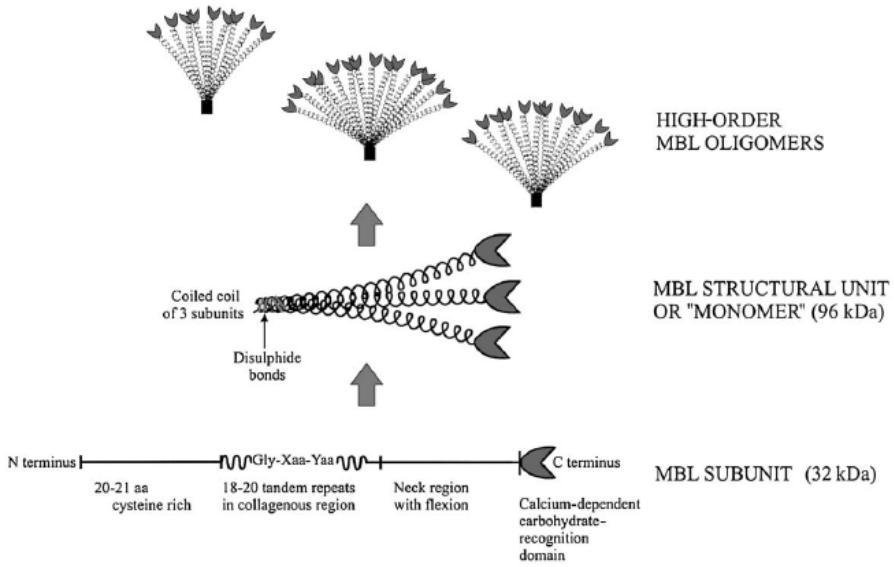


**Figura 2** - Subunidade característica da MBL e montagem em unidade estrutural e de ordem elevada, oligômeros de MBL funcional (Eisen e Minchinton, 2003).

Essa proteína de fase aguda e de origem hepática é capaz de se ligar multivalentemente à manose terminal, N-acetilglicosamina, glicose e frutose, em leveduras, bactérias gram-negativas e gram-positivas, vírus, fungos e protozoários (Worthley *et al.*, 2005). Após se ligar a um patógeno, a MBL sofre uma mudança conformacional ativando moléculas associadas, como as serino proteases associadas à MBL (MASP-1, MASP-2, MASP-3 e sMAS/MAP19), resultando na iniciação da ativação do complemento (SC) pela terceira via,

conhecida como a via das lectinas (Figura 3). A interação entre MBL e MASPs ocorre através do domínio de colágeno da MBL (Ip *et al.*, 2004; Selander *et al.*, 2006).

A MBL é a única colectina conhecida capaz de ativar o SC (iniciando a via das lectinas) e, desse modo, promover a fagocitose dos microorganismos sem o envolvimento de anticorpos (Zimmermann-Nielsen *et al.*, 2002; Fiane *et al.*, 2005; Zimmermann-Nielsen *et al.*, 2005). Complexos de MBL-MASP-2 são ativados quando ligados a superfícies microbianas com disposições apropriadas de açúcar. A MASP-2, então, expressa atividade enzimática idêntica à esterase C1, que resulta em uma seqüência de clivagem de C4 e C2. O fragmento C4b gerado se liga covalentemente à superfície microbiana e, subseqüentemente, interage com componentes C2 que são também clivados por MASP-2. O complexo C4b2a criado ativa a convertase C3. Múltiplos fragmentos C3b são gerados e se ligam à superfície do organismo. Tais fragmentos são reconhecidos pelo receptor CR1 (CD35) do fagócito. Alguns C3b são convertidos em iC3b e estes são reconhecidos pelos receptores CR3. A MBL também pode interagir diretamente com receptores da superfície celular, promovendo opsonofagocitose independente de complemento. Isso sugere que a MBL tem um importante papel nas primeiras horas/dias de qualquer resposta imune primária (Super *et al.*, 1989; Dommett *et al.*, 2006).

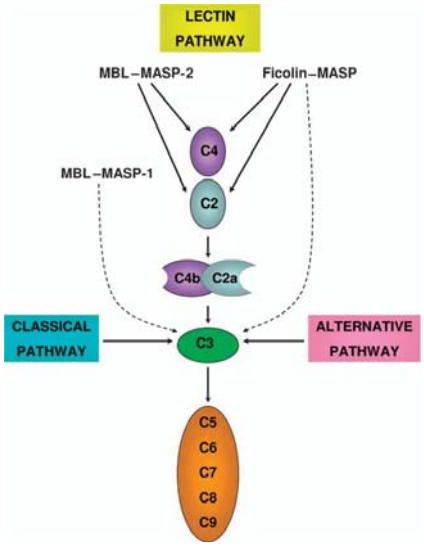


**Figura 3** - Ativação do complemento (Dommett et. al, 2006)

### 1.7.1. Caráter duplo da MBL

A MBL reconhece os padrões de carboidratos que se encontram na superfície de um grande número de microorganismos patogênicos, incluindo bactérias, vírus, protozoários e fungos. Essa proteína é considerada um paradoxo da imunidade inata, sendo referenciada por muitos autores como uma “faca-de-dois-gumes” e apelidada de “*Jekyl-and-Hyde*”. A MBL tem um papel duplo na pato-fisiologia humana: de um lado, baixos níveis de MBL estão associados com maior susceptibilidade a infecções por microorganismos extracelulares, pois a MBL reconhece esses patógenos e desencadeia resposta imune, eliminando-os. Por outro lado, estes mesmos baixos níveis de MBL têm caráter protetor contra infecções por microorganismos intracelulares, pois a proteína desencadeia a opsonização e a fagocitose desses microorganismos, transportando-os para dentro da célula, o que facilita a ação e aumenta a infectividade de alguns patógenos que atuam dentro das células (Ezekowitz, 2003; Fiane *et al.*, 2005; Worthley *et al.*, 2005; Bouwman *et al.*,

2006). Além disso, altos níveis e alta atividade de MBL têm sido associados com doenças inflamatórias, rejeição a transplantes e nefropatia diabética (Bouwman *et al.*, 2006). Em conclusão, em geral o ideal seria o indivíduo apresentar níveis intermediários de MBL, já que tanto níveis muito elevados quanto níveis deficientes podem estar relacionados com maior susceptibilidade a determinado tipo de patógeno, trazendo prejuízos à saúde.

### 1.7.2. Gene *MBL2*

Há dois genes humanos de MBL, no entanto, *MBL1* é um pseudogene e somente *MBL2* codifica um produto protéico. O gene *MBL2*, localizado no cromossomo 10, região 10q11.2-q21, codifica a Proteína de Ligação à Manose (Ip *et al.*, 2004). O éxon 1 codifica a região rica em cisteína e parte da região colagenosa rica em glicina, o éxon 2 codifica o restante da região colagenosa, o éxon 3 codifica a estrutura helicoidal, conhecida como a região do pescoço e o éxon 4 codifica o CRD (Figura 4). A região promotora do gene contém elementos regulatórios que afetam a transcrição da proteína (Dommett *et al.*, 2006).

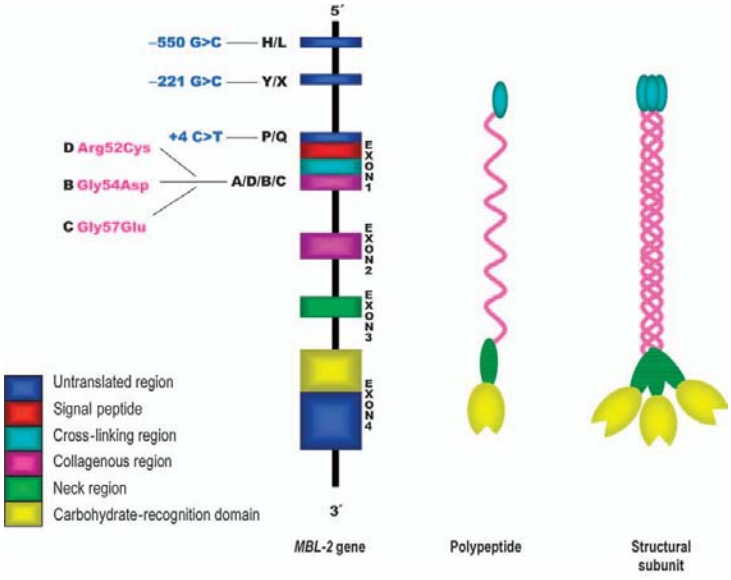


**Figura 4 -** Estrutura do gene *MBL2* e o produto protéico codificado e indicação das mutações que serão abordadas posteriormente neste texto (Dommett et al., 2006)

Polimorfismos de um único nucleotídeo (SNPs) localizados no éxon 1 desse gene estão relacionados com alterações na conformação da proteína, resultando na deformação da estrutura em hélice da região colagenosa e, conseqüentemente, interferindo na formação dos oligômeros. Esse prejuízo na polimerização leva a baixos níveis séricos da proteína, os quais estão associados com deficiência na fagocitose e susceptibilidade aumentada a doenças infecciosas e auto-imunes (Fgura 5) (Thio *et al.*, 2005; Bouwman *et al.*, 2006; Maury *et al.*, 2007).

As regiões polimórficas mais estudadas encontradas no éxon 1 compreendem os seguintes polimorfismos: R52C, que representa a troca de uma arginina por uma cisteína no códon 52, causada pela substituição CGT → TGT, denominado alelo "*D*" (rs5030737); G54D, que envolve a troca de uma glicina por um aspartato no códon 54, representando a substituição GGC →

GAC, e é denominado alelo "*B*" (rs1800450); e G57E, caracterizando a troca de uma glicina por um glutamato no códon 57, devido à substituição GGA → GAA, denominado de alelo "*C*" (rs1800451). Uma região codificadora contendo qualquer uma das três variantes não selvagens é designada "*0*", enquanto a presença simultânea das variantes selvagens nos três sítios polimórficos caracteriza o alelo "*A*" (Wiertsema *et al.*, 2006; Muller *et al.*, 2007).

Todos os três alelos mutantes têm um efeito dominante nos níveis de MBL no soro, diminuindo os níveis séricos de MBL funcional em até 90%. As proteínas MBL formadas com essas variantes são instáveis, facilmente degradadas para reduzir formas oligoméricas (com baixa avidez para ligantes e incapacidade de ativar o sistema complemento) e potencialmente apresentam menor meia-vida na circulação. O que leva a redução na função devido à grande diminuição de seus níveis na circulação (Garred, 2008).

Três outros polimorfismos foram descritos na região promotora do gene da MBL, também relacionados com a diminuição dos níveis de MBL sérica (Madsen *et al.*, 1995). Estes sítios polimórficos estão localizados nos nucleotídeos -550 (alelos H/L, substituindo G→C, rs11003125), -221 (alelos X/Y, com a troca G→C, rs7096206) e +4 (alelos P/Q, onde, C→T, rs7095891). O locus -221, portando a variante X, tem o efeito mais forte na regulação da diminuição dos níveis de MBL, entre as três variantes do promotor. Em análises funcionais das variantes dessa região, foi demonstrado que os haplótipos LX, LY e HY estão relacionados com baixa, intermediária e alta atividade promotora, de acordo com os níveis séricos da proteína (Garred, 2008). Estes três *loci* estão intimamente ligados e, devido ao forte desequilíbrio de ligação, apenas sete haplótipos (HYPA, LYQA, LYPA, LXPA, LYPB, LYQC e HYPD)

são comumente encontrados (Madsen *et al.*, 1998; Parrella *et al.*, 2007; Wang *et al.*, 2007).

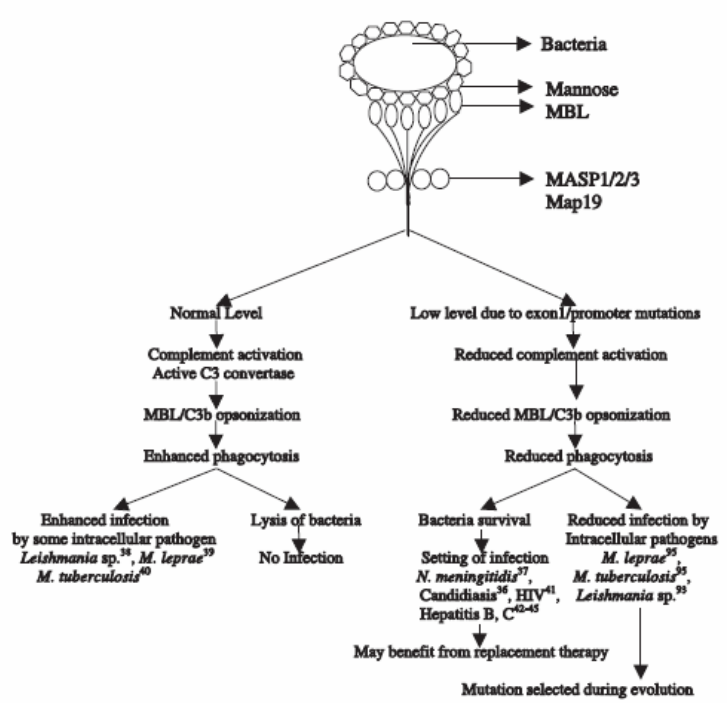


**Figura 5 -** Influência dos polimorfismos do gene *MBL2* na defesa do organismo contra infecções por patógenos intracelulares e extracelulares (Gupta *et al.*, 2008).

### 1.7.3. MBL e Susceptibilidade a Doenças

Os níveis de MBL já foram relacionados a diferentes patologias, como infecções, inflamações, complicações pós-transplantes, auto-imunidade, principalmente em pessoas com o sistema imune comprometido (crianças, portadores de fibrose cística ou infectadas com HIV, pacientes sob quimioterapia, por exemplo). O papel da MBL como modulador de inflamação é complexo e seu mecanismo de ação ainda não é totalmente conhecido. Uma possível explicação para a associação das variantes de MBL com diferentes doenças leva em conta que a MBL seria capaz de ativar citocinas pró-

inflamatórias, induzindo sua liberação a partir de monócitos. A indução de liberação de TNF-α, IL-1β e IL-6 a partir de monócitos, mediada por MBL, foi confirmada em concentrações de MBL abaixo de 4mg/ml. No entanto, altas concentrações desta mesma proteína suprimem esta mesma expressão (Dommett *et al.*, 2006).

Uma observação interessante é que pacientes com septicemia apresentam habilidade reduzida em ativar o sistema complemento pela via da MBL, quando comparados com controles saudáveis, mostrando que a MBL parece ser um modulador importante da cascata inflamatória através da liberação de citocinas pró-inflamatórias (necessárias para uma resposta imune eficaz contra doenças infecciosas) (Eisen *et al.*, 2006). Foi demonstrado que a MBL aumenta a produção do TNF pelas células mononucleares ligadas aos microorganismos, diminuindo a possibilidade de disseminação da infecção (Valdimarsson *et al.*, 2004).

A deficiência da MBL também tem sido associada com susceptibilidade a infecções bacterianas severas (Eisen e Minchinton, 2003). Por exemplo, portadores de variantes alélicas da MBL foram associados com maior susceptibilidade ao desenvolvimento de Doença Meningocócica em pacientes pediátricos e ao desenvolvimento de Pneumonia Pneumocócica em adultos (Roy *et al.*, 2002; Eisen *et al.*, 2006; Eisen, 2010). Deficiências nos níveis séricos de MBL também parecem contribuir para infecções de pele recorrentes, e algumas formas de doenças inflamatórias de pele (Miller *et al.*, 2010).

Outro estudo demonstrou que a MBL representa uma importante molécula antiviral com um papel protetor nos primeiros estágios de infecção pelo vírus da Hepatite C. Assim, polimorfismos do éxon 1 do gene *MBL2* foram

relacionados com progressão da infecção pelo HCV para inflamação do fígado e fibrose (Segat *et al.*, 2007; Koutsounaki *et al.*, 2008).

Essas variantes também parecem influenciar a susceptibilidade ao Lúpus Eritematoso Sistêmico. Um estudo feito por nosso grupo demonstrou um papel da variante R52C no desenvolvimento da doença, mas sem envolvimento com a expressão clínica do Lúpus (Takahashi *et al.*, 2005; Monticielo *et al.*, 2010).

Além disso, variantes da MBL foram associadas com Doença Celíaca e risco aumentado de desenvolver doenças auto-imunes (Boniotto *et al.*, 2005). Outro estudo demonstrou que mulheres grávidas portadoras da variante G54D têm um risco aumentado de desenvolver Diabetes Mellitus gestacional e seus filhos nascem com peso acima da média (Megia *et al.*, 2004). Outro estudo desenvolvido por nosso grupo analisou as variantes da MBL na gravidez, concluindo que mulheres com os genótipos relacionados com baixos níveis séricos de MBL têm maior probabilidade de desenvolver pré-eclampsia, demonstrando o envolvimento inflamatório desta molécula (Vianna *et al.*, 2010).

Entretanto, todos os estudos citados acima são de caráter exploratório, e ainda apresentam algumas divergências, sendo necessários estudos clínicos confirmatórios para uma avaliação mais específica do papel da MBL em diferentes patologias.

### 1.8. Relação entre MBL e AIDS

Além dos estudos que têm mostrado a relação dos haplótipos HLA e os polimorfismos do gene CCR5 com a transmissão do HIV e progressão da

AIDS, o efeito da heterogeneidade genética na susceptibilidade à infecção com o HIV e progressão da doença é pouco compreendido. Contudo, a MBL poderia ter um papel direto na infecção com o HIV por diversas razões, que serão discutidas a seguir (Garred *et al.*, 1997). A MBL pode reconhecer estruturas de superfície de carboidratos de diversas bactérias, fungos e vírus, incluindo o HIV-1 (Neth *et al.*, 2000). A interação entre os microrganismos e a MBL pode iniciar a ativação do complemento e fagocitose, assim como a indução de resposta inflamatória de citocinas (Alagarasu *et al.*, 2007).

Estudos sugeriram que a MBL pode estar envolvida no reconhecimento do HIV. A proteína do envelope gp120 do HIV-1 é altamente glicosilada com carboidratos, permitindo potencialmente que a MBL realize ligação, opsonização e neutralização do HIV-1 (Bouwman *et al.*, 2006). A MBL se liga seletivamente a células infectadas pelo HIV e inibe a infecção do vírus nas células CD4+ (Worthley *et al.*, 2005). Baseado nos estudos existentes pode-se sugerir que, em algumas circunstâncias, a MBL possa agir promovendo ativação das células inflamatórias, e por meio disso, acelerar a taxa de depleção das células T CD4+ (Dommett *et al.*, 2006).

Dados recentes indicam que a MBL pode opsonizar o HIV, mas não induzir neutralização até níveis normalmente presentes no soro. De qualquer forma, a ligação e a opsonização do HIV pela MBL altera o trajeto do vírus e apresentação do antígeno viral durante a infecção do HIV (Dommett *et al.*, 2006).

Dados de um estudo em pacientes com AIDS demonstram que a sobrevivência foi significantemente reduzida nos pacientes com mutações no gene da MBL (Turner, 2003). Outro estudo sugere que a MBL pode estar

envolvida na patogênese da doença, induzindo resposta inflamatória persistente e potencialmente prejudicial. De fato, foi sugerido que a MBL, na presença de manose, associada com infecções fúngicas, pode aumentar a replicação do HIV (Heggelund *et al.*, 2005).

No Brasil, um estudo avaliou os polimorfismos do éxon 1 do gene *MBL2* e demonstrou que a presença da variante "*B"* está associada com níveis elevados de carga viral plasmática, sugerindo sua importância na evolução clínica da infecção pelo HIV-1. Além disso, este estudo concluiu que a caracterização dos polimorfismos da MBL em pacientes HIV positivos pode ser um importante marcador biológico auxiliar na avaliação da progressão da doença e na associação com a contagem de células CD4+ e carga viral plasmática (Vallinoto *et al.*, 2006). Outros dois estudos brasileiros confirmaram a associação entre a presença dos polimorfismos do éxon 1 da MBL e a infecção pelo HIV-1 em crianças expostas no período perinatal, indicando que a presença do alelo *"0"* confere maior risco de transmissão vertical da infecção pelo HIV-1 (Boniotto *et al.*, 2003; Arraes *et al.*, 2006).

Qualquer que seja o mecanismo de interação da MBL com o HIV, a maioria dos estudos já realizados sugere que a deficiência da MBL é fator de risco para a infecção pelo HIV. Porém, esses achados não se repetem em todas as populações e alguns estudos falham em demonstrar um papel da MBL na infecção pelo HIV (Worthley *et al.*, 2005; Dommett *et al.*, 2006). Além disso, não existem estudos abordando as diferentes variantes do gene *MBL2*, ou seja, incluindo as variantes do promotor e do éxon 1.

Foi estimado que diariamente em torno de 6.800 pessoas são infectadas com o HIV e 5.700 morrem em decorrência da AIDS. A pandemia do HIV permanece um grave desafio para a saúde pública (Unaids/Who, 2007).

Apesar dos enormes avanços, problemas consideráveis persistem. Primeiro, o acesso e utilização de intervenções efetivas continua limitado. Segundo, não foi identificada cura para o HIV-1, primariamente devido à nossa inabilidade de eliminar o reservatório latente de células nas quais o HIV-1 tem se integrado dentro do genoma do hospedeiro. Finalmente, pouco progresso foi alcançado no desenvolvimento de vacina para o HIV-1, dado o entendimento incompleto da imunidade da infecção pelo HIV-1 e indução de potente resposta imune protetiva (Cohen *et al.*, 2008).

Considerando essas situações, juntamente com a grande incidência e prevalência da doença, o envolvimento de fatores genéticos ainda não completamente determinados e a importância do sistema imune na susceptibilidade à infecção pelo HIV, estamos propondo a caracterização de indivíduos HIV positivos, através de uma abordagem imunogenética.

# 2. OBJETIVOS

## 2.1. Objetivo Geral

O objetivo geral do presente trabalho visa verificar a presença e freqüência das variantes alélicas do gene da proteína de Ligação à Manose em amostras de pacientes portadores de HIV e de indivíduos controle.

## 2.2. Objetivos Específicos

- Analisar os polimorfismos G54D, G57E e R52C do éxon 1 do gene *MBL2* em pacientes infectados pelo HIV e controles.
- Genotipar os polimorfismos L/H e X/Y localizados no promotor do gene *MBL2*, nas mesmas amostras.
- Estimar os haplótipos derivados da combinação dos referidos polimorfismos em pacientes e controles.
- Comparar as freqüências genotípicas e haplotípicas entre pacientes e controles.
- Comparar as freqüências destes polimorfismos entre os subgrupos dos pacientes portadores do HIV sem co-infecção e co-infectados com hepatite B e hepatite C.

## 3. ARTIGO CIENTÍFICO

**Versão com as sugestões dos revisores já incorporadas. Manuscrito submetido ao periódico AIDS (ver as considerações dos revisores do periódico em anexo),**

# THE ROLE OF MANNOSE-BINDING LECTIN GENE POLYMORPHISMS IN THE SUSCEPTIBILITY TO HIV-1 INFECTION IN SOUTHERN BRAZILIAN PATIENTS

Running head: *MBL2* POLYMORPHISMS IN HIV INFECTION

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## ABSTRACT

**Objective:** This study investigates the role of Mannose-binding lectin (MBL) in the susceptibility to HIV-1 infection through the analysis of polymorphisms located at the *MBL2* promoter and exon 1 regions.

**Materials and Methods:** We investigated the prevalence of the variant alleles in 410 HIV-1 infected patients from the South Brazilian HIV Cohort, and in 345 unexposed uninfected healthy individuals. The promoter variants were genotyped using PCR-SSP and the exon 1 variants were analyzed by Real-time PCR using a melting temperature assay and were confirmed by PCR-RFLP. Genotypic and allelic frequencies in the two groups analyzed, namely HIV-1 infected subjects and controls, were compared using Chi-square-tests.

**Results:** The analyses were performed subdividing the individuals according to their ethnic origin. Among Euro-derived individuals a higher frequency of the LX/LX genotype was observed in patients when compared to controls ($p<0.001$). The haplotypic analysis also showed a higher frequency of the haplotypes associated with lower MBL levels among HIV-1 infected patients ($p=0.0001$). Among Afro-derived individuals the frequencies of LY/LY and HY/HY genotypes were higher in patients when compared to controls ($p=0.009$ and $p=0.02$).

**Conclusions:** An increased frequency of genotypes associated with low MBL levels was observed in Euro-derived patients, suggesting a potential role for MBL in the susceptibility to HIV-1 infection in Euro-derived individuals.

Key Words: MBL, polymorphisms, immunogenetics, HIV, ethnicity.

## INTRODUCTION

The Acquired Immune Deficiency Syndrome (AIDS) became known worldwide after the 1980's, when the first epidemic signals appeared. Without treatment, life expectancy of patients diagnosed with AIDS is around two years [1, 2]. Interestingly, humans show a remarkable variation in vulnerability to HIV infection, probably due to genetic and immunologic factors. HIV transmission depends both on infectivity factors (for example, the higher the viral load, the greater the chance of transmission) as well as on host susceptibility [3].

There are two different HIV types, HIV-1 and HIV-2, though in Brazil HIV-1 is much more prevalent than HIV-2 [4, 5]. Infectivity depends on HIV-1 concentration and presence and number of infected cells with the virus in body fluids, although the viral and cell requirements for HIV-1 transmission remain poorly understood [5]. By 2008, there were 33.4 million people living with HIV worldwide. In this scenario, new cases add up to 2.7 million and 2 million died in 2008 [6]. In Brazil, by 2009 approximately 544 thousand cases of the disease had been reported, since the first case in 1980 [2]. The resistance to infection reflects the combination of genetic factors, innate and acquired immunoresistance. Among the genetic factors associated with HIV resistance, homozygosis of the delta 32 allele of the *CCR5* gene is by far the best known [5].

In recent years, there has been an emerging interest in mannose-binding lectin (MBL) due to its central role as a recognition molecule in the complement system. MBL is a member of a family of proteins called collectins and is characterized by the presence of both a collagenous region and a lectin domain [7, 8]. This protein is produced by the liver and is capable of binding mannan,

gram-negative bacteria and envelope glycoproteins of viruses [9]. After binding to a pathogen, MBL goes through a conformational change and activates associated molecules, such as MBL-associated serine proteases (MASP-1, MASP-2 and MASP-3), resulting in complement activation by the lectin pathway [10, 11]. Since HIV gp120 protein is highly glicosylated, it has been suggested that MBL could be involved in binding and opsonization of HIV-1 [12].

MBL is also known as a paradox of innate immunity. High MBL serum levels are useful in the immune response against extracellular pathogens. MBL recognizes pathogens and triggers phagocytosis, opsonization and elimination of the microorganism, but conversely, MBL-facilitated opsonization and phagocytosis may increase the infectivity of some intracellular pathogens. Thus, in this case, low MBL levels would be advantageous to the host by hindering the infection by intracellular microorganisms [9, 12-14]. The human mannose-binding lectin gene (*MBL2*) is located on chromosome 10 (q11.2-q21) and contains four exons. Polymorphisms located at exon 1 are reported to interfere with protein conformation, resulting in altered collagenous regions and, as a consequence, inhibiting oligomerization. This leads to low serum MBL levels and impaired protein function [12, 15, 16]. *MBL2* gene exon 1 region contains three functional single nucleotide polymorphisms (SNPs), at codon 52 (CGT to TGT, Arg→Cys, referred as “D” allele), at codon 54 (GGC to GAC, Gly→Asp, “B” allele) and at codon 57 (GGA to GAA, Gly→Glu, “C” allele). A wild type sequence is called allele “A”, and the presence of any of these variants is called collectively as allele “0” [17, 18]. Additional polymorphisms in the promoter and 5’UTR regions of the *MBL2* are also associated to reduced serum MBL levels [19]. The H/L polymorphism is located at -550 bp, X/Y is found at -221 bp (both

are G to C nucleotide substitutions), and P/Q is located at position +4 of the 5'UTR (C/T). These three *loci* are in strong linkage disequilibrium with exon 1 SNPs, in such a way that seven haplotypes are commonly found – HYPA, LYQA, LYPA, LXPA, LYPB, LYQC and HYPD [7, 20-22].

Several mechanisms link MBL and HIV infection. MBL can promote activation of inflammatory cells and, in doing so, induce the depletion of T CD4+ cells [23]. It has been shown that HIV-infected patients carrying *MBL2* mutations had a significantly shorter lifespan after AIDS diagnosis [24]. Heggelund *et al.* (2005) suggested that MBL can increase HIV replication, inducing persistent inflammatory response [25]. Also, the "B" allele was associated to disease progression, and two studies found association of exon 1 genotypes with HIV vertical transmission [26-28]. Although the mechanism of MBL-HIV interaction has not as yet been fully understood, most studies suggest that MBL deficiency is a risk factor to HIV infection. However, these findings were not replicated in all populations analyzed, and some studies do not point to a role of MBL in HIV infection [9, 23]. In the present work we genotyped a total of 410 samples from HIV-positive individuals and 345 healthy blood donors, both from Southern Brazil, in search for a possible association between these data and HIV infection.

## MATERIALS AND METHODS

### Patients and Controls

HIV-infected patients were consecutively enrolled at the South Brazilian HIV Cohort (SOBRHIV) from January, 2004 through November, 2005, in Porto Alegre, capital of Brazil's southernmost state [29]. The inclusion criteria were as follows: (1) asymptomatic HIV-infected individuals on HAART for at least one year (HAART prescribed according to Brazilian guidelines at that time) [2]; (2) HIV-1 RNA load < 50 copies/mL determined by the technical Versant HIV-1 RNA 3.0 assay / bDNA automation system in 340 bDNA Analyzer (Bayer, Germany); and (3) over 18 years of age. Exclusion criteria were: (1) pregnancy, (2) present use of drugs that could be associated to body changes such as corticosteroids or anabolizing steroids, and (3) mental illness. Written informed consent was signed by all individuals and the protocol was approved by the Hospital de Clínicas de Porto Alegre Committee on Ethics in Research.

A total of 410 samples from HIV-infected individuals, 224 men and 186 women, with ages ranging from 19 to 73 years were obtained. Patients were classified as European- or African-derived according to phenotypic characteristics of individuals and ethnicity data of parents/grandparents reported by the participants in an appropriate questionnaire. The issue concerning skin color-based classification criteria adopted in Brazil is well documented and has been already assessed by our group in previous studies [30, 31]. In total, 239 individuals were classified as European-derived and 171 were classified as African-derived.

The control group was formed by 345 unexposed, uninfected healthy blood donors, 244 being European-derived and 101 African-derived, from the urban

population of Porto Alegre, the capital of the southernmost state of Brazil. The demographic characteristics of both groups are described in Table 1. All patients and controls participating in this study gave their written informed consent. The genomic DNA for molecular characterization was obtained from 5-ml peripheral blood samples collected with EDTA and purified through a salting-out procedure as described by Lahiri and Nurnberger (1991) [32].

**Exon 1 genotyping**

The exon 1 polymorphisms genotyping was performed by a melting temperature assay described by Arraes *et al.* (2006) [27]. After melting temperature genotyping, all the A/0 and 0/0 individuals were confirmed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay, as previously described by our group [33], and the specific presence of “B”, “C” and “D” alleles was defined.

**Promoter region genotyping**

The promoter region of the *MBL2* gene spanning the -550 (L or H) and -221 (X or Y) polymorphisms was amplified by Polymerase Chain Reaction (PCR) with sequence-specific primers (PCR-SSP) as described by Neonato *et al*. (1999), using specific primers named: L forward, X reverse, H forward and Y reverse [34]. Four simultaneous reactions were performed for each sample, using different combinations of primers (LX, LY, HX and HY), to identify the haplotypes. The amplified fragments were visualized in 2% agarose gels. In addition, a control gene (cytochrome P450 debrisoquine, *CYP2D6*) was amplified in all the reactions.

**Statistical analysis**

*MBL2* genotypic distribution was determined by direct counting. The genotypic frequencies were compared to Hardy–Weinberg expectations using Chi-Square tests. *MBL2* allelic frequencies were compared between patients and controls using the Chi-square-test and adjusted residuals were also calculated. The significance level was set at $\alpha$ =0.05 (two-tailed). All statistical analyses were performed with SPSS 15.0 and WinPepi 10.0 softwares.

**RESULTS**

Data from the literature concerning *MBL2* polymorphic variant frequencies revealed a high level of interethnic diversity. For this reason, our analyses were performed subdividing the individuals according to ethnic origin [33, 35]. The European-derived group was composed by 244 healthy individuals and 239 HIV+ patients. In this group, we first compared the individuals for exon 1 *MBL2* polymorphisms (Table 2). The frequency of *MBL2* 0/0 homozygote genotype was 0.053 in controls and 0.042 in HIV+ patients; no statistical difference was found. All A/0 and 0/0 individuals were then genotyped in order to identify “B”, “C” and “D” alleles. The analyses of the allelic frequencies showed a higher frequency of the “D” allele in HIV+ patients, when compared to controls (p=0.002).

We also compared the frequency of polymorphisms in the promoter region of the *MBL2* gene (Table 3). Among healthy individuals, 0.041 were genotyped as X/X against 0.122 of the HIV+ patients (p=0.001). The analysis of the haplotypes showed a higher frequency of the LY haplotype in the control group, when compared to HIV-infected patients (p=0.048). The haplotypic combination frequencies were also compared between cases and controls, showing a higher frequency of the LX/LX genotype in the HIV-infected patients as compared to healthy individuals (0.122 and 0.041, respectively; p=0.001).

Finally, we put together the genotypes from both exon 1 and the promoter region of the *MBL2* and grouped them in three classes, associated to the potential level of serum MBL (as described in the literature): high serum MBL levels (HYA/A and LYA/A), intermediate serum MBL levels (LXA/LXA, HYA/0 and LYA/0) and deficient serum MBL levels (LXA/0 and 0/0) [12]. These results

are shown in Table 4. The frequency of genotypes associated with high serum MBL levels was 0.574 in healthy individuals and 0.460 in HIV+ patients (p=0.012). Conversely, frequency of genotypes associated with lower serum MBL levels was 0.053 in healthy individuals and 0.174 in HIV+ patients (p<0.0001).

The same approach was used to analyze the African-derived individuals (101 healthy individuals and 171 HIV + patients). Concerning exon 1 polymorphisms (grouped as allele “A” and allele “0”), similar genotype frequencies were observed in both patients and controls (Table 2). When the analysis was conducted using “B”, “C” and “D” alleles separately, we observed an increased frequency of “B” allele in patients (0.136) as compared to controls (0.065; p=0.009). Conversely, an increased frequency of “C” allele was detected in controls in comparison to patients (0.144 and 0.075, respectively; p=0.01) (Table 2).

Concerning the *MBL2* promoter region, a higher frequency of the LY/LY haplotypic combination was observed among healthy individuals (0.390), compared with HIV+ subjects (0.158; p<0.001); also, an increase in the LY/HY and HY/HY genotypes in patients (respectively 0.316 and 0.164) was observed in comparison with controls (0.18 and 0.07, respectively) (p=0.015 and p=0.026, in that order). These findings were reflected on haplotypic frequencies [see, for example, a frequency of 0.60 of the LY haplotype in the controls against 0.39 in HIV+ patients (p<0.001), and the frequency of 0.37 of the HY haplotype in patients, compared with 0.19 in controls (p<0.001)]. When genotypes were examined, H/L and H/H were more frequent in HIV+ individuals (0.42 and 0.16), compared to 0.24 and 0.07 in healthy individuals (p=0.003 and p=0.026), while

the L/L genotype had a frequency of 0.69 among controls and 0.41 in patients ($p<0.0001$) (Table 3). When the genotypes from exon 1 and the promoter region of the *MBL2* were grouped and analyzed, no statistically significant differences were observed.

We also performed analysis subdividing HIV-infected individuals according to co-infection with hepatitis B and C, considering that these infections have similar routes of transmission, making co-infection a common event. In the HIV-infected individuals group, there were 17 individuals co-infected with Hepatitis B Virus (0.04) and 103 individuals co-infected with Hepatitis C Virus (0.25). Yet, we did not find statistical differences (data not shown).

## DISCUSSION

In the present study, we investigated the association between *MBL2* gene polymorphisms and HIV infection in patients and ethnicity-matched controls from Southern Brazil. The ethnic classification used by our group (based on phenotypic characteristics of individuals and ethnicity data of parents/grandparents reported by the participants) is widely adopted in our country. However, we must admit that individuals classified as European-derived or African-derived can present a certain degree of admixture. A recent study by Santos *et al.* (2010), which assessed individual interethnic admixture using a 48-insertion-deletion Ancestry-Informative Marker panel, identified a very high level of European contribution (94%) and far fewer Native American (5%) and African (1%) genes in a sample of 81 European-derived individuals from southern Brazil [36]. Therefore, the subgrouping of the studied individuals according to the criteria employed in the present work seems to reflect the actual ethnic/genetic background of this human population. Since MBL is an important molecule in the first line defense of the organism, and considering its ability to bind to HIV-1 gp120 glycoprotein, several studies have evaluated *MBL2* polymorphisms in different populations in the context of HIV infection, although the conclusions were somewhat conflicting [37-39]. In view of the variability in the frequency of allelic variants of MBL in different human populations, our analyses were performed subdividing the individuals according to their ethnic origin [40].

Among European-derived individuals we observed a higher frequency of the “D” allele in HIV+ patients as compared to controls. Nevertheless, considering “B”, “C” and “D” alleles grouped under the designation of “0” allele,

similar frequencies were observed in patients and controls. Studies with Spanish and Colombian HIV+ individuals were done and also reported no statistical differences considering the polymorphisms of the *MBL2* structural region [41, 42]. The importance of considering the ethnic origin of the analyzed population becomes evident when we observe that other studies found different results, with the frequencies of the 0/0 genotype increased among HIV+ individuals or associated with disease progression in children [27, 43-47]. Also, it is important to point out that neither the study with Spanish nor the investigation in Colombian individuals approached the *MBL2* promoter region polymorphisms.

The analyses of the *MBL2* promoter region polymorphisms showed a frequency of 0.122 of the LX/LX haplotypic combination in HIV+ individuals, compared to 0.041 in healthy individuals, indicating a higher frequency of the genotype related with low MBL levels in patients. Promoter haplotypes and genotypes frequencies also suggested a tendency of increased frequency of the LX haplotype and of the X/X genotype in HIV positive individuals as compared to controls, both indicating the increased presence of the X allele, which is related to low serum MBL levels in patients. Taken together, these results point to a possible role for these SNPs, and consequently for low MBL levels, in HIV infection.

It is interesting to note that other studies also suggest a role of the X/X genotype in the context of HIV infection. For instance, the X/X genotype was already associated with a rapid rate of HIV infection progression [40], and homozygosity for the XA/XA genotype was suggested as an important genetic

determinant of HIV-1 acquisition through vertical transmission and the pathogenesis of pediatric HIV/AIDS [45].

Although most studies with HIV patients do not analyze simultaneously both exon 1 and promoter polymorphisms of *MBL2*, it is clear that this approach is able to provide much more information about a real role of MBL in HIV infection than comparisons made only with the promoter or the coding variants. The exon 1 variants disturb protein polymerization, not only resulting in reduced serum MBL levels, but also functionally altering its ligand binding and complement activation capacities. The SNPs in the promoter region can modulate the serum concentration of the protein [48, 49]. Therefore, through a combination of structural and promoter gene polymorphisms, MBL concentrations may present a wide variation.

When promoter and exon 1 gene polymorphisms are considered together, we found a significant difference between patients and controls among the European-derived individuals. The haplotypic combinations related to high MBL levels (LYA/A and HYA/A) were more frequent in controls (0.57) than in HIV+ individuals (0.46), and the genotypes of haplotypes related with deficient MBL levels (LXA/0 and 0/0) were more frequent in HIV+ patients (0.17) when compared to controls (0.05). These data are in agreement with a study in Hispanic individuals, where HIV positive children, under two years of age and who have *MBL2* genetic variants resulting in lower MBL levels were at a significantly greater risk of disease progression and central nervous system impairment as compared to the other patients tested [46]. Other studies found that HIV+ pediatric patients who are carriers of the “B” allele and/or have low MBL levels progress more rapidly to AIDS. Also, adult HIV+ patients with this

pattern exhibit a significantly shorter survival time after an AIDS diagnosis [44, 50, 51].

The importance of considering the ethnic origin of the analyzed population is again evidenced when we analyzed the African-derived patients. Results from exon 1 polymorphisms (grouped as “0” allele) showed similar frequencies of the genotypes between patients and controls, although we observed an increased frequency of the “B” allele among patients and, oppositely, an increased frequency of the “C” allele in controls. It is important to point out that the *MBL2* gene “C” allele is relatively frequent among individuals of African ancestry, which can explain its higher frequency observed among our controls [52, 53]. Another study, in a South African population, observed that in the absence of intervention, infants with *MBL2* genetic variants were more likely to acquire HIV from their mothers than infants with normal *MBL2* genes [54].

The analyses of promoter polymorphisms showed an increased frequency of the LY/HY and HY/HY haplotypic combinations in patients, when compared to controls, as well as a lower frequency of LY/LY. The analysis of *MBL2* haplotypes confirmed this higher frequency of the LY haplotype in controls, and of the HY haplotype in HIV positive individuals. Since the LY haplotype is related to intermediate MBL serum levels and the HY haplotype is related with high MBL serum levels, the results indicate that African-derived patients have genotypes related to high MBL levels. Some studies with African-derived population suggested an association of MBL deficiency with resistance against leprosy and leishmaniasis [55, 56]. The high prevalence of infections caused by intracellular pathogens in Africa may explain the differences in the *MBL2* allelic frequency observed between European and African populations [53]. Two

studies carried out with sub-Saharan and Indian populations showed that low MBL levels were related with susceptibility to HIV infection, but high levels of MBL may be involved in the pathogenesis of tuberculosis in these individuals, indicating that MBL may exert opposing effects, suggesting the need of a balance, modulated by different pathogens [51, 57].

In the present study only asymptomatic HIV-infected individuals that have been on HAART for one year and that have an HIV RNA of <50 were included. However it can be argued that these criteria restrict the analyses to a specific group, since MBL levels and/or alleles have been associated with progression of HIV disease in infected individuals, it also lends more robustness to our results. Actually, we observed a marked difference in the SNPs frequencies in terms of ethnicity, evidenced in European-derived individuals — for whom the majority of HIV positive patients have genotypes related to low MBL serum levels, when compared to healthy individuals. Although the results in African-derived individuals point to an opposite effect for MBL in HIV infection, we believe that other genetic factors that reflect the selection exercised by the exposure of this ethnic group to a different environment through time are responsible for these differences. In conclusion, since low serum MBL concentrations are associated with inefficient phagocytosis and opsonization, our data suggest that *MBL2* polymorphisms — and consequently low MBL levels — could lead to an inefficient response against viral infection in individuals with European ancestry. Thus, we suggest that individuals carrying *MBL2* variants, and who are consequently less efficient in clear pathogens with mannose surface residues, such as HIV-1, would be more susceptible to HIV-1 infection.

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## TABLES

**Table 1** – Demographic characteristics of the study group

| Characteristics | HIV-infected individuals | Control group |
|---|---|---|
| Gender (male/females) | 224/186 | 243/102 |
| Age (years ± S.D. and min-max) | 42.98 ± 9.42 (19-73) | 43.43 ± 7.78 (20-62) |
| Ethnicity (European/African-derived) | 239/171 | 244/101 |
| HCV co-infection (n, %) | 103 (25.1%) | NA |
| HBV co-infection (n, %) | 17 (4.1%) | NA |

NA – Not apply.

Table 2 – Distribution of *MBL2* exon 1 polymorphisms allelic and genotypic frequencies in HIV-1 infected individuals and controls, according to ethnicity

| | | European-derived | | African-derived | |
|---|---|---|---|---|---|
| | | Control | HIV+ | Control | HIV+ |
| | | Freq (n) | Freq (n) | Freq (n) | Freq (n) |
| Genotypes | | n=244 | n=239 | n=101 | n=171 |
| A/A | | 0.607 (148) | 0.544 (130) | 0.584 (59) | 0.526 (90) |
| A/0 | | 0.340 (83) | 0.414 (99) | 0.366 (37) | 0.409 (70) |
| 0/0 | | 0.053 (13) | 0.042 (10) | 0.050 (5) | 0.064 (11) |
| | | $\chi^2$ p=0.233 | | $\chi^2$ p=0.630 | |
| Genotypes | | n=244 | n=229 | n=101 | n=167 |
| | A/A | 0.607 (148) | 0.568 (130) | 0.584 (59) | 0.539 (90) |
| A/0 | A/B | 0.246 (60) | 0.223 (51) | 0.099 (10) | 0.192 (32) |
| A/0 | A/C | 0.037 (9) | 0.039 (9) | 0.218 (22) | 0.132 (22) |
| A/0 | A/D | 0.057 (14) | 0.127 (29) | 0.050 (5) | 0.072 (12) |
| 0/0 | B/C | 0.020 (5) | 0.000 (0) | 0.010 (1) | 0.006 (1) |
| 0/0 | B/D | 0.008 (2) | 0.018 (4) | 0.000 (0) | 0.018 (3) |
| 0/0 | C/D | 0.000 (0) | 0.004 (1) | 0.000 (0) | 0.012 (2) |
| 0/0 | B/B | 0.025 (6) | 0.013 (3) | 0.010 (1) | 0.030 (5) |
| 0/0 | C/C | 0.000 (0) | 0.004 (1) | 0.030 (3) | 0.000 (0) |
| 0/0 | D/D | 0.000 (0) | 0.004 (1) | 0.000 (0) | 0.000 (0) |
| No statistical comparison was performed on these data due to the small number of individuals in each subgroup. | | | | | |
| Alleles | | n=488 | n=468 | n=202 | n=334 |
| | A | 0.777 (379) | 0.767 (359) | 0.767 (155) | 0.736 (246) |
| | B | 0.162 (79) | 0.130 (61) | 0.065 (13)[b] | 0.138 (46)[b] |
| | C | 0.029 (14) | 0.026 (12) | 0.144 (29)[c] | 0.075 (25)[c] |
| | D | 0.033 (16)[a] | 0.077 (36)[a] | 0.025 (5) | 0.051 (17) |

| | | |
|---|---|---|
| | $\chi^2$ p=0.016<br>[a] Adjusted residual, p=0.003 | $\chi^2$ p=0.003<br>[b] Adjusted residual, p=0.009<br>[c] Adjusted residual, p=0.01 |

0 allele = B+C+D alleles; A allele = wild type

A/A genotype: high serum MBL levels

A/0 genotype: intermediate serum MBL levels

0/0 genotype: lower serum MBL levels

**Table 3 –** *MBL2* promoter polymorphisms frequencies in patients and controls, according to ethnicity

| | European-derived | | African-derived | |
|---|---|---|---|---|
| | Control | HIV + | Control | HIV + |
| | Freq (n) | Freq (n) | Freq (n) | Freq (n) |
| Haplotypic combination | n=244 | n=237 | n=100 | n=171 |
| **LX/LX** | 0.041 (10)[a] | 0.122 (29)[a] | 0.060 (6) | 0.105 (18) |
| **LX/LY** | 0.193 (47) | 0.135 (32) | 0.240 (24) | 0.152 (26) |
| **LX/HY** | 0.102 (25) | 0.110 (26) | 0.060 (6) | 0.105 (18) |
| **LY/LY** | 0.180 (44) | 0.169 (40) | 0.390 (39)[b] | 0.158 (27)[b] |
| **LY/HY** | 0.336 (82) | 0.291 (69) | 0.180 (18)[c] | 0.316 (54)[c] |
| **HY/HY** | 0.148 (36) | 0.173 (41) | 0.070 (7)[d] | 0.164 (28)[d] |
| | $\chi^2$ p=0.018 | | $\chi^2$ p<0.001 | |
| | [a] Adjusted residual, $\chi^2$ p=0.001 | | [b] Adjusted residual, $\chi^2$ p<0.001 | |
| | | | [c] Adjusted residual, $\chi^2$ p=0.015 | |
| | | | [d] Adjusted residual, $\chi^2$ p=0.026 | |
| Haplotypes | n=488 | n=474 | n=200 | n=342 |
| **LX** | 0.188 (92)[e] | 0.245 (116)[e] | 0.210 (42) | 0.234 (80) |
| **LY** | 0.445 (217)[f] | 0.382 (181)[f] | 0.60 (120)[g] | 0.392 (134)[g] |
| **HY** | 0.367 (179) | 0.373 (177) | 0.19 (38)[h] | 0.374 (128)[h] |
| | $\chi^2$ p=0.054 | | $\chi^2$ p<0.001 | |
| | [e] Adjusted residual, $\chi^2$ p=0.034 | | [g] Adjusted residual, $\chi^2$ p<0.001 | |
| | [f] Adjusted residual, $\chi^2$ p=0.048 | | [h] Adjusted residual, $\chi^2$ p<0.001 | |
| Genotypes | n=244 | n=237 | n=100 | n=171 |
| **L/L** | 0.414 (101) | 0.426 (101) | 0.690 (69)[j] | 0.415 (71)[j] |
| **H/L** | 0.438 (107) | 0.401 (95) | 0.240 (24)[l] | 0.421 (72)[l] |
| **H/H** | 0.148 (36) | 0.173 (41) | 0.070 (7)[m] | 0.164 (28)[m] |
| | $\chi^2$ p=0.626 | | $\chi^2$ p<0.0001 | |
| | | | [j] Adjusted residual, $\chi^2$ p<0.0001 | |
| | | | [l] Adjusted residual, $\chi^2$ p=0.003 | |

| | | | [m] Adjusted residual, $\chi^2$ p=0.026 | |
|---|---|---|---|---|
| **X/X** | 0.041 (10)[l] | 0.122 (29)[l] | 0.060 (6) | 0.105 (18) |
| **X/Y** | 0.295 (72) | 0.245 (58) | 0.300 (30) | 0.258 (44) |
| **Y/Y** | 0.664 (162) | 0.633 (150) | 0.640 (64) | 0.637 (109) |
| | $\chi^2$ p=0.004 | | $\chi^2$ p=0.390 | |

[i] Adjusted residual, $\chi^2$ p=0.001

Genotype LX: lower serum MBL levels

Genotype LY: intermediate serum MBL levels

Genotype HY: high serum MBL levels

**Table 4 –** *MBL2* haplotypic combination related to MBL serum levels in HIV-1 patients and healthy controls, according to ethnicity

| | Euro-descendant | | Afro-descendant | |
|---|---|---|---|---|
| | Control Freq (n) | HIV + Freq (n) | Control Freq (n) | HIV + Freq (n) |
| Haplotypes: | n=244 | n=235 | n=100 | n=167 |
| High | 0.574 (140)[a] | 0.460 (108)[a] | 0.540 (54) | 0.443 (74) |
| Intermediate | 0.373 (91) | 0.366 (86) | 0.290 (29) | 0.395 (66) |
| Deficient | 0.053 (13)[b] | 0.174 (41)[b] | 0.170 (17) | 0.162 (27) |
| | $\chi^2$ p<0.0001 | | $\chi^2$ p=0.202 | |

[a] Adjusted residual, p=0.012

[b] Adjusted residual, p<0.0001

Haplotypes:

High serum MBL levels (LYA/A, HYA/A)

Intermediate serum MBL levels (LXA/LXA, LYA/0, HYA/0)

Deficient serum MBL levels (LXA/0, 0/0)

## 4. CONCLUSÕES GERAIS

A infecção pelo HIV-1 tem sido estudada extensivamente por muitos grupos, e o que continua intrigando os pesquisadores é a capacidade que o vírus tem de infectar as células e “enganar” o sistema imune, não sendo reconhecido, e conseqüentemente, não induzindo uma adequada resposta imune (Adamson e Freed, 2010; An e Winkler, 2010; Blankson, 2010; Casado *et al.*, 2010). Por essas razões, decidimos investigar a influência do gene *MBL2* na infecção pelo HIV-1. Como já foi dito, o gene *MBL2* codifica a proteína MBL, que está envolvida no reconhecimento de carboidratos presentes na superfície de microorganismos, desencadeando a resposta imune para eliminação desses patógenos (Ip *et al.*, 2004; Fiane *et al.*, 2005; Worthley *et al.*, 2005; Dommett *et al.*, 2006; Selander *et al.*, 2006). Dentre os microorganismos que a MBL é capaz de reconhecer encontra-se o HIV (Neth *et al.*, 2000; Hart *et al.*, 2003; Ying *et al.*, 2004; Bouwman *et al.*, 2006). Muitos estudos foram feitos na tentativa de explicar a relação da MBL com a infecção pelo HIV-1, mas até agora os resultados foram inconclusivos (Madsen *et al.*, 1995; Garred *et al.*, 1997; Maas *et al.*, 1998; Malik *et al.*, 2003; Heggelund *et al.*, 2005; Dzwonek *et al.*, 2006; Garcia-Laorden *et al.*, 2006; Vallinoto *et al.*, 2006; Singh *et al.*, 2008; Tan *et al.*, 2009).

Dessa maneira, analisamos os polimorfismos presentes no gene *MBL2* em um grupo de pacientes infectados pelo HIV-1 e em um grupo de indivíduos controles não infectados. Nosso estudo demonstrou que os polimorfismos do gene *MBL2*, que causam diminuição dos níveis séricos da proteína, estão em maior freqüência em indivíduos Euro-descendentes HIV-1-positivos, quando comparados com o grupo controle, indicando que indivíduos Euro-

descendentes portadores das variantes do gene *MBL2*, e conseqüentemente, de baixos níveis de MBL, podem apresentar dificuldade em eliminar patógenos com resíduos de manose na superfície, incluindo o HIV-1, aumentando a susceptibilidade à infecção pelo HIV-1. Outro achado importante foi a notável heterogeneidade genética existente entre os dois grupos étnicos analisados, já que os resultados encontrados no grupo de indivíduos Euro-descendentes foi oposto ao encontrado no grupo dos indivíduos Afro-descendentes. No grupo de Afro-descendentes, nossos achados demonstraram maior freqüência dos genótipos relacionados com altos níveis de MBL em pacientes infectados pelo HIV, quando comparado com controles. Nesse grupo de indivíduos a MBL parece ter um papel oposto, como já foi descrito, há uma grande prevalência de infecções por patógenos intracelulares na África, e isso pode explicar as diferenças observadas nas freqüências alélicas de MBL entre as populações Euro e Afro-descendentes. A presença de genótipos relacionados com baixos níveis de MBL pode significar um fator protetor contra essas infecções, e dessa maneira, como os pacientes apresentaram genótipos relacionados com níveis altos de MBL, eles podem estar mais susceptíveis à infecção por esses patógenos. Mas como a análise combinada dos polimorfismos do éxon 1 e do promotor não demonstrou diferença na comparação entre pacientes e controles, outros fatores genéticos que refletem a exposição desse grupo étnico à um ambiente diferente devem ser responsáveis pelos mecanismos envolvidos na infecção pelo HIV-1 em indivíduos Afro-descendentes.

Nosso trabalho avaliou proteínas relacionadas com o reconhecimento antigênico no sistema imune, tentando elucidar os mecanismos imunogenéticos usados pelo HIV-1 para infectar o sistema imune sem induzir uma resposta

imunológica. Nossos resultados indicam a existência de um papel dos polimorfismos do gene *MBL2* na infecção pelo HIV. No entanto, nosso estudo desvenda apenas uma pequena parcela dos diversos mecanismos envolvidos na defesa do nosso organismo contra infecções virais.

Durante o desenvolvimento desse estudo foi publicado um artigo pelo nosso grupo, onde foi evidenciada a associação entre os polimorfismos do gene do Antígeno Leucocitário Humano–G (HLA-G) em pacientes com Anemia Falciforme co-infectados com hepatite C (Cordero *et al.*, 2009). A partir dessa informação surgiu a idéia de analisarmos a influência dos polimorfismos do gene HLA-G em nossa amostra, já que o HIV e o HCV possuem rotas de transmissão similares, tornando a co-infecção um evento comum (Soriano *et al.*, 2010). O gene HLA-G situa-se no Complexo de Histocompatibilidade Principal (MHC), uma região que contém grande número de genes com funções importantes na regulação do sistema imune (Carosella *et al.*, 1999). A molécula HLA-G é capaz de inibir a resposta imune em diferentes níveis e tipos celulares, o que é de grande interesse para alguns patógenos, já que eles podem usar mecanismos semelhantes para escapar da vigilância imunológica (Van Der Ven *et al.*, 2000; Larsen e Hviid, 2009; Menier *et al.*, 2009; Veit e Chies, 2009). Além disso, foi descrito que a infecção pelo HIV-1 pode aumentar os níveis de expressão da molécula HLA-G (Lozano *et al.*, 2002; Tripathi e Agrawal, 2007). Dessa maneira analisamos dois polimorfismos que estão relacionados com os níveis de expressão de HLA-G. Foi encontrada maior freqüência dos alelos relacionados com menor expressão de HLA-G nos indivíduos infectados pelo HIV-1, em comparação com o grupo controle, dentro do grupo de indivíduos Afro-descendentes. Novamente foi observada uma

variedade étnica, já que encontramos associações dentro do grupo dos indivíduos Afro-descendentes que não se repetiram nos indivíduos euro-descendentes, onde não foi encontrada nenhuma associação com os polimorfismos da molécula HLA-G.

Nossos achados discordam de alguns dados da literatura, que encontraram níveis aumentados de HLA-G em pacientes infectados pelo HIV, demonstrando que altos níveis de HLA-G solúvel podem contribuir para o escape viral do sistema imune devido à ação imunossupressora dessa molécula (Lozano *et al.*, 2002; Matte *et al.*, 2004; Lajoie *et al.*, 2006; Tripathi e Agrawal, 2007). Embora estes resultados indiquem que a expressão de altos níveis de HLA-G possa suprimir o sistema imunológico, permitindo a infecção pelo vírus, nós especulamos um papel diferente do HLA-G durante a infecção pelo HIV.

Já foi descrito que a molécula HLA-G desempenha atividade anti-viral, potencialmente através da ativação das células *natural killer*. A ativação dessas células pode induzir citocinas pró-inflamatórias, que quando liberadas, podem ativar mais células NK (Rajagopalan *et al.*, 2006; Van Der Meer *et al.*, 2007). Assim, nossos dados aliados às informações acima sugerem um possível papel da HLA-G na defesa contra infecção viral pela ativação da imunidade inata. Podemos supor que indivíduos HIV-positivos com genótipos relacionados à baixa expressão de HLA-G são mais suscetíveis à infecção pelo HIV e também à co-infecção com hepatite C (o artigo em preparação para submissão está em anexo).

O sistema imune é composto por uma rede de interações simultâneas, que resultam em cascatas de ativação e inibição. Ao mesmo tempo em que o

sistema imunológico elimina patógenos, ele também deve evitar respostas que produzam danos aos tecidos próprios ou que eliminem micróbios comensais benéficos. O sistema imune inato inclui barreiras físicas, proteínas solúveis, citocinas, quimiocinas, receptores de membrana, etc. É preciso estudar a influência de cada um desses fatores para elucidar o papel do sistema imune no desenvolvimento de cada patologia (Chaplin, 2010).

A resposta imune contra o HIV é modulada por múltiplos determinantes genéticos, muitos deles relacionados direta ou indiretamente com o reconhecimento do vírus [receptores de quimiocinas, HLA, receptores de células T, anticorpos, MBL, receptores tipo imunoglobulina das células NK, receptores *Toll-like* (TLR)], tráfico de células imunes (quimiocinas e receptores, moléculas de adesão), e amplificação da resposta imune (genes de citocinas) (Kaur e Mehra, 2009).

A compreensão dos efeitos dos componentes citados acima pode levar ao desenvolvimento de vacinas anti-HIV e estratégias para aumentar as defesas do hospedeiro contra a infecção.

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## 6. ANEXOS

### ANEXO 1

28 Jun 2010

TO: Mr Jose A Chies
Porto Alegre, RS
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E-mail: jabchies@terra.com.br
Fax: 555133087311

Dear Jose Chies,
Manuscript reference number: AIDS-D-10-00652
Title: The role of Mannose-Binding Lectin gene polymorphisms in the susceptibility to HIV-1 infection in Southern Brazilian patients
Article type: Original paper (Basic Science)

Thank you for submitting your paper to AIDS.
The Editors have reviewed it and have decided in light of the reviewers' comments that the paper is not suitable for publication in its present form. However, should you decide to resubmit, having revised your manuscript and making major changes according to the referees' comments, the Editors will reconsider its suitability for publication. Resubmitted papers may be subject to further peer review, and can be rejected without further written explanation. Please ensure that limits to length of structured abstracts (250 words) and titles (120 characters) are not exceeded, and that authorship is limited to those who have made a substantial contribution to the paper - justification of more than 10 names should be submitted to the Editors. More than 12 authors is not acceptable.
I look forward to hearing from you.
Yours sincerely,
Madiha Chaudry
Editorial Co-ordinator, AIDS
Email: Madiha.Chaudy@wolterskluwer.com

1. To submit a revision, go to http://aids.edmgr.com/ and log in as an Author using your username (Your username is: XXXX) and password (Your password is: XXXX). You will see a menu item called Submission Needing Revision. You will find your submission record there. Click on 'Submit revision' to begin the resubmission process.
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4. In this document state how you have responded to the referee(s) comments.
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6. The title must be no longer than 120 characters with a running head of no more than 40 characters.
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8. At the end of the manuscript, provide a description of the role of each of the authors in the study reported.
9. Please note that the inclusion of a signed copyright transfer form will be required for resubmission. This form is available on the AIDS Editorial Manager home page and the Instructions for Authors page.

Reviewers' comments:

Reviewer #1: da Silva et al. investigate the association of mannose-binding lectin (MBL) gene polymorphisms and allele frequencies with HIV positivity among a population-based cohort in southern Brazil. They reveal a range of specific polymorphisms and alleles that are associated with a higher likelihood of HIV infection and are unique to particular ethnic subdivisions in the cohort. They provide an interesting interpretation of those genetic alterations in the context of HIV infection and immune phenotype. Finally the authors posit a role for natural selection in the geographic/ethnic variation in MBL genotypes observed in this and other studies.
General comments:
1. The manuscript text suffers from a wide variety of spelling, grammar, and usage errors, as well as overall organizational issues most apparent in the Discussion section. These errors are distracting and detract from the overall message of the paper; a thorough revision of the text is critical.
2. In the Discussion, the authors determine specific genetic changes associated with HIV positivity but do not provide an adequate interpretation of those specific changes and their potential role in promoting HIV infection. Without such interpretation, the functional significance of these findings is unclear.
3. The main findings of the manuscript are contingent on multiple chi-square statistical tests, although the authors make no mention of correcting the p-values of these tests for multiple comparisons. In the context of genetic analysis with iterative statistical testing, such correction is essential and may negate a number of the significant p-values.
4. A demographic comparison of the patient and control groups is wholly lacking in the manuscript. The authors should describe these two groups in a separate table (typically Table 1) and/or provide evidence of the exchangeability of these two populations for factors such as age, gender, ethnicity, geographic location. Such factors could serve as potential confounders explaining both susceptibility to HIV infection as well as MBL genotype variation and distribution.
5. The Discussion does not contain a well-defined description of limitations, although there are a number of limitations both with this study in particular as well as statistical genetics studies in general. The authors should add this section to the Discussion.
Specific comments:
1. In the Introduction, the sentence "HIV-1 is much more prevalent in Brazil due to both the origin of the infection in the country?" is unclear and misleading.
2. In the Introduction, the sentence "conversely, MBL-facilitated opsonization and phagocytosis may increase the infectivity of some intracellular pathogens.

In this case, low MBL levels would be better." is vague. The authors should define what is meant by "better."
3. In Methods, the sentence "The issue arisen on the skin color-based classification criteria that is used in Brazil is well documented and has been already assessed by our group in previous studies?" is unclear. The authors should expand on the issue/limitation and include this in a limitations section of the Discussion.
4. The Methods section should provide a clear means of correction of statistical tests for multiple hypothesis testing per (3) above.
5. The Discussion section is poorly organized and the focus of a number of the paragraphs is unclear. For example, the paragraph on p.13 that begins, "Therefore, through a combination of structural and promoter gene polymorphisms, MBL concentrations can present a wide variation." then proceeds to report two unrelated lines of reasoning: the potential phenotypes of MBL-variation found in the study (and their relation to HIV positivity), as well as the observation by others that MBL phenotype is related to HIV disease progression (not positivity). Similarly, the paragraph that begins, "Also, another study, in a South African population, observed that in the absence of intervention, infants with MBL2 genetic variants were more likely to acquire HIV from their mothers than were infants with normal MBL2 genes?" proceeds to describe specific haplotype markers of infection susceptibility that were revealed in the current study (and may have no relation to the SA finding in infants). Restructuring of these paragraphs is critical as the Discussion does not follow a clear logical sequence.

Reviewer #2: This manuscript details a cross-sectional retrospective study of frequency of MBL polymorphisms in HIV infected persons in southern Brazil. Several significant relationships between HIV-infected persons and alleles associated with low MBL levels were noted. Overall this is a well-written study.
1. One important issue that needs to be explained in the materials and methods is the rationale for the inclusion criteria. Only asymptomatic HIV-infected persons that have been on HAART for one year and that have an HIV RNA of <50 were included. Thus, infected persons that progressed would not be included. Since MBL levels and/or alleles have been associated with progression of HIV disease, this would skew the MBL types of the HIV-infected population that is analyzed.
2. The paper should include a discussion of the 2003 study in Immunogenetics (Malik et al.).

**ANEXO 2**

**Manuscrito em preparação para submissão ao periódico AIDS.**

# INFLUENCE OF HLA-G POLYMORPHISMS IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION AND HEPATITIS C VIRUS CO-INFECTION IN BRAZILIAN HIV POSITIVE PATIENTS

Running head: HLA-G polymorphisms in HIV and HCV co-infection

Gabriela Kniphoff da SILVA[1], Priscila VIANNA[1], Tiago Degani VEIT[1], Vanessa Suñé MATTEVI[2], Rosmeri Kuhmmer LAZZARETTI[3], Eduardo SPRINZ[3], Regina KUHMMER[3], José Artur Bogo CHIES[1].

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## ABSTRACT

**Objective:** This study aimed to investigate the role of Human leukocyte antigen (HLA)-G in the susceptibility to HIV-1 infection through the analysis of HLA-G gene polymorphisms 14bp insertion/deletion and +3142 (C/G), located at 3' UTR region.

**Materials and Methods:** We investigated the prevalence of the variant alleles in 408 HIV-1 infected patients from the South Brazilian HIV Cohort, and in 545 unexposed uninfected healthy individuals. The HLA-G polymorphisms were genotyped using PCR-RFLP assay. Genotypic and allelic frequencies in both groups analyzed, namely HIV-1 infected subjects and controls, were compared using Chi-square or Fischer exact tests. Also, haplotypic frequencies were estimated using MLocus software.

**Results:** All analysis were performed subgrouping the individuals according to their ethnic origin. Concerning the Euro-derived individuals the allelic and genotypic frequencies between patients and controls did not show statistical differences. However, Afro-derived HIV-infected individuals presented the higher frequency of G/ins haplotype, when compared to controls ($p=0.005$). We also evaluated the influence of HCV co-infection among these patients and we found high frequency of the ins/ins genotype in African-derived individuals ($p=0.024$).

**Conclusions:** Our data point out to an increased frequency of genotypes associated with low HLA-G expression among African-derived patients, suggesting a potential role for HLA-G in the susceptibility to HIV-1 infection and HCV co-infection in African-derived individuals.


Key Words: HLA-G, 14bp, +3142, HIV, ethnicity.

## INTRODUCTION

The human immune system involves a complex array of mechanisms to distinct pathogens or toxins from host cells. This discrimination is essential to allow the host to eliminate the injury without damaging its own tissues [1]. It was already described that highly polymorphic molecules encoded by genes of the Major Histocompatibility Complex (MHC) play a key role in immune system functions. The human leukocyte antigen (HLA)-G is a nonclassical HLA class Ib molecule able to suppress immune responses, contributing to immune escape or tolerance [2]. This immunomodulatory molecule was first characterized by its expression at maternal–fetal interface, protecting the fetus against the maternal immune system [3-5]. Over the years, emerging studies have shown the relevance of the HLA-G molecule in pathologic conditions, such as transplant rejection, autoimmunity, cancer, viral infection and inflammation [6-9].

The *HLA-G* gene encodes seven protein isoforms, including four membrane-bound (G1 to G4) and three soluble molecules (G5 to G7), generated by alternative splicing. Its coding region displays a quite low allelic polymorphism, with only 14 protein variants described to date. However, its 5’ regulatory region, as well as its 3’ region, is highly polymorphic. The 3' untranslated region (UTR) seems to play an important role in regulating HLA-G expression [7]. A 14 bp insertion/deletion polymorphism (rs1704) located at this region (exon 8) was shown to play a role in alternative splicing and was also associated with different levels of HLA-G in plasma. Transcripts with the 14 nucleotides sequence could undergo an additional splicing step which removes 92 bases including the region where this sequence is located. Although the 14 bp insertion allele was associated with a more stable subpopulation of *HLA-G*

mRNAs, in vitro studies showed that this allele also presents a decreased HLA-G expression [9, 10]. A C/G SNP (single nucleotide polymorphism) located less than 200 bp away from the 14 bp polymorphic site, at position +3142 (rs1063320), is thought to influence microRNA (miRNA) binding, thus influencing RNA turnover and miRNA-mediated repression of translation. In this polymorphic site, the G allele favors the targeting of three miRNAs to the binding site and therefore directs a reduction on the HLA-G expression. Both polymorphisms are in linkage disequilibrium [7, 9].

The HLA-G role in the immune system is extensive since this molecule is able to inhibit immune responses in different levels, acting in different immune cell types. The immnunossupressive HLA-G activity is of interest to some pathogens, since they might use these same mechanisms to escape immune surveillance [11]. It was already described that Human Immunodeficiency Virus (HIV) infection leads to increased HLA-E and HLA-G expression, indicating that the virus can develop a strategy for controlling HLA-G expression taking advantage of its specific immune tolerance function [12, 13].

The Acquired Immune Deficiency Syndrome (AIDS) became worldwide known after the 1980´s, when the first epidemic signals appeared, and until now, there is no cure or effective vaccine for this disease. Without treatment, life-expectancy of patients diagnosed with AIDS is around two years [14, 15]. Interestingly, humans show a notably variation in vulnerability to HIV infection, probably due to genetic and immunologic factors. HIV transmission depends both on factors of infectivity as well as on host susceptibility [16].

There are two different HIV types, HIV-1 and HIV-2, however HIV-1 is much more prevalent in Brazil [17, 18]. The infectivity depends on HIV-1

concentration, virus presence and on the number of infected cells in body fluids. However, the viral load and cell used for HIV-1 transmission remain poorly understood [18]. Until 2008 there were 34.4 million HIV-infected people worldwide and 2.0 million deaths during 2008 [19]. In Brazil, about 462 thousand cases of the disease were registered since the first case in 1980 until 2008 [15]. The resistance to HIV infection reflects a combination of genetic factors, innate and acquired immunoresistance. Among the genetic factors associated with HIV resistance, homozygosis of the delta 32 allele of the *CCR5* gene is by far the best known [18].

Considering that not all mechanisms used by the HIV-1 to infect cells and to escape immune surveillance are known, the role of HLA-G as an immunomodulatory molecule in HIV infection was investigated. In the present work, we evaluated the 14bp insertion/deletion and +3142C/G *HLA-G* polymorphisms in samples from HIV-infected individuals and unexposed, uninfected blood donors from Southern Brazil, seeking a possible association of these polymorphisms with HIV infection.

## MATERIALS AND METHODS

### Patients and Controls

HIV-infected patients were consecutively enrolled at the South Brazilian HIV Cohort (SOBRHIV) from January, 2004 through November, 2005, in Porto Alegre, capital of Brazil's southernmost state [20]. The inclusion criteria for the patients group were: (1) asymptomatic HIV-infected individuals on High activity Anti-Retrovirus Therapy (HAART) for, at least, one year (prescribed according to Brazilian guidelines at that time) [15]; (2) an HIV-1 RNA load < 50 copies/mL, determined by the technical Versant HIV-1 RNA 3.0 assay / bDNA automation system in 340 bDNA Analyzer (Bayer, Germany); and (3) older than 18 years. The exclusion criteria included: (1) pregnancy, (2) present use of drugs that could be associated to body changes such as corticosteroids or anabolizing steroids and (3) mental illness.

A total of 408 samples from HIV-infected individuals, 222 men and 186 women, with ages ranging from 19 to 73 years old were obtained. Patients were classified as European or African-derived according to phenotypic characteristics of individuals and ethnicity data of parents/grandparents reported by the participants, filling an appropriate inventory. The issue arisen on the skin color-based classification criteria that is used in Brazil is well documented and has been already assessed by our group in previous studies [21-23]. We classified 237 individuals as European-derived and 171 as African-derived.

The control group was formed by 545 unexposed, uninfected healthy blood donors, 235 being European-derived and 310 African-derived, from the urban population of Porto Alegre, the capital of the southernmost state of Brazil.

The inclusion criteria for controls were: good health, ages between 18 and 65 years, weigh over 50 kilograms. The exclusion criteria included: fever, cold or flu, pregnancy, puerperium, use of some medicines, people who adopt risky behavior for sexually transmitted diseases, people who underwent surgical procedure, vaccinations or tattoos in the last months and use of illicit drugs. Beyond the clinical trials performed do indentify blood-borne diseases: hepatitis, AIDS (HIV virus), HTLV virus, Chagas disease and malaria.

The demographic characteristics of both groups are described in Table 1. All patients and controls participating to this study gave their written informed consent and the protocol was approved by the Hospital de Clínicas de Porto Alegre Committee on Ethics in Research. The genomic DNA for molecular characterization was obtained from samples of 5ml of peripheral blood, collected with EDTA, and purified through a salting-out procedure as described by Lahiri and Nurnberger (1991) [24].

**Genotyping of *HLA-G* gene polymorphisms**

The 14bp insertion/deletion and +3142 polymorphisms were genotyped through Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay, and different reactions were performed for each polymorphism, as previously described by our group [25]. The results for the 14bp insertion/deletion polymorphism were analyzed by electrophoresis in a 6% polyacrilamide gel whereas the results for the +3142 C/G polymorphism were analyzed by electrophoresis in a 1.5% agarose gel, both stained with ethydium bromide and visualized under UV light.

**Statistical analysis**

*HLA-G* genotypic distribution was determined by direct counting. The genotypic frequencies were compared to Hardy–Weinberg expectations using Chi-Square tests. *HLA-G* genotypic and allelic frequencies were compared between patients and controls using the Chi-square-test or Fischer exact test. Haplotype frequencies were estimated with the MLocus software, which uses an expectation maximization algorithm. The significance level was set at $\alpha$ =0.05 (two-tailed). All statistical analyses were performed with SPSS 15.0 and WinPepi 10.0 softwares.

## RESULTS

In the present study we performed all analyses by subgrouping the individuals according to their ethnic origin, because of the known existing difference in HLA-G polymorphic allelic frequencies between European and African-derived populations [6]. The European-derived group was composed by 235 uninfected individuals and 237 HIV-infected patients. The African-derived group was composed by 310 uninfected individuals and 171 HIV-positive individuals.

Patient and control groups had their genotypic frequencies compared to Hardy–Weinberg expectations. No significant differences were observed (data not shown). We then compared genotypic and allelic frequencies of the 14bp insertion/deletion polymorphism between patients and controls (Table 2). No significant differences were observed between the frequency of the ins/ins homozygote genotype in European-derived control and HIV-infected individuals (0.187 *versus* 0.143, respectively; p=0.391). Concerning the frequency of the insertion allele we also did not find statistical difference between controls and patients in the European-derived group (0.423 *versus* 0.382, respectively; p=0.207) (Table 2). In the African-derived group, however, the frequency of the ins/ins genotype in HIV-infected individuals was higher as compared to controls (0.205 versus 0.139, respectively; p=0.013). Also, evaluating the ins allele frequency we observed a higher frequency in patients as compared to uninfected controls (0.468 versus 0.373, respectively; p=0.005; Table 2).

Concerning the +3142C/G polymorphism, we observed similar frequencies between European-derived HIV-infected and uninfected subjects. The frequencies of the G/G genotype and G allele were 0.277 and 0.528 for

uninfected individuals and 0.228 and 0.504 for patients, respectively (p=0.469 and p=0.298). Since the 14bp insertion/deletion and +3142 polymorphisms are in linkage disequilibrium, we evaluated the estimated haplotype frequencies between the studied groups. We did not find statistical differences comparing patients and controls (p=0.422; Table 3).

When comparing the genotypic frequencies of the +3142 C/G polymorphism in the African derived group, we observed a higher frequency of the G/G genotype in patients as compared to uninfected subjects (0.427 versus 0.332) although not significant (p=0.107). However, the frequency of the G allele was significantly increased in patients, as compared to controls (0.649 *versus* 0.578, respectively p=0.039; Table 2). At the haplotypic level, a higher frequency of the G/ins haplotype was observed in African-derived HIV-infected individuals as compared to healthy individuals of the same ethnic origin (0.456 *versus* 0.346; residual p<0.001; Table 3).

We also analysed Hepatitis C virus (HCV) co-infection and other clinical symptons, in order to evaluate the possible influence of the HLA-G polymorphisms on clinical features that affect the HIV positive individuals (Table 4). Analyzing the European-derived group, we found 43 individuals co-infected with HCV and 185 without HCV co-infection. Comparing these individuals in respect to the two *HLA-G* polymorphisms studied, the genotypic and allelic frequencies between the co-infected and non-co-infected groups were similar (ins/ins genotype: 0.130 in HIV+/HCV- patients *versus* 0.186 in HIV+/HCV+ patients, p=0.632; ins alleles: 0.368 in HIV+/HCV- *versus* 0.407 in HIV+/HCV+, p=0.537). Regarding the frequencies for the G/G genotypes and G allele, no significant differences were observed between the groups (0.222 and

0.497 in HIV+/HCV- and 0.256 and 0.523 in HIV+/HCV+; p=0.885 and p=0.720, respectively).

Among African-derived individuals, 60 HCV co-infected subjects and 105 patients without HCV co-infection were described. It is interesting to point out that the proportion of co-infected patients was higher in this group as compared to the European-derived group (0.364 versus 0.189, P<0.001). Concerning the 14bp polymorphism, no statistically significant differences were observed concerning allelic frequencies between the HIV+/HCV+ and HIV+/HCV- patients (ins allele: 0.525 *versus* 0.429; p=0.108). However, an increased frequency of the ins/ins genotype in HCV co-infected individuals was observed as compared to HCV-HIV+ patients (0.317 versus 0.143; p=0.024). Concerning the +3142C/G polymorphism, no significant differences were observed between HIV+/HCV+ and HIV+/HCV- patients, neither concerning the G/G genotype nor the G allele frequencies (G/G genotype: 0.433 *versus* 0.438, respectively, p=0.887 and G allele 0.642 *versus* 0.657, respectively, p=0.811).

## DISCUSSION

The HIV infection has been extensively studied, however, the ability of the virus to infect cells and "trick" the immune system remains a puzzle for researchers. Although HIV initially induces an immune response, it later escapes and evades the immune system allowing a successful infection [13]. HLA-G is an immunomodulatory molecule able to suppress immune responses and this feature has attracted attention of researchers, since it could be involved in HIV escape from the immune surveillance. Several works suggested that there is an increased expression of HLA-G after HIV infection [12, 13, 26].

In the present work we investigated the association between the 14bp insertion/deletion and the +3142 G/C *HLA-G* gene polymorphisms with HIV infection in patients and ethnic-matched controls from Southern Brazil. We observed an increased frequency of the genotypes related to low HLA-G expression in African-derived-HIV-infected as compared to the healthy individuals. Therefore, we suggest that low HLA-G levels play a role in HIV infection.

Evaluating the European-derived individuals, we found similar genotypic, allelic and haplotypic frequencies comparing patients and controls, suggesting that the studied *HLA-G* polymorphisms have no influence in HIV infection in those patients. However, analyzing the African-derived individuals, we found an increased frequency of the ins/ins genotype and ins allele in HIV-infected individuals comparing to controls. In addition, the analysis of the +3142 G/C polymorphism resulted in a higher frequency of the G allele in HIV-positive patients also indicating lower HLA-G expression in HIV-infected individuals. Finally, haplotype analysis pointed to a similar situation, where the G/ins

haplotype was more frequent among patients as compared to controls. Taken together, our results suggested that among African-derived HIV infected individuals the genotypes, alleles and haplotypes related to lower HLA-G expression are associated with HIV-infection.

Nevertheless, other studies have suggested a relation between high *HLA-G* expression and HIV infection, suggesting that this molecule may contribute to a tolerogenic environment that favors viral immune escape and dissemination [12, 13, 26, 27, 28]. Although these findings indicated that high HLA-G expression can suppress the immune system, allowing the virus infection, we can speculate a different role of HLA-G during HIV infection. It was already described that the expression of MHC-I molecules is required to initiate and sustain an efficient anti-viral immunity. The soluble forms of HLA-G have the ability to present viral peptides to cytotoxic CD8+ T cells, suggesting that this class Ib molecule could also play a role during early phases of viral infections [29, 30]. Confirming the HLA-G participation in innate immunity, it was shown that soluble forms of HLA-G are also able to bind activation killer cell immunoglobulin-like receptors [31], such as KIR2DL4, promoting NK cell activation. The NK cell activation can induce IFN-γ and TNF-α release and this pro-inflammatory cytokines can activate more NK cells [32, 33]. Moreover, IFN-γ is required for immune protection, promoting up regulation of MHC and enhancing T cell recruitment to sites of virus challenge [34]. It was also described that soluble HLA-G forms could activate NF-kappa B in NK cells [35, 36]. Taking together, these findings point out to a possible role for HLA-G in protecting the host against viral infections by activating innate immunity. Considering this, we can hypothesize that HIV-infected individuals with

genotypes related to low HLA-G expression could be more susceptible to HIV-infection.

Derrien *et. al*. (2004) found that HLA-G1 cell surface expression is decreased after HIV-infection through a Vpu-dependent mechanism [37]. In the same way, a study evaluating HIV vertical transmission in Brazilian children and 14bp *HLA-G* polymorphism, observed an increased frequency of the ins allele among HIV-positive children, indicating a protective role for the del allele [38].

We also investigated the influence of HLA-G polymorphisms in HIV-positive individuals co-infected with HCV. Analyzing African-derived individuals, we observed an increased frequency of the ins/ins genotype in HIV-infected patients. This ins/ins genotype is related to low HLA-G levels. Evaluating HCV co-infection in sickle cell disease patients, we have previously described genotypes related to low HLA-G expression in co-infected individuals [25]. These findings may reflect the same mechanism described above, in which Individuals with lower HLA-G expression could be more susceptible to viral infections.

The precise role of HLA-G in immune modulation of HIV infection probably depends on many aspects including HLA-G tissue distribution, the cell type involved, the type of HLA-G receptor expressed, the HLA-G protein levels, the isoforms of the molecule and the formation of HLA-G dimers [33, 39]. Here, we investigated the influence of two HLA-G polymorphisms in European and African-derived Brazilian subjects suggesting a possible role for this molecule in orchestrating the innate immunity against HIV and HCV infection in African-derived individuals. It will be thus important that future studies do not neglect

any possible interaction between low HLA-G levels in the HIV-infected patients and the disease profile.

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**Table 1** – Demographic characteristics of the study group

| Characteristics | HIV-infected individuals | Control group |
|---|---|---|
| Gender (male/females) | 224/186 | 250/102* |
| Age (years ± S.D. and min-max) | 42.98 ± 9.42 (19-73) | 38.45 ± 10.37 (18-62) |
| Ethnicity (European/African-derived) | 239/171 | 235/310 |
| HCV co-infection (n, %) | 103 (25.1%) | NA |
| HBV co-infection (n, %) | 17 (4.1%) | NA |

NA – Not apply. *Missing data.

**Table 2 –** Genotypic and allelic frequencies of 14bp and +3142 polymorphisms in HIV + individuals and healhy controls, according to ethnicity.

| | European-derived | | African-derived | |
|---|---|---|---|---|
| | Control Freq (n) | HIV + Freq (n) | Control Freq (n) | HIV + Freq (n) |
| Genotypes 14bp | n=235 | n=237 | n=310 | n=171 |
| **del/del** | 0.340 (80) | 0.380 (90) | 0.394 (122)* | 0.269 (46)* |
| **ins/del** | 0.472 (111) | 0.477 (113) | 0.468 (145)* | 0.526 (90)* |
| **ins/ins** | 0.187 (44) | 0.143 (34) | 0.139 (43)* | 0.205 (35)* |
| | $\chi^2$ p=0.391 | | * $\chi^2$ p=0.013 | |
| Alleles | n=470 | n=474 | n=620 | n=342 |
| **del** | 0.577 (271) | 0.618 (293) | 0.627 (389)* | 0.532 (182)* |
| **Ins** | 0.423 (199) | 0.382 (181) | 0.373 (231)* | 0.468 (160)* |
| | Fischer p=0.207 | | * Fischer p=0.005 | |
| Genotypes +3142 | n=235 | n=237 | n=262 | n=171 |
| **C/C** | 0.200 (47) | 0.219 (52) | 0.176 (46) | 0.129 (22) |
| **C/G** | 0.523 (123) | 0.553 (131) | 0.492 (129) | 0.444 (76) |
| **G/G** | 0.277 (65) | 0.228 (54) | 0.332 (87) | 0.427 (73) |
| | $\chi^2$ p=0.469 | | $\chi^2$ p=0.107 | |
| Alleles | n=470 | n=474 | n=524 | n=342 |
| **C** | 0.462 (217) | 0.496 (235) | 0.422 (221)* | 0.351 (120)* |
| **G** | 0.538 (253) | 0.504 (239) | 0.578 (303)* | 0.649 (222)* |
| | Fischer p=0.298 | | * Fischer p=0.039 | |

**Table 3 –** Haplotypic frequencies estimated considering both polymorphisms together, between patients and controls, according to ethnicity.

| | European-derived | | African-derived | |
|---|---|---|---|---|
| | Control Freq (n) | HIV + Freq (n) | Control Freq (n) | HIV + Freq (n) |
| Haplotypes | n=470 | n=474 | n=627 | n=342 |
| **del/C** | 0.451 (212) | 0.492 (233) | 0.391 (245) | 0.339 (116) |
| **del/G** | 0.125 (59) | 0.127 (60) | 0.236 (148) | 0.193 (66) |
| **ins/C** | 0.110 (5) | 0.004 (2) | 0.270 (17) | 0.012 (4) |
| **ins/G** | 0.413 (194) | 0.378 (179) | 0.346 (217)* | 0.456 (156)* |
| | $\chi^2$ p=0.412 | | $\chi^2$ p=0.005 | |
| | | | * Residual p<0.001 | |

**Table 4 –** Genotypic and allelic frequencies of 14bp and +3142 polymorphisms in HIV + individuals with or without HCV co-infection, according to ethnicity.

| | European-derived | | African-derived | |
|---|---|---|---|---|
| | HIV + HCV - Freq (n) | HIV + HCV + Freq (n) | HIV + HCV - Freq (n) | HIV + HCV + Freq (n) |
| Genotypes 14bp | n=185 | n=43 | n=105 | n=60 |
| **del/del** | 0.394 (73) | 0.372 (16) | 0.286 (30)* | 0.267 (16)* |
| **ins/del** | 0.476 (88) | 0.442 (19) | 0.571 (60)* | 0.417 (25)* |
| **ins/ins** | 0.130 (24) | 0.186 (8) | 0.143 (15)* | 0.317 (19)* |
| | $\chi^2$ p=0.632 | | * $\chi^2$ p=0.024 | |
| Alleles | n=370 | n=86 | n=210 | n=120 |
| **del** | 0.632 (234) | 0.593 (51) | 0.571 (120) | 0.475 (57) |
| **ins** | 0.368 (136) | 0.407 (35) | 0.429 (90) | 0.525 (63) |
| | Fischer p=0.537 | | Fischer p=0.108 | |
| Genotypes +3142 | n=185 | n=43 | n=105 | n=60 |
| **C/C** | 0.227 (42) | 0.209 (9) | 0.124 (13) | 0.150 (9) |
| **C/G** | 0.551 (102) | 0.535 (23) | 0.438 (46) | 0.417 (25) |
| **G/G** | 0.222 (41) | 0.256 (11) | 0.438 (46) | 0.433 (26) |
| | $\chi^2$ p=0.885 | | $\chi^2$ p=0.887 | |
| Alleles | n=370 | n=86 | n=210 | n=120 |
| **C** | 0.503 (186) | 0.477 (41) | 0.343 (72) | 0.358 (43) |
| **G** | 0.497 (184) | 0.523 (45) | 0.657 (138) | 0.642 (77) |
| | Fischer p=0.720 | | Fischer p=0.811 | |

## ANEXO 3

## Documento de aprovação do Comitê de Ética do HCPA

# HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
## Grupo de Pesquisa e Pós-Graduação
COMISSÃO CIENTÍFICA E COMISSÃO DE PESQUISA E ÉTICA EM SAÚDE

## RESOLUÇÃO

A Comissão Científica e a Comissão de Pesquisa e Ética em Saúde, que é reconhecida pela Comissão Nacional de Ética em Pesquisa (CONEP)/MS como Comitê de Ética em Pesquisa do HCPA e pelo Office For Human Research Protections (OHRP)/USDHHS, como Institucional Review Board (IRB00000921) analisaram o projeto:

**Projeto:** 05-295

| Pesquisador Responsável: |
|---|
| EDUARDO SPRINZ |

**Título:** IDENTIFICAÇÃO DE MARCADORES GENÉTICOS ASSOCIADOS A EFEITOS ADVERSOS EM PACIENTES COM SÍNDROME DA IMUNODEFICIÊNCIA ADQUIRIDA SOB TERAPIA ANTI-RETROVIRAL

**Data da Versão:**

**ADENDO** 06/10/2008

Este documento referente ao projeto acima foi Aprovado em seus aspectos éticos e metodológicos, de acordo com as Diretrizes e Normas Internacionais e Nacionais, especialmente as Resoluções 196/96 e complementares do Conselho Nacional de Saúde.

Porto Alegre, 09 de outubro de 2008.

Profª Nadine Clausell
Coordenadora do GPPG e CEP-HCPA

Porto Alegre, 06 de outubro de 2008

Ao Grupo de Pesquisa e Pós-Graduação do HCPA

Projeto: 05-295

Pesquisador responsável: Eduardo Sprinz

Prezados Sr(a)s,

Segue em anexo adendo ao projeto de pesquisa 05-295, intitulado "Proteína de Ligação à Manose como potencial alvo para terapia na Síndrome da Imunodeficiência Adquirida – um estudo imunogenético".

Atenciosamente,

Prof. Dr. Eduardo Sprinz

Serviço de Medicina Interna