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FREQUÊNCIA DE POLIMORFISMOS E MUTAÇÕES DE RESISTÊNCIA NOS GENES NS3, NS5A E NS5B E BARREIRA GENÉTICA AOS ANTIVIRAIS DE AÇÃO DIRETA EM SEQUÊNCIAS DEPOSITADAS NO BANCO DE DADOS EUROPEU DE HEPATITE C

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Tese submetida ao Programa de Pós-Graduação em Hepatologia da Fundação Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Doutor.

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RESUMO

Introdução: A infecção pelo vírus da hepatite C (HCV) afeta mais de 180 milhões de pessoas em todo o mundo. O HCV tem ampla diversidade genética, existindo no sangue de pessoas infectadas como quasispécies. O desenvolvimento de novos antivirais de ação direta (DAA) resultou em terapias orais sem necessidade do uso de interferon, com três alvos terapêuticos principais: NS3/4A protease, NS5B polimerase e inibidores do complexo NS5A.

Objetivo: O objetivo deste trabalho foi analisar o impacto da variabilidade genética do HCV na barreira genética para o desenvolvimento de resistência aos DAA's e a ocorrência de polimorfismos e mutações de resistência nas regiões que codificam as proteínas virais NS3, NS5A e NS5B, com base em sequências genéticas de HCV depositadas em bancos de dados.

Métodos: O estudo baseou-se na análise de sequências de HCV oriundas de pacientes não tratados depositadas no *European Hepatitis C Virus database* (euHCVdb). Foram analisadas todas as sequências que codificam as proteínas NS3, NS5A e NS5B do HCV, depositadas no euHCVdb. Foram excluídas sequências incompletas, sequências contendo erros e/ou *gaps* e sequências de amostras de pacientes tratados com DAAs, resultando, para análise final, em 798 sequências da região NS3, 708 da região NS5A e 535 da região NS5B. A barreira genética foi quantificada com base no número e no tipo de mutação necessária para gerar resistência.

Resultados: Foram identificadas alterações genéticas nas sequências de HCV analisadas, as quais apresentam relação com a resistência aos antivirais. Variantes que requerem somente uma transversão nos subtipos 1a e 1b incluem NS3 F43S, R80K, R155K/G e A156T. A barreira genética à resistência mostra diferenças entre os subgenótipos na posição 155 da região que codifica NS3, onde somente uma transição é necessária no subtipo 1a. Na região da NS5A, a variante L31M requer ao menos uma transversão em todos genótipos, exceto em 0,28% das sequências. Para os inibidores da NS5B, a barreira genética nas posições que conferem resistência foi praticamente idêntica nos subtipos 1a e 1b. As posições C316Y, Y448H e S556 G/N/R e D requerem somente uma transição em todos genótipos em

até 98,8% das sequências analisadas. Uma única variante na posição 448 pode conferir alguma proteção no subtipo 1a porque requer duas transversões para tornar-se a variante resistente 448H. Com relação à ocorrência de polimorfismos, a variante Q80K na região NS3 foi a mais prevalente, sendo encontrada em 44,66% do subtipo 1a e em 0,25% do 1b. Outras substituições frequentes observadas em mais do que 2% na região NS3 foram I170V (3,21%) no subtipo1a; Y56F (15,93%), V132I (23,28%) e I170V (65,20%) no 1b. Para NS5A, as seguintes alterações que conferem resistência foram encontradas: P58S em 2,21% das sequências do subtipo 1a; R30Q em 5,95% do subtipo 1b; Q30R em 15,79% das sequências do subtipo 2a; L31M em 23,08% das sequências do subtipo 2b, e M31L em 23,08% das sequências do subtipo 3a. Para NS5B, a variante de resistência V321L foi identificada em 0,60% e em 0,32% das sequências dos subtipos 1a e 1b, respectivamente.

Conclusão: Mesmo com uma baixa frequência global de mutações observadas nos dados apresentados, esta população resistente é altamente provável de ser selecionada em pacientes que sejam submetidos a tratamento com DAA's. As variantes do HCV resistentes a uma das classes de DAA permanecem suscetíveis às outras classes, mas a terapia combinada pode falhar, devido à seleção de cepas de HCV com substituição de resistência, especialmente porque a análise de barreira genética revelou que em 14 de 16 posições a conversão para uma variante resistente requer somente a substituição de um nucleosídeo.

Palavras-chave: HCV; antivirais de ação direta; hepatites virais; resistência viral; quasispécies.

ABSTRACT

Background & Aims: Hepatitis C virus (HCV) infection affects around 180 million people worldwide. HCV has enormous genetic diversity in infected hosts, existing in blood as quasispecies. The development of new direct-acting antiviral (DAA) drugs resulted in oral interferon-free therapies, with three main therapeutic targets: the NS3/4A protease, NS5B polymerase, and NS5A replication complex. The aim of this study was to analyze the impact of genetic variability on the genetic barrier to drug resistance to DAA's and the occurrence of polymorphisms and resistant mutations in the genome regions that code for NS3, NS5A and NS5B proteins in HCV sequences deposited in databanks.

Methods: The study included sequences of HCV, from samples of treatment-naïve patients, deposited in theEuropean Hepatitis C Virus database (euHCVdb). All sequences coding for NS3, NS5A and NS5B were analyzed. Sequences containing errors and/or gaps or incomplete sequences, and from patients previously treated with DAAs were excluded; the final analyses included 798 sequences for the NS3 region, 708 for NS5A and 535 for NS5B. The genetic barrier was quantified based on the number and type of nucleotide mutations required to impart resistance.

Results: Genetic alterations related to antiviral resistance were identified in the HCV sequences analyzed. Variants that require only one transversion in the NS3 region of subtypes 1a and 1b include F43S, R80K, R155K/G and A156T. The genetic barrier to resistance shows subtypic differences at position 155 of the NS3 region, where a single transition is necessary in subtype 1a. In the NS5A region, the L31M variant required at least one transversion in all subtypes, except in 0.28% of subtype 1b sequences. For the NS5B inhibitors, the genetic barrier at positions conferring resistance was nearly identical in subtypes 1a and 1b. The positions C316Y, Y448H and S556 G/N/R and D required only one transition in all genotypes for up to 98.8% of the sequences analyzed. A single variant in position 448 can confer some protection in genotype 1a because it requires two transversions to become the resistance variant 448H. Regarding the occurrence of polymorphisms, the Q80K variant in the NS3 region was the most prevalent, found in 44.66% of subtype 1a and in 0.25% of subtype 1b sequences; other amino acid substitutions observed in more

than 2% of the NS3 sequences were: I170V (3.21%) in genotype 1a, and Y56F (15.93%), V132I (23.28%) and I170V (65.20%) in 1b. For the NS5A regions the substitutions observed were: P58S in 2.21% of the subtype 1a sequences, R30Q in 5.95% of subtype 1b, Q30R in 15.79% of subtype 2a, L31M in 23.08% of subtype 2b sequences, and in subtype 3a, 23.08% of the sequences had M31L resistant variants. For the NS5B region, the V321L resistance-associated variant was identified in 0.60% and 0.32% of genotypes 1a and 1b sequences, respectively.

Conclusions: Despite the overall low frequency of mutations as observed in our data, this resistant population is likely to be able to be selected in the patients undergoing therapy with DAAs. HCV variants resistant to DAA targeting one viral protein remain susceptible to DAAs targeting another viral protein, but combination therapy could failure due to selection of HCV with resistance substitutions, especially because the genetic barrier analysis revealed that in 14 of 16 positions conversion to a drug-resistant variant of HCV required only single nucleotide substitutions.

Key-words: HCV; direct acting antivirals; viral hepatitis; virus resistance; quasispecies.

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LISTA DE ABREVIATURAS

DNA: Ácido Desoxiribonucleico

RNA: Ácido Ribonucleico

HCV: Vírus da Hepatite C

CHC: carcinoma hepatocelular

GBV-B: GB vírus B

ORF: *Open Reading Frame*

UTR: *Untranslated Region*

IRES: *internal ribosome entry site*

miR: micro RNA

U: uracil

UC: uracil citosina

NS5B: proteína não estrutural 5B

E1: proteína do envelope 1

E2: proteína do envelope 2

NS2: proteína não estrutural 2B

NS3: proteína não estrutural 3

NS4A: proteína não estrutural 4A

NS4B: proteína não estrutural 4B

NS5A: proteína não estrutural 5A

HVR: região hipervariável

HVR-1: região hipervariável 1

HVR-2: região hipervariável 2

igVR: intergenotypic variable region

IFN: interferon

IRF3: fator regulador do interferon 3

LCS1: *low complexity sequences* 1

LCS2: *low complexity sequences* 2

D1: domínio 1

D2: domínio 2

D3: domínio 3

IFN – ISDR: região determinante de sensibilidade ao IFN

dsRNA: *doble stranded* RNA

LDL: *Low Density Lipoprotein*

CLDN1: claudina 1

OCLN: occludina

EGF: fator de crescimento endotelial

DAA: antivirais de ação direta

NGS: *Next-generation sequencing*

EPLD: *end-point limiting dilution*

HIV: Vírus da Imunodeficiência Humana

AASLD: Associação Americana para Estudo das Doenças do Fígado

IDSA: Sociedade Americana de Doenças Infecciosas

euHCVdb: European Hepatitis C Virus database

EASL: Associação Europeia para Estudo do Fígado

SUMÁRIO

1 REVISÃO DA LITERATURA

1.1 O Vírus da Hepatite C

Quando surgiram os primeiros testes sorológicos para hepatite A e hepatite B, durante os anos 70, logo ficou evidente que a maioria dos casos de hepatite associados à transfusão era causada por outro agente, levando à criação do termo “hepatite não A - não B”. Em 1989, um clone de DNA complementar, construído a partir de plasma infectado com o agente até então não caracterizado da hepatite não A - não B, possibilitou o isolamento de um vírus cujo genoma é composto por uma molécula de RNA senso positivo de aproximadamente 10 mil nucleotídeos, que foi chamado de vírus da hepatite C (HCV)(1). Esse achado rapidamente levou à identificação completa do genoma viral (2) e a outras descobertas importantes, como a propensão desse vírus em estabelecer uma infecção persistente e a sua forte associação com hepatite crônica, cirrose e carcinoma hepatocelular (CHC).

O HCV é um vírus grosseiramente esférico, envelopado, de aproximadamente 55 nm de diâmetro (3-8). Sua estrutura, organização genômica e replicação suportam sua classificação como membro da família *Flaviviridae.* Por ser suficientemente distinto do gênero *Flavivirus,* ao qual pertencem os vírus da febre amarela, febre do Nilo Oriental, dengue e zika, além de muitos vírus causadores de encefalite. O HCV está classificado em um gênero separado, o *Hepacivirus* (9, 10), junto com o GB vírus B (GBV-B) e os hepacivirus de não primatas encontrados em roedores, cachorros, cavalos e morcegos (11-16).

1.2 Organização do Genoma Viral

O genoma do HCV consiste de cadeia de RNA de fita única, senso positivo, de aproximadamente 9,6 kb de tamanho. Ao contrário de um RNA eucariótico típico, o HCV não possui *cap* na extremidade 5’ e nem uma cauda com poliadenilação na extremidade 3’, além de apresentar uma única fase de leitura (ORF, *Open Reading Frame*) que codifica uma única grande poliproteína de aproximadamente 3.010

aminoácidos. Essa grande ORF é flanqueada pelas regiões 5’ e 3’ não traduzidas (UTR, *Untranslated Regions*) e altamente conservadas que tem papel na tradução e na replicação do RNA viral (figura 1).

1.2.1 Segmentos não traduzidos do RNA

A região 5' UTR tem aproximadamente 341 nucleotídeos de comprimento e apresenta uma extensa estrutura secundária e terciária de RNA, contendo duas regiões sobrepostas funcionais. Os 125 nucleotídeos terminais dessa região são essenciais para a replicação viral, provavelmente pelo reconhecimento do RNA viral pela replicase, enquanto o restante da 5' UTR parece desempenhar um papel acessório no processo (17, 18). Uma sobreposição de um segmento de aproximadamente 300 nucleotídeos age com um sítio interno de entrada no ribossomo (IRES, *Internal Ribosome Entry Site*), direcionando a tradução da ORF viral independentemente da cápsula (19-26). Uma característica altamente incomum de replicação do HCV que envolve a região 5’ UTR é a ligação desta região com o uma molécula de microRNA humana complementar a essa região – o microRNA 122, que é específico do fígado (miR-122); tal interação tem sido considerada necessária para a replicação do HCV e contribui para o tropismo do HCV pelo fígado, o que também torna os microRNAs alvos terapêuticos promissores(27-29).

A região 3' UTR consiste de um segmento relativamente variável de 30 a 60 nucleotídeos posteriores ao segmento do códon de terminação que é seguido por uma cauda poli U/UC altamente variável composta de 50 a 100 nucleotídeos. Seguindo-se a essa cauda, há uma sequência de 98 bases denominada região 3’X que é o segmento mais conservado do genoma do HCV. Experimentos *in vitro* indicam que a interação entre as estruturas de RNA da região 3’-X e da região codificante NS5B, tanto quanto os 33 resíduos U na cauda poli U/UC, são absolutamente necessários para a replicação viral (30-33).

1.2.2 Poliproteína

Uma ORF de aproximadamente 9 kb codifica uma poliproteína que é traduzida em pelo menos 10 proteínas. Essas proteínas incluem o nucleo capsídeo, o core ou

cerne, e duas proteínas do envelope (E1 e E2); duas proteínas essenciais à produção de vírions, mas que não são necessárias para a replicação do RNA viral (p7 e NS2); e cinco proteínas não estruturais que formam o complexo de RNA replicase (NS3, NS4A, NS4B, NS5A e NS5B). O processamento dessas proteínas é dirigido por proteases celulares e virais. Quatro distintas sequências sinalizadoras dentro do terceiro aminoácido da poliproteína dirigem a translocação da proteína nascente dentro do retículo endoplasmático, tendo como resultado a clivagem da poliproteína pela peptidase nas junções C/E1, E1/E2, E2/p7 e p7/NS2. A proteína NS2/NS3 inicial é uma cisteíno-protease, que cliva a junção NS2/NS3, enquanto a NS3 possui atividade de serino-protease que catalisa o restante, originando uma poliproteína *trans* clivada através das proteínas não estruturais. A completa expressão da atividade protease da NS3 requer a formação de um complexo dentro da proteína NS4.

O segmento de 191 aminoácidos no final da poliproteína do HCV é clivado do polipetídeo inicial pela peptidase sinalizadora, formando um núcleo proteico altamente básico que possui atividade de ligação do RNA. Uma segunda clivagem ocorre um pouco antes da sequência dos peptídeos sinalizadores, dirigida pela peptidase dentro das membranas do retículo endoplasmático, produzindo um núcleo maduro de 173 aminoácidos que é transferido para gotículas de lipídeos, onde é associado com a NS5A (34-38). A proteína do núcleo é imunogênica e tanto ela quanto os anticorpos contra ela estão tipicamente presentes no soro de indivíduos infectados.

Peptidases sinalizadoras direcionam a clivagem da poliproteína do HCV nos resíduos 383 e 746 (posições baseadas na cepa de referência H77), produzindo as proteínas E1 e E2 do envelope, respectivamente (39). Elas são secretadas no retículo endoplasmático, dobradas na sua estrutura tridimensional, e permanecem ancoradas à membrana por uma sequência âncora carboxil-terminal hidrofóbica. Informações detalhadas relativas à estrutura das proteínas do envelope permanecem incertas, já que predições a partir de ligação de receptores, ligação de anticorpos, mutagênese ou desenhos computadorizados não têm gerado um modelo coerente,

mas ainda assim alguns domínios foram identificados. Um segmento altamente variável de aproximadamente 30 aminoácidos em tamanho próximos à extremidade amino-terminal da proteína E2 tem sido chamado de “região hipervariável” (HVR,*Hypervariable Region*) 1 (40-43) e é considerado o segmento com maior variabilidade genética do envelope; a extensão da heterogeneidade dentro da HVR-1 indica que existem poucas limitações relacionadas à sequência na sua função (44-46).

Foi sugerido que a HVR-1 pode funcionar como um decodificador imunológico durante a infecção por mascarar uma estrutura mais profunda e altamente conservada dentro do envelope, como um sítio de reconhecimento para o receptor celular (47) e que a deleção dessa região reduz, mas não elimina a infectividade do vírus (48). Adiante da região HVR-1 estão a HVR-2 e a região intergenotípica variável (igVR, *intergenotypic variable region*) que, apesar da sua variabilidade, pode estar mais diretamente envolvida na entrada do vírus na célula (49). Estas regiões são intercaladas entre os determinantes de ligação ao CD 81 e outras moléculas do hospedeiro que são necessárias para a entrada viral (50-53).

As proteínas p7 e NS2 são necessárias para a reunião das partículas virais ou para a saída da célula, mas nenhuma delas é necessária para a replicação do RNA (54). A clivagem de uma peptidase sinalizadora próxima à extremidade carboxi-terminal da E2 gera a proteína p7. A proteína NS2 é uma cisteíno-protease dimérica associada à membrana com dois sítios ativos de material composto que fazem a mediação da clivagem nas junçõesNS2/NS3 (55-59).As estruturas do domínio transmembrana e da protease da NS2são essenciais para a produção de vírions infecciosos em cultura de células, enquanto a atividade de protease não é (54). Análises estruturais e *in vitro* sugerem que a NS2 possa servir como uma ponte associada a gotículas lipídicas ligando as proteínas do envelope com a p7 e com a NS3.

1.2.3 Proteínas não estruturais envolvidas na replicação do RNA

A porção dentro da poliproteína que abrange desde a região que codifica a

NS3 até a região que codifica a NS5B é necessária para a replicação do RNA e para o agrupamento em um complexo de replicase associado à membrana interna do citoplasma das células infectadas. A NS3, quando completamente madura e totalmente ativa, requer a associação não covalente da proteína NS3 com a NS4A, a qual se torna uma parte integral da estrutura da protease (60-62). Estruturas com resoluções em nível atômico desses domínios têm sido elucidados separadamente e também em conjunto, servindo como base para o desenvolvimento de drogas antivirais (63-65). A NS3 pode se ligar à sequência 3'poli U/UC e desempenhar atividade helicase na direção de 3' para 5' (60, 66). Nenhum sítio de clivagem foi identificado entre as regiões da NS3 com atividade protease e helicase, e estudos funcionais sugerem que esses domínios são independentes (67). Adicionalmente, o domínio helicase da NS3 interage diretamente com as proteínas do núcleo durante a produção de vírions (68).

Foi demonstrado que a atividade da NS3 proteásica interfere com a sinalização mediada pelo interferon (IFN) através do bloqueio da fosforilação ativada por vírus do fator regulador do IFN 3(IRF3), proporcionando um mecanismo pelo qual o HCV pode evadir as defesas antivirais celulares inatas (69, 70). A natureza multifuncional da NS3, incluindo o processamento da poliproteína, o seu papel na RNA replicase e a sua contribuição para a evasão imune, é típica das proteínas de vírus pequenos de RNA de cadeia positiva, como é o caso do HCV.

A proteína NS4A age como um cofator para a NS3 protease. Um segmento amino-terminal da proteína ancora o complexo NS3/4A às membranas intracelulares, enquanto a NS4A também interage com a NS5A como um componente crítico para o complexo replicase. A proteína NS4B é uma proteína hidrofóbica associada à membrana que aparentemente media as modificações na membrana do retículo endoplasmático que ocorrem em associação com a reunião da replicase e que também podem inibir o caminho secretório normal do retículo endoplasmático para o complexo de Golgi (71-76).

A NS5A é uma fosfoproteína ligada ao RNA e ancorada à membrana

citoplasmática, que possui um papel na replicação do RNA, embora sua função exata permaneça obscura (77-80). Ela contém uma região de ancoragem à membrana na extremidade amino-terminal, seguida por três domínios (D1 a D3) separados por sequências de baixa complexidade (LCS1 e LCS2, *low complexity sequences*). A replicação do genoma viral depende da ligação do RNA e da dimerização mediada pelas regiões que se estendem de D1 até D2, enquanto a região D3 interage com as proteínas do núcleo nas gotículas lipídicas sendo essencial para a reunião viral (81). As porções D2 e D3 interagem com múltiplas proteínas, incluindo a ciclofilina A e uma quinase lipídica, e cada uma dessas funções é considerada um alvo terapêutico para as novas medicações (82, 83).

Estudos têm mostrado que polimorfismos dentro de um segmento curto da NS5A (região determinante de sensibilidade ao IFN – ISDR,*IFN sensitivity determining region*) apresentam correlação com resistência ao tratamento com interferon em alguns genótipos do HCV, e que isso pode ser mediado pela interação da NS5A com o domínio catalítico da proteína quinase R ativada pelo dsRNA e induzida pelo interferon.

A NS5B é uma proteína ligada à membrana que contém um motivo característico Gly-Asp-Asp de RNA polimerases dependentes de RNA e é considerada como sendo o núcleo catalítico do complexo replicase. Tal como acontece com as atividades enzimáticas da proteína NS3, a polimerase de RNA NS5B tem provado ser um alvo útil para o desenvolvimento de drogas antivirais como os inibidores análogos dos nucleosídeos/nucleotídeos e não nucleosídeos, bem como análogos de ciclosporina A.

1.3 Replicação do Vírus da Hepatite C

Os detalhes do ciclo viral do HCV foram inicialmente examinados usando replicons com RNA subgenômico competente em cultura de células e, mais recentemente, com um sistema de cultura celular que completa o ciclo de multiplicação do vírus, desde sua entrada na célula até a liberação dos vírions infectantes. Esses estudos e algumas analogias razoáveis com outros vírus com

cadeia de RNA senso positivo sugerem que o vírus entra na célula através da interação com múltiplos receptores celulares de superfície, incluindo CD81, receptor de LDL, NPC1L1, receptor EGF, DC-SIGN, L-SIGN, receptor humano de limpeza SR-B1 e CLDN1e OCLN (84-97). Após a ligação, penetração e absorção num endossoma celular, a acidificação altera a conformação das proteínas do envelope, resultando na fusão com a membrana endossomal. O RNA viral é liberado para o citoplasma, onde atua como RNA mensageiro dirigindo a tradução cap-independente da poliproteína viral (34, 98-100). A tradução do RNA viral ocorre nos ribossomos do retículo endoplasmático rugoso pelo processo de entrada do ribossoma interno e a poliproteína é submetida a uma série de novas clivagens proteolíticas cotranslacionais.

Após a clivagem do peptídeo sinal pela peptidase sinalizadora, a proteína do core permanece no citoplasma, enquanto E1 e E2 são secretadas para o lúmen do retículo endoplasmático, permanecendo ligadas à membrana e tornando-se altamente glicosiladas. O complexo de replicase, composto pelas proteínas NS3, NS4A, NS4B, NS5A eNS5B, forma agregados citoplasmáticos de "teias membranosas" derivados do retículo endoplasmático associados com gotículas lipídicas (75, 76). O complexo de replicase reconhece a extremidade 3' do RNA genômico e, subsequentemente, dirige a síntese de uma cópia de cadeia negativa do genoma. A molécula de RNA de fita dupla resultante provavelmente serve como um molde para a síntese subsequente de cópias múltiplas do RNA genômico de cadeia positiva, após o reconhecimento da extremidade oposta do genoma pela replicase. ORNA viral é empacotado em novas partículas virais, que são provavelmente exportadas para o retículo endoplasmático conduzindo à liberação do vírus através da via secretora vesicular.

Embora o fígado pareça ser a fonte primária do HCV presente no sangue, existem poucos dados que apoiam diretamente esta conjectura. De fato, antígenos específicos do HCV e tanto RNA viral de cadeia negativa como de cadeia positiva foram identificados dentro dos hepatócitos, indicando que a replicação ocorre neste tipo de célula através de uma cadeia negativa intermediária (101-104). No entanto,

dados adicionais sugerem que o vírus pode replicar também dentro das células mononucleares periféricas de origem linfoide ou, talvez, na medula óssea (105-107).

Modelos matemáticos de cinética viral sugerem que a meia-vida do HCV é de aproximadamente 45 minutos para os vírions na corrente sanguínea e que até 10^{12} vírions são produzidos cada dia em um paciente com infecção crônica (108, 109). Comparado com o HIV, a taxa de produção do HCV é 10 a 100 vezes maior; entretanto, estudos sugerem que o mecanismo de eliminação possa ser parecido para ambos os vírus (109-111).

1.4 Diversidade Genética

A alta taxa de replicação viral, acompanhada da ausência de controle da replicação através de prova ou revisão de leitura pela NS5B polimerase resulta na rápida geração de mutações virais. Análises do plasma e dos hepatócitos de indivíduos infectados revelam um "enxame" de múltiplas variantes de HCV geneticamente distintas infectando um mesmo indivíduo; tais variantes são chamadas de "quasispécie" (43, 112). Durante a replicação do RNA, mutações ocorrem aleatoriamente em aproximadamente todo o genoma, enquanto a fixação de uma substituição dentro da população de quasispécies (evolução) depende de quanto essa substituição influencia a aptidão (*fitness*) viral no sentido da funcionalidade da proteína e estrutura do RNA, da capacidade de replicação do vírus e da interação vírus-hospedeiro.

Fortes pressões seletivas, tais como respostas imunológicas do hospedeiro, conduzem à evolução do HCV *in vivo*. Por exemplo, mutações espontâneas dentro do segmento HVR1 da proteína E2 podem ser favoráveis para a sobrevivência no hospedeiro quando reduzem a ligação de anticorpos neutralizantes ao envelope viral; entretanto, em vírus cujos hospedeiros não geram anticorpos anti-E, tais mutações são raras ou inexistentes(113-120). Também há evidência de que a resposta imune celular possa ser direcionada para a seleção de variantes específicas dentro das quasispécies, embora também isso possa incorrer em um custo no *fitness* viral, como, por exemplo, redução na eficiência da enzima viral (121-127). Assim,

quasispécies variantes recuperadas do sangue refletem um equilíbrio da produção e das forças seletivas.

Embora as substituições de nucleotídeos identificadas em vírus circulantes representem apenas uma fração de todas as mutações geradas durante a replicação viral, essas mutações são estimadas para ocorrer em um taxa global de 0,9 a 1,92 x 10^3 substituições de bases por sítio por ano durante a infecção crônica (128-130). Variações dentro das quasispécies instantaneamente e através do tempo podem estar ligadas à extensão da doença e à duração da infecção (131-133). Isso é consistente com a hipótese de que a resposta imunológica afeta tanto a extensão da progressão da doença como o ritmo de modificação na sequência genética. Diferenças nas quasispécies do HCV presentes no fígado e no sangue têm sido descritas, sugerindo que as diferenças no tropismo possam influenciar a variação genética, embora tais estudos careçam de um maior número de amostras (107, 134, 135).

A extensão da diversidade genética varia enormemente ao longo do genoma do HCV, sendo maior nos segmentos que codificam a extremidade amino-terminal da segunda proteína do envelope (E2), dentro da HVR1, e menor no centro do gene e nos segmentos não traduzidos 5' e 3' do genoma (136-142). O alto grau de conservação em alguns *loci* sugere uma "coação" funcional (a mutação seria fatal ou suficientemente desvantajosa para a replicação, de modo que é indetectável entre as populações virais sobreviventes).

1.5 Genótipos do HCV

As cepas de HCV têm sido agrupadas em sete genótipos, numerados de 1 a 7, com 30% de divergência entre as sequências, e um grande numero de subtipos, classificados como *a, b, c* e assim por diante, com 20% de divergência entre as sequências (143, 144). Diferenças são observadas também na distribuição geográfica, com o genótipo 1 dominando nas Américas (70% dos casos), Japão (75% dos casos) e Europa (50-70% dos casos); os genótipos 2 e 3 são também prevalentes nessas regiões. Os genótipos 3 e 6 são muito difundidos no sul e

sudeste da Ásia, enquanto os genótipos 4 e 5 são mais comuns na África e também têm se espalhado pela Europa. O genótipo 7 foi recentemente encontrado em alguns pacientes da África Central, mas até então não apresenta uma maior importância clínica. (143, 145)

Seguindo o padrão de distribuição nas Américas, o Brasil apresenta maior prevalência do genótipo 1, seguido do genótipo 3 (146). Em estudo realizado utilizando amostras obtidas em diferentes estados brasileiros, observou-se que a média de prevalência do genótipo 1 nas regiões brasileiras é de 64%, do genótipo 2 é de 4%, do genótipo 3 de 31%, e dos genótipos 4 e 5 é inferior a 1% (147).

A associação com doença hepática é extremamente similar entre os genótipos; entretanto, um maior risco de esteatose hepática e doença hepática progressiva (148) é associada ao genótipo 3. Por razões ainda obscuras, regimes baseados em IFN levavam a uma resposta virológica sustentada em aproximadamente 80% dos pacientes infectados com os genótipos 2 e 3, mas em apenas 50% nos casos de infecções pelos genótipos 1 e 4 (149), sendo que os casos envolvendo os genótipos 5 e 6 apresentam uma resposta virológica intermediária. Essa variabilidade genética permanece importante na era dos antivirais de ação direta.

A primeira geração de antivirais inibidores da protease (IP), que tinham como alvo a NS3 e a NS4A, apresentava eficácia dependente do genótipo, e foi aprovada apenas para uso em pacientes infectados pelo genótipo 1. Há um direcionamento seletivo para o genótipo 1, devido à sua alta prevalência em países industrializados e ao uso de replicons baseados no genótipo 1b como padrão para o desenvolvimento de drogas pela indústria farmacêutica. A segunda geração de IP tem importantes vantagens quando comparada com a primeira, em termos de efeitos adversos e posologia (150). Os inibidores da NS3/NS4A podem ser divididos em duas classes: inibidores macrocíclicos e inibidores lineares. Ambas as classes ligam-se dentro do sítio ativo da protease, inibindo o processamento da poliproteína e a replicação do HCV. Entretanto, os aminoácidos dentro do gene NS3/4A com que cada composto

interage são discretamente diferentes para cada classe e, como resultado, as substituições associadas à resistência também diferem (151).

Os inibidores do complexo NS5A são caracterizados por alta potência antiviral, mesmo com baixas doses, mas a eficácia varia entre os genótipos. No entanto, diversos fatores contribuem para que esses inibidores possuam uma baixa barreira à resistência (152). Mutações no domínio I frequentemente são observadas em estudos *in vitro*, com diferenças dependendo do genótipo viral. Também tem sido mostrado que uma única transição é suficiente para gerar uma variante mutante nessa região (21, 155), tornando o vírus resistente às drogas dessa classe.

Os inibidores da polimerase são categorizados como análogos nucleosídeos ou nucleotídeos e não nucleosídeos. Os inibidores da polimerase não nucleosídeos constituem a classe mais fraca de compostos contra o HCV, por apresentarem uma baixa barreira à resistência. A maioria das drogas dessa classe são principalmente ativas contra o genótipo 1b e, em menor extensão, contra o genótipo 1a. Esses inibidores estão sendo desenvolvidos para serem usados em combinação com outros DAA's, principalmente IP e inibidores do complexo NS5A; além disso, diferentes domínios da polimerase podem servir como alvo de inibidores não nucleosídeos e, ao menos em teoria, o uso de uma combinação de drogas dessa classe é possível, já que não há resistência cruzada entre drogas cujo alvo são os diferentes domínios da polimerase (153).

Os análogos nucleosídeos são ativos contra todos os genótipos do HCV e têm alta barreira à resistência. Variantes virais resistentes aos análogos nucleosídeos podem emergir, mas apresentam um *fitness* (capacidade de modificação do vírus) muito baixo e não se replicam rapidamente; mutações observadas nessas variantes causam o término da tradução do HCV, de modo que a população resistente não é mantida.

1.6 Ferramentas de Sequenciamento (Análise Genotípica)

Variantes resistentes podem se desenvolver após o tratamento com os DAAs

ou já estarem presentes como quasispécies virais (154-157). A diferenciação entre variantes pré-existentes (isto é, anteriores ao uso dos DAA's) ou que surgiram após a exposição a essas drogas é desafiadora no presente momento, já que variantes resistentes em pacientes não expostos ao tratamento podem existir em um nível tão baixo que torna impossível a detecção com as técnicas de sequenciamento padrão. Ferramentas de genotipagem estão disponíveis para determinar o modelo de substituições das quasispécies presentes em um paciente em um dado momento. Esses métodos incluem sequenciamento populacional (também chamado de sequenciamento direto) (155, 158), sequenciamento clonal (159, 160) ou nova geração de sequenciamento (NGS, *Next-generation sequencing*), atualmente baseada em sequenciamento profundo (*deep sequencing*) (161). A sensibilidade para detecção com essas três técnicas é de aproximadamente 25%, 5% e 0,5%, respectivamente, e a presença de mutantes virais abaixo desses níveis não seria detectada (162, 163). A relevância de um método ou outro depende do contexto ou do objetivo da pesquisa.

1.6.1 Sequenciamento populacional

O método padrão atualmente empregado para análise genotípica rotineira da presença de variantes resistentes do HCV é o sequenciamento populacional, através do método de Sanger. O sequenciamento populacional pode ser facilmente realizado em amostras clínicas para gerar uma sequência consenso, que mostra, com sensibilidade apropriada, qual variante dominante está presente na amostra. Entretanto, por causa do seu alto nível de divergência entre genótipos e subtipos virais, *primers* específicos para cada genótipo precisam ser usados para garantir a amplificação correta e adequada dos genes alvos (NS3, NS5A ou NS5B)(162).

1.6.2 Sequenciamento clonal

Por muitos anos, o estudo de quasispécies virais foi baseado na separação de variantes individuais através da clonagem genética ou de titulação (EPLD,*end-point limiting dilution*), seguida pelo sequenciamento por Sanger. O sequenciamento era realizado em clones individuais de uma amostra clínica após a inserção da

quaisispécie viral em um plasmídeo vetor e a multiplicação em células bacterianas, sendo que a sequência de cada clone representa uma única variante populacional presente na mistura. Entretanto, o número de clones que pode ser analisado é limitado, e o método é caro e extremamente trabalhoso. Sequenciamento clonal está, atualmente, sendo substituído por métodos de sequenciamento de nova geração (162, 164, 165).

1.6.3 Sequenciamento de Nova Geração – NGS

NGS refere-se a tecnologias de sequenciamento de alto rendimento e tem demonstrado enorme potencial em muitos campos da virologia, incluindo a análise de resistência viral do HCV. As plataformas disponíveis atualmente diferem entre si pelos reagentes empregados, tamanho da sequência genética que a plataforma é capaz de sequenciar e capacidade de rendimento. As mais recentes técnicas, como a amplamente usada tecnologia *Illumina*, geram centenas de milhões de sequências (chamadas leituras) em uma única corrida. As técnicas NGS estão tendo seu uso ampliado em laboratórios de virologia, tanto para pesquisa básica como para aplicações diagnósticas (166-170).

Há duas estratégias possíveis para se estudar o genoma do HCV através de NGS: sequenciamento do genoma completo ou análises de quasispécies com um gene-alvo específico. Quando se foca em regiões curtas, a NGS proporciona uma grande sensibilidade para a detecção de populações virais menores. As leituras são usualmente alinhadas com a sequência de referência do genótipo específico, como por exemplo, a cepa H77 para o genótipo 1a. A escolha do limite de detecção depende da variabilidade do método em níveis muito baixos. Variantes que representam menos do que 0,5% das quasispécies virais são usualmente excluídas, por causa do risco de falso-positivos associados com as etapas de amplificação e sequenciamento. Além disso, assim como em outras plataformas de sequenciamento, é fundamental a experiência em bioinformática para análise da enorme quantidade de sequências geradas.

1.7 Testes de Resistência Viral na Prática Clínica

O monitoramento de resistência é fundamental durante o desenvolvimento de drogas para o tratamento da hepatite C. Em contraste, a utilidade de realizar sistematicamente testes de resistência ao HCV na prática clínica ainda permanece em debate. Diferentemente da infecção pelo HIV, em que as células infectadas albergam variantes resistentes por períodos prolongados (171), no HCV essas variantes podem não ter consequências clínicas a longo prazo, dependendo do gene, do aminoácido substituído e da droga envolvida.

Testes de resistência ao HCV antes do primeiro tratamento não são atualmente recomendados (172). De fato, as taxas de resposta virológica sustentada são bem altas em pacientes com e sem a detecção de mutações de resistência pré-existentes e a detecção dessas mutações muitas vezes não parece influenciar a decisão de tratamento, mas pode em alguns casos influenciar a escolha das drogas usadas. A única exceção a essa recomendação é a testagem da substituição Q80K no gene da protease do HCV genótipo 1a, que confere resistência ao simeprevir e que é recomendada por *guidelines* internacionais quando está se considerando a possibilidade de tratamento com simeprevir (173).

Testes de resistência podem ser úteis em pacientes experimentando falha virológica ou em recidivas pós-tratamento, particularmente naqueles tratamentos que incluem inibidores da NS5A. De fato, mutações de resistência do gene NS5A podem permanecer detectáveis por muitos anos após a suspensão do tratamento (174). As diretrizes da Associação Americana para Estudo das Doenças do Fígado (AASLD) e da Sociedade Americana de Doenças Infecciosas (IDSA) recomendam testagem para mutações de resistência do gene da NS3 e da NS5A para retratamento de pacientes cirróticos ou para outros pacientes que necessitem tratamento urgente quando esses pacientes tiverem história de falha a regimes que continham inibidores da NS5A (175).

1.8 Bases de Dados com Sequências Genéticas do HCV

A quantidade de sequências genéticas obtidas através do método de Sanger disponíveis em bancos de dados públicos possibilita que sejam distinguidas características genômicas entre os diferentes genótipos e que essas informações sejam relacionadas a fenótipos resistentes aos novos DAA's.

Três bases de dados estão atualmente disponíveis e são capazes de proporcionar informações relevantes dentro das áreas de biologia básica, imunologia e evolução do vírus: a base de dados japonesa *(Hepatitis Virus DataBase Server*, http://s2as02.genes.nig.ac.jp), a base de dados europeia (*European Hepatitis C Virus database,* euHCVdb, http://euhcvdb.ibcp.fr) e a base de dados americana de Los Alamos (*The Hepatitis C Virus (HCV) Database Project,* http://hcv.lanl.gov). Atualmente há mais de 1.684 sequências de genomas completos, além de 6.567 sequências do gene NS3, 6.819 do gene NS5A e 1.877 sequências do gene NS5B.

2 JUSTIFICATIVA

Os avanços no conhecimento na replicação do ciclo de vida do HCV levaram a descobertas de inúmeras moléculas que especificamente bloqueiam as proteínas virais. Esses compostos são chamados de antivirais de ação direta (DAA's) e agem em diferentes proteínas alvos do HCV, como a NS3/4A protease, a NS5B polimerase e a proteína NS5A. O genoma do HCV é altamente variável como consequência da baixa fidelidade da polimerase viral, devido à ausência de uma revisão adequada da fita de RNA secundária por essa enzima. Em média, 10^{-3} a 10^{-5} substituições incorretas de nucleotídeos ocorrem no genoma do HCV a cada ciclo replicativo. Esse achado, associado à alta taxa de replicação do HCV, estimada em torno de 10^{12} virions produzidos por dia em um indivíduo infectado, resultam na geração de um grande número de variantes virais. Enquanto algumas dessas variantes apresentam falhas no seu genoma que impedem sua replicação, outras permanecerão competentes e constituirão o que é conhecido como quasispécies virais.

Um indivíduo com infecção crônica pelo HCV alberga cepas virais dominantes ou selvagens (*wild-type*), bem como variantes que circulam em menor frequência. Essas quasispécies virais estão constantemente evoluindo e, dependendo do ambiente em que a replicação ocorre, a variante dominante pode ser alterada. O uso de um DAA irá favorecer a persistência de variantes do HCV dentro das quasispécies que possuem alguma mutação que confira resistência a esse DAA. Após a seleção de uma variante resistente ao DAA, pode se seguir a seleção de variantes com substituições que aumentem a capacidade de replicação dessa variante resistente. A existência de variantes do HCV com resistência aos DAA's em um indivíduo infectado é, sem dúvida alguma, uma preocupação que se leva em conta quando se vai prescrever um tratamento que será afetado por essas substituições. Ao longo da história da medicina, toda medicação antimicrobiana foi afetada em maior ou menor grau por cepas resistentes, e há muitos casos de surgimento de microrganismos pan-resistentes, devido à resistência adquirida e também casos de transmissão dessa resistência.

A abundância de informações disponíveis a partir das sequências depositadas nos bancos de dados públicos permite a identificação de inúmeros polimorfismos em pacientes não submetidos a tratamento e, através da análise desses dados, é possível determinar a barreira genética e a presença de variantes com resistência aos DAA's entre sequências genéticas do HCV. Isso fornecerá informações sobre a resistência aos medicamentos, que podem afetar o sucesso dos regimes de tratamento.

3 OBJETIVOS

3.1 Objetivo Geral

- Avaliar a ocorrência de polimorfismos nos genes NS3, NS5A e NS5B nas sequências genéticas do vírus da hepatite C depositadas na *European Hepatitis C Virus database* (euHCVdb) em indivíduos não submetidos ao tratamento com antivirais de ação direta.

3.2 Objetivos secundários

- Estabelecer a barreira genética dos antivirais de ação direta recomendados pelas diretrizes da Associação Europeia para Estudo do Fígado (EASL) para tratamento da Hepatite C.
- Determinar a prevalência das mutações de resistências nas regiões NS3, NS5A e NS5B nas sequências genéticas do vírus da hepatite C depositadas no euHCVdb.
- Comparar a prevalência de mutações nas sequências do euHCVdb com dados publicados a partir da análise da base de dados de Los Alamos (*The Hepatitis C Virus Database Projec*t).

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5. ARTIGOS

5.1 Artigo 1: artigo submetido à revista *Plos One*

Genetic Barrier to Direct Acting Antivirals in HCV sequences deposited in the European Databank

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Abstract:

- Background & Aims: Development of resistance results from mutations in the viral genome, and the presence of selective drug pressure leads to the emergence of a resistant virus population. The aim of this study was to analyze the impact of genetic variability on the genetic barrier to drug resistance to DAAs.

- Methods: The genetic barrier was quantified based on the number and type of nucleotide mutations required to impart resistance, considering full-length HCV NS3, NS5A and NS5B regions segregated by genotype into subtypes 1a, 1b, 2a, 2b and 3a. This study analyzeds 789 NS3 sequences, 708 sequences and 536 NS5B sequences obtained by Sanger sequencing deposited in the European Hepatitis C Virus Database, in the following resistance-associated positions: NS3 (5 positions): F43/I/L/S/V, Q80K/R, R155K/G, A156G/S/T and D168A/C/E/G/H/N/T/V/Y; NS5A (5 positions): L/M28A/T/V, Q30E/H/R, L31F/I/M/V, H58D or P58S and Y93C/F/H/N/S; NS5B (6 positions): S282P/R/T, C316H/N/Y, S368T, Y448C/H, S556G/R, D559R.

- Results: Variants that require only one transversion in NS3 were found in 4 positions and include F43S, R80K, R155K/G and A156T. The genetic barrier to resistance shows subtypic differences at position 155 of the NS3 gene where a single transition is necessary in subtype 1a. In the NS5A gene, 5 positions where only one nucleotide change can confer resistance were found, such as L31M which requires one transversion in all subtypes, except in 0.28% of 1b sequences; and R30H, generated by a single transition, which was found in 10.25% of the sequences of genotype 1b. Other subtypic differences were observed at position 58, where resistance is less likely in genotype 1a because a transversion is required to create the variant 58S. For the NS5B inhibitors, the genetic barrier at positions conferring resistance was nearly identical in subtypes 1a and 1b, and single transitions or transversions were necessary in 5 positions to generate a drug-resistant variant of HCV. The positions C316Y and S556D required only one transition in all genotypes, Y448H and S556 G/N/R positions required only one transition for up to 98.8% of the sequences analyzed. A single variant in position 448 in genotype 1a is less likely to become the resistance variant 448H because it requires two transversions. Also, in the position

559D a transversion and a transition were necessary to generate the resistance mutant D559H.

- Conclusion: Results revealed that in 14 out of 16 positions, conversion to a drug-resistant variant of HCV required only one single nucleotide substitutions threatening direct acting antivirals from all three classes.

Introduction:

First discovered in 1989, hepatitis C virus (HCV) is a major health problem worldwide (1). The percentage of people who are seropositive for anti-HCV antibodies worldwide is estimated to have increased from 2.3% to 2.8% between 1990 and 2005 (2). Most patients (80–85%) who become acutely infected cannot clear the virus and progress to chronic infection. Current data states that more than 170 million people are chronically infected by HCV; the outcomes of chronic infection are cirrhosis, portal hypertension, hepatic decompensation, and the development of hepatocellular carcinoma, causing approximately 350,000 deaths per year (3).

HCV contains a positive-sense, single-stranded 9,600 kb RNA genome. A single HCV polyprotein of 3,011 amino acids is translated, and then cleaved by cellular and viral proteases into three structural proteins (core, E1 and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (4) Additionally, HCV has enormous genetic diversity in infected hosts, existing in blood as a swarm of closely related individual genotypes that may code for subtly distinct phenotypes, known as quasispecies. One of the phenotypes potentially selectable from the quasispecies is drug resistance. HCV diversity derives from an error-prone viral polymerase, rapid replication and natural selection within each host to antibody and cellular immune responses, and now, increasingly, to antiviral drugs (5).

There are two major models explaining the development of drug resistance mutations: the deterministic model and the stochastic model. If the viral effective population size is relatively small, drug resistance mutations might emerge “stochastically” under the selection pressure during treatment with the antirretroviral, so in this case the genetic barriers of codon changes may affect the development of drug resistance mutations. On the other hand, the deterministic model is based on effective virus population that are large enough to infer that all drug resistance mutations pre-exist and can be seen; this model can be used if there is enough sampling depth (6-8). The model to be used in drug resistance studies depend on the pathogen and population size; there are few studies calculating the effective

population size in HCV (6, 9).

Factors favoring the emergence of resistant variants include high viral replicative load with prolonged and rapid viral turnover; high intrinsic viral mutation rates; degree of selective drug pressure, which is higher with prolonged or repeated courses of drug therapy, particularly with suboptimal doses; and an antiviral target that can mutate without adversely affecting viral fitness (10).

Advances in our knowledge of the molecular biology of the HCV replication life cycle have led to the development of several molecules that specifically inhibit HCV enzymatic activities that are essential for replication (11, 12). These compounds are called direct-acting antiviral agents (DAA) and target viral non-structural proteins, including the NS3/4A protease, the NS5B polymerase, and the NS5A protein (13). Resistance to DAAs is driven by the selection of mutations in the non-structural proteins (14-16). Each compound or drug family induces a specific mutation profile that is also influenced by the HCV genotype/subtype. Furthermore, each class of DAAs is characterized by a difference in the genetic barrier to resistance; though this general characterization differs for individual agents in the class. Cross-resistance between compounds in the same inhibitor class is of greatest concern for NS3 protease and NS5A inhibitors.

The genetic barrier to resistance, defined as the number of viral mutations required for replication in the presence of drug-selective pressure, is an important factor in HCV treatment. The huge variability between HCV genotypes and subtypes at the nucleotide level could impact the effectiveness of the genetic barrier and therefore, the likelihood of drug resistance development. It has been recognized that despite an identical amino acid at certain positions within the NS3 protease of HCV subtype 1a and 1b the probability/frequency with which a treatment-induced resistance mutation is detected was different between these HCV subtypes. For example, the alteration of arginine at codon position 155 to lysine, which confers resistance to SMV and PTV, is 6 times more frequent in genotype 1a than in 1b (17-19). This is explained, in part, by different nucleotide codons encoding the same

amino acid. For instance, two nucleotide changes are required at codon position 155 for generation of R155K in HCV subtype 1b isolates; whereas, in HCV subtype 1a isolates one alteration is sufficient. As a consequence, distinct resistance frequencies are observed in HCV subtype 1a and 1b infected patients after failure to a protease inhibitor based antiviral therapy (20, 21). Similar differences have been observed also for the generation of other resistance-associated variants (RAVs) in other HCV genes (22, 23).

Beside the number of nucleotide changes required for an amino acid exchange as definition of the genetic barrier to resistance, also the type of exchange seems to be of importance in HCV infection. A model previously described for HIV, HBV, or HCV proposed a score of 1 for transitions and 2.5 for transversions, because it was based on an initial report that addressed the issue of quantifying the genetic barrier for development of drug resistance substitutions between subtypes (24), where it was reported that transitional replacement of a purine by another purine or of a pyrimidine by another pyrimidine are sterically more favorable and therefore occur 2.5-fold more frequently than transversional replacement of a purine by a pyrimidine and vice-versa. RNA structural effects which are much more frequentently behold in HCV than HIV or HBV may also alter the propensity for mutants to occur. The HCV NS5B RNA polymerase was shown to favor the generation of nucleotide transitions in comparison to transversions (22). This may explain that some RAVs are rarely observed at all or generated only after a longer DAA exposure (for example S282T within NS5B leading to resistance to sofosbuvir (SOF) or L31M within NS5A) causing resistance to ledipasvir (LDV) (22, 25). In the case of S282T substitution, the deficit in viral fitness and the low frequencies of transversions over transitions within the diversity of viral quasispecies found by deep-sequencing analyses of HCV samples from treatment-naive patients represent an overall high barrier to the selection of 282T *in vivo* (26, 27) and there is little *in vivo* evidence of drug resistance to nucleo(s/t)ide inhibitors and, when detected, resistant variants with 282T rapidly revert to the wild type as soon as the treatment is interrupted (28, 29).

Currently, there are more than 1,684 complete HCV genome sequences, and 6,567, 6,819 and 1,877 sequences of NS3, NS5A and NS5B, respectively, obtained by Sanger sequencing available on public databanks. However, the European Hepatitis C Virus database have not been related to genetic barrier to resistance (30). Three HCV databases are currently available to provide insights into the basic biology, immunology, and evolution of the virus: the Hepatitis Virus DataBase Server (http:// s2as02.genes.nig.ac.jp), the European Hepatitis C Virus database (http://euhcvdb.ibcp.fr) and The Hepatitis C Virus (HCV) Database Project (http://hcv.lanl.gov).

Considering the genetic diversity, the quality and quantity of nucleotide changes and the selection of mutations leading to resistance to DAA's, the aim of this study was to analyze the impact of genetic variability on the genetic barrier to development of substitutions causing drug resistance to DAAs, quantifying the genetic barrier from the number and type of nucleotide mutations required to impart resistance after analysis of a large number of HCV sequences of all genotypes deposited in European HCV databank.

Methods

HCV Database

The sequences analyzed in this study were downloaded in November 2015 from the European Hepatitis C Virus database (https://euhcvdb.ibcp.fr/euHCVdb/). This databank provides key data about the HCV sequences (e.g. genotype, genomic region, viral proteins and their functions, known 3-dimensional structures) and ensures consistency of the annotations, which enables reliable keyword queries. Any user can extract subsets of sequences matching particular criteria or enter their own sequences and analyze them with various bioinformatics programs available on the same server. The euHCVdb is mainly oriented towards protein sequence, structure and function analyses and structural biology of HCV, and is updated every month from a database by an automated process (31).

The search was performed for full-length HCV NS3 protease, NS5A inhibitors and NS5B polymerase sequences segregated by genotype into subtypes 1a, 1b, 2a, 2b and 3a. These subtypes were chosen due to their worldwide prevalence and

presence in drug trials, specifically genotype 1 with protease inhibitors and genotype 3 with Polymerase Inhibitor. Reference strains for the three genotypes were obtained (1a: AF009606, 1b: D90208, 2a: D00944, 2b: D10988 and 3a: D17763). Sequences that contain missing data, such as gaps and sequencing errors or were incomplete and sequences from patients previously treated with DAAs were excluded from the analysis. To ensure the quality of the data, sequences were excluded from the analysis if they contained stop codons in the NS5B gene or contained ambiguities consisting of more than 2 bases per nucleotide position or more than 2 ambiguities per codon at individual drug resistance-associated position.

Alignment and edition of the sequences

Sequence alignment was performed with MEGA 6.06 MAC (32) for editing and excluding sequences with missing data, and for translating the genetic information into amino acids. The resulting protein sequences were then analyzed using BioEdit 7.2.5. software to identify mutations previously associated with resistance (33).

Genetic barrier calculation

The genetic barrier for each drug resistance substitution was calculated according to a model previously described elsewhere. In summary, transitions (A↔G and C↔T) were assigned a score of 1 and transversions (A↔C, A↔T, G↔C, and G↔T) were assigned a score of 2.5, since transitions have been generally shown to occur for steric reasons on average 2.5 times more frequently than transversions (24, 34, 35). Briefly, due to the degeneracy of the genetic code, most amino acids associated with drug resistance can be encoded by more than one codon. Therefore, starting from the wild-type codon detected in drug-naive patients, we calculated a numerical score by summing the number of nucleotide transitions and/or transversions required to generate a specific resistance substitution. As a result, we obtained different scores for each pathway of nucleotide mutations required to generate a resistance substitution in response to a given drug. The minimal genetic barrier score for each drug resistance substitution analyzed was considered.

The genetic barrier for each drug resistance substitution within 789 NS3 sequences was investigated: 313 from genotype 1a, 405 from genotype 1b, 18 from

genotype 2a, 25 from genotype 2b, and 28 from genotype 3a. We evaluated 708 sequences in the NS5A data set: 274 from genotype 1a, 361 from genotype 1b, 19 from genotype 2a, 26 from genotype 2b, and 28 from genotype 3a. Furthermore, we compiled 536 HCV NS5B sequences: 166 from genotypes 1a, 308 from genotype 1b, 20 from genotype 2a, 24 from genotype 2b, and 18 from genotype 3a. It was included only positions that have been described in previous studies to be associated *in vivo* with treatment failure and/or have been shown *in vitro* phenotypic assays to confer a >2-fold change in replication in comparison to the wildtype reference strain in the presence of the following HCV DAAs: simeprevir (SMV), sofosbuvir (SOF), paritaprevir (PTV), daclatasvir (DCV), ledipasvir (LDV), ombitasvir (OMV), dasabuvir (DSV), grazoprevir (GZR) and elbasvir (EBR). Based on the drug usage recommendations, the following resistance-associated mutations were analysed: NS3: F43/I/L/S/V, Y56H, Q80K/R, R155K/G, A156G/S/T and D168A/C/E/G/H/N/T/V/Y; NS5A: L/M28A/T/V, Q30E/H/R, L31F/I/M/V, P32L, H58D or P58S and Y93C/F/H/N/S; NS5B: C316H/N/Y, S368T, Y448C/H, S556G/R and D559R (36-40).

Results

Genetic variability among HCV genotypes impacts the calculation of the genetic barrier to development of resistance substitutions. Considering that the available protease inhibitors are less effective against genotypes other than genotype 1 due to natural polymorphisms in their NS3 region, the analyses have taken this into consideration and the discussion focus on the genotype 1 dataset; nevertheless the results of the other genotypes are shown in Table 1. Although some rarely observed NS3 PI resistance variants require transversions or multiple changes, many of the commonly observed changes consist of a single transition to become a resistance mutation (Table 1). Variants that require only one transversion in subtype 1a and 1b include NS3 F43S, R80K, R155K/G and A156T. Subtypic differences were observed at position 155 where a single transition is necessary in subtype 1a but are rare in subtype 1b, where most of the variants require both a transversion and a transition. Some differences in the variants at position 156 are also observed where a transition

is necessary to generate the variant 156T but a transversion is necessary to generate 156G or S.

Table 1: Codon variability at HCV NS3 positions associated with major drug resistance to IP and its impact on the genetic barrier to drug resistance development in HCV genotypes 1 to 3

NS3 position	Amino acid	Codon	Frequencies of codon in each genotype					Score
			1a (n=313)	1b (n=405)	2a (n=18)	2b (n=25)	3a (n=28)	
43	F	TTC	283 (90.41%)	395 (97.53%)	18 (100%)	23 (92%)	28 (100%)	1.0
		TTT	29 (9.27%)	10 (2.47%)		2 (8%)		1.0
	S	**TCC**	**1 (0.32%)**					
80	Q	CAA	151 (48.24%)	53 (13.09%)			3 (10.71%)	2.5
		CAG	16 (5.11%)	325 (80.25%)			25 (89.29%)	2.5
	G	GGC				2 (8%)		4.5
		GGA			4 (22.28%)	5 (20%)		2.0
		GGG			14 (77.78%)	18 (72%)		2.0
	R	AGG	1 (0.32%)					1.0
		CGA	2 (0.64%)					3.5
		CGG	0	1 (0.25%)				3.5
	L	CTA	2 (0.64%)	1 (0.25%)				5.0
		CTG	0	24 (5.93%)				5.0
	K	**AAA**	**86 (27.48%)**					
		AAG	**55 (17.57%)**	**1 (0.25%)**				
155	R	AGA	49 (15.65%)			24 (96%)	1 (3.57%)	1.0
		AGG	262 (83.71%)	1 (0.25%)		1 (4%)	27 (96.43%)	1.0
		CGA		18 (4.44%)	1 (5.56%)			2.5 - 155G
		CGG		384 (94.81%)	17 (94.44%)			2.5 - 155G
	P	CCG		2 (0.49%)				5.0
	K	**AAG**	**2 (0.64%)**					
156	**G**	**GGC**						
		GGA						
		GGG						
	A	GCT		338 (83.46%)		1 (4%)	23 (82.14%)	1.0 - 156T
		GCC	313 (100%)	66 (16.30%)			5 (17.86%)	2.5 - 156G/S
		GCA			17 (94.44%)	4 (16%)		1.0 - 156T
		GCG		1 (0.25%)	1 (5.56%)	20 (80%)		1.0 - 156T
	T	**ACA**						

		ACG						
	D	GAT	12 (3.83%)	28(6.91%)	14 (77.78%)			2.5
		GAC	299 (95.53%)	373 (92.10%)	4 (22.28%)	25 (100%)	28 (100%)	2.5
	Q	CAG					28 (100%)	2.5
168	G	GGC	1 (0.32%)					3.5
	A	GCC		1 (0.25%)				5.0
	E	**GAA**	**1 (0.32%)**	**4 (0.99%)**				

Amino acids and nucleotides in bold are associated with resistance.

Only codons with at least one sequence found in the database are shown, except for the resistance amino acids.

The score was calculated considering the minimal change necessary to generate a resistant variant. In cases where more than one variant can be resistant, the nucleotide used as reference is indicated following the score.

The genetic barrier for resistance to NS5A inhibitors appears similar to that for the NS3 PIs, with the majority of variants requiring a single transition in two of the five positions analyzed (Table 2). Exceptions include the NS5A L31M variant, which requires at least one transversion in all subtypes (except in 0.28% of the 1b sequences). Also, in 10.25% of the sequences of genotype 1b, at position 30 a single transition is necessary to generate the mutation R30H which causes resistance to DCV, LDV and OMV. Other subtypic differences were observed at position 58 where genotype 1a seems to confer some protection against development of resistance, because a transversion is required to create the variant 58S, which leads to resistance to DCV, compared to single transitions in other subtypes.

Table 2: Codon variability at HCV NS5A positions associated with major drug resistance to NS5A inhibitors and its impact on the genetic barrier to drug resistance development in HCV genotypes 1 to 3

NS5A position	**Amino Acid**	**Codon**	**Frequencies of codon in each genotype**					**Score**
			1a (n=274)	**1b (n=361)**	**2a (n=19)**	**2b (n=26)**	**3a (n=28)**	
28	M	ATG	259 (94.53%)	10 (2.77%)			27 (96.48%)	1.0
	V	GTG	12 (4.38%)	2 (0.55%)				2.0
	I	ATT	1 (0.36%)					1.0
		ATA					1 (3.57%)	1.0
	F	TTT			3 (15.79%)			3.5

		TTC			16 (84.21%)			3.5
	L	TTG		10 (2.77%)				5.0
		CTT		1 (0.28%)		2 (7.69%)		3.5
		CTC				24 (92.31%)		3.5
		CTA		6 (1.66%)				3.5
		CTG		332 (91.97%)				3.5
	T	**ACG**	**2 (0.73%)**					
30	Q	CAA	260 (94.89%)	3 (0.83%)				2.5
		CAG	9 (3.28%)	20 (5.54%)				2.5
	R	AGG		1 (0.28%)	1 (5.26%)			6.0
		CGT		1 (0.28%)				1.0
		CGC		1 (0.28%)				3.5
		CGA	1 (0.36%)	37 (10.25%)				3.5
		CGG		290 (80.33%)				3.5
	K	AAA		4 (1.11%)	4 (21.05%)	24 (92.31%)		5.0
		AAG			14 (73.68%)	2 (7.69%)		5.0
	L	TTG					1 (3.57%)	6.0
		CTG		1 (0.28%)				5.0
	M	ATG		1 (0.28%)				6.0
	A	GCA					2 (7.14%)	7.5
		GCG					25 (89.29%)	7.5
	H	**CAT**	**1 (0.36%)**					
		CAC	**3 (1.09%)**					
31	L	TTA		184 (50.97%)				3.5
		TTG	19 (6.93%)	104 (28.81%)				2.5
		CTT	5 (1.82%)	1 (0.28%)				5.0
		CTC	4 (1.46%)				28 (100%)	5.0
		CTA	1 (0.36%)	28 (7.76%)				3.5
		CTG	242 (88.32%)	30 (8.31%)	3 (15.79%)	6 (23.08%)		2.5
	I	ATA		1 (0.28%)				1.0
	M	**ATG**	**3 (1.09%)**	**12 (3.32%)**	**16 (84.21%)**	**20 (76.92%)**		
58	H	CAT	7 (2.55%)					2.5
		CAC	253 (92.34%)					2.5
	C	TGT	4 (1.46%)					3.5
	P	CCT	3 (1.09%)	2 (0.55%)	16 (84.21%)		27 (96.48%)	1.0 - 585
		CCC	3 (1.09%)	7 (1.94%)	2 (10.53%)			5.0
		CCA		298 (82.55%)		22(84.62%)		1.0 - 585
		CCG		29 (8.03%)		3 (11.54%)		1.0 - 585

	R	CGT					1 (3.57%)	3.5
		CGC	1 (0.36%)					3.5
	Y	TAT	1 (0.36%)					2.5
	T	ACA		5 (1.39%)				2.5 - 58
		ACG		1 (0.28%)				2.5 - 58
	Q	CAA		2 (0.55%)				5.0
	A	GCA		1 (0.28%)				2.5 - 58
	L	CTA		3 (0.83%)				2.0 - 58
	S	**AGC**						
		AGT						
		TCT		**1 (0.28%)**	**1 (5.26%)**	**1 (3.85%)**		
		TCA		**12 (3.32%)**				
93	Y	TAT	17 (6.20%)	4 (1.11%)	2 (10.63%)		3 (10.71%)	1.0
		TAC	254 (92.70%)	338 (93.63%)	17 (89.47%)	26 (100%)	25 (89.29%)	1.0
	R	CGT		3 (0.83%)				1.0
	C	**TGT**		**1 (0.28%)**				
		TGC	**1 (0.36%)**					
	H	**CAT**		**15 (4,25%)**				
		CAC	**2 (0.73%)**					

Amino acids and nucleotides in bold are associated with resistance.

Only codons with at least one sequence found in the database are shown, except for the resistance amino acids.

The score was calculated considering the minimal change necessary to generate a resistant variant. In cases where more than one variant can be resistant, the nucleotide used as reference is indicated following the score.

The genetic barrier differs for the various NS5B inhibitor classes (nucleo(s/t)ide, Palm site, Thumb site, Finger-loop) (Table 3). For the NS5B inhibitors, while subtypic differences in activity are known, the genetic barrier at positions conferring resistance were nearly identical in subtypes 1a and 1b, and the majority were single transitions or transversions. The positions C316Y and S556D where palm inhibitors NS5B take action required only one transition in all genotypes, Y448H and S556 G/N/R positions required only one transition for up to 98.8% of sequences analyzed. A single variant with a glutamic acid replacing the tyrosine in position 448 can confer some protection in genotype 1a because it then necessitates two transvertions to become the

resistance variant 448H. Also, in the position 559D a transvertion and a transition were necessary to become a resistance mutant D559H.

Table 3: Codon variability at HCV NS5B positions associated with major drug resistance to NS5B inhibitors and its impact on the genetic barrier to drug resistance development in HCV genotypes 1 to 3

NS5B position	Amino acid	Codon	Frequencies of codon in each genotype					Score
			1a (n=166)	1b (n=308)	2a (n=20)	2b (n=24)	3a (n=18)	
282	S	AGC	164 (98.8%)	293 (95.13%)	19 (95%)	24 (100%)	3 (16.67%)	2.5
		AGT	1 (0.66%)	15 (4.87%)	1 (5%)		14 (77.78%)	2.5
	P	CCG					1 (5.56%)	2.5
	R	AGG	1 (0.66%)					2.5
	T	**ACA**						
		ACG						
316	C	TGT	152 (91.57%)	7 (2.27%)		24 (100%)	1 (5.56%)	1.0
		TGC	14 (8.43%)	185 (60.06%)	20 (100%)		17 (94.44%)	1.0
	R	CGC		1 (0.32%)				2.0
	N	AAT		3 (0.97%)				2.5
		AAC		111 (36.04%)				2.5
	Y	**TAT**						
		TAC		**1 (0.32%)**				
368	S	TCT	40 (24.10%)	5 (1.62%)			4 (22.22%)	2.5
		TCC	123 (74.10%)	298 (96.75%)			12 (66.67%)	2.5
		TCA		3 (0.97%)	19 (95%)	24 (100%)	2 (11.11%)	2.5
		TCG	3 (1.81%)	1 (0.32%)	1 (5%)			2.5
	P	CCC		1 (0.32%)				2.5
	T	**ACA**						
		ACG						
448	Y	TAT	2 (1.20%)	18 (5.84%)	2 (10%)	9 (37.50%)		1.0
		TAC	162 (97.59%)	290 (94.16%)	18 (90%)	15 (62.50%)	18 (100%)	1.0
	E	GAA	1 (0.66%)					5.0
		GAG	1 (0.66%)					5.0

	H	**CAT**						
		CAC						
	S	AGT		270 (87.66%)				1.0
		AGC	166 (100%)	5 (1.62%)				1.0
556	D	GAC		3 (0.97%)				1.0
	G	**GGC**		**24 (7.79%)**	**20 (100%)**	**24 (100%)**	**18 (100%)**	
	N	**AAC**		**6 (1.95%)**				
	D	GAT	1 (0.66%)					3.5
		GAC	165 (99.34%)	307 (99.68%)	19 (95%)	24 (100%)	18 (100%)	3.5
559	N	AAC		1 (0.32%)				3.5
	H	**CAT**						
		CAC			**1 (5%)**			

Amino acids and nucleotides in bold are associated with resistance.

Only codons with at least one sequence found in the database are shown, except for the resistance amino acids.

The score was calculated considering the minimal change necessary to generate a resistant variant.

Regardless of HCV genotype the analysis revealed that in 14 of 16 positions conversion to a drug-resistant variant required only single nucleotide substitutions (Table 4). That is, one transition with a genetic barrier score of 1 (F43S, Q80R, R155K or G, A156T in NS3 gene; H or P58S, Y93H in NS5A; C316Y and S556N in NS5B), or one transversion with a genetic barrier score of 2.5 (Q80K, A156S, D156E in NS3 gene; and S368T in NS5B gene).

Table 4: Resistance level to DDA at HCV NS3, NS5A and NS5B positions and level of genetic barrier

Gene	Drug	Mutation	Codon	Frequencies in each genotype					Resistance level
				1a	1b	2a	2b	3a	
NS3	Simeprevir	Q80K	CAA	151 (48.24%)	53 (13.09%)			3 (10.71%)	low
			CAG	16 (5.11%)	325 (80.25%)			25 (89.29%)	
		R155K	AGA	49 (15.65%)					high
			AGG	262 (83.71%)	1 (0.25%)				
			CGA		18 (4.44%)				
			CGG		384 (94.81%)				
		D168E	GAT	12 (3.83%)	28(6.91%)	14 (77.78%)			low-intermediate
			GAC	299 (95.53%)	373 (92.10%)	4 (22.28%)	25 (100%)		
	Paritaprevir	R155K	AGA	49 (15.65%)					high
			AGG	262 (83.71%)	1 (0.25%)				
			CGA		18 (4.44%)				
			CGG		384 (94.81%)				
		D168E	GAT	12 (3.83%)	28(6.91%)	14 (77.78%)			low-intermediate
			GAC	299 (95.53%)	373 (92.10%)	4 (22.28%)	25 (100%)		
	Grazoprevir	A156T	GCT		338 (83.46%)		1 (4%)	23 (82.14%)	low-intermediate
			GCC	313 (100%)	66 (16.30%)			5 (17.86%)	
			GCA			17 (94.44%)	4 (16%)		
			GCG		1 (0.25%)	1 (5.56%)	20 (80%)		
		D168E	GAT	12 (3.83%)	28(6.91%)	14 (77.78%)			low-intermediate
			GAC	299 (95.53%)	373 (92.10%)	4 (22.28%)	25 (100%)		
NS5A	Ledipasvir	L31M	TTA		184 (50.97%)				low-high
			TTG	19 (6.93%)	104 (28.81%)				
			CTT	5 (1.82%)	1 (0.28%)				
			CTC	4 (1.46%)				28 (100%)	
			CTA	1 (0.36%)	28 (7.76%)				
			CTG	242 (88.32%)	30 (8.31%)				
		Y93H	TAT	17 (6.20%)	4 (1.11%)	2 (10.63%)		3 (10.71%)	high
			TAC	254 (92.70%)					
	Daclatasvir	M28T	ATG	259 (94.53%)	10 (2.77%)			27 (96.48%)	intermediate
		Q30H	CAA	260 (94.89%)	3 (0.83%)				low-high
			CAG	9 (3.28%)	20 (5.54%)				
		Y93H	TAT	17 (6.20%)	4 (1.11%)	2 (10.63%)		3 (10.71%)	high

			TAC	254 (92.70%)				
		M28T	ATG	259 (94.53%)	10 (2.77%)		27 (96.48%)	intermediate
		Y93H	TAT	17 (6.20%)	4 (1.11%)	2 (10.63%)	3 (10.71%)	high
			TAC	254 (92.70%)				
		M28T	ATG	259 (94.53%)	10 (2.77%)		27 (96.48%)	intermediate
	Elbasvir	Q30H	CAA	260 (94.89%)	3 (0.83%)			low-high
			CAG	9 (3.28%)	20 (5.54%)			
		L31M	TTA		184 (50.97%)			low-high
			TTG	19 (6.93%)	104 (28.81%)			
			CTT	5 (1.82%)	1 (0.28%)			
			CTC	4 (1.46%)			28 (100%)	
			CTA	1 (0.36%)	28 (7.76%)			
			CTG	242 (88.32%)	30 (8.31%)			
		Y93H	TAT	17 (6.20%)	4 (1.11%)	2 (10.63%)	3 (10.71%)	high
				254 (92.70%)				
			TAC					
NS5B	Sofosbuvir	S282T	AGC	164 (98.8%)	293 (95.13%)	19 (95%)	24 (100%) 3 (16.67%)	high
			AGT	1 (0.66%)	15 (4.87%)	1 (5%)	14 (77.78%)	
			TCT					
			TCC					
			TCA					
			TCG					
	Dasabuvir	C316Y	TGT	152 (91.57%)		20 (100%)	24 (100%) 1 (5.56%)	high
					7 (2.27%)			
			TGC	14 (8.43%)	185 (60.06%)		17 (94.44%)	
		C316N	AAT	152 (91.57%)	7 (2.27%)	20 (100%)	24 (100%) 1 (5.56%)	low
			AAC	14 (8.43%)	185 (60.06%)		17 (94.44%)	
		S556G	AGT		270 (87.66%)			intermediate
			AGC	166 (100%)	5 (1.62%)			
		S556N	AGT		270 (87.66%)			
			AGC	166 (100%)	5 (1.62%)			

legend:

one transition	score 1.0
one transversion	score 2.5
one transition + one transversion	score 3.5
two transversions	score 5.0

Discussion

Besides the genetic variability and natural presence of drug resistance substitutions in selected genotypes prior to treatment, another factor that can be associated with probability of success of a DAA-based regimen is the genetic barrier for the development of resistance. This can be broadly defined as the number and type of nucleotide mutations required for the generation of a specific resistance substitution, starting from the wild-type genetic background of the virus (26). Previous studies have shown that genetic variability among HIV, HBV, or HCV genotypes can in some cases facilitate the development of specific resistance variants (24, 26, 27, 34, 35, 41). For instance, it has recently been proposed that the high degree of HCV genetic variability makes HCV genotypes, and even subtypes, differently prone to the development of PI resistance substitutions, with important clinical implications for tailoring individualized and appropriate regimens (34). These findings support that genetic barrier is higher for genotype 1b than to 1a in the NS3 gene. These results are consistent with available HCV experimental and clinical treatment observations (42, 43). Within NS3, many sequences had shown the Q80K mutation (44.7% for genotype 1a, 0.25% for genotype 1b), which can cause resistance to SMV, PTV and ASV (Table 1 and 4). The R155K mutation was rarely observed in subtype 1b viruses, where two changes (one transition and one transversion) are required, while subtype 1a needs only one transition. A similar profile has also been observed for other PIs such as SMV, FDV, ASV and PTV/r, to which subtype 1a is more prone to acquire RAVs (Table 4).

Regarding NS5A RAVs, Y93H in genotype 1b was the most commonly variant identified (4.25%), followed by L31M (3.40%), whereas other NS5A RAVs occurred at low frequencies. Furthermore, the overall number of sequences with some NS5A RAVs was higher compared to other genes but also the genetic barrier in this class is higher (requiring changing in more than one nucleotide). Differences in the level of resistance depending on the HCV subtype were seen in all position except in 93. The genetic barrier to DAA's acting in NS5B gene was lower than for NS5A, with exception of positions 448 and 559 where at least two mutations were shown to be

necessary to generate a RAV. Similarly, two substitutions, 316Y and 448H, with low genetic barriers were shown to strongly reduce HCV susceptibility to DAA's; accordingly, the 316Y variant was described to confer resistance to DSV (44, 45). Finally, the major resistance variant S282T, selected *in vitro* by SOF, requires only a single G-to-C transversion (score 2.5) and is rarely, if ever, seen in clinical isolates (16, 26), because it alters the conformation of the enzyme catalytic site (46) and severely compromises viral fitness among different HCV genotypes (47, 48).

All RAVs found in this study were identified in näıve-treatment patients. However, considering that the present analysis is based on a databank, this findings are only correlative rather than conclusive and the clinical relevance of this data is yet to be confirmed by additional longitudinal follow-up studies with DAAs involving patients infected with distinct HCV genotypes, nevertheless the results found that in 14 of 16 positions, conversion to a drug-resistant variant required only single nucleotide substitutions are interesting and can contribute with additional data about HCV resistance to DAAs.

Given that all oral HCV DAA therapies are associated with high costs, resistance testing at baseline, which is also a significant cost, may nevertheless be worthwhile to identify the best DAA treatment option for each patient depending on the HCV genotype and preexisting polymorphisms in the NS3 and NS5A/B (49). However, which frequencies of pre-existing RAVs within the HCV quasispecies and which level of resistance of pre-existing RAVs may contribute to treatment failure is not completely clarified. The presence in DAA-naive patients of natural polymorphisms at resistance positions in selected genotypes, together with a broad low genetic barrier for the development of resistance represents indeed an important issue in the global approach for the management and treatment of HCV-related disease.

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5.2 Artigo 2**:** artigo submetido à revista *World Journal of Gastroenterology*

Occurrence of polymorphisms and resistant mutations in the NS3, NS5A and NS5B genes of HCV based on sequences deposited in the European Hepatitis C Virus Database (euHCVdb)

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Abstract

Chronic Hepatitis C Virus (HCV) infection affects around 180 million people worldwide and is a significant cause of liver-related morbidity and mortality. The main therapeutic targets for HCV infection are the NS3/4A protease, NS5B polymerase, and NS5A replication complex. Pre-existence of resistance associated variants (RAVs) to direct antiviral agents (DAAs) reduces sustained virologic response (SVR) rates. The objective of this analysis was to evaluate the occurrence of polymorphisms and resistant mutations in NS3, NS5A and NS5B regions in treatment-naïve HCV sequences deposited in the European Hepatitis C Virus database (euHCVdb). The search resulted in 798 NS3, 708 NS5A and 535 NS5B sequences from HCV genotypes 1a, 1b, 2a, 2b and 3a, after the exclusion of sequences containing errors and/or gaps or incomplete sequences, and sequences from patients previously treated with DAAs. The Q80K variant in the NS3 gene was the most prevalent mutation, being found in 44.66% of the subtype 1a and 0.25% of the subtype 1b; other frequent mutations observed in more than 2% of the NS3 sequences were: I170V (3.21%) in genotype 1a, and Y56F (15.93%), V132I (23.28%) and I170V (65.20%) in genotype 1b. For the NS5A, 2.21% of the genotype 1a sequences have the P58S mutation, 5.95% of genotype 1b sequences have the R30Q mutation, 15.79% of subtypes 2a sequences have the Q30R mutation, 23.08% of subtype 2b sequences have a L31M mutation, and in subtype 3a sequences, 23.08% have the M31L resistant variants. For the NS5B, the V321L RAV was identified in 0.60% of genotype 1a and in 0.32% of genotype 1b sequences, and the N142T variant was observed in 0.32% of subtype 1b sequences. The C316Y, S556G, D559N RAV were identified in 0.33%, 7.82% and 0.32% of genotype 1b sequences, respectively, and were not observed in other genotypes. Despite the overall low frequency of mutations observed in our data, this resistant population is likely to be selected and maintained in the patients undergoing therapy with DAAs, and over multiple rounds of replication mutated variants can acquire additional substitutions to increase viral fitness. Even though HCV variants resistant to DAA targeting one viral protein remain susceptible to DAAs targeting another viral protein, combination therapy could fail due to selection of HCV with resistance substitutions in multiple targets.

Introduction

Chronic HCV infection affects around 180 million people worldwide and is a significant cause of liver-related morbidity and mortality (1). Until recently, Interferon-α in combination with Ribavirin was the mainstream treatment regimen but eligibility and safety of the interferon-based therapies were low, and consequently the overall effectiveness of the treatment was very limited. Fortunately, the development of new direct-acting antiviral (DAA) drugs against HCV has progressed significantly and resulted in oral interferon-free therapies (2).

The three main therapeutic targets for HCV infection are the NS3/4A protease, the NS5B polymerase, and the NS5A replication complex. The first series of interferon-free regimens, including combinations of simeprevir (SMV), sofosbuvir (SOF), paritaprevir (PTV), daclatasvir (DCV), ledipasvir (LDV), ombitasvir (OMV), dasabuvir (DSV), grazoprevir (GZR) and elbasvir (EBR), have already been approved and recommended by the European Association for the Study of the Liver (EASL) and by the American Association for Study of Liver Diseases (AASLD) (3, 4).

HCV variants infecting the human population show extreme genetic diversity, which is partly explained by the long evolutionary association between the virus and its human host. HCV exists in the host as a swarm of related quasispecies. This diversity is a result of the error-prone viral polymerase combined with rapid viral replication, which enable the virus to rapidly adapt to the host humoral and cellular immune responses, as well as to antiviral drugs (5). The selection of resistance-associated amino-acid variants (RAVs) from HCV quasispecies is dependent on drug-, host- and virus-related factors. The potency of the drug itself is primarily influenced by viral susceptibility, previous exposure to the drug and by the genetic barrier to resistance. The ability of a RAV to persist and to induce treatment failure (relapse, non-response or viral breakthrough) is related to its fitness or its replication capacity as compared to the wildtype virus (6, 7).

Resistance to DAAs is driven by the selection of mutations at different positions in the NS3 protease, NS5B polymerase and NS5A protein (8, 9). Each compound or drug family induces a specific mutation profile that may be characteristic of the viral genotype/subtype. Furthermore, each class of DAAs is characterized by a difference in the genetic barrier to resistance. Even though the specific resistance mutation for each

individual agent in the drug class differs, there is a great concern about the possibility of cross-resistance between compounds in the same inhibitor class, especially for the NS3 protease and NS5A inhibitors (10).

The ability to detect RAVs depends primarily on the different types of the sequencing technologies used, including population-based sequencing, clonal sequencing and deep sequencing. The sensitivities for detection by these three approaches were reported to be approximately 25%, 5% and 0.5%, respectively, and the presence of viral mutants below the detection levels might be missed (11). For HCV the frequency of routine testing of drug resistance prior to the use of the new treatment regimens is not known. Some guidelines (4) suggest that routine monitoring for HCV drug resistance–associated variants during therapy is not recommended and there is no consensus on the utility of pre-treatment resistance testing.

Currently, there are a number of HCV sequences obtained by Sanger sequencing available on public's databanks, however, they have not been used to analyze distinct viral genotypes and HCV genomic characteristics to relate them to resistance phenotypes (11). Three HCV databases are currently available to provide insight into the basic biology, immunology, and evolution of the virus: the Japanese database (http:// s2as02.genes.nig.ac.jp), the European database (http://euhcvdb.ibcp.fr) and the American database (Los Alamos National Laboratory) (http://hcv.lanl.gov). The present analysis will provide insights into the levels of circulating drug resistance, which may affect the success of the therapeutic regimens.

The objective of this analysis is to evaluate the occurrence of polymorphisms and resistant mutations in the NS3, NS5A and NS5B regions in treatment-naïve HCV sequences deposited in the European Hepatitis C Virus database (euHCVdb).

Methods

HCV Database

The sequences were downloaded from the European Hepatitis C Virus database (https://euhcvdb.ibcp.fr/euHCVdb/). This bank provides key data about the HCV sequences (e.g. genotype, genomic region, viral proteins and their functions, known 3-

dimensional structures) and ensures consistency of the annotations, which enables reliable keyword queries. User can extract subsets of sequences obtained by Sanger sequencing matching particular criteria or enter their own sequences and analyze them with various bioinformatics programs available on the same server. The euHCVdb is mainly oriented towards protein sequence, structure and function analyses and structural biology of HCV, and is re-built every month from an up-to-date database by an automated process (12).

The search was performed for full-length NS3 protease, NS5A and NS5B polymerase sequences of HCV separated by genotypes 1a, 1b, 2a, 2b and 3a. These subtypes were chosen due to their worldwide prevalence and presence in drug trials, specifically genotype 1 with protease inhibitors (PI) and genotype 3 with polymerase inhibitor. Reference strains for the three genotypes were obtained (1a: AF009606, 1b: D90208, 2a: D00944, 2b: D10988 and 3a: D17763). Sequences containing missing data, such as gaps and sequencing errors, and sequences from patients previously treated with DAAs were excluded from the analysis. To ensure the quality of the analysis, sequences with stop codons in the NS5B gene or with ambiguities consisting of more than 2 bases per nucleotide position or more than 2 ambiguities per codon at individual drug resistance-associated position were also excluded.

Alignment and edition of the sequences

The sequence alignment was performed with MEGA 6.06 MAC (13) using followed by sequence editing, exclusion of sequences with missing data, and translation of the nucleic acids sequences into amino acids. The resulting protein sequences were then analyzed using the BioEdit 7.2.5. software for mutations associated with resistance (14).

Analysis of natural polymorphisms

Known mutations associated with resistance of protease, NS5A complex and polymerase inhibitors were used to search for polymorphism patterns between HCV genotypes (15). It was included only positions that have been described in previous studies to be associated *in vivo* with treatment failure and/or have been shown *in vitro* phenotypic assays to confer a more than 2-fold change in replication in comparison to the wildtype reference strain.

Results

Database Search

The search resulted in 831 NS3, 869 NS5A and 6,065 NS5B sequences from HCV genotypes 1a, 1b, 2a, 2b and 3a. After the exclusion of incomplete sequences and those containing errors and/or gaps, and from patients previously treated with DAAs, 798 sequences were included in the NS3 data set. There were 313 from genotype 1a, 412 from genotype 1b, 19 from genotype 2a, 26 from genotype 2b and 28 from genotype 3a. There were 699 sequences identified in the NS5A data set, with 272 from genotype 1a, 353 from genotype 1b, 19 from genotype 2a, 26 from genotype 2b and 29 from genotype 3a. For the NS5B polymerase there were 535 HCV sequences: 165 from genotypes 1a, 307 from genotype 1b, 19 from genotype 2a, 24 from genotype 2b and 20 from genotype 3a. Notably, the NS5B region has more than 5,300 incomplete sequences deposited into this databank.

Mutation analyses

Mutation analyses were performed for positions where resistance-associated amino acid substitutions have been described in the literature for conferring resistance to DAA's. Amino acid substitutions related to HCV resistance to DAAs are described below.

Frequency of resistance-associated variants

a) NS3/4A protease inhibitors (Table 1)

Considering that the available PI are less effective against genotype non-1 group due to natural polymorphisms in their NS3 region, the analyses have taken this into consideration and the discussion focus on the genotype 1 dataset; nevertheless the results of the other genotypes are shown in Table 1. The Q80K variant was the most prevalent mutation, found in 44.66% of the subtype 1a, and in 0.25% of the 1b subtype sequences, despite that variant V80L was also observed in 6.39% of the sequences from this subtype. Other positions with frequencies higher than 2% were I170V (3.21%) in genotype 1a, and Y56F (15.93%), V132I (23.28%) and I170V (65.20%) in genotype 1b.

The V36L and V36M RAVs were identified in 1.60% and 0.32% of genotype 1a sequences, respectively, and in 0.74% and 0% of genotype 1b, respectively. The T54S variant was observed in 0.97% of genotype 1a and in 0.5% of genotype 1b sequences. The R155K variant was observed in 0.64% of genotype 1a sequences and was not observed in genotype 1b. There were two genotype 1b sequences (0.5%) with P substitution at position 155. Finally, no RAV A156T mutation was found in all 831 NS3 sequences analyzed.

The prevalence of resistant variants for the PI were found to be low in this data set. Variants resistance to these drugs were observed at the NS3 position 168; the most frequent mutation was D168E which was found in 0.32% of subtype 1a and in 0.98% of subtype 1b sequences. Another mutation, Q80K, was present in 44.66% of subtype 1a and in 0.25% of subtype 1b sequences. The prevalence of known NS3 variants enriched for by GZR was found to be low: F43S (0.31%) and Y56H (0%) in the whole data set; the NS3 Q41R mutation was not observed.

b) NS5A replication complex inhibitors (Table 2)

For subtype 1a there were a total of 272 NS5A sequences in our data set. Mutations L23M (0.37%), M28T (0.75%), Q30H (1.47%), Q30R (0.37%), L31M (1.12%), P58S (2.21%) and Y93C (0.37%) were observed, whereas no variants were observed at NS5A position 32. For subtype 1b, of 353 sequences analyzed 0.28% had the L23I mutation, 2.27% had L28M mutation, 5.95% had R30Q mutation, 3.40% had M31L mutation, 3.68% had P58S mutation, and 4.25% had the Y93H mutation. Of 19 subtype 2a sequences analyzed, one (5.26%) sequence had the Q30R mutation, 3 (15.79%) sequences had the M31L mutation, and one (5.26%) sequence had the H58P mutation. For subtype 2b a total of 26 sequences were analyzed, 6 (23.08%) with the L31M and one (3.85%) with the S58P mutation. In subtype 3a, for which 28 sequences were analyzed, the resistant variants M28I, A30L and P58R were found, each in a different sequence (3.57%) of the data set. Only M31L was found in more than one sequence (23.08%) for this subtype. No mutation was found in the NS5A sequence at position 32 of any subtype.

c) NS5B polymerase inhibitors (Table 3)

The NS5B S96T, C223H/Y, and S282T variants were not observed in any sequence in the present study, and the NS5B N142T variant was observed in 0.32% of the subtype 1b sequences. The V321L RAV was identified in 0.60% of genotype 1a sequences and in 0.32% of genotype 1b sequences.

The C316Y, S556G, and D559N RAVs were identified in 0.33%, 7.82% and 0.32% of genotype 1b sequences, respectively, and were not observed in other genotypes. The M414T and Y448H RAVs were not found in any of the 535 NS5B sequences analyzed.

Variants at NS5B positions 495 and 496 were not observed; on the other hand, the NS5B A421V and V499A substitutions were found in both subtypes 1a and 1b. The A421V mutation occurred in 9.64% of subtype 1a and in 4.55% of subtype 1b sequences. The V499A variant was the dominant amino acid substitution in subtype 1a sequences (95.15%), but for subtype 1b it was observed in 9.74% of the sequences but there has been no reported evidence for negative clinical impact of the V499A.

There were no variant mutations at positions 422, 494 and 554 of NS5B and only one variant was found in position 486 in all the sequences analyzed.

Discussion

The resistance to direct antiviral therapy has been a major problem in a number of chronic viral infections; while much attention has been given to HIV infection and resistance to antiviral therapy (16), the extent of mutations in the development of drug resistance in infection by HCV is less studied. The presence of HCV mutations is mainly due to factors such as selection pressure, error-prone replication (because of RNA polymerase's poor fidelity) and the high replication capacity of the virus. It is believed that every possible mutant can be generated continuously in a HCV-infected patient (17). Hence, selected variants are considered to be pre-existent mutations generated during the natural HCV life cycle. The incidence of resistant variants is variable and depends on the binding domain, as well as on the different HCV populations, genotypes and subtypes. With the exception of NS5B nucleoside analogues, the current DAAs target the NS3, as well as the allosteric sites of NS5B and NS5A, which all have a low threshold of resistance (10, 18). Data from both replicon analysis and from clinical trials

have consistently identified viral mutations that can be associated with antiviral treatment failure (19). A recently published analysis found that 58.7% of the HCV sequences deposited in the GenBank harbored at least one dominant resistance variant (20).

The present study investigated the prevalence of baseline polymorphism/resistance mutations in the NS3, NS5a and NS5b regions of HCV, based on viral sequences deposited in the euHCVdb and that were from samples of treatment-naïve patients infected with HCV, including genotypes 1a, 1b, 2a, 2b or 3a .

Overall, the prevalence of patients with variants resistant to protease, NS5A, or polymerase inhibitors was low, with exception of Q80K in genotype 1a, found in 44.66% of all sequences. The frequency of NS5A inhibitor-resistant variants was also low (0 to 4.25%). Similar to the previous reports, the primary polymerase inhibitor resistant variants were observed mainly in genotype 1b and occurred in 0 to 7.82% (palm site), 0 to 2.41% (thumb site), and 0 to 9.74% (finger-loop site) of the sequences. These data are very similar to other studies reported in the literature (15, 21), and a comparasion with an analysis performed in Los Alamos databank showed similar results (Table 4).

NS3/4A protease inhibitors

The first PI were primarily designed for the treatment of genotype 1 infection and have no or only moderate activity on other genotypes. Pre-existing dominant resistance mutations in the NS3 region are more common in treatment-naïve patients infected with genotype 1a (cumulative incidence 8.6% vs. 1.4%) (22). Within NS3, the resistant Q80K mutation, which is based on available data only relevant for SMV and ASV, was the most prevalent (44.66% genotype 1a, 0.25% genotype 1b) and this result corroborates the recent findings of Pol et al. with European patients (1, 23).

The mutation I170V, present in 3.21% of genotype 1a and 65.20% of the genotypes 1b sequences analyzed, has been reported as not showing any influence on protease inhibitor activity (24). Other observed mutations, though found in lower frequencies, have been reported to confer resistance to PI: the R155K variant confers resistance to all PI ranging from 4-fold to 360-fold change, while mutation D168E has been associated to a higher-level of resistance (17-fold to 2591-fold changes) to non-covalent inhibitors. Therefore, considering the actual recommendations in EASL and

AASLD guidelines, up to 45% of patients with genotypes 1 have resistance mutations that can lead to treatment fail using PI.

NS5A inhibitors

The HCV NS5A protein has no known enzymatic activity, yet it plays essential roles in both viral genome replication and virus particle assembly, and may be a mediator between the two events. The prevalence of resistant variants in the context of the NS5A inhibitors is highly dependent on viral subtype due to several of the positions having different baseline amino acids in each subtype (15). Resistance against DVC, OMV, LDV is more common in genotype 1b (up to 4.25% of the sequences), but it can also occur in genotype 1a in less than 1.5% of the sequences. Furthermore, a broad cross-resistance between NS5A inhibitors is expected by the selection of mutations at codons 31 and/or 93 causing a loss in susceptibility to the majority of these compounds (24). Other researchers also determined Y93H as most frequent baseline NS5A RAV in genotype 1b (6–23%), followed by L31M (3–4%) (24, 25), whereas NS5A RAVs occurred at low frequencies in genotype 1a .

Across HCV genotypes, variation is observed at several of the residues identified as important sites for resistance, and the substitutions M28L, Q30R, H58P that were found in genotype 1b; M28F, Q30K, L31M, H58P in genotype 2a; M28L, Q30K, L31M, H58P in genotype 2b and Q30A in genotype 3a could be defined as natural polymorphisms that distinguish those genotypes from 1a.

NS5B inhibitors

In contrast to NS3 PI, NS5B non-nucleoside and NS5A-inhibitors where resistance mutations are subtype-dependent, little is known about NS5B nucleos(t)ide analogs genotype- and subtype-dependent resistance mutations. Several nucleotide analogs have shown very promising results and SOF is the first DAA in this family to gain regulatory approval (9, 26), followed by DSV In the present analysis, NS5B RAVs were not detected in genotype 1a, whereas in genotype 1b, NS5B RAVs were found in more than one third of the individuals (C316N in 37.02% and S556G in 7.82%) conferring low to medium resistance to DSV. In mixed cohorts consisting of American and European patients, while the S556G mutation was observed in frequencies of 0.5–16%, the

C316N RAVs occurred in frequencies lower than observed in this study (11–18%), at baseline in genotype 1b samples (15).

The S282T is the *in vitro* signature resistance mutation that conveys decreased susceptibility to SOF in the replicon system. Although the S282T substitution requires only a single nucleotide change, this variant was not found in any of the NS5B sequences analyzed in this study, neither in a previous study based on sequences from the Los Alamos databank (11); in a study that analyzed 1459 HCV sequences from GenBank, this mutation was found in only one sequence (20).

DAA-resistant variants generally demonstrate reduced replicative capacity compared to 'wildtype' (or DAA-susceptible) variants (14). This low fitness, typically of resistant viral strains, makes the detection of RAVs more difficult in the viral population. Nonetheless, despite the overall low frequency of mutations as observed in our data, this resistant population is likely to be able to be selected and maintained in the patients undergoing therapy with DAAs. It is possible that over multiple rounds of replication mutated variants can acquire additional substitutions to increase viral fitness. Nevertheless, it has been demonstrated that removal of the DAA generally results in restoration of the susceptible/more fit variants as the dominant species within the quasispecies. HCV variants resistant to DAA targeting one viral protein remain susceptible to DAAs targeting another viral protein. However, combination therapy remains susceptible to failure due to selection of HCV with resistance substitutions in multiple targets.

With the currently in-use DAA recommended by EASL and AASLD guidelines, our analyses suggest that it is possible that virologic failure could occur in half of the patients with genotype 1a receiving SPV in combinations with PEG-IFN and Ribavirin. In addition, more than 7% of the patients with genotype 1b receiving DSV could also fail to respond to treatment, and resistant variants in NS5A region should affect almost 5% of the treated individuals. The main concern of an analysis of a public databank is whether the sequences truly reflect the prevalence of these RAV in the population, but on the other hand the abundance of information deposited in databank's sequences allows one to identify potentially unknown polymorphisms in populations not submitted to new HCV treatments. Since it is impossible to correlate criteria of inclusion in

databanks with population data, epidemiological studies are necessary to determine the prevalence of RVA in the population.

Although the ability of the majority of the amino acid substitutions identified in our analyses to confer resistance is not known, the ability of HCV to rapidly evolve under drug selection pressure and the presence of baseline natural polymorphisms associated with resistance to DAAs should be considered as possible threats to the success of these new therapies.

In summary, there are many relevant clinical questions that still need to be answered, this is mainly due to the limited available data and the large number of DAAs approved or soon to be approved for clinical use. Perhaps resistance mutations in the new interferon-free DAA era may not have significant clinical impact initially (27, 28), nonetheless the presence of a minor drug-resistance population will likely affect the successes of the therapy upon the expansion and prolonged use of the DAA regimens, and the relevance of pre-existing resistance mutations for responses to Interferon-free DAA therapies needs to be further investigated. Therefore, testing for drug resistance variants prior to the initiation of treatment will be needed in the very near future in order to help guide the selection of the most optimized treatment option.

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Tabela 1: HCVNS3 Amino Acids positions according with genotype

HCV NS3 Amino Acids positions according with genotype (%)					
genotype	1a	1b	2a	2b	3a
Position	wt	wt	wt	wt	wt
16	C 99.35 S 0.65	C 99.50 S 0.25 T 0.25	T 31.58 S 5.26 A 63.12	A 100	T 100
36	V 98.08 **L 1.6** M 0.32	**L 0.74** I 0.25 V 99.01	L 100	L 100	L 100
41	Q 99.35 H 0.65	Q 99.50 H 0.50	Q 100	Q 100	Q 100
43	F 99.69 **S 0.31**	F 100	F 100	F 100	F 100
54	T 99.03 **S 0.97**	T 99.50 **S 0.50**	T 100	T 100	T 100
55	V 98.39 A 0.64 I 0.97	V 99.50 I 0.50	V 100	V 100	V 100
56	Y 100	Y 84.07 F 15.93	Y 100	Y 100	Y 100
80	Q 54.37 **K 44.66** R 0.97	Q 93.37 **K 0.25** L 6.39	G 100	G 100	Q 100
107	V 100	V 100	V 100	V 100	V 100
109	R 100	R 100	R 100	R 100	R 100
122	S 95.51 **G 4.49**	S 82.84 **G 9.07** T 4.41	N 3.43 S	S	S
132	I 98.71 T 0.32 V 0.97	I 76.47 I 23.28 L 0.25	L 94.44 I 5.56	L 100	L 89.29 I 10.71
138	S 100	S 99.75 P 0.25	S 100	S 100	S 100
155	R 99.36 **K 0.64**	R 99.50 P 0.50	R 100	R 100	R 100
156	A 100	A 100	A 100	A 100	A 100
168	D 99.36 **E 0.32** G 0.32	D 98.77 A 0.25 **E 0.98**	D 100	D 100	Q 100
170	I 96.79 V 3.21	I 34.80 V 65.20	I 100	I 91.67 V 8.33	I 96.43 V 3.57
175	L 100	M 99.50 L 0.50	L 100	L 92.31 I 7.69	L 100

Amino acids in bold are associated with resistance.

Tabela 2: HCV NS5A Amino Acids positions according with genotype

HCV NS5A Amino Acids positions according with genotype (%)																				
Gen	1a								1b											
Position	wt								wt											
23	L	99.63	M	0.37					L	99.72	I	0.28								
28	M	94.38	I	0.37	**T**	**0.75**	V	4.49	L	97.17	M	2.27	V	0.52						
30	Q	98.16	**H**	**1.47**	**R**	**0.37**			R	92.35	K	1.13	L/M	0.28	Q	5.95				
31	L	98.88	**M**	**1.12**					L	96.03	M	3.40	I	0.57						
32	P	100							P											
58	H	95.96	C	1.10	P	2.21	R/Y	0.37	P	92.92	A	0.28	L	0.85	**S**	**3.68**	T	1.70	Q	0.52
93	Y	98.90	**C**	**0.37**	**H**	**0.74**			Y	95.75	**H**	**4.25**								

HCV NS5A Amino Acids positions according with genotype (%)												
Gen	2a				2b				3a			
Position	wt				wt				wt			
23	L	100			L	100			L	100		
28	F	100			L	100			M	96.46	I	3.57
30	K	94.74	R	5.26	K	100			A	96.46	L	3.57
31	M	84.21	L	15.79	M	76.92	L	23.08	L	100		
32	P	100			P	100			P	100		
58	P	94.74	S	5.26	P		S	0.85	P	96.46	R	3.57
93	Y	100			Y	100			Y	100		

Amino acids in bold are associated with resistance.

Tabela 3: HCV NS5B Amino Acids positions according with genotype

HCV NS5B Amino Acids positions according with genotype (%)					
Gen	1a	1b	2a	2b	3a
Position	wt	wt	wt	wt	wt
95	H	H 99.68 L 0.32	H	H	H
96	S	S	S	S	S
142	N	N 89.90 S 9.77 T 0.32	N	N	N
223	C	C 99.68 R 0.32	C	C 95.83 W 4.17	C
282	S 99.40 R 0.60	S	S	S	S
316	C	N 37.02 C 62.34 **R/Y 0.32**	C	C	C
321	V 99.40 I 0.60	V 99.68 I 0.32	V	V	V
365	S	S	S	S	S
368	S	S 99.68 P 0.32	S	S	S
414	M 99.40 **V 0.60**	M 99.68 I 0.32	Q	Q	M
419	L	L 99.03 I 0.97	I 90 V 10	I	I
421	A 88.76 M 0.60 V 9.64	A 95.13 V 4.55 S 0.32	V 63.16 A 36.84	V	V
422	R	R	R	R	R
423	M 96.99 **I 2.41** **V 0.60**	M	M	M	M
424	I 98.80 V 1.20	I 90.58 V 9.42	V	V	V
426	M 91.57 A 0.60 L 7.83	M 97.08 L 2.92	M	M	M
445	C 99.40 G 0.60	C 99.68 W 0.32	F	F	F
448	Y 99.40 E 0.60	Y	Y	Y	Y
452	Y 99.40 G 0.60	Y 98.38 **H 1.62**	Y	Y	Y
482	I	I 99.68 L 0.32	L	L	L
486	A	A	A	A	A 93.75 S 6.25
494	V	V	A	A	C
495	P	P	P	P	P
496	P	P	P	P	P
499	A 95.15 T 3.03 V 1.82	V 86.04 A 9.74 T 4.22	A 85 T 10 V 5	A	A
554	G	G	G	G	G
556	S	N 89.24 D 0.98 **G 7.82** N 1.95	G	G	G
559	D	D 99.68 N 0.32	D 95 H 5	D	D

Amino acid in bold are associated with resitance.

Tabela 4: Resistance Associated Variants (RAV) conferring resistance to DAAs recommended by the EASL and AASLD guidelines 2015.

	euHCVdb		**Los Alamos**	
	1a	1b	1a	1b
DAA	RAV (%)	RAV (%)	RAV (%)	RAV (%)
NS3				
simeprevir	V36M (0.32%)		V36M (0.44%)	
	Q80K (44.66%)	Q80L (6.39%)	Q80K (36.62%)	Q80L (6.02%)
	S122G (4.49%)	S122G (9.07%)	n.a	n.a
	R155K (0.64%)		R155K (0.88%)	
	D168E (0.32%)	D168E (0.98%)	D168E (0.29%)	D168E (0.80%)
				D170T (0.20%)
paritaprevir	R155K (0.64%)		R155K (0.88%)	
	D168E (0.32%)	D168E (0.98%)	D168E (0.29%)	D168E (0.80%)
grazoprevir	A156T (0.00%)	A156T (0.00%)	A156T (0.00%)	A156T (0.00%)
	D168E (0.32%)	D168E (0.98%)	D168E (0.29%)	D168E (0.80%)
NS5A				
ledipasvir	M28T (0.75%)		n.a	n.a
	Q30H (1.47%)			
	Q30R (0.37%)			
	L31M (1.12%)			
	Y93H (0.74%)	Y93H (4.25%)		
	Y93C (0.37%)			
daclatasvir	M28T (0.75%)		n.a	n.a
	Q30H (1.47%)			

	Q30R (0.37%)			
	Y93H (0.74%)	Y93H (4.25%)		
ombitasvir	M28V (4.49%)	Y93H (4.25%)	n.a	n.a
elbasvir	Q30H (1.47%)			
	L31M (1.12%)			
	Y93H (0.74%)	Y93H (4.25%)		
NS5B				
sofosbuvir	S282T (0.00%)	S282T (0.00%)	S282T (0.00%)	S282T (0.00%)
dasabuvir		C316N (37.02%)		C316N (36.17%)
		C316Y (0.32%)		C316Y (0.30%)
		N556G (7.82%)	N556G (0.42%)	N556G (8.21%)

6 CONCLUSÃO

Globalmente, a prevalência de pacientes com variantes resistentes aos inibidores da protease, do complexo NS5A ou dos inibidores da polimerase foi baixa neste estudo, com exceção da Q80K no genótipo 1a que foi encontrada em 44,66% de todas as sequências depositadas no *European Hepatitis C Virus database* (euHCVdb). A prevalência de variantes resistentes aos inibidores do complexo NS5A também foi baixa, com frequência entre 0 a 4,25%. Semelhante a outros estudos já publicados, as variantes resistentes aos inibidores nucleosídeos e nucleotídeos foram observadas em 7,82% das sequências, principalmente no genótipo 1b, sendo observadas em frequências entre 0 e 7,82% no sítio “palma”, 0 a 2,41% no sítio “polegar” e 0 a 9,74% no sítio “curva do dedo”. Esses dados são bastante semelhantes aos encontrados nos estudos publicados que avaliaram as sequências depositadas no banco de dados de *Los Alamos.*

Independentemente do genótipo do HCV, a presente análise revelou que em quatorze das 16 das posições a conversão para uma variante resistente ao antiviral de ação direta requer somente uma única substituição de nucleotídeo, isto é, uma transição com escore 1 para a barreira genética nas posições F43S, Q80R, R155K ou G, A156T no gene NS3; H ou P58S, Y93H no gene NS5A; C316Y e S556N no gene NS5B, ou uma transversão com um escore para barreira genética de 2,5 nas posições Q80K, A156S, D156E no gene NS3 e S368T no gene NS5B.

A presença de polimorfismos naturais em posições capazes de conferir resistência aos antivirais de ação direta em pacientes virgens de tratamentos, junto com uma baixa barreira genética para o desenvolvimento de resistência em todos os genes do genoma do HCV representa uma importante preocupação na abordagem global para o manejo e tratamento da hepatite C crônica e de doenças relacionadas ao HCV.

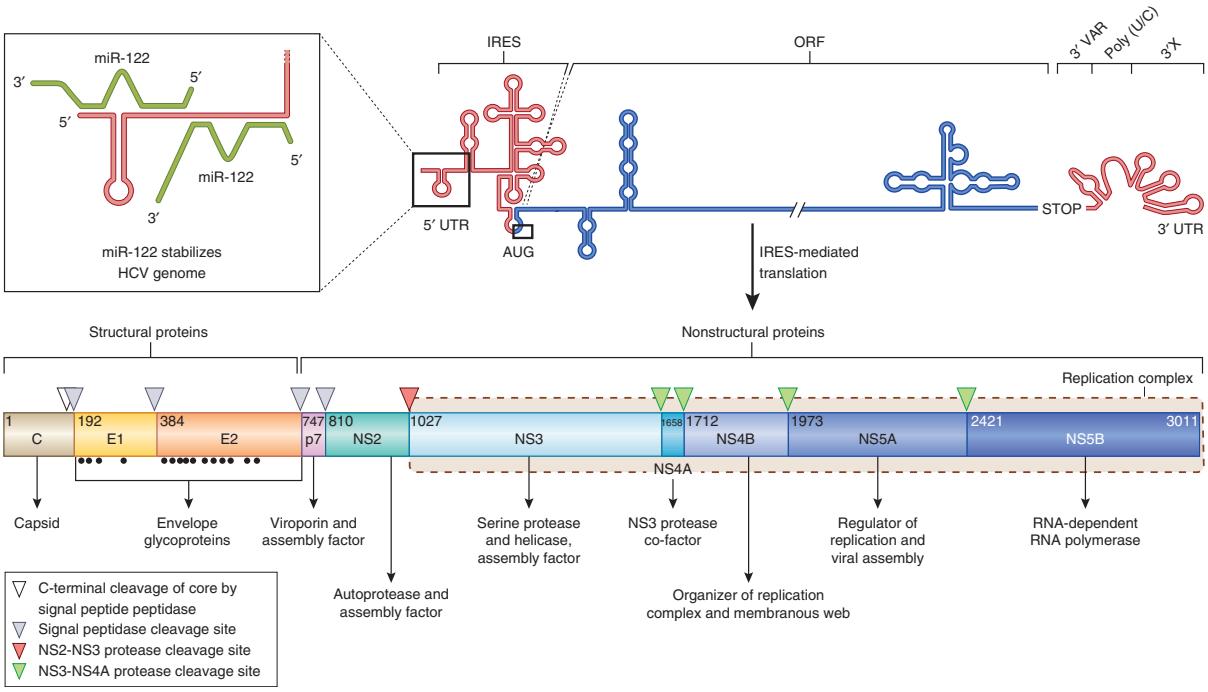
7 PERSPECTIVAS FUTURAS

Pretendemos avaliar, futuramente, a presença de polimorfismos em sequências genéticas de HCV, a partir de amostras de sangue de pacientes infectados pelo HCV com ou sem coinfecção com HIV atendidos no ambulatório de Hepatites Virais do Hospital Nossa Senhora da Conceição (HNSC), através do uso de sequenciamento por *Next Generation Sequencing* (NGS) pela plataforma Illumina.

Na primeira fase de experimentos, já em andamento, estamos utilizando *primers* para amplificar as regiões NS3 e NS5 e, a partir dessas regiões amplificadas, realizaremos o sequenciamento genético; tentaremos realizar o sequenciamento do genoma completo do HCV.

A segunda fase de experimentos avaliará a resposta ao tratamento com os DAA's disponíveis no Brasil e correlacionar a presença de mutações de resistência detectadas através do uso de sequenciamento por NGS na plataforma Illumina com a resposta virológica sustentada. Também serão avaliados os pacientes que apresentarem falha virológica ou recidiva ao tratamento com novas coletas de sangue durante a falha ou recidiva, para subsequente sequenciamento viral e melhor entendimento da dinâmica viral do HCV em cepas sem resposta ao tratamento.

Figura 1: O genoma do HCV e o processamento da poliproteína



Fonte: Scheel TK, Rice CM. Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. Nat Med. 2013;19:837-49.