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ANÁLISE DO POLIMORFISMO 2848 G/A DO GENE *TLR9* EM INDIVÍDUOS HIV^{+}, HCV^{+} E COINFECTADOS

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RESUMO

As infecções por HIV e HCV configuram graves problemas de saúde pública no Brasil. Variantes genéticas do hospedeiro já foram associadas a diferenças na suscetibilidade a ambas as infecções e progressão à AIDS, assim como à coinfecção por estes vírus. Os TLRs (do inglês, *Toll-Like Receptors*) são importantes componentes da resposta imune inata e agem reconhecendo PAMPs e DAMPs. O TLR9, codificado pelo gene sinônimo localizado no cromossomo 3, está presente na porção interior de endossomos, e reconhece moléculas de DNA não metilado. Esse receptor pode participar da resposta à infecção pelo HIV reconhecendo o DNA formado a partir da atividade da enzima transcriptase reversa sobre o RNA viral. Além disso, a interação da proteína viral gp120 com células dendríticas inibe respostas inatas mediadas a partir do TLR9, sugerindo um papel desse receptor na resposta contra o HIV. Uma variante do gene *TLR9*, o polimorfismo de nucleotídeo único 2848 G/A (rs352140), é alvo de estudos de associação com a infecção por HIV, e seu papel ainda não foi claramente elucidado na infecção pelo HCV. Da mesma forma, há uma controvérsia a respeito deste SNP na coinfecção por HIV/HCV. Dito isso, o presente trabalho tem como objetivo avaliar as frequências da variante 2848 G/A (TLR9) em indivíduos HCV^+, HIV^+ e coinfectados, provenientes da região Sul do Brasil, estratificando-os em diferentes grupos étnicos. Foram genotipados um total de 1182 indivíduos, divididos nos grupos: controle (n = 409); HCV^+ (n = 376); HIV^+ (n = 296); HCV^+/HIV^+ (n = 101). As sequências de interesse foram amplificadas por Reação em Cadeia da Polimerase (PCR) convencional, utilizando um par de iniciadores específico para a região analisada. Os amplicons gerados por PCR foram submetidos à digestão enzimática com endonuclease (*Bsh*1236I). A verificação dos genótipos foi realizada em gel de agarose 3% com brometo de etídeo sob luz UV. Foram calculadas as frequências genotípicas e alélicas do polimorfismo. Os grupos estudados foram comparados para verificar a existência de influência da variante na susceptibilidade à infecção pelo HIV, pelo HCV e coinfecção por HIV/HCV. Foi utilizado o teste de qui-quadrado de Pearson para comparação dos dados, e valores de p menores que 0,05 foram definidos como estatisticamente significativos. O estudo foi aprovado pelos comitês de ética da Universidade Federal do Rio Grande do Sul (UFRGS), Hospital de Clínicas de Porto Alegre e Universidade Luterana do Brasil (ULBRA). Todos os participantes da pesquisa assinaram um termo de consentimento desenvolvido de acordo com a Resolução No. 466 do Ministério da Saúde. A partir dos resultados do trabalho, observamos que não há uma diferença estatisticamente significativa entre os grupos (p>0,05 em todas as comparações). Dessa forma, o estudo sugere que a variante 2848 G/A não influencia as infecções por HCV e HIV e a coinfecção por ambos os vírus na população do Sul do Brasil.

Palavras-chave: Receptor Toll-Like 9. HIV. HCV. Polimorfismo. Coinfecção.

ABSTRACT

HIV and HCV infections constitute important public health problems in Brazil. Host genetic variants have already been associated with differences in susceptibility to both infections and progression to AIDS, as well as coinfection by both viruses. TLRs (Toll-like Receptors) are important components of the innate immune response and act recognizing PAMPs and DAMPs. TLR9, formed from the synonymous gene located on chromosome 3, is present in the inner portion of endosomes, and recognizes molecules of unmethylated DNA. This receptor may participate in the response to HIV infection by recognizing the DNA formed from the activity of the reverse transcriptase enzyme on viral RNA. In addition, the interaction of the gp120 viral protein with dendritic cells inhibits innate responses mediated by TLR9, suggesting a role for that receptor in the response to HIV. A variant of the *TLR9* gene, the 2848 G/A single nucleotide polymorphism (rs352140), is targeted in association studies with HIV infection, and its role has not yet been clearly elucidated in HCV infection. Likewise, there is controversy regarding this SNP in HIV/HCV coinfection. The aim of this study was to evaluate the frequencies of the 2848 G/A variant (*TLR9*) in HCV^+, HIV^+ and coinfected individuals from the southern region of Brazil, stratifying them in different ethnic groups. A total of 1182 individuals were genotyped, divided into groups: control (n = 409); HCV^+ (n = 376); HIV^+ (n = 296); HCV^+/ HIV^+ (n = 101). The sequences of interest were amplified by conventional Polymerase Chain Reaction (PCR) using a pair of specific primers for the analyzed variant. With the amplicons generated by PCR, the samples were cleaved using specific restriction enzyme (*Bsh*1236I). Genotype verification was performed on 3% agarose gel with ethidium bromide under UV light. The genotypic and allelic frequencies of the polymorphism were calculated. The groups studied were compared to verify the influence of the variant on susceptibility to HIV infection, HCV and HCV/HIV coinfection. Pearson's chi-square test was used for comparison of the data, and *p*-values lower than 0.05 were defined as statistically significant. The study was approved by the ethics committees of the *Universidade Federal do Rio Grande do Sul* (UFRGS), *Hospital de Clínicas de Porto Alegre* and *Universidade Luterana do Brasil* (ULBRA). All study participants signed a consent form developed according to Resolution No. 466 of Ministério da Saúde. From the results of the study, we observed that there is no statistically significant difference between the groups ($p > 0.05$ in all cases). Thus, the study suggests that the 2848 G/A variant does not influence HCV and HIV infections and coinfection by both viruses in the southern Brazilian population.

Keywords: Toll-Like Receptor 9. HIV. HCV. Polymorphism. Coinfection.

SUMÁRIO

1 INTRODUÇÃO

1.1 VÍRUS DA IMUNODEFICIÊNCIA HUMANA (HIV)

O Vírus da Imunodeficiência Humana, mais conhecido pela sigla HIV (do inglês *Human Immunodeficiency Virus*), é extensamente investigado desde o início da década de 1980, época em que o mundo começava a se preocupar com uma epidemia de AIDS (do inglês *Acquired Immunodeficiency Syndrome*). Classicamente, tal síndrome é caracterizada por uma grande debilitação do sistema imune, levando ao aparecimento de diversas patologias secundárias, como infecções por patógenos oportunistas e alguns tipos de neoplasias (GOTTLIEB et al., 1981; BRODER; GALLO, 1984). Em seguida serão discutidos marcos históricos relacionados à pandemia de HIV/AIDS, características biológicas do vírus e aspectos epidemiológicos e clínicos da AIDS.

1.1.1 História do HIV e AIDS

Nos repositórios digitais de artigos científicos, são encontrados registros de pacientes com AIDS desde 1981, época em que a causa da síndrome ainda era desconhecida. Os primeiros casos foram notificados pelo *Centers for Disease Control and Prevention* (CDC), que relataram cinco indivíduos que apresentavam pneumonia por *Pneumocystis carinii* (atualmente referido como *Pneumocystis jirovecii*), patógeno que classicamente acomete apenas indivíduos imunodeprimidos. Os cinco pacientes eram homossexuais e foram diagnosticados com candidíase oral e infecção por citomegalovírus, doenças oportunistas comuns, levando à conclusão de que os cinco apresentavam algum tipo de imunossupressão (CDC, 1981). A partir desse relatório, outros casos começaram a ser notificados pelas agências de saúde, demarcando o início das pesquisas epidemiológicas.

Foi no ano seguinte que a hipótese de que a AIDS poderia ser causada por um retrovírus começou a surgir, depois que fora constatado que outros retrovírus poderiam causar imunodeficiências – como o FeLV, causador de leucemia em felinos (BRODER; GALLO, 1984; HARDY JUNIOR et al., 1976). Um ano depois, em 1983, a hipótese foi endossada por evidências empíricas mostrando a presença de anticorpos contra peptídeos do envelope – descritos inicialmente como peptídeos membranares de células infectadas – do HTLV-I em uma amostra de pacientes com AIDS. Além disso, um retrovírus não identificado foi isolado de um paciente com sintomatologia coerente com AIDS (BRODER; GALLO, 1984; ESSEX et al., 1983; BARRE-SINOUSSI et al., 1983).

Nos primeiros anos de investigação, o vírus foi chamado por diferentes nomes – LAV, IDAVI, IDAVI2 e HTLV-III – e finalmente, alocado em uma família diferente da que se encaixa hoje: a do HTLV (*Human T-cell Leukemia/Lymphoma Virus*). Isso se deu por uma série de semelhanças entre as partículas virais de membros deste grupo, tais como: (1) possuir capacidade de infectar células $CD4^+$; (2) apresentar enzimas transcriptase reversa semelhantes; (3) conter antígenos com reatividade cruzada; (4) possuir proteínas de capsídeo viral de tamanhos similares; (5) exibir homologia entre as sequências de nucleotídeos; e, por fim, (6) apresentar uma sequência pX na extremidade 3' dos genomas. (BRODER; GALLO, 1984; POPOVIC et al., 1984; GALLO et al., 1984). No entanto, determinadas características da AIDS não poderiam ser explicadas por uma infecção por um HTLV. Enquanto que esses vírus possuem baixa replicação nas células, o mesmo não era observado nos pacientes imunodeprimidos (MIYOSHI et al., 1981). Além disso, os HTLV causam um aumento no número de linfócitos – característica clássica de uma neoplasia do sangue, ao passo que havia uma queda brusca na contagem de linfócitos T $CD4^+$ em pacientes com AIDS (POIESZ et al., 1980; SUGAMURA; HINUMA, 1993). Portanto, o vírus não se adequava aos padrões estabelecidos para os HTLV, e foi em 1986 que o Comitê Internacional sobre Taxonomia de Vírus atribuiu o nome de HIV ao causador da AIDS (COFFIN et al., 1986).

Com o estabelecimento do HIV como o agente etiológico, as pesquisas com o vírus foram intensificadas em todo o mundo, a fim de descobrir uma possível cura para a doença. Mesmo com todo o direcionamento dado, porém, ainda não foi encontrada uma cura, mas houve muito avanço. O tratamento para pacientes HIV^+ se tornou bastante avançado e eficaz em atingir níveis indetectáveis de carga viral em boa parte dos indivíduos. No Brasil, os fármacos são amplamente distribuídos pelo Sistema Único de Saúde (SUS) a partir do diagnóstico positivo para a infecção. Com o imenso progresso, hoje a infecção pelo HIV já pode ser considerada uma doença crônica, visto que episódios agudos de doença são incomuns e a progressão para a AIDS pode ser bastante retardada e até evitada.

1.1.2 Biologia do HIV e da Infecção

O HIV é um *Lentivirus*, pertencente à família *Retroviridae* e ordem *Ortervirales*. Assim como os outros membros da família, é um vírus envelopado de cerca de 100 nm de

diâmetro, com capsídeo viral cônico, como pode ser visualizado na figura 1, tendo RNA como seu material genético e genoma apresentando um tamanho de cerca de 10 kb (LEVY, 2010). Existem duas espécies de HIV: HIV-1 e HIV-2. O HIV-1 é o responsável pela pandemia verificada atualmente, enquanto que o HIV-2 é encontrado majoritariamente na África e parece ser menos transmissível e virulento (ROYLE et al., 2014; MARLINK et al., 1994). Por esse motivo, trataremos aqui apenas do HIV-1.

Há uma heterogeneidade de características genéticas das cepas encontradas de HIV, decorrentes da replicação sujeita a erro mediada pela enzima transcriptase reversa (COFFIN, 1995). Caracteristicamente, um retrovírus possui três genes com funções essenciais: *gag*, *pol* e *env*. O gene *gag* é responsável por codificar as proteínas do capsídeo viral – p16 (MA), p24 (CA), p9 (NC); a enzima transcriptase reversa é derivada do gene *pol*; e o gene *env* codifica as glicoproteínas do envelope viral – gp120 e gp41. (BRODER; GALLO, 1984; GALLO, 1984; KRÄUSSLICH et al., 1993; MERVIS et al., 1988). As proteínas virais são derivadas de uma única poliproteína, a Gag p55, que é clivada pela protease viral (GOMEZ; HOPE, 2005). Algumas das principais proteínas produzidas a partir da protease são a transcriptase reversa, responsável pela produção da fita de cDNA a partir do RNA viral, e a integrase, capaz de integrar o provirus ao genoma do hospedeiro (COFFIN, 1992).

Na infecção pelo HIV verifica-se uma grande depleção de células $CD4^+$, dentre as quais destaca-se o linfócito T, elemento essencial para o início da grande maioria das respostas imunes antígeno-específicas (GOTTLIEB et al., 1981; BRODER; GALLO, 1984; MONTAGNIER, L. et al., 1984; DALGLEISH et al., 1984). Isso acontece porque a molécula CD4 é o principal receptor em que a partícula viral se liga para realizar a penetração, por meio da proteína de superfície viral gp120 (KLATZMANN et al., 1984; RYU et al., 1990). Após essa ligação, a proteína gp120 é deslocada, deixando livre a proteína viral gp41, que realiza uma ligação necessária com um correceptor celular – sendo os correceptores mais importantes o CCR5 (C-C Chemokine Receptor 5) e o CXCR4 (C-X-C Chemokine Receptor 4). A partir dessas interações, ocorre finalmente a fusão vírus-célula (MOORE et al., 1990;. BERSON et al., 1996; ALKHATIB, 2009).

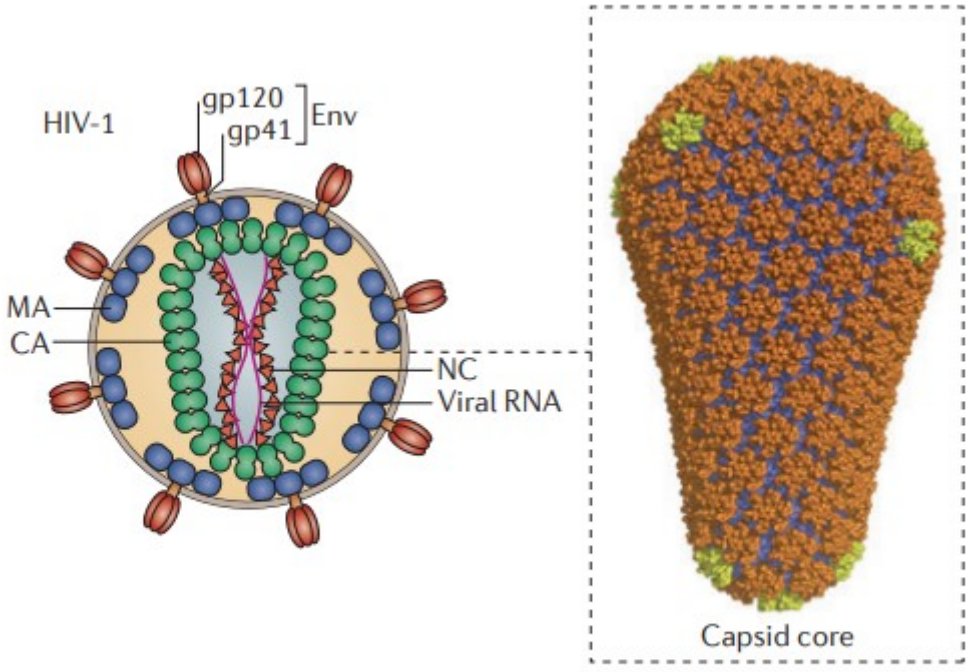

Figura 1. Estrutura simplificada do vírion (adaptado de CAMPBELL, 2015).

Com a fusão, o envelope viral é clivado, e o conteúdo interno da partícula é deslocado para o citoplasma da célula infectada. Assim, a transcriptase reversa entra em ação, produzindo fitas de cDNA virais, que posteriormente serão deslocadas para o núcleo e então serão inseridas no genoma hospedeiro, etapa mediada pela integrase viral. O vírus se integra ao genoma do hospedeiro na forma de DNA – sendo chamado de provirus. Flanqueando o provirus existem sequências repetitivas terminais longas que possibilitam o início da transcrição viral (BRODER; GALLO, 1984). A partir dessa etapa, se torna possível a geração de fitas de mRNA virais e consequentemente a produção das poliproteínas virais. Então, a partir desse ponto, são geradas novas partículas virais, que podem sofrer brotamento e posteriormente infectar novas células, ou até mesmo serem transferidas para outras células por contato direto (LEHMANN-CHE; SAÏB, 2004; SATO et al., 1992). A figura 2 retrata as etapas de entrada e replicação viral do HIV (RAMBAUT et al., 2004).

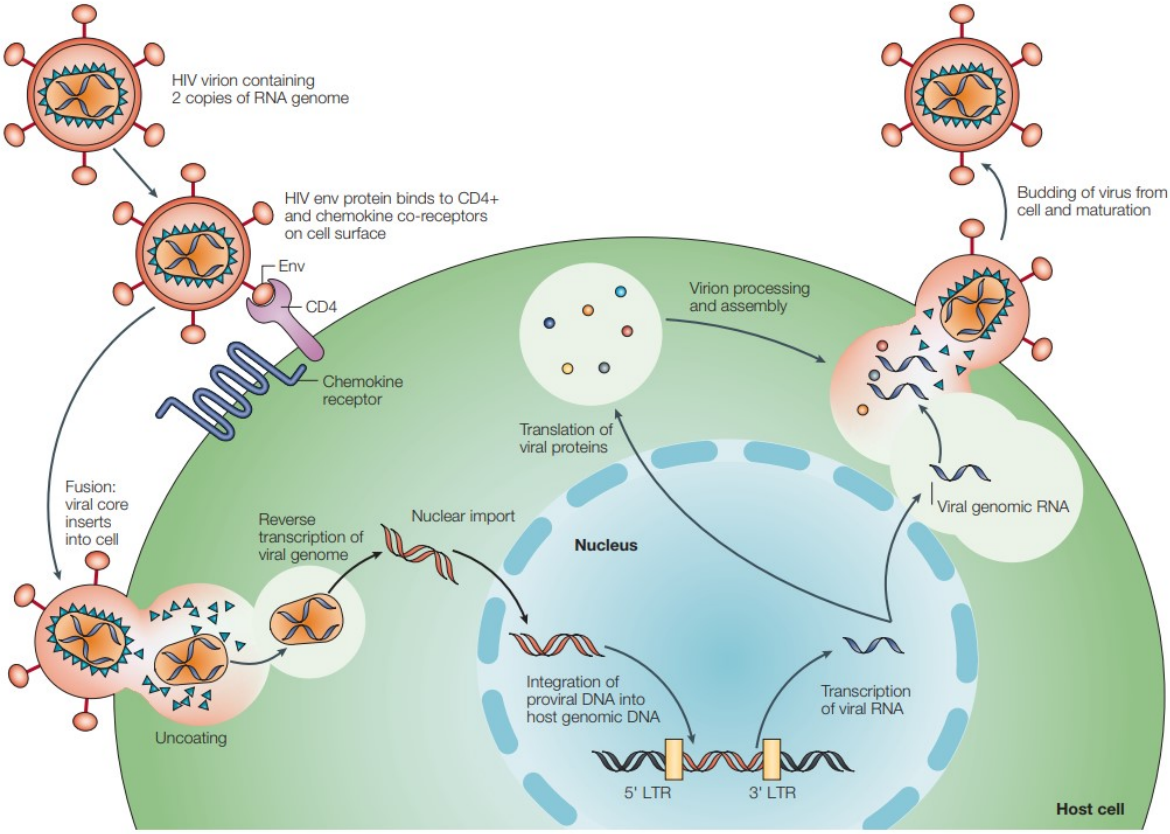

Figura 2. Ciclo de replicação do HIV (RAMBAUT et al., 2004).

1.1.3 Transmissão do HIV

Diversos são os meios de transmissão do HIV. Destacam-se o sexo vaginal, anal ou oral sem preservativo; o uso de seringas compartilhadas; uso de instrumentos perfurocortantes não esterilizados; a transmissão vertical da mãe infectada para o filho durante a gestação, o parto ou na amamentação. Ainda, há casos documentados de infecção por transfusão sanguínea, salientando-se que atualmente o risco dessa via de transmissão é minimizado devido à rigorosa investigação de patógenos por parte dos bancos de sangue (MINISTÉRIO DA SAÚDE, 2017a).

1.1.4 Dados Epidemiológicos

De acordo com o Ministério da Saúde, de 2007 a 2017 foram registrados 194.217 casos de infecção pelo HIV no Brasil, sendo 131.969 casos em homens e 62.198 em mulheres. Dentre o grupo de infectados, a faixa etária de 20 a 34 anos se destaca com o maior número de casos de infecção. A taxa de infecção pelo HIV tem aumentando a cada ano. No

entanto, a taxa de indivíduos diagnosticados com AIDS diminui. Isso se deve ao resultado da efetividade da terapia antirretroviral (ARV) e pelas políticas de saúde pública adotadas no Brasil. Desde 2013, recomenda-se que todos os indivíduos diagnosticados com HIV iniciem ARV imediatamente (MINISTÉRIO DA SAÚDE, 2017b). A partir desse período até 2015, foi observada uma redução de 7,2% na taxa de óbitos causados pela AIDS no Brasil. Mesmo assim, ainda não existe uma cura efetiva para a doença, e esforços científicos ainda são extremamente necessários para que as taxas de ocorrência da AIDS diminuam. Importante ressaltar que Porto Alegre é a capital brasileira com maior taxa de detecção da AIDS, segundo dados de 2016 (figura 3) (MINISTÉRIO DA SAÚDE, 2017b).

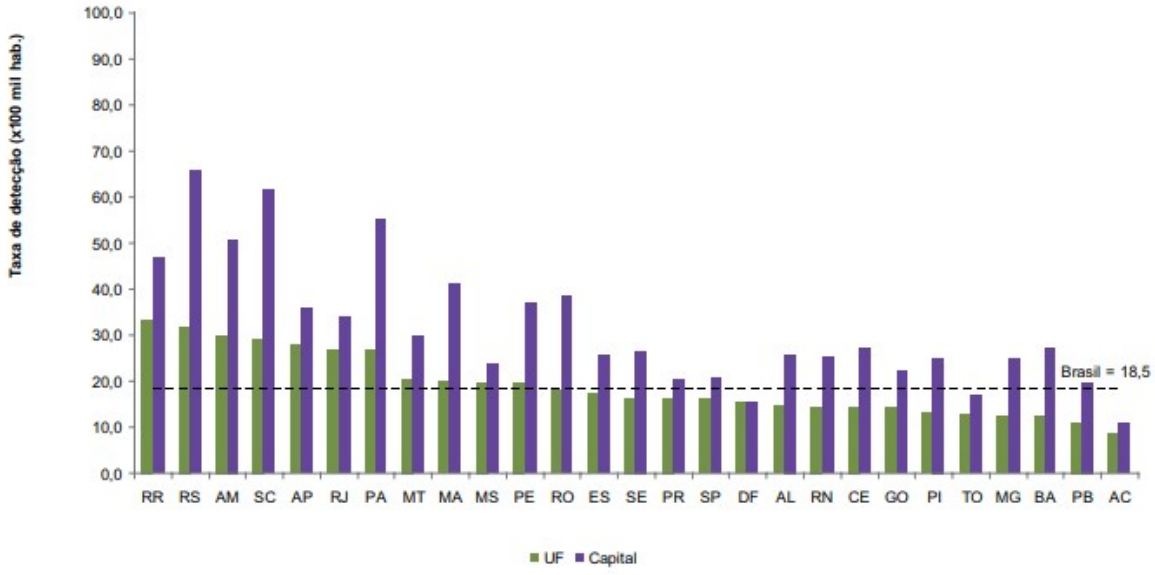

Figura 3. Taxa de detecção de AIDS (/100 mil hab.) segundo UF e capital de residência. Brasil no primeiro semestre de 2016 (adaptado do MINISTÉRIO DA SAÚDE, 2017b).

1.1.5 Aspectos Clínicos da AIDS

Indivíduos recém infectados pelo HIV podem apresentar sintomas clínicos leves, como febre, dores de cabeça, dores musculares e erupções eritematosas não pruriginosas. Esses sinais e sintomas inespecíficos podem levar a um diagnóstico errôneo, como o de uma simples gripe (COOPER et al., 1985). No entanto, alguns pacientes podem exibir sintomatologia mais complexa, como candidíase oral, úlceras esofágicas e vaginais, encefalite e pneumonia (RABENECK et al., 1990; ROUTY et al., 2000). Essa é a chamada fase de

infecção aguda, e em alguns pacientes pode até mesmo apresentar-se assintomática (LEVY, 2010).

Após a fase de infecção aguda, inicia-se uma fase de latência clínica, também conhecida como janela imunológica, em que os exames laboratoriais tendem à normalidade, dificultando o diagnóstico. À medida que a infecção progride sem tratamento, alguns achados se tornam mais comuns, como linfadenopatia, contagem de células $CD4^+$ diminuída e diagnóstico de algumas patologias associadas à imunodepressão. A partir do momento em que infecções oportunistas e neoplasias são observadas com frequência, o indivíduo é diagnosticado com AIDS (MINISTÉRIO DA SAÚDE, 2018).

O diagnóstico laboratorial da infecção pelo HIV é baseado em imunoensaio. Em casos de suspeita de infecção recente (aguda) ou resultados inconclusivos, é utilizado teste molecular baseado em detecção do RNA viral. Além destes, testes complementares como *western blot* e *imunoblotting* podem ser utilizados. Durante a janela imunológica esses testes podem apresentar resultados negativos, sendo necessário, portanto, repetir os testes em casos de suspeita. A partir do diagnóstico da infecção pelo HIV, deve-se iniciar ART (MINISTÉRIO DA SAÚDE, 2018a).

Atualmente no Brasil, três são os fármacos utilizados para tratamento em indivíduos adultos: a lamivudina (3TC), um inibidor de transcriptase reversa; o tenofovir (TDF), outro inibidor da transcriptase reversa; e o dolutegravir (DTG), um inibidor da integrase viral. Em crianças e adolescentes, recomenda-se o uso da lamivudina conjugada à zidovudina (AZT). Além disso, atualmente são disponibilizadas duas terapias de profilaxia: a de pré-exposição (PrEP) e de pós-exposição (PEP). A PrEP é composta por um comprimido que conjuga tenofovir e entricitabina (FTC) – o conhecido Truvada, e é indicada quando existe risco à infecção pelo HIV, normalmente sendo limitada a alguns grupos populacionais – como homossexuais e outros homens que fazem sexo com homens, pessoas trans e profissionais do sexo. Importante ressaltar que o risco aumentado não se dá apenas por comportamento de risco, mas também por serem alvos de preconceito e estigma, o que contribui fortemente para a vulnerabilidade à infecção. O uso da PrEP de forma adequada tem efetividade quase total na proteção contra a infecção pelo HIV (FONNER et al., 2016).

Já a PEP pode ser utilizada quando o indivíduo foi exposto ao HIV de forma acidental ou não. A PEP é comumente usada em casos de violência sexual, acidentes de trabalho envolvendo objetos perfurocortantes ou amostras biológicas, e em casos de relações sexuais sem uso de preservativo ou com rompimento do mesmo. O esquema farmacológico da PEP é

o mesmo da ARV utilizada em indivíduos já diagnosticados (TDF+3TC+DTG) (MINISTÉRIO DA SAÚDE, 2018).

1.2 VÍRUS DA HEPATITE C (HCV)

A infecção pelo HCV configura um grave problema de saúde pública no Brasil, sendo uma das maiores causas de doença hepática crônica, assim como de cirrose e carcinoma hepatocelular (LINGALA; GHANY, 2015; FAN et al., 2017). A seguir, serão explorados a descoberta do vírus, sua biologia e direcionamento da infecção, suas vias de transmissão, além de aspectos epidemiológicos e clínicos.

1.2.1 História do HCV

A compreensão da história do vírus da hepatite C começa a partir de 1975, quando cientistas perceberam que a maioria das infecções transmitidas por transfusão sanguínea na época não poderiam ser causadas pelos já identificados Vírus da Hepatite A (HAV) e Vírus da Hepatite B (HBV). Inicialmente, a infecção foi chamada de Hepatite Não-A e Não-B (NANBH, *Non-A, Non-B Hepatitis*), e pouco se sabia sobre o agente causador desta infecção. Os primeiros estudos mais robustos foram realizados a partir da inoculação de material de pacientes com NANBH em chipanzés, a fim de acompanhar todas as etapas do processo infeccioso. Mesmo utilizando modelo animal, os resultados foram confusos, e os pesquisadores responsáveis sugeriram que o vírus responsável poderia ser um novo subtipo de HBV ou até mesmo vários patógenos diferentes não identificados (ALTER et al., 1978; HOLLINGER et al., 1978; HOUGHTON, 2009).

O HCV foi identificado pela primeira vez em 1989. Diversos foram os métodos empregados no caminho – houve tentativas de isolamento por cultivo celular, modelos animais e testes moleculares, mas todos sem sucesso. Foi com a construção de uma biblioteca de cDNA viral derivada de material infectado, a fim de aumentar as concentrações de antígenos virais, que se tornou possível a identificação do patógeno, que foi então denominado Vírus da Hepatite C. A figura 4 ilustra o processo de identificação do vírus (HOUGHTON, 2009; CHOO et al., 1989). A partir da determinação do agente etiológico, se tornou possível a geração de testes eficazes para a detecção do patógeno em amostras

biológicas – principalmente em bolsas de sangue para transfusão – e as ferramentas diagnósticas foram se tornando cada vez mais robustas. Em 1990 surgiu o primeiro teste sorológico a ser utilizado nos bancos de sangue, o qual foi responsável por uma queda brusca nas taxas de infecção por transfusão sanguínea (HOUGHTON, 2009).

Como consequência da identificação do HCV, o tratamento para os pacientes infectados também pôde ser desenvolvido e será explorado adiante. Atualmente, a hepatite C é uma doença curável, e esforços científicos são realizados no mundo todo para que seja produzida uma vacina eficaz contra a infecção, como já existe para o HBV. Apesar disso, o HCV ainda é um patógeno que afeta cerca de 3% da população mundial, mostrando a importância de estudos sobre o assunto (WORLD HEALTH ORGANIZATION, 2018).

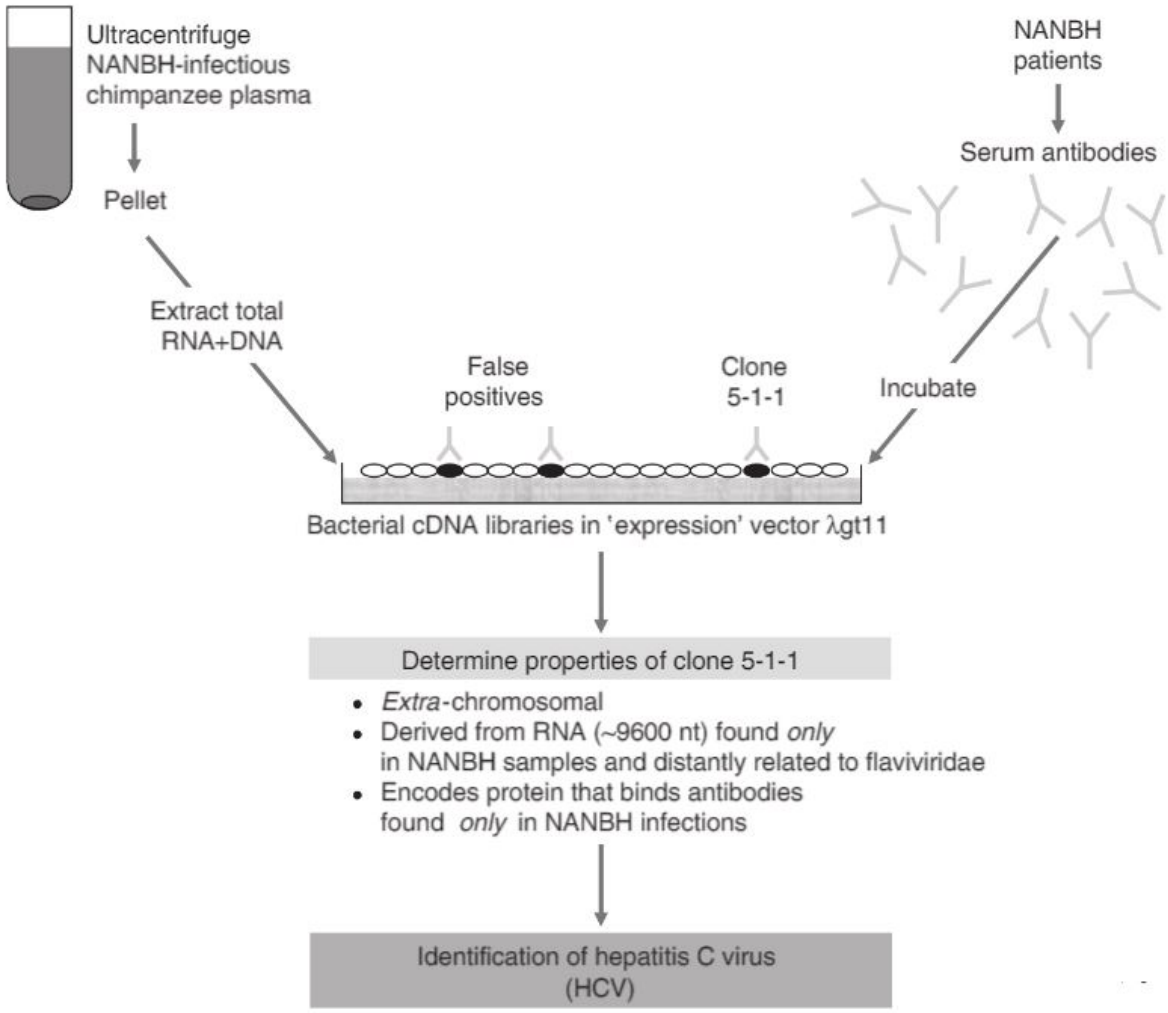

Figura 4. Esquema metodológico responsável pela identificação do HCV (HOUGHTON, 2009)

1.2.2 Biologia do HCV e da Infecção

O HCV pertence ao gênero *Hepacivirus* e família *Flaviviridae* (ROBERTSON et al., 1998). Possui RNA fita simples senso positiva [(+)ssRNA] como seu material genético, e tem um tamanho de cerca de 9,6 kb (CHOO et al., 1989). São encontrados sete genótipos virais diferentes – HCV-1, HCV-2, HCV-3, HCV-4, HCV-5, HCV-6 e HCV-7, sendo os seis primeiros mais importantes (o HCV-7 foi identificado recentemente em indivíduos na África Central). Além disso, já foram identificados 67 subtipos do HCV (ELLWANGER et al., 2017).

Assim como no HIV, seu transcrito gera uma única poliproteína viral, que é clivada por proteases virais e por peptidases da célula hospedeira. Os dez produtos proteicos gerados são tanto estruturais – como as proteínas do capsídeo viral – como não estruturais (LINDENBACH; RICE, 2005; FAN et al., 2017). As características biológicas gerais do HCV estão esquematizadas na figura 5 (ELLWANGER et al., 2017).

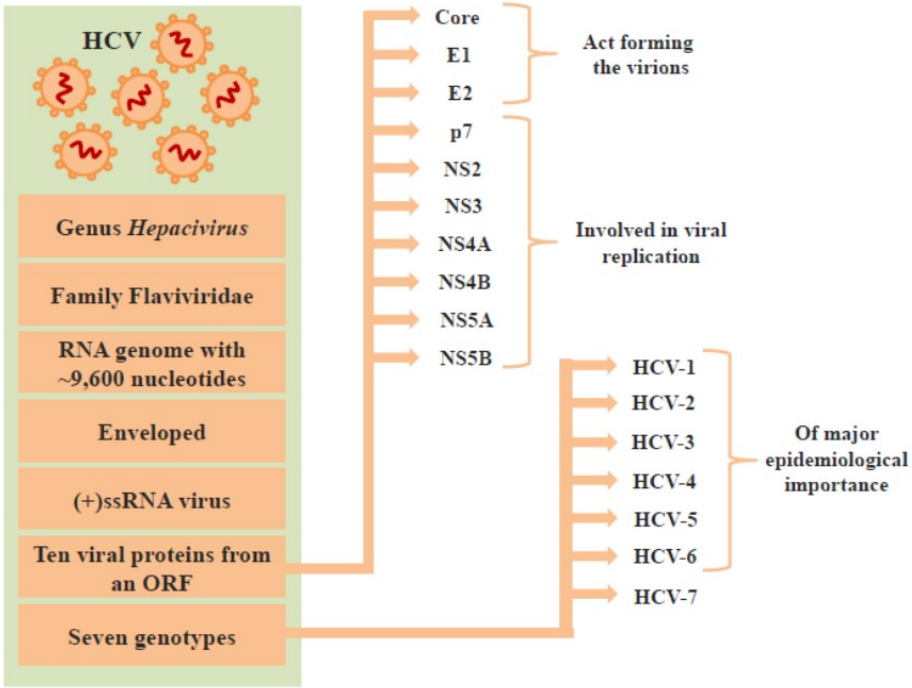

Figura 5. Características biológicas do HCV (ELLWANGER et al., 2017).

O HCV penetra nas células hospedeiras por meio de endocitose mediada por receptor. Diversas moléculas já foram identificadas como receptores ou co-receptores para o HCV, sendo estes divididos em receptores de ligação e de pós-ligação (BLANCHARD et al., 2006). Os receptores de ligação mais importantes do envelope viral são a apolipoproteína E (apoE) e

a fosfatidilserina (PS), que se ligam a proteoglicanos de heparan sulfato e à proteína TIM-1 na superfície de hepatócitos. Já os co-receptores de pós-ligação mais relevantes incluem as proteínas CD81, claudina-1, ocludina, SCARB-1 e LDLR, que interagem com as glicoproteínas E1 e E2 do envelope viral (FAN et al., 2017).

O processo infeccioso do HCV pode ocorrer de duas formas: por brotamento de partículas virais e penetração em outras células ou por transmissão direta para células vizinhas. O primeiro modo é o principal, enquanto que o segundo parece ser o responsável pela infecção persistente, por ser um meio de escapar do sistema imune do hospedeiro (FAN et al., 2017). Se tratando de resposta imunológica, moléculas do sistema imune inato expressas pelos hepatócitos se tornam a primeira linha de defesa contra o vírus, e são indispensáveis para o controle da resposta imune e da patogenia do HCV (Figura 6). Ainda assim, o vírus consegue evadir o sistema imune eficientemente, podendo levar a um quadro de hepatite crônica (HORNER; GALE, 2013).

Antígenos do HCV são reconhecidos por receptores específicos do sistema imune inato, como os *Toll-Like Receptors* (TLRs), que reconhecem padrões moleculares associados a patógenos (PAMPs, *Pathogen Associated Molecular Patterns)*, levando à produção de moléculas pró-inflamatórias que agem no recrutamento da resposta adaptativa e limitam a replicação e a propagação viral (HORNER; GALE, 2013). Mais de um TLR foi associado à resposta contra o HCV, como o TLR3, o TLR7 e o TLR9. Foi visto que células dendríticas plasmocitóides produzem grandes quantidades de interferon I a partir do reconhecimento de antígenos virais pelos três TLRs citados (TAKAHASHI et al., 2010).

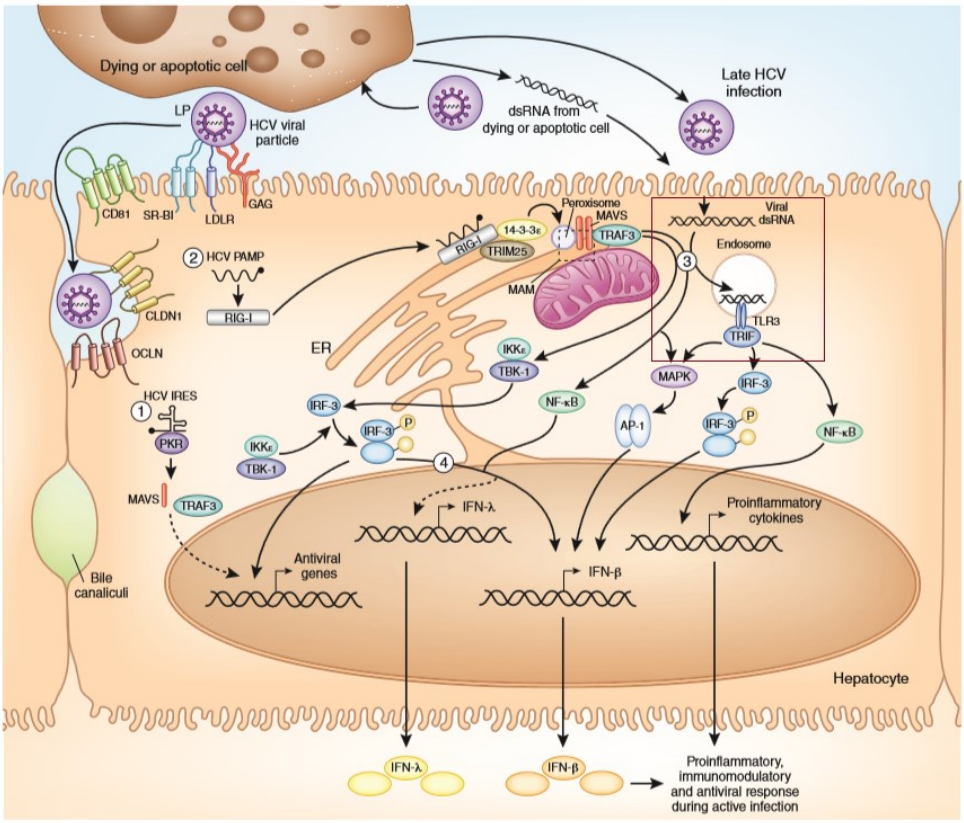

Figura 6. Ilustração esquemática da resposta imune inata ao HCV. Em destaque, o reconhecimento da molécula de dsRNA viral por um TLR – TLR3 (adaptado de HORNER; GALE, 2013).

1.2.3 Epidemiologia do HCV

A infecção pelo HCV atinge cerca de 71 milhões de pessoas no mundo, sendo responsável pelo óbito de aproximadamente 400 mil indivíduos por ano (WORLD HEALTH ORGANIZATION, 2018). No Brasil, até 2016 foram notificadas em torno de 1.032.000 pessoas sororreagentes para HCV, sendo que 657.000 desses casos são virêmicos e necessitam de tratamento. Desde 1996, a notificação da infecção pelo HCV é compulsória, dada a importância desta doença em nível populacional. Dentre os genótipos do vírus, o HCV-1 é o de maior importância no Brasil, seguido pelo HCV-3, que é mais detectado na região Sul (MINISTÉRIO DA SAÚDE, 2018b).

1.2.4 Aspectos Clínicos da Hepatite C

Na maioria dos casos, indivíduos recém infectados pelo HCV não apresentam sintomas perceptíveis. Em cerca de 20% dos pacientes, há a presença de sintomas de hepatite

aguda, como icterícia, náusea e dor abdominal. Ao longo do tempo, é possível uma resolução espontânea da infecção – de 15 a 45% dos casos − e o restante apresenta uma progressão para hepatite crônica, podendo evoluir para casos de cirrose e carcinoma hepatocelular (HCC). A hepatite crônica é diagnosticada quando se detecta RNA viral mesmo seis meses após início da hepatite aguda, e acomete até 85% dos indivíduos com sintomas agudos. A resolução espontânea em casos de hepatite C crônica é rara (LINGALA; GHANY, 2015).

Na maioria dos indivíduos, é possível realizar o diagnóstico utilizando ferramentas moleculares (detecção de RNA viral) duas semanas após a infecção, enquanto que um imunoensaio é capaz de detectar anticorpos anti-HCV apenas doze semanas após a infecção. Testes bioquímicos também se tornam interessantes nesse contexto, já que se pode observar níveis elevados da enzima alanina aminotransferase (ALT) nos pacientes em cerca de oito semanas pós-infecção (LINGALA; GHANY, 2015). No Brasil, é recomendado que o diagnóstico seja realizado por pelo menos dois testes, o molecular e o imunoensaio. Além disso, recomenda-se a genotipagem do vírus para uma escolha adequada do tratamento a ser realizado (MINISTÉRIO DA SAÚDE, 2018b).

O tratamento de primeira linha para a hepatite C foi durante muitos anos o interferon-alfa (IFN-α) peguilado conjugado à ribavirina (RBV), um fármaco que atua inibindo a síntese proteica viral (MEDSCAPE, 2018). Entretanto, o tratamento com IFN se mostrou bastante desagradável para os pacientes em razão dos efeitos adversos, e não era muito eficaz para alguns genótipos do vírus (HORNER; GALE, 2013). Em 2015, porém, foi implementado no Brasil o tratamento com antivirais de ação direta (DAA, *Direct Acting Antivirals*), sendo estes disponibilizados pelo SUS. Para o direcionamento correto dos fármacos a serem utilizados, é necessária a avaliação do estado da doença – se há fibrose avançada ou cirrose − visto que esses achados patológicos influenciam na eficácia dos medicamentos (MINISTÉRIO DA SAÚDE, 2018b).

Os fármacos disponibilizados pelo SUS para o tratamento da hepatite C crônica são: (1) daclatasvir (DCV), um inibidor do complexo de replicação viral; (2) simeprevir (SIM), inibidor da protease viral; (3) associação de ombitasvir (3D), um inibidor da replicação viral, desabuvir, um inibidor da polimerase, veruprevir, inibidor da protease viral e ritonavir, um potencializador farmacológico; (4) associação de ledipasvir (LED), um inibidor do complexo de replicação viral e sofosbuvir (SOF), um inibidor da polimerase; (5) associação de elbasvir (EBR) um inibidor do complexo de replicação viral e grazoprevir (GZR), inibidor da protease viral (MINISTÉRIO DA SAÚDE, 2018b).

Em casos de pacientes que já apresentam um quadro de cirrose, é recomendado o uso da ribavirina em associação ao DAA utilizado. Esse esquema também pode ser utilizado em indivíduos com baixa resposta ao tratamento de primeira linha. Em pacientes gestantes, todo e qualquer tratamento contra a hepatite C deve ser interrompido, visto que a ribavirina possui propriedades teratogênicas e os DAA não possuem estudos suficientes nesse grupo para garantir segurança (MINISTÉRIO DA SAÚDE, 2018b).

1.3 COINFECÇÃO POR HIV/HCV

A infecção concomitante pelo HIV e o HCV pode facilitar a progressão à AIDS e agravar doenças crônicas hepáticas, como cirrose e carcinoma hepatocelular (PIROTH et al., 1998; HERNANDEZ; SHERMAN, 2011). Por apresentarem vias de transmissão bastante similares, não é incomum observar ambas as infecções em indivíduos com comportamentos de risco – como o uso de drogas injetáveis (WIESSING et al., 2011). Além disso, parece haver uma relação entre a presença de infecção por HIV-1 e manifestações extra-hepáticas em pacientes HCV^{+} (RADKOWSKI et al., 2002).

Alguns dados se mostram bastante interessantes em relação à influência de uma infecção sobre a outra. Temos como exemplo o fato de que indivíduos expostos à glicoproteína viral gp120 do HIV apresentam replicação do HCV aumentada e níveis elevados de TGF-β1 – um mediador importante para a fibrogênese (LIN; WEINBERG; CHUNG, 2013). Em modelo de cultura celular, foram encontrados níveis de apoptose maiores em cultivos inoculados com ambos os vírus em comparação a cultivos monoinfectados por HCV ou HIV (JANG et al., 2011). Além disso, várias interações entre moléculas do HCV e proteínas do HIV já foram relatadas, reafirmando a interferência de uma infecção sobre a outra (ELLWANGER et al., 2017).

1.4 RECEPTORES DO TIPO TOLL (TLRs)

Os TLRs (do inglês, *Toll-Like Receptors*) são importantes componentes da resposta imune inata e agem principalmente reconhecendo padrões moleculares associados a patógenos (PAMPs, *Pathogen Associated Molecular Patterns*) (LIEN; INGALLS, 2002). A partir do estímulo desses receptores, cascatas de sinalização complexas são ativadas, resultando na produção de moléculas pró-inflamatórias. Em humanos, já foram descritos 10

diferentes TLRs, sendo alguns deles expressos na superfície celular e outros em membranas endossomais (KAWAI; AKIRA, 2010).

1.4.1 História dos TLRs

Os Receptores do tipo Toll foram identificados pela primeira vez na década de 1990 (MEDZHITOV; PRESTON-HURLBURT; JANEWAY, 1997; ROCK et al., 1998). Essas moléculas começaram a ser investigadas devido à similaridade com os receptores Toll de *Drosophila*, que são importantes mediadores da embriogênese nesses animais. Além disso, pesquisadores verificaram que os receptores Toll também possuíam alguma função de resposta imune nos insetos, visto que a ativação desses estimula a produção de peptídeos antifúngicos. Com essa descoberta, o pesquisador responsável, Jules Hoffmann, foi reconhecido como um dos cientistas pioneiros nos estudos sobre imunidade inata, sendo um dos contemplados do Prêmio Nobel de Fisiologia ou Medicina de 2011 (LEMAITRE et al., 1996; ANDERSON, 2000).

Com a descoberta de genes homólogos aos genes Toll de Drosophila em mamíferos, então denominados TLRs, extensas pesquisas foram realizadas a fim de elucidar os papéis de cada um, principalmente em humanos e murinos, e devido a esses estudos, muitos outros receptores de padrões associados a patógenos foram identificados, como os do tipo NOD e RIG (KAWAI; AKIRA, 2010). Em 1998, outro pesquisador destacado fez uma descoberta interessante: ao investigar que receptores poderiam ter o lipopolissacarídeo (LPS), componente principal de membranas externas bacterianas, como ligantes, Bruce Beutler e seu grupo identificaram o gene TLR4, ao verificarem que camundongos com respostas defectivas a estímulos com LPS possuíam mutações nesse gene. A partir da descoberta de um TLR em mamíferos e da sua importante função no reconhecimento de antígenos microbianos, Bruce Beutler também foi um dos ganhadores do Nobel de Fisiologia ou Medicina de 2011 (POLTORAK, 1998).

1.4.2 Biologia dos TLRs

Os TLRs são importantes moléculas da imunidade inata, já que fazem o reconhecimento de PAMPs e de antígenos relacionados a dano (DAMPs). É a partir dessa primeira resposta que diversos mecanismos imunológicos mais robustos podem ser ativados,

incluindo a estimulação da resposta imune adaptativa (AKIRA; TAKEDA; KAISHO, 2001). Esses receptores são proteínas transmembranares que são ancoradas na superfície celular ou em membranas endossomais – sendo que os TLRs determinados são específicos para um dos dois locais (ANDERSON, 2000).

Os TLRs são expressos por uma ampla variedade de células – desde células clássicas do sistema imune até adipócitos e miócitos (AKIRA; TAKEDA; KAISHO, 2001). A estrutura dos TLRs é dividida em três porções: a externa, rica em leucinas e responsável pelo reconhecimento dos PAMPs e DAMPs; a transmembranar, que ancora os receptores em seus sítios; e a citosólica, também chamada de domínio TIR (*Toll–interleukin 1 (IL-1) receptor*), por sua similaridade com o receptor de interleucina-1 (IL-1R), responsável pela transdução da sinalização celular (KAWAI; AKIRA, 2010).

Dez TLRs já foram identificados na espécie humana (TLR1 – TLR10), tendo cada um deles uma especificidade no reconhecimento de antígenos e na localização celular. Vários são os possíveis ligantes dos TLRs, como lipídeos, proteínas e até mesmo ácidos nucleicos. Os sítios de ancoramento de cada TLR influenciam também o reconhecimento de antígenos específicos, sendo que os TLRs endossomais reconhecem principalmente ácidos nucleicos e os de superfície celular reconhecem moléculas maiores. Além disso, os diferentes TLRs podem desencadear cascatas de sinalização diferentes, possuindo domínios TIR relacionados a moléculas adaptadoras distintas (KAWAI; AKIRA, 2010). A figura 7 retrata os sítios de ancoramento dos TLRs em humanos e seus principais ligantes (ONEILL; GOLENBOCK; BOWIE, 2013).

A partir do conhecimento de que diferentes TLRs reconhecem diferentes moléculas em diferentes compartimentos celulares, percebe-se que estes receptores respondem usualmente a microrganismos específicos, em momentos do processo infeccioso distintos, além de determinados processos inflamatórios gerados por necrose. Por exemplo, em uma infecção por uma bactéria gram-negativa, é possível o reconhecimento de LPS da membrana externa pelo TLR4 antes da penetração do patógeno na célula hospedeira. Após a entrada da bactéria na célula ou fagocitose da mesma, é possível o reconhecimento da molécula de DNA bacteriano pelo TLR9 em compartimentos endossomais (AKIRA; UEMATSU; TAKEUCHI, 2006). Dessa forma, o conjunto dos TLRs pode ser considerado uma ferramenta robusta de reconhecimento de patógenos.

Outra questão a ser abordada é a dispersão dos genes *TLRs* pelo genoma. No cromossomo 4, se localizam os genes *TLR1, TLR2, TLR3* e *TLR6*; o *TLR4* é encontrado no

cromossomo 9; o *TLR5* no cromossomo 1; *TLR7* e *TLR8* no cromossomo X; e o *TLR9* é localizado no cromossomo 3 (AKIRA; TAKEDA; KAISHO, 2001). Diversos polimorfismos desses genes já foram descritos. Além disso, já foram verificadas muitas associações entre tais polimorfismos e diferenças nas respostas imunes a patógenos e doenças inflamatórias. Um papel mais extenso do gene *TLR9* e seus polimorfismos será mais discutido a seguir.

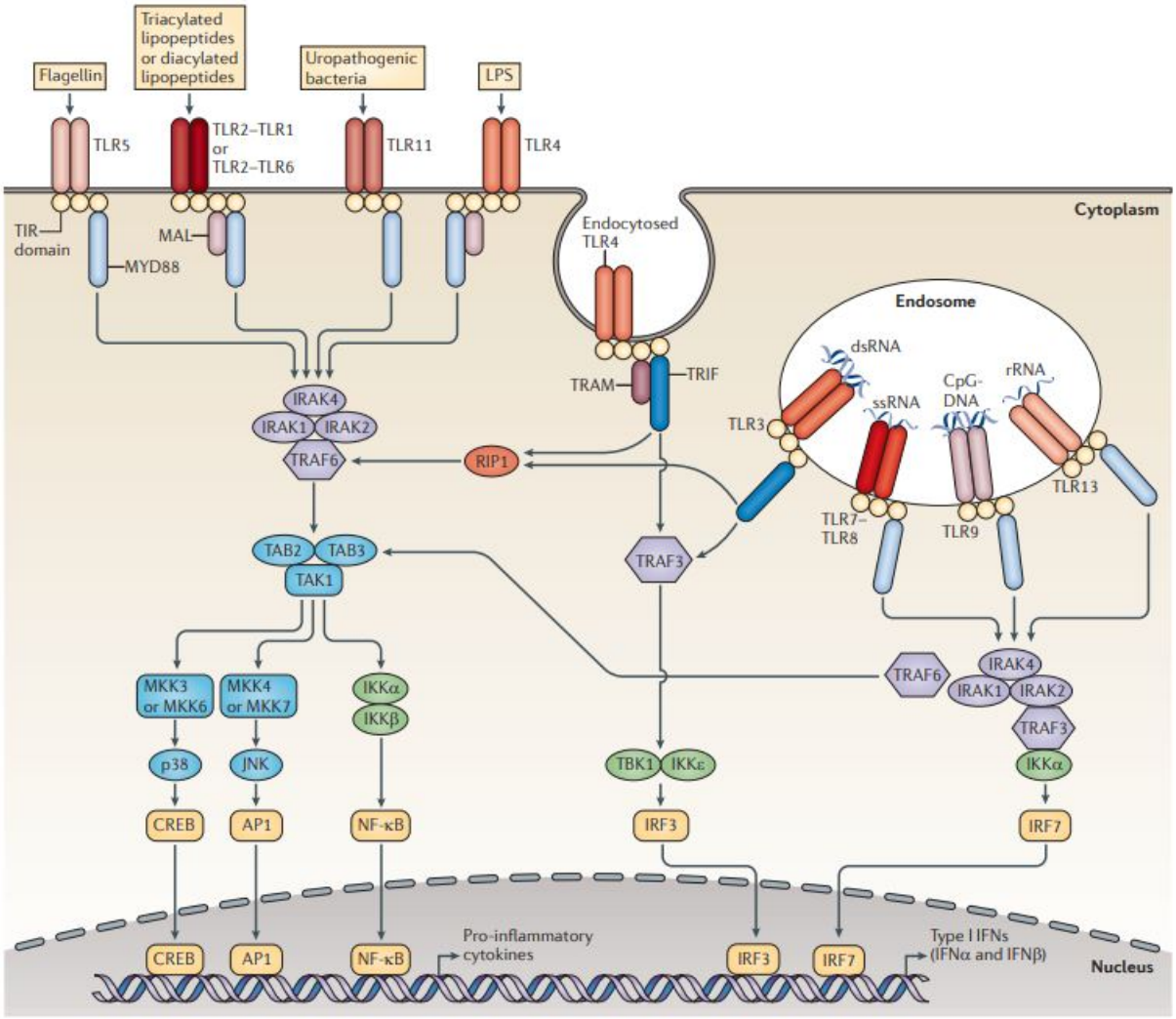

Figura 7. TLRs e seus sítios de ancoramento, bem como seus ligantes e vias de sinalização (ONEILL; GOLENBOCK; BOWIE, 2013).

1.4.3 O Gene *TLR9* e o polimorfismo 2848 G/A

A proteína TLR9, formada a partir do gene sinônimo localizado no cromossomo 3, está presente na porção interior de endossomos e reconhece moléculas de DNA não metilado e DNA bacteriano (AKIRA; TAKEDA; KAISHO, 2001; HEMMI et al., 2000). O

reconhecimento de antígenos pelo TLR9 ativa uma cascata de sinalização celular que culmina na produção de citocinas pró-inflamatórias, além de ter um papel importante na indução da resposta celular do tipo Th1 e na proliferação de células B (KUMAGAI; TAKEUCHI; AKIRA, 2008).

Sabe-se que diversos vírus também possuem a capacidade de gerar respostas imunes a partir da ativação do TLR9. Com a ligação desses antígenos virais, a produção de interferon do tipo I (IFN) é estimulada em células dendríticas plasmocitóides (pDCs) que expressam TLR9 (KRUG, 2004). Essa estimulação é mediada por fatores de transcrição como o fator nuclear κB (NF-κB) e a proteína ativadora-1 (AP-1) (KUMAGAI; TAKEUCHI; AKIRA, 2008). A figura 8 ilustra as redes de sinalização estimuladas a partir da interação do ligante com o TLR9.

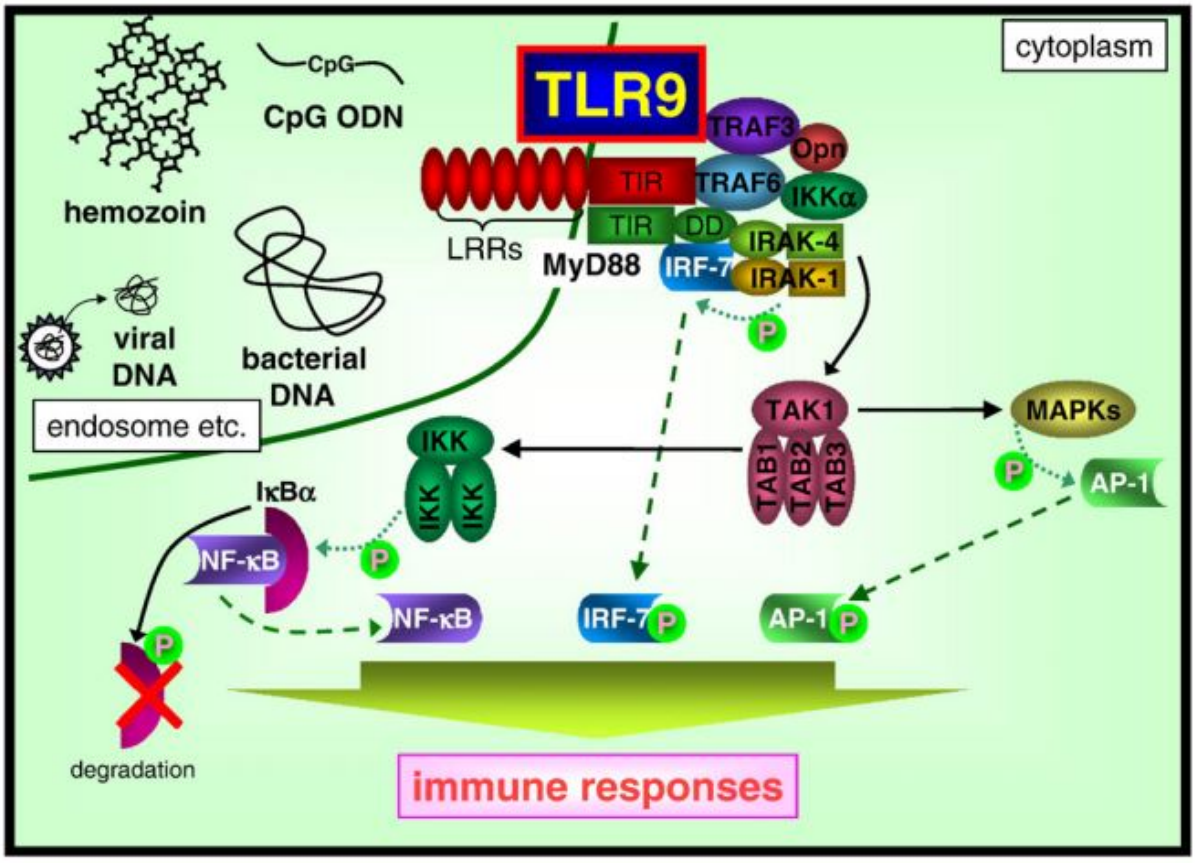

Figura 8. Vias de sinalização estimuladas a partir da interação entre os possíveis ligantes (à direita) com o TLR9 (KUMAGAI; TAKEUCHI; AKIRA, 2008).

Esse receptor pode participar da resposta à infecção pelo HIV reconhecendo o DNA formado a partir da atividade da enzima transcriptase reversa (RT) sobre o RNA viral (MOGENSEN et al., 2010). Além disso, a interação da proteína viral gp120 com células

dendríticas inibe respostas inatas mediadas a partir do TLR9, sugerindo um papel desse receptor na resposta contra o HIV (MARTINELLI et al., 2007). Dois SNPs do gene *TLR9*, 2848 G/A (rs352140) e +1174G/A (rs352139), já foram relacionados a uma progressão acelerada à AIDS (BOCHUD et al., 2007).

O papel do SNP 2848 G/A na infecção pelo HIV ainda é alvo de discussão. Essa variante consiste em uma troca de uma guanina (alelo selvagem) por uma adenina na posição 2848 (éxon 2) do gene, sendo um polimorfismo sinônimo. Apesar da correlação já mencionada, em estudo recente considerando efeitos desse polimorfismo na susceptibilidade à infecção por HIV, nenhum resultado estatisticamente significativo foi detectado (VALVERDE-VILLEGAS et al., 2017). Nesse mesmo estudo, foi verificada uma associação entre o polimorfismo e susceptibilidade a coinfecção por HCV/HIV. No entanto, o impacto da variante 2848 G/A sobre a infecção pelo HIV e HCV ainda é pouco compreendido. Portanto, mais estudos são necessários para o melhor entendimento do papel da variante na infecção pelo HIV e coinfecção por HIV/HCV.

Especificamente na infecção pelo HCV, a influência de polimorfismos do TLR9 ainda é escassamente entendida. Sabe-se que apenas DNA se liga ao receptor, sendo que o HCV possui RNA como seu material genético, de forma que o reconhecimento e ativação de resposta se torna confuso. No entanto, efeitos antivirais já foram observados a partir da ativação de TLR9 em indivíduos HCV^{+} (BROERING et al., 2008). Além disso, níveis elevados do receptor foram identificados nesses pacientes em comparação a um grupo controle (HU; WANG; WANG, 2011). Desse modo, imagina-se que o TLR9 tenha alguma participação na resposta imune ao HCV, mas sua função específica ainda é desconhecida e deve ser explorada (BROERING et al., 2008).

2 JUSTIFICATIVA

O polimorfismo 2848 G/A (rs352140) do gene *TLR9* já foi investigado em diversos contextos. No entanto, seu papel nas infecções pelo HIV, HCV e indivíduos coinfectados ainda é controverso, justificando a realização de estudos adicionais abordando o impacto desta variante sobre essas infecções. Dada a relevância social e epidemiológica das infecções pelo HIV e HCV no Brasil e no mundo, o estudo de variantes genéticas que possam influenciar a suscetibilidade a essas infecções e a progressão das mesmas é bastante pertinente.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Este trabalho tem como objetivo avaliar a influência do polimorfismo 2848 G/A do gene *TLR9* nas infecções por HCV, HIV e na coinfecção por HCV/HIV em uma amostra da população do sul do Brasil.

3.2 OBJETIVOS ESPECÍFICOS

1) Genotipar o polimorfismo 2848 G/A em amostras do material genético de indivíduos HCV^+, HIV^+ e HCV^+/HIV^+, descrevendo as frequências alélicas e genotípicas da variante nos grupos avaliados.

2) Investigar a influência da variante genética estudada sobre a suscetibilidade à infecção pelo HCV, HIV e coinfecção HCV/HIV.

3) Avaliar o impacto do polimorfismo sobre as doenças relacionadas a infecção pelo HCV e coinfecção HIV/HCV.

4 TRABALHO EXPERIMENTAL NA FORMA DE ARTIGO CIENTÍFICO

O trabalho apresentado a seguir encontra-se na forma de *short communication* a ser submetido para a revista *Infection, Genetics and Evolution* (ISSN: 1567-1348).

Evaluation of the 2848 G/A variant (*TLR9* gene) in HCV, HIV, and HCV/HIV Brazillian-infected individuals

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Abstract

Host immunologic and genetic factors have crucial roles in susceptibility to infections and their outcomes. In this context, Toll-like receptors and their polymorphisms are being targeted in a variety of studies. The 2848 G/A variant of *TLR9* gene is a synonymous polymorphism associated with an increased expression of TLR9. This variant has been explored in diverse situations, such as viral infections. In HIV^+, HCV^+ and HCV^+/HIV^+ individuals, its role is still controversial. Therefore, the aim of the present study was to investigate the 2848 G/A polymorphism in individuals affected by those infections. A total of 1182 individuals from southern Brazil were genotyped (Control group = 409; HCV^+ = 376; HIV^+ = 296; HCV^+/HIV^+ = 101). The genotypic and allelic frequencies were very similar among the groups, showing no statistical significant difference between them (p>0.05 in all comparisons). Stratification of individuals by ethnicity did not modify such result. In conclusion, we did not find an influence of the 2848 G/A variant on the susceptibility to HIV infection, HCV infection or co-infection by both viruses in our sample.

Keywords: Hepatitis C virus, Human immunodeficiency virus, HCV/HIV co-infection, TLR9, rs352140, Immunogenetics.

1. Introduction

The Toll-Like Receptor 9 (TLR9) is an essential molecule of the innate immune system, being responsible for the recognition of unmethylated CpG DNA and triggering of inflammatory response (Hemmi et al., 2000). This receptor is expressed by a variety of cells, primarily immune cells, like dendritic and B-cells, but also by hepatocytes, Kupffer cells, and hepatic stellate cells (Hemmi et al., 2000; Akira, 2001; Meli, 2014). Some polymorphisms of the *TLR9* gene have been identified and associated to several clinical outcomes, including in viral infections (Broering et al., 2008; Said et al., 2014; Lai et al., 2013; Mozer-Lisewska et al., 2010). The 2848 G/A variant (rs352140) is a synonymous single nucleotide polymorphisms (SNP) located in the exon 2 of the gene, characterized by the presence of A instead of the wild-type G nucleotide. Although this SNP does not have a direct impact on the protein structure, it has been related to another polymorphism in the promoter region, the -1237 C/T variant (rs5743836), showing modest linkage disequilibrium, especially in Europeans (Roszak et al., 2012; Valverde-Villegas et al., 2017). This promoter variation causes an increased binding of NFκB transcription factor and consequently an increased expression of TLR9 (Hamann et al., 2016; Ng MTH et al., 2010).

Since host genetic factors strongly influence the clinical outcome and susceptibility to infections the investigation of genetic polymorphisms in the context of viral infections is quite relevant (Ellwanger et al., 2017). The 2848 G/A variant has already been explored in HCV^+, HIV^+ and HCV^+/HIV^+ individuals, but its role is still controversial in these three contexts. In a previous study by our group, Valverde-Villegas et al. (2017) have observed an association of this polymorphism with susceptibility to HCV/HIV co-infection in African descendants, leading to a discussion about the impacts of the ethnic background on viral infections. Regarding the association between the 2848 G/A variant and HIV infection, controversial results exist in the literature, some showing lack of association and some detecting influences on the clinical course of HIV infection (Said et al., 2014; Valverde-Villegas et al., 2017; Soriano-Sarabia et al., 2008). In HCV-infected individuals, little progress has been made in the investigation of the 2848 G/A *TLR9* polymorphism. Taking into consideration this scenario, the present study aims to investigate the allelic and genotypic frequencies of 2848 G/A variant in HCV^+, HIV^+ and HCV^+/HIV^+ Brazillian individuals and in a control group, and then evaluate the influence of this variant in the susceptibility to such infections.

2. Methods

Samples and ethical aspects

All participants of this study donated blood samples for DNA extraction on a voluntary basis. Samples were collected in the city of Porto Alegre (South of Brazil). In Table 1 we summarized the four groups evaluated in this study, totalizing 1182 Brazillian individuals. The control group is composed of healthy blood donors (tested negative for HIV and HCV). This study was approved by the Ethics Committees of UFRGS, ULBRA, and *Hospital de Clínicas de Porto Alegre*, Brazil. All participants signed an informed consent following the Resolution No. 466 from *Ministério da Saúde* (Brasil, 2012).

Genotyping

DNA samples were amplified following a PCR-RFLP protocol using the restriction endonuclease *Bsh1236*I (BstUI) as described by Cheng et al. (2007). The amplicons were visualized in 3% agarose gel under UV light. Negative and positive controls were included in all reactions.

Statistical analysis

We verified the Hardy-Weinberg equilibrium in all groups using the chi-square test (χ^2). The allelic and genotypic frequencies were compared between groups through the Pearson's chi-square test, considering Yates's correction in 2x2 tables. Once the groups were not homogenous regarding the ethnic background, we stratified the individuals by ethnicity (Caucasians or Non-Caucasians, based on skin-color and self-declaration) and then repeated the analyses. A p-value <0.05 was set as statistically significant. When appropriate, p-values were adjusted for multiple comparisons by Benjamini-Hochberg step-up False discovery rate (FDR). The analyzes were performed with the aid of WINPEPI (Abramson, 2011).

3. Results

The genotypic and allelic frequencies are shown in Table 2. All groups were in Hardy-Weinberg equilibrium ($p>0.05$ in all analyzes). All groups were homogenous regarding both

genotype and allele frequencies. No comparison between groups resulted in a statistically significant difference (p>0.05, Table 3).

Table 4 shows the allele and genotype frequencies of individuals stratified by ethnicity. In Caucasian individuals, the allele and genotype frequencies remained very similar between the groups. Considering the Non-Caucasian sample, the HIV^+/HCV^+ group presented a slightly higher frequency of the A allele. Following these analyzes, we made comparisons between all groups (Table 5). However, no statistically significant result was observed after p-values were adjusted for multiple comparisons (p>0.05).

4. Discussion

The present study evaluated the distribution of the 2848 G/A variant in HIV^+, HCV^+ and HIV^+/HCV^+ individuals, comparing the frequencies between these groups and a non-infected control group. No effect of the polymorphism was observed in susceptibility or protection to the infections. As mentioned before, AA genotype was already associated with susceptibility to HCV/HIV co-infection in African descendants (Valverde-Villegas et al., 2017), however our study does not corroborated this finding.

When investigating the role of TLR9 and its variants in HIV e HCV infections, some findings should be highlighted. An antiviral effect against HCV infection by TLR9 activation was already proposed, although the mechanism linked to this activation is not well understood (Fischer et al., 2017). Also, reduced spontaneous clearance of HCV infection was associated with the -1237 C/T variant of *TLR9*, which is in low linkage disequilibrium with the 2848 G/A variant in African descendants, and presents a modest linkage disequilibrium with this same polymorphism in European descendants (Valverde-Villegas et al., 2017; Fischer et al., 2017). It has been suggested that C allele of the -1237 C/T variant is associated to increased IL-6 production and B-cell proliferation, which may be correlated to enhanced liver fibrosis (Fischer et al., 2017; Carvalho et al., 2011).

As briefly described before, the impact of 2848 G/A variant on HIV infection is an open question. A higher frequency of the G allele of the 2848 G/A variant was found in rapid progressors when compared to other HIV^+ individuals (Bochud et al., 2007). Some studies have analyzed viral load and $CD4^+$ cell counts in HIV^+ patients and compared to the variant frequencies, but the results are conflicting (Said et al., 2014; Valverde-Villegas et al., 2017; Soriano-Sarabia et al., 2008). In this study, we did not find an influence of 2848 G/A variant

on the susceptibility to HIV infection, but studies evaluating the role of this polymorphism on the progression of HIV infection are still needed.

5. Conclusion

Our study aimed to verify a potential association between the 2848 G/A variant and susceptibility or protection to HIV and HCV infections, as well as HIV/HCV co-infection. No statistically significant result was found, suggesting that this polymorphism does not directly influence those infections in the population from South of Brazil. Stratification of individuals according to ethnicity did not modify the results.

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Table 1. Demographic data of the individuals included in this study.

Demographic data	Control group *n*=409	HCV+ group *n*=376	HIV+ group *n*=296	HCV+/HIV+ group *n*=101	*p*-value*
Sex, *n* (%)					
Female	185 (45.2)	183 (48.7)	143 (48.3)	38 (37.8)	$p>0.05^{b}$
Male	224 (54.8)	193 (51.3)	153 (51.7)	63 (62.2)	
Ethnicity [a], *n* (%)					
Caucasians	307 (75.1)	257 (68.4)	186 (62.8)	40 (39.6)	$p<0.05^{b}$
Non-Caucasians	102 (24.9)	119 (31.6)	110 (37.2)	61 (60.4)	

n, sample number. SD, standard deviation. [a] Based-on color-skin self-declaration. [b]All groups, compared to control group. *Pearson's Chi-square with Yates's correction.

Table 2. Distribution of the TLR9 genotypes/alleles.

TLR9 genotypes/alleles	Control group (*n*=409)	HCV+ group (*n*=376)	HIV+ group (*n*=296)	HCV+/HIV+ group (*n*=101)
GG, *n* (genotypic freq.)	116 (0.28)	87 (0.23)	79 (0.27)	26 (0.26)
GA, *n* (genotypic freq.)	198 (0.48)	194 (0.52)	150 (0.5)	49 (0.48)
AA, *n* (genotypic freq.)	95 (0.23)	95 (0.25)	67 (0.23)	26 (0.26)
G, *n* (allelic freq.)	314 (0.525)	281 (0.49)	229 (0.52)	75 (0.5)
A, *n* (allelic freq.)	293 (0.47)	289 (0.51)	217 (0.48)	75 (0.5)

n, sample number. GG, wild homozygote genotype. GA, heterozygote genotype. AA, variant homozygote genotype.
Allelic frequencies = (2x *n* individuals GG/AA)+(*n* individuals GA)/(2x *n* total individuals).

Table 3. Comparisons of genotype and allele frequencies (detailed in Table 2) between groups.

Comparision	**Considering**	**Chi-square***	***p*-value**
HCV+ group *versus* Control group	Genotypes	2.801	0.246
	Alleles	0.601	0.438
HIV+ group *versus* Control group	Genotypes	0.378	0.828
	Alleles	0.004	0.951
HCV+/HIV+ group *versus* Control group	Genotypes	0.416	0.812
	Alleles	0.083	0.773
HCV+/HIV+ group *versus* HCV+ group	Genotypes	0.383	0.826
	Alleles	0.004	0.951
HCV+/HIV+ group *versus* HIV+ group	Genotypes	0.406	0.816
	Alleles	0.036	0.849

*Pearson's Chi-square.

Table 4. Distribution of the TLR9 genotypes/alleles (stratified by ethnicity).

	TLR9 genotypes/alleles	Control group	HCV+ group	HIV+ group	HCV+/HIV+ group
Caucasians	Sample size	*n*=307	*n*=257	*n*=186	*n*=40
	GG, *n* (genotypic freq.)	74 (0.24)	53 (0.21)	41 (0.22)	13 (0.325)
	GA, *n* (genotypic freq.)	151 (0.49)	133 (0.52)	92 (0.5)	15 (0.375)
	AA, *n* (genotypic freq.)	82 (0.27)	71 (0.27)	53 (0.28)	12 (0.3)
	G, *n* (allelic freq.)	225 (0.49)	186 (0.46)	133 (0.47)	28 (0.51)
	A, *n* (allelic freq.)	233 (0.51)	204 (0.54)	145 (0.53)	27 (0.49)
Non-Caucasians	Sample size	*n*=102	*n*=119	*n*=110	*n*=61

	GG, *n* (genotypic freq.)	42 (0.41)	34 (0.29)	38 (0.34)	13 (0.21)
	GA, *n* (genotypic freq.)	47 (0.46)	61 (0.51)	58 (0.53)	34 (0.56)
	AA, *n* (genotypic freq.)	13 (0.13)	24 (0.20)	14 (0.13)	14 (0.23)
	G, *n* (allelic freq.)	89 (0.64)	95 (0.54)	96 (0.61)	47 (0.49)
	A, *n* (allelic freq.)	60 (0.36)	85 (0.46)	72 (0.39)	48 (0.51)

n, sample number. GG, wild homozygote genotype. GA, heterozygote genotype. AA, variant homozygote genotype.
Allelic frequencies = (2x *n* individuals GG/AA)+(*n* individuals GA)/(2x *n* total individuals).

Table 5. **Comparisons of genotype and allele frequencies (detailed in Table 4) between groups (stratified by ethnicity).**

Ethnicity	**Comparision**	**Considering**	**Chi-square**	***p*-value**	**Adjusted *p*-value***
Caucasians	HCV+ group *versus* Control group	Genotypes	0.979	0.613	0.681
		Alleles	0.121	0.728	-
	HIV+ group *versus* Control group	Genotypes	0.347	0.841	0.841
		Alleles	0.069	0.793	-
	HCV+/HIV+ group *versus* Control group	Genotypes	2.147	0.342	0.570
		Alleles	0.011	0.915	-
	HCV+/HIV+ group *versus* HCV+ group	Genotypes	3.678	0.159	0.398
		Alleles	0.092	0.762	-
	HCV+/HIV+ group *versus* HIV+ group	Genotypes	2.528	0.283	0.566
		Alleles	0.072	0.788	-
Non-Caucasians	HCV+ group *versus* Control group	Genotypes	4.647	0.098	0.398
		Alleles	1.330	0.249	-
	HIV+ group *versus* Control group	Genotypes	1.089	0.580	0.681
		Alleles	0.124	0.724	-
	HCV+/HIV+ group *versus* Control group	Genotypes	7.581	**0.023**	0.230
		Alleles	2.076	0.150	-
	HCV+/HIV+ group *versus* HCV+ group	Genotypes	1.115	0.573	0.681
		Alleles	0.156	0.693	-
	HCV+/HIV+ group *versus* HIV+ group	Genotypes	3.794	0.150	0.398
		Alleles	1.146	0.284	-

*Benjamini-Hochberg step-up False discovery rate (by genotypes).

5 CONCLUSÃO E PERSPECTIVAS

O presente trabalho investigou a variante 2848 G/A do gene *TLR9* em indivíduos HCV^+, HIV^+ e HCV^+/HIV^+. Características interessantes de ambas as infecções foram revisadas, assim como o contexto de coinfecção. Além disso, aspectos importantes dos TLRs foram retomados, dando uma atenção especial ao TLR9. Ao investigar experimentalmente o papel do polimorfismo nas infecções por HIV e HCV e na coinfecção na amostra do sul do Brasil, as seguintes conclusões e perspectivas podem ser apontadas:

a) A variante 2848 G/A não mostrou influência na susceptibilidade às infecções;
b) Nossos resultados não foram modificados quando as características étnicas da população estudada foram consideradas nas análises;
c) Através da discussão dos nossos resultados com base na literatura disponível, sugere-se que a ativação do TLR9 parece levar a uma resposta imune importante contra a infecção pelo HIV;
d) Para um melhor entendimento do papel da variante 2848 G/A nos desfechos clínicos e resposta a fármacos em indivíduos HCV^+, HIV^+ e HCV^+/HIV^+, mais estudos são necessários.

Dessa forma, o estudo realizado se mostra importante para a construção do conhecimento acerca da influência de variantes genéticas em infeções virais. Como perspectivas para além do trabalho atual, pretende-se desenvolver análises semelhantes em outros polimorfismos genéticos de TLRs, além de estender as análises considerando haplótipos, desfechos clínicos e outros.

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ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA *INFECTION, GENETICS AND EVOLUTION*

INFECTION, GENETICS AND EVOLUTION

Journal of Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases (MEEGID)

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DESCRIPTION

Infectious diseases constitute one of the main challenges to medical science in the coming century. The impressive development of molecular megatechnologies and of bioinformatics have greatly increased our knowledge of the evolution, transmission and pathogenicity of infectious diseases. Research has shown that host susceptibility to many infectious diseases has a genetic basis. Furthermore, much is now known on the molecular epidemiology, evolution and virulence of pathogenic agents, as well as their resistance to drugs, vaccines, and antibiotics. Equally, research on the genetics of disease vectors has greatly improved our understanding of their systematics, has increased our capacity to identify target populations for control or intervention, and has provided detailed information on the mechanisms of insecticide resistance.

However, the genetics and evolutionary biology of hosts, pathogens and vectors have tended to develop as three separate fields of research. This artificial compartmentalisation is of concern due to our growing appreciation of the strong coevolutionary interactions among hosts, pathogens and vectors.

Infection, Genetics and Evolution and its companion congress MEEGID (for **Molecular Epidemiology** and **Evolutionary Genetics** of Infectious Diseases) are the main forum acting for the cross-fertilization between **evolutionary science** and biomedical research on infectious diseases.

Infection, Genetics and Evolution is the only journal that welcomes articles dealing with the **genetics** and **evolutionary biology** of hosts, pathogens and vectors, and coevolution processes among them in relation to infection and disease manifestation. All infectious models enter the scope of the journal, including **pathogens** of humans, animals and plants, either parasites, fungi, bacteria, viruses or prions. The journal welcomes articles dealing with genetics, population genetics, **genomics**, **postgenomics**, **gene expression**, evolutionary biology, population dynamics, mathematical **modeling** and **bioinformatics**. We also provide many author benefits, such as free PDFs, a liberal copyright policy, special discounts on Elsevier publications and much more. Please click here for more information on our author services .

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AUDIENCE

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INTRODUCTION
Infectious diseases constitute one of the main challenges to medical science in the coming century. The impressive development of molecular megatechnologies and of bioinformatics have greatly increased our knowledge of the evolution, transmission and pathogenicity of infectious diseases. Research has shown that host susceptibility to many infectious diseases has a genetic basis. Furthermore, much is now known on the molecular epidemiology, evolution and virulence of pathogenic agents, as well as their resistance to drugs, vaccines, and antibiotics. Equally, research on the genetics of disease vectors has greatly improved our understanding of their systematics, has increased our capacity to identify target populations for control or intervention, and has provided detailed information on the mechanisms of insecticide resistance.

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