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INFLUÊNCIA DOS POLIMORFISMOS NOS GENES DOS RECEPTORES *TLR4* e*TLR9* NO DESENVOLVIMENTO DA FIBROSE HEPÁTICA ASSOCIADA À INFECÇÃO PELO VÍRUS DA HEPATITE C

ANDRÉA MONTEIRO TARRAGÔ

MANAUS
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Tese apresentada ao Programa de Pós-Graduação em Imunologia Básica e Aplicada da Universidade Federal do Amazonas como avaliação para o exame de defesa de Doutorado para a obtenção do título de Doutor em Imunologia na área de concentração "*Imunologia Básica e Aplicada*".

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Orientadora: Profª. Dra. Adriana Malheiro Alle Marie

Coorientador: Prof. Dr. Antônio Carlos Rosário Vallinoto

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Ao amor da minha vida, Alexandre Tarragô,
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Concluir o doutorado é uma vitória nossa**!**

Dedico

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.”

Arthur Schopenhauer

Porque eu, o Senhor teu Deus, te tomo pela tua mão direita; e te digo: Não temas, eu te ajudo.

Isaías 41:13

RESUMO

A hepatite C é um problema de saúde pública que afeta aproximadamente 3% da população mundial. É uma doença de prognóstico muito variável que pode evoluir para a cura ou para o desenvolvimento de hepatite C crônica, cirrose hepática, carcinoma hepatocelular e morte. Vários polimorfismos nos genes receptores tipo Toll estão associados a alterações clínicas hepaticas e distúrbios estão relacionados à inflamação. Neste estudo investigou-se a influência dos polimorfismos nos genes dos receptores *TLR4* e *TLR9* e sua associação sobre o perfil de citocinas em pacientes com doença hepática crônica. Os SNPs de *TLR4* e *TLR9* foram genotipados por PCR-RFLP em 151 pacientes com doença hepática crônica causada pelo HCV oriundos da Fundação de Medicina Tropical Doutor Heitor Vieira Dourado e 206 doadores de sangue da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas. Destes, 45 indivíduos foram selecionados de forma randomica para o ensaio de cultura, sendo 15 amostras foram provenientes de doadores saudáveis e 30 de pacientes com doença hepática crônica (15 ≤ F2 e 15 ≥ F2), tratados com DAAs e resposta virológica sustentada (RVS) confirmada pela ausência de RNA viral por testes moleculares quantitativos. A dosagem de citocinas IL-6, TNF, IL-10, IL-2, IFN-γ, IL-4 e IL17A foram realizadas pela técnica de Citometria de Fluxo, utilizando o Kit CBA (*Cytometric Bead Array*). Dentre os resultados obtidos relatamos a presença do genótipo 4,oriundo de um paciente do estado do Pará, descrito pela primeira vez no estado do Amazonas.Nenhum dos polimorfismos de TLR4 e TLR9 analisados neste estudo apresentou associação significativa com doença hepática crônica. As variantes TLR4 A299G A/A + A/G e TLR4 T399I C/C + C/T são mais frequentemente na população estudada. Observamos também que a combinação das variantes TLR9 -1237T/T e TLR9 -1486C/T foi maior nos grupos estudados em comparação com outras combinações de genótipos.Em seguida,estudamos a influência dos genótipos em combinação para as variantes TLR9 TLR9 -1237T/T e -1486C/T sobre perfil de citocinas séricas e em sobrenadante de cultura estimulada com LPS. Assim, observamos que pacientes portadores dos genótipos combinados de TLR9 -1237T/T e -1486C/T demonstraram aumento significativo de IL-6 (p = 0,005) e IL-4 (p = 0,0007) no soro em comparação com inivíduos saudáveis.Um aumento significativo de IL-10 foi observado em pacientes com HCV ≥ F2 (p = 0,028) em comparação com ≤ F2, enquanto as citocinas IL-6, TNF, IL-2, IFN-γ, IL-4 e IL17A não foram significativas quando comparadas entre pacientes com diferentes estágios de doença hepática. Não foi observada diferença significativa em IL-6, TNF, IL-10, IL-2, IFN-γ e IL-4 em cultura de PBMC estimulada com LPS. Apenas a IL-17A apresentou aumento significativo nos pacientes ≥ F2 (p = 0,043) em comparação com ≤ F2. Ao final pudemos observar a mudança do perfil de citocinas para T_H17 sob o estímulo de LPS especialmente entre os pacientes com doença hepática crônica avançada ainda que tenham atingido resposta virológica sustentada. Concluímos que os polimorfismos associados ao risco de desenvolver câncer podem ajudar a selecionar melhor os pacientes para o tratamento do HCV.

Palavras-chaves: Hepatite C; Fibrose hepática; Polimorfismos; Receptores da Imunidade Inata, Antivirais de ação direta.

ABSTRACT

Hepatitis C is a public health problem that affects approximately 3% of the world's population. It is a very variable prognosis disease that can progress to cure or to the development of chronic hepatitis C, liver cirrhosis, hepatocellular carcinoma and death. Several polymorphisms in the Toll receptor genes are associated with clinical hepatic changes and disorders are related to inflammation. In this study we investigated the influence of polymorphisms on the TLR4 and TLR9 receptor genes and their association on the cytokine profile in patients with chronic liver disease. TLR4 and TLR9 SNPs were genotyped by PCR-RFLP in 151 patients with chronic liver disease caused by HCV from the Tropical Medicine Foundation Doctor Heitor Vieira Dourado and 206 blood donors from the Hospital Hematology and Hemotherapy Foundation of Amazonas. Of these, 45 subjects were randomly selected for the culture assay, 15 of which were from healthy donors and 30 from patients with chronic liver disease (15 ≤ F2 and 15 ≥ F2), treated with DAAs and sustained virological response) confirmed by the absence of viral RNA by quantitative molecular tests. The cytokines IL-6, TNF, IL-10, IL-2, IFN-γ, IL-4 and IL17A were performed using the Flow Cytometry technique using the Ctom Kit (Cytometric Bead Array). Among the results obtained, we report the presence of genotype 4, from a patient from the state of Pará, described for the first time in the state of Amazonas. None of the TLR4 and TLR9 polymorphisms analyzed in this study were significantly associated with chronic liver disease. The TLR4 A299G A / A + A / G and TLR4 T399I C / C + C / T variants are more frequently found in the study population. We also observed that the combination of the TLR9 -1237T / T and TLR9 -1486C / T variants was higher in the studied groups compared to other genotype combinations. Next, we studied the influence of the genotypes in combination for TLR9 TLR9 -1237T / T variants and -1486C / T on serum cytokine profile and in LPS-stimulated culture supernatant. Thus, we observed that patients with the combined TLR9 -1237T / T and -1486C / T genotypes showed a significant increase in serum IL-6 (p = 0.005) and IL-4 (p = 0.0007) compared to healthy subjects A significant increase in IL-10 was observed in patients with HCV ≥ F2 (p = 0.028) compared to ≤ F2, whereas the cytokines IL-6, TNF, IL-2, IFN-γ, IL-4 and IL17A did not were significant when compared among patients with different stages of liver disease. No significant difference was observed in IL-6, TNF, IL-10, IL-2, IFN-γ and IL-4 in LPS-stimulated PBMC culture. Only IL-17A presented a significant increase in patients ≥ F2 (p = 0.043) compared to ≤ F2. At the end, we could observe a change in the cytokine profile for TH17 under the LPS stimulus, especially among patients with advanced chronic liver disease, even if they had sustained virological response. We conclude that the polymorphisms associated with the risk of developing cancer may help to better select patients for the treatment of HCV.

Keywords: Hepatitis C; Hepatic fibrosis; Polymorphisms; Receptors of Innate Immunity, direct-acting antivirals.

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LISTA DE ABREVIATURAS E SIGLAS

RNA	Ácido Ribonucléico
HLA I	Antígeno leucocitário humano de classe I
HLA II	Antígeno leucocitário humano de classe II
LTA	Ácido Lipoteicóico
APRI	AST to Platelet Ratio Index
AST	Aspartato aminotransferase
ALT	Alanina aminotransferase
CBA	*Cytometric Bead Array*
CLDN-1	Claudina -1
CTL	Linfócitos T citotóxicos
CTGF	Fator de crescimento do tecido conjuntivo
HCC	Carcinoma Hepatocelular
iDC	Célula Dendrítica Imatura
FIB4	*Fibrosis-4*
FMT-HVD	Fundação de Medicina TropicaL Dr. Heitor Vieira Dourado
HBV	Vírus da Hepatite B
HCV	Vírus da Hepatite C
HIV	Vírus da Imunodeficiência Humana
HEMOAM	Fundação Hospitalar em Hematologia e Hemoterapia do Amazonas
HSC	células estreladas hepáticas
HTLV	Vírus Linfocitário Humano
G	Gravidade
IFN-γ	Interferon-γ
IL-10	Interleucina 10
IL-12	Interleucina 12
IL-2	Interleucina 2
IL-4	Interleucina 4
IL-5	Interleucina 5
IL-6	Interleucina 6
IRF-3	Fator regulador do interferon -3
IRF-7	Fator regulador do interferon -7
JAK-STAT	Janus Kinase /transdutor de sinal

µg	Micrograma
µL	Microlitro
LDL	Lipoproteína de baixa densidade
LDL-R	Receptor de Lipoproteína de baixa densidade
mL	Mililitro
MEC	matriz extracelular
MMP	metaloproteinases de matriz
MyD88	Molécula adaptadora fator de diferenciação mielóide 88
NK	Natural killer
NFkB	Fator nuclear kappa B
OCLDN	Ocludina
OMS	Organização Mundial de Saúde
PAMP	Padrões moleculares associados a patógenos
PDGF	Fator de crescimento derivado de plaquetas
KPR	receptor de proteína kinase
RIG-1	Receptor ácido retinóico I
rpm	Rotações por minuto
RVS	Resposta Virológica Sustentada
SOCS-3	Supressor da sinalização de citocinas- 3
TCR	Receptor de Células T
T_H	Células T Helper
TIMPS	inibidores de tecidos de metaloproteinases
TLR-3	Receptor do Tipo Toll 3
TLR-4	Receptor do Tipo Toll 4
TLR-7	Receptor do Tipo Toll 7
TLR-8	Receptor do Tipo Toll 8
TLR-9	Receptor do Tipo Toll 9
Treg	Células T Regulatórias
TNF-α	Fator de Necrose Tumoral-α
TGF-β1	Fator de transformação do crescimento β
UFAM	Universidade Federal do Amazonas
VEGF	Fator de crescimento do endotélio vascular
αSMA	α-actina do músculo liso

SUMÁRIO

Introdução

INTRODUÇÃO

A Hepatite C crônica é uma inflamação hepática que resulta da resposta inflamatória contra o vírus da Hepatite C (HCV). Estima-se que aproximadamente 71 milhões de pessoas no mundo estejam cronicamente infectadas pelo HCV, dos quais 399.000 pessoas morrem ao ano por desenvolverem cirrose hepática e câncer de fígado como consequência da doença (WHO, 2017).

Na maioria das pessoas infectadas pelo HCV, a doença geralmente se apresenta de forma assintomática ou oligossintomática, o que dificulta o diagnóstico precoce e reduz as chances de tratar os indivíduos infectados no início do processo infeccioso. Isto se deve à complexidade da interação hospedeiro-patógeno que pode estar associada tanto a composição genética do hospedeiro quanto ao genótipo do vírus, que podem promover uma resposta imune favorável tanto para a cura quanto para o desenvolvimento da doença hepática crônica (FERWEDA et al., 2007; COELHO-CASTELO et al., 2009).

O processo de cronificação desta doença ocorre em consequência da falha na resposta imune em eliminar o agente etiológico (HCV) que pode resultar na destruição hepática ou agressão progressiva dos hepatócitos, modificando a arquitetura do fígado e comprometendo sua funcionalidade (FRIDMAN, 2004; FRIDMAN, 2008).

Sabe-se que a imunidade inata é a primeira linha de defesa do hospedeiro em resposta aos patógenos. Dentre os receptores existentes, os receptores do tipo Toll (TLRs) reconhecem os patógenos invasores através de padrões moleculares associados aos patógenos (PAMPs) e desempenham um papel fundamental na imunidade inata do hospedeiro. Em humanos, dos 10 TLRs funcionais descritos, seis deles reconhecem diferentes moléculas presentes no HCV que são capazes de estimular a sinalização dos TLR, desempenhando um papel importante no *clearance* viral através da produção de citocinas pró-inflamatórias (MACHIDA et al.,2006; SEKI et al, 2008; RAMASAWMY et al., 2009).

A desregulação da resposta imune na doença hepática pode ocorrer a partir da sinalização alterada de citocinas como consequência de variações genéticas dentro dos genes de receptores do tipo Toll e estão associados à suscetibilidade a infecções, podendo comprometer os resultados das intervenções terapêuticas e propiciar o desenvolvimento da forma mais grave da doença hepática (NIETO et al.,

2014;PIÑERO et al.,2017). Contudo, não é bem conhecido em que ponto esses polimorfismos contribuem para o desenvolvimento do carcinoma hepatocelular na nova era da terapia livre de interferon atribuída aos antivirais de ação direta (DAAs).

Em 2015, o Ministério da Saúde (MS) incorporou os DAAs para o tratamento da hepatite C, no âmbito do Sistema único de Saúde (SUS). Sabe-se que estes medicamentos apresentam menos efeitos colaterais, tempo de tratamento menor que as terapias anteriores e expectativa de cura acima de 90% por serem capazes de inibir a replicação viral tendo como alvo as proteínas não estruturais NS3-4A, NS5A e NS5B do vírus da hepatite C (BRASIL, 2018).

Nesse aspecto, o controle da infecção pelo HCV e acompanhamento da doença hepática são importantes, principalmente em países de média renda, em virtude da sua alta prevalência e complicações graves decorrentes da cronificação da doença. No estado do Amazonas essa realidade é ainda mais preocupante. A alta demanda de atendimentos de casos de pacientes infectados pelo HCV advindos das mais diversas localidades do estado comprovam a fragilidade na acessibilidade aos testes que auxiliem no acompanhamento da doença hepática disponíveis na rede pública, bem como no acompanhamento dos pacientes crônicos nas cidades mais distantes da capital, por conta da infra-estrutura precária de cada município do Amazonas[1].

Assim, investigamos a influência dos polimorfismos nos genes dos receptores *TLR4* e *TLR9* no desenvolvimento da doença hepática crônica, em pacientes tratados com DAAs que podem estar influenciando no desenvolvimento das formas mais graves da doença mesmo após o paciente ter alcançado resposta virológica sustentada (RVS).

[1] Entre os anos de 1975 a 2015, foram confirmados 8.538 casos de hepatites virais, sendo 3.416 do tipo A, 2.825 do tipo B, 1.662 do tipo C e 635 do tipo D. As maiores incidências foram nos municípios de Manaus, Eirunepé, Coari, Atalaia do Norte, Lábrea, Boca do Acre, Guajará, Manacapuru, Fonte Boa e São Paulo de Olivença. Informação fornecida pela Fundação de Vigilância em Saúde do Amazonas. Disponível em: http://www.fvs.am.gov.br/index.php/9-noticiais/178-campanha-pretende-prevenir-contra-as-hepatites-virais-em-manaus. Acesso em:19 de novembro de 2017.

Revisão Bibliográfica

1 REVISÃO BIBLIOGRÁFICA

1.1Hepatite C: Aspectos Gerais

A infecção pelo vírus da Hepatite C é um problema crescente de saúde pública em todo mundo. A Hepatite C é uma doença endêmica em muitos países e é subdiagnosticada em virtude da ausência de sintomas ou, muitas vezes, por apresentar sintomas inespecíficos. Contudo, nos casos mais graves, pode-se desenvolver a longo prazo, cirrose e carcinoma hepatocelular (HCC) (BRASIL, 2011a; BRASIL, 2017d; WHO,2017).

Segundo dados fornecidos pela Organização Mundial de Saúde (OMS) estima-se que aproximadamente 71 milhões de pessoas no mundo estejam cronicamente infectadas pelo vírus da hepatite C (HCV) sob o risco de desenvolverem cirrose hepática e câncer de fígado (WHO, 2017).

1.2 O vírus da Hepatite C

O vírus da Hepatite C (HCV) está classificado dentro do gênero *Hepacivirus* e membro da família *Flaviviridae* (CHOO et al., 1989). Esta partícula viral é constituída por um envelope lipídico (no qual se encontram as glicoproteínas E1 e E2), um capsídeo protéico (formado por capsômeros e o ácido ribonucléico de fita simples (ssRNA), com polaridade positiva de cerca de 9.600 bases que codificam as proteínas estruturais (Core, E1 e E2), as proteínas não estruturais (NS:NS3, NS4A, NS4B, NS5A e NS5B) e a membrana peptídica p7, que separa as proteínas estruturais das não estruturais, contribui na montagem de partículas virais e liberação de vírions infecciosos(TANG& GRISÉ, 2009; ASHFAQ et al., 2011) (Figura 01).

O HCV apresenta seis genótipos bem caracterizados, e cada um pode ser dividido em vários subtipos. A distribuição global dos genótipos do HCV é diversificada, o que reflete nas diferenças epidemiológicas e variabilidade étnica. Os genótipos 1, 2 e 3 têm ampla distribuição geográfica, enquanto que genótipos 4, 5 e 6 são geralmente confinados em regiões geográficas específicas conforme ilustrado na Figura 02 (HAJARIZADEH; GREBELY; DORE, 2013). Existem relatos na literatura sobre um sétimo genótipo (7), que foi primeiramente descrito em pacientes canadenses e belgas, que possivelmente foram infectados na África central (MURPHY et al., 2015).

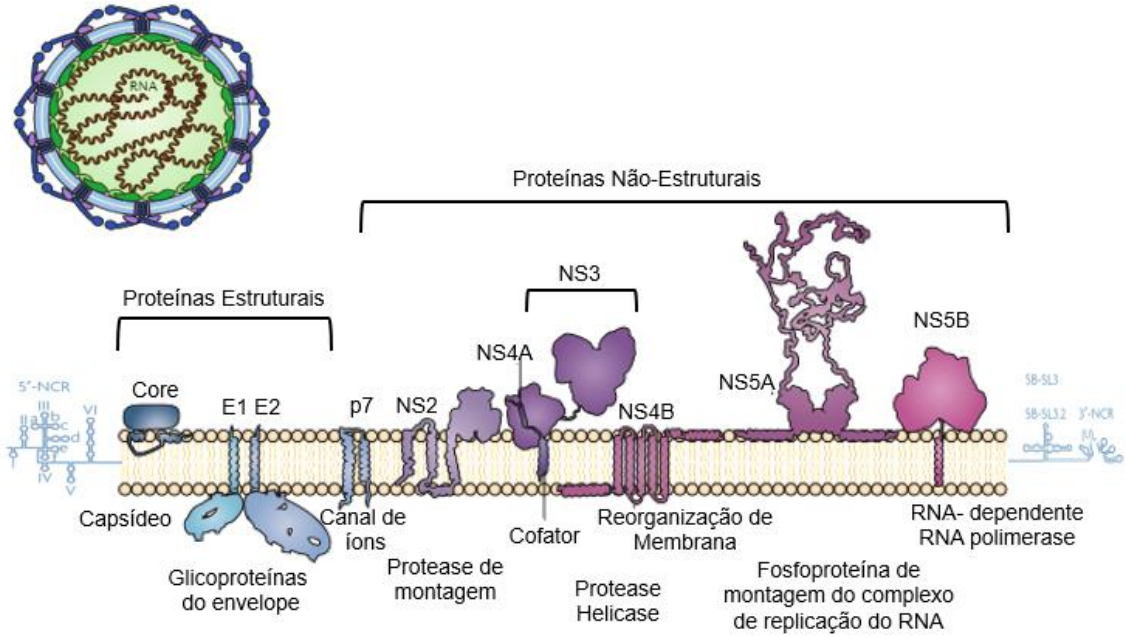

Figura 01: Genoma do Vírus da Hepatite C.
Fonte: Adaptado de LINDENBACHET & RICE, 2013;BARTENSCHLAGER et al., 2013.

No Brasil, são encontrados, principalmente, os genótipos 1 (64,9%), seguido do genótipo 3 (30,2%), 2 (4%), 4 (0,2%) e 5 (0,1%). Na Região Norte, observou-se o predomínio do genótipo 1, seguido do genótipo 3 e genótipo 2 (CAMPIOTTO, 2005; PARANÁ et al.,2017).

No estado do Amazonas, estudos desenvolvidos em nosso grupo de pesquisa[2] apontam maior prevalência do genótipo 1, seguido do genótipo 3 e 2 tanto em doadores de sangue atendidos pela Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM) quanto em pacientes atendidos pela Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (FMT-HVD) (TORRES et al., 2009; ARAÚJO et al., 2011). O genótipo 4 também foi descrito recentemente pelo nosso grupo de pesquisa, o que nos leva a crer que nossa região seja uma porta de entrada para variantes incomuns do genótipo do vírus (TARRAGÔ et al., 2017).

[2] Grupo de pesquisa “Imunologia Básica e Aplicada”, liderado pela Profa. Dra. Adriana Malheiro Alle Marie com desenvolvimento de pesquisas na Universidade Federal do Amazonas, certificado pela instituição segundo as normas vigentes da CNPq e CAPES.

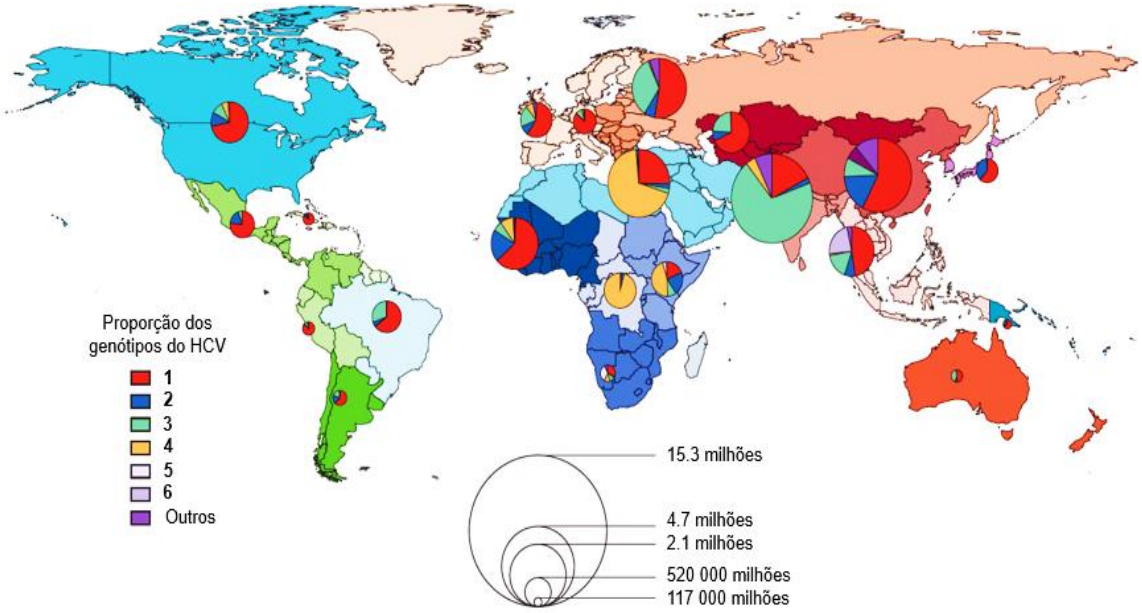

Figura 02: Distribuição global dos genótipos do Vírus da Hepatite C
Fonte: Adaptado da WHO, 2016.

1.3 Transmissão e Aspectos Clínicos

A transmissão do vírus da Hepatite C ocorre principalmente por via parenteral, por meio de transfusão de sangue (hemocomponentes e/ou hemoderivados) contaminados, transplante de órgãos de doadores infectados, uso de drogas ilícitas injetáveis, hemodiálise, exposição ocupacional ao sangue, infecção perinatal e sexual, bem como por secreções orgânicas como saliva, urina, líquido ascítico, bile e mucosa intestinal (CAVALHEIRO et al., 2009; CAVALHEIRO et al., 2010; MARTINS; NARCISO-SCHIAVON; SCHIAVON, 2012). Além disso, outros fatores de riscos inerentes às atividades humanas estão associados à transmissão do vírus, tais como tatuagem, piercing, rituais de escarificação, acupuntura, utilização de equipamentos perfurocortantes, tratamentos odontológicos, endoscopia digestiva alta e produtos para realização de procedimentos de higiene pessoal (escovas de dente, alicates, lâminas de barbear e de depilar, entre outros) CAVALHEIRO et al., 2010; MARTINS; NARCISO-SCHIAVON; SCHIAVON, 2011).

A doença se apresenta de forma assintomática, oligossintomática ou associada a sintomas inespecíficos. Geralmente o diagnóstico é incidental, ocorrendo, algumas vezes, durante triagem sorológica para doação de sangue ou exames médicos de rotina. Também podemos reportar que existem importantes manifestações extra-hepáticas relacionadas à infecção pelo HCV, sendo a maioria destas síndromes

associadas a estados autoimunes ou linfoproliferativos (LAUER; WALKER, 2001; BRASIL, 2017).

Em virtude da história natural da doença, muitas pessoas assintomáticas infectadas pelo HCV transmitiram o vírus, preferencialmente, através de transfusão de sangue e/ou hemoderivados (antes de 1993), hemodiálise e compartilhamento de utensílios para uso de drogas injetáveis (LAUER; WALKER, 2001; BOTELHO et al., 2008; MARTINS; NARCISO-SCHIAVON; SCHIAVON, 2012; BRASIL, 2017d). Segundo análise realizada por Amaku et al., (2016), observou-se que a notificação da infecção pelo HCV acomete principalmente em indivíduos acima de 40 anos, o que nos leva a crer que muitos se infectaram há pelo menos entre 10 e 20 anos.

Estima-se que 55 à 85% dos casos de infecção pelo HCV evoluem para a Hepatite C crônica, desencadeando complicações como esteatose, fibrose hepática, aparecimento de nódulos regenerativos que podem resultar em insuficiência hepática, cirrose e descompensação hepática, caracterizada por alterações sistêmicas e hipertensão portal, evoluindo com ascite, varizes esofágicas e encefalopatia hepática (FRIEDMAN; 2004; BATALLER; BRENNER, 2005; FRIEDMAN; 2008). Cerca de 1 a 5% dos pacientes desenvolvem carcinoma hepatocelular, enquanto que apenas 15 a 45% dos indivíduos infectados conseguem eliminar o vírus espontaneamente (GALE; FOY,2005; SHEPARED et al., 2005; LAVANCHY,2009; LAVANCHY,2011).

1.4 Diagnóstico laboratorial

O diagnóstico laboratorial para confirmação do vírus da Hepatite C preconizado pelo Ministério da Saúde inclui o método imunocromatográfico, usando antígenos sintéticos e recombinantes imobilizados na membrana para identificação seletiva de anti-HCV, imunoensaios para a detecção de anticorpos anti-HCV no soro e os testes moleculares que foram incluídos para permitir a detecção do RNA viral, bem como, caracterização dos genótipos e dos subtipos descritos do HCV (BRASIL, 2011b; BRASIL,2017d).

A presença de anti-HCV, isoladamente, não define a presença de infecção ativa e deve ser interpretada como contato prévio com o vírus. O resultado reagente desse marcador deve ser confirmado por teste de amplificação de ácido nucléico (NAT) para detecção de ácidos nucléicos do HCV ampliando a segurança transfusional (DESMET et al., 1994).

Vale ressaltar que pacientes imunossuprimidos e indivíduos que foram expostos ao vírus em um período inferior a cento e cinquenta dias podem não apresentar sorologia reagente (anti-HCV), em virtude da diminuição ou ausência da produção de anticorpos. Nesses casos, o diagnóstico deve ser realizado por meio de testes moleculares (BATTS KP,1995).

1.5 Avaliação do Estadiamento da Doença Hepática e Identificação de Cirrose Descompensada

O grau de acometimento hepático pode ser estabelecido por meio da avaliação da biópsia hepática (padrão ouro), elastografia hepática transitória e também por meio da aplicação de índices APRI (*AST to Platelet Ratio Index*) e FIB-4 (*Fibrosis-4*). O APRI e o FIB-4 são métodos não invasivos, recomendados pela Organização Mundial de Saúde desde 2014 e podem ser obtidos por meio do cálculo matemático simples utilizando os resultados de exames laboratoriais (Figura 03). Os resultados de fibrose ausente/inicial ou avançada, são obtidos por meio de pontos de corte preestabelecidos conforme observados no Quadro 01 (WHO, 2014; BRASIL 2017d).

Cálculo do APRI:

$$APRI = \frac{\frac{\text{Valor de AST (UI/L)}}{\text{Limite Superior Normal de AST (UI/L)}}}{\text{Contagem de Plaquetas } (10^9)} \times 100$$

Cálculo do FIB4:

$$FIB4 = \frac{\text{Idade (anos) X AST (UI/L)}}{\text{Contagem de Plaquetas } (10^9) \times \sqrt{\text{ALT (UI/L)}}} \times 100$$

Figura 03: Cálculo matemático do APRI e FIB-4.
Fonte: BRASIL, 2017d.

METAVIR	APRI (baixo corte)	APRI (alto corte)	FIB4 (baixo corte)	FIB4 (alto corte)
METAVIR F2 (fibrose moderada)	0,5	1,5	1,45	3,25
METAVIR F4 (cirrose)	1,0	2,0	-	-

Quadro 01: Detecção de cirrose e fibrose moderada conforme valores de alto e baixo corte de APRI e FIB-4.
Fonte:BRASIL, 2017d.

Existem diversos sistemas de classificação para graduação e estadiamento das hepatites crônicas. Recomenda-se o uso da classificação METAVIR, que considera os aspectos histológicos básicos das hepatites crônicas: atividade periportal, lobular e fibrose (BRASIL, 2011; BRASIL, 2017). O escore de Child-Turcotte-Pugh (Child-Pugh), utilizado para avaliar o grau de deterioração da função hepática, é calculado somando-se os pontos dos cinco fatores abaixo, variando entre 5 e 15. As classes de Child-Pugh são A (escore de 5 a 6), B (7 a 9) ou C (acima de 10)(BRASIL, 2017d).

1.6 Entrada e replicação do HCV nas células hospedeiras

O HCV circula em várias formas no hospedeiro infectado, circulando livremente ou associado diretamente a lipoproteínas de baixa densidade (LDL) agindo como um dos fatores de ligação inicial (PRINCE, et al., 1996; AGNELLO et al., 1999; PATRICE et al., 2005).

Várias moléculas de superfície celular medeiam a ligação do HCV para células hospedeiras. As moléculas de glicosaminoglicanos (GAGs) e os receptores de lipoproteína de baixa densidade (LDL-R) facilitam a fixação inicial, interagindo com as glicoproteínas do HCV e lipoproteínas associadas aos vírions, respectivamente. Após o passo inicial de ligação, o vírus interage com o receptor scavenger classe B tipo I e a molécula CD81. Logo, as proteínas de junção claudina-1 (CLDN-1) e ocludina (OCLDN) contribuem para a absorção, ligação e internalização do HCV por endocitose (EVANS, et al., 2007; DAO THI, et al., 2011) (Figura 04).

Após a entrada do vírion, o endossoma acidificado induz a fusão das glicoproteínas virais à membrana da célula e em seguida o RNA é liberado do nucleocapsídeo para replicação e tradução das proteínas que compõe o vírus (MORADPOUR et al.,2007). O processo de formação do nucleocapsídeo acontece dentro do retículo endoplasmático. Posteriormente, o nucleocapsídeo envelopado matura-se no complexo de Golgi e novos vírions são produzidos e liberados por exocitose, iniciando um novo ciclo (PENIN et al., 2004).

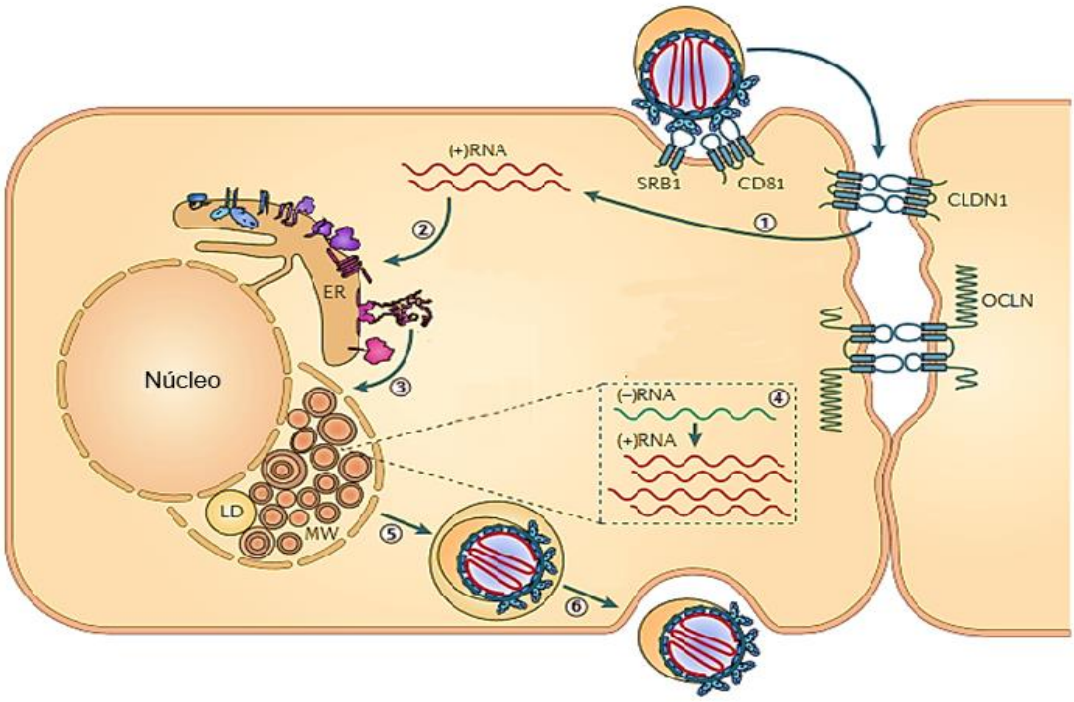

Figura 04: Ciclo de Replicação do Vírus da Hepatite C.
Fonte: Adaptado de BARTENSCHLAGER et al., 2013.

1.7 Mecanismos imunes inatos e adaptativos no desenvolvimento da fibrose hepática

Na infecção por HCV, a lesão hepática aguda e crônica promovem a ativação da resposta pro-inflamatória, que resulta na produção de citocinas inflamatórias e no recrutamento de leucócitos que contribuem para um desequilíbrio no processo de remodelação da matriz extracelular (MEC), induzindo progressivamente a deformação de septos, e anéis de cicatriz que envolvem nódulos hepáticos (FRIDMAN, 2004).

A resposta imune na infecção por HCV inicia-se através da ativação de receptores do tipo Toll por proteínas e RNA do HCV. Os TLRs podem reconhecer uma variedade de elementos provenientes de bactérias, fungos, protozoários e vírus, que podem ser categorizados em componentes de lipídeos, proteínas e ácidos nucléicos. O reconhecimento pelos TLR através da interação dos padrões moleculares associados a patógenos – receptores do tipo Toll (PAMP-TLR) induz a ativação de fatores de transcrição, como fatores reguladores de interferon 3/ 7(IRF3/7) e factor nuclear kappa B (NF-kB), que convergem para a ativação de genes alvo do IFN tipo I (α e β), resultando na inibição da replicação viral, produção de citocinas pró-inflamatórias, como TNF-α, IL-12, IL-1, IL-6, quimiocinas endoteliais (CCL2, CXCL8), além de promover a elevação das células inflamatórias para o

fígado (MACHIDA et al., 2006;SATO et al., 2007;EKSIOGLU, et al., 2011; DENTAL et al., 2012;BUONAGURO et al., 2012).

As proteínas do Core e a proteína NS3 são reconhecidas pelo TLR-2 associado ao TLR-1/TLR-6 em células mononucleares do sangue periférico (SATO, 2007). Enquanto NS5A ativa TLR-4 e induz a produção de IFN-β em células infectadas e tecido circundante (MACHIDA, 2006). No processo de replicação do HCV, seu RNA também estimula TLR-3, 7/8 e 9, que contribui para o controle da replicação viral, reduzindo os níveis de RNA do HCV e ampliando o processo inflamatório (ZANG, 2009) (Figura 05).

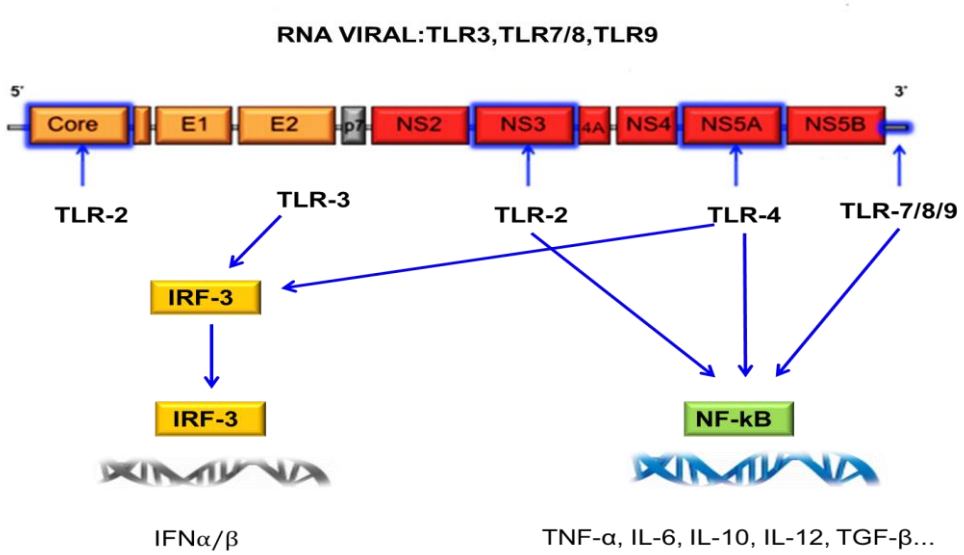

Figura 05: Reconhecimento das proteínas do Vírus da Hepatite C e ativação das vias de sinalização.
Fonte: Adaptado de HOWELL et al., 2013.

Este processo inicial de reconhecimento do patógeno é feito pelas células dendríticas imaturas (iDC) que reconhecem o material genético do vírus por meio dos receptores do tipo Toll e, em seguida, migram para os linfonodos onde tornam-se células dendríticas maduras e ativam linfócitos T auxiliares (linfócitos T *helper, naive*), por meio da ligação do antígeno leucocitário humano de classe II (HLA II) e moléculas co-estimulatórias (CD80 e CD86). Simultaneamente, as células dendríticas ativam linfócitos T citotóxicos (CTLs) através da ligação do HLA I e moléculas co-estimulatórias. Por conseguinte, as células dendríticas mieloides produzem IL-12, modulando uma resposta imune celular T_H1. Estas, por sua vez secretam IL-2, TNF-α e IFN-γ que induzem a ativação e proliferação de linfócitos T citotóxicos (CTLs) específicos ao HCV. Os CTLs sensibilizados são estimulados a deixarem os linfonodos e migrarem para o fígado. Eles reconhecem a infecção por

meio da ligação do receptor de células T (TCR) aos epítopos virais associados à molécula de HLA de classe I na superfície de hepatócitos infectados, e erradica-os através da injúria causada nesses hepatócitos por liberação de perforinas, granzimas, ligação de FAS/FASL e TNF-α, iniciando assim, o processo inflamatório (Figura 06) (HIROISHI et al., 2008; HIROISHI et al., 2010; PARK et al., 2014).

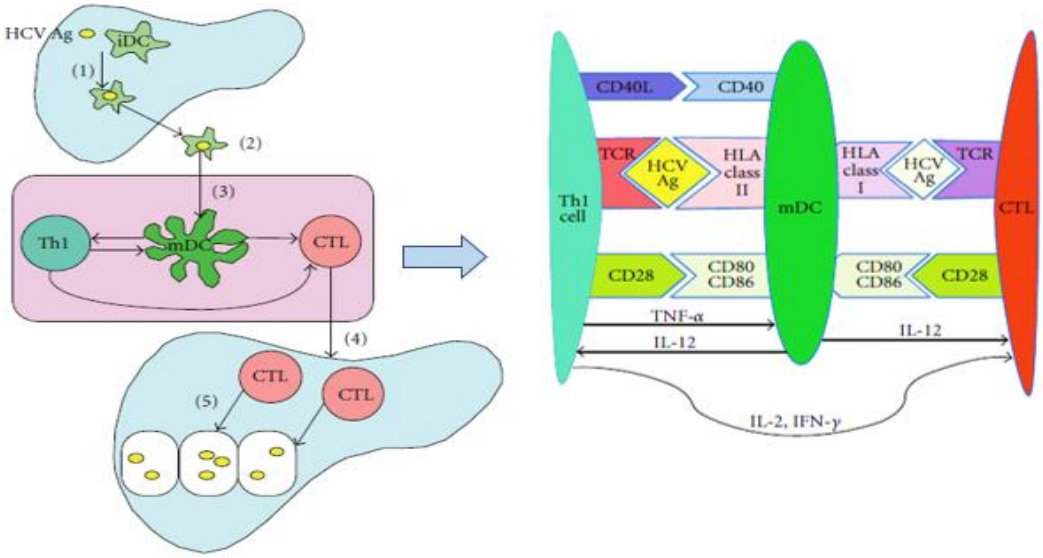

Figura 06: Mecanismos das células imunes no reconhecimento e eliminação do Vírus da Hepatite C.
Fonte: Adaptado de HIROISHI et al., 2010.

Em caso de eliminação espontânea do vírus, uma forte resposta celular devido aos linfócitos T $CD4^+$ e T $CD8^+$ é observada na fase inicial da infecção. Em seguida, os anticorpos neutralizantes são rapidamente expressos. No entanto, na maioria dos casos, a imunidade adaptativa do hospedeiro é incapaz de controlar a infecção pelo HCV e evolui para uma forma crônica da doença. Os anticorpos específicos para as proteínas virais E1/ E2 são ineficientes devido à seleção rápida das variantes resultantes da mutação do vírus, promovendo a falha no reconhecimento e a eliminação viral por células T $CD8^+$ específicas (JEULIN et al., 2013).

Para continuar seu processo de replicação, o HCV interfere em várias vias de sinalização das respostas imunes antivirais. Uma das proteínas que participa desse processo é a NS3/4, que bloqueia a ativação do gene induzível pelo ácido retinóico I (RIG-1) e a tradução do fator regulador de IFN-3 (IRF-3). A proteína Core interfere na via janus-cinase de transdutores de sinais e ativadores de transcrição (JAK-STAT) pela ativação dos supressores da sinalização de citocinas-3 (SOCS-3). A

proteína E2 inibe o receptor de proteína cinase (PKR) e as funções das células natural killer (NK) (ASHFAQ et al., 2011).

A NS5A tem propriedades importantes no escape da ação antiviral dos IFNs (ASHFAQ et al., 2011). Uma explicação para isto vem da observação de que a NS5A pode induzir a expressão e secreção de IL-8 cujas ações inibem a produção dos IFNs (GALE & FOY, 2005).

Para proteger o dano hepático causado pelo processo de persistência viral, as células reguladoras dos linfócitos T desempenham um papel central pela supressão da atividade imune excessiva através da expressão de citocinas anti-inflamatórias, como TGF-β1 e IL-10 (CABRERA et al., 2004; WARD et al. 2007, JUNG; SHIN, 2016).

No entanto, o TGF-β1 é um forte indutor de fibrogênese, que regula a α-actina do músculo liso (αSMA), promove a transdiferenciação de células estreladas hepáticas (HSCs) em miofibroblastos além de induzir a síntese de colágeno de tipo I nestas células (LU & INSEL, 2014, TSOU et al., 2014).

Quando a doença crônica se estabelece, a capacidade de regeneração das células do parênquima é prejudicada e os hepatócitos mortos são substituídos por uma acumulação abundante da matriz extracelular, segregada principalmente pelas células estreladas hepáticas ativadas (miofibroblastos). Isto se deve ao fato de que os macrófagos têm uma capacidade para degradar a matriz cicatricial recentemente sintetizada através da secreção de metaloproteinases de matriz (MMPs), mas a atividade da protease é inibida pela produção concomitante de inibidores de tecidos de metaloproteinases (TIMPs) por miofibroblastos e macrófagos, o que resulta na acumulação excessiva de componentes da matriz extracelular (MEC), como colágeno e fibronectina, que vão progressivamente evoluir para fibrose em ponte e finalmente cirrose (PELLICORO et al., 2014).

Além das citocinas já mencionadas, outros mediadores pró-inflamatórios que são gerados por danos celulares e por estimulação celular dentro ambiente hepático.Dentre eles, podemos incluir o fator de crescimento derivado de plaquetas (PDGF), fator de crescimento do tecido conjuntivo (CTGF) e fator de crescimento do endotélio vascular (VEGF), cooperam para a proliferação dos miofibroblastos, para a formação de novos vasos e para proliferação compensatória de hepatócitos (Figura 07) (AOYAMA et al., 2010).

A cirrose está definida histologicamente como uma forma difusa do processo caracterizado por fibrose e a conversão da arquitetura normal do fígado em nódulos estruturalmente anormais (BATALLER; BRENNER,2005; BERARDIS et al.,2015).

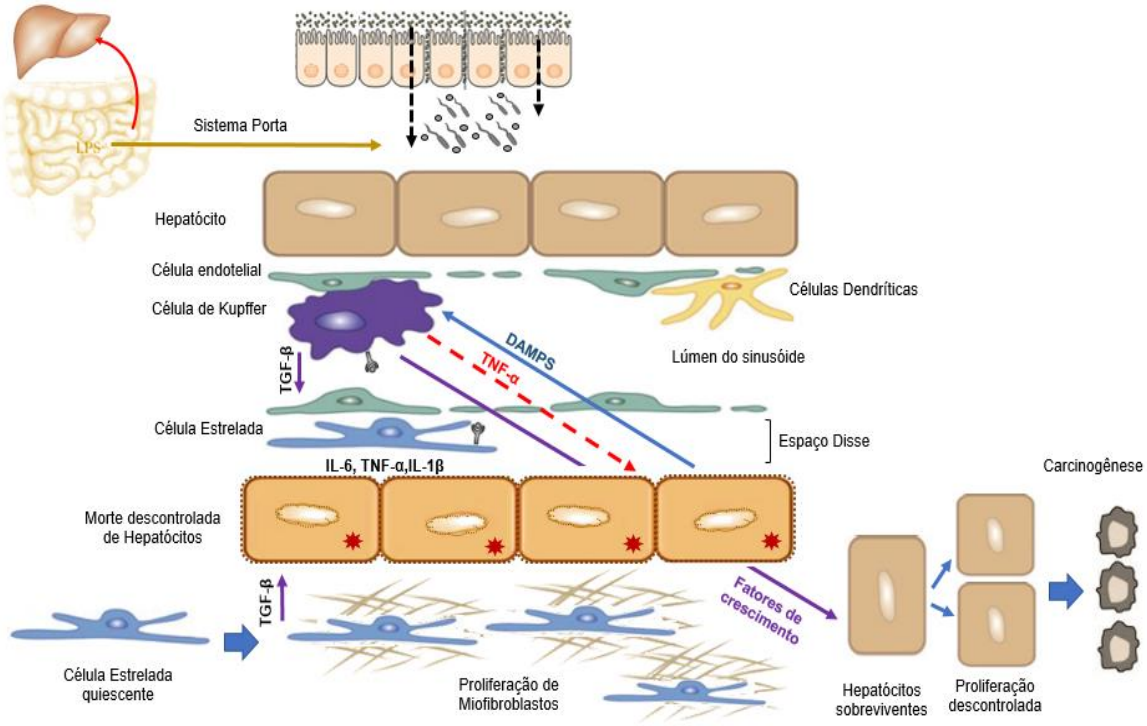

Figura 07: Cascata de sinalização de dano Hepático.
Fonte: Adaptado de AOYAMA et al., 2010; YU et al., 2017.

A progressão da doença hepática crônica aumenta a resistência intra-hepática ao fluxo e está associado à translocação bacteriana. A conexão anatômica através da veia porta se permite a exposição frequente a bactérias intestinais e produtos bacterianos e contribui para a ativação de TLR-4 nas células do fígado, como células de Kupffer e células estreladas hepáticas. Contudo, a resposta imune hepática é extremamente regulada para evitar uma resposta nociva em condições fisiológicas (SEKI et al., 2008; AOYAMA et al., 2010; YU; SCHWABLE,2017).

Curiosamente, níveis mais elevados de bactérias/PAMPs que translocam do intestino para a circulação portal em pacientes com doenças hepáticas e podem induzir o aumento da sinalização TLR, contribuindo para a progressão da doença hepática crônica para seu estágio final, o carcinoma hepatocelular. Esse fenômeno pode estar relacionado à diminuição da motilidade intestinal, ao crescimento excessivo de bactérias patogênicas, à inflamação do trato gastrointestinal, ao estresse oxidativo da mucosa e hipertensão portal (WEISS, et al., 2012; SCHWADE; JOBIN, 2013; MEHTA et al., 2014).

Outro receptor importante que também participa neste processo de evolução da doença é o TLR-9 que reconhece o DNA bacteriano rico em motivos CpG. Watanabe et al., (2007) demostraram que o DNA desnaturado de origem do hospedeiro proveniente de hepatócitos apoptóticos estimula HSCs através de TLR-9 na fibrose hepática.

Clinicamente, o carcinoma hepatocelular está fortemente associado à fibrose hepática e cirrose. Contudo, ainda não se sabe ao certo em que momento estes mecanismos podem propiciar o desenvolvimento do carcinoma hepatocelular.

1.8 Impacto do polimorfismo de nucleotídeo único (SNP) em genes de receptores do tipo toll (TLRs)

A interação do sistema imunológico com agentes infecciosos ocorre dinamicamente, com sofisticados mecanismos de controle da infecção. A pressão de seleção de patógenos leva a altas taxas consideráveis de polimorfismo genético nos TLR, e muitos deles afetam a função genética. Fato este que despertou um grande interesse pela comunidade científica em explorar as relações dos polimorfismos nos TLRs e os aspectos clínicos da doença. (SMIRNOVA et al., 2000; FERWEDA et al., 2007; COELHO-CASTELO et al., 2009; HOWELL et al.,2013).

No caso da hepatite, alguns polimorfismos nos genes dos TLRs são importantes no desfecho da doença. O receptor TLR-3, localizado no 4q35.1, reconhece RNA de cadeia dupla (dsRNA), enquanto o TLR-7 e TLR-8 reconhecem RNA de cadeia simples (ssRNA). O dsRNA é produzido pela maioria dos vírus durante a sua replicação e induz a síntese de interferon de tipo I (IFN-α/β), que exerce atividades antivirais e imunoestimuladoras (TAKEDA & AKIRA, 2005).

Vários estudos mostraram que as variações genéticas no gene *TLR3* estão associadas a suscetibilidade e/ou resistência a várias doenças infecciosas e podem estar relacionadas com a proliferação/angiogênese no desenvolvimento do hepatocarcinoma (HOWELL et al.,2013; YUAN et al., 2015). Al-Anazi et al., (2017), relataram que o SNP rs78726532 do *TLR3* estava fortemente associado à infecção pelo HCV no estudo caso-controle, na Arábia Saudita, enquanto o SNP rs5743314 com progressão da doença hepática devido ao HCV (cirrose e CHC). Sá et al., (2015) sugeriram que polimorfismos no gene *TLR3* (rs5743305 e rs3775291) podem estar associados a danos nas vias biliares durante o curso da infecção pelo HCV, contribuindo para o aumento do dano hepático.

O gene *TLR7* é expresso em compartimentos intracelulares e estão envolvidos na regulação da resposta imune inata através de cascatas de sinalização pro-inflamatória dependente de MyD88 (DU et al., 2000). Recentemente, Zhu et al., (2017) descreveram o impacto de três SNPs no *TLR7* envolvidos com a suscetibilidade e progressão da infecção pelo vírus da hepatite B em adultos chineses. Os três polimorfismos rs179010, rs2074109 e rs179009, localizados em Xp22.2, abrangendo três exons, não causam alterações nos aminoácidos, mas parecem influenciar a expressão de TLR-7 e resultam em diferenças na citocina, como o IFN-α, sugerindo que esses SNPs são funcionais. Yue et al., (2014) demonstraram que o genótipo GG de rs179009 estava associado a um risco aumentado de infecção pelo HCV entre mulheres chinesas e correlacionado com um nível mais baixo de IFN-α. Os IFNs são importantes para modular uma resposta imune efetiva dependente de T_H1 contra o vírus da hepatite C (YUE et al., 2014; ZHU et al.,2017).

Piñero et al., (2017) analisaram vários SNPs em genes TLRs e observaram que o rs4696480 (*TLR2*), rs4986790 (*TLR4*) e o rs187084 (*TLR9*) estavam associados a níveis séricos significativamente elevados de ácido lipoteicóico (LTA), lipopolissacarídeo (LPS) e DNA bacteriano. Além disso, os níveis séricos de TNF-α, IL-6 e óxido nítrico estavam significativamente diminuídos em todos os pacientes portadores dessas variantes. Esses SNPs também estão associados a um aumento no fator de circulação de antígenos e a uma diminuição na resposta pró-inflamatória em pacientes com cirrose. Esta imunodeficiência geneticamente derivada pode ter consequências na depuração do antígeno bacteriano e contribuir para as complicações clinicamente relevantes que são freqüentemente desenvolvidas em pacientes com cirrose (PIÑERO, 2017).

Indivíduos com doença hepática crônica avançada causada pela infecção do HCV e sob constante exposição à microbiota derivada do intestino podem levar à produção inapropriada de citocinas pró-inflamatórias, podendo evoluir para doença hepática grave associada à expressão anormal de TLR em células estreladas de hepatócitos e células de Kupffer (KIZILTAS et al., 2016).

Evidências crescentes sugerem que o rs4986790 *TLR4* leva a uma alteração de aminoácidos (*Asp299Gly*) alterando a estrutura da proteína podendo afetar a ligação dos ligantes TLR-4 à estabilidade da proteína e causar recrutamento deficiente de MYD88 e TRIF, mas eles não têm efeito sobre a expressão do receptor

TLR-4 (OTHO et al., 2012; FIGUEROA et al.,2014). Curiosamente, dois estudos mostraram uma hiporresponsividade imunológica à ligação de LPS em variantes de *TLR4* em monócitos / macrófagos humanos (HOLD et al., 2014; SCHMITT et al., 2002). Esses SNPs podem contribuir para o aumento do risco de infecções bacterianas e uma diminuição na resposta pró-inflamatória em pacientes com cirrose.

Vários estudos dedicaram-se a estudar possíveis associações entre os polimorfismos do gene *TLR9* e o risco de câncer (MOLLAKI et al., 2009; KUTIKHIN et al., 2011). De acordo com Novak et al., (2007) e Ng et al., (2010), o alelo T é transcrito de forma mais eficaz sob condições basais em comparação com o alelo C variante. Uma das possíveis conseqüências funcionais do SNP *TLR9 -1237T/C* é a adição de um sítio de ligação transcricional de NF-kB que pode levar ao aumento da produção de citocinas pró-inflamatórias.

Carvalho et al., (2011) mostraram que a IL-6 regula positivamente a expressão de TLR-9, levando a respostas celulares exacerbadas ao CpG-DNA, incluindo produção de IL-6 e proliferação de células B em células mononucleares portadoras do SNP *TLR9 -1237 T/C* (CARVALHO et al., 2011). Além disso, o aumento da atividade transcricional de *TLR9* em células mononucleares de pacientes portadores de SNP *TLR9 -1237 T/C* suporta um efeito funcional deste polimorfismo na suscetibilidade ao linfoma não-Hodgkin (CARVALHO et al., 2012). Roszak et. al., (2012) sugerem que o heterozigoto *TLR9 -1486C/T* pode ser um fator de risco genético para o câncer do colo do útero. Tao et al., (2007) confirmam que o alelo C do *TLR9 -1486C/T* SNP contribui para diminuir a expressão de TLR-9 e também está estreitamente correlacionado com o risco de lúpus eritematoso sistêmico (TAO et al., 2007; ROSZAK et al., 2012).

Sabe-se que os efeitos da infecção pelo HCV na sinalização de TLRs são complexos e que a supressão da sinalização de TLRs em células efetoras imunes podem favorecer a inibição da inflamação, a persistência viral e a infecção crônica. Evidências preliminares sugerem que os polimorfismos nos TLRs oferecem uma ferramenta em potencial para a predição dos resultados adversos relacionados ao HCV e outras infecções.

1.9 Tratamento da Hepatite C

O tratamento da Hepatite C tem como objetivo redução da atividade inflamatória nos hepatócitos por meio da interrupção da replicação do agente infecioso e está indicado para todos os pacientes, independentemente do estadiamento da fibrose hepática (BRASIL,2018).

Atualmente, o Sistema Único de Saúde (SUS) disponibilizou um arsenal terapêutico de fármacos, inibidor de protease que são capazes de inibir o complexo enzimático NS5A (daclatasvir/ombitasvir), análogo de nucleotídeo que inibe a polimerase do HCV (sofosbuvir), inibidor não nucleosídico da polimerase NS5B (dasabuvir), inibidor de protease NS3/NS4A (simeprevir/veruprevir) e o potencializador farmacocinético (ritonavir) (Brasil,2017). Estes agentes mostraram efeitos colaterais mínimos, boa eficácia, com tempo de tratamento menor e altas taxas de resposta virológica sustentada (RVS) (AHMED & FELMLEE, 2015).

No entanto, estudos recentes têm discutido o perfil de segurança dessas novas drogas. Vários estudos sugeriram um aumento na incidência e recorrência de carcinoma hepatocelular (CHC) em pacientes cirróticos que atingiram a RVS (STRAZZULLA et al., 2016; SYED et al., 2018; MAIDA et al., 2018). Existem indícios na literatura que o tratamento promove uma interrupção da vigilância imunológica durante a terapia antiviral com os DAAs e a persistência do processo inflamatório através da resposta do estresse de retículo endoplasmático e inibição da expresão da proteína p53, mesmo após o tratamento (AYDIN et al., 2017).

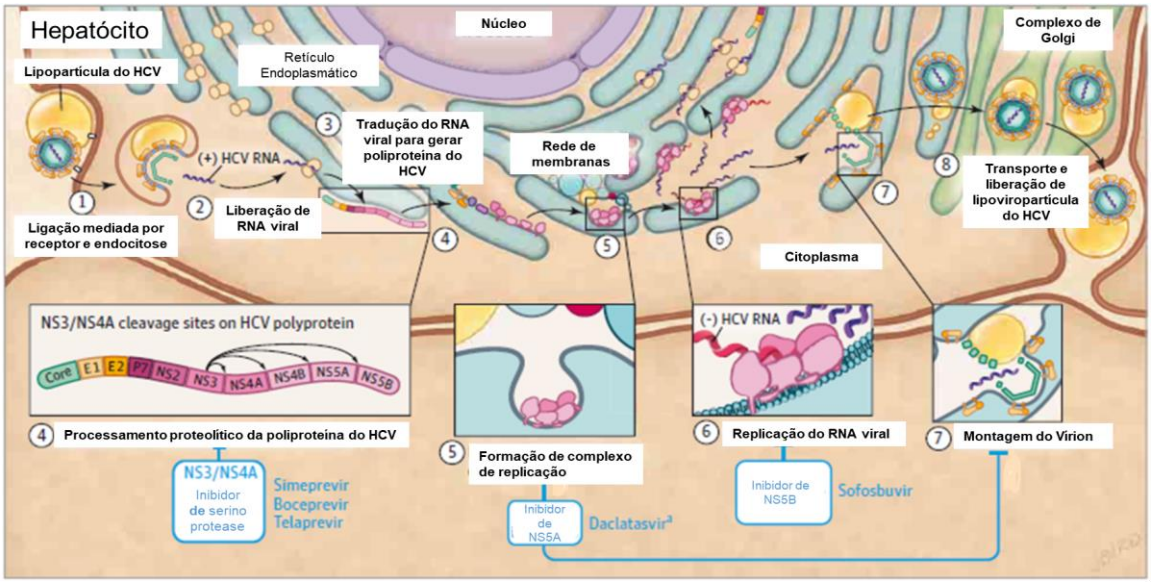

Figura 08: Etapas do ciclo de vida do vírus da hepatite C alvo de agentes antivirais de ação direta.
Fonte: Adaptado de KOLHI et al., 2014.

Além disso, estudos recentes demostram que há uma desregulação persistente de citocinas e quimiocinas (IP-10, MCP-1, MIP-1β, e IL-18) durante/pós tratamento (CARLIN et al.,2015; HENGST et al.,2016), bem como no perfil celular de $CD4^+/CD8^+$ (MEISSNER et al., 2017) e células T invariáveis associadas à Mucosa (HENGST et al.,2016).Observou-se também um aumento dos níveis de VEGF durante o tratamento (VILLANI et al.,2016) e uma hipercoagulabilidade transitória marcada pela elevação do fator VIII e pelo potencial de trombina endógena gerada durante o tratamento (RUSSO et al., 2018).

Outro fato importante a ser considerado é que essas populações que recebem DAAs têm, comumente, idade mais avançada e com uma porcentagem maior de fibrose avançada ou cirrose podendo contribuir para um estado inflamatório crônico acompanhado de alterações hormonais e adiposas (MICHAUD et al., 2013). Todos esses fatores juntos somados ao patrimônio genético que cada um carrega podem favorecer ao desenvolvimento do hepatocarcinoma mesmo depois de tratamento. Diferente do IFN exógeno que desempenha um papel protetor na incidência de CHC através do *clearance* viral, ação imunomodulatória, antiangiogênica e efeito antitumoral indireto, comparado aos DAAs, que erradicam o HCV sem controlar os clones neoplásicos e reduzindo a resposta anti-inflamatória (STRAZZULLA et al., 2016).

Diante do que foi exposto, decidimos avaliar a influência dos polimorfismos nos genes *TLR4* e *TLR9* em pacientes tratados com antivirais de ação direta e que atingiram resposta virológica sustentada.

Objetivos

3 OBJETIVOS

3.1 Objetivo Geral

- Descrever a influência dos polimorfismos nos genes dos receptores *TLR4* e *TLR9* e sua associação sobre o perfil de citocinas em pacientes com doença hepática crônica.

3.2 Objetivos Específicos

- Determinar a frequência dos polimorfismos encontrados, através da genotipagem nos genes dos receptores do tipo Toll *TLR4* (rs4986790 e rs4986791) e *TLR9* (rs187084 e rs5743836);
- Descrever os genótipos variantes circulantes do vírus da Hepatite C na população de pacientes estudados;
- Investigar a associação dos polimorfismos nos genes dos receptores do tipo Toll com o grau de fibrose hepática, relacionando as alterações laboratoriais e clínicas;
- Avaliar a relação desses polimorfismos dos receptores do tipo Toll sobre o perfil citocinas circulantes e em sobrenadante de cultura envolvidas na progressão da fibrose hepática após tratamento com antivirais de ação direta (DAAs).

Material e Métodos

4 MATERIAL E MÉTODOS

4.1 Modelo de Estudo

Foi realizado um estudo descritivo, do tipo caso-controle e transversal, retrospectivo realizado em pacientes diagnosticados com Hepatite C crônica, atendidos no ambulatório de Hepatopatia da Fundação de Medicina Tropical- Doutor Heitor Vieira Dourado (FMT-HVD).

O presente estudo constitui subprojeto de um projeto maior da linha de pesquisa sobre Hepatites do Grupo de Pesquisa “Imunologia básica e Aplicada”[3], realizado em pacientes com Hepatite C crônica atendidos no ambulatório de hepatites virais da (FMT-HVD) em Manaus, Amazonas.

4.2 População de estudo e critérios de inclusão e exclusão

Para a coleta das informações do objeto de pesquisa, foram utilizadas amostras biológicas de sangue total dos seguintes segmentos populacionais:

I) Foram incluídos no estudo pacientes diagnosticados com Hepatite C, advindos de qualquer localidade, e que procuraram atendimento na FMT-HVD, no período de dezembro de 2015 a julho de 2017, localizada na cidade de Manaus, Estado do Amazonas.

II) Para o grupo controle, foram selecionados candidatos a doadores de sangue da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM), de ambos os sexos, com idade mínima de 18 anos residentes em Manaus, que não apresentaram reatividade para Hepatite B (anti-HBc, HBsAg), HCV (anti-HCV), HIV (anti-HIV-1 e 2), confirmados por NAT-HBV/HCV/HIV, HTLV (anti-HTLV I e II), doença de Chagas (ELISA), sífilis (VDRL).

Foram excluídos pacientes que apresentaram co-infecção com os vírus da hepatite B (anti-HBc), vírus da imunodeficiência adquirida-HIV (anti-HIV 1 e 2), HTLV (anti-HTLV I e II), doença de Chagas (ELISA), sífilis (VDRL) pacientes que apresentaram quadro clínico-laboratorial de cirrose hepática descompensada, pacientes diabéticos descompensados, pacientes com relato de consumo diário de

[3] “*Identificação e Seleção de Peptídeos Miméticos Reativos aos soros de pacientes HCV⁺ e sua influência no desenvolvimento da fibrose hepática in vitro”*.

bebida alcoólica, pacientes com distúrbios psiquiátricos, pacientes renais crônicos e pacientes com síndrome plurimetabólica. Não foram incluídos grávidas e indígenas.

4.3 Participantes do Estudo

A amostragem foi composta por 206 candidatos a doadores de sangue e por 151 pacientes diagnosticados com Hepatite C e em acompanhamento pós-tratamento, coletados por demanda espontânea no Ambulatório de Hepatopatia da (FMT-HVD).

4.4 Critérios de Avaliação dos Pacientes infectados pelo vírus da Hepatite C com fibrose ausente ou inicial e fibrose avançada

Os pacientes infectados pelo vírus da Hepatite C foram avaliados pela equipe médica da FMT-HVD e foram classificados de acordo com a presença de doença hepática estabelecidos por meio da aplicação dos índices APRI (*AST to Platelet Ratio Index*) e FIB4 *(Fibrosis-4)* mediante a correlação dos resultados dos referidos índices com a escala Metavir.

4.5 Aspectos Éticos

As amostras utilizadas neste estudo foram obtidas a partir dos projetos aprovados pelos CEPs intitulados "*Identificação e Seleção de Peptídeos Miméticos Reativos aos soros de pacientes HCV*$^{+}$ *e sua influência no desenvolvimento da fibrose hepática in vitro*" e "*Avaliação do perfil Celular, humoral e molecular em doadores de sangue com sorologia reativa ao vírus da hepatite B (HBV) na Cidade de Manaus*", coordenados pela Dra. Adriana Malheiro Alle Marie e Dra. Laura Patrícia Viana Chaves, respectivamente. Vale ressaltar que do segundo projeto utilizamos apenas as amostras controles.

Ambos projetos foram aprovados pelo Comitê de Ética em Pesquisa da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas sob os respectivos números de CAAE: 49652815.8.0000.0009 e CAAE 0024.112.000-10.

4.6 Procedimentos

4.6.1 Coleta e conservação das amostras

As amostras analisadas neste estudo foram coletadas através de punção venosa. O volume total de 30 mL de sangue periférico foram distribuídos em dois tubos: 5 mL em tubo PPT (*Plasma Preparation Tube, Vacuntainer BD*) para procedimentos referentes à determinação dos polimorfismos das citocinas

propostas, 5 mL em um tubo sem anticoagulante e 4 tubos contendo heparina (BD Vacutainer® PST™**)** para realização de cultura de células mononucleares do sangue periférico, no qual foi reservado para dosagem de citocinas previstas pelos projetos.

As amostras foram transportadas ao Laboratório Multidisciplinar da Fundação HEMOAM, onde o soro e o plasma foram separados por centrifugação a 3.000 (rpm) por 5 minutos, e, juntamente com a fração celular, congelado à -80 °C. As etapas descritas neste estudo foram realizadas conforme o fluxograma abaixo (Figura 08).

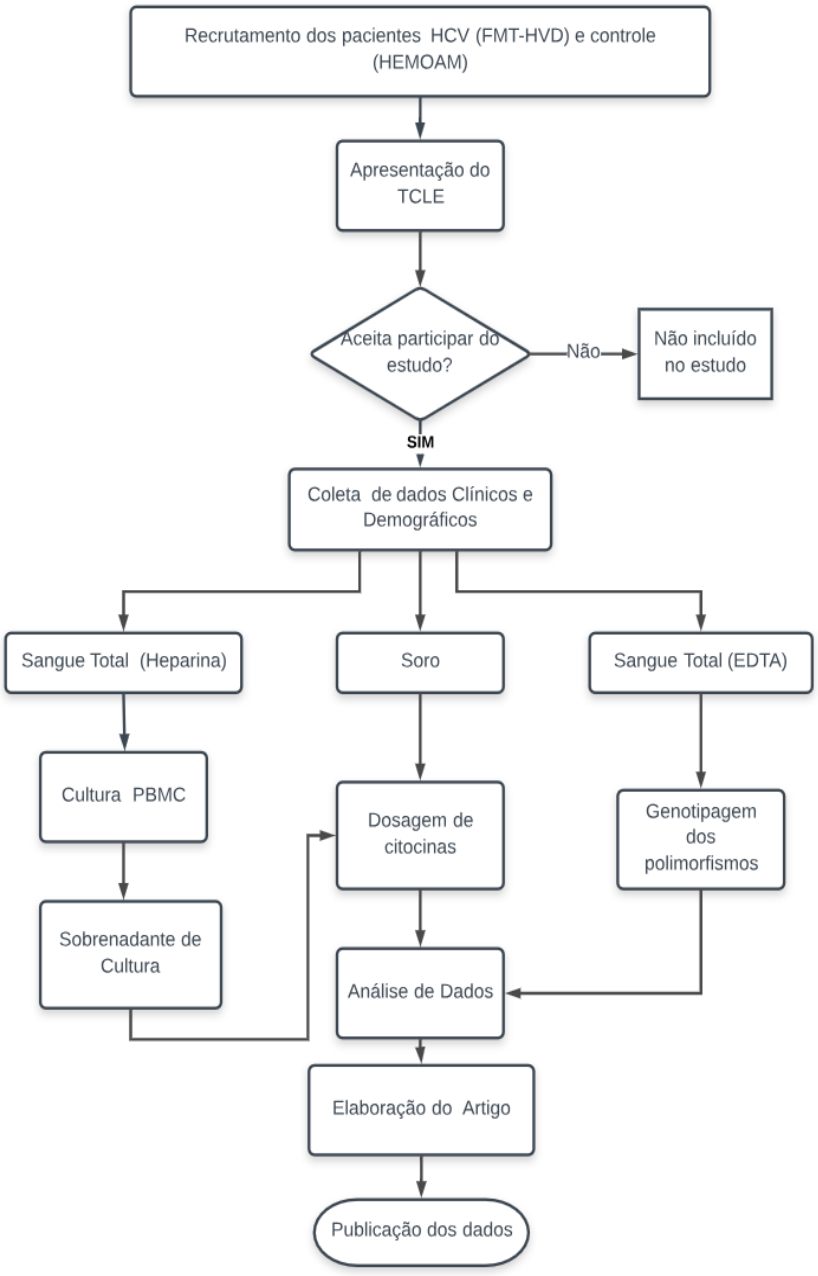

Figura 09: Fluxograma demonstrativo das etapas do projeto.

4.6.2 Extração do DNA

A extração de DNA genômico foi realizada com o kit comercial *QIAmp DNA kit* (*QIAGEN, Chatsworth, CA, USA*). A extração foi realizada segundo as recomendações descritas no protocolo do fabricante. Após a extração as amostras foram congeladas a -20°C até a execução da Reação em Cadeia da Polimerase (PCR) .

4.6.3 Genotipagem dos genes dos receptores do tipo Toll por Reação em Cadeia da Polimerase (PCR) associada à análise de polimorfismos por Fragmentos de Restrição (RFLP)

Os polimorfismos nos genes dos receptores do tipo Toll foram genotipados pela técnica de PCR-RFLP descritas por Leoratti et al., (2008), Ramasawmy et al., (2009) e Costa et al, (2017).

Resumidamente, a reação de PCR para cada SNP consistiu em 1 µL de DNA genômico (~20ng) adicionado a 24 µL de mix de PCR contendo 0,2 µL (2U) Platinum™ Taq polimerase (Thermo Fisher Scientific), 2,5 µL de tampão 10x (100 40 mmol/L Tris-HCl [pH 8,3] e 500 mmol/L de KCl), 1 µL de $MgCl_2$ (1,5 mmol/L), 1 µL de dNTPs (40 mmol/L), 0,5 µL de primers forward e reverse (0,25 pmol/L) e 18,3 µL de dH2O ultrapura. O processo de amplificação foi realizado no termociclador Veriti® 96 Well Thermal Cycler (Applied Biosystems, Carlsbad, USA). Em seguida, foi utilizado 10 µL de produto de PCR para a reação de restrição, adicionado de 5U da respectiva enzima de restrição (New England Biolabs, Ipswich, MA, EUA) diluída em tampão enzimático de acordo com as instruções do fabricante. Os primers, as condições de ciclagem para PCR e as endonucleases de restrição estão descritos na Tabela 01. Os fragmentos gerados pela reação de PCR-RFLP foram separados por eletroforese em um gel de agarose a 2% - 4% corado com GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, EUA) e visualizados em luz Ultravioleta (UV) no aparelho de fotodocumentação Molecular Imager Gel Doc™ XR (Bio-Rad Corporation, Hercules, CA, EUA), como demonstrado nas Figuras 10-13. Descrição dos genes, referência dos SNPs e primers utilizados na genotipagem estão demonstrados na tabela 01.

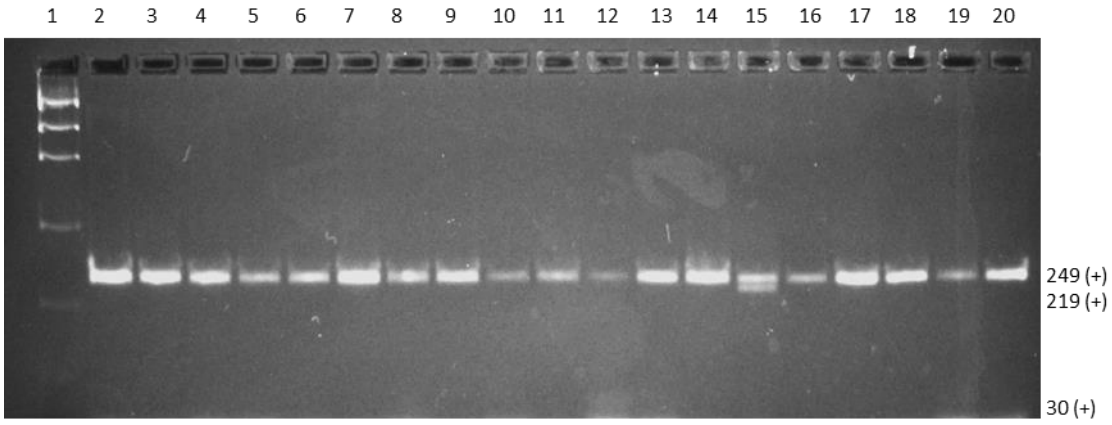

Figura 10: Análise do Polimorfismo TLR4 A299G em indivíduos com hepatite C por PCR-RFLP.Alelo selvagem do gene *TLR4 A299G* (249pb) e alelo variante (219 e 30pb). Poço 1: Ladder de 100pb ; Poço 2-14/ 16 -20: homozigoto selvagem (A/A); Poço 15 : Heterozigoto (A/G).

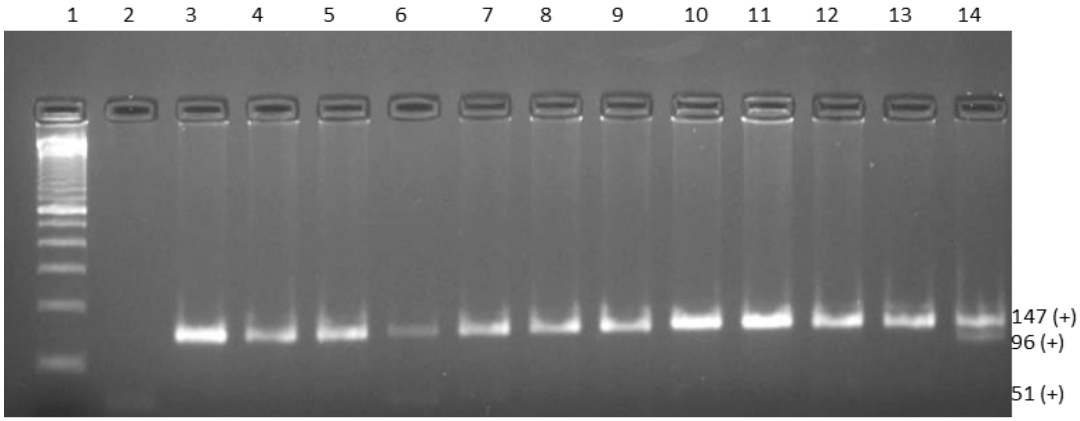

Figura 11: Análise do Polimorfismo TLR4 T399I em indivíduos com hepatite C por PCR-RFLP. Alelo selvagem do gene *TLR4 T399I* (147pb) e alelo variante (96 e 51pb) . Poço 1: Ladder de 100pb;Poço 2; controle negativo; Poço 3-13: homozigoto selvagem (C/C); Poço 14 : Heterozigoto (C/T).

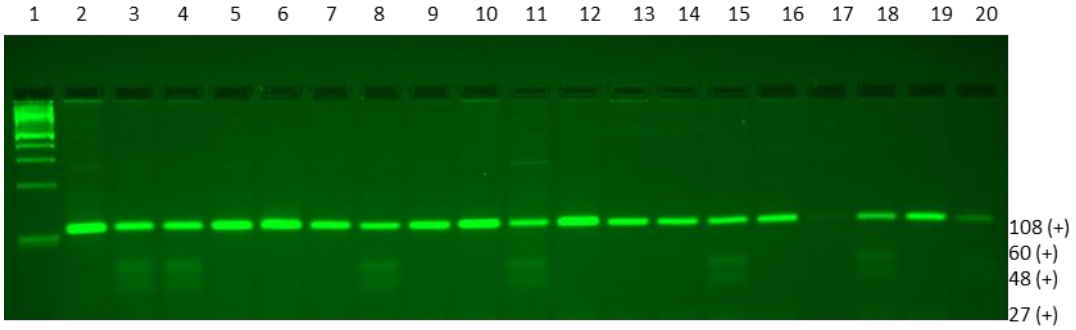

Figura 12: Análise do Polimorfismo TLR9 -1237C/T em indivíduos com hepatite C por PCR-RFLP.
Alelo selvagem do gene *TLR9 -1237C/T* (60,48 e 27pb) e alelo variante (108 e 27pb). Poço 1: Ladder de 100pb; Poço 2,5,6,7,9-10,12-14,15-16,19: homozigoto mutado (T/T); Poço 3,4,8,11,15,18e 20 : Heterozigoto (C/T).

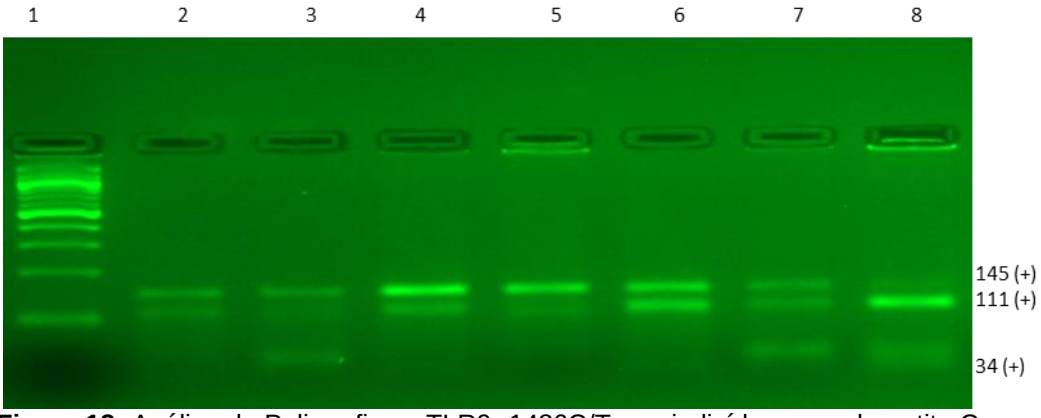

Figura 13: Análise do Polimorfismo TLR9 -1486C/T em indivíduos com hepatite C por PCR-RFLP. **Alelo selvagem do gene *TLR9 -1486C/T*(145pb), alelo variante (111 e 34pb) Poço 1: Peso molecular de 100pb; Poço 2-7: Heterozigoto (C/T). Poço 8: Homozigoto mutado (T/T).**

4.6.4 Isolamentos de células mononucleares do sangue periférico (PBMC) e cultura celular

O material coletado foi processado na Universidade Federal do Amazonas - Laboratório de Imunologia Celular (LIC), dentro da capela de fluxo laminar nível II, previamente esterilizado por 20 minutos sob a luz U.V. O sangue periférico obtido foi adicionado em um tubo falcon de 50 mL contendo Ficoll-Hypaque, submetido à centrífugação por 800g durante 20 minutos a 18 °C.

Após a centrífugação, a massa leucocitária formada por células mononucleares de sangue periferico foi transferida para um tubo falcon de 15 mL, onde foi acrescentado o volume de 15 mL de meio de cultivo RPMI não suplementado (Roswell Park Memorial Institute-1640- *Sigma-Aldrich*) e centrífugado por 250g por 10 minutos a 15º C. O sobrenadante foi desprezado e o *pellet* foi ressuspendido em 10 mL de RPMI (Sigma – Aldrich) e centrífugado novamente. Ao descartar o sobrenadante, as células foram ressupensas em 5 mL de RPMI suplementado (RPMI, 1% de estreptomicina + 10 % de soro bovino fetal). As células foram contadas em câmera de Neubauer utilizando a solução de Turk e para verificar a viabilidade celular, utilizamos o corante vital Azul de Tripan (GIGI et al.,2008).

O volume final de 4 mL de células mononucleares ($2x10^6$/mL) foram distribuídos em tubos de polipropileno/fosco de fundo em U com tampa. O tubo 1 foi preenchido com 500 µL de suspensão celular, 500 µL de RPMI completo (controle negativo). No no tubo 2 foi preenchido com 500 µL de suspensão celular, 500 µL RPMI completo, 100µL de endotoxina exógena (LPS/1µg/mL). Cada estímulo foi realizado em duplicata. Os tubos foram submetidos a um período de incubação de

12 horas em estufa a 5% de CO_2 a 37º C. Após a incubação foi realizada a coleta do sobrenadante da cultura para dosagem de citocinas utilizando o kit CBA e ELISA, e coleta das células para marcação com anticorpos específicos para citometria de fluxo.

Tabela 01: Descrição dos genes, referência dos SNPs e primers utilizados na genotipagem.

Polimorfismos	Iniciadores	Protocolo de PCR	Enzimas de Restrição	Alelos e Fragmentos (bp)
TLR4 A299G *(rs4986790)*	F: 5'-ATACTTAGACTACTACCTCCATG-3' R: 5'-AAACTCAAGGCTTGGTAGATC-3'	95°C for 5min, 35x (95°C for 30s, 56°C for 30s, 72°C for 30s), 72°C for 7min	*NcoI*	A: 259 G: 239+20
TLR4 T399I *(rs4986791)*	F: 5'-GCTGTTTTCAAAGTGATTTTGGGAGAA-3' R: 5'-CACTCATTTGTTTCAAATTGGAATG-3'	95°C for 5min, 35x (95°C for 30s, 60°C for 30s, 72°C for 45s), 72°C for 5min	*Hinf-I*	C: 147 T: 96+51
TLR9 -1237C/T *(rs5743836)*	F: 5'-CTGCTTGCAGTTGACTGTGT-3' R: 5'-ATGGGAGCAGAGACATAATGGA-3'	95°C for 5min, 40x (95°C for 30s, 59°C for 30s, 72°C for 45s), 72°C for 7min	*BstNI*	C: 108+27 T: 60+48+27
TLR9 -1486C/T *(rs187084)*	F: 5'-TATCGTCTTATTCCCCTGCTGGAATGT-3' R: 5'-TGCCCAGAGCTGACTGCTGG-3'	95°C for 5min, 40x (95°C for 30s, 59°C for 30s, 72°C for 30s), 72°C for 7min	*AflII*	C: 145 T: 111+34

4.6.5 Dosagem de Citocinas por *Cytometric Bead Array* (CBA)

A dosagem de citocinas das amostras de soro e sobrenadante de cultura dos pacientes e controles foram realizadas pela técnica de Citometria de Fluxo, utilizando o kit CBA (Cytometric Bead Array) com o Kit BDTM Human TH1/TH2/TH17 Cytokine (marca BD® Biosciences, San Diego, CA, USA), seguindo as orientações descritas pelo fabricante.

As citocinas quantificadas foram IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ e IL17A.O Kit BDTM CBA utiliza uma série de partículas (microesferas ou *beads*) de tamanho conhecido e com intensidade de fluorescência distinta para detectar simultaneamente, através de uma superfície de captura as várias citocinas solúveis. Cada *bead* de captura está conjugada com um anticorpo específico para cada citocina. A detecção das citocinas presentes na amostra foi realizada através de anticorpos conjugados ao fluorocromo ficoeritrina (PE) que fornecem um sinal fluorescente em proporção a quantidade de citocina da amostra ligada a bead. Os complexos formados de bead de captura+citocina da amostra + anticorpo de detecção foram quantificados através da Citometria de Fluxo. A intensidade da Fluorescência PE de cada complexo revela a concentração em pg/mL de cada citocina. Para a aquisição das amostras foi utilizado o Citômetro de Fluxo FACSCanto II (Becton, Dickinson and Company, San Jose, CA, USA) da Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM).

Para o cálculo das concentrações em pg/mL e Intensidade Média de Fluorescência (MFI) de cada citocina foi utilizado o software FCAP-ArrayTM (v3.0.1).

4.6.6 Análise dos Resultados

A comparação entre os grupos foi realizada com o teste qui-quadrado (χ^2) ou exato de Fisher com intervalo de confiança de 95% [IC]. O equilíbrio de Hardy-Weinberg (HWE) foi determinado pela comparação da freqüência do número de genótipos observados e esperados. Os testes para o equilíbrio de Hardy-Weinberg foram realizados por uma aplicação online (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl). O software Graphpad Prism v.5 (San Diego, CA, EUA) foi utilizado para análises comparativas de citocinas entre os genótipos combinados dos SNPs estudados. As variáveis contínuas apresentaram distribuição não normal e os dados foram analisados com teste não paramétrico de Mann-Whitney para comparar os pares.

O teste de correlação de Spearman foi realizado para avaliar a associação entre os níveis de cada citocina testada. As correlações positivas e negativas são consideradas significativas quando $p < 0,05$. O índice de correlação (r) foi utilizado para categorizar a força de correlação como fraca ($r \leq 0,35$), moderada ($r \geq 0,36$ a $r \leq 0,67$) ou forte ($r \geq 0,68$), como descrito anteriormente (TAYLOR,1990). Redes foram montadas para avaliar as associações entre as citocinas circulantes. Correlações significativas foram compiladas usando o software de acesso aberto Cytoscape v3.3 (Cytoscape Consortium, San Diego, CA), conforme relatado anteriormente (SOUZA-CRUZ et. al, 2016; COSTA et al., 2018). A significância estatística foi considerada em todos os casos com $p < 0,05$.

Resultados e Discussão

Capítulo 1

5 RESULTADOS E DISCUSSÃO

CAPÍTULO I:

Case Report: Sofosbuvir and daclatasvir combination therapy for current hepatitis C virus genotype 4 achieves SVR: a case report of HCV genotype 4 from the Amazon.

RELATO DE CASO PUBLICADO NA REVISTA DA SOCIEDADE BRASILEIRA DE MEDICINA TROPICAL 50(6):861-863, November-December, 2017
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Abstract

Hepatitis C is a worldwide endemic disease. However, hepatitis C virus genotype 4 (HCV GT-4) has rarely been reported in Brazil. HCV GT-4 demonstrates high sustained virological response (SVR). Here, we report the case of a 62-year-old HCV GT-4 positive woman complaining of a headache, nausea, and arthralgia. The patient was treated according to the protocol for genotype 4 (12 weeks administration of 400mg sofosbuvir and 60mg daclatasvir daily) and achieved SVR. Although this is not an Amazonas autochthonous case, the presence of genotype 4 is rarely reported in the region.

Keywords: Hepatitis C. Genotype 4. Sustained viral response.

Introduction

According to the World Health Organization (WHO), hepatitis C virus (HCV) affects millions of people worldwide;it is estimated that approximately 71 million people are chronically infected with HCV, causing a serious global public health problem[1].

There are seven HCV genotypes including 67 subtypes, which are geographically distributed throughout the world2. In Brazil, the genotype distribution pattern consists of genotype 1, followed by genotypes 3, 2, 4, and 5[3-4].

The treatment recommended by the Brazilian Ministry of Health for chronic HCV infection involves oral combinations of direct-acting antivirals (DAAs); sofosbuvir in association with simeprevir or daclatasvir. These drug combinations are well tolerated and have been reported to increase sustained virological response (SVR) rates to approximately 90%[5-7].

Case Report

A 62-year-old woman was admitted to the *Fundação de Medicina Tropical Doutor Heitor Vieira Dourado* (FMT-HVD) in November 2015. Upon admission, the patient complained of a headache, nausea, and an extrahepatic manifestation of neurological motor impairment. The patient had received blood transfusion eight years prior in Alenquer, during an upper digestive endoscopy procedure. Follow-up tests prior to treatment are detailed in **Table 1**. The patient was treated according to the protocol for genotype 4 (12 weeks administration of 400mg sofosbuvir and 60mg daclatasvir daily); SVR was achieved and hepatic markers values returned to normal six months after the end of treatment (**Table 1**).

Discussion

Hepatitis C virus genotype 4 (HCV GT-4) represents approximately 15 18 million cases of the total global HCV infections. It is prevalent in lower income countries in Northern and Equatorial Africa, the Middle East, and Caribbean and Indian regions[8,9].

The frequency of the geographical distribution of the genotypes in the Amazon region is similar to the pattern in Brazil and other world regions; a higher prevalence of genotypes 1 (64.9%) and 3 (30.2%) and a lower prevalence of genotypes 2 (4.6%), 4 (0.2%), and 5 (0.1%)3. However, genotype 4 is rarely reported in the State of Amazonas[10].

The current global distribution of HCV genotypes has undoubtedly been infl uenced by historical events and modified by contemporary human migration trends. Although genotype 1 has been established as the most prevalent genotype worldwide, including in the Amazon region, we must be aware of the introduction of other genotypes into the region.

Table 01: Laboratory tests test results

Laboratory tests	Admission	Post -Treatment**	Reference values
Hemoglobin	12.9 g/dL	13.73 g/dL	12 g/dL -18 g/dL
Hematocrit	39.8 %	41.1%	37%-52%
Platelets	196.000 /mm^3	239.000/mm^3	130.000/mm^3-400.000/mm^3
Leukocytes	6190 /mm^3	6340/mm^3	5.2 /mm^3-12.4/mm^3
Albumin	5.1 g/dL	5.0 g/dL	3.5 - 5.0 g/dL
INR	1.13	1.12	1.0
TGO/AST	81 IU/L	20 IU/L	2 IU/L – 38 IU/L
TGP/ALT	116 IU/L	20 IU/L	2 IU/L - 44 IU/L
HCV-RNA	541978 (Log= 5.73)	undetectable	12 IU/ mL (Log=1.08) – 100 $_X$ 10^6 IU/mL (Log=8)
FIB-4*	2,38	1,20	FIB 4 > 3,25 (≥ F2)
Child pugh score	A5	A5	A-C

INR:international normalized ratio; TGO/AST: transaminase glutamic-oxalacetic/aspartate aminotransferase; TGP/ALT: transaminase glutamic-pyruvic/alanine aminotransferase; HCV-RNA: hepatitis C virus- ribonucleic acid; IU/mL: international units/milliliter; FIB-4: index for liver fi brosis; APRI: AST-to-platelet ratio index;METAVIR: score that quantify the degree of infl ammation and fi brosis using the results of a liver biopsy. *Post -Treatment tests were performed six months after the end of treatment. **The treatment is indicated for HCV mono infected patients with APRI > 1.5 or FIB-4 > 3.25, characterizing METAVIR ≥F2. HCV.

In this case, the patient is a native of Tracuateua, a small City in Pará, in the Amazon region; however, she lives in Alenquer, another city in Pará closer to Manaus. Thus, this is not an autochthonous case as the patient temporarily moved to Manaus for HCV treatment. Migratory fl ow may contribute to the dissemination of genotype 4 originating from other states in the region into Amazonas via virus carriers prior to SVR.

The origin of the region of Tracuateua is linked to the construction of the Belém-Bragança railroad (completed in 1908), during which many African descent, Portuguese, and Spanish immigrants began to colonize the vicinity. Although this migratory phenomenon is not recent, it has been increasing; thus, new and unusual genotypes may be more prevalent than thought[11].

Because of the low prevalence of HCV GT-4 in Brazil, this genotype has not been studied extensively in prospective trials evaluating treatment outcomes. The lack of genotype 4 in our samples indicates that it is absent or rarely present in our population. Because many people in Bahia are of African descent and Brazil is a

country with continental proportions, it is possible that uncommon Brazilian HCV strains in other regions of Brazil may be the result of multiple introductions of genotype 4[12].

Therefore, we believe that HCV patient numbers are inexact in some municipalities of the state of Amazonas, a poor region of the country affected by this endemic disease, where the only public access to advanced technology for diagnosis and treatment is at the FMT-HVD and Araújo Lima Ambulatory, the Federal University of Amazonas. In addition, the natural history of hepatitis C and the limited education of the population in some of these municipalities hinder patient notifi cation and treatment.

This case report may contribute to demonstrate that there is HCV GT-4 in the North of Brazil and these individuals carrying this genotype have developed response to the DAAs.

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Conflict of Interest

The authors declare that there is no confl ict of of interest.

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Capítulo 2

CAPÍTULO II:

REVISÃO DA LITERATURA NA FORMA DE ARTIGO CIENTÍFICO
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Variants of toll-like receptors in the development of liver fibrosis: the importance of genetic background in the clinical management of patients with hepatitis c in the new era of interferon-free terapy.

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Abstract

Hepatitis C is a public health problem and affects approximately 3% of the world's population. HCV infection has a wide spectrum of clinical manifestations that ranges from spontaneous clearance in the period of six months after infection of the virus to the development of chronic hepatitis C, liver cirrhosis hepatic decompensation, hepatocellular carcinoma and death. Several variants in the genes of the toll-like receptors are cited to influence the clinical outcomes of HCV infection. This minireview describes the influence of polymorphisms of toll-like receptors in the development of liver fibrosis associated with Hepatitis C virus and highlights the importance of genetic background in the new era of interferon- free treatment.

Keywords: Hepatitis C, fibrosis, immune response, Toll -like receptors, polymorphisms, genetic background

Introduction

Hepatitis C is a liver disease caused by the hepatitis C virus (HCV). HCV can cause acute or chronic hepatitis. HCV infection has a wide spectrum of clinical manifestations that ranges from spontaneous clearance of the virus to the development of chronic hepatitis C, liver cirrhosis, hepatocellular carcinoma and to severe liver disease leading to death. It is estimated that nearly 71 million people have chronic infection and 399 000 people die each year from hepatitis C, mainly cirrhosis and hepatocellular carcinoma[1].

The hepatitis C usually presents asymptomatic or oligosymptomatic [2], which makes early diagnosis difficult and reduces the chances of treating infected individuals at the onset of infection. Immunological reactions underlying the fibrosis process mediate the course of the disease that can lead to dysregulated organ architecture and function [3-4].

Innate immunity is the host's first line of defense in response to pathogens and orchestrate the innate immune response and the subsequent adaptive immune response to keep in check the pathogen. The innate immune response needs to strike an appropriate balance, resulting in an inflammatory response sufficient to controll the infection and preserve the integrity of the tissue [5]. Toll-like receptors (TLRs) recognize invading pathogens through distinct pathogens associated molecular patterns (PAMPs) and play a key role in the innate immunity of the host. In humans, of the 10 functional TLRs described, six of them recognize different molecules present in HCV that are capable of stimulating TLR signaling. TLR- 2,3, 4 and 7- 9 play important role in viral immune clearance through activation of transcription factors and the production of proinflammatory cytokines. An excess activation of intracellular signaling pathways via TLRs may lead to high levels of proinflammatory mediators that may contribute to the host tissue damage [6-8]. The host-pathogen interaction is complex and dependent on the host's genetic makeup and the virus genotype and depending on the intensity of the immune response, it either can promote a favorable immune response for healing or for the development of liver disease. Of all the patients with the chronic form of the disease (60-80%), only 15-30% progress to the severe form in 20 years [1]. This minireview outlines recent advances made in the understanding of the mechanisms underlying liver

inflammation in HCV infection and how the genetic background of the host may contribute to the progression of the disease or spontaneous clearance of the virus.

Innate immune Mechanisms in development of Hepatic fibrosis

In HCV infection, the acute and chronic liver injury promote the activation of proinflammatory response. The release of inflammatory cytokines together with the recruitment of leucocytes to the site of injury contributes to an unbalance in the process of remodeling of the components of extracellular matrix (EMC) that, progressively, leads to a septum formation and rings of scar that surround nodules of hepatocytes [9].

The immune response in HCV infection initiates through the activation of TLRs by viral proteins (Core and non-structural proteins) and HCV-RNA that trigger a cascade of signaling leading to the activation of transcription factors, such as Interferon regulatory factor 3/7 (IRF3/7) and Nuclear factor kappa B (NF-kB). IRF3/7 transcribe IFN type I (IFN-α and β) target genes to inhibit viral replication and NFk-B transcribes the proinflammatory cytokines (TNF-α, IL-12, IL-1 and IL-6), chemokines (CCL2 and CXCL8), endothelial adhesion molecules and costimulatory molecules [10-11].

HCV Core and non-structural protein 3 (NS3) are recognized by TLR2 associated with TLR1/TLR6 in peripheral blood mononuclear cells [12]. NS5A activates TLR4 and induces IFN-β production in infected cells and surrounding tissue [6]. HCV-RNA stimulates TLR3, 7/8 and 9 to impede the HCV replication, reducing HCV-RNA levels and the intensity of the inflammatory process [12-13].

TLRs have different effects upon TLR pathway stimulation in various cellular compartments. The activation of TLR2, TLR3 and TLR4 in monocytes, dendritic cells, Hepatocytes, Kupffer cells and endothelial cells promote upregulation of inflammatory cells into the liver [6]. The TLRs activation induces CD4+T cells to polarize to a Th1 profile through the activation of Natural Killer cells, Natural killer T cell and CD8+T cells, essential for HCV elimination in infected hepatocytes [14].

The humoral immune response is essential for the neutralization of viral particles of HCV. Specific antibodies are detectable in the serum of infected individuals after the appearance of the cellular immune response and remain throughout the course of chronic infection. However, its actions in chronic individuals appear to have no effect against viral particles [15].

Despite the existence of neutralizing antibodies to the HCV envelope E2 glycoprotein, specific antibodies are inefficient due to the rapid selection of variants result of virus scape mutation, promoting failure in recognition and viral elimination by cells [16-17]

HCV interferes in several signaling pathways to escape the antiviral immune responses. The core protein interferes in the transit pathway of transducers and transcription activators (JAK-STAT) by the so-called cytokine-3 signaling (SOCS-3) [18]. HCV E2 protein was reported to bind to the CD-81 receptor acting on NK cells and to inhibit its actions of cytotoxicity and IFN production through the protein kinase (PKR) receptor [19]. NS3/4A protease promotes blockade of the activation of the retinoic acid induction gene I (RIG-1) and Toll receptor proteins (TLR-3). It was further observed that this protease is host, including a mitochondrial antiviral signaling protein (MAVS) and the TIR domain containing interferon-β adapter-inducer (TRIF), downregulate IRF-3/7 activation and induction of IFN [20-21]

HCV viral persistence leads to liver damage through continuous production of proinflammatory cytokines. To counter balance the exacerbating response, T regulatory cells play a central role by suppressing the excessive immune activity through the expression of anti-inflammatory cytokines such as TGF-β1 and IL-10 [22-24]. Of note, TGF-β1 is a strong inducer of fibrogenesis, which regulates α-smooth muscle actin (αSMA) and induces collagen synthesis by hepatic stellate cells (HSCs) [25-26].

Once the chronic disease is established, the regeneration capacity of the parenchyma cells is impaired, and the dead hepatocytes are replaced by an abundant accumulation of the extracellular matrix, secreted mainly by the activated liver stellate cells (myofibroblasts). The excessive accumulation of components of extracellular matrix (ECM), such as collagen (I, III and IV) fibronectin, elastin, laminin, hyaluronan and proteoglycans leads to the development of fibrosis[5].

The continuous activation of the hepatic stellate cells leads to collagen fibers deposition that transform the normal liver architecture into structurally abnormal nodules [25-27]. This process results from the concomitant secretion of matrix metalloproteinase (MMPs) by macrophages and tissue inhibitor metalloproteinases (TIMPs) by both macrophages and myofibroblast. Normally, MMPs degrade the newly synthesized scar matrix, but as its protease activity is inhibited by the concomitant production of TIMP, there is an excessive accumulation of extracellular

matrix (ECM) components, such as collagen and fibronectin, that may gradually progress to cirrhosis [28-29].

In addition to the cytokines, other proinflammatory mediators such as growth factors including platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF) vascular endothelial growth factor (VEGF), cooperate for the proliferation of myofibroblasts, for the formation of new vessels and for compensatory proliferation of hepatocytes are generated due to cellular damage and stimulate the immune cells [30].

The continuous fibrotic process stablished in cirrhosis rises due to intrahepatic resistance to flow and is associated with bacterial translocation. The anatomical connection via the portal vein exposes itself frequently to intestinal bacteria and bacterial products that contributes to the activation of TLRs in liver cells. The gut permeability and gut microbiota may contribute to the progression of the chronic liver disease, hepatocellular carcinoma [31-33].

Interestingly, higher levels of bacteria/ molecular patterns associated with a microorganism (MAMPs) translocate from the gut and portal circulation in patients with cirrhosis, compared with healthy individuals, may induce increased TLR4 signaling and may contribute to the progression of chronic liver disease. This phenomenon may be related to decreased intestinal motility, overgrowth of intestinal bacteria, oxidative stress of the mucosa, intestinal inflammation, portal hypertension and/or compromised tight junctions [34-35].

Another important receptor that also participates in this process of disease evolution is TLR-9 that recognizes the cpG-rich DNA of bacteria. Watanabe et al., 2007 demonstrated that host-derived denatured DNA from apoptotic hepatocytes stimulates HSCs through TLR-9 in hepatic fibrosis [35]. Clinically, hepatocellular carcinoma is strongly associated with liver fibrosis and cirrhosis. However, it is not yet known at what point these mechanisms may promote the development of hepatocellular carcinoma [36].

Impact of single-nucleotide polymorphisms in TLRs genes and clinical outcome prediction

The interaction of the immune system with infectious agents is a dynamic process, with sophisticated mechanisms of infection control and escape [3]. Several studies have explored the relationships between TLR polymorphisms in human populations and infectious diseases [34-37].

In case of hepatitis, some polymorphisms in TLRs genes are important on the outcome of the disease. TLR3 recognize double-stranded RNA (dsRNA), while bothTLR7 and TLR8 recognize single-stranded RNA (ssRNA). The dsRNA is produced by most viruses during their replication and induces the synthesis of interferon type I (IFN-α/β), which exert antiviral and immunostimulatory activities [38]. Several studies have shown that genetic variations in TLR3 gene are associated with susceptibility and/or resistance to various infectious diseases and may be related to proliferation/angiogenesis in the development of hepatocellular carcinoma [39-43].Al-Anazi et al., (2017) reported that the SNP rs78726532 of TLR3 was strongly associated with HCV infection in case-control study, in Saudi Arabia, while the SNP rs5743314 with progression of liver disease due to HCV (cirrhosis and HCC) [44].Sá et al., (2015) suggested that polymorphisms in the TLR3 gene (rs5743305 and rs3775291) might be associated to biliary damage during the course of HCV infection contributing to increased liver damage [45].

Recently, Zhu et al., (2017) described the impact of three TLR7 SNPs on susceptibility and disease progression of Hepatitis B virus infection in Chinese adults. The three polymorphisms rs179010, rs2074109 and rs179009, is located at Xp22.2, spanning three exons, do not cause change in amino acids but appear to influence TLR7 expression and result in difference in cytokine, such as IFN-α, suggesting that these SNPs are functional [46]. Yue et al. (2014), demonstrated that the GG genotype of rs179009 was associated with an increased risk of HCV infection among Chinese women and correlated to a lower level of IFN-α. The IFNs are important to modulate an effective Th1 -dependent immune response against hepatitis virus [47].

Individuals with chronic HCV advanced liver disease and under constant exposure to gut-derived microbiota may lead to inappropriate production of proinflammatory cytokines may progress to severe liver disease associated with abnormal TLR expression in hepatocytes stellated cells and Kupffer cells [48].

Increasing evidences suggests that the rs4986790 of TLR4 that leads to an amino acid change (Asp299Gly) altering the protein structure may affect the binding of the TLR4 ligands the protein stability and cause deficient recruitment of MYD88 and TRIF, but they have no effect on TLR4 receptor expression [49-50]. Interestingly two studies showed an immunological hyporesponsiveness to the binding of LPS in TLR4 variants in human monocyte/macrophages [51-52]. These SNPs might contribute

to the increase risk of bacterial infections and a decrease in the proinflammatory response in patients with cirrhosis.

Piñero et al., (2017) analyzed several SNPs in TLRs genes and observed that the TLR-2 rs4696480, TLR-4 rs4986790 and TLR-9 rs187084 were associated with significantly elevated serum levels of lipoteichoic acid (LTA), lipopolysaccharide (LPS) and bacterial DNA [39]. Furthermore, serum levels of TNF-α, IL-6 and nitric oxide were significantly decreased in all patient carriers of these variants. These SNPs are also associated with an increase in the antigen circulation factor and a decrease in the proinflammatory response in patients with cirrhosis. This genetically derived immunodeficiency may have consequences in the clearance of bacterial antigen and contribute to the clinically relevant complications that are frequently developed in patients with cirrhosis [53-56].

Several studies devoted to possible associations between *TLR9* gene polymorphisms and cancer risk [57-58]. According to Novak et al.,2007 and Ng et al.,2010, agree that the T allele is transcribed more effectively under basal conditions compared to the variant C allele. Possible functional consequences of the TLR9 -1237T/C SNP an additional NF-kB transcriptional binding site that may lead to increased production of proinflammatory cytokines [59-60].

Carvalho et al., (2011) showed that IL-6 up-regulates TLR9 expression, leading to exacerbated cellular responses to CpG- DNA, including IL-6 production and B-cell proliferation in mononuclear cells carrying the TLR9 -1237T/C SNP [61]. Further, the increased transcriptional activity of TLR9 in mononuclear cells from patients harboring TLR9 -1237T/C SNP supports a functional effect of this polymorphism on non-Hodgkin lymphoma susceptibility [62]. Roszak et. al.,2012 suggests that TLR9 -1486C/T heterozygote may be a genetic risk factor for cervical cancer [63]. Tao et al., 2007 confirm that the C allele of TLR9 -1486C/T SNP contribute to down regulate TLR9 expression and is also closely correlated with the risk of systemic lupus erythematosus [64].

Tumor Risk during Anti-HCV Treatment: the new era of all-oral interferon-free treatment

The incorporation of direct-acting antivirals (DAAs) in the treatment of chronic hepatitis C has been a remarkable transformation in next-generation DAAs. These agents have shown minimal side effects, good efficacy with shortened courses of treatment and high sustained viral response (SVR) rates. DAAs inhibit HCV

replication by targeting the virus encoded non-structural proteins, NS3-4A, NS5A and NS5B [65].

However, recent studies have argued the safety profile of these new drugs. Several studies have suggested an increased incidence of hepatocellular carcinoma (HCC) occurrence and recurrent HCC in cirrhotic patients that achieved SVR [66-68].

A proposed hypothesis is the disruption of immune surveillance during DAAs antiviral therapy and the persistence of the inflammatory process through the ER-stress response even after treatment [69-76]. A summary of these immunological dysregulation is outlined in Table 1.

In fact, populations receiving DAAs are commonly older and with a higher percentage of advanced fibrosis or cirrhosis. This different background may contribute to a chronic inflammatory state accompanied by hormonal and adipose changes [77]. Moreover, exogenous IFN plays a protective role in HCC incidence through viral clearance, immunomodulatory, antiangiogenic, and antitumoral actions indirect effect, compare to DAAs, that eradicate HCV without controlling neoplastic clones [67] and reducing the anti-inflammatory response.

The genetic background of the host may contribute to the progression of the disease in the presence of the inflammatory process after virologic immune response achieved by DAAs protocol. The discovery of the free interferon therapy pro-oncogenic effects and the polymorphisms associated with the risk of developing cancer may help to better select patients for treatment of HCV, taking into consideration the current high cost of the all-oral regimen and the favorable prognosis. Patients with advanced liver disease may be candidates for closer surveillance.

Table 01: Immunological dysregulation induced by DAAs.

Autor, year, ref	Treatment combination	Type study	Effect
Carlin et al.,2015[69]	Sofosbovir + Ribavirin	case-control study	Temporal dynamics of IP-10, MCP-1, MIP-1β, and IL-18 during HCV infection and treatment.
Hengst et al.,2016[70]	Sofosbovir + Ribavirin	case-control study	Temporal dynamics of IP-10, MIP-1β, and IL-18 during HCV infection and treatment.
Hengst et al.,2016[71]	Sofosbovir + Ribavirin	cohort study/ *in vitro*	MAIT cell-dysfunction
Villani et al.,2016[72]	DAA +Ribavirin	cohort study/ *in vitro*	Increase serum VEGF Level during the treatment
Meissner et al.,2017[73]	ledipasvir/sofosbuvir ledipasvir/sofosbuvir + GS-9669 ledipasvir/sofosbuvir + GS-9451	cohort study	$CD4^+/CD8^+$ population increase, but not $CD4^+/CD8^+(HLADR^+/CD38^+)$ population; IP-10 increased
Russo et al.,2018[74]	SOF+RBV, SOF+Ledipasvir+RBV, SOF+ Daclatasvir+ RBV, Ombitasvir + Paritaprevir + Ritonavir+3D + RBV.	cohort study	FVIII decrease, PC and EPT increase at the end of treatment
Olveira et al., 2018[75]	DAA +Ribavirin	cohort study	Persistent alteration of liver tests after SVR.
Aydin et al.,2017[76]	ledipasvir+ sofosbuvir	*In vitro*	Decreased expression of p53 in cirrhotic liver

IP-10: inducible protein-10; MCP-1: monocyte chemotactic protein-1, MIP-1β: macrophage inflammatory protein-1 beta, IL-18: interleukin -18, FVIII: Factor VIII; PC: Protein C and EPT: Endogenous thrombin potential;

Conclusion

HCV infection on TLR signaling is complex. The suppression of TLR signaling in immune effector cells may favor an inhibition of inflammation, viral persistence and chronic infection. Preliminary evidence suggests that polymorphisms in TLRs are a potential tool for predicting adverse outcomes related to HCV infection and bacterial – derived complications of cirrhosis. This may suggest that there is a decrease in the innate immune response in these patients with variants of TLRs. In our opinion, the study of patients' genetic background ally the age of the patient, stage of liver disease in the new era of the DAAs, may be useful to predict the different outcomes, especially those who have advanced chronic liver disease treated with DAAs (Figure 01).

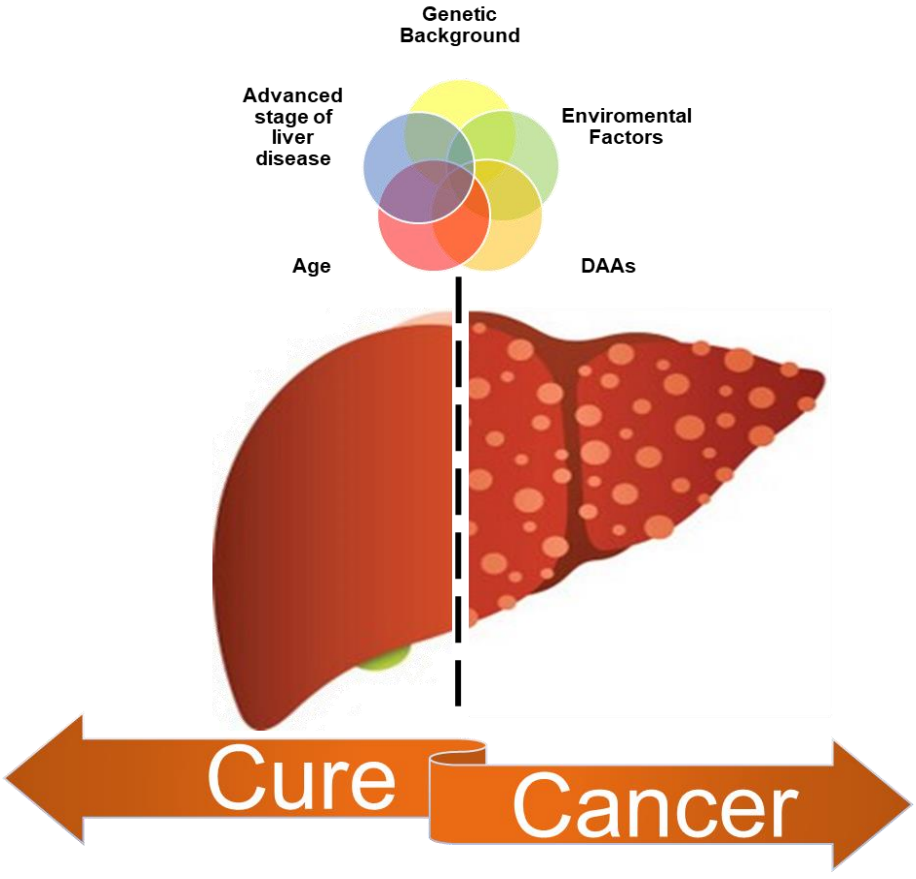

Figure 1: Factors that may influence the outcome of chronic liver disease in the new era of treatment for hepatitis C.

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Capítulo 3

CAPÍTULO III:

Combination of genetic polymorphisms in TLR influence cytokine profile in HCV patients treated with DAAs in State of Amazonas.

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Abstract

Hepatitis C is a public health problem and affects approximately 3% of the world's population. HCV infection has a wide spectrum of clinical manifestations and several single nucleotide polymorphisms (SNPs) in the genes of the toll-like receptors are cited to influence the clinical outcomes. A cross-sectional study was conducted in the Amazonas State, Brazil. SNPs in *TLR4* and *TLR9* genes were genotyped by PCR-RFLP in 151 HCV chronic hepatic disease patient and 206 health donors. Circulating cytokines IL-6, TNF, IL-10, IL-2, IFN-γ, IL-4 and IL17A were measured by cytometric bead array (CBA). The combined genotypes of *TLR9 -1237T/T* and *-1486C/T* seem to influence the cytokine profile under LPS stimulus to Th17 profile, especially among patients with advanced chronic liver disease, treated with DAAs.

Key words: Hepatitis C, polymorphisms, Toll like receptor, carcinoma hepatocellular, DDAs

Introduction

Chronic hepatitis C (CHC) is a major global health problem worldwide and more than 71 million people have chronic hepatitis C infection [1]. The liver damage during chronic infection is commonly attributed to mechanisms mediated by the host immune response associated with marked release of inflammatory mediators, favoring changes in the patterns of adhesion molecule expression and recruitment of cells to the hepatic inflammatory tissue microenvironment [2]. Immunological events linked to chronic hepatitis C can lead to dysregulated architecture and function of the liver and increases intestinal permeability through the release of cytokines that alter the portal circulation and intestinal epithelial junctions, allowing the lipopolysaccharide (LPS) derived from the intestinal microbiota to enter the portal circulation [3-5].

Previous studies showed that TLR-4 and TLR-9 pathway can activate hepatic stellated cells (HSCs) in patients with advanced liver disease through increased of LPS levels in systemic/portal vein blood and DNA from dying hepatocytes, respectively [6-9]. Several studies investigated the association between TLR single nucleotide polymorphisms (SNPs) and different outcomes in HCV infection (10). Direct acting antivirals (DAAs) are known to inhibit the tumor suppressor p53 in patients with successful clearance of HCV, while the inflammatory process continues to persist and placed patients at risks of developing HCC [11].

Clearance of HCV in patients treated with interferon-free therapy and the genetic background of the host may contribute to the progression of the disease in the persistence of the inflammatory process after virologic immune response [12-17].

In this study, we analyzed whether polymorphisms in *TLR4 A299G* A/G rs4986790, *TLR4 T399I* C/T rs4986791, *TLR9 -1237C/T* rs5743836 and *TLR9 -1486C/T* rs187084 are associated with susceptibility to HCV infection and cytokine profile changes after treatment with DAAs in the Amazon population.

Material and Methods

Ethics approval

This study was approved by the Ethics Committee of the Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM) (1.405.965/2015 and 00240112000-10/2010). Participants read and signed the written informed consent

form prior to the enrollment in the study, according to Declaration of Helsinki and Resolution 466/12 of the Brazilian National Health Council for research involving human subjects. All patients were treated according to the recommendations of the Brazilian Ministry of Health [18].

Samples and clinical data

The study was carried out at the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD) in Manaus, the capital city of the Amazonas State, during 2016-2017. The study population was a nonprobability sample of convenience consisting of 206 Healthy donor's candidates (HD) and 151 HCV infected patients. All participants were submitted to a serological screening at HEMOAM, recommended to monitor blood borne pathogens by Brazilian Blood Donor Bank Authorities, that includes serological analysis for the Hepatitis B and C virus, HIV, DENV, HTLV, Syphilis and Chagas' Disease. Among the HCV infected patients, 84 and 67 had ≤ F2 and ≥F2, respectively.

Genomic DNA extraction

10 mL of blood were collected by venipuncture from each participant in two tubes, one containing EDTA (BD Vacutainer® EDTA K2) and one tube containing Gel separator (Gel BD SST® II Advance®) for carrying out the genotyping of polymorphisms and assay of circulating cytokines respectively. Sera were kept at -80°C until cytokines were assayed. Genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's instructions. DNA samples were quantified with a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) to evaluate the concentration, and purity of nucleic acids.

Genotyping of TLR

The following polymorphisms, *TLR4 A299G* (rs4986790), *TLR4 T399I* (rs4986791), *TLR9 -1237C/T* (rs5743836) and *TLR9 -1486C/T* (rs187084) were investigated. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used for allelic discrimination as described previously [19-21]. Briefly, the PCR reaction for each SNP consisted of 1µL genomic DNA (~20ng) added to 24µL amplification mix containing 0.2µL (2U) Platinum™Taq polymerase (Thermo Fisher Scientific), 2.5µL10x buffer (100 mmol/L Tris-HCl (pH 8.3) and 500

mmol/L KCl), 1µL MgCl2 (1.5 mmol/L), 1µLdNTPs (40 mmol/L), 0.5µL each of forward and reverse primer (0.25 pmol/L) and 18.3µL ultrapure dH_2O. A total of 10µL of PCR product was digested with 5 U of respective restriction endonuclease NcoI, HinfI, BstNI, and AfIII (New England Biolabs, Ipswich, MA, USA) in enzyme buffer according to the manufacturer's instructions. The primers, PCR cycling conditions, and restriction endonucleases are shown in ST1 Table. The fragments generated by PCR-RFLP were separated by electrophoresis in either a 2% - 4% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA), and visualized with the UV light Gel Doc™ XR +System (Bio-Rad Corporation, Hercules, CA, USA) with a photo documentation system.

Isolations of peripheral blood mononuclear cells (PBMC) and cell culture

PBMC were collected from 45 individuals for culture assay as described previously [22]. 15 samples were from Healthy donors (HD) and 30 from HCV infected patients with chronic liver disease (15 ≤ F2 and 15 ≥F2), treated with DAAs and sustained virological response (SVR) confirmed by the absence of viral RNA by quantitative molecular tests (Figure 01). PBMC were isolated from 20 ml of venous venipuncture from each participant in four tubes containing Heparin (BD Vacutainer® PST™), using the Ficoll-Hypaque protocol. After centrifugation, PBMC were collected from the interface and washed with RPMI 1640 medium. After washing two times, the supernatant was discarded and the PBMC were resuspended at a concentration of 2×10^6 cells/ml in 4mL of RPMI supplemented with 1% streptomycin and 10% fetal bovine serum and incubated in 5%CO_2 at 37 °C for 12 hours. LPS stimulus was performed in duplicate using 100 µL of exogenous endotoxin (LPS / 1 µg / mL).

Cytokine assay

The cytokines IL-6, TNF, IL-10, IL-2, IFN-γ, IL-4 and IL17A in human blood serum and supernatant of PBMC culture were measured by Cytometric Bead Array (CBA), Kit BD™ Human Th1/Th2/Th17 Cytokine (Cat: Nº 560484, Lot:29132, BD®Biosciences, San Diego, CA, USA) according to the manufacturer's technical guidelines and protocols. A FACSCanto II flow cytometer (BD® Biosciences) at the HEMOAM was used for sample acquisition. The FCAP-Array software v3 (Soft Flow inc, USA) was used to calculate the cytokine levels.

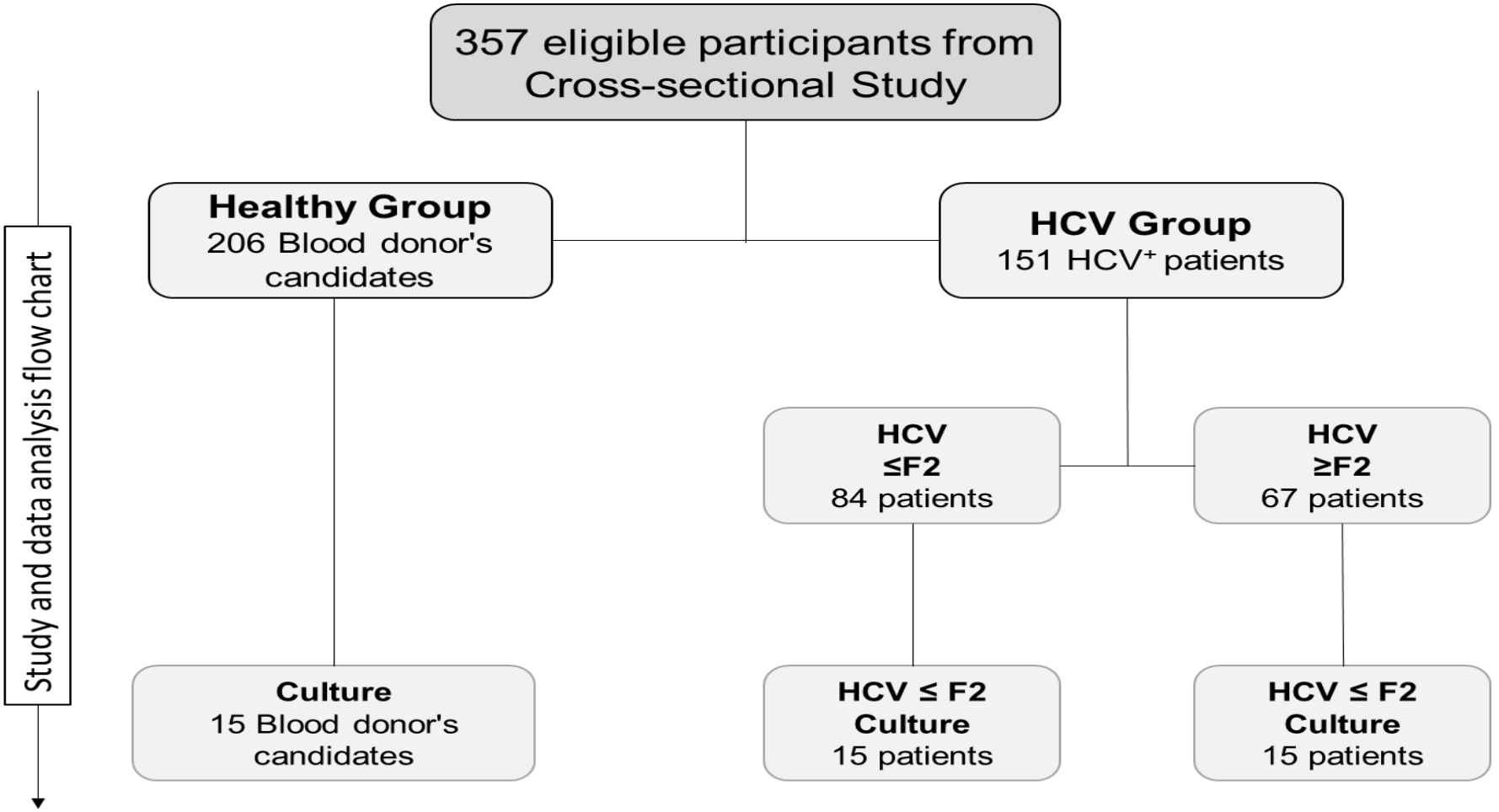


Figure 1: Study and data analysis flow chart. We included 357 eligible participants:Study and data analysis flow chart.

We included 357 eligible participants:206 Healthy blood donors and 151 that were infected with and were attended at FMT-HVD. Of these, thirty biological samples from patients with chronic hepatic disease, treated with direct-acting antivirals (DAAs) and sustained virological response (SVR) were collected for cell culture.

Genotype association test and Statistical analyzes

.Comparison between groups was performed with the chi-squared (χ^2) or Fisher's exact test with 95% confidence interval (CI). The Hardy-Weinberg equilibrium (HWE) was determined by comparing the frequency of the observed and expected number of genotypes. Tests for Hardy-Weinberg equilibrium were performed by an online application (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Graphpad Prism v.5 software (San Diego, CA, USA) was used for comparative analyzes of cytokines among the genotypes combination of the SNPs studied. The continuous variables presented a non-normal distribution and data was analyzed with non-parametric Mann-Whitney test to compare pairs. A Spearman correlation test was performed to assess the association between the levels of each cytokine tested. The positive and negative correlations are considered significant when $p < 0.05$. The correlation index (r) was used to categorize the correlation strength as weak ($r \leq 0.35$), moderate ($r \geq 0.36$ to $r \leq 0.67$), or strong ($r \geq 0.68$), as previously described [23]. Networks were assembled to assess the associations among the circulating cytokines. Significant correlations were compiled using the open access software Cytoscape v3.3 (Cytoscape Consortium, San Diego, CA), as previously reported [24-25]. Statistical significance was considered in all cases at $p < 0.05$.

Results

Baseline demographic, Laboratorial and clinical characteristics of the study population

The median age of the healthy blood donors and HCV patients were 32 and 57 years, respectively (p<0,0001). In both groups, male subjects were predominant (65% and 55%). At the time of blood collection, the HCV group were ≤ F2 (55.6%) and ≥F2 (44.4.5%), presenting statistical differences in follow-up markers (AST p<0,0317 and ALT p<0,0001). The data are shown in Table 01.

Table 01: Clinical and demographics characteristics of the study population

Variables	Healthy Donor´s (n = 206)	HCV patients		
		All (n = 151)	≤F2 (n = 84)	≥F2 (n = 67)
Age (mean ±SD)	32 ±11	58 ±11	55 ±11	61 ±9
Gender (male/female)	134/72	83/68	45/39	38/29
ALT (IU/L)	-	69.2 ±56.8	59.8 ±44.6	80.9 ±67.4
AST (IU/L)	-	63.2 ±43.3	44.1 ±25.8	86.9 ±48.7

Polymorphisms in *TLR4 and TLR9* are not associated in Chronic hepatic disease patients

To systematically examine association of TLR4 and TLR9 SNPs with HCV infection, allelic and genotypic frequencies of two TLR4 SNPs (rs4986790/rs4986791) and two TLR9 SNPs (rs5743836/rs187084) were analyzed and shown in Table 2. Homozygosity for TLR4 A299G G/G and TLR4 T399I T/T were absent in the population studied. The genotype distributions for TLR4 A299G G/G, TLR4 T399I T/T, TLR9 -1237 C/T and TLR9 -1486C/T were not significantly different between the groups. Carriers of the TLR9 -1237 T/T and TLR9 -1486 C/T variants confer 40% of the studied population. All of the SNPs were in HWE, except TLR9 -1486C/T SNP deviated slightly, in both HCV patients (p=0.002) and HD (p=0.04).

Lack of Association of genotypic combinations of polymorphisms in *TLR9 -1237T/T* and *-1486C/T* with Chronic hepatic disease

None of the polymorphisms of TLR4 and TLR9 analyzed in this study, exhibited any significant association with Chronic hepatic disease. TLR4 A299G A/A + A/G and TLR4 T399I C/C + C/T variants are most frequently in the population study 97% + 3% and 96% + 4%, respectively. We, therefore, proposed the influence of the combination genotypes for TLR9 variants TLR9 -1237T/T and -1486C/T on the cytokines profile in HCV patients treated with DAAs. Statistical analysis revealed that combination of TLR9 -1237T/T and TLR9 -1486C/T variants were higher in HD and HCV group compared to others genotypes combinations between the groups Table 3.

Influence of genotypic combinations of polymorphisms in *TLR9 -1237T/T* and *-1486C/T* on the circulating profile of cytokines in HCV patients

Human antiviral response against HCV is characterized by induction of Th1 cytokines profile. Analysis of genotypic combinations over the Th1, Th2, Th17 and Treg cytokines profiles among the studied groups indicated a different profile between the HCV patients over the liver damage. A significant increase of IL-6 (p=0.005) and IL-4 (p=0.0007) (Fig. 2A and 2F) were observed in the serum of these patients compared with HD. The cytokines TNF, IL-10, IL-2, IFN-γ and IL17A were not significant when compared between HCV patients and HD (Fig. 2B-2E and 2G). A significant increase of IL-10 were observed in HCV ≥F2 patients (p=0.028) compared to ≤ F2 whereas the cytokines IL-6, TNF, IL-2, IFN-γ, IL-4 and IL17A were not significant when compared between HCV patients with different stages of liver disease (Fig. 3A).

Influence of genotypic combinations over cytokines profile in the culture supernatant stimulated with Lipopolysaccharide (LPS)

No significant difference in IL-6, TNF, IL-10, IL-2, IFN-γ and IL-4, (Fig. 3B) were observed on stimulated with LPS PMBC culture. Only, the IL-17A showed significant increase in HCV ≥F2 patients (p=0.043) compared to ≤ F2 (Fig. 3B).

Lipopolysaccharide (LPS) change the interaction of cytokines in HCV patients

To test the relationship between altered levels of cytokines after LPS stimulus, a series of correlation analyzes were performed (Fig.4). The numbers of interactions between the cytokines analyzed were also different according to the hepatic gravity in HCV patients. No interactions were observed in HD group carrying the same genotypes combinations as HCV group that exhibited a strong positive relation between IL-6, IL- 10, IL-2, IL-17A (Fig.4A). A moderate relation between IL-6, TNF and IL-10 also were observed in HCV group. HCV ≤ F2 patients exhibited a strong positive relation between IL-6, IL-10 and TNF. In addition, in HCV ≥F2 patients, a strong positive relation between IL-6, IL-10, IL-2 and IL17A was preserved, while IL-6, IL17A and IL-10 exhibited a strong negative relation (Fig.4B).

Table 02: Genotypes and alleles association of TLRs polymorphisms in HCV patients.

Polymorphism, Genotype or Allele	Healthy Donor´s (n = 206)	HCV patients (n = 151)	OR (IC 95%)	(p) value	HCV ≤F2 (n = 84)	HCV ≥F2 (N = 67)	OR (IC 95%)	(p) value	
***TLR4 A299G* (rs4986790)**									
A/A	196 (95%)	147 (97%)	0.656 (0.219-1.96)	0.447	81 (96%)	66 (99%)	0.656 (0.042-4.025)	0.429	A/A vs A/G
A/G	10 (5%)	4 (3%)			3 (4%)	1 (1%)			
G/G	-	-			-	-			
A	402 (97%)	298 (99%)	0.662 (0.224-1.958)	0.452	165 (98%)	133 (99%)	0.414 (0.043-4.021)	0.660	A vs G
G	10 (3%)	4 (1%)			3 (2%)	1 (1%)			
***TLR4 T399I* (rs4986791)**									
C/C	199 (96%)	146 (96%)	0.952 (0.296-3.066)	0.934	80 (95%)	66 (99%)	0.303 (0.033-2.777)	0.264	C/C vs C/T
C/T	7 (4%)	5 (4%)			4 (5%)	1 (1%)			
T/T	-	-			-	-			
C	405(98%)	297 (98%)	0.953 (0.299-3.035)	0.935	164 (98%)	133 (99%)	0.308 (0.034-2.791)	0.399	C vs T
T	7(2%)	5 (2%)			4 (2%)	1 (1%)			
***TLR9 -1237C/T*(rs5743836)**									
T/T	137 (66%)	99 (65%)	0.286 (0.055-1.496)	0.115	56 (67%)	43 (64%)	1.204 (0.195-7.420)	0.551	C/C vs C/T+TT
C/T	67 (33%)	47(31%)	0.281 (0.052-1.508)	0.117	25 (30%)	22 (33%)	1.320 (0.202-8.639)	0.771	C/C vs C/T
C/C	2(1%)	5 (4%)	0.289 (0.055-1.520)	0.120	3 (3%)	2 (3%)	*0.868 (0.139-5.428)*	*0.01*	C/C vs T/T
T	341 (83%)	245(81%)	0.895 (0.609-1.316)	0.572	137 (82%)	108 (80%)	1.064 (0.596-1.898)	0.833	C vs T
C	71(17%)	57(19%)			31 (18%)	26 (20%)			
TLR9 -1486C/T (rs187084)									
C/C	26 (13%)	17 (11%)	1.065 (0.548-2.069)	0.851	8 (10%)	9 (13%)	0.678 (0.247-1.866)	0.400	C/C vs C/T+TT
C/T	114(55%)	93 (62%)	1.190 (0.600-2.360)	0.617	54 (64%)	39 (60%)	0.642 (0.227-1.812)	0.400	C/C vs C/T
T/T	66 (32%)	41 (27%)	0.856 (0.407-1.801)	0.682	22 (26%)	19 (27%)	0.768 (0.247-2.384)	0.647	C/C vs T/T
C	166 (40%)	127 (42%)	0.893 (0.652-1.223)	0.479	70 (42%)	57 (43%)	0.965 (0.609-1.529)	0.878	C vs T
T	246 (60%)	175 (58%)			98 (58%)	77 (57%)			

Table 03: Association of genotypic combinations of deferent polymorphisms with HCV patient's.

Polymorphism, genotypic combinations	Healthy Group (n = 206)	HCV patients (n = 151)	OR (IC 95%)	(p) value	HCV ≤F2 (n = 84)	HCV ≥F2 (n = 67)	OR (IC 95%)	(p) value
***TLR9 -1237C/T* (rs5743836) / *TLR9 -1486C/T* (rs187084)**								
TT/TT	41(20%)	23(15%)	1.38 (0.78 – 2.42)	0.267	14(17%)	9(13%)	1.28 (0.52-3.19)	0.652
TT/CC	18 (9%)	12(8%)	1.10 (0.51-2.37)	0.848	4(5%)	8(12%)	0.36 (0.10-1.28)	0.134
CT/CT	35 (17%)	29(19%)	0.86 (0.49-1.48)	0.675	16(19%)	13(19.5%)	0.97 (0.43-2.20)	1.000
TT/CT	78(38%)	64(42%)	0.82 (0.53-1.27)	0.443	38(45%)	26(39%)	1.30 (0.67-2.50)	0.507
CT/CC	8 (4%)	5 (3%)	1.18 (0.37-3.68)	1.000	4(5%)	1(1.5%)	3.30 (0.35-30.26)	0.382
CC/TT	1 (0.5%)	4(3%)	0.17 (0.01-1.62)	0.166	3(3%)	1(1.5%)	2.44 (0.24- 24.07)	0.629
CC/CC	-	-	-	-	-	-	-	-
CC/CT	1(0.5%)	1(1%)	0.73 (0.04-11.80)	1.000	-	1(1.5%)	0.26 (0.01- 6.54)	0.443
CT/TT	24 (11%)	13 (9%)	1.40 (0.68-2.84)	0.384	5(6%)	8 (12%)	0.46 (0.14- 1.50)	0.246

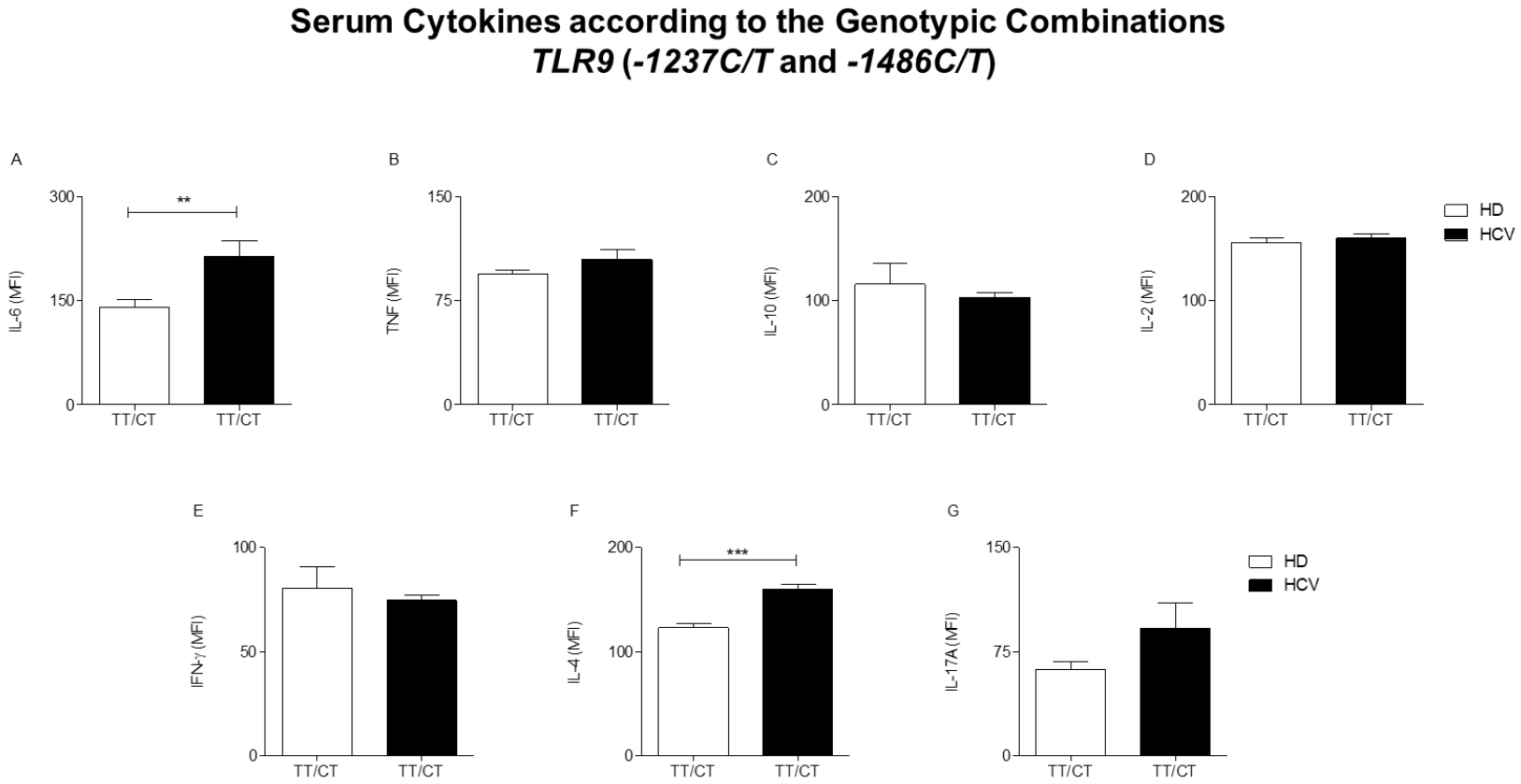
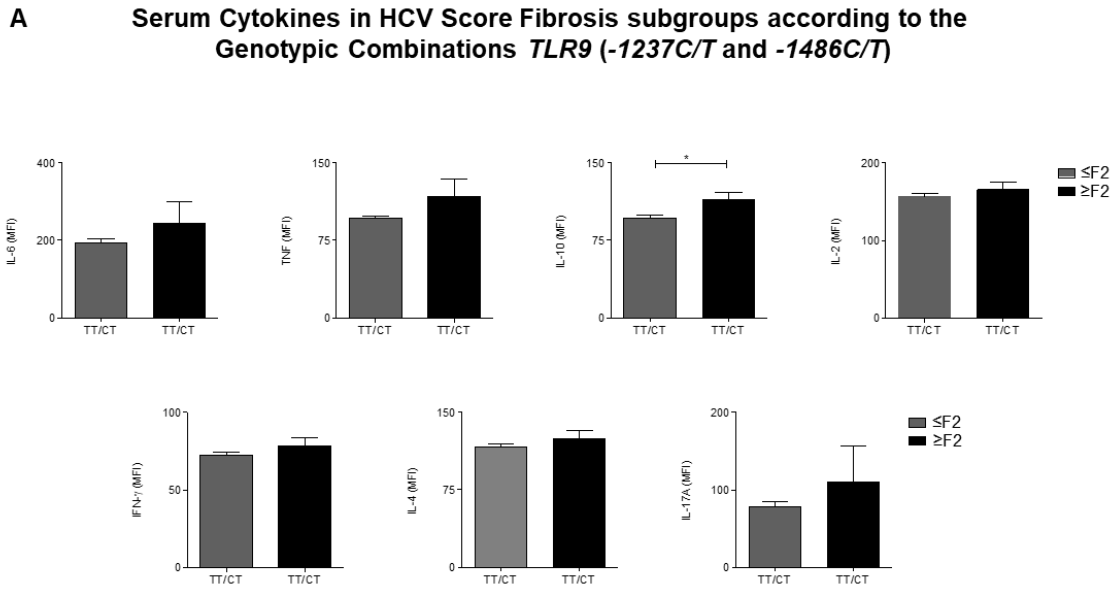


Figure 2: Concentration in Mean Fluorescence Intensity (MIF) of serum cytokines according to the genotypic combinations (rs5743836/ rs187084) between the control group (HD) and HCV patient group (HCV).

Results are expressed as median and standard deviation. Statistical analyzes were performed using the non-parametric Mann Whitney test. A significant statistical difference was considered when $p < 0.05$, represented by "*".



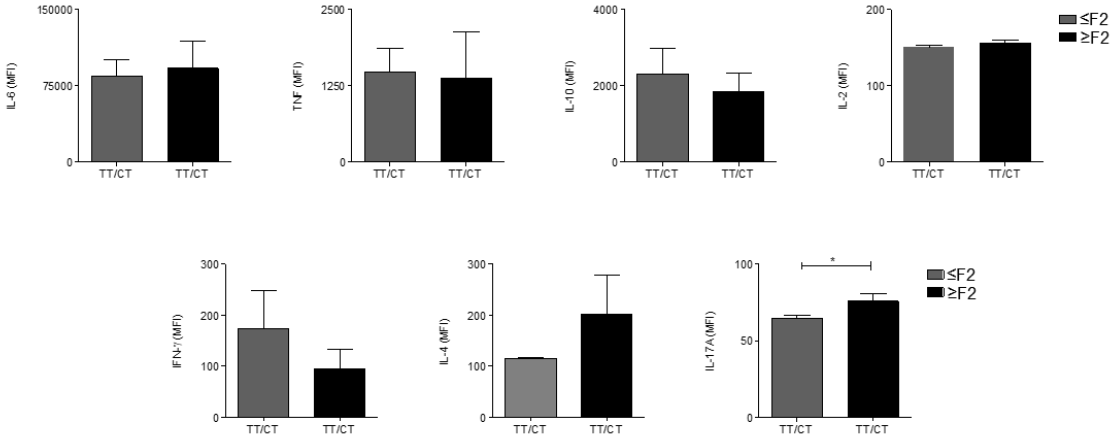


Figure 3: Concentration in Mean Fluorescence Intensity (MIF) of serum cytokines.

(A) according to the genotypic combinations (rs5743836/ rs187084) between HCV patient group (≤F2 and ≥F2). (B) Concentration in Mean Fluorescence Intensity (MIF) in the culture supernatant stimulated with Lipopolysaccharide (LPS) (1 µg/ml), according to the genotypic combinations (rs5743836/ rs187084) between HCV patient group (≤F2 and ≥F2). Results are expressed as median and standard deviation. Statistical analyzes were performed using the non-parametric Mann Whitney test. A significant statistical difference was considered when $p < 0.05$, represented by "*".

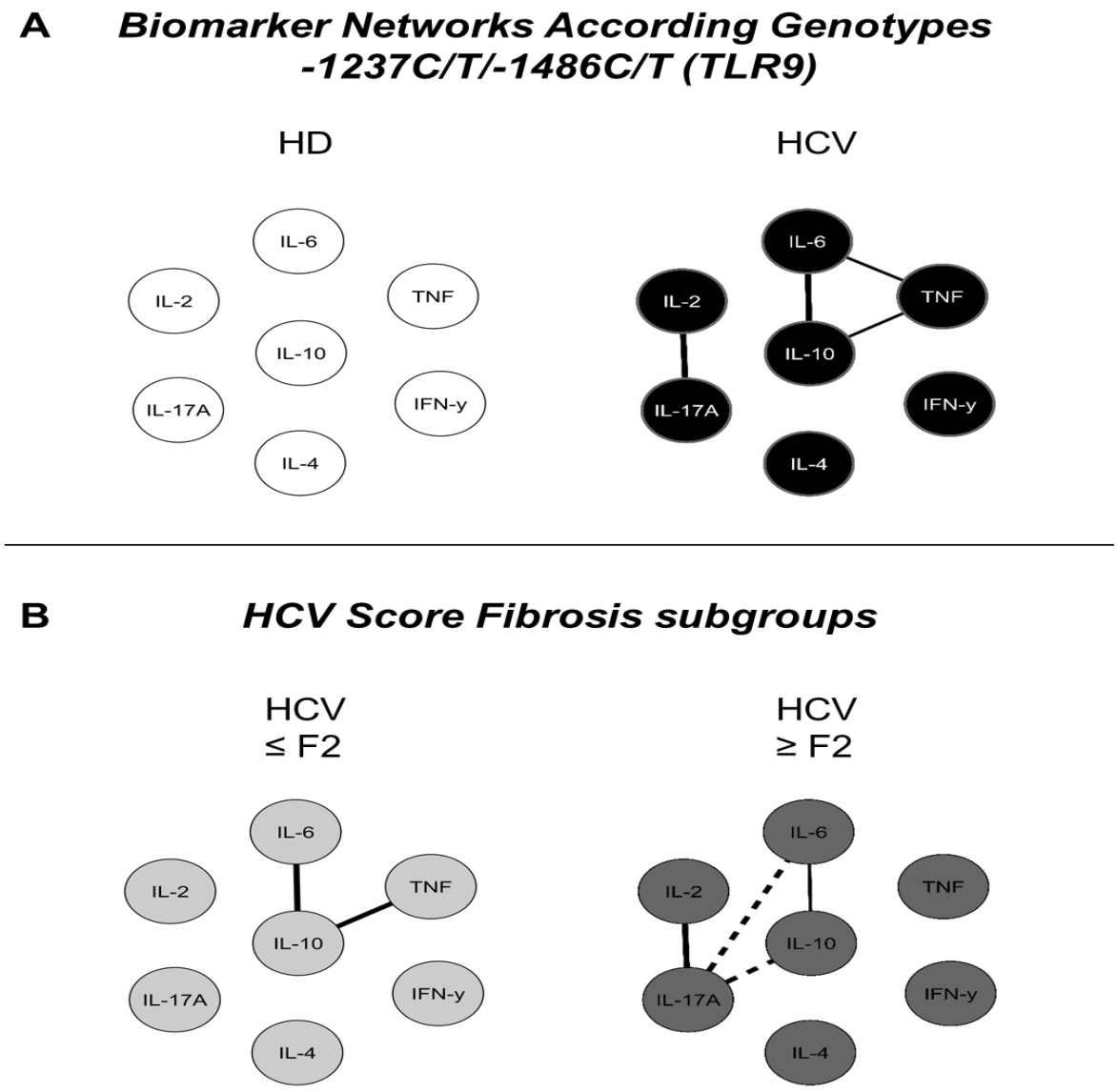


Figure 4: "Cytokine Networks" in the HCV patients according to the fibrosis score.

(A). Concentration in Mean Fluorescence Intensity (MIF) in the culture supernatant stimulated with Lipopolysaccharide (LPS) (1 µg/ml), according to the genotypic combinations (rs5743836/ rs187084) between the control group (HD) and HCV patient group (HCV) (A) and patient group (≤F2 and ≥F2) (B). Customized biomarker network layouts were built to identify the relevant association between proinflammatory IL-6, TNF, IL-2 and IL17A cytokines, modulatory IL-10 axis, using a clustered distribution of nodes. Significant Spearmam's correlations at $P < 0.05$ were represented by connecting edges to highlight positive [strong ($r \geq 0.68$; thick continuous line) or moderate ($0.36 \geq r \leq 0.67$; thin continuous line)] and negative [strong ($r \leq -0.68$; thick dashed line) or moderate ($-0.36 \geq r \leq -0.67$; thin dashed line)] as proposed by Taylor [18]. The overall statistic analysis of the network node neighborhood connections point out for an almost linear-chain pattern in the HCV groups with a clear shift towards a more imbricate profile in HCV patients ≥F2. A persistent IL-6/IL-10 loop was observed in all HCV subgroups with differential neighborhood connections for the IL-17A node in HCV patients ≥F2.

Discussion

The results of the present study revealed the low frequency of polymorphic alleles in *TLR4* (rs4986790 and rs4986791) in the Amazon population. Studies conducted with other North Brazilian samples have reported frequencies of heterozygotes similar to ours [19-21,26-27]. Increasing evidences suggest that the rs4986790 of *TLR4* that leads to an amino acid change (Asp299Gly) might interfere with the interaction between the receptor-ligand and the protein stability. This may cause deficiency in the recruitment of Myeloid differentiation primary response 88 (MYD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) and contribute to the increase risk of bacterial infections and a decrease in the proinflammatory response in patients with cirrhosis [28-35]. Our data does not show any association of these SNPs with susceptibility to HCV infection and hepatic impairment.

Another important receptor that also participates in this process of hepatic disease evolution is TLR-9 that recognizes the CpG-rich DNA of bacteria. Watanabe et al., 2007 demonstrated that host-derived denatured DNA from apoptotic hepatocytes stimulates HSCs through TLR-9 in hepatic fibrosis [9]. Several studies showed possible associations between *TLR9* gene SNPs and cancer risk [10,36]. The T allele of the TLR9 rs5743836 is transcribed more effectively under basal conditions compared to the variant C allele [37-38].

IL-6 up-regulates TLR9 expression, leading to exacerbated cellular responses to CpG, including IL-6 production and B-cell proliferation in mononuclear cells carrying the TLR9 -1237C/T SNP [39]. Further, the increased transcriptional activity of TLR9 in mononuclear cells from patients harboring TLR9 -1237T/C SNP supports a functional effect of this polymorphism on non-Hodgkin lymphoma susceptibility [40]. It was suggested that TLR9 -1486C/T heterozygote may be a genetic risk factor for cervical cancer [41]. The C allele of TLR9 -1486C/T SNP showed lower TLR9 expression and correlated with the risk of systemic lupus erythematosus [42]. TLR9 expression by fibroblast-like cells was shown to be significantly correlated with shortened overall survival in patients with hepatocellular carcinoma [43].

Successful treatment of hepatitis C is associated with sustained virological response and declining liver inflammation through decrease cytokines and chemokines levels during treatment of hepatitis C [44]. The direct-acting antivirals

(DAAs) dramatically increased number of patients that will be able to clear hepatitis C virus infection with this new medication. However, risk of hepatocellular carcinoma (HCC) after DAAs still a great concern among patients with advanced cirrhosis. What we know is that the ER-stress response and restores p53 are normalized after HCV clearance induced by interferon-alpha-based antiviral therapies, whereas HCV clearance by DAAs does not restore p53. The p53 is a tumor suppressor and coordinates diverse cellular responses to stress, damage and in the suppression of autoimmune and inflammatory diseases [11,45].

TLRs may play an important role in a wide range of human diseases by triggering early events in the immunological response. The polymorphisms studied have been associated to cancer risk and the most frequently genotype combination in our population is TLR4 299A/A, TLR4 299C/C, TLR9 -1237T/T and TLR9 -1486C/T.

The data presented in this study suggest the influence of combination of *TLR9 (-1237T/T* and *-1486C/T*) on the production of circulating cytokines IL-6 and IL-4 in HCV patients compared to health donors. Furthermore, these combined polymorphisms also affect the production of circulating IL-10 in patients with moderate fibrosis. These cytokines are important in chronic liver disease caused by chronic HCV infection. Nieto et al., (2014) observed a spontaneous increase of L-6 and IL-10 production in patients with cirrhosis carrying the wild-type variants of *TLR4 A299G* and/or *T399I*. Yet, the production of IL-6, TNF-α and IL-10 after TLR stimulation was similar between the patients. This different pattern of cytokine production could influence in development of cirrhosis complications [46].

An increase of IL-17A in PBMC from patients with greater severity of hepatic disease treated with DAAs was observed in this study. LPS can up-regulates the expression of several TLRs and nuclear factor kappa B (NF-kB), extracellular-signal-regulated kinase (ERK) and p38 kinase signal pathways. This phenomenon might explain the synergy between bacterial DNA and LPS in activating macrophages to invading bacteria [47-48].

LPS also downregulated the expression of p53 protein in mouse Kupffer cell and RAW 264.7 macrophage cell line [49]. This observation may contribute to the hypothesis that the p53 inhibition perpetuate through TLR4 activation by LPS in patients with moderated fibrosis. In fact, p53 tumor suppressor protein is related to

carcinogenesis and is suggested to cause prolonged NF-κB activation at a late stage after LPS stimulation and possibly to sustain prolonged proinflammatory responses [49]. Several inflammatory factors encoded by NF-κB target genes and IL-6 are important activator of transcription 3 (STAT3). NF-κB and STAT3 regulate the expression of numerous oncogenic and inflammatory mediators such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatic growth factor (HGF), IL-10, IL-17 and IL-6, favoring the emergence of tumor [50]. The interactions between the cytokines analyzed seem in this study to change after LPS stimulation. A strong positive relation between IL-6, IL-10, IL-2 and IL17A was observed.

The present study reveals different interactions between cytokines in HCV patients according to polymorphisms combinations and liver damage. The changes of the cytokines dynamic connections during the disease associated with these genotypes may lead to a progressive oncogenic milieu. This would be signaling an increased risk of cancer in patients treated with DAAs who carry the combination of *TLR9* (*-1237T/T* and *-1486C/T*) even after successful treatment of viral clearance (Figure 05). In addition, understanding the influence of these polymorphisms may be useful in clinical practice for designing new therapeutic scenarios.

This study has some limitations. The study population size is small and may influence in the levels of associations with HCV infection and disease severity. The small sample size does not allow intra-comparison of the genotype's combination studied with cytokines profile. However, it showed that the combinations of these polymorphisms seem to influence in the chronic hepatic disease. Further studies are needed to confirm this preliminary finding.

Conclusion

Altogether, our findings demonstrated that *TLR9 -1237T/T* and *-1486C/T* variants may change the cytokine correlations between IL-6, IL-10,IL-2 and IL17A, especially among patients with advanced chronic liver disease, treated with DAAs. The possibility of these patients presenting an unresolved ER stress after HCV clearance by DAAs linked to the genetic background, and the pathology already installed could increase the risk for HCC development. Our results suggest the relevance of these set of biomarkers for clinical investigations as a potential tool for

predicting adverse outcomes in patients with chronic hepatic disease caused by HCV infection.

Acknowledgements

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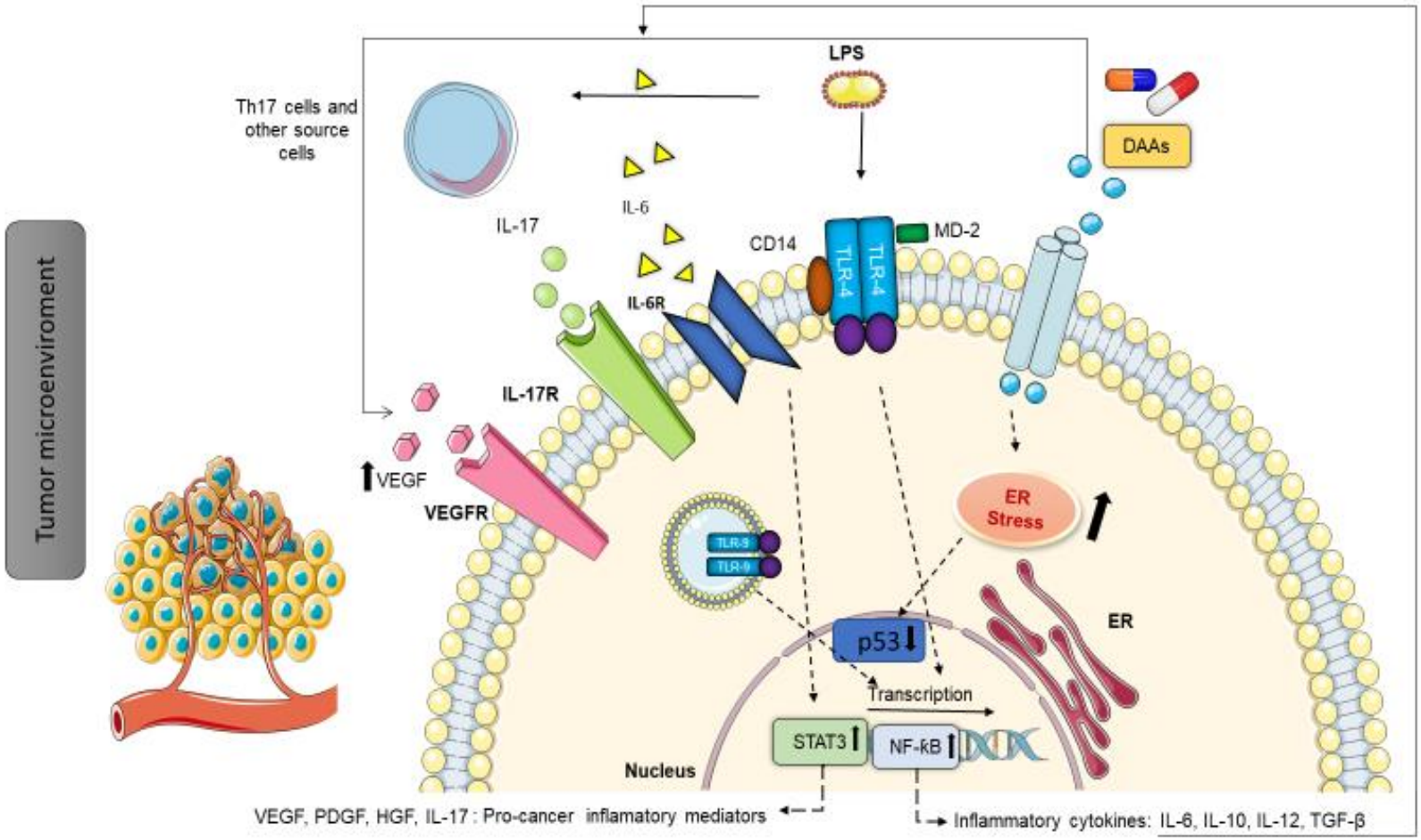

Figure 5: Schematic diagram for the role of the interplay between effects caused by DAAs and sever chronic hepatic disease.

Supporting information

Table Supplementary 01: Description of polymorphisms, primer sequences, PCR protocols, restriction enzymes, and fragments generated during the SNP identification study.

Polymorphisms	Primers	PCR Protocols	Restriction enzymes	Alleles and fragments (bp[¤])
TLR4 A299G (rs4986790)	F: 5'-ATACTTAGACTACTACCTCCATG-3' R: 5'-AAACTCAAGGCTTGGTAGATC-3'	95°C for 5min, 35x (95°C for 30s, 56°C for 30s, 72°C for 30s), 72°C for 7min	*NcoI*	A: 259 G: 239+20
TLR4 T399I (rs4986791)	F: 5'-GCTGTTTTCAAAGTGATTTTGGGAGAA-3' R: 5'-CACTCATTTGTTTCAAATTGGAATG-3'	95°C for 5min, 35x (95°C for 30s, 60°C for 30s, 72°C for 45s), 72°C for 5min	*Hinf-I*	C: 147 T: 96+51
TLR9 -1237C/T (rs5743836)	F: 5'-CTGCTTGCAGTTGACTGTGT-3' R: 5'-ATGGGAGCAGAGACATAATGGA-3'	95°C for 5min, 40x (95°C for 30s, 59°C for 30s, 72°C for 45s), 72°C for 7min	*BstNI*	C: 108+27 T: 60+48+27
TLR9 -1486C/T (rs187084)	F: 5'-TATCGTCTTATTCCCCTGCTGGAATGT-3' R: 5'-TGCCCAGAGCTGACTGCTGG-3'	95°C for 5min, 40x (95°C for 30s, 59°C for 30s, 72°C for 30s), 72°C for 7min	*AflII*	C: 145 T: 111+34

*rs: Reference sequence; [#]F: Forward; [¥]R: Reverse; [¤]bp: Base pairs.

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Conclusão

6 CONCLUSÃO

Os resultados deste trabalho demonstraram achados relevantes sobre a influência dos polimorfismos nos receptores da imunidade inata (*TLR4* e *TLR9*) sobre o perfil de citocinas em pacientes com doença hepática crônica, apontando que:

- Homozigotos para TLR4 A299G G / G e TLR4 T399I T / T apresentara-se ausente na população estudada. As distribuições de genótipo para TLR4 A299G G / G, TLR4 T399I T / T, TLR9 -1237C / T e TLR9 -1486C / T não foram significativamente diferentes entre os grupos. Pacientes que carregam as variantes TLR9 -1237 T / T e TLR9 -1486C / T representam 40% da população estudada.
- Nenhum dos polimorfismos de TLR4 e TLR9 analisados neste estudo exibiu associação significativa com doença hepática crônica. As variantes TLR4 A299G A / A + A / G e TLR4 T399I C / C + C / T foram as mais frequente na população estudada - 97% + 3% e 96% + 4%, respectivamente. Portanto, avaliamos a influência da combinação genótipos das variantes -1237T / T e -1486C / T do TLR9 na citocina perfil em pacientes com HCV tratados com DAAs. A análise estatística revelou essa combinação das variantes TLR9 -1237T / T e TLR9 -1486C / T foi maior nos grupos HD e HCV quando comparado a outro genótipo combinações entre os grupos;
- As variantes TLR9 -1237T/T e -1486C/T podem alterar as correlações de citocinas entre IL-6, IL-10, IL-2 e IL17A, especialmente entre pacientes com doença hepática crônica avançada e tratados com antivirais de ação direta;

Nossos resultados sugerem a relevância deste conjunto de biomarcadores para investigações clínicas como uma ferramenta potencial para prever desfechos adversos em pacientes com doença hepática crônica causada por infecção pelo HCV. No entanto, estudos adicionais devem ser realizados para confirmar a influência da genética do hospedeiro na resposta imune no estadiamento da doença hepática.

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Anexos

8 ANEXOS

Anexo 01: Parecer do Comitê de Ética em Pesquisa -CEP-HEMOAM (CAAE:49652815.8.0000.0009).

FUNDAÇÃO DE HEMATOLOGIA E HEMOTERAPIA DO

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: IDENTIFICAÇÃO E SELEÇÃO DE PEPITÍDEOS MIMÉTICOS REATIVOS AOS SOROS DE PACIENTES HCV+ E SUA INFLUÊNCIA NO DESENVOLVIMENTO DA FIBROSE HEPÁTICA IN VITRO

Pesquisador: ANDRÉA MONTEIRO TARRAGÔ

Área Temática:

Versão: 1

CAAE: 49652815.8.0000.0009

Instituição Proponente: Fundação de Hematologia e Hemoterapia do Amazonas - HEMOAM

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.406.965

Apresentação do Projeto:

Projeto de Pesquisa: IDENTIFICAÇÃO E SELEÇÃO DE PEPITÍDEOS MIMÉTICOS REATIVOS AOS SOROS DE PACIENTES HCV+ E SUA INFLUÊNCIA NO DESENVOLVIMENTO DA FIBROSE HEPÁTICA IN VITRO

Trata-se de um estudo descritivo, transversal constituindo subprojeto de um estudo maior da linha de pesquisa sobre Biotecnologia & Saúde

intitulado: "Desenvolvimento de tecnologias aplicadas ao diagnóstico e terapia de doenças infecciosas tropicais", que será desenvolvido pelos

grupos de Pesquisa "Virologia" da Universidade Federal do Pará – UFPA, em parceria com os grupos "Imunologia Básica e Aplicada" da

Universidade Federal do Amazonas e "Nanotecnologia" da Universidade Federal de Uberlândia- UFU. Ainda teremos apoio das Universidade da

California (UCDAVIS) e da Universidade de Miami(UM). Aprovado pelo Edital Pró-Amazônia n. 047/2012,

Objetivo da Pesquisa:

Objetivo Primário:

• Determinar a reatividade em soro de indivíduos HCV+ para peptídeos miméticos ao HCV identificados pela técnica phage display e caracterizar o

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FUNDAÇÃO DE HEMATOLOGIA E HEMOTERAPIA DO

perfil de citocinas envolvidas no desenvolvimento de fibrose hepática em cultura de células

Objetivo Secundário:

• Selecionar e identificar peptideos miméticos reativos a soros de pacientes HCV+ com fibrose hepática;

• Sequenciar os clones isolados e realizar análises de bioinformática;

• Descrever o perfil de citocinas envolvidos no desenvolvimento da fibrose em cultura de células mesenquimais e hepatócitos;

• Pré–validar os clones selecionados através de ensaios de Phage-ELISA utilizando proteinas totais de pacientes com fibrose hepática;

Avaliação dos Riscos e Beneficios:

Riscos:

Risco de contaminação dos colaboradores da pesquisa com sangue contaminado com o vírus da Hepatite C

Benefícios: Conseguir desenvolver peptídeos que possam ser utilizados posteriormente em biossensores capazes de detectar proteínas presente em soro de

pacientes que possam predizer e servir de biomarcadores para diagnóstico de fibrose.

Comentários e Considerações sobre a Pesquisa:

Pesquisa Relevante que busca detectar marcadores que auxiliem na detecção de peptideos miméticos ao virus da hepatite C capazes de predizer fibrose utilizando tecnologias combinadas em engenharia genética, como Phage Display com o intuito de gerar estratégias direcionadas para a investigação do processo do desenvolvimento da fibrose.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos foram apresentados

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Aprovado

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações	PB_INFORMAÇÕES_BASICAS_DO_P	09/09/2015		Aceito

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FUNDAÇÃO DE HEMATOLOGIA E HEMOTERAPIA DO

Continuação do Parecer: 1.406.965

Básicas do Projeto	ETO_579290.pdf	15:35:04		Aceito
Folha de Rosto	folhaderosto.pdf	09/09/2015 15:32:42	ANDRÉA MONTEIRO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	09/09/2015 15:30:02	ANDRÉA MONTEIRO TARRAGÔ	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.pdf	09/09/2015 15:26:05	ANDRÉA MONTEIRO TARRAGÔ	Aceito
Declaração de Pesquisadores	anuencias.pdf	09/09/2015 15:21:14	ANDRÉA MONTEIRO	Aceito
Declaração de Pesquisadores	anuencias.docx	09/09/2015 14:46:19	ANDRÉA MONTEIRO	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

MANAUS, 12 de Fevereiro de 2016

Assinado por:
Elisa Brosina de Leon
(Coordenador)

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Anexo 02: Parecer do Comitê de Ética em Pesquisa -CEP-HEMOAM (CAAE:0024.0.112.000-10).

Fundação de Hematologia e Hemoterapia do Amazonas
HEMOAM
GOVERNO DO ESTADO DO AMAZONAS

COMITÊ DE ÉTICA EM PESQUISA - CEP
CAAE- 0024.0.112.000-10 - VERSÃO 002/10

IDENTIFICAÇÃO	
Título:	Avaliação do perfil celular, humoral e molecular em doadores de sangue com sorologia reativa ao vírus da hepatite B (HBV) na cidade de Manaus.
Instituição:	Fundação HEMOAM
Pesquisador:	Laura Patricia Viana Maia
Data da Reapresentação:	06.10.2010

OBJETIVOS	
Geral:	Avaliar o perfil celular, humoral e molecular em doadores de sangue com sorologia reativa ao vírus da hepatite B (HBV) na cidade de Manaus.
Específico:	Avaliar a soroprevalencia de doadores de sangue com sorologia reativa ao vírus da hepatite B, através dos marcadores HBsAg e/ou anti-HBc; Analisar o perfil celular dos linfócitos T CD3+/CD4+ e CD3+/CD8+; Analisar a sub-população de leucócitos ativadas através do marcdor CD69+ (linfócitos, eosinófilos, basófilos e monócitos; Analisar a sub-população linfócitos T reguladores através do marcador CD25+; Avaliar o perfil de citocinas TH1 (IL12 e IFN-y); citocinas Th2 (IL-4); citocina supressora (IL-10) e citocinas inflamatórias (IL-6, IL-8 e TNF – a); f – Caracterizar os genótipos virais na população de doadores e correlacionar com a resposta imune encontrada.

SUMÁRIO DO PROJETO	
Metodologia:	Trata-se de um estudo observacional descritivo do tipo não-probabilístico que será realizado com doadores de sangue com sorologia positiva para HBsAg e/ou anti-HBc no período de novembro de 2010 a abril de 2011, com uma amostra de 123 doações reativas.
Caracterização da Amostra:	Trata-se de um estudo observacional descritivo do tipo não-probabilística que será realizado com todos os doadores de sangue com sorologia positiva para HBsAg e/ou anti-HBc do vírus da hepatite B no período de setembro de 2010 a setembro de 2011, totalizando 1.396 doadores (valor estimado).
Critérios de Inclusão	Doadores de sangue com sorologia positiva para HBsAg e/ou anti-HBc, de ambos os sexos, com idade entre 18 e 65 anos e os que apresentarem

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	co-infecção (sífilis, doença de chagas, HCV, HIV e HTLV).
Critérios de Exclusão	Doadores que apresentarem resultados negativos para ambos os marcadores da hepatite B na segunda amostra de sangue.

COMENTÁRIOS DO CEP FRENTE À RESOLUÇÃO CNS 196/96 E RESOLUÇÕES COMPLEMENTARES
Na análise do protocolo observou-se que os autores responderam aos questionamentos descritos no parecer anterior.

PARECER DO CEP
Projeto Aprovado. Conforme determinado pela resolução CNS 196/96 os pesquisadores deverão apresentar a este CEP relatórios parcial e/ou final, ficando determinado desde então, o prazo para apresentação de relatório: 27/04/2011. Eventuais modificações ou emendas ao protocolo devem se apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. O termo de Consentimento deverá ser aplicado em duas vias sendo que uma delas ficará com o sujeito da pesquisa.

SITUAÇÃO	**DATA DO PARECER**
APROVADO	26.10.2010

Anexo 03: Produção científica relacionada a Tese

Rev Soc Bras Med Trop 50(6):861-863, November-December, 2017
doi: 10.1590/0037-8682-0094-2017

Case Report

Sofosbuvir and daclatasvir combination therapy for current hepatitis C virus genotype 4 achieves SVR: a case report of HCV genotype 4 from the Amazon

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Abstract

Hepatitis C is a worldwide endemic disease. However, hepatitis C virus genotype 4 (HCV GT-4) has rarely been reported in Brazil. HCV GT-4 demonstrates high sustained virological response (SVR). Here, we report the case of a 62-year-old HCV GT-4 positive woman complaining of a headache, nausea, and arthralgia. The patient was treated according to the protocol for genotype 4 (12 weeks administration of 400mg sofosbuvir and 60mg daclatasvir daily) and achieved SVR. Although this is not an Amazonas autochthonous case, the presence of genotype 4 is rarely reported in the region.

Keywords: Hepatitis C. Genotype 4. Sustained viral response.

INTRODUCTION

According to the World Health Organization (WHO), hepatitis C virus (HCV) affects millions of people worldwide; it is estimated that approximately 71 million people are chronically infected with HCV, causing a serious global public health problem[1].

There are seven HCV genotypes including 67 subtypes, which are geographically distributed throughout the world[2]. In Brazil, the genotype distribution pattern consists of genotype 1, followed by genotypes 3, 2, 4, and 5[3-4].

The treatment recommended by the Brazilian Ministry of Health for chronic HCV infection involves oral combinations of direct-acting antivirals (DAAs); sofosbuvir in association with simeprevir or daclatasvir. These drug combinations are well tolerated and have been reported to increase sustained virological response (SVR) rates to approximately 90%[5-7].

CASE REPORT

A 62-year-old woman was admitted to the *Fundação de Medicina Tropical Doutor Heitor Vieira Dourado* (FMT-HVD) in November 2015. Upon admission, the patient complained of a headache, nausea, and an extrahepatic manifestation of neurological motor impairment. The patient had received blood transfusion eight years prior in Alenquer, during an upper digestive endoscopy procedure. Follow-up tests prior to treatment are detailed in **Table 1**. The patient was treated according to the protocol for genotype 4 (12 weeks administration of 400mg sofosbuvir and 60mg daclatasvir daily); SVR was achieved and hepatic markers values returned to normal six months after the end of treatment (**Table 1**).

DISCUSSION

Hepatitis C virus genotype 4 (HCV GT-4) represents approximately 1518 million cases of the total global HCV infections. It is prevalent in lower income countries in Northern and Equatorial Africa, the Middle East, and Caribbean and Indian regions[8,9].

The frequency of the geographical distribution of the genotypes in the Amazon region is similar to the pattern in Brazil and other world regions; a higher prevalence of genotypes 1 (64.9%) and 3 (30.2%) and a lower prevalence of genotypes 2 (4.6%), 4 (0.2%), and 5 (0.1%)[3]. However, genotype 4 is rarely reported in the State of Amazonas[10].

The current global distribution of HCV genotypes has undoubtedly been influenced by historical events and modified by contemporary human migration trends. Although genotype 1 has been established as the most prevalent genotype worldwide, including in the Amazon region, we must be aware of the introduction of other genotypes into the region.

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TABLE 1: Patient laboratory test results.

Laboratory tests	Admission	Post-treatment*	Reference values
Hemoglobin	12.9g/dL	13.73g/dL	12g/dL–18g/dL
Hematocrit	39.8%	41.1%	37%–52%
Platelets	196,000/mm³	239,000/mm³	130,000/mm³–400,000/mm³
Leukocytes	6,190/mm³	6,340/mm³	5.2/mm³–12.4/mm³
Albumin	5.1g/dL	5.0g/dL	3.5–5.0g/dL
INR	1.13	1.12	1.0
TGO/AST	81IU/L	20IU/L	2IU/L–38IU/L
TGP/ALT	116IU/L	20IU/L	2IU/L–44IU/L
HCV-RNA	541,978 (Log= 5.73)	Undetectable	12IU/mL (Log=1.08) – $100_{,}10^{6}$ IU/mL (Log=8)
FIB-4**	2.38	1.20	FIB 4 > 3.25 (≥ F2)
Child pugh score	A5	A5	A-C

INR: international normalized ratio; **TGO/AST:** transaminase glutamic-oxalacetic/aspartate aminotransferase; **TGP/ALT:** transaminase glutamic-pyruvic/alanine aminotransferase; **HCV-RNA:** hepatitis C virus- ribonucleic acid; **IU/mL:** international units/milliliter; **FIB-4:** index for liver fibrosis; **APRI:** AST-to-platelet ratio index; **METAVIR:** score that quantify the degree of inflammation and fibrosis using the results of a liver biopsy. *Post -Treatment tests were performed six months after the end of treatment. **The treatment is indicated for HCV mono infected patients with APRI > 1.5 or FIB-4 > 3.25, characterizing METAVIR ≥F2. HCV.

In this case, the patient is a native of Tracuateua, a small City in Pará, in the Amazon region; however, she lives in Alenquer, another city in Pará closer to Manaus. Thus, this is not an autochthonous case as the patient temporarily moved to Manaus for HCV treatment. Migratory flow may contribute to the dissemination of genotype 4 originating from other states in the region into Amazonas via virus carriers prior to SVR.

The origin of the region of Tracuateua is linked to the construction of the Belém-Bragança railroad (completed in 1908), during which many African descent, Portuguese, and Spanish immigrants began to colonize the vicinity. Although this migratory phenomenon is not recent, it has been increasing; thus, new and unusual genotypes may be more prevalent than thought[11].

Because of the low prevalence of HCV GT-4 in Brazil, this genotype has not been studied extensively in prospective trials evaluating treatment outcomes. The lack of genotype 4 in our samples indicates that it is absent or rarely present in our population. Because many people in Bahia are of African descent and Brazil is a country with continental proportions, it is possible that uncommon Brazilian HCV strains in other regions of Brazil may be the result of multiple introductions of genotype 4[12].

Therefore, we believe that HCV patient numbers are inexact in some municipalities of the state of Amazonas, a poor region of the country affected by this endemic disease, where the only public access to advanced technology for diagnosis and treatment is at the FMT-HVD and Araújo Lima Ambulatory, the Federal University of Amazonas. In addition, the natural history of hepatitis C and the limited education of the population in some of these municipalities hinder patient notification and treatment.

This case report may contribute to demonstrate that there is HCV GT-4 in the North of Brazil and these individuals carrying this genotype have developed response to the DAAs.

Acknowledgments

We are greatly thankful to the *Fundação de Medicina Tropical Dr. Heitor Vieira Dourado* and *Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas* for providing technical support for the development and implementation of this study.

Conflict of interest

The authors declare that there is no conflict of of interest.

Financial support

Fundação de Amparo à Pesquisa, Programa de Apoio a Núcleos emergentes de Pesquisa (PRONEN 009/2011).

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Combination of genetic polymorphisms in TLR influence cytokine profile in HCV patients treated with DAAs in the State of Amazonas

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ABSTRACT

Hepatitis C is a public health problem and affects approximately 3% of the world's population. HCV infections have a wide spectrum of clinical manifestations, and several single nucleotide polymorphisms (SNPs) in the genes of the toll-like receptors are cited to influence the clinical outcomes. A cross-sectional study was conducted in the Amazonas State, Brazil in which SNPs in *TLR4* and *TLR9* genes were genotyped by PCR-RFLP in 151 HCV chronic liver disease patients and 206 healthy donors. The circulating cytokines IL-6, TNF, IL-10, IL-2, IFN-γ, IL-4 and IL-17A were measured by cytometric bead array (CBA) which revealed that the combined genotypes of *TLR9*-1237T/T and -1486G/T seem to influence the cytokine profile under lipopolysaccharide (LPS) stimulation of the Th17 profile, especially among patients with advanced chronic liver disease when treated with DAAs.

1. Introduction

Chronic hepatitis C (CHC) is a major global health problem worldwide and in 2018 more than 71 million people suffered chronic hepatitis C infections [1]. The liver damage which occurs during chronic infection is commonly attributed to mechanisms mediated by the host immune response associated with a marked release of inflammatory mediators, which favors changes in the patterns of adhesion molecule expression and recruitment of cells to the hepatic inflammatory tissue microenvironment [2]. Immunological events linked to chronic hepatitis C can lead to dysregulated architecture and function of the liver and increases intestinal permeability through the release of cytokines that alter the portal circulation and intestinal epithelial junctions, which allows the LPS derived from the intestinal microbiota to enter the portal circulation [3–5].

Previous studies showed that TLR-4 and TLR-9 pathways can activate hepatic stellated cells (HSCs) in patients with advanced liver disease through an increase of LPS levels in systemic/portal vein blood and DNA from dying hepatocytes, respectively [6–9]. Several studies have already investigated the association between TLR single nucleotide polymorphisms (SNPs) and different outcomes in HCV infection [10], and direct-acting antivirals (DAAs) are known to inhibit the tumor suppressor p53 in patients with successful clearance of HCV, though the inflammatory process continues to persist and places patients at risks of developing HCC [11].

Clearance of HCV in patients treated with interferon-free therapy and the genetic background of the host may contribute to the progression of the disease via the persistence of the inflammatory process after virologic immune response [12–17].

In the present study, we analyzed whether the polymorphisms in *TLR4* rs4986790 (*A299G* A/G), *TLR4* rs4986791 (*T399I* C/T), *TLR9* rs5743836 (*-1237C/T*) and *TLR9* rs187084 (*-1486C/T*) are associated with susceptibility to HCV infection and cytokine profile changes after treatment with DAAs in the sample group of the population from the Amazonas state.

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2. Material and methods

2.1. Ethics approval

This study was approved by the Ethics Committee of the Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas (HEMOAM) (1.405.965/2015 and 00240112000-10/2010). Participants read and signed the written informed consent form prior to the enrollment in the study, in accordance with the Declaration of Helsinki and Resolution 466/12 of the Brazilian National Health Council regarding research involving human subjects. All patients were treated according to the recommendations of the Brazilian Ministry of Health [18].

2.2. Samples and clinical data

The study was carried out at the Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD) in Manaus, the capital city of the Amazonas State, during 2016–2017. The study population was a non-probability convenience sample that consisted of 206 healthy blood donors (HD) who were randomly included during donations at HEMOAM and 151 HCV infected patients treated at the FMT-HVD. All the 357 participants were submitted to a serological screening at HEMOAM, which is recommended by Brazilian Blood Donor Bank Authorities in order to monitor blood borne pathogens and includes serological analysis for the Hepatitis B and C virus, HIV, DENV, HTLV, Syphilis and Chagas Disease. Among the HCV infected patients, 84 and 67 had ≤F2 and > F2, respectively.

2.3. Genomic DNA extraction

10 mL of blood were collected by venipuncture from each participant in two tubes, one containing EDTA (BD Vacutainer® EDTA K2) and one tube containing Gel separator (Gel BD SST® II Advance®) for carrying out the genotyping of polymorphisms and assay of circulating cytokines, respectively. Sera samples were kept at −80 °C until cytokines were assayed. Genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's instructions. DNA samples were quantified with a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) to evaluate the concentration and purity of nucleic acids.

2.4. Genotyping of TLR

The following polymorphisms, *TLR4* rs4986790, *TLR4* rs4986791, *TLR9* rs5743836 and *TLR9* rs187084 were investigated. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used for allelic discrimination as described previously [19–21]. The PCR reaction for each SNP consisted of 1 μL genomic DNA (~20 ng) added to 24 μL amplification mix containing 0.2 μL (2U) Platinum®Taq polymerase (Thermo Fisher Scientific), 2.5μL10x buffer (100 mmol/L Tris-HCl (pH 8.3) and 500 mmol/L KCl), 1 μL MgCl2 (1.5 mmol/L), 1μLdNTPs (40 mmol/L), 0.5 μL each of forward and reverse primer (0.25 pmol/L) and 18.3 μL ultrapure dH_2O. A total of 10 μL of PCR product was digested with 5 U of respective restriction endonuclease: NcoI, HinfI, BstNI, and AflII (New England Biolabs, Ipswich, MA, USA) in enzyme buffer, according to the manufacturer's instructions. The primers, PCR protocols conditions, and restriction endonucleases are shown in Supplementary Table 1. The fragments generated by PCR-RFLP were separated by electrophoresis in either a 2% or 4% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA), and visualized with the UV light Gel Doc™ XR + System (Bio-Rad Corporation, Hercules, CA, USA) with a photo documentation system.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2020.155052.

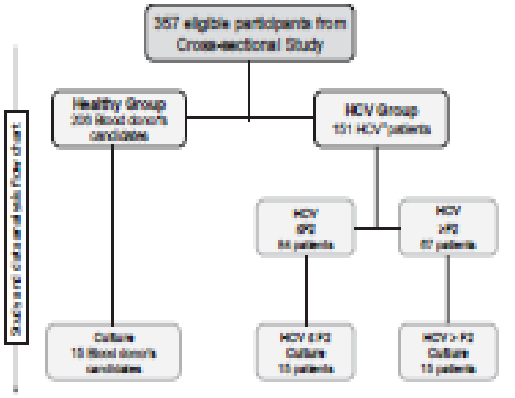

Fig. 1. Study and data analysis flow chart. We included 357 eligible participants:206 Healthy blood donors and 151 that were infected with HCV and were attended by FMT-HVD. Of these, thirty biological samples were collected for cell culture after being obtained from patients with chronic hepatic disease who were treated with direct-acting antivirals (DAAs) and sustained virological response (SVR).

2.5. Isolation of peripheral blood mononuclear cells (PBMCs) and cell culture

PBMCs were collected from 45 individuals for culture assay as described previously [22]. 15 samples were from healthy donors (HD) and 30 from HCV infected patients with chronic liver disease (15 ≤ F2 and 15 > F2) who were treated with DAAs and sustained virological response (SVR) confirmed by the absence of viral RNA by quantitative molecular tests (Fig. 1). PBMCs from each participant were isolated from 20 mL of venous venipuncture in four tubes containing Heparin (BD Vacutainer® PST™), using the Ficoll-Hypaque protocol. After centrifugation, PBMCs were collected from the interface and washed with RPMI 1640 medium. After washing twice, the supernatant was discarded and the PBMCs were resuspended at a concentration of 2×10^6 cells/ml in 4 mL of RPMI supplemented with 1% streptomycin and 1.0% fetal bovine serum and incubated in 5% CO_2 at 37 °C for 12 h. LPS stimulus was performed in duplicate using 100 μL of exogenous endotoxin (LPS/1 μg / mL).

2.6. Serum cytokine assay

The cytokines IL-6, TNF, IL-10, IL-2, IFN-γ, IL-4 and IL-17A in human blood serum and supernatant of PBMC culture were measured by Cytometric Bead Array (CBA), Kit BD® Human Th1/Th2/Th17 Cytokine (Cat: N° 560484, Lot: 29132, BD® Biosciences, San Diego, CA, USA) according to the manufacturer's technical guidelines and protocols. A FACSCanto II flow cytometer (BD® Biosciences) at the HEMOAM was used for sample acquisition. The FCAP-Array software v3 (Soft Flow Inc, USA) was used to calculate the cytokine levels.

2.7. Genotype association test and statistical analysis

Comparison between groups was performed with the chi-squared (χ^2) or Fisher's exact test with 95% confidence interval (CI). The Hardy-Weinberg equilibrium (HWE) was determined by comparing the frequency of the observed and expected number of genotypes. Tests for Hardy-Weinberg equilibrium were performed using an online application (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Graphpad Prism v.5 software (San Diego, CA, USA) was used for comparative analyses of cytokines among the genotype combination of the SNPs studied. The continuous variables presented a non-normal distribution and data were analyzed with a non-parametric Mann-Whitney test to compare

Table 1
Clinical and demographics characteristics of the study population.

Variables	Healthy Donors	HCV patients		
		All	≤F2	> F2
	(n = 206)	(n = 151)	(n = 84)	(n = 67)
Age (mean ± SD)	32 ± 11	58 ± 11	55 ± 11	61 ± 9
Gender (male/female)	134/72	83/68	45/39	38/29
ALT (IU/L)	–	69.2 ± 56.8	59.8 ± 44.6	80.8 ± 67.4
AST (IU/L)	–	63.2 ± 40.3	44.1 ± 25.8	86.8 ± 48.7

pairs. A Spearman correlation test was performed to assess the association between the levels of each cytokine tested. The positive and negative correlations are considered significant when $p < 0.05$. The correlation index (r) was used to categorize the correlation strength as weak ($r \leq 0.35$), moderate ($r \geq 0.36$ to $r \leq 0.67$), or strong ($r \geq 0.68$), as previously described [23]. Networks were assembled to assess the associations among the circulating cytokines. Significant correlations were compiled using the open access software Cytoscape v3.3 (Cytoscape Consortium, San Diego, CA), as previously reported [24,25]. Statistical significance was considered $p < 0.05$ in all cases.

3. Results

3.1. Baseline demographic, laboratorial and clinical characteristics of the study population

The median age of the healthy blood donors and HCV patients were 32 and 57 years, respectively ($p < 0.0001$). In both groups, male subjects were predominant (65% and 55%). Of note, the healthy blood donors were randomly selected and are younger than the HCV patients since the development of the disease may take 15–20 years to occur. At the time of blood collection, the HCV group were ≤F2 (55.6%) and > F2 (44.4%), and presented statistical differences in follow-up markers (AST $p < 0.0317$ and ALT $p < 0.0001$), as shown in Table 1.

3.2. Polymorphisms in TLR4 and TLR9 are not associated in patients with chronic liver disease

In order to systematically examine the association of TLR4 and TLR9 SNPs with HCV infection, allelic and genotypic frequencies of two TLR4 SNPs (rs4986790 and rs4986791) and two TLR9 SNPs (rs5743836 and rs187084) were analyzed and the results are shown in Table 2. Homozygosity for TLR4 A299G G/G and TLR4 T399I T/T was absent in the studied population. The genotype distributions for TLR4 A299G G/G, TLR4 T399I T/T, TLR9 -1237C/T and TLR9 -1486C/T were not significantly different between the groups. Carriers of the TLR9 -1237 T/T and TLR9 -1486C/T variants represent 40% of the studied population. All the SNPs studied were in HWE, only TLR9

Table 2
Genotypes and alleles association of TLRs polymorphisms in HCV patients.

Polymorphism, Genotype or Allele	Healthy Donor's	HCV patients	OR (CI 95%)	(p) value	HCV ≤F2	HCV > F2	OR (CI 95%)	(p) value	
	(n = 206)	(n = 151)			(n = 84)	(N = 67)			
TLR4 A299G (rs4986790)									
A/A	196 (95%)	147 (97%)	0.656 (0.229–1.86)	0.447	81 (96%)	66 (99%)	0.656 (0.043–4.022)	0.429	A/A vs A/G
A/G	10 (5%)	4 (3%)			3 (4%)	1 (1%)			
G/G	–	–			–	–			
A	402 (97%)	298 (99%)	0.662 (0.224–1.958)	0.452	165 (98%)	133 (99%)	0.414 (0.043–4.021)	0.660	A vs G
G	10 (3%)	4 (1%)			3 (2%)	1 (1%)			
TLR4 T399I (rs4986791)									
C/C	199 (96%)	146 (96%)	0.852 (0.296–2.064)	0.834	80 (95%)	66 (99)	0.303 (0.033–2.777)	0.264	C/C vs C/T
C/T	7 (4%)	5 (4%)			4 (5%)	1 (1%)			
T/T	–	–			–	–			
C	405(98%)	297 (98%)	0.853 (0.299–2.032)	0.835	164 (98%)	133 (99%)	0.308 (0.034–2.791)	0.309	C vs T
T	7(2%)	5 (2%)			4 (2%)	1 (1%)			
TLR9 -1237C/T (rs187084)									
T/T	137 (66%)	99 (65%)	0.286 (0.032–1.496)	0.115	56 (67%)	43 (64%)	1.204 (0.105–7.420)	0.521	C/C vs C/T + TT
C/T	67 (33%)	47(31%)	0.281 (0.032–1.508)	0.117	25 (30%)	22 (33%)	1.320 (0.202–8.629)	0.771	C/C vs C/T
C/C	2(1%)	5 (4%)	0.289 (0.032–1.520)	0.120	3 (3%)	2 (3%)	0.868 (0.139–5.420)	0.07	C/C vs T/T
T	341 (83%)	245(81%)	0.892 (0.609–1.314)	0.572	137 (82%)	108 (80%)	1.064 (0.596–1.898)	0.833	C vs T
C	71(17%)	57(19%)			31 (18%)	26 (20%)			
TLR9 -1486C/T (rs187084)									
C/C	26 (13%)	17 (11%)	1.065 (0.548–2.069)	0.851	8 (10%)	9 (13%)	0.678 (0.247–1.864)	0.400	C/C vs C/T + TT
C/T	114(55%)	93 (62%)	1.100 (0.600–2.360)	0.617	54 (64%)	39 (60%)	0.642 (0.222–1.872)	0.400	C/C vs C/T
T/T	66 (32%)	41 (27%)	0.824 (0.407–1.801)	0.632	22 (26%)	19 (27%)	0.768 (0.247–2.384)	0.647	C/C vs T/T
C	166 (40%)	127 (42%)	0.893 (0.653–1.223)	0.479	70 (42%)	57 (43%)	0.965 (0.609–1.529)	0.878	C vs T
T	246 (60%)	175 (58%)			98 (58%)	77 (57%)			

Table 3
Association of genotypic combinations of different polymorphisms with HCV patient's susceptibility.

Polymorphism, genotypic combination	Healthy Group	HCV patients	OR (CI 95%)	(p) value	HCV <F2	HCV > F2	OR (CI 95%)	(p) value
	(n = 206)	(n = 151)			(n = 84)	(n = 67)		
TLR9 -1237C/T (rs5743836) / TLR9 -1486C/T (rs187084)								
TT/TT	43(20%)	22(15%)	1.38 (0.78–2.42)	0.267	14(17%)	9(13%)	1.28 (0.52–3.19)	0.622
TT/CC	18 (9%)	12(8%)	1.10 (0.51–2.37)	0.848	4(5%)	8(12%)	0.26 (0.10–1.28)	0.124
CT/CT	35 (17%)	20(13%)	0.86 (0.49–1.48)	0.675	16(19%)	12(18.5%)	0.97 (0.43–2.20)	1.000
TT/CT	78(38%)	64(42%)	0.82 (0.53–1.27)	0.443	38(45%)	26(39%)	1.30 (0.67–2.50)	0.507
CT/CC	8 (4%)	5 (3%)	1.18 (0.37–3.68)	1.000	4(5%)	1(1.5%)	3.30 (0.35–30.26)	0.382
CC/TT	1 (0.5%)	4(3%)	0.17 (0.01–1.62)	0.166	3(3%)	1(1.5%)	2.44 (0.24–24.07)	0.628
CC/CC	–	–	–	–	–	–	–	–
CC/CT	1(0.5%)	1(1%)	0.73 (0.04–11.80)	1.000	–	1(1.5%)	0.26 (0.01–6.54)	0.443
CT/TT	24 (11%)	13 (9%)	1.40 (0.68–2.84)	0.384	5(6%)	8 (12%)	0.46 (0.14–1.50)	0.246

-1486C/T SNP deviated slightly, in both HCV patients (p = 0.002) and HD (p = 0.04).

3.3. Lack of association of genotypic combinations of polymorphisms in *TLR9 -1237T/T* and *-1486C/T* with chronic liver disease

None of the polymorphisms of TLR4 and TLR9 analyzed in this study exhibited any significant association with chronic liver disease. TLR4 A299G A/A + A/G and TLR4 T399I C/C + C/T variants were the most frequent in the study population – 97% + 3% and 96% + 4%, respectively. We therefore assessed the influence of the combination genotypes for TLR9 variants -1237T/T and -1486C/T on the cytokine profile in HCV patients treated with DAAs. Statistical analysis revealed that combination of TLR9 -1237T/T and TLR9 -1486C/T variants was higher in the HD and HCV groups when compared to other genotype combinations between the groups, as can be seen in Table 3.

3.4. Influence of genotypic combinations of polymorphisms in TLR9 -1237T/T and -1486C/T on the circulating profile of cytokines in HCV patients

Human antiviral response against HCV is characterized by induction of Th1 cytokines profile. Analysis of genotypic combinations in regard to the Th1, Th2, Th17 and Treg cytokines profiles among the studied groups indicated a different profile between the HCV patients in regard to liver damage. A significant increase of IL-6 (p = 0.005) and IL-4 (p = 0.0007) (Fig. 2A and F) was observed in the serum of these patients compared with HD. The cytokines TNF, IL-10, IL-2, IFN-γ and IL-17A did not show any differences between HCV patients and HD group (Fig. 2B-E and G). A significant increase of IL-10 was observed in HCV > F2 patients (p = 0.028) compared to ≤F2, whereas the cytokines IL-6, TNF, IL-2, IFN-γ, IL-4 and IL-17A were not significant when compared between HCV patients with different stages of liver disease (Fig. 3A).

3.5. Influence of genotypic combinations regarding cytokine profiles in the culture supernatant stimulated with Lipopolysaccharide (LPS)

No significant difference in IL-6, TNF, IL-10, IL-2, IFN-γ and IL-4, (Fig. 3B) was observed on stimulated PMBCs with LPS. Only, IL-17A showed a significant increase in the HCV > F2 patients (p = 0.043) when compared to the ≤F2 group (Fig. 3B).

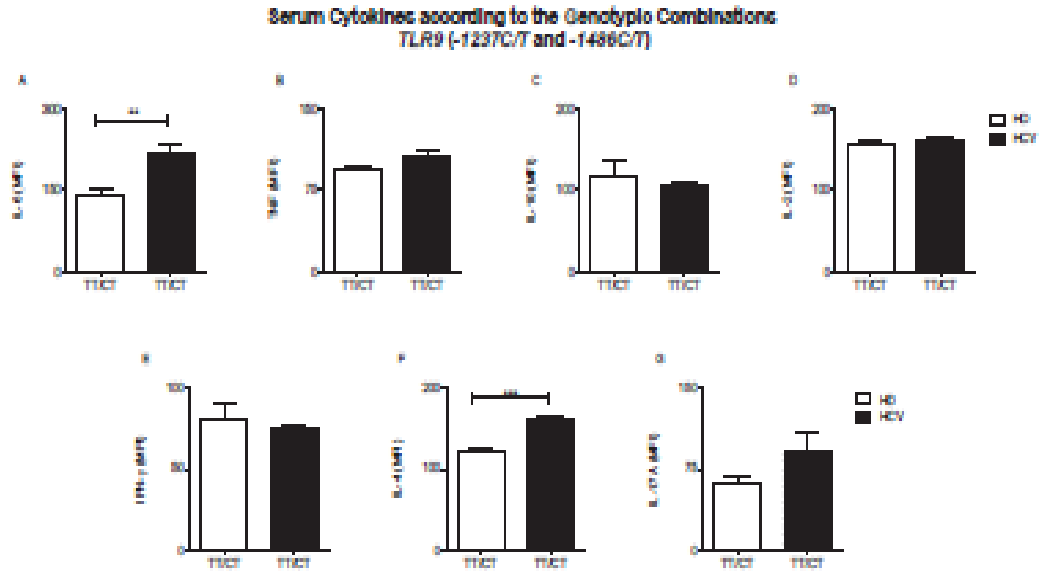

Fig. 2. Concentration in Mean Fluorescence Intensity (MFI) of serum cytokines according to the genotypic combinations (rs5743836 / rs187084) between the control group (HD) and HCV patient group (HCV). Results are expressed as median and standard deviation. Statistical analyses were performed using the non-parametric Mann Whitney test. Significant statistical difference was considered when *p < 0.05, **p < 0.01 and *** < 0.0001.

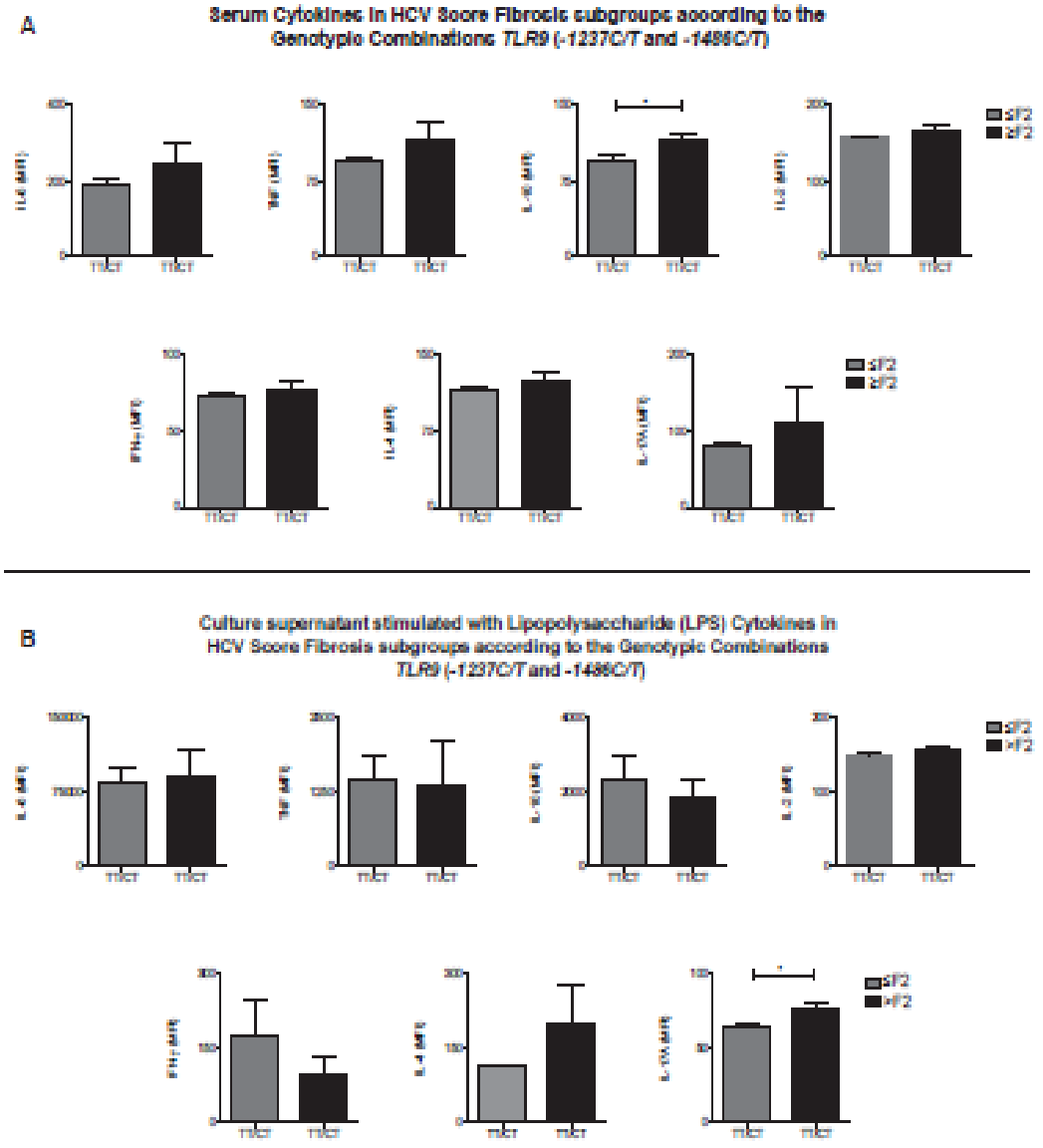

Fig. 3. (A) Concentration in Mean Fluorescence Intensity (MFI) of serum cytokines according to the genotypic combinations (rs5743836/ rs187084) between HCV patient group (≤ F2 and > F2). (B) Concentration in Mean Fluorescence Intensity (MFI) in the culture supernatant stimulated with Lipopolysaccharide (LPS) (1 μg/ml), according to the genotypic combinations (rs5743836/ rs187084) in HCV patient group (≤F2 and > F2). Results are expressed as median and standard deviation. Statistical analysis: were performed using the non-parametric Mann Whitney test. Significant statistical difference was considered when *p < 0.05, **p < 0.01 and *** < 0.0001.

3.6. Lipopolysaccharide (LPS) changes the interaction between of cytokines in HCV patients

To test the relationship between altered levels of cytokines after LPS stimulus, a series of correlation analyses was performed (Fig. 4). The numbers of interactions between the cytokines analyzed were also different according to the hepatic score in HCV patients. No interactions were observed in the HD group carrying the same genotype combinations as the HCV group, which exhibited a strong positive relation between IL-6, IL- 10, IL-2 and IL-17A (Fig. 4A). A moderate relation between IL-6, TNF and IL-10 was also observed in the HCV group. HCV ≤F2 patients exhibited a strong positive relation between IL-6, IL-10 and TNF. In addition, in HCV > F2 patients, a strong positive correlation between IL-6, IL-10, IL-2 and IL-17A was preserved, while IL-6, IL-17A and IL-10 exhibited a strong negative correlation (Fig. 4B).

4. Discussion

The results of the present study revealed the low frequency of polymorphic alleles in *TLR4* (rs4986790 and rs4986791) in the sample group of the population from the Amazon state. Studies conducted using other Northern Brazilian samples have reported frequencies of

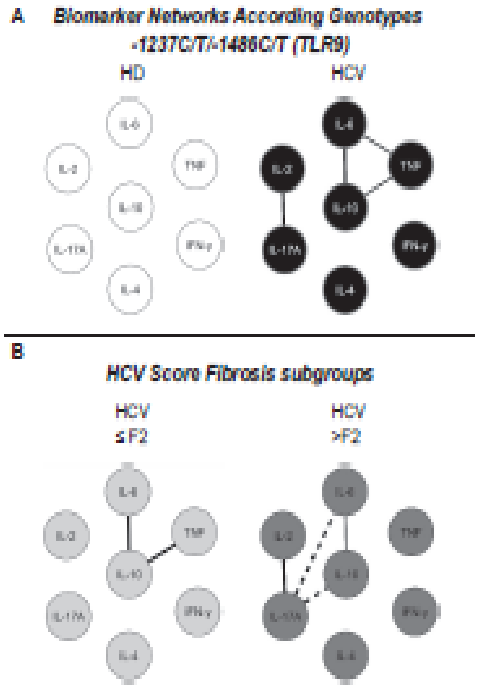

Fig. 4. "Cytokine Networks" in the HCV patients according to the fibrosis score. A. Concentration in Mean Fluorescence Intensity (MFI) in the culture supernatant stimulated with Lipopolysaccharide (LPS) (1 μg/ml), according to the genotypic combinations (rs5743836/ rs187084) between the control group (HD) and HCV patient group (HCV) (A) and patient group (≤ F2 and > F2) (B). Customized biomarker network layouts were built to identify the relevant association between proinflammatory IL-6, TNF, IL-2 and IL-17A cytokines, modulatory IL-10 axis, using a clustered distribution of nodes. Significant Spearman's correlations at $P < 0.05$ were represented by connecting edges to highlight positive (strong ($r \geq 0.68$; thick continuous line) or moderate ($0.36 \geq r \leq 0.67$; thin continuous line) and negative (strong ($r \leq -0.68$; thick dashed line) or moderate ($-0.36 \geq r \leq -0.67$; thin dashed line) as proposed by Taylor (18). The overall statistical analysis of the network node neighborhood connections point to an almost linear-chain pattern in the HCV groups with a clear shift towards a more intricate profile in HCV patients > F2. A persistent IL-6/IL-10 loop was observed in all HCV subgroups with differential neighborhood connections for the IL-17A node in HCV patients > F2.

heterozygotes similar to those found in our study [19–21,26,27]. Increasing evidence suggests that the rs4986790 allele of *TLR4* that leads to an amino acid change (Asp299Gly) might interfere with the interaction between the receptor-ligand and the protein's stability. This may cause deficiency in the recruitment of Myeloid differentiation primary response: 88 (MyD88) and TIR-domain-containing adaptor-inducing interferon-β (TRIF), and contribute to the increase risk of bacterial infections and a decrease in the proinflammatory response in patients with cirrhosis [28–35]. Our data does not show any association between these SNPs and susceptibility to HCV infection and hepatic impairment.

Another important receptor that also participates in this process of hepatic disease evolution is TLR-9, which recognizes the CpG-rich DNA of bacteria. Watanabe et al., 2007 demonstrated that host-derived denatured DNA from apoptotic hepatocytes stimulates HSCs through TLR-9 in hepatic fibrosis [9]. Several studies showed possible associations between *TLR9* gene SNPs and a greater risk of cancer [10,36]. The T allele of the TLR9 rs5743836 is transcribed more effectively under basal conditions compared to the variant C allele [37,38].

IL-6 up-regulates TLR9 expression and leads to exacerbated cellular responses to CpG, including IL-6 production and B-cell proliferation in mononuclear cells carrying the TLR9 -1237C/T SNP [39]. Furthermore, the increased transcriptional activity of TLR9 in mononuclear cells from patients harboring TLR9 -1237C/T SNP supports a functional effect of this polymorphism on non-Hodgkin lymphoma susceptibility [40]. It has been suggested that TLR9 -1486C/T heterozygote may be a genetic risk factor for cervical cancer [41]. The C allele of TLR9 -1486C/T SNP showed lower TLR9 expression and correlated with the risk of systemic lupus erythematosus [42]. TLR9 expression by fibroblast-like cells has been shown to be significantly correlated with shortened overall survival in patients with hepatocellular carcinoma [43].

Successful treatment of hepatitis C is associated with SVR and declining liver inflammation through a decrease in cytokine and chemokine levels during the treatment of hepatitis C [44] and the DAAs dramatically increased the number of patients that will be able to clear a hepatitis C virus infection with the use of this new medication. However, risk of developing hepatocellular carcinoma (HCC) after DAAs is still a great concern among patients with advanced cirrhosis. It is known that the ER-stress response and p53 are normalized after HCV clearance when induced by interferon-alpha-based antiviral therapies, whereas HCV clearance by DAAs does not restore p53. The p53 is a tumor suppressor and coordinates diverse cellular responses to stress, damage, and in the suppression of autoimmune and inflammatory diseases [11,45].

TLRs may play an important role in a wide range of human diseases by triggering early events in the immunological response. The polymorphisms studied have been associated with greater risks of cancer and the most frequently found genotype combination in our population is TLR4 299A/A, TLR4 299C/C, TLR9 -1237T/T and TLR9 -1486C/T.

The data presented in this study suggest the influence of combination of *TLR9* (-1237T/T and -1486C/T) on the production of circulating cytokines IL-6 and IL-4 in HCV patients when compared to healthy donors. Furthermore, these combined polymorphisms also affect the production of circulating IL-10 in patients with moderate fibrosis. These cytokines are important in chronic liver disease caused by chronic HCV infection. Nisto et al., (2014) observed a spontaneous increase in IL-6 and IL-10 production in patients with cirrhosis which carried the wild-type variants of *TLR4* A299G and/or T399I [46]. Yet, the production of IL-6, TNF-α and IL-10 after TLR stimulation was similar between the patients. This different pattern of cytokine production could be a factor in the development of complications in cirrhosis [46].

An increase of IL-17A in PBMCs in patients who had a greater severity of hepatic disease and were treated with DAAs was observed in this study. LPS can up-regulate the expression of several TLRs and nuclear factor kappa B (NF-kB), extracellular-signal-regulated kinase (ERK) and p38 kinase signal pathways. This phenomenon might explain the synergy between bacterial DNA and LPS in activating macrophages against invading bacteria [47,48].

LPS also downregulated the expression of p53 protein in mouse Kupffer cell and RAW 264.7 macrophage cell line [49]. This observation may contribute to the hypothesis that the p53 inhibition perpetuates through TLR4 activation by LPS in patients with moderated fibrosis. In fact, the p53 tumor suppressor protein is related to carcinogenesis and is suggested to cause prolonged NF-κB activation at a late stage after LPS stimulation and possibly sustain prolonged proinflammatory responses [49]. Several inflammatory factors encoded by NF-κB target genes and IL-6 are important activators of transcription 3 (STAT3). NF-κB and STAT3 regulate the expression of numerous oncogenic and inflammatory mediators, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatic growth factor (HGF), IL-10, IL-17 and IL-6, which favor the emergence of tumors [50]. In this study, the interactions between the cytokines analyzed seem to change after LPS stimulation. A strong positive correlation between IL-6, IL-10, IL-2 and IL-17A was observed.

The present study reveals different interactions between cytokines in HCV patients according to polymorphism combinations and liver damage. During the disease, the changes in the cytokines' dynamic

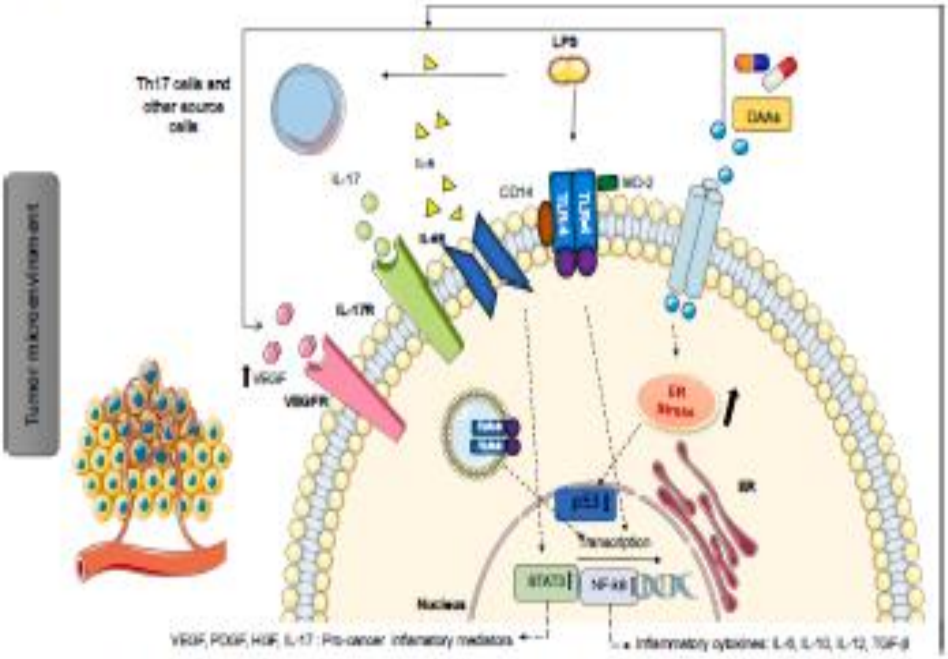

Fig. 5. Schematic diagram for the role of the interplay between the effects caused by DAAs and sever chronic hepatic disease.

connections during the disease associated with these genotypes may lead to a progressive oncogenic milieu. This would signal an increased risk of cancer in patients treated with DAAs who carry the combination of TLR9 (-1237T/T and -1486C/T) even after successful viral clearance treatment (Fig. 5). In addition, understanding the influence of these polymorphisms may be useful in clinical practice for designing new therapeutic scenarios.

This study has some limitations. The recruitment of the group of healthy donors served randomly as a normal parameter for analysis purposes. Nevertheless, age discrepancy between patients and healthy donors is still a limitation. The study population size is small and may influence in the levels of associations with HCV infection and disease severity. The small sample size does not allow intra-comparison of the genotype combination when studied with the profile of cytokines. However, it did show that the combinations of these polymorphisms seem to influence chronic hepatic disease, although more studies are needed in order to confirm this preliminary finding.

5. Conclusion

Altogether, our findings demonstrated that TLR9 -1237T/T and -1486C/T variants may change the cytokine correlations between IL-6, IL-10, IL-2 and IL-17A, especially among patients with advanced chronic liver disease, and who are treated with DAAs. The possibility of these patients presenting an unresolved ER stress after HCV clearance by DAAs is linked to the genetic background, and the pathology already installed could increase the risk of HCC development. Our results suggest the relevance of these sets of biomarkers for further clinical investigations, with a view to being used as a potential tool for predicting adverse outcomes in patients with chronic hepatic disease caused by HCV infection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Anexo 04: Produção científica colaborativa

COSTA, AG; RAMASAWMY, RAJENDRANATH ; VAL, FERNANDO FONSECA ALMEIDA; IBIAPINA, HIOCHELSON NAJIBE SANTOS; OLIVEIRA, AMANDA CARVALHO; **TARRAGÔ, ANDREA MONTEIRO**; GARCIA, NADJA PINTO; HECKMANN, MARIA IZABEL OVELLAR ; MONTEIRO, WUELTON MARCELO; MALHEIRO, ADRIANA; LACERDA, MARCUS VINÍCIUS GUIMARÃES. Polymorphisms in TLRs influence circulating cytokines production in Plasmodium vivax malaria. CYTOKINE , v. 104, 2018.

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