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GUILHERME RODRIGUES FERNANDES CAMPOS

Estudo de mutações de resistência ao tratamento com Drogas de Ação Direta em pacientes infectados pelo Vírus da Hepatite C genótipo 3.

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

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RESUMO

O tratamento do HCV com antivirais de ação direta (DAAs) resulta em uma resposta virológica sustentada (RVS) que varia de acordo com o genótipo viral. Dentre os genótipos, o genótipo 3 é o segundo mais prevalente no Brasil e o que apresenta a menor RVS. É o responsável por quase 24% dos casos de infecção no país e a literatura possui poucos dados que expliquem essa menor resposta e maior taxa de escape ao tratamento. Dessa forma, o objetivo deste estudo foi analisar os vírus do genótipo 3 circulantes em pacientes brasileiros, sob tratamento com Daclatasvir (DCV) e/ou Sofosbuvir (SOF), para analisar mutações já descritas associadas à resistência e identificar novas mutações que confiram resistência dentro da NS5A e NS5B, investigando in vitro o comportamento dessas substituições. Dos pacientes inclusos no trabalho, a taxa de recidiva foi de 11,9%, onde a falha no tratamento foi relacionada com diversos fatores, e a presença de mutações associadas à resistência foi um deles. Mutações de resistência descritas na literatura foram observadas para a NS5A nos sítios 30, 62 e 93, onde o último apresentou mutações somente após a administração medicamentosa, indicando uma possível ação da pressão seletiva. Para a NS5B, as substituições foram detectadas nas posições 159, 421 e 554, onde somente a primeira foi mantida após o tratamento. Entre as mutações não conhecidas, S98G se mostrou promissora devido a sua alta frequência em pacientes recidivantes, antes e após o tratamento. As análises *in vitro* revelaram que S98G apresenta efeito compensatório na replicação viral, sem fenótipo de resistência quando isolada, mas quando presente em conjunto com Y93H, aumentou ainda mais a resistência ao DCV. Utilizando uma nova abordagem *in vitro* a partir da clonagem do domínio I da NS5A proveniente das amostras clínicas, mutações descritas e não descritas foram selecionadas pela pressão do tratamento com DCV. Os resultados desse estudo abrem possibilidades para compreender um pouco melhor o comportamento viral durante o tratamento, além de entender melhor a dinâmica de seleção de mutações. Dessa forma é possível mapear a frequência de circulação de mutações descritas na literatura e descrever possíveis novas mutações de resistência, delineando assim estratégias terapêuticas mais seguras para evitar o escape do tratamento.

Palavras-chave: HCV. Genótipo 3. Mutação de resistência. Daclatasvir. Sofosbuvir.

ABSTRACT

The HCV treatment with direct acting antivirals (DAAs) results in a sustained virological response (SVR) that varies according to the viral genotype. Among all genotypes, genotype 3 is the second most prevalent in Brazil and the one that presents the lowest SVR. It is responsible for almost 24% of infected cases in the country, and literature does not have enough data that can explain this lower response and higher treatment failure rate. Thus, the aim of this study was to analyze genotype 3 viruses circulating in Brazilian patients, under treatment with Daclatasvir (DCV) and/or Sofosbuvir (SOF), to observe resistance associated substitutions (RAS) already described in literature and to identify novel mutations that could confer resistance inside NS5A and NS5B, investigating the in vitro behavior of these substitutions. Among the patients included in this work, the relapse rate was 11.9%, where the treatment failure was related with many factors, and RAS presence was one of them. Described RAS were observed inside NS5A in the sites 30, 62 and 93, where the last one presented mutations only after drug administration, indicating a possible selective pressure. For NS5B, substitutions were detected in positions 159, 421 and 554, where only the first one was maintained after treatment. Regarding undescribed mutations, S98G showed promising data due to its high frequency in relapsing patients, before and after treatment. *In vitro* analysis revealed that S98G presents a compensatory effect on viral replication, with no resistance phenotype when isolated, but when present concomitantly with Y93H, led to an increasing on resistance to DCV. Using a brand new *in vitro* tool, based on cloning of NS5A domain I derived from clinical samples, described RAS and undescribed novel mutations were selected after DCV selective pressure. The results of this study enable the comprehension of viral behavior under DAA treatment, leading to a better understanding on the dynamics of RAS selection. These approaches enable the frequency mapping of already known RAS and the description of possible novel resistance associated mutations, designing safer treatment strategies to avoid treatment failure.

Keywords: HCV. Genotype 3. Resistance associated mutations. Daclatasvir. Sofosbuvir.

SUMÁRIO

1. Capítulo I

1.1 INTRODUÇÃO

Durante muitos anos, os vírus das Hepatites A e B foram considerados os maiores causadores de hepatite, incluindo casos associados à transfusão. Entretanto, mesmo após o estabelecimento de métodos de prevenção e diagnóstico para esses vírus, casos de hepatite associados à transfusão continuaram ocorrendo, esses casos foram denominados hepatite não-A, não-B (Kato, 2001). Em 1989 Choo et al isolou parte do genoma deste vírus desconhecido, que ficou conhecido como vírus da Hepatite C (HCV), a partir do plasma de um chimpanzé cronicamente infectado. (Choo, Kuo et al., 1989)

A prevalência da infecção por HCV pode flutuar de acordo com a região geográfica, variando de 2.3% e 1.5% nas regiões leste mediterrânea e europeia, respectivamente, à 0.5% em outras regiões do globo (Who, 2019). Em números, a estimativa é de aproximadamente 71 milhões de pessoas soropositivas no mundo (Alter, 2007; Who, 2019). O HCV replica-se preferencialmente nos hepatócitos (Hoofnagle, 2002), porém já se conhecem outros tipos celulares permissivos à infecção por esse vírus, como é o caso dos colangiócitos, células responsáveis por revestir os ductos biliares (Ralphs e Khan, 2013; Fletcher, Humphreys et al., 2015).

1.1.1 Fatores de Risco e Transmissão

O HCV é transmitido pelo contato percutâneo com sangue contaminado. Os fatores de risco associados à infecção incluem transfusão de sangue, transplante de órgãos, usuários de drogas injetáveis, injeções terapêuticas não seguras, exposição ocupacional a sangue, perinatal e sexual (Mello, 2006; Alter, 2007). Outras maneiras de transmissão pela utilização de procedimentos percutâneos, como piercing, tatuagem e circuncisão, podem ocorrer caso seja utilizado material inadequadamente esterilizado (Who, 2000).

Durante muito tempo a transfusão de sangue foi a principal forma de transmissão da hepatite C. Entretanto, com o implemento de testes anti-HCV nos bancos de sangue, esta forma de transmissão vem reduzindo. Ainda assim, a incidência de hepatite relacionada à transfusão ainda é alta em alguns lugares do mundo. Por exemplo, um estudo realizado no Brasil, no banco de sangue de Santa Catarina, mostrou uma queda significante no risco de aquisição de HCV, porém o índice de 1:13720 ainda se mostrou alto quando comparado com o de países desenvolvidos (Sy e Jamal, 2006).

Segundo a Organização Mundial da Saúde (OMS), é estimado que, das 71 milhões de pessoas infectadas pelo HCV no mundo, cerca de 400 mil pessoas chegam a óbito por ano. Grande parte dos infectados desconhece o diagnóstico (Lavanchy, 2009; Mohd Hanafiah, Groeger et al., 2013; Who, 2014). Dados do Ministério da Saúde brasileiro indicam que aproximadamente 700 mil pessoas estão infectadas pelo HCV no país. De todos os casos notificados a cada ano, 88% deles se encontram nas regiões sul ou sudeste (Brasil, 2018).

1.1.2 Sintomatologia e Prognóstico

O período de infecção, antes do aparecimento de sintomas clínicos, é de 15 a 150 dias. Na infecção aguda os sintomas mais comuns são fadiga e icterícia, porém na maioria dos casos, de 60% a 70%, são assintomáticos, mesmo nos casos que desenvolvem infecção crônica (Who, 2000).

Estima-se que mais de 75% dos indivíduos soropositivos desenvolvam uma infecção crônica que pode levar a cirrose hepática e em alguns casos ao carcinoma hepatocelular (Rosenberg, 2001). Dos indivíduos com infecção persistente 20% irão desenvolver cirrose hepática e o carcinoma hepatocelular ocorre em até 2,5% dos casos (Bowen e Walker, 2005).

1.1.3 Diagnóstico

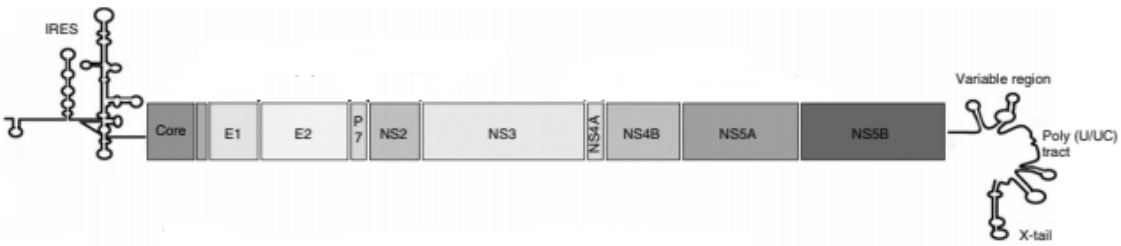
O diagnóstico da infecção pelo HCV ocorre por meio de testes de triagem seguidos de teste confirmatório. Os testes rápidos e por imunoensaio, que são testes de triagem, detectam anticorpos anti-HCV e indicam contato prévio com o HCV. A confirmação diagnóstica é possível por meio de testes moleculares que detectam ácido nucleico viral. A hepatite C crônica é caracterizada por um exame que indique ácido ribonucleico (RNA) do HCV detectável associado ao anti-HCV reagente há mais de seis meses (Brasil, 2015). Segundo a OMS, cerca de somente 19% da população infectada (13,1 milhões de pessoas) sabem sobre seu diagnóstico (Who, 2019).

1.1.4 O Vírus

O vírus da hepatite C (HCV) é um vírus de RNA fita simples, com polaridade positiva, pertencente à família Flaviviridae. O genoma do HCV, que possui 9,6Kb, é composto por uma região 5’ não traduzida (5’ UTR), uma open reading frame (ORF) que codifica

uma única poliproteína de aproximadamente 3000 aminoácidos e uma região 3’ não traduzida (3’ UTR). Essa poliproteina é clivada por proteases virais e do hospedeiro para gerar pelo menos 10 proteínas na seguinte ordem, iniciando pela extremidade amino terminal: core (C), envelope (E1; E2 e p7), não estruturais (NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Figura 1) (Kato, 2001; Lindenbach e Rice, 2005; Dustin e Rice, 2007; Suzuki, Ishii et al., 2007).

Figura 1: Estrutura do genoma do Vírus da Hepatite C (HCV), evidenciando as regiões responsáveis por codificar cada proteína viral e as estruturas secundárias do RNA nas regiões não traduzíveis (Kao, Yi et al., 2016). IRES: Internal Ribosome Entry Site



A região 5’ UTR é altamente conservada e contém um sítio de entrada ribossomal (IRES) essencial para a tradução do RNA viral. (Lyra, Fan et al., 2004). A região 3’ UTR é composta por uma pequena região variável, uma cauda poli (U/UC) e uma região altamente conservada de 98 nucleotídeos essenciais para a replicação viral (Moradpour e Blum, 2004).

A proteína core é a primeira a ser traduzida e está envolvida na formação do nucleocapsídeo (Lemon, Walker et al., 2007). As proteínas do envelope, E1 e E2, são glicoproteínas associadas, liberadas da poliproteína por peptidases de sinal do hospedeiro, são componentes chave para a entrada do vírus na célula. Ambas possuem regiões hipervariáveis envolvidas na evasão da resposta imune (Lemon, Walker et al., 2007; Li, Ma et al., 2014). A proteína p7 é transmembranica e funciona como canal de íons, ela se mostrou essencial para a infectividade em chimpanzés (Sakai, Claire et al., 2003; Chandler, Penin et al., 2012). A proteína NS2 é uma proteína de membrana com atividade na montagem de partículas virais, e antes de sua clivagem possui papel proteolítico, clivando a junção NS2-NS3, sendo os primeiros 180 nucleotídeos da NS3 também necessários para esta quebra (Boson, Granio et al., 2011; Popescu, Callens et al., 2011). A NS3 é uma proteína multifuncional, que possui um domínio serina-protease e um domínio helicase. A NS3 forma um complexo não covalente com a NS4A

que é um polipeptídeo ancorado na membrana. A NS4A age como um co-fator da NS3 e também a estabiliza (Yao, Reichert et al., 1999; Morikawa, Lange et al., 2011). A proteína NS4B provoca alterações específicas na membrana do retículo endoplasmático, produzindo um ambiente favorável à replicação viral, a chamada “rede membranosa” (Egger, Wolk et al., 2002; Gosert, Egger et al., 2003). A proteína NS5A é uma fosfoproteína multifuncional que tem atraído atenção considerável por seu potencial papel de modulação da resposta ao interferon (IFN), atua como parte do complexo replicativo e em outros processos da célula do hospedeiro (Enomoto, Sakuma et al., 1995; Enomoto, Sakuma et al., 1996; Kim, Welsch et al., 2011). A NS5B é uma proteína enzimática com função de RNA polimerase dependente de RNA (RdRp), sendo, portanto responsável pela replicação viral (Lohmann, Korner et al., 1997; Simister, Schmitt et al., 2009).

O estudo de sequências de nucleotídeos de HCV, de variantes identificadas em diferentes indivíduos e regiões geográficas, revelou a existência de pelo menos sete genótipos. Os genótipos possuem variantes mais relacionadas, os subtipos, classificados alfabeticamente (1a, 1b, 3a, 3e, etc). Em média, os genótipos diferem, no genoma completo, de 30% a 35% com relação aos nucleotídeos enquanto os subtipos diferem de 20% a 25% (Simmonds, Holmes et al., 1993; Simmonds, Bukh et al., 2005; Murphy, Chamberland et al., 2007).

Os genótipos 1, 2 e 3 e seus subtipos têm distribuição mundial, enquanto que o genótipo 4 está presente na África, o genótipo 5 na África do Sul e o genótipo 6 principalmente na Ásia. Acredita-se que o genótipo 7 tenha origem na África Central, onde foi primeiramente descrito (Silva, Parana et al., 2000; Murphy, Chamberland et al., 2007).

No Brasil, um estudo recente utilizando quase 30 mil amostras coletadas entre março de 2016 e março de 2018 atualizou a distribuição genotípica do vírus no país. De forma geral, a distribuição foi de 40,9% para o genótipo 1a, 30,2% para o genótipo 1b, 23,8% para o genótipo 3, 3,8% para o genótipo 2, 0,7% para o genótipo 4, 0,1% para o genótipo 5 e 0,6% para infecções genotípicas múltiplas. Juntos, genótipos 1 e 3 correspondem a 94,9% de todas as infecções por HCV no Brasil (Nutini, Hunter et al., 2020).

Um importante fato que contribui para a variabilidade genética viral é a falta de atividade corretiva da RdRp NS5B, e como resultado, trocas de bases podem ser

introduzidas aleatoriamente no genoma viral (Bowen e Walker, 2005; Argentini, Genovese et al., 2009). A frequência média de mutações de nucleotídeos varia de 1,4 x 103 a 1,9 x 103 substituições por nucleotídeo por ano (Le Guillou-Guillemette, Vallet et al., 2007). O HCV circula, portanto no hospedeiro como um conjunto de variantes virais geneticamente semelhantes chamadas de quasispecies (Martell, Esteban et al., 1992; Davis, 1999). Esta característica confere ao vírus um maior potencial de se adaptar a cada novo hospedeiro e aos desafios da infecção possibilitando o surgimento de mutações de escape tanto para evadir a resposta imune quanto para os tratamentos antivirais (Eigen e Biebricher, 1988; Eigen, 1993; Coffin, 1995; Domingo e Holland, 1997; Forns e Bukh, 1999; Mizokami, Ohno et al., 1999).

O primeiro tratamento utilizado para Hepatite C foi o Interferon (IFN), uma citocina componente da resposta imune inata, que atua induzindo a expressão de vias antivirais, mais precisamente dos genes ISGs (do inglês IFN-Stimulated Genes), que codificam proteínas atuantes na modulação da resposta imune e na atividade antiviral (De Veer, Holko et al., 2001). Células não infectadas expressam o interferon normalmente, porém uma vez na célula, o HCV interfere na expressão desta citocina, e consequentemente, dos genes ISGs, facilitando o estabelecimento da infecção (Raychoudhuri, Shrivastava et al., 2010; Chowdhury, Kim et al., 2014). Este tratamento apresenta diversos efeitos colaterais, como anemia, distúrbios autoimunes, diarreia, eritema, retinopatia e perda de peso (Brasil, 2011).

A utilização do interferon-α (INF-α) como tratamento para a Hepatite C iniciou por volta de 1986, antes mesmo da descoberta do agente etiológico da doença. Foi utilizado primeiramente como uma monoterapia, o que resultava em uma resposta virológica sustentada (RVS) de 20% a 30% (Hoofnagle, Mullen et al., 1986; Romeo, Rumi et al., 1995; Shiratori, Kato et al., 1997). Com o intuito de aumentar a eficácia do tratamento, o INF-α foi combinado à Ribavirina, proporcionando valores de RVS de 60%, para os genótipos 2 e 3 e 40% para genótipo 1 (Davis, Esteban-Mur et al., 1998; Mchutchison, Gordon et al., 1998; Poynard, Marcellin et al., 1998; Kato, 2001). A modificação do interferon com polietilenoglicol (pegINF) associado à ribavirina aumentou a RVS em até 40% (Manns, Mchutchison et al., 2001; Fried, Shiffman et al., 2002).

Com o desenvolvimento de antivirais de ação direta (DAA – Direct acting antiviral), como por exemplo, os inibidores de protease Boceprevir e Telaprevir, foi desenvolvida

a chamada terapia tripla, que consistia na associação de um desses inibidores com os dois componentes do tratamento utilizado até então (pegINF-α e Ribavirina) (Ghany, Nelson et al., 2011). Nesta terapia, tanto o Boceprevir quanto o Telaprevir atuavam sobre a protease viral NS3/4A, que possui papel crucial para o completo ciclo viral. Outros inibidores de protease, como o Simeprevir e o Grazoprevir, foram aprovados para substituir a primeira geração de inibidores de NS3/4A. Esse fato ocorreu principalmente devido ao efeito pan-genotípico dessas drogas contra os genótipos 1-6 (Summa, Ludmerer et al., 2012; Gentile, Buonomo et al., 2014; Rosenquist, Samuelsson et al., 2014), enquanto que Boceprevir e Telaprevir atuavam somente contra o genótipo 1 (Kumada, Toyota et al., 2012; Hayashi, Izumi et al., 2014; Hayashi, Seto et al., 2014; Izumi, Hayashi et al., 2014). Outra maneira para aumentar o espectro de ação do tratamento contra todos os genótipos foi o desenvolvimento de antivirais direcionados para outras proteínas virais, como a multifuncional NS5A e a RNA polimerase viral NS5B (Poveda, Wyles et al., 2014). Essas drogas possuem atividade contra todos os genótipos e dentre elas podemos destacar o inibidor de polimerase Sofosbuvir (Lawitz, Lalezari et al., 2013) e os inibidores de NS5A Daclatasvir e Velpatasvir (Gao, Nettles et al., 2010; Link, Taylor et al., 2019).

No Brasil, segundo protocolo publicado em julho de 2015, a administração de alfapeguinterferon (α-pegINF) e ribavirina ocorre somente em alguns casos, e o uso dos inibidores de protease de primeira geração (Boceprevir e Telaprevir) foi descontinuado. Em seu lugar, adicionaram-se outras drogas de ação direta contra o vírus, como o Sofosbuvir, o Simeprevir e o Daclatasvir. Essa mudança apresenta vantagens para os pacientes e profissionais da saúde, como menor duração do tratamento, menos efeitos colaterais, maior facilidade na posologia e principalmente melhora nos resultados quando comparado ao tratamento anterior (Kohli, Shaffer et al., 2014; Brasil, 2018). O tratamento hoje consiste em geral de terapia combinada, levando em consideração o genótipo viral, estado clinico do paciente e grau de comprometimento do fígado para a definição do tempo de tratamento e de quais drogas serão utilizadas.

Apesar dos melhores resultados obtidos com a administração do novo tratamento, relatos de variantes virais resistentes aos novos medicamentos já existiam antes mesmo da sua aprovação e distribuição no Brasil. Trabalhos in vitro e in vivo descreveram mutações relacionadas à resistência ao inibidor de NS5B Sofosbuvir (Lam, Espiritu et al., 2012; Gane, Stedman et al., 2013; Gerber, Welzel et al., 2013; Lawitz, Poordad et

al., 2014) enquanto outros relataram a resistência viral ao tratamento com o inibidor de NS5A Daclatasvir (Plaza, Soriano et al., 2012; Mcphee, Hernandez et al., 2014). A alta diversidade genética encontrada no hospedeiro, proveniente de alta taxa mutacional do vírus e da característica de circular como quasispecies, leva a uma diversidade populacional no hospedeiro elevada, que aliada a diferentes pressões seletivas impostas permite uma rápida adaptação (Cruz-Rivera, Carpio-Pedroza et al., 2013; Honegger, Kim et al., 2013). Esse preceito ajuda a entender como ocorre o surgimento de variantes resistentes a novos antivirais, onde o processo de evolução molecular possui papel fundamental (Ralston, Jacobson et al., 2011; Preciado, Valva et al., 2014).

Dentre os genótipos virais, o genótipo 3 tem se mostrado o menos susceptível ao tratamento com os novos antivirais de ação direta, com taxas de resposta abaixo dos outros genótipos. Essa resposta é ainda menor quando se trata de pacientes infectados pelo genótipo 3 e que apresentam um grau de progressão da doença bem avançado, como é o caso de pacientes com cirrose hepática. Estudos mostraram que pacientes infectados pelo genótipo 3 apresentam maior evasão ao tratamento do que pacientes infectados pelo genótipo 1, com RVS de 83% e de 96%, respectivamente (Lawitz, Poordad et al., 2013; Dore, Lawitz et al., 2015), e quando avaliado o grau de lesão hepática, a RVS cai para aproximadamente 60% em pacientes cirróticos infectados pelo genótipo 3 (Nelson, Cooper et al., 2015).

1.1.5 Daclatasvir

Formalmente denominado de BMS-790052, o antiviral de ação direta Daclatasvir é um inibidor de NS5A com potencial de ação em escala μM/L e amplo espectro de ação, atuando contra todos os genótipos virais. Foi descoberto por meio de um screening com a droga BMS-858, um fraco, porém específico, inibidor de replicação viral. BMS-858 interage com a porção N-terminal da NS5A, e após refinamento químico para melhorar a resposta antiviral e a cobertura pan-genotípica, desenvolveu-se o Daclatasvir (Gao, Nettles et al., 2010; Lemm, Leet et al., 2011; Nakamoto, Kanda et al., 2014).

O alvo do Daclatasvir é a NS5A, uma proteína multifuncional que, apesar de não possuir nenhuma atividade enzimática conhecida até o momento, está envolvida em funções essenciais para o ciclo replicativo do HCV, o que a torna um alvo interessante para o desenvolvimento de antivirais de ação direta (Macdonald e Harris, 2004; Tellinghuisen, Marcotrigiano et al., 2005; Hwang, Huang et al., 2010). De acordo com a

literatura, as mutações associadas à resistência ao Daclatasvir já conhecidas e descritas se concentram na porção inicial da proteína, dentro do Domínio I, que corresponde exatamente à região entre os aminoácidos 28 e 213 (Gao, Nettles et al., 2010). O Domínio I tem papel fundamental na replicação do HCV, sendo caracterizado por possuir motifs de ligação de RNA e ZN2+, e após sua analise por cristalografia, pode-se observar uma organização estrutural dimérica que é essencial para a replicação viral (Tellinghuisen, Marcotrigiano et al., 2004; Tellinghuisen, Marcotrigiano et al., 2005; Love, Brodsky et al., 2009). É nessa região que acontece a ligação do Daclatasvir com a proteína viral, onde o antiviral se liga precisamente com sua face dimérica. Essa interação com o Daclatasvir desregula o processo de hiperfosforilação da NS5A, o que resulta em alteração na localização celular da proteína, inibindo a formação e ativação do complexo replicativo, além de bloquear síntese de RNA viral, a montagem de novos vírions e a subsequente liberação dessas partículas (Lee, Ma et al., 2011; Qiu, Lemm et al., 2011; Guedj, Dahari et al., 2013).

1.1.6 Sofosbuvir

O Sofosbuvir, também conhecido como GS-7977, é um inibidor de polimerase de efeito pan-genotípico (Stedman, 2014). Utilizando a polimerase viral NS5B como alvo, o Sofosbuvir é conhecido por atuar como um análogo de nucleosídeo, causando a terminação da cadeia nucleotídica durante a replicação do HCV. Entretanto, diferente de outros inibidores nucleosídicos, o Sofosbuvir é um pró-farmaco, derivado do 2’-deoxy-2’-fluoro2’-C-metiluridina monofosfato, que é ativado somente nos hepatócitos (célula alvo do HCV), atuando diretamente no foco da infecção (Murakami, Tolstykh et al., 2010).

Diferentemente da NS5A, não há uma porção da polimerase onde as mutações de resistência se concentram. Elas se encontram distribuídas por toda a proteína viral, desde a posição 159 até o aminoácido 559, sendo a mutação S282T identificada como a mutação mais selecionada, in vitro, em trabalhos com diferentes replicons tratados com Sofosbuvir (Lam, Espiritu et al., 2012; Cento, Chevaliez et al., 2015; Cuypers, Li et al., 2015). Entretanto, essa mutação se mostra pouco presente em pacientes não tratados, além de possuir baixo fitness replicativo. Essa característica indica que outras substituições podem ocorrer de forma a proporcionar um escape ao Sofosbuvir com

menor influência na capacidade replicativa do vírus, além de mutações com caráter compensatório para a S282T (Kuntzen, Timm et al., 2008).

1.2 JUSTIFICATIVA

O novo esquema de tratamento para pacientes infectados pelo vírus da Hepatite C apresenta vantagens, como menos efeitos colaterais, maior facilidade na administração dos medicamentos, menor tempo de duração e melhora significativa na resposta ao tratamento (Brasil, 2018). Entretanto, é preciso entender como funciona a dinâmica entre os genótipos virais, o grau de progressão da doença no paciente e a resposta ao tratamento, fatores esses intrinsicamente relacionados. A utilização dos DAAs aumentou consideravelmente a resposta virológica sustentada porém o genótipo 3 apresentou a maior taxa de evasão ao tratamento, especialmente quando associado a um quadro clínico mais severo, como cirrose hepática. Em geral, essa diminuição da resposta ao tratamento está diretamente relacionada com mutações de resistência no genoma viral, fazendo com que, após o tratamento, pacientes não-respondedores apresentem variantes virais resistentes de forma majoritária (Lawitz, Poordad et al., 2014; Mcphee, Hernandez et al., 2014; Preciado, Valva et al., 2014). Apesar do genótipo 3 ter a menor taxa de resposta aos DAAs, os estudos sobre mutações de resistência disponíveis na literatura focam principalmente no genótipo 1, pois este apresenta a maior prevalência mundial. No Brasil, estima-se que o genótipo 3 corresponda a 23,8% dos casos de Hepatite C consistindo em um importante problema de saúde pública. Considerando o exposto, esse estudo teve como foco analisar a frequência de mutações de resistência já descritas na literatura para as regiões NS5A e NS5B em pacientes infectados pelo HCV genótipo 3, além de investigar a ocorrência de mutações ainda não descritas em indivíduos que não tenham respondido ao tratamento com Daclatasvir, avaliando, em cultura de células, tanto o comportamento replicativo das novas mutações como o grau de resistência contra inibidores da proteína viral NS5A.

1.3 OBJETIVO GERAL

Avaliar a associação entre resposta ao tratamento e presença de mutações no Vírus da Hepatite C, genótipo 3, em pacientes submetidos ao esquema de tratamento com os novos antivirais de ação direta Sofosbuvir e Daclatasvir.

1.4 OBJETIVOS ESPECÍFICOS

- Analisar a frequência de mutações de resistência descritas na literatura contra os antivirais Daclatasvir e Sofosbuvir em amostras de pacientes infectados por HCV genótipo 3 antes e após o tratamento.

- Investigar a ocorrência de mutações ainda não descritas em indivíduos que não tenham respondido ao tratamento com Daclatasvir.

- Avaliar, em cultura de células, o impacto dessas possíveis novas mutações na eficiência replicativa do HCV e na aquisição de resistência contra diferentes inibidores de NS5A.

1.5 METODOLOGIA

1.5.1 Amostra e População

Foram incluídos no estudo amostras de pacientes adultos maiores de 18 anos infectados pelo Vírus da Hepatite C genótipo 3 submetidos ao esquema de tratamento com os antivirais de ação direta (DAA), como descrito no Protocolo Clínico e Diretrizes Terapêuticas para Hepatite C e Coinfecções, número 171, de julho de 2015, e que estejam em acompanhamento no Hospital das Clinicas da Faculdade de Medicina de Ribeirão Preto (HCFMRP-USP). Tanto pacientes virgens de tratamento quanto pacientes que não atingiram Resposta Virologica Sustentada com tratamentos prévios foram convidados a participar do estudo. Foram excluídos do estudo pacientes co-infectados pelo HIV e/ou HBV, além de indivíduos que se recusaram a assinar o Termo de Consentimento Livre e Esclarecido (TCLE). O trabalho foi submetido e aprovado pelo Comitê de Ética do HCFMRP-USP com número de parecer 2.461.935.

Os pacientes foram divididos em dois grupos, o grupo A (pacientes tratados com Sofosbuvir, Ribavirina e Interferon peguilado por 12 semanas) e o grupo B (para pacientes com restrição ao uso de Interferon e Ribavirna, tratados com Sofosbuvir e Daclatasvir por 12 semanas).

1.5.2 Pontos de Coleta

Com o intuito de se identificar possíveis mutações de resistência no genoma viral, coletas de sangue dos pacientes foram realizadas antes (*baseline*) e após o tratamento farmacológico. Foram coletadas, para todos os pacientes, amostras antes do tratamento e 12 semanas após o início do tratamento, que marca o seu final, para observação da resposta ao tratamento. Os pacientes que não responderam aos medicamentos tiveram mais uma amostra coletada com 24 semanas após o término do tratamento para a investigação de mutações de resistência selecionadas pelo tratamento.

O sangue foi coletado em tubo à vácuo de 8,5 ml adequado para a separação do soro. Em seguida este foi centrifugado à 3000 rpm para a separação do soro, sendo este transferido para criotubos e armazenado -80 ºC.

1.5.3 Extração de RNA e Síntese de DNA complementar

A extração de RNA viral foi realizada a partir do plasma dos pacientes utilizando o protocolo de extração baseado em TRIzol Reagent (Thermo Fisher Scientific). Em tubos de 1,5 ml, para cada amostra a ser analisada, foram adicionados 250 µl de soro e 750 µl de Trizol, seguido de homogeneização e incubação por 5 min, à temperatura ambiente. Foi adicionado 200 µl de clorofórmio, homogeneizado e incubado por 3 min à temperatura ambiente, seguido por centrifugação à 4ºC, 14.000 rpm por 20 min. A fase aquosa foi transferida para um novo tubo de 1,5 ml e misturada à 500 µl de isopropanol absoluto. Foi realizada uma nova incubação à temperatura ambiente, por 10 min, prontamente seguida de uma centrifugação à 4ºC, 14.000 rpm por 15 min. O sobrenadante foi descartado e o pellet de RNA foi lavado com etanol DEPEC (Dietilpirocarbonato) 75%, seguido de centrifugação à 4ºC, 10.000 rpm por 5 min. O sobrenadante foi descartado, o RNA foi submetido a secagem à temperatura ambiente por 5 min e eluído em 50 µl água DEPEC, com posterior armazenagem em freezer -150ºC. O DNA complementar (cDNA) foi sintetizado utilizando o High Capacity cDNA Archive Kit (LifeTechnologies) segundo as instruções do fabricante.

1.5.4 Amplificação

As regiões genômicas que correspondem às proteínas alvo dos DAAs (NS5A e NS5B) foram amplificadas por Reação em Cadeia da Polimerase (PCR), utilizando a estratégia de NESTED-PCR. A região NS5A foi amplificada em amostras de pacientes do Grupo B e a região NS5B em amostras de pacientes dos Grupos A e B. Para a amplificação dos primeiros 300 nucleotídeos da porção amino terminal da região NS5A de HCV genótipo 3 foram utilizados *primers* específicos para este genótipo já descritos na literatura (H.NS5AP-F4, HNS5AP-R4, H.NS5AN-F4, H.NS5AN-R4) (Bittar, Jardim et al., 2013). Foram desenhados *primers* para a amplificação da região NS5B completa, específicos para HCV genótipo 3. A reação de PCR foi realizada utilizando a enzima com atividade corretiva *Long PCR Enzime Mix* (ThermoFisher Scientific) e a reação foi otimizada de acordo com as instruções do fabricante, alterando somente as temperaturas de anelamento de acordo com a temperatura ideal para cada par de *primers* utilizado. A positividade para a amplificação da região de interesse foi obtida a partir de análise por eletroforese em gel de agarose corado com brometo de etídeo.

1.5.5 Sequenciamento direto das amostras clínicas

As amostras amplificadas foram purificadas utilizando os kits de purificação de produto de PCR *Zymoclean™ Gel DNA Recovery Kit* (Zymo Research) e *DNA Clean & Concentrator -5* (Zymo Research). Após a purificação, foi realizada a reação de sequenciamento utilizando *BigDye Terminator v3.1* (Life Technologies) e *primers* específicos para cada região.

As mutações de resistência para o Daclatasvir concentram-se na região N-terminal da proteína NS5A, no domínio I. Dessa forma, foi sequenciado um fragmento que compreende os primeiros 300 nucleotídeos do gene utilizando os mesmos *primers* da segunda reação de PCR (H.NS5AN.F4 e H.NS5AI-R1).

Com relação ao Sofosbuvir, as mutações de resistência não se concentram um uma determinada região da proteína viral. A literatura descreve mutações ao longo de toda a proteína, desde o aminoácido 159 até o aminoácido 559. Pacientes infectados por HCV genótipo 3 tratados com este medicamento apresentam menor RVS. Assim, foram desenhados *primers* para sequenciar a região NS5B completa em pacientes HCV genótipo 3 positivos visando identificar possíveis mutações de resistência ao SOF. Por se tratar de um fragmento de aproximadamente 1600 bases, foi necessário dividir em duas reações de sequenciamento. Portanto, além do sequenciamento com os *primers* do NESTED-PCR, *primers* internos foram desenhados e utilizados para certificar que toda a região codificante da polimerase NS5B fosse sequenciada e analisada.

Após as reações de sequenciamento, as leituras das sequências foram realizadas no equipamento *ABI 3130 XL Genetic Analyzer* (Life Technologies), e as análises das sequências, observando mutações de resistência já descritas na literatura e procurando possíveis novas mutações associadas à evasão aos antivirais, foram realizadas utilizando o *software* BioEdit 7.1.11 (Hall, 1999). Essa plataforma permitiu o alinhamento, tradução e comparação das sequências obtidas com a sequência referência de genótipo 3 NZL1 (número de acesso no GenBank D17763) em busca das mutações.

1.5.6 Analises in vitro da mutação selecionada

As análises *in vitro* da mutação selecionada foram realizadas durante o intercâmbio científico na *University of Leeds*, sob supervisão do Prof. Dr. Mark Harris. O laboratório do Prof. Harris possuía toda a infraestrutura necessária para a realização dos testes *in vitro*.

1.5.7 Construções HCV genótipo 3

Os experimentos *in vitro* realizados nesse trabalho foram baseados em dois replicons subgenômicos de genótipo 3 de diferentes variantes, o SGR-luc-S52, derivado do replicon subgenômico S52/SG-neo (Saeed, Scheel et al., 2012) e o SGR-luc-DBN3a (Ward et al., a ser publicado). Como controle negativo foram utilizados os replicons defectivos com mutações inativando a RNA polimerase dependente de RNA NS5B: GDD→GNN. Todas as construções são constituídas pela região não estrutural do HCV, pelo gene repórter da luciferase *firefly* e pelo gene de resistência a neomicina.

1.5.8 Cultura de células

Os ensaios in vitro foram realizados em células Huh 7.5, uma linhagem de carcinoma hepatocelular, cultivada em meio DMEM (*Dulbecco's modified Eagles Medium*) suplementado com 10% de soro fetal bovino (FBS), 100 IU de penicilina ml-1, 100 µg de estreptomicina ml-1. As células foram incubadas à 37ºC e 5% de CO2.

1.5.9 Replicação

Mutações sítio-dirigidas foram realizadas utilizando o *kit Q5® Site-Directed Mutagenesis* (New England Biosciences) de forma a inserir a mutação selecionada nos replicons de genótipo 3 (SGR-luc-S52 e SGR-luc-DBN3a), seguido de ensaios de replicação para analisar o impacto no *fitness* viral e a resistência aos inibidores de NS5A na presença dessa mutação. Os replicons selvagens, controles negativos e mutantes foram eletroporados em células Huh 7.5 utilizando o *Gene Pulser Xcell Electroporation System* (Bio-Rad) seguindo o protocolo *square wave* (260V, 25ms, 1 pulse e cubeta de 4mm). Para cada eletroporação, 2x106 células foram adicionadas à cubeta e, após o procedimento, 10^4 células foram distribuídas por poço em uma placa branca de 96 poços. Após o período de incubação, as células foram lisadas utilizando o *Passive Lysis Buffer* (PLB) e a replicação foi obtida pela medição da atividade da luciferase utilizando o *Luciferase Assay System Kit* (Promega). As análises foram feitas 24h, 72h e 96h após a eletroporação para a variante S52, enquanto que para o DBN3a foram realizadas após 24h, 48h e 72h. Essa diferença no período de incubação se deu por conta da alta eficiência de replicação de SGR-luc-DBN3a (Ramirez, Mikkelsen et al., 2016).

1.5.10 Resistência aos DAAs

Para avaliar a resistência contra os inibidores de NS5A, ensaios antivirais foram realizados para determinar a concentração capaz de inibir 50% da replicação viral (EC50). Replicons subgenômicos (mutantes e selvagens), de ambas as variantes de genótipo 3, foram eletroporadas em Huh 7.5 como descrito anteriormente e 10^4 células foram distribuídas por poço em placa branca de 96 poços. Após 4h, os DAAs foram adicionados em diferentes faixas de concentração de acordo com a eficiência do DAA (inibidores de NS5A foram testados em escala pM logarítmica, enquanto que o inibidor de NS5B foi testado em escala nM logarítmica) (Incepta Pharmaceuticals). Células foram incubadas por 96h para S52 e 72h para DBN3a e todos os ensaios foram realizados em triplicata com três eventos independentes.

1.5.11 Análises *in vitro* do domínio I da NS5A proveniente das amostras clínicas

Com o intuito de avaliar o domínio I da proteína NS5A proveniente das amostras clínicas, modificações no replicon S52/SG-neo foram feitas pelo grupo do Prof. Dr. Mark Harris de forma a inserir sítios de restrição únicos (Bsu36I e PspXI) flanqueando um pequeno oligonucleotídeo substituto inserido no lugar da região codificante do domínio I, chamando esse replicon de S52t-SF. Essa abordagem permitiria a inserção do domínio I da NS5A derivado de virus circulantes em amostra clínicas no replicon S52t-SF, substituindo o oligonucleotídeo. Como consequência, o replicon subgenômico seria biologicamente funcional somente após a inserção do domínio I, uma vez que a presença do olignucleotídeo incapacita a replicação.

A partir de amostras pré-tratamento de cada um dos pacientes recidivantes inclusos em nosso trabalho, o domínio I foi clonado no replicon S52t-SF. Para isso, *primers* específicos foram delineados para amplificar o domínio I da NS5A pelo método de NESTED-PCR onde os *primers* para a segunda reação carregavam, em suas regiões 5', o mesmo sítio de restrição encontrado em S52t-SF. Amostras foram amplificadas utilizando a enzima *Phusion High-Fidelity DNA Polymerase* (New England Biosciences), de acordo com o protocolo do fabricante, e os produtos do NESTED-PCR, juntamente com o vetor S52t-SF, foram digeridos com as enzimas de restrição Bsu36I e PspXI (New England Biosciences) por 2h à 37ºC. Uma eletroforese em gel de agarose 0.8% foi realizada e os fragmentos de interesse da digestão foram purificados utilizando o *kit Monarch DNA Gel Extraction* (New England Biosciences). Os insertos do domínio I foram clonados no replicon S52t-SF pela *DNA ligase T4* (New England

Biosciences) em uma incubação à temperatura ambiente por 4h, transformados em *E. coli* DH5α competentes e semeadas em placas com meio de cultura LB, contendo ampicilina, com incubação durante à noite. As colônias obtidas após a transformação foram coletadas e purificadas conjuntamente, em um mesmo tubo, de forma a produzir um *pool* de replicons para cada amostra clínica.

Células Huh 7.5 foram eletroporadas com esse *pool* de replicons e um ensaio transiente de replicação foi realizado. As células eletroporadas foram divididas em dois grupos, um tratado com Daclatasvir na concentração de 1 nM (valor correspondente ao EC90 do replicon selvagem – WT) e outro, denominado grupo controle, incubado somente com meio de cultura fresco. Células foram lisadas usando o *Passive Lysis Buffer* (PLB) (Promega) e o nível de replicação foi obtido 72h e 96h após a eletroporação por meio da medição da atividade da luciferase. Após incubação, para os *pools* que apresentaram resistência, o RNA viral foi extraído das células tratadas utilizando TRIzol de acordo com o descrito anteriormente e reações de RT-PCR foram realizadas, utilizando os mesmos *primers* específicos para NS5A. O fragmento do domínio I obtido foi então submetido ao sequenciamento de Sanger para análise das sequencias e substituições presentes.

1.5.12 Análise estatística

Os dados obtidos nos ensaios de replicação *in vitro* foram analisados pelo software GraphPad Prism 5. A média e o desvio padrão foram determinados e todas as análises estatísticas foram realizadas pelo teste ANOVA e pelo teste de comparação múltipla de Dunnett, considerando $P < 0,05$ como significativo.

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2. Capítulo II

Selection dynamics of HCV genotype 3 resistance associated substitutions under daclatasvir and sofosbuvir therapy pressure in a real Brazilian cohort

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2.1 ABSTRACT

The HCV treatment with direct acting antivirals (DAAs) presents sustained virological response (SVR) that varies according to viral genotype. Among HCV genotypes, genotype 3 is the second most prevalent in Brazil and presents the lowest SVR. Since data about this higher therapy escape rate is scarce in literature, this study aimed to analyze HCV genotype 3 in a real cohort from Brazil, evaluating the safety and efficiency of the offered treatment schemes and associating the SVR achievement with different clinical factors. The circulation dynamics of resistance associated mutations (RAS) in this population, before and after selective pressure of the DAAs (Daclatasvir and Sofosbuvir), was also investigated. A cohort of 121 patients was analyzed and the overall SVR of the study was 88.1%. The treatment schemes with Sofobuvir + peguilated Interferon + Ribavirin (SOF + pegIFN + RBV) and Sofosbuvir + Daclatasvir ± Ribavirin (SOF + DCV ± RBV) presented close SVR efficiency (SVR rates of 80% and 87.6%, respectively). However, therapy regimen with pegIFN presented a considerably higher risk of treatment interruption due to adverse events, while RBV use showed to be related to the occurrence of mild adverse drug reactions (ADRs). From all clinical factors analyzed, cirrhosis, previous treatment with pegIFN + RBV and presence of baseline RAS at NS5A were strongly linked with treatment failure. Some RASs at NS5A (A30K, A62S/T) and NS5B (L159F and G554A) were detected at baseline level. After DAA treatment, in relapsing patients, mutations in NS5A were apparently selected (combination A30K+A62T) while others emerged that were not detected before treatment (Y93H and E92D). For NS5B, L159F was maintained after SOF administration. Our findings enables the better comprehension of genotype 3 behavior under DAA treatment, and the RAS selection dynamics here described allows the design of safer and more efficient therapeutic strategies to avoid treatment failure.

2.2 INTRODUCTION

For many years, hepatitis C was known as non-A, non-B hepatitis, mainly due to the lack of knowledge about the disease causing agent (1). This changed in 1989, when the unknown viral genome was isolated from the plasma of a chronically infected chimpanzee (2). This newly described virus was named hepatitis C virus (HCV), and after its discovery, the virus has been target of many different studies due to its public health importance. This enabled the description of many viral characteristics, such as the positive single stranded RNA genome, its high variability and high mutation rate (2-6). It is estimated that the average frequency for nucleotide mutation varies from 1.4 x 10^3 to 1.9 x 10^3 substitutions per nucleotide, per year (6), and this fact is responsible for a very important HCV feature: the ability to circulate in the host as quasispecies, a group of genetically close variants (7, 8).

The quasispecies characteristic confers to the virus a greater potential to adapt to each new host and the challenges of infection, allowing the emergence of escape mutations both to evade immune response and antiviral therapies (9-14). The selective pressure imposed by them can change the panorama of the quasispecies population, selecting resistance associated substitutions (RAS) that were circulating at a baseline level, in a minority frequency (15, 16). This precept helps to comprehend the occurrence of RAS and how fundamental is the molecular evolution role (17, 18). The association of all these factors hinders the discovery of a vaccine and makes constant the development of new antivirals as the current ones become obsolete with the emergence of new RAS (19, 20).

While HCV is responsible for the infection of 71 million people around the globe, with a death rate of approximately 400.000 deaths per year, in Brazil the infection number is around 1.5 million people, being a huge issue for the Brazilian health system (21, 22). Genotypes 1 and 3 are the most prevalent, being responsible for 70% and 24% of the cases in the country, respectively (23). Even though it is not the most prevalent, genotype 3 has lower susceptibility to treatment with the direct acting antivirals (DAAs) when compared to other genotypes. Patients infected with genotype 3 presents sustained virological response (SVR) between 80 to 90%, while for genotype 1 this number reaches 96%, and this response is even worse in severe cases, like hepatic cirrhosis, decreasing SVR to 60% (24-26).

The treatment for HCV genotype 3, in Brazil, was restrict to the use of interferon and ribavirin until 2015, when DAAs effective against this genotype were introduced in the treatment schemes (27). Those DAAs were the NS5A inhibitor Daclatasvir (DCV) and the NS5B RNA polymerase Sofosbuvir (SOF). Despite the great improvement on the SVR, *in vitro* and *in vivo* reports showed viral variants resistant to these DAAs even before their approval and distribution in the country (28-33). Thus, the genotype 3 lower response to treatment is directly related to RAS in the viral genome, causing relapsing patients to present resistant viral variants in a major frequency after DAA selective pressure (16, 18, 31, 33).

Although HCV genotype 3 presents the highest treatment resistance among all genotypes, studies about RAS in the literature are mainly focused on genotype 1, once this presents the highest prevalence around the world. Due to the scarce data about genotype 3 and RAS emergence, our study aimed to analyze the frequency of RAS in the NS5A and NS5B regions of HCV genotype 3 infected patients, before and after treatment with DCV and SOF. Furthermore, the association with the success and safety of DAAs was performed to observe the impact of these mutations into therapy effectiveness.

2.3 MATERIALS AND METHODS

2.3.1 Ethical aspects for study design

This work was performed as a collaboration between the University Hospital of the Ribeirão Preto Medical School, inside University of São Paulo (HCFMRP-USP) and the Genomic Studies Laboratory of the Institute of Bioscience, Language and Exact Science of the São Paulo State University (IBILCE/UNESP). The prospective cohort study was performed in the first location, while the second one was responsible for all molecular experiments and analysis. The study was submitted to the Research Ethics Committee of HCFMRP-USP and approved under the number 2.461.935.

2.3.2 Clinical Samples

In this study, only patients above 18 years old were included. All of them were infected by HCV genotype 3 and submitted to the DAA treatment scheme defined by the Clinical Protocol and Therapeutic Guidelines for Hepatitis C and Coinfections, number 171 from July of 2015. Both naïve patients and individuals who did not achieve SVR

with previous treatments were invited for the study. On the other hand, patients coinfected by HIV and/or HBV were excluded from the cohort.

The patients were divided in two groups, the first one comprehending individuals treated with Sofosbuvir, Ribavirin and peguilated Interferon for 12 weeks (SOF + pegIFN + RBV), and the second one containing patients with restrictions to the usage of Interferon, treated with Sofosbuvir and Daclatasvir for 12 weeks, with or without ribavirin (SOF + DCV ± RBV).

2.3.3 Collection of clinical data

Since patients were followed during the whole therapy, data were collected before, during and after treatment. In the pre-treatment period, sociodemographic and anthropometric data were collected in parallel with health history information like hypertension, diabetes mellitus, obesity, liver fibrosis staging, hepatic encephalopathy, ascites, esophageal varices, history of HCC, history of antiviral treatment, alcohol ingestion and tobacco smoking. Laboratory tests were performed for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total serum bilirubin (TB), international normalized ratio (INR), creatinine and HCV viral load. Cirrhosis progression was measured following the Child-Turcotte-Pugh score and Model for End-Stage Liver Disease (MELD). In our study, were considered obese those patients with over 30 Kg/m^2 on the Body Mass Index (BMI) (34).

For liver fibrosis measurement we adopted both invasive (biopsy – METAVIR score) and non-invasive methods (point shear wave elastography – pSWE; or transient elastography). The individuals that presented splenomegaly, ascites and esophageal varices were classified as F4.

To evaluate the safety of the treatments, the incidence of adverse drug reactions (ADRs) such as anemia, neutropenia and thrombocytopenia was observed. The respective thresholds for these analysis were: serum hemoglobin levels < 13.9 g/dL (men) and < 12 g/dL (women); neutrophils < 1,700/mm^3; and platelets < 130,000/mm^3.

The effectiveness of treatment was obtained by HCV viral load determination at the end of treatment and 24 weeks after the end. Quantification was performed using the qRT-PCR method, considering the limit of detection < 12 UI/mL.

2.3.4 Serum collection for RAS prevalence analysis.

To observe the frequency of RAS on the viral genome before and after the treatment, serum samples were collected on the pre- (Baseline) and post-treatment points. For all patients, serum samples were collected before treatment, and for those that relapsed treatment, samples were collected 24 weeks after the end of antiviral administration. The collection points are described on **Figure 1**.

The viral targets were determined according to the treatment regimen for each patient. NS5B was analyzed in all samples once both treatment groups were treated with Sofosbuvir, while NS5A was investigated on those patients included in the group treated with Daclatasvir.

2.3.5 RNA extraction and viral targets amplification

Viral RNA extraction was performed using TRIzol. For each sample, 250 µl of serum were added to 750 µl of TRIzol (Thermo Fisher Scientific) followed by homogenization and incubation at room temperature for 5 min. Following the manufacturer's protocol, RNA was separated, precipitated and eluted in nuclease-free water. At this point, RNA was used as template for cDNA synthesis using the High Capacity cDNA Archive Kit (Life Technologies) according to the manufacturer's guidance protocol.

After cDNA synthesis, viral targets NS5A and NS5B were amplified by Nested-PCR technic using the Long PCR Enzyme Mix (Thermo Fisher Scientific) following the manufacturer's instructions. For NS5B, the entire gene was amplified since RAS are found distributed across the entire protein, and specific primers were designed for this process **(Supplementary table 1)**. For NS5A, primers from the literature were used (35) to amplify the first 300 nucleotides since NS5A RAS are restricted to the domain I.

2.3.6 Sanger sequencing and RAS frequency analysis

After amplifications, the PCR products were than submitted to the sequencing reaction using the BigDye Terminator v3.1 (Life Technologies). For NS5A, the primers for the second PCR reaction were used for sequencing, while for NS5B, besides the internal Nested-PCR primers, an additional pair of internal primers were used to sequence the whole fragment **(Supplementary table 2)**.

The sequencing was performed using the ABI 3130 XL Genetic Analyzer (Life Technologies) and the obtained sequences were aligned by Clustal W nested in BioEdit 7.1.11 package (36, 37). The analysis of RAS occurrence was performed by the comparison of the sequences with the reference sequence for genotype 3, NZL1 (GeneBank accession number D17763).

2.3.7 Statistical analysis

For the quantitative variables, data was presented as mean and standard deviation (SD) for normal distribution, while median and interquartile range was used when distribution was not normal. For qualitative variables, data was categorized and frequencies (absolute and relative) were described. The chi-square test and the Relative Risk (RR) calculation were performed to compare SVR and categorized variables and to analyze de relation between adverse drug reactions and treatment regimen. Global SVR results were considered by the intention to treat. A 5% of significance level was established for all the association analysis and the Statistical Packaged for Social software (SPSS Inc., Version 17.1.0) was used to perform the tests.

2.4 RESULTS

2.4.1 Cohort characteristics

A total of 121 patients chronically infected by HCV genotype 3 were included in our study, 82 (67.8%) men and 39 (32.2%) women. The average age was 56.4 ± 8.5 years. Hepatic cirrhosis was present in 93 (76.9%) patients from which 76 (81.7%) presented compensated liver disease. About the treatment schemes, 105 individuals (86.6%) were treated with SOF + DCV ± RBV, 15 (12.4%) used SOF + pegIFN + RBV and one patient (0.8%) was treated with SOF + DCV + pegIFN + RBV. All demographic and clinical data collected in this study are described into Table 1**.**

2.4.2 Treatment outcomes

For the group treated with SOF + DCV ± RBV, 92 patients reached SVR (87.6%), 10 relapsed the treatment (9.5%) and 3 died during the therapy (2.9%). For the group administrated with SOF + pegIFN + RBV, 12 reached SVR (80%), with no relapsing individuals and no deaths, but 3 patients discontinued treatment due to adverse reactions (20%). The analysis of treatment response, considering only patients with available

response data, indicated SVR rates of 88.1% for the whole study population and 90.2% for the SOF + DCV ± RBV group. About SOF + pegIFN + RBV group, all patients presented therapy response data and the SVR was 100% when considering only the individuals that reached the end of the treatment. The treatment outcomes of all patients included in the study and treated with SOF + DCV ± RBV or SOF + pegIFN + RBV are described in **Figure 2**.

The patient that treated with SOF + DCV + pegIFN + RBV for 24 weeks relapsed the pharmacological therapy. The three individuals that died during treatment were cirrhotic, while two of them presented a compensated liver disease diagnosis.

The comparison of SVR between the indicated treatments (SOF + DCV ± RBV x SOF + pegIFN + RBV) showed no significant difference (RR = 1.128; CI 95% = 0.869-1.464).

2.4.3 Treatment safety and tolerability

The evaluation of adverse drug reactions (ADRs) on the treatment discontinuity showed that patients from SOF + pegIFN + RBV group presented a higher risk of treatment interruption due to adverse events (RR = 35.000; IC 95% = 4.382-279.528). In total, 6 patients interrupted the treatment due to ADRs, 5 from SOF + pegIFN + RBV group and 1 from SOF + DCV ± RBV group. This individual used RBV associated with the DAAs and therapy was discontinued due to gastrointestinal events (nausea, vomiting, diarrhea and abdominal pain). About the other 5 patients, two had therapy interruption due hepatic decompensation, one due to dermatological reaction (rash and pruritus), one patient presented kidney injury and the last one showed both hepatic decompensation and kidney injury.

Considering the SOF + DCV ± RBV group, the association among RBV administration and the most frequent ADRs (≥ 10%) was investigated. Individuals treated with RBV presented a considerably higher risk to develop anemia (RR = 12.500; CI 95% = 1.878-83.220). A single patient treated with SOF + DCV presented a mild anemia (Hb ≥ 10 g/dL). Asthenia e fatigue (RR = 3.417; CI 95% = 0.9222-12.661), nausea and/or vomit (RR = 1.917; CI 95% = 0.503-7.302) and headache (RR = 1.417; CI 95% = 0.364-5.516) were other adverse events highly prevalent in patients under RBV treatment. The absence of statistical relevance is probably due to the reduced number of patients

included into these three subgroups. None of the patients treated with SOF + DCV presented dizziness or loss of appetite. Thrombocytopenia was the only ADR observed in more than 10% of the patients treated with SOF + DCV, showing no statistical difference in comparison with SOF + DCV + RBV subgroup. Our results suggest that the treatment free of IFN and RBV is safer in comparison with the other therapies here evaluated.

The data for ADRs prevalence in the studied cohort, as well as the analysis of ADRs incidence in the different studied groups, are described on Table 2.

2.4.4 Baseline RASs prevalence

To analyze the prevalence of RAS in the studied population, serum samples were collected pre- (all patients) and post-treatment (relapsing patients) and submitted to RNA extraction, cDNA synthesis, Nested-PCR and Sanger sequencing. For viral protein NS5A, a fragment of 589 bp was amplified and analyzed. The total of 94 samples (79 pre- and 15 post-treatment) were collected, with an amplification efficiency of 92.6% (NS5A amplified from 87 samples). For NS5B, a fragment of 1615 bp was amplified. The total of 106 samples (91 pre- and 15 post-treatment) were collected and the amplification success rate was 80.2% (NS5B amplified from 85 samples).

The frequency analysis of baseline RAS (at pre-treatment period) showed that the most prevalent site for RAS in the NS5A protein was position 62 (63.3% of clinical samples from SOF + DCV ± RBV group presented mutation A62S and 24.1% presented mutation A62T). For NS5B, position 554 was the most prevalent with RAS G554A, found in 3 patients (3.7% of the clinical samples amplified for this region). The prevalence of all baseline RAS, in each treatment group and each viral target, are described in Table 3**.**

2.4.5 RASs and treatment response

One of the aims of our study was to analyze de RAS selective process under the DAA treatment. For this, an evaluation of RAS frequency pre- and post-treatment was performed. As observed before, our data evidenced an association between baseline RAS presence into NS5A and SVR. For mutation A62T, even though no statistical relevance was observed due to the small number of relapsing patients, the results suggest 20% more chances to achieve SVR in patients that did not present this RAS

before therapy start. Substitution A30K was detected in 6 patients, always followed by another change at position 62. All patients where viral variants were bearing both A30K and A62T mutations failed the treatment. On the other hand, those individuals whose samples presented A30K and A62S concomitantly achieved SVR. When evaluated alone, there was no evidence of relation between A62S (the most prevalent RAS) and treatment failure.

When observing the post-treatment period, mutation Y93H was detected in 4 clinical samples. However, this mutation was not observed in any pre-treatment sample. Mutation E92D showed the same pattern, being detected in one relapsing patient after the end of treatment, but without being observed at the baseline level. Patients where A30K + A62T association was found in pre-treatment also presented these mutations at post-treatment period. Two individuals were submitted to a second treatment scheme with SOF + DCV + RBV for 24 weeks, a longer period, and did not achieve SVR. The first one presented three RAS in NS5A (A30K, A62T and E92D) after the second treatment, while the second one showed A62T inside NS5A at baseline level and Y93H was observed in this clinical sample after the treatment with SOF + DCV + RBV for 12 weeks.

For NS5B, mutation L159F was the only RAS detected after treatment end. It was observed in a single relapsing patient and in this case, L159F was present in both pre- and post-treatment samples.

The frequency results for post-treatment RAS, with other clinical data for the relapsing patients, are described on Table 4.

2.4.6 Factors associated with SVR

Overall, 14 patients relapsed treatment, presenting detectable HCV RNA at 12 or 24 weeks after treatment. Data for the association analysis between clinical variables and SVR and the SVR rates according to each variable are described on Table 5.

The results showed that hepatic cirrhosis, history of previous treatment with pegIFN + RBV and presence of baseline RAS at NS5A viral protein are factors associated with a higher risk of treatment failure. A significant relation between absence of cirrhosis and SVR was observed in the whole cohort and in the SOF + DCV ± RBV group. About the other treatment group (SOF + pegIFN + RBV), the analysis showed that the cirrhotic

subgroup presents a 50% higher risk of treatment failure. In this case, the absence of statistical relevance is probably related to the reduced number of individuals in this therapy group.

The association between absence of SVR and previous treatment with pegIFN + RBV was observed in the whole studied population, while the link between treatment failure and presence of baseline RAS inside NS5A, was evidenced in the SOF + DCV ± RBV group. All patients whose clinical samples did not present any RAS before treatment achieved SVR.

2.5 DISCUSSION

With the progress of the pharmaceutical researches and development of new antiviral therapies, it is natural that treatment schemes would evolve focusing on two main pillars, the efficiency improvement and the decrease of adverse drug events. In the era of antiviral drugs that can act directly against HCV components, like proteins and genome, it was just a matter of time that these promising drugs would replace interferon-based treatments. Our study was performed in Brazil during this transition period, between IFN-based therapies discontinuation and DAAs treatment free of IFN initial distribution.

In our results, no significant difference on achieving SVR was observed when comparing groups treated with or without IFN. These results corroborate with two different studies that showed high SVR rates for genotype 3 infected patients treated with SOF + pegIFN + RBV, with no difference for DAAs regimens (38, 39). However, patients treated with IFN in our cohort presented higher risk of treatment interruption, where the treatment led to severe adverse events such as decompensated liver disease. These is supported by literature data, where the use of DAAs combination, replacing the usage of IFN, showed higher tolerability, with mild adverse drug events and no deaths, being highly recommended in severe cases (40, 41). The issue about genotype 3 is that, compared to other genotypes, the chances of disease progression to severe liver damage are higher (42), so the usage of IFN-free therapies shows to be a safer option (43, 44). For RBV, the administration was highly related to the appearance of some ADRs, especially anemia and fatigue, similar to the observed in other studies (38, 45, 46).

Some clinical factors were involved with higher risk of treatment failure. Similar to our findings, a Spanish cohort study, with 137 individuals, also related the hepatic cirrhosis as an important contributor for therapy escape (47). This was observed too in a Polish published article in 2018 (38). A huge study, including almost 16,000 individuals, reinforces this data and includes previous IFN-based treatment as an important risk factor, as observed in our results. The same paper cites cirrhosis as a hinder for treatment success too (48). Other factors are described to impact SVR achievement, like thrombocytopenia (49), but this was not observed here. Literature also indicates that genotypic characteristics of HCV genotype 3 have influence on this process (48). This is mainly related to the association of RAS presence, especially NS5A RAS and previous contact with NS5A inhibitors (47), showing that RAS evaluation prior to DAA therapy definition could be a way to reduce treatment failure for genotype 3. The dynamics for RAS prevalence under the selective pressure of the DAAs therapy was observed in our study. In general, the majority of RAS here detected were in the NS5A region. In contrast with other DAAs HCV targets (NS3 and NS5B), RAS into NS5A tend to persist more across time, while in the other viral proteins these mutations rapidly disappear in the population. This higher maintenance is related to the better replicative fitness presented by these substitutions (50).

Although mutation A62S was the most prevalent in the pre-treatment samples, when analyzing the post-treatment we could observed that, at this site, A62T became the most prevalent. The data here shown suggests that the change for a threonine (T) was selected after treatment with DCV. It is described in the literature that mutations at this position confer resistance to NS5A inhibitors, but at low levels (51-53). Mutation A30K was also present at pre-treatment point and was maintained after DCV treatment. Substitutions at this position are frequently present at baseline level in relapsing patients and are strongly selected after antiviral pressure (51). Also, studies with other genotypes highlight this site as one of the most important for resistance acquisition (54, 55). Our findings show that this RAS was always associated with a mutation at site 62 (A62T or A62S) and, interestingly, only the clinical samples that presented A30K+A62T at baseline failed the treatment, showing that possibly there is a compensatory effect between these RASs.

Mutation Y93H is the most described RAS into NS5A. In our pre-treatment analysis, Y93H was not detected. However, after DCV selective pressure, Y93H was highly

frequent in the relapsing clinical samples. This could be explained by the fact that, despite this RAS is described to confer high levels of resistance for almost all HCV genotypes (54-57), it is also known for its defective replication fitness (58). Probably Y93H was circulating, before treatment, in a lower frequency in the infected population, not being possible the detection by the Sanger method, but became prevalent after DAA administration. In this scenario, the change on host environment arranged by DCV selective pressure possibly benefitted variants bearing Y93H.

Another mutation that showed the same pattern was E92D, being detected only after treatment in relapsing patients. Despite some mutations at this site are known to confer resistance (53, 59), there is no information about this particular substitution and its connection with DCV resistance. Further assays are necessary to determine the role of this mutation on resistance acquisition.

For NS5B, only one RAS was maintained after SOF administration. L159F was detected at the baseline level and was present in one relapsing clinical sample. Studies show that L159F is capable to confer resistance against SOF and this probably happens by the structural interaction of this RAS position with residue S282, situated on the catalytic site of the NS5B RNA polymerase. Observing the tertiary structure of the viral protein, L159F and S282 are only 4 angstrom distant from each other, and a possible interaction between them could lead to a change on the catalytic site conformation, influencing on SOF activity (60, 61).

This study enables the understanding of how HCV genotype 3 behaves under the selective pressure of DCV and SOF treatment. The associations of clinical variables with SVR achievement, as well as the early detection of RASs combined with the comprehension of selection dynamics, are important tools to design more suitable treatment schemes and reduce antiviral therapy failure.

AUTHOR CONTRIBUTIONS: GRFC and JPVR performed the experiments, AdLCM, FFS, LRLP, CB and PR supervised the study. GRFC, JPVR, CB and PR wrote the manuscript.

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2.6 TABLES

Table 1: Demographic and clinical characteristics of patients treated with antiviral therapy.

Characteristics	All patients (n = 121)*	SOF + DCV ± RBV (n = 105)	SOF + pegIFN + RBV (n = 15)
Age (years), mean (SD)	56.4 (8.5)	56.8 (8.3)	53.1 (9.2)
Male, n (%)	82 (67.8)	68 (64.8)	13 (86.7)
Race/Ethnicity, n (%)			
White	104 (86.0)	89 (84.8)	14 (93.3)
Other	17 (14.0)	16 (15.2)	1 (6.7)
Diabetes Mellitus, n (%)	35 (28.9)	30 (28.6)	4 (26.7)
Hypertension, n (%)	50 (41.3)	44 (41.9)	6 (40.0)
Obesity, n (%)	36 (29.8)	32 (30.5)	3 (20.0)
Dyslipidemia, n (%)	26 (21.5)	22 (21.0)	4 (26.7)
Liver fibrosis stage, n (%)			
F0/F1	6 (5.0)	5 (4.8)	1 (6.7)
F2	10 (8.3)	9 (8.6)	1 (6.7)
F3	12 (9.9)	8 (7.6)	4 (26.7)
F4	93 (76.9)	83 (79.0)	9 (60.0)
Child-Turcotte-Pugh score, n (%)**			
A	76 (81.7)	68 (81.9)	8 (88.9)
B	13 (14.0)	12 (14.5)	1 (11.1)
C	2 (2.2)	2 (2.4)	0 (0.0)
Data not available	2 (2.2)	1 (1.2)	0 (0.0)
MELD score, n (%)**			
≤ 9	50 (53.8)	44 (53.0)	6 (66.7)
10-19	37 (39.8)	34 (41.0)	3 (33.3)
20-29	4 (4.3)	4 (4.8)	0 (0.0)
≥ 30	0 (0.0)	0 (0.0)	0 (0.0)
Data not available	2 (2.2)	1 (1.2)	0 (0.0)
HCV-RNA, mean x 10^6 UI/mL (SD)	1.1 (1.2)	1.1 (1.3)	1.1 (0.9)
AST ≥ 3x ULN, n (%)	36 (29.8)	32 (30.5)	4 (26.7)
ALT ≥ 3x ULN, n (%)	41 (33.9)	36 (34.3)	4 (26.7)
Platelets < 100.000 mm^3, n (%)	41 (33.9)	37 (35.2)	3 (20.0)
History of HCC, n (%)	13 (10.7)	13 (12.4)	0 (0.0)
Antiviral treatment history, n (%)			
IFN or pegIFN + RBV	53 (43.8)	43 (41.0)	9 (60.0)
SOF	3 (2.5)	2 (1.9)	0 (0.0)
DCV + SOF	2 (1.7)	2 (1.9)	0 (0.0)
Treatment duration, n (%)			

12 weeks	94 (77.7)	79 (75.2)	15 (100.0)
24 weeks	27 (22.3)	26 (24.8)	0 (0.0)
Use of RBV, n (%)	106 (87.6)	90 (85.7)	15 (100.0)
eGFR (CKD-EPI equation), n (%)			
G1 (> 90 ml/min/1,73m^2)	61 (50.4)	49 (46.7)	11 (73.3)
G2 (60-89 ml/min/1,73m^2)	43 (35.5)	40 (38.1)	3 (20.0)
G3a (45-59 ml/min/1,73m^2)	5 (4.1)	4 (3.8)	1 (6.7)
G3b (30-44 ml/min/1,73m^2)	7 (5.8)	7 (6.7)	0 (0.0)
G4 (15-29 ml/min/1,73m^2)	2 (1.7)	2 (1.9)	0 (0.0)
G5 (< 15 ml/min/1,73m^2)	3 (2.5)	3 (2.9)	0 (0.0)
Hepatic steatosis, n (%)	39 (32.2)	33 (31.4)	5 (33.3)
Extrahepatic manifestations[***], n (%)	11 (9.1)	8 (7.6)	3 (20.0)
History of alcohol use, n (%)[****]			
Heavy drinker	50 (41.3)	43 (41.0)	7 (46.7)
Light drinker	40 (33.1)	34 (32.4)	5 (33.3)
Tabacco smoking			
Smoker	25 (20.7)	23 (21.9)	2 (13.3)
Ex-smoker	38 (31.4)	35 (33.3)	3 (20.0)

ALT: alanine aminotransferase; AST: aspartate aminotransferase; CKD-EPI: chronic kidney disease epidemiology collaboration; DCV: daclatasvir; eGFR: estimated glomerular filtration rate; Obesity, Body Mass Index ≥ 30,0 Kg/m^2; MELD: model for end-stage liver disease; HCC: hepatocellular carcinoma; IFN: interferon; pegIFN: pegylated interferon; RBV: ribavirin; SD: standard deviation; SOF: sofosbuvir; ULN: upper limit of normal.

[*]One patient treated with SOF + DCV + pegIFN + RBV.

[**]Cirrhotic patients from each group (n = 93, n = 83, n = 9).

[***]Cryoglobulinemia, porphyria cutanea tarda, lichen planus, neuropathy, glomerulonephritis, vasculitis.

[****]Heavy drinker: consumption history over 40g/day of alcohol for men and 20g/day for women.

Fonte: Própria, 2020

Table 2: Most common adverse drug reactions (ADRs) in the studied cohort.

	All patients (n = 121)*	**SOF + DCV ± RBV (n = 105)**	**SOF + pegIFN + RBV (n = 15)**	
	n (%)	**n (%)**	**n (%)**	**RR (95% CI)**
Anemia	91 (75.2)	76 (72.4)	14 (93.3)	0.776 (0.648-0.928)
Neutropenia	16 (13.2)	6 (5.7)	10 (66.7)	0.086 (0.036-0.202)
Thrombocytopenia	25 (20.7)	14 (13.3)	11 (73.3)	0.182 (0.102-0.323)
Lymphopenia	14 (11.6)	9 (8.6)	5 (33.3)	0.257 (0.100-0.665)
Asthenia/fatigue	52 (43.0)	43 (41.0)	8 (53.3)	0.768 (0.454-1.300)
DCL	13 (10.7)	8 (7.6)	5 (33.3)	0.229 (0.086-0.608)
Skin reactions	14 (11.6)	10 (9.5)	4 (26.7)	0.357 (0.128-0.996)
Headache	22 (18.2)	19 (18.1)	2 (13.3)	1.357 (0.351-5.250)
Dry cough	7 (5.8)	4 (3.8)	3 (20.0)	0.190 (0,047-0,769)
Epigastric pain	10 (8.3)	8 (7.6)	2 (13.3)	0.571 (0.134-2.441)
Nausea and/or vomiting	28 (23.1)	25 (23.8)	3 (20.0)	1.191 (0.409-3.465)
Flu-like reactions	9 (7.4)	1 (1.0)	8 (53.3)	0.018 (0.002-0.133)
Dizziness	16 (13.2)	13 (12.4)	2 (13.3)	0.929 (0.232-3.717)
Somnolence	9 (7.4)	7 (6.7)	2 (13.3)	0.500 (0.114-2.186)
Appetite loss	22 (18.2)	13 (12.4)	9 (60.0)	0.206 (0.107-0.397)
Neuropsychiatric events	7 (5.8)	3 (2.9)	4 (26.7)	0,107 (0.027-0.433)

DCL: decompensated liver disease; Neuropsychiatric events includes irritability, anxiety, emotional lability, agitation; Skin reactions includes rash and/or pruritus; Flu-like reactions include concomitant occurrence of at least two events such as fever, headache, chills, sweating, myalgia, indisposition and arthralgia;
Fonte: Própria, 2020

Table 3: Baseline RAS frequency.

	SOF + DCV ± RBV (n = 79)*	**SOF + pegIFN + RBV**
NS5A	n (%)	n (%)
M28	0 (0.0)	---
P29	0 (0.0)	---
A30K	6 (7.6)	---
A30T	2 (2.5)	---
A30S	1 (1.3)	---
L31	0 (0.0)	---
P32	0 (0.0)	---
P58T	1 (1.3)	---
A62F	2 (2.5)	---
A62I	1 (1.3)	---
A62L	1 (1.3)	---
A62T	19 (24.1)	---
A62S	50 (63.3)	---
E92D	1 (1.3)	---
Y93	0 (0.0)	---
	SOF + DCV ± RBV (n = 70)*	**SOF + PEG + RBV (n = 11)***
NS5B	n (%)	n (%)
L159F	1 (1.4)	0 (0.0)
S282	0 (0.0)	0 (0.0)
C316	0 (0.0)	0 (0.0)
V321	0 (0.0)	0 (0.0)
S368	0 (0.0)	0 (0.0)
N411	0 (0.0)	0 (0.0)
M414	0 (0.0)	0 (0.0)
V421A	1 (1.4)	0 (0.0)
Y448	0 (0.0)	0 (0.0)
P495	0 (0.0)	0 (0.0)
V553	0 (0.0)	0 (0.0)
G554A	3 (4.3)	0 (0.0)
G556	0 (0.0)	0 (0.0)
D559	0 (0.0)	0 (0.0)

DCV: daclatasvir; pegIFN: pegylated interferon; RBV: ribavirin; SOF: sofosbuvir; ---: Not applied.
*Number of amplified samples according to the viral genome region.
Fonte: Própria, 2020

Table 4: Demographic and clinical characteristics of patients who did not achieve SVR.

Case	Age	Sex	Cirrhosis	DM	CKD stage	INF/PEG + RBV treatment history	DAA treatment history	Drugs used in the study	Therapy duration	Pre-therapy RAS NS5A	Pre-therapy RAS NS5B	Post-therapy RAS NS5A	Post-therapy RASs NS5B
1	42	F	Yes	No	1	Yes	No	SOF+DCV+ RBV	12 W	A62S	---	A62S Y93H	---
2	48	M	Yes	No	1	Yes	No	SOF+DCV+ RBV	12 W	A62S	L159F	A62S Y93H	L159F
3	67	M	Yes	No	3a	Yes	No	SOF+pegIFN +RBV	5 days*	NA	---	NA	NA
4	66	M	Yes	Yes	4	No	No	SOF+DCV	12 W	A30K A62T	None	A30K A62T	---
5	62	M	Yes	Yes	1	Yes	No	SOF+DCV+ RBV	12 W	A62S	---	A62S Y93H	---
6	56	F	Yes	Yes	1	No	No	SOF+DCV+ RBV	12 W	A62S	None	---	---
7	55	M	Yes	No	2	No	No	SOF+DCV+ RBV	12 W	---	---	---	---
8	62	M	Yes	Yes	1	Yes	SOF	SOF+DCV+ PEG+RBV	24 W	---	---	A62T	---
9	51	F	Yes	No	1	No	No	SOF+pegIFN +RBV	2 W*	NA	None	NA	NA
10	62	M	Yes	Yes	1	Yes	No	SOF+pegIFN +RBV	3 W*	NA	None	NA	NA
11	49	M	Yes	No	2	Yes	No	SOF+DCV+ RBV	12 W	A30K A62T	None	A30K A62T E92D	None
12	50	M	Yes	No	2	Yes	SOF + DCV	SOF+DCV+ RBV	24 W	A30K A62T E92D	None	A30K A62T	None
13	54	M	Yes	No	2	Yes	No	SOF+DCV+ RBV	12 W	---	---	A62T Y93H	None
14	57	M	Yes	No	2	Yes	SOF + DCV	SOF+DCV+ RBV	24 W	A62T	None	---	---

CKD: chronic kidney disease; DAA: direct acting antiviral; DCV: daclatasvir; DM: Diabetes Mellitus; IFN: interferon; NA: not applied; PEG: pegylated interferon; RAS: resistance associated substitutions; RBV: ribavirin; SOF: sofosbuvir; SVR: sustained virologic response; ---: Serum sample not collected/not amplified.

*Discontinuation due to adverse drug reaction. Fonte: Própria, 2020

Table 5: SVR rates by clinical variables subgroups and association analysis of variables with SVR.

	All patients*		SOF + DCV ± RBV		SOF + pegIFN + RBV	
	SVR (%)	RR (95% CI)	SVR (%)	RR (95% CI)	SVR (%)	RR (95% CI)
Cirrhosis		1.184 (1.084-1.294)		1.143 (1.052-1.242)		1.500 (0.945-2.381)
No cirrhosis	100.0		100.0		100.0	
Cirrhosis	84.4		87.5		66.7	
Cirrhosis stage**		1.009 (0.785-1.295)		1.057 (0.808-1.381)		0.625 (0.365-1.069)
Compensated	85.3		88.1		62.5	
Decompensated	84.6		83.3		100.0	
Diabetes Mellitus		1.040 (0.891-1.214)		1.003 (0.871-1.155)		1.091 (0.581-2.050)
No	89.2		90.3		81.8	
Yes	85.7		90.0		75.0	
CKD stage***		0.966 (0.791-1.180)		0.991 (0.812-1.209)		---
Non-severe CKD	87.9		90.1		80.0	
Severe CKD	90.9		90.9		---	
Treatment duration		NA		0.984 (0.850-1.139)		NA
12 weeks	NA		89.9		80.0	
24 weeks	NA		91.3		NA	
Use of RBV		NA		1.034 (0.880-1.216)		NA
No	NA		92.9		NA	
Yes	NA		89.8		80.0	
Previous therapy with IFN/pegIFN + RBV		1.157 (1.002-1.336)		1.134 (0.981-1.310)		1.071 (0.650-1.767)
No	93.9		94.9		83.3	
Yes	81.1		83.7		77.8	
Baseline RAS****						
NS5A RAS		NA		1.125 (1.037-1.221)		NA
No	NA		100.0		NA	
Yes	NA		88.9		NA	
A62T (NS5A RAS)		NA		1.200 (0.929-1.550)		NA
No	NA		93.3		NA	
Yes	NA		77.8		NA	
A62S (NS5A RAS)		NA		0.932 (0.785-1.106)		NA
No	NA		85.7		NA	
Yes	NA		92.0		NA	
NS5B RAS		1.133 (0.727-1.767)		1.152 (0.739-1.797)		---
No	90.7		92.2		81.8	
Yes	80.0		80.0		---	

CI: confidence interval; CKD: chronic kidney disease; DCV: daclatasvir; IFN: interferon; NA: not applied; pegIFN: pegylated interferon; RAS: resistance associated substitutions; RBV: ribavirin; RR: relative risk; SOF: sofosbuvir; SVR: sustained virologic response. ---: not calculated due to the absence of individuals in a subgroup of the analyzed variable.
*Total n includes one patient treated with SOF + DCV + pegIFN + RBV.
**Compensated, Child-Pugh A; Decompensated, Child-Pugh B or C.
***CKD non-severe, CKD stage 1-3a; CKD severe, CKD stage 3b-5. ****Analysis considered amplified samples. Fonte: Própria, 2020

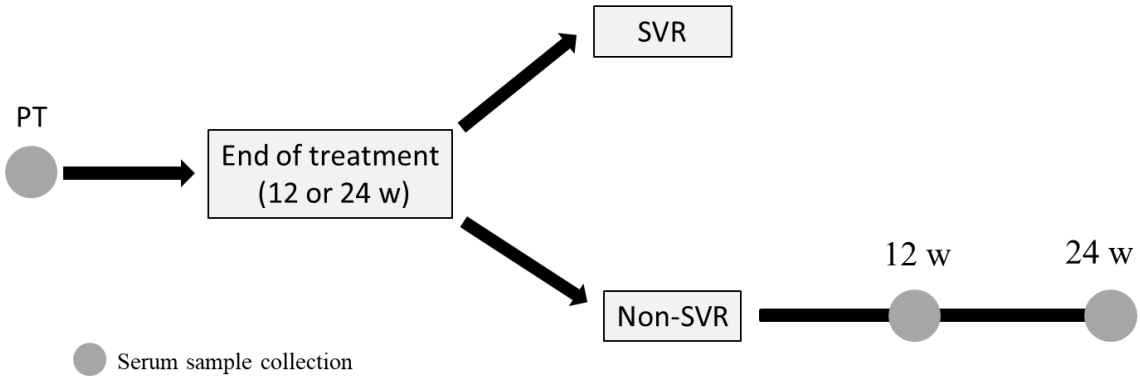
2.7 FIGURE SUBTITLES

Figure 1: Serum collection scheme for RAS analysis. PT: pre-treatment; SVR: sustained virological response; W: weeks of treatment;

Figure 2: Cohort treatment outcomes for both therapy regimens.

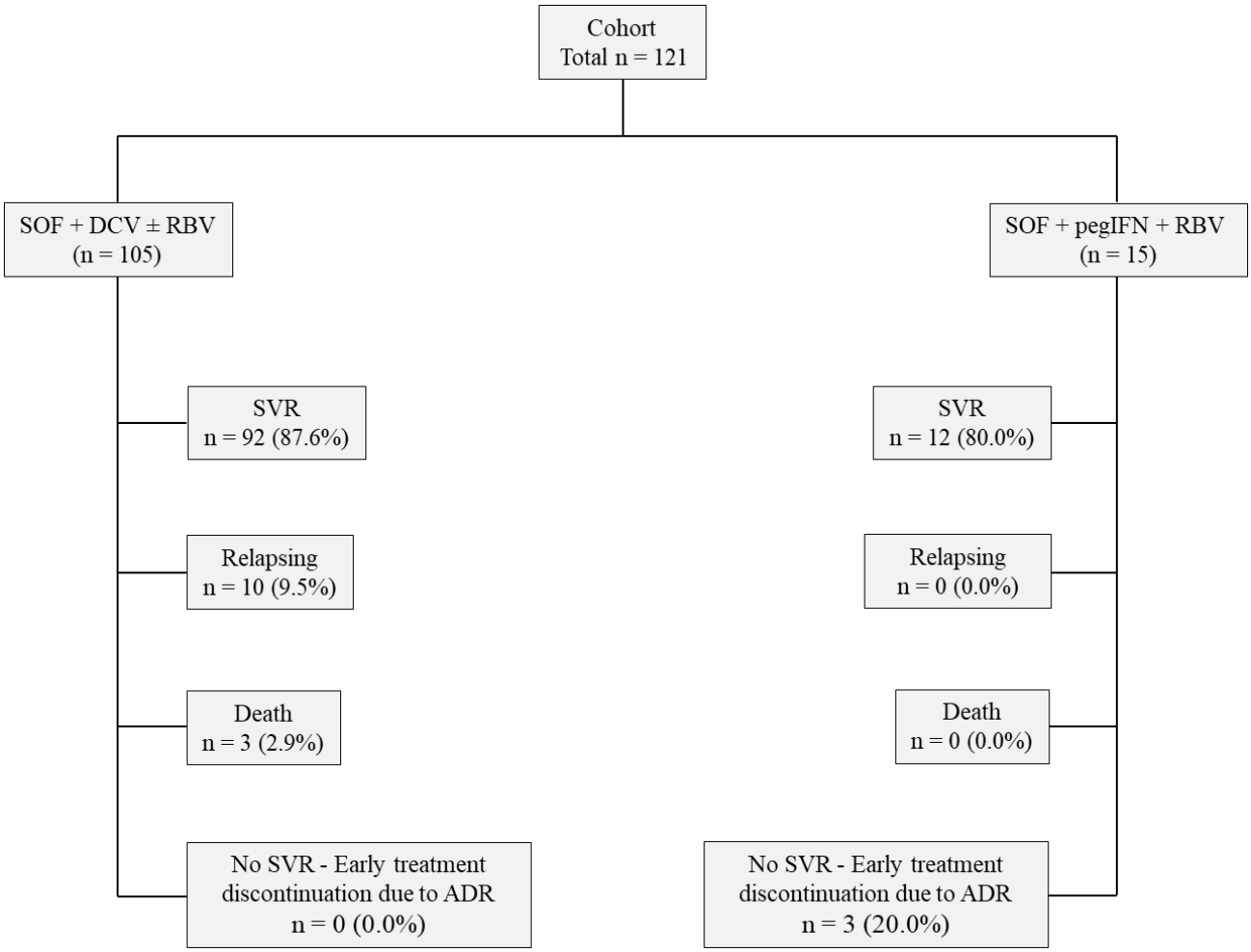
2.8 FIGURES

Figure 1



Fonte: Própria, 2020

Figure 2



Fonte: Própria, 2020

2.9 SUPPLEMENTARY MATHERIAL

	Reação	Nomenclatura do *Primer*	Sequência 5'- 3'	TºC de anelamento
NS5A	PCR	H.NS5AP-F4 (*Foward*)	TTGCCCGCCATACTATCT	47ºC
		H.NS5AI.R2 (*Reverse*)	TTCTTGAAACACTCTGCAGC	
	Nested	H.NS5AN-F4 (*Foward*)	GTVCAGTGGATGAACAG	45ºC
		H.NS5AI.R1 (*Reverse*)	CACGGACACTTGAGCTCATC	
NS5B	PCR	G3.NS5BP.F (Foward)	GATAACACCATGTAGTGCTGAGG	50ºC
		G3.NS5BP.R (Reverse)	AGAAAGATGCCTACCCCTAC	
	Nested	G3.NS5BN.F (Foward)	TATTCAACGTCGTCTAGAAGCGC	53ºC
		G3.NS5BN.R (Reverse)	AAAGCAGCAAATGGCGGGTT	

Supplementary table 1: *Primers* and annealing temperatures used in each PCR reactions for NS5A (35) and NS5B. Fonte: Própria, 2020

NS5B 5' → 3' *Primers* NESTED	**NS5B 5' → 3' *Primers* Internos**
G3.NS5BN.F	**G3.NS5BI.F**
TATTCAACGTCGTCTAGAAGCGC	GGTATAGTGCGAAGGACGT
G3.NS5BN.R	**G3.NS5BI.R**
AAAGCAGCAAATGGCGGGTT	GAGAGTAACTGTGGAGCGT

Supplementary table 2: *Primers* used in sequencing reactions for NS5B region amplified from clinical samples. Fonte: Própria, 2020

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3. Capítulo III

A novel resistance-associated substitution in NS5A enhances resistance of hepatitis C virus genotype 3 to daclatasvir.

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3.1 ABSTRACT

Hepatitis C virus (HCV) genotype 3 presents a high level of both baseline and acquire resistance to direct acting antivirals (DAAs), particularly those targeting the NS5A protein. To understand this resistance we studied a cohort of Brazilian patients treated with the NS5A DAA daclatasvir and the nucleoside analogue, sofosbuvir. We observed a novel resistance associated substitution (RAS) at NS5A amino acid residue 98 (serine 98 to glycine (S98G)) in patients who relapsed post-treatment. This substitution was evaluated using two genotype 3 subgenomic replicons to evaluate its effect on both replication fitness and resistance to DAAs. S98G had a modest effect on replication, but in combination with the previously characterised RAS, Y93H, resulted in a significant increase in daclatasvir resistance. This result suggests that combinations of RAS may drive high level of DAA resistance and provide some clues to the mechanism of action of NS5A targeting DAAs.

3.2 INTRODUCTION

Hepatitis C virus (HCV) infects 70 million individuals worldwide. In 85% of cases infection leads to chronic liver disease: fibrosis, cirrhosis and hepatocellular carcinoma (HCC). HCV has a positive-strand RNA genome and is the most variable human virus, classified into 7 genotypes (GTs), exhibiting >30% nucleotide sequence divergence, with each GT being further divided into subtypes [1]. GT3 is the second most common GT (after GT1) and accounts for 30% of global HCV cases [2, 3]. The variability of the virus results from a lack of proofreading activity of the viral RNA-dependent RNA polymerase (NS5B) and importantly enables the emergence of mutations that can lead to evasion of both the immune response and antiviral treatments.

The development of direct-acting antivirals (DAAs) has revolutionized the treatment of HCV infection. In Brazil, since 2015, the administration of pegylated interferon-α (pegIFN-α) and ribavirin occurs only in few cases, and the first generation of DAAs (telaprevir and boceprevir) has been discontinued. A second generation of DAAs is currently in use and comprises: simeprevir (NS3 protease inhibitor), daclatasvir (NS5A inhibitor) and sofosbuvir (NS5B inhibitor). However, despite the high efficiency obtained with this therapy, resistance is still reported in about 10% of the patients, depending on the viral genotype and the disease progression level. Resistance is invariably linked to the presence of resistance associated substitutions (RAS). Generally, these substitutions can be found easily and in high frequency after treatment failure, but in some cases they can be detected pre-treatment and rapidly increase in frequency due to the selective pressure of DAAs. Since the circulation of these pre-existing substitutions can impact on the efficacy of antiviral treatment, studying the phenotype of these substitutions with regard to DAA resistance and viral fitness is important to understand DAA failure.

Among all HCV genotypes, GT3 exhibits the greatest resistance to DAA therapy, with lower response rates compared with other genotypes. This response is even lower in patients with advanced liver disease, such as cirrhosis [3]. GT3 is the second most prevalent genotype in Brazil, being responsible for approximately 24% of the infections and is thus an important public health problem [4]. In this regard, this study focused on resistance to the NS5A-targeting DAA daclatasvir (DCV) with a view to characterizing the RAS associated with treatment failure in GT3 infected patients. We observed a novel RAS at amino acid residue 98 in NS5A, serine 98 to glycine (S98G) that was

associated with relapsed patients, and we characterised the phenotype of this substitution in the context of both replication fitness and resistance to DAAs, using two subgenomic replicon (SGR) systems recently developed for GT3.

3.3 MATERIALS AND METHODS

3.3.1 Clinical samples. The population in this study consists a cohort of HCV GT3 infected patients under treatment with the NS5A inhibitor daclatasvir (DCV) and the NS5B inhibitor sofosbuvir (SOF). Serum samples from these patients were collected at Ribeirão Preto Medical School, University of São Paulo, Brazil. The samples were collected in two different time points, before and after treatment, where treatment response was assessed. The individuals that did not present sustained virological response (SVR) after treatment were considered non-responders, while those whose viral load was detected 24 weeks after treatment end were considered relapsers. Individuals whose viral load was not detected during treatment and at this last time point were classified as responders.

3.3.2 Molecular analysis. RNA was extracted from serum using TRIzol according to manufacturer's instructions (Thermo Fisher Scientific), followed by cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Considering that DCV interaction with NS5A occurs in domain I, likely between amino acids 28 and 93 [5], we performed the amplification and sequencing of the first 300 nucleotides of the NS5A coding region. Amplification of the target region was performed by nested PCR using the Long PCR Enzyme Mix (Thermo Fisher Scientific) and specific primers (sequences available upon request). Sequencing was performed using the Sanger method using BigDye Terminator v.3.1 (Applied Biosystems) using the same primers as the second PCR. Readings were performed in a 3130xl Genetic Analyzer (Applied Biosystems).

3.3.3 Cell culture. Huh7.5 cells, a human hepatocellular carcinoma cell line, were cultured in Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin ml-1 and 100 µg streptomycin ml-1. Cells were incubated at 37^{o}C and 5% CO_2.

3.3.4 SGR constructs. The *in vitro* approaches in this study were based on two GT3a SGRs from different isolates, SGR-luc-S52 [6], derived from S52/SG-neo [7], and SGR-luc-DBN3a (Ward et al, manuscript in preparation), derived from the DBN3a

infectious clone [8]. As a negative control, we used the replication defective constructs with inactivating mutations in the RNA-dependent RNA polymerase, NS5B: GDD→GNN. This, and the other mutations, were introduced using the Q5® Site-Directed Mutagenesis Kit (New England Biosciences).

3.3.5 SGR replication assays. SGR RNA was produced by *in vitro* transcription and electroporated in Huh7.5 cells with the Gene Pulser Xcell Electroporation System (Bio-Rad), using the square wave protocol (260V, 25 ms, 1 pulse and 4 mm cuvette). For each electroporation, 2 x 10^6 cells were added to the cuvette and, after the procedure, 10^4 cells were seeded in each well, in a 96 well white plate. Cells were lysed using Passive Lysis Buffer (PLB) and replication was obtained by luciferase activity measurement using the Luciferase Assay System Kit (Promega). The assay was analyzed at 24, 72 and 96 h post electroporation (hpe) for S52, and 24, 48 and 72 hpe for DBN3a. This difference in incubation period is due to the more efficient replication of DBN3a [8]. For DAA assays, at 4 hpe DAAs were added to the media at the appropriate concentration ranges and incubated for 96 h (S52) or 72 h (DBN3a). All assays were in triplicate and three independent assays were performed.

3.3.6 Statistical analysis. Data were analyzed using GraphPad Prism 5 software. Mean and standard deviation were determined, and all statistical analyses were performed by ANOVA test and Dunnett's multiple comparison test, considering $P<0.05$ as significant.

3.4 RESULTS

3.4.1 Identification of a novel resistance associated substitution (RAS) in NS5A

This study analysed a cohort of 82 patients infected with HCV genotype 3 who were treated with a combination of sofosbuvir (SOF: NS5B inhibitor) and daclatasvir (DCV: NS5A inhibitor) for a period of 12 weeks. Serum samples from these patients were collected at Ribeirão Preto Medical School, at the University of São Paulo (USP), Brazil. The sustained virological response (SVR) rate of this cohort was 92.7%. Although all the patients responded to therapy, six of them then relapsed after the end of treatment. In an attempt to understand the molecular basis for relapse, we performed Sanger sequence analysis of NS5A domain I (corresponding to amino acids 28-100) both pre- and post-treatment. We detected a number of substitutions within the NS5A domain I coding sequence when compared to the consensus genotype 3 (NZL1) (see Table 1), however the most prevalent was S98G which was present in 14.45% of all

pre-treatment samples. What was more interesting however, was that this substitution was present in 66.6% of the patients who subsequently relapsed, compared to 9.63% of responders (Figure 1A). In the post-treatment samples S98G was present in the relapsed patients at a frequency of 50% (Figure 1B). A comparison with 2915 genotype 3 isolates from different geographical regions showed that S98G is present at a frequency of 14.5%, corresponding with our pre-treatment frequency. We were intrigued that S98G seemed to correlate with relapse after DCV treatment and we therefore set out to analyse the effect of this substitution on viral fitness and DAA sensitivity *in vitro*.

3.4.2 *In vitro* replication phenotype of S98G

To evaluate the fitness impact of this substitution during viral genome replication, site directed mutagenesis was performed to generate S98G derivatives of two different genotype 3 SGR: S52 and DBN3a. The SGR-luc-S52 was described previously [6], the DBN3a SGR was derived from the DBN3acc infectious clone [8] and will be described elsewhere (Ward et al, manuscript submitted). Both SGR contained a modified firefly luciferase reporter with an engineered low frequency of CpG/UpA dinucleotides, we [6] and others [9] have previously shown that this enhanced the replication of the SGR by an as yet undefined mechanism.

Huh7.5 cells were electroporated with SGR-luc-S52 or SGR-luc-DBN3a, including the corresponding polymerase-inactive GNN mutants as negative controls, and luciferase levels assayed in lysates harvested between 4-96 hpe to measure genome replication. For both SGRs, the S98G substitution was tolerated and allowed a similar replication level to WT. However, for S52 there was some evidence that S98G resulted in a modest enhancement of replication. As expected, S52 replicated poorly (Figure 2A), although the input value (4 h) for S98G was significantly lower than WT, by 96 h both S98G and WT exhibited the same luciferase value, suggesting that S98G was able to replicate more efficiently. This was confirmed by the normalized data (normalized to the 4 h value) (Figure 2B), showing that S98G did indeed replicate at a level that was statistically significantly higher than wildtype. In contrast, the DBN3a SGR replicated more efficiently than S52, and in this case all values for both S98G and WT were indistinguishable. We conclude that S98G can confer a modest increase in replicative fitness in HCV GT3a, however in the context of an already efficiently replicating SGR this enhancement was not observed.

3.4.3 S98G resistance phenotype against DAAs

As S98G was associated with relapsing patients we investigated the effect of this substitution on the response to two NS5A inhibitors: DCV (used in the clinical study) and velpatasvir (VEL) a third-generation DAA with potent pan-genotypic activity that was not available in Brazil whilst this patient cohort was being treated. Huh7.5 cells electroporated with SGR were treated with DAAs at different concentrations to determine the effective concentration 50% (EC_{50}). As a control we also determined the EC_{50} for the NS5B inhibitor, SOF, a nucleoside analogue. For S52, the S98G substitution did have a modest effect on the DCV EC_{50} with an increase from 11.5 to 20 pM (Figure 3A), however this would be unlikely to contribute to clinical resistance. S98G had no effect on the VEL or SOF EC_{50} values for S52 (Figure 3B, C). In contrast, for DBN3a (Figure 4), the S98G substitution had no effect on the response to any of the DAAs tested.

3.4.4 S98G phenotype in the presence of Y93H

This result was somewhat surprising and did not explain why S98G was strongly associated with the relapsing phenotype as it appeared to confer very little selective advantage, either in the context of replicative fitness or DAA resistance. We reasoned that S98G might therefore confer some advantage when combined with other substitutions. To address this, we focused our attention on the well characterised RAS, Y93H. We had previously demonstrated that, in our hands, Y93H resulted in a 71,000-fold increase in the DCV EC_{50} for the S52 SGR [6].

Although Y93H was not detected in pre-treatment samples, it was detected in 66.6% of the post-treatment samples from the relapsed patients and, in half of these, this substitution occurred in conjunction with S98G. To test the potential phenotype of S98G in the context of Y93H, we generated SGR-luc-DBN3a derivatives with either Y93H alone, or the combination of Y93H/S98G. For this we focused on DBN3a as the replication levels were higher than S52, making it easier to identify any subtle differences in replication. Huh7.5 cells were electroporated with SGRs, including the polymerase-inactive GNN mutant as negative control, and harvested over a 72 h time-period (Figure 5). The data show that both Y93H and Y93H/S98G replicated to a similar level as WT. Although the luciferase values throughout for both were slightly lower that WT, this is most likely due to lower transfection efficiency as evidenced by the 4 h timepoint. Importantly the rate of replication for all 3 SGRs between 24-72 h

was indistinguishable, clearly demonstrating that neither substitution impacted on replication fitness.

We therefore evaluated the effect of Y93H and Y93H/S98G on the response of SGR-luc-DBN3a to DCV, VEL and SOF. As expected, Y93H resulted in a dramatic increase in resistance of SGR-luc-DBN3a to DCV, increasing the EC_{50} from 16 pM to 194 nM (Figure 6A). Reassuringly, the presence of S98G further increased the EC_{50} to 455 nM. The DBN3a SGR was highly sensitive to VEL, and again Y93H resulted in an increase of the EC_{50} from 0.014 pM to 0.195 nM (Figure 6B). However, in this case S98G did not result in the acquisition of further resistance. Neither Y93H nor the combination of Y93H/S98G resulted in resistance to SOF compared to WT (Figure 6C). Although the effect of S98G was modest, it did provide an explanation for the selection of S98G in samples from patients who relapsed following DCV treatment.

3.5 DISCUSSION

In this study we identify a novel RAS, S98G, in NS5A of GT3a infected patients who relapsed following DAA treatment. The frequency of this RAS was significantly higher in relapsed patients post-therapy compared to pre-treatment and we considered that it might be directly contributing to treatment failure. If this was the case S98G would need to maintain replicative fitness and result in an increase in DCV EC_{50}. To evaluate whether this was the case, we investigated the phenotype of the corresponding mutation in the context of two GT3a SGRs derived from the S52 [7] and DBN3a [8] infectious clones.

This analysis revealed that S98G had no deleterious effect on the replicative capacity of either SGR, indeed in the context of the poorly replicating S52 SGR it exhibited a modest enhancement of replication (Figure 2A, B). Thus, there would be no selective disadvantage of S98G, and S98G could persist in a viral population as it had no replicative defect.

S98G had little or no effect on the EC_{50} for DCV in the context of either SGR (Figs 3 and 4). Although there was a modest increase in the S52 EC_{50} (from 11.5 – 20 pM) this would not be expected to have a clinically significant effect. The plasma concentration of DCV in the 24 h period following a single daily dose of 60 mg was between 270-2700 nM [10]. Although modelling analysis suggests that tissue concentrations are 10-fold lower [11], this would still give a concentration of between 27-270 nM in the liver.

This did not explain the apparent selection of S98G in relapsed patients. We therefore considered that S98G might act additively, or synergistically, with other RAS to further increase EC_{50} values. An obvious candidate was Y93H, which was also observed in the relapsed patient post-treatment samples. To test this, we analysed the phenotype of S98G when combined with Y93H in the context of the DBN3a SGR and compared this with either WT or Y93H. SGR-luc-DBN3a Y93H or Y93H/S98G replicated as well as WT (Figure 5), indicating that the combination of the two RAS did not adversely affect replicative capacity and could therefore persist in a viral population. Reassuringly, compared to Y93H, the combined Y93H/S98G SGR exhibited a further increase in DCV EC_{50}, from 194 to 455 nM (Figure 6). Although this is only a ~2-fold increase we believe that it is potentially clinically significant – if the maximal DCV concentration in the liver is 270 nM, then this level of DCV would be predicted to inhibit both WT and Y93H, but would be less effective against Y93H/S98G. In contrast, S98G had no effect on the EC_{50} for VEL – both Y93H and Y93H/S98G exhibited similar EC_{50} for this DAA (Figure 6). This has implications for the choice of DAA regime – patients with baseline Y93H/S98G RAS should be treated with a DAA combination that contains VEL, not DCV. As the two RAS are only 15 nucleotides apart it should be relatively easy to identify linkage via either next-generation or conventional Sanger sequencing approaches. Interestingly, S98G was observed in a subset of the relapsed patients in the absence of Y93H (Table 1), suggesting that it might also contribute to resistance in cooperation with other RAS (eg A30K or A62T).

So how might S98G cooperate with Y93H to drive high level resistance to DCV? To obtain some insight into this we mapped the two RAS on to the three-dimensional structure of domain I of genotype 1b NS5A [12, 13] (Figure 7). Although close to each other in the primary amino acid sequence (Figure 7A), the two residues are not proximal within the structure. In particular, residue 93 is surface exposed whereas residue 98 is partially buried (Figure 7B). However, it is still plausible that both residues form key contact points for DCV binding, alternatively S98G might have an allosteric effect on the structure of NS5A. The other implication of the data is that the potential interaction of VEL with NS5A is subtly different than DCV and does not involve residue 98. VEL is clearly a more potent DAA in comparison to DCV but equally affected by Y93H (Figure 6), it is likely therefore that VEL contacts other residues within NS5A, perhaps residues that are more functionally constrained and therefore cannot mutate to confer resistance.

In conclusion we propose that resistance of HCV GT3 to NS5A-targeting DAAs is driven by multiple RAS and varies with respect to the specific DAA being administered to the patient. Consideration must be taken to baseline RAS when deciding on clinical approaches. The identification of novel RAS such as S98G may contribute to our understanding of the molecular mechanisms of NS5A-targeted DAA and resistance, and may also be useful tools to investigate NS5A functions in the virus lifecycle. In this context, it will be intriguing to study the phenotype of S98G in the context of infectious virus, particularly in light of our previous observation that NS5A domain I plays a role in virus assembly [14]. Such experiments are planned in our laboratory for when work can recommence following the COVID-19 lockdown.

AUTHOR CONTRIBUTIONS: GRFC, JW and SC performed the experiments, JPVR, AdLCM, FFS and LRLP provided the clinical samples, CB, PR and MH supervised the study. GRFC, JW, CB, PR and MH wrote the manuscript.

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3.6 TABLES

Substitution	Pre-treatment frequency	Post-treatment Frequency
D3N	2.4% (2/82)	16.6% (1/6)
A75V	6.1% (5/82)	16.6% (1/6)
T79M	6.1% (5/82)	16.6% (1/6)
T94F	0%	16.6% (1/6)
S98G	14.45% (12/82)	50% (3/6)
A30K	6.1% (5/82)	33.3% (2/6)
A62T	24.4% (20/82)	50% (3/6)
Y93H	0%	66.6% (4/6)
A30K/S98G	2.4% (2/82)	16.6% (1/6)
A62T/S98G	8.5% (7/82)	33.3% (2/6)
Y93H/S98G	0%	33.3% (2/6)

Table 1: Resistance associated substitutions (RAS) identified in this study. RAS in **bold** are previously unidentified. Fonte: Própria, 2020

3.7 FIGURE SUBTITLES

Figure 1: Frequency of S98G in the studied population. A) S98G frequency in pre-treatment samples. B) S98G frequency in post-treatment samples.

Figure 2: Replication phenotype of S98G. A) S52 SGR RNAs (WT, S98G and NS5B GNN) were electroporated into Huh7.5 cells and harvested at the indicated times for luciferase assay. B) S52 SGR replication values were normalized to the 4 hpe values (corresponding to translation of input RNA. $P<0.01$. C) DBN3a SGR RNAs (WT, S98G and NS5B GNN) were electroporated into Huh7.5 cells and harvested at the indicated times for luciferase assay.

Figure 3: DAA resistance phenotype of S98G. S52 SGR RNAs (WT or S98G) were electroporated into Huh7.5 cells, treated with DCV (A), VEL (B) or SOF (C) at the indicated concentrations for 96 h prior to harvest for luciferase assay. Values were normalized to the no DAA controls. EC_{50} values for WT and S98G are indicated on the right.

Figure 4: DAA resistance phenotype of S98G. DBN3a SGR RNAs (WT or S98G) were electroporated into Huh7.5 cells, treated with DCV (A), VEL (B) or SOF (C) at the indicated concentrations for 72 h prior to harvest for luciferase assay. Values were normalized to the no DAA controls. EC_{50} values for WT and S98G are indicated on the right.

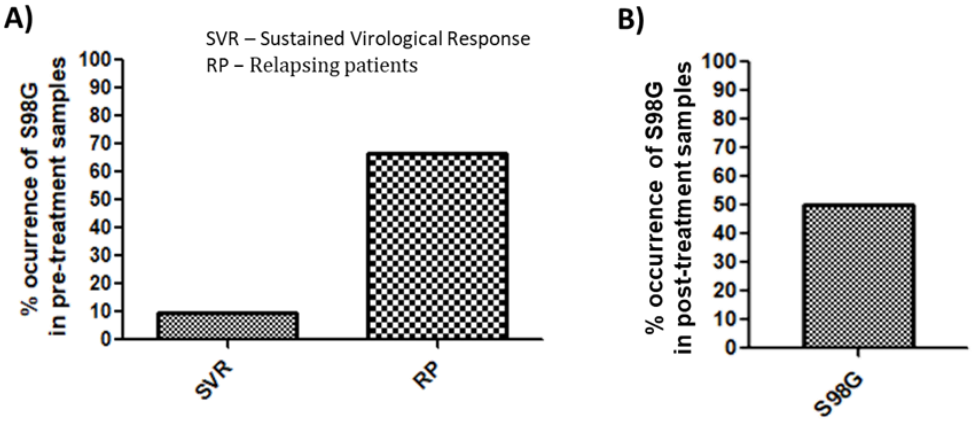
Figure 5: Replication phenotype of S98G in combination with Y93H. DBN3a SGR RNAs (WT, Y93H, Y93H/S98G and NS5B GNN) were electroporated into Huh7.5 cells and harvested at the indicated times for luciferase assay.

Figure 6: DAA resistance phenotype of S98G in combination with Y93H. DBN3a SGR RNAs (WT, Y93H or Y93H/S98G) were electroporated into Huh7.5 cells, treated with DCV (A), VEL (B) or SOF (C) at the indicated concentrations for 72 h prior to harvest for luciferase assay. Values were normalized to the no DAA controls. EC_{50} values for WT and S98G are indicated on the right.

Figure 7: NS5A domain I monomer tertiary structure and surface characterization, showing the localization of Y93 (in blue) and S98 (in red).

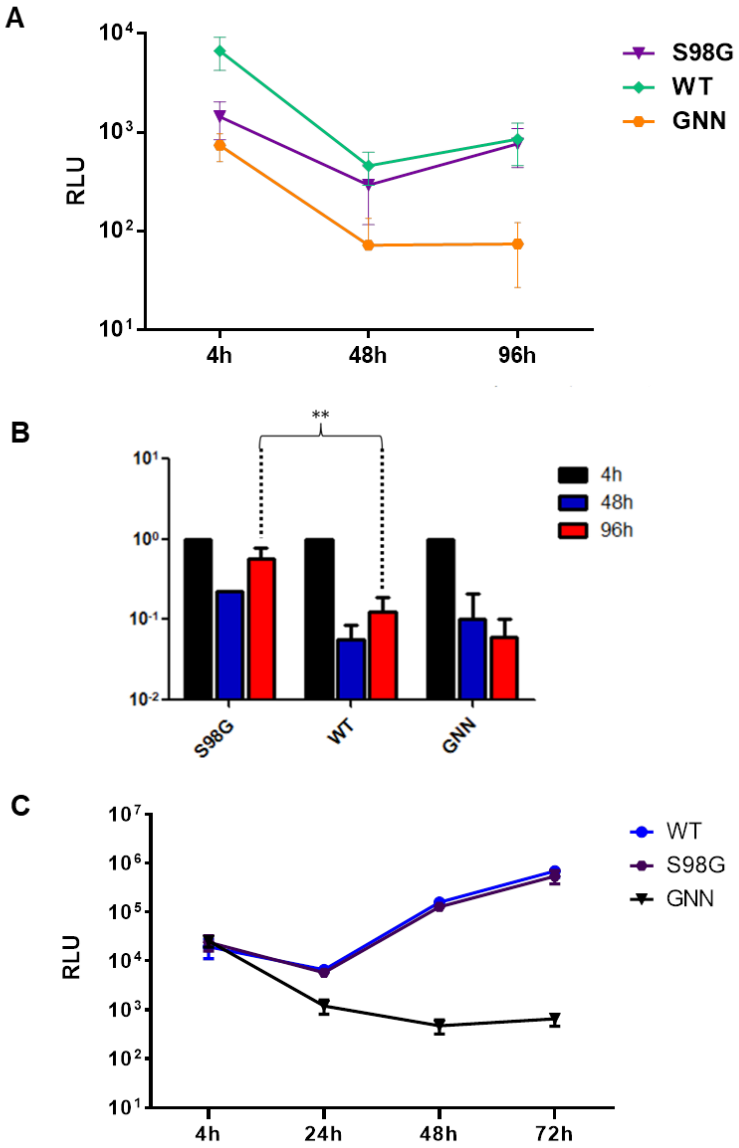
3.8 FIGURES

Figure 1



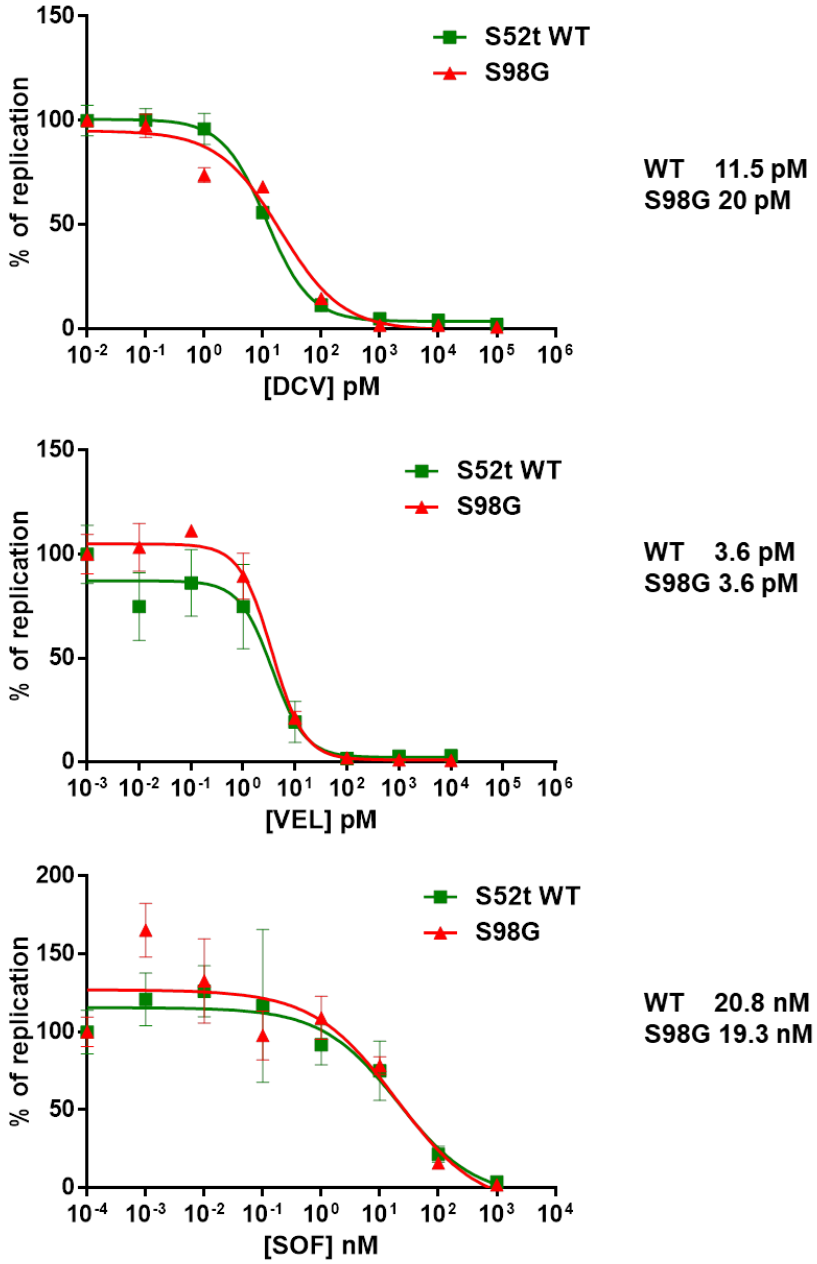
Fonte: Própria, 2020

Figure 2



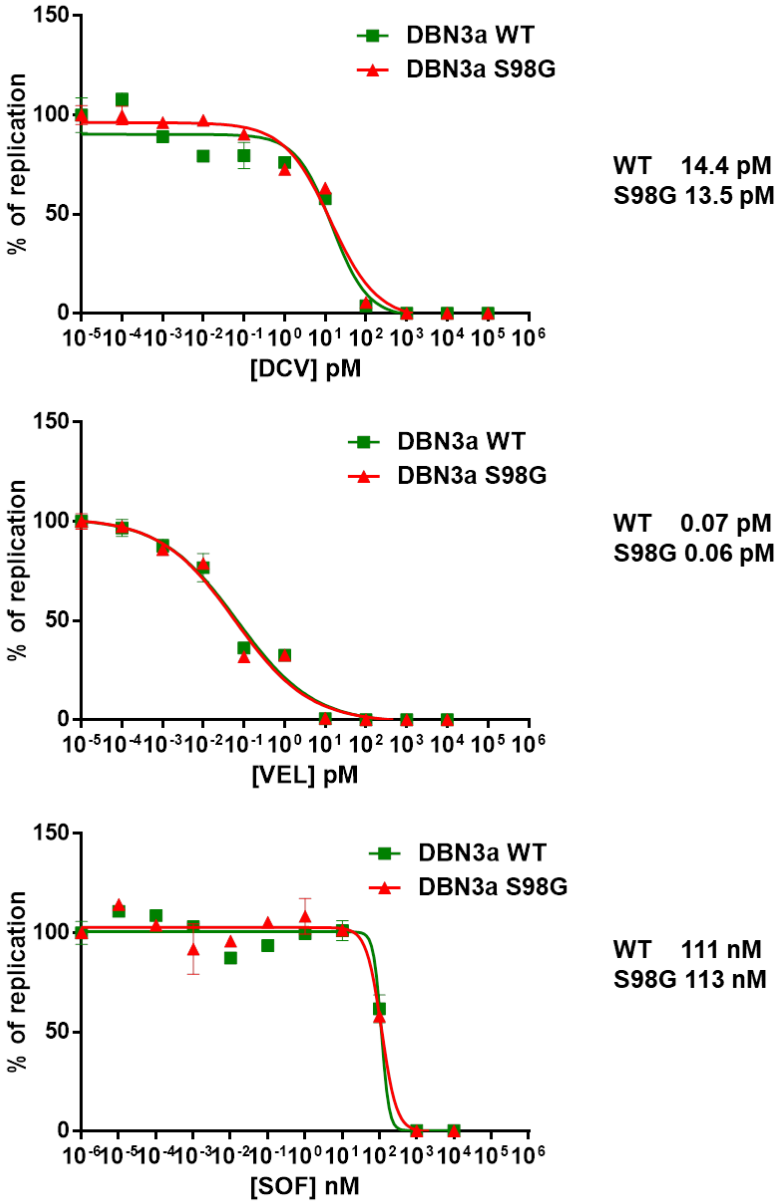
Fonte: Própria, 2020

Figure 3



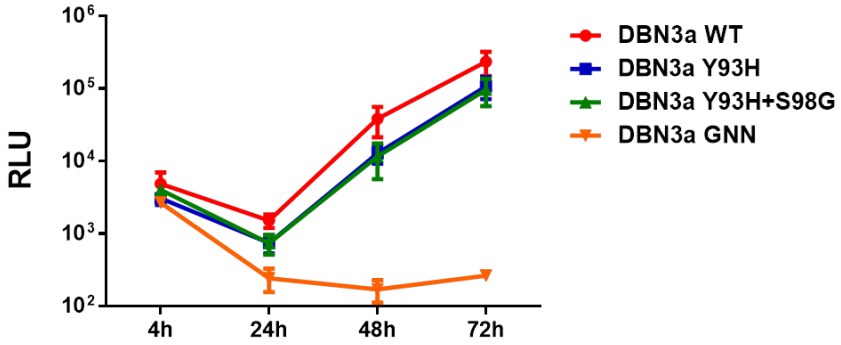
Fonte: Própria, 2020

Figure 4



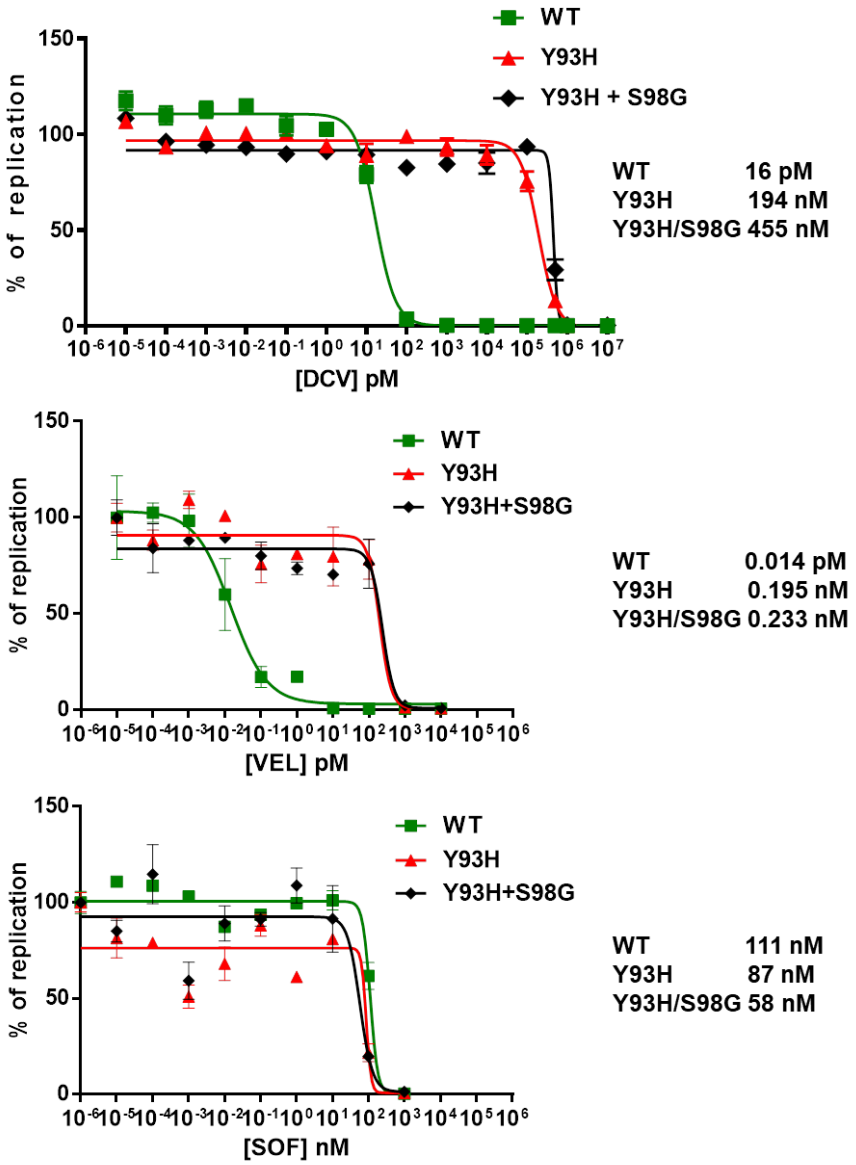
Fonte: Própria, 2020

Figure 5



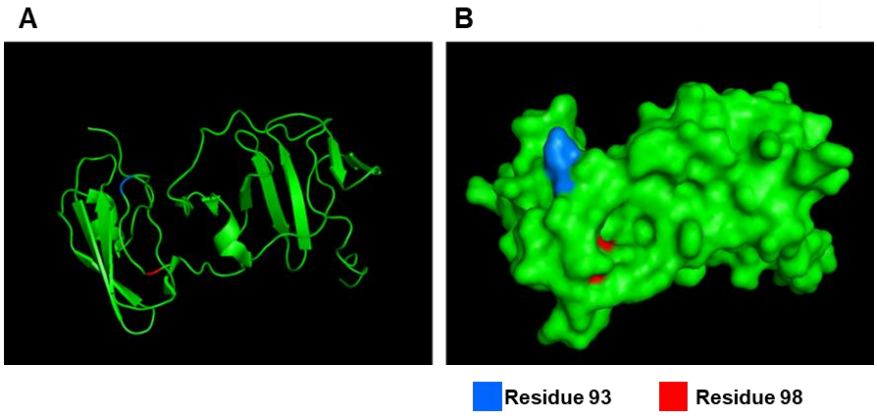
Fonte: Própria, 2020

Figure 6



Fonte: Própria, 2020

Figure 7



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4. Capítulo IV

Description of a novel *in vitro* tool for the investigation of RAS into hepatitis C virus genotype 3 NS5A protein

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4.1 ABSTRACT

Hepatitis C virus (HCV) genotype 3 is, among all HCV genotypes, the most difficult to treat, presenting the lowest sustained virological response (SVR), especially in severe cases such as cirrhosis and hepatocellular carcinoma. In general, it is unknown all the variables that can contribute to the genotype 3 lower susceptibility to direct-acting antiviral (DAA) treatment, but it is described that the presence of resistance associated substitutions (RAS) is the main factor for treatment failure. In addition, genotype 3 *in vitro* systems for RAS study are scarce and not so developed when compared to other genotypes. Due to these facts, our study focused on the description of a new *in vitro* tool that can be used to detect, from clinical samples, already described RAS and possible novel mutations into NS5A under DAA selective pressure, evaluating concomitantly resistance level and fitness cost of these substitutions. Samples from 6 relapsing patients were included in this study. NS5A domain I was amplified from these clinical samples and cloned into a genotype 3 subgenomic replicon. A pool of replicons, containing domain I from different variants, was prepared for each clinical sample, inserted into Huh 7.5 cells and treated with DCV for 72h and 96h. Our data shows that insertion of NS5A domain I, derived from clinical samples, was biologically functional, with no impact on replication level when compared to wild-type (WT). The treatment with DCV presented selective activity for the replicons bearing domain I from clinical samples, independent on the incubation period, and next generation sequencing (NGS) analysis showed both the selection of baseline RAS and possible novel resistance associated mutations. Here we describe a new *in vitro* approach that can be useful in the study of HCV resistance acquisition. This tool mimics the host quasispecies environment, including cell and DAA selective pressures, enabling the study of viral genome regions derived from clinical samples.

Key words: HCV; Genotype 3; *In vitro* RAS investigation;

4.2 INTRODUCTION

The discovery of hepatitis C virus (HCV) in 1989 led to the pursuit of different ways to cure patients infected with this virus [1]. The genetic variability of HCV makes difficult to produce vaccines in order to prevent infection [2], so antiviral therapy has been the main tool for infection treatment since then [3].

At the beginning, treatment was based on the administration of Interferon (IFN), an antiviral molecule naturally produced by human cells whose production is blocked by HCV infection [4-7]. However, due many side effects and lower response to treatment [8], other strategies showed to be necessary, like the combination of IFN and ribavirin [8,9]. The science progress and increased knowledge about the viral structure, proteins and lifecycle enabled that new antiviral approaches could be developed. The direct-acting antivirals (DAAs) era for HCV started in 2011 with two NS3 protease inhibitors, Boceprevir and Telaprevir. Both were specific against HCV genotype 1 and were used in combination with the previous treatment, in a triple therapy, reaching high sustained virological response (SVR) [10]. Since the first generation of DAAs were specific for genotype 1, the pursuit of broad spectrum DAAs was necessary, leading to the development of many other DAAs. These new drugs are classified into three different categories according to their target: NS3 protease inhibitors; NS5A inhibitors; and NS5B RNA-dependent RNA polymerase [11]. In Brazil, since 2015, three DAAs are distributed by the Brazilian health system, Simeprevir, Daclatasvir and Sofosbuvir, inhibitors of NS3, NS5A and NS5B, respectively. These antivirals improved SVR to all genotypes and showed more advantages like shorter period of treatment (12 weeks) and fewer side effects [12,13].

Even with all these improvements, antiviral treatments have some negative points that need to be considered. Genotypes present different response rates. Genotype 3, for example, has the lowest response to treatment among all genotypes [14-16], so designing broad spectrum antivirals with high efficiency for all genotypes is a big challenge. Since HCV has a high genetic variability due to its high mutation rate [17,18], some substitutions can confer resistance to these antivirals, and this mutations can naturally circulate in the population, at a baseline level [19-22], or can be selected by treatment pressure [23,24]. In addition to these aspects, among all viral targets, NS5A has the most crucial role on resistance. It is described that, when failure to

treatment with regimens based on NS5A inhibitors occurs, in almost 90% of the cases it is due to the presence of resistance associated substitutions (RAS) in this protein [23,24]. Also, differently from RAS present on the other viral proteins (NS3 and NS5B), those detected into NS5A are very fit and usually persist for a longer period [25]. Once all these characteristics can decrease the effectiveness of current antivirals, it is indispensable the continuous development of new DAAs.

The development of new antivirals is a hard and slow process, taking many years to reach the population after the first tests, and the occurrence of resistance associated mutations is a dynamic event that is happening during this period. Therefore, some procedures are necessary to avoid resistance and maintain the current treatment with the highest possible SVR rates. In this work, we describe a new *in vitro* tool for the study of NS5A RAS that enables the validation of novel resistance mutations detected in clinical samples, evaluating fitness cost and resistance level, as well as understanding how these mutations behave under treatment selective pressure.

4.3 MATERIALS AND METHODS

4.3.1 Clinical samples

Serum samples from patients infected with hepatitis C virus genotype 3 that relapsed the treatment with Daclatasvir were used in this study. These samples were obtained at the Ribeirão Preto Medical School, in Brazil, situated in the University of São Paulo (USP). Serum samples were collected before the beginning of the treatment and response was assessed 24 weeks after the end of it, considering relapsing patients the ones that presented detectable viral load at this last time point.

4.3.2 Cell culture

All *in vitro* assays were performed using a hepatocellular carcinoma cell line called Huh 7.5. The cells were cultured in Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 IU penicillin ml^{-1}, 100 µg streptomycin ml^{-1}, followed by incubation at 37ºC and 5% CO_2.

4.3.3 HCV constructs

To perform the experiments, two genotype 3 subgenomic replicons were used, S52t and S52t-SF (Supplementary Figure 1). Both are derived from the S52/SG-neo [26] and as a

negative control, the replication defective mutant S52t-GNN was used. All constructs included in this study carry the subgenomic region of HCV genotype 3, the firefly luciferase reporter gene for replication measurement and the neomycin resistance gene.

4.3.4 Cloning of NS5A domain I from clinical samples into S52t-SF subgenomic replicon

In order to evaluate NS5A domain I derived from clinical samples, modifications of the S52/SG-neo replicon were made by the group headed by Prof. Mark Harris in order to insert unique restriction sites (Bsu36I and PspXI) flanking a small stuffer fragment replacing the coding sequence for NS5A domain I, calling this new replicon as S52t-SF. With this approach, the NS5A domain I from viruses circulating in patients could be cloned inside S52t-SF by RT-PCR, replacing the stuffer fragment. As a consequence, the subgenomic replicon would be biologically functional only with domain I insertion, once the presence of the stuffer fragment disables the replication.

NS5A domain I from pre-treatment samples from each relapsing patients containing putative resistance associated mutations was cloned into S52t-SF. For this, specific primers were designed to amplify the NS5A domain I by NESTED-PCR (Supplementary Table 1), where the primers for the second PCR reaction were carrying, in the 5' region, the same restriction sites found in S52t-SF replicon (Bsu36I and PspXI). Amplifications were performed using the Phusion High-Fidelity DNA Polymerase (New England Biosciences) according to enzyme's protocol and the NESTED-PCR products, with the S52t-SF vector, were double digested using Bsu36I and PspXI restriction enzymes (New England Biosciences) for 2h at 37ºC. An electrophoresis in agarose gel 0.8% was performed and the desired fragments obtained by digestion were purified using Monarch DNA Gel Extraction Kit (New England Biosciences). NS5A domain I inserts were cloned into S52t-SF backbone by T4 DNA Ligase (New England Biosciences). After a 4h incubation at room temperature the plasmid was transformed in *E. coli DH5α* competent cells and plated in LB agar plates, treated with ampicillin, overnight. The colonies obtained by this transformation were collected and purified together in the same tube, resulting in a pool of replicons for each clinical sample.

4.3.5 Replication and resistance analysis for replicons carrying NS5A domain I from clinical samples

Huh 7.5 cells were electroporated with this pool of constructs and a transient replication assay was performed, where the electroporated cells were divided into two groups, one treated with 1 nM of Daclatasvir (the EC90 for Wild Type replicon - WT) and the other, called control group, was incubated only with fresh media. Cells were lysed using Passive Lyses Buffer (PLB) (Promega) and the replication level was analyzed 72h and 96h after electroporation by luciferase activity measurement. After *in vitro* biological validation and antiviral incubation, RNA was extracted from treated cells using TRIzol protocol and RT-PCR, with the same primers for NS5A (Supplementary Table I), was performed. The NS5A domain I obtained fragment was submitted to NGS analyzes.

4.4 RESULTS

4.4.1 Relapsing clinical samples

Once we aimed to validate this tool in order to study the selection of resistance mutations against DCV, samples from patients that did not respond to treatment with this DAA were necessary. Six clinical samples (P11, P45, P112, P175, P189 and P192) were obtained from different patients from the same geographic region. All of the patients responded well during DCV administration, with no detectable viral load at this period, but 24 weeks after the end of treatment, viral load was detected again by RT-qPCR. Due to this, these patients were classified as relapsing patients and the pre-treatment serum samples, from each one, were processed and submitted to the next steps.

4.4.2 Replication analysis of replicons harboring NS5A domain I from clinical samples

In order to evaluate if the insertion of NS5A domain I into the S52t-SF subgenomic replicon was biologically functional, we performed a replication assay. For this procedure, we cloned NS5A domain I from a clinical sample into S52t-SF and electroporated the pool of replicons generated after transformation process in Huh 7.5 cells. Replication was evaluated 72h and 96h post-electroporation. We could observe that the replicons harboring NS5A domain I from clinical sample presented the same replication level as WT replicon, and both were higher than GNN defective mutant. This was observed regardless of the incubation time (72h and 96h) (Figure 1a and 1b), showing that replicons obtained by NS5A domain I cloning are biologically functional.

4.4.3 Resistance phenotype of NS5A domain I derived from clinical samples

In order to test the selective pressure of DCV treatment on NS5A domain I, we performed an *in vitro* approach where S52t-SF subgenomic replicon carrying the domain I derived from clinical samples were electroporated in Huh 7.5 cells, followed by 1nm DCV treatment. P189 presented the highest resistance phenotype among all clinical samples, independent on the incubation period (72h or 96h). The other clinical samples also showed to be more resistant than WT, however the difference was not high and consistent between incubation periods like P189 (Figure 2a and 2b).

4.4.5 NGS sequencing analysis evaluating potential novel RAS

Since P189 subgenomic replicons presented a higher replication level when compared to WT after treatment, and showed the highest replication level among all clinical samples, being consistent in both incubation periods, we decided to proceed to sequencing analysis with this sample. For each treatment group RNA was extracted and submitted to RT-PCR in order to amplify the NS5A domain I from this replicon pool for sequencing analysis. The obtained sequences were compared to genotype 3 S52 strain (GenBank Access number GU814263) (Table 1).

The NGS data showed some slight differences between control and DCV group. Some mutations were detected in different frequencies depending on the treatment group, like A62T, frequent in 100% of the isolates from control group and in 97.55% from DCV treated group. T64A presented a similar pattern, with 100% and 96.71% frequencies for control and DCV treated groups, respectively. Mutations S14T and T21S behaved the same way with 100% at control and 97.4% after treatment. On the other hand, H85Y showed an opposite situation, with frequencies for control and DCV groups of 53.57% and 79%, respectively, showing an increase in the population frequency after treatment. Substitution M79T presented this same pattern, reaching frequencies of 51.5% for control group and 75.6% after incubation with DCV.

Mutation S98G was also increased after incubation with DCV. The NGS data showed that this substitution was present in 46.4% in control group, while in DCV treated group the frequency increased to 59.7%.

For mutation Y93H, the sequencing analysis showed that, even with DCV treatment, this mutation was not enriched. Y93H was present in a frequency of 10.7% in the control group. However, after DCV incubation, this mutation was not observed.

4.5 DISCUSSION

HCV treatment, based on direct acting antivirals, never reached such a high efficiency rate like the reported in the last few years. However, many variables can influence the cure of infected patients like DAA metabolism, genetic background, level of disease progression and viral resistance against antivirals. This last one is considered the main cause of decrease in DAA efficiency along treatment [27,28]. Due to genetic variability, the viral populations called quasispecies can naturally carry mutations that can confer resistance to a specific DAA, and these substitutions, present at a baseline level, can be circulating in different frequencies inside this population. Usually, these mutations, at this point, are present in minor frequencies since frequently they have a negative impact on viral fitness [29]. However, even with this situation, they can be maintained in the population by some viral replication strategies like transcomplementation [30,31].

At treatment beginning, during DAAs usage, this panorama changes and a selective pressure process starts. These viral variants carrying mutations related with less sensitivity to DAAs can be now selected due to antiviral administration and, in this specific situation, can become the major variants in that population, even if it has a fitness cost, defining the viral resistance. Concomitantly, mutations with no relation with DAA resistance acquisition but with a role on viral fitness improvement can occur, during replication or also at a baseline level, impacting on rapid outgrowth of these resistant and adapted novel variants [29].

Two main methods are used to detect resistance associated substitutions and both are based on the sequencing of viral regions aimed by the antivirals. The first one is performed by Sanger sequencing method, while the second one, more accurate, is based on Next Generation Sequencing (NGS) protocols. Sanger sequencing allows the detection of most frequent variants on the population, with prevalence higher than 20%. On the other hand, NGS is more sensitive and can also identify the minor variants circulating in less than 1% of the population [32]. However, regarding RAS report and description procedures, the clinical cutoff is a prevalence of, at least, 15% [33-35].

Thus, even using advanced sequencing techniques that can screen minor variants, this proposed standardization can let minor RAS escape from detection and description.

In our work, our new *in vitro* tool can be used as an ally in the identification of possible novel RAS and maybe solve this cutoff issue. In our results, using a subgenomic replicon that carries two unique restriction sites, we were able to insert the NS5A domain I from relapsing clinical samples and analyze the originated replicons in cell culture. Our first concern was if they were biologically functional after NS5A domain I insertion and we could see that, after electroporation in Huh 7.5 cells, they were replicating at the same level as WT replicons. Since these restriction sites were generated *in vitro* and NS5A domain I was amplified using primers carrying these same restriction sites, there was a care to perform this cloning preserving the correct reading frame. Also, it is described in literature that, for RNA viruses, synonymous mutations in a codon can lead to different translation efficiency due to codon usage bias, affecting viral replication. For HCV the same pattern is observed, where some codons are preferred when compared to others [36-38]. However, our replication data shows that the restriction sites present in the cloned replicons have no impact on replication efficiency, being biologically functional. In our NGS analysis we can see that, after treatment with DCV, mutation frequencies were influenced and some of them were selected. As discussed before, HCV has a huge genetic variability and, due to this, circulates in the host as quasispecies. This characteristic allows some RAS to circulate in different frequencies, even maintaining those with high fitness cost in minor variants. However, there is a consensus by the standardized protocols for RAS description that neglects baseline mutations lower than 15% as they usually do not influence on resistance acquisition [29]. Since our *in vitro* approach clones NS5A domain I from clinical samples and submit the originated replicon pools to DCV pressure, this technic consists in a way to try to avoid losing these minor variants that would be discarded at direct sequencing analysis. Also, our choice to use a replicon pool, with mixed variants derived from the same clinical sample, is a good way to mimic the quasispecies population found in the infected patient. Thus, when treating with DAAs, we try to create a selective pressure as close as the one observed *in vivo*.

About the mutations observed by NGS, some already described substitutions were maintained after DCV treatment, like A62T. It is known that at position 62 some substitutions related to resistance can appear and, for genotype 3, eight different amino

acids were already detected in this position. A62T was observed in our population in 97.55% of the variants after DCV treatment, while other study describes for this mutation a frequency of 28.8%, showing some sort of enrichment in our samples [39]. The point is that A62T was already predominant at our control group, possibly suggesting this as a genomic characteristic of this population that differs from the cohort of the other study. In any case, the fact that already described RAS were selected in our approach reinforces it as a useful tool for resistance.

On the other hand, other mutation observed, Y93H, an important RAS that confers high resistance level against NS5A inhibitors, behaved differently from A62T. Y93H was detected in the control samples in a frequency of 10.7% and surprisingly, after DCV treatment, it was not detected anymore. For this assay we treated the infected cells with DCV at 1 nM, the WT EC90 concentration, and we believe that probably it was not high enough to maintain Y93H due to its fitness cost [40]. Probably, the panorama would be different using higher concentrations of DCV.

Substitutions with unknown relation with resistance also suffered some kind of selection after DCV treatment. M79T and H85Y presented the same pattern, being enriched after DAA administration, changing the frequency from 51.5% and 53.5% before treatment to 75.6% and 79% after treatment, respectively. Other mutation detected in high frequency after treatment was S98G, improved from 46.4% in control samples to 59.7% in DCV treated samples. Interestingly, these mutations were maintained after treatment, suggesting some kind of selection, but other assays are necessary to determine the resistance level associated with each substitution and the fitness impact of them on viral replication.

Here we describe a new *in vitro* approach that can be useful in the study of HCV resistance acquisition. This procedure allows, in cell culture, the investigation of viral regions derived from infected patients, aiming the already described RAS emergence, circulation of them at baseline level and description of possible novel substitutions related with antiviral failure. In addition to being biologically viable, this tool also tries to mimic an ambiance similar to what happens with the quasispecies in the host during treatment, including cell and DAA selective pressures, and improves detection efficiency to avoid the loss of any substitution regardless the frequency in the population.

AUTHOR CONTRIBUTIONS: GRFC and JW performed the experiments, JPVR, AdLCM, FFS and LRLP provided the clinical samples, CB, PR and MH supervised the study. GRFC, CB, PR and MH wrote the manuscript.

CONFLICTS OF INTEREST: The author(s) declare that there are no conflicts of interest.

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ACKNOWLEDGEMENTS: We thank Charles Rice (Rockefeller University, New York) for the S52 subgenomic replicon.

4.6 TABLES

Nuclotide Position	Reference	Variant	Coding Change	Codon Change	AA Change	AA Position	% of Variant in Control Population	% of Variant in DCV treated Population
39	CTCG	TACA	Coding	TCG>ACA	Serine>Threonine	S14T	100%	97.4%
57	CAAGACG	TAAATCA	Silent/Coding	ACG>TCA	Threonine>Serine	T21S	100%	97.4%
180	GGCAT	AGCAA	Silent/Silent/Coding	TCA>ACA	Serine>Threonine	S62T	100%	97.55%
189	CACT	AGCC	Silent/Coding	ACT>GCC	Threonine>Alanine	T64A	100%	96.71%
236	TG	CA	Coding	ATG>ACA	Methionine>Threonine	M79T	51,50%	75.6%
253	C	T	Coding	CAC>TAC	Histidine>Tyrosine	H85Y	53,57%	79%
277	TAC	CAT	Coding	TAC>CAT	Tyrosine>Histidine	Y93H	10,70%	0%
292	A	G	Coding	AGC>GGC	Serine>Glycine	S98G	46,40%	59.7%

Table 1: Panel of NS5A domain I substitutions detected at NGS analysis for clinical sample P189. RNA was extracted 72 hours after electroporation in Huh 7.5 Cells and submitted to Nested RT-PCR and NGS. Frequency at the population was determined for Control and DCV treated groups in percentage. AA: Amino Acid; Fonte: Própria, 2020

4.7 FIGURES SUBTITLES

Figure 1: Replication assay in Huh 7.5 cells for biological validation of genotype 3 subgenomic replicon S52t-SF carrying NS5A domain I derived from clinical samples. **A)** Incubation for 72 hours post-electroporation; **B)** Incubation for 96 hours post-electroporation. CS: Clinical Sample; WT: Wild Type; RLU: Relative Luminescence Unit;

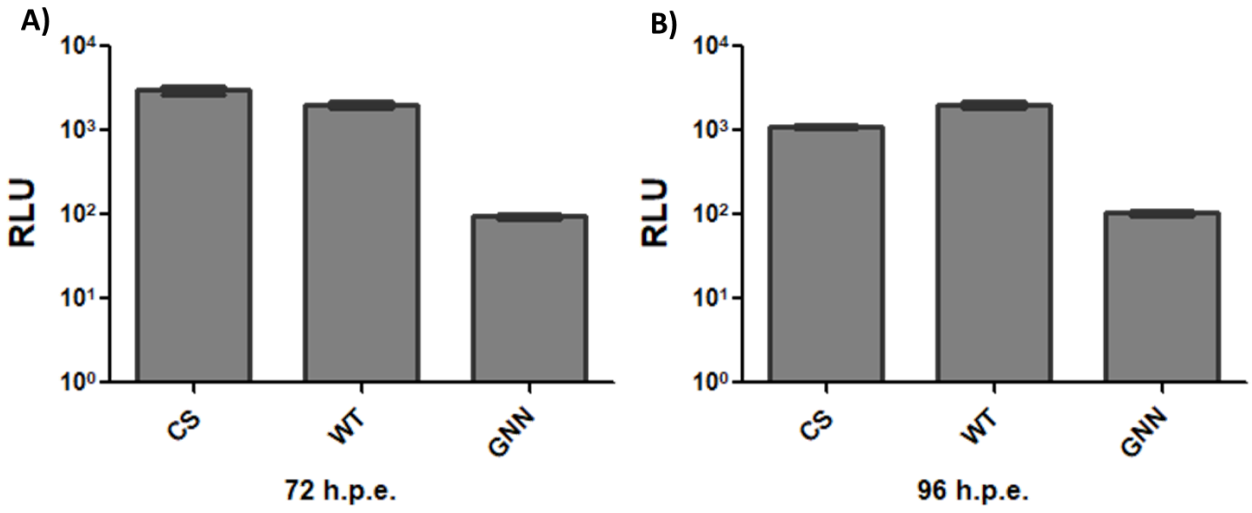
Figure 2: Resistance phenotype assay of NS5A domain I derived from six relapsing patients in Huh 7.5 cells Electroporated cells were incubated for **A)** 72h and **B)** 96h in two different groups, with or without Daclatasvir (Control and DCV).

Supplementary Figure 1: Genotype 3 subgenomic replicons S52t and S52t-SF. Both have the unique restriction sites Bsu36I and PspXI, but S52t-SF has a stuffer fragment replacing NS5A domain I.

Supplementary Table 1: Primers used for NESTED-PCR and RT-PCR of NS5A domain I derived from clinical samples.

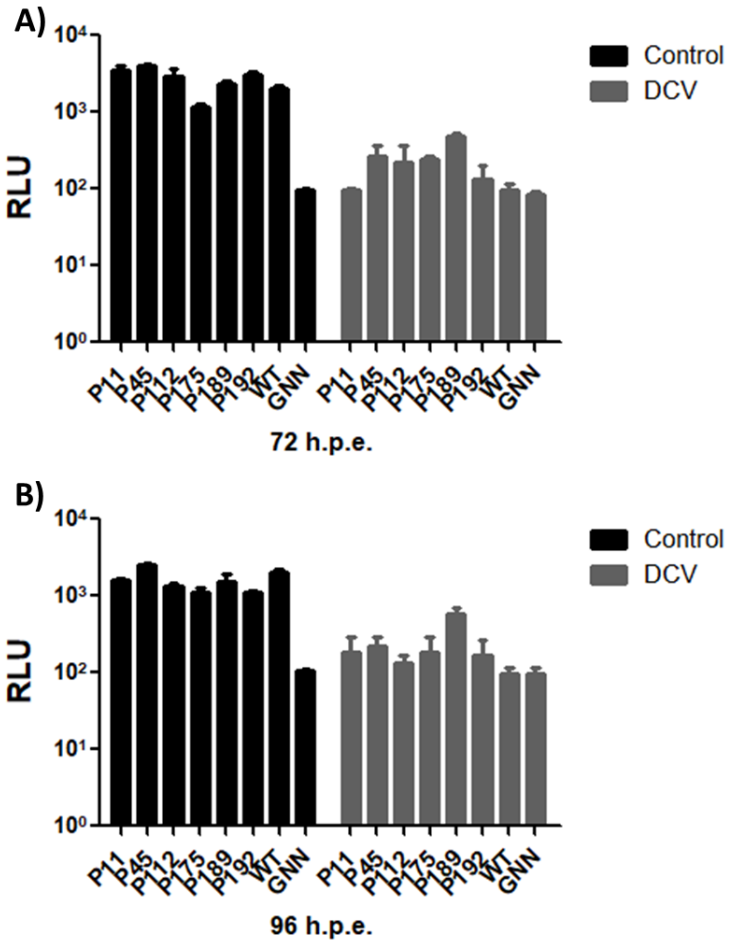
4.8 FIGURES

Figure 1



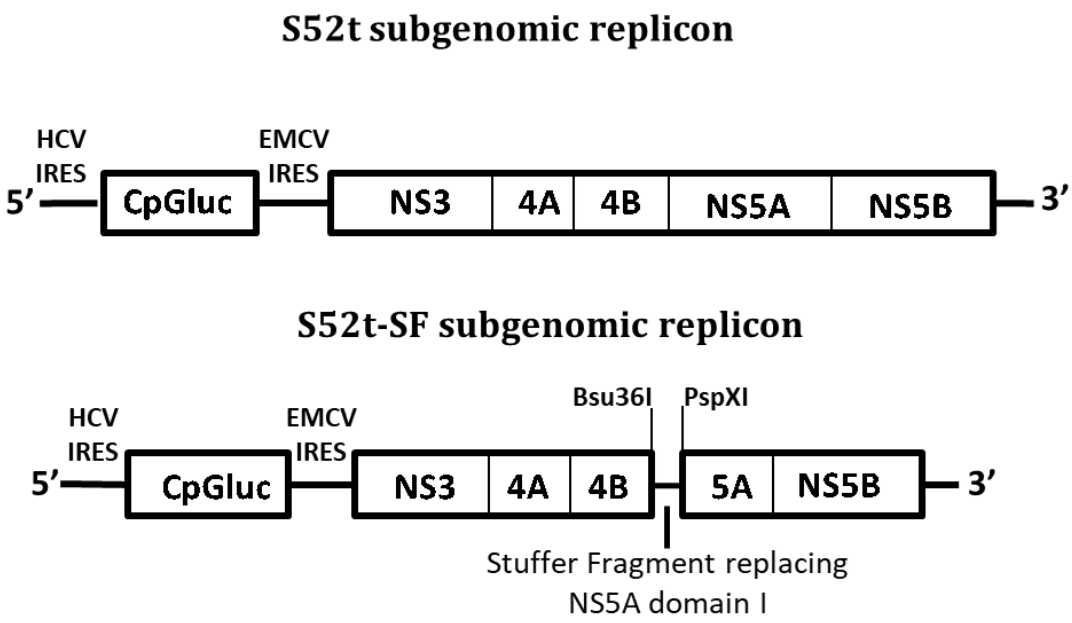
Fonte: Própria, 2020

Figure 2



4.9 SUPPLEMENTARY MATHERIAL

Supplementary Figure 1



Fonte: Própria, 2020

Supplementary Table 1

Primer name	5' -> 3'
NS5A_1stPCR_Rev	CCCATCTCTTGCCGCCATAACAAGTT
NS4B_1stPCR_Fwd	ATACTACGTCGACACGTAGGACCTG
Bsu36I_Fwd	TCTCCTCAGGCGGTTACACAAGTGGATCAA
PspXI_Rev	GACCCTCGAGCAAGGCGGCGCGCTGCCGT

Fonte: Própria, 2020

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Anexo A

In-frame Opal stop codon presence at hepatitis C virus genotype 3 NS5A protein is not determinant for premature translation interruption

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ABSTRACT

Hepatitis C virus (HCV) is a positive single stranded RNA virus and, due to this positive sense characteristic, its genome can be promptly translated once in the hepatocyte cytoplasm. The translation process originates a polyprotein that will be later cleaved originating the individual viral proteins. NS5A, a multifunctional viral protein, has its gene located right before the RNA dependent RNA viral polymerase NS5B, which is responsible for the viral replication. A molecular evolution analysis of the NS5A protein, based on a cohort under treatment with Interferon and Ribavirin, showed the recurrence, in viruses from different patients and time-points, of mutations introducing the Opal stop codon (UGA) into the NS5A genomic region of HCV genotype 3. In order to investigate the impact of this codon in position 111 of NS5A protein, site-directed mutagenesis were performed in subgenomic replicons from GT3 (SGR-luc-S52 and SGR-luc-DBN3a) and GT2 (SGR-feo-JFH-1) carrying the firefly luciferase gene reporter. Their replication efficiency was accessed in Huh 7.5 cells and compared to the wild-type controls. Replication assay results showed that the Opal readthrough occurs in GT3, while in GT2 the replication is completely abrogated in the stop codon presence. Our data also shows that some readthrough signals, like a cytosine present right after Opal and secondary structures downstream stop codon site, are present in genotype 3 but not in genotype 2, reinforcing that Opal readthrough at this site could be specific for genotype 3. Since Opal mutants replicate poorly when compared to wild-types and transcomplementation was not observed in our results, the Opal maintenance mechanism in the cohort is still unclear. However, our findings showed that Opal presence, in genotype 3, is not determinant for the early translation interruption. The description of this process could help to understand some unique molecular features of this genotype that make it different from the others.

Key words: HCV; Genotype 3; Opal stop codon; Readthrough

INTRODUCTION

Hepatitis C is a liver inflammation disease caused by hepatitis C virus (HCV) infection. It is believed that approximately 71 million people in the world are affected with this disease (1). HCV infection is divided in two phases, where the symptoms can vary from muscle pain, fever and jaundice in the acute phase to cirrhosis, fibrosis and hepatocellular carcinoma in the chronic phase (2-4).

HCV, a member of the *Flaviviridae* family, is a single-stranded positive-sense RNA encapsulated virus, and its genome encodes a polyprotein, which is processed by viral and host proteases, originating 10 individual viral proteins (5-7). Due to its high replication rate and lack of proof reading activity from the viral RNA dependent RNA polymerase NS5B, HCV has high genetic heterogeneity, being classified into different genotypes and several subtypes (8-11).

NS5A, one of the viral non-structural proteins, is a phosphoprotein with nearly 447 amino acids subdivided in three structural domains (I, II and III) and an amphipathic α-helix located in the N-terminal region (12-14). Domain I plays a role in viral RNA replication (15), while domains II and III may possibly act as immune response antagonist and in viral particles assembly, respectively (14, 16-18). The N-terminal region acts by anchoring at the endoplasmic reticulum membrane and the C-terminal region interacts with core protein in viral particle assembly (14, 19). This protein is reported to influence interferon (IFN) activity (20, 21), conferring resistance to this natural antiviral molecule mainly due to its interaction with cellular signaling pathways, such as PKR protein kinase, which is induced by IFN and is inhibited by NS5A during infection (22). Some authors describe the interaction between this viral proteins and proapoptotic factors, such as p53, where NS5A hinders the apoptotic process (23-26).

Some studies from our group, based on the genetic variability of HCV NS5A in clinical samples of infected patients undergoing treatment, have identified stop codons in some variants (27, 28). Frequent stop codons, especially Opal stop codon (UGA), were reported in the same position in different patients and in different sampling time-points of the same patient (28, 29). One of the mutations exchanged a guanine at nucleotide 333 for an adenosine, creating a stop codon at position 111 of the protein, a highly conserved position throughout genotypes (29). The occurrence of stop codons in the middle of eukaryotic genes is common and well described (30), but for viruses it is still

an unknown territory. Corroborating our findings with HCV infected patients, studies with dengue virus (DENV), another *Flaviviridae* family member, also identified strains containing stop codons in the same position; these samples were collected from distinct individuals and hosts (humans and mosquitoes) during an outbreak (31, 32), and this phenomenon is observed in other viruses (33).

Regarding HCV RNA translation process, this finding would imply that the polyprotein translation would abnormally stop in NS5A preventing NS5B translation. Due to the intriguing recurrence of a possible defective genome, we decided to investigate and evaluate, in cell culture, the impact of a UGA codon presence at position 111 in hepatitis C virus NS5A protein (originally UGG) in the translation of the protein and in the replication process.

MATERIALS AND METHODS

Constructs

In order to investigate the impact of Opal stop codon in the replication of hepatitis C virus, genotype 2 subgenomic replicon derived from JFH1 strain (SGR-feo-JFH-1) (34) and two genotype 3 subgenomic replicons from different strains, SGR-luc-S52 (35), a subgenomic replicon derived from S52/SG-neo (36) and SGR-luc-DBN3a (Ward et al, manuscript in preparation), were used. As negative controls, we used the replication defective constructs SGR-feo-JFH-1-GNN for genotype 2 and S52t-GNN SGR-luc-DBN3a-GNN for genotype 3. All replicons are constituted by the non-structural region of HCV, the firefly luciferase reporter gene and the neomycin resistance gene.

Cell culture

The *in vitro* assays were performed in Huh 7.5 cells, a hepatocellular carcinoma cell line, cultivated in Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 IU penicillin ml^{-1}, 100 µg streptomycin ml^{-1}. Cells were incubated at 37ºC and 5% CO_2.

In vitro evaluation of Opal stop codon into NS5A position 111

To insert the Opal stop codon inside the coding region of NS5A viral protein of the replicons, a site directed mutagenesis was performed using Q5® Site-Directed Mutagenesis Kit (New England Biosciences) according to manufacturer's protocol. This

procedure was performed in all constructs here used (genotype 2 and 3). The same process was used to change the nucleotides located after the stop codon site.

Subgenomic replicons were electroporated in Huh 7.5 cells following the square wave protocol (260V, 25ms, 1 pulse and 4mm cuvette) using the Gene Pulser Xcell Electroporation System (Bio-Rad). For each electroporation, $2x10^6$ cells were added to the cuvette and, after the procedure, 10^4 cells per well were seeded in 96 well white plates and lysed using Passive Lysis Buffer (PLB) (Promega) after different incubation periods, according to the constructs replication efficiency (4h, 24h and 48h for JFH-1; 4h, 48h and 96h for SGR-luc-S52; and 4h, 24h, 48h and 72h for SGR-luc-DBN3a). Lysed cells were submitted to Luciferase Assay System (Promega) and replication was determined by the relative luminescence unit (RLU) log levels. All the assays were performed in triplicate, with three independent repetitions.

Transcomplementation assays

To analyze how the Opal stop codon was maintained in the population, we performed two transcomplementation assays. The first one consisted on the electroporation, using the same protocol described before, of SGR-luc-DBN3a Opal mutant and GNN defective replicon in Huh 7.5 cell line stably expressing the DBN3a-neo replicon that carries no reporter gene, only the neomycin resistance gene. In a 96 well white plate, 10^4 cells were seeded in triplicate, incubated for 72h, lysed with 1x Passive Lyses Buffer (PLB) (Promega) and submitted to Luciferase Assay System (Promega) for replication measurement. The other one consisted in the concomitant transfection, using Lipofectamine 2000 (Invitrogen), of Opal mutants and GNN with the pSGR-DBN3a-GFP, a replicon that carries the green fluorescent protein (GFP) instead of firefly luciferease reporter gene. In this case, as a control, transfections of Opal mutant and GNN with Yeast tRNA were performed to ensure that RNA concentrations transfected in the cells were the same when testing these mutants without WT. RNA combinations (1.5 μg of each one) were incubated, in the same tube, with Lipofectamine 2000 for 15 min. Meanwhile, cells were washed with 1x PBS and incubated with Opti-MEM (Gibco). The mix containing RNA and Lipofectamine was added to the cells and incubated at 37ºC for 4h. After incubation, media was changed and fresh DMEM was added. In both cases, cells were incubated for 72h, lysed using 1x Passive Lysis Buffer (Promega) and submitted to luciferase measurement by Luciferase Assay System Kit

(Promega). In the second approach, instead of using the conventional cell incubator, cells were incubated using the IncuCyte Live Cell Analysis Systems (Sartorius), an equipment that enabled us to evaluate the GFP expression during all incubation time, without the need of lysing or fixing the cells before the end of incubation.

RESULTS

Opal replication phenotype

To evaluate the impact of the Opal presence inside NS5A on viral replication, Opal stop codon was inserted by site direct mutagenesis into replicons from genotypes 2 and 3. The obtained data shows that the genotype 3 Opal mutant (SGR-luc-S52) (Figure 1a), at 96h time point, can replicate while the genotype 2 Opal mutant (SGR-feo-JFH-1) (Figure 1b) cannot, showing a continuous decrease in replication until reaching the GND level. The same process was performed using a more replicative efficient genotype 3 replicon, the SGR-luc-DBN3a, and the same pattern was observed, where Opal mutant can replicate higher than GNN at 72h after electroporation, corroborating with the data observed for S52t, and these values are maintained, as a plateau, when increasing the incubation to 96h, showing that this slight difference in replication level of Opal mutant and GNN is consistent (Figure 1c).

Impact of downstream cytosine on Opal replication phenotype

To understand why genotype 3 Opal mutants are capable to replicate while genotype 2 Opal mutant replication is abrogated, readthrough signaling patterns that could explain this difference between HCV genotypes were tested. Thus, the original Opal subsequent nucleotides from genotype 2 and genotype 3 replicons were exchanged (A → C in SGR-feo-JFH-1; C → A in SGR-luc-S52) (Figure 2a), observing the impact of these substitutions in viral replication. When cytosine was changed for adenine in genotype 3, the replication was completely abrogated (Figure 2b), and when the opposite was done to genotype 2, replication was not recovered (Figure 2c), showing that cytosine enables readthrough for genotype 3 but not for genotype 2.

Differences in RNA secondary structure may influence Opal readthrough

Once the Opal readthrough occurs in genotype 3 but not in genotype 2, the pursuit of a reason behind this difference was necessary. One of the observed readthrough triggers

was the presence of secondary structures on the RNA molecule, downstream of the stop codon position. Using the genotype 2 and genotype 3 genomes, some RNA secondary structure predictions were performed for the NS5A region with the *mfold* web server for nucleic acid folding and hybridization prediction (37). The analysis was performed in the same region, around the site where Opal was found, and two stem-loops downstream Opal site were detected, for both genotypes. For genotype 3, all predicted stem-loops presented high affinity among their interactions (defined by the red, orange and yellow dots), which means that these interactions are more likely to happen. For genotype 2, only one was found with this strong affinity characteristic, while the other presented week interactions, with a lower probability to happen (defined by blue, purple and green dots) (Figure 3a and 3b).

Opal mutant is not maintained by transcomplementation

Considering the HCV capability of circulation as quasispecies and the ability to maintain defective variants by transcomplementation, the reason why Opal was recurrent in the studied population was investigated. Two different transcomplementation assays were performed to check if the Opal replication was recovered by the WT replicon. In the first one, using a Huh 7.5 stable cell line carrying the DBN3a-neo subgenomic replicon, the data shows that the WT was not able to recover the replication of both electroporated replicons, also reducing the relative luminescence units (RLU) value for Opal (2067 RLU to 290 RLU) (Figure 4a and 4b).

For the second transcomplementation approach, WT carrying the GFP reporter gene and defective replicons with luciferease reporter gene (Opal and GNN) were inserted in the cells at the same time, instead of using a stable cell line expressing the WT replicon. The obtained results show that there was no recover in replication of Opal or GNN, showing no signal of transcomplementation (Figure 5). This is corroborated by the fact that GFP expression was detected in the cells, showing that WT was replicating in both double transfections (WT+Opal and WT+GNN) (data not shown).

DISCUSSION

In conventional translation processes, the mRNA is read and each codon codifies a specific amino acid, while stop codons are responsible for determining translation stops

and protein synthesis endings. As introduced before, studies on NS5A genetic variability reported the recurrence of stop codons at the same position in more than one patient (27-29, 38). These codons in these positions undergoing traditional translation would imply an early translation termination at NS5A and the consequent absence of NS5B. However, the obtained results showed that, for HCV genotype 3, the UGA codon at position 111 of the NS5A protein was not determinant for translation stop. This pattern was observed for both genotype 3 subgenomic replicons (SGR-luc-S52 and SGR-luc-DBN3a), where the mutants containing the stop codon were able to maintain replication levels higher than GNN negative control until 96h after electroporation, while the same was not detected for genotype 2, where Opal presence abrogated the replication.

The mechanism that could explain this phenomenon is called “readthrough”. This process consists of the non-recognition of a stop codon, leading to protein extension, and this is mainly due to decoding of the codon by some tRNA, almost always a cognate or suppressor, thus extending the peptide chain to the next stop codon (39). The importance of the process can be observed in some plant viruses, such as *Luteovirus*, where it occurs in order to facilitate transmission by aphids (40), and in members of *Alphavirus*, where the event is needed in viral replicase synthesis (41). Despite this, the stop codons in these cases are not results of mutations.

Considering the occurrence of readthrough for Opal stop codon in HCV NS5A protein and that this happened only in genotype 3, our data suggests that this process could be genotype specific, and some signals on viral RNA could explain this difference. The first one is that, in some cases, the presence of a cytosine right after stop codon site could act as a readthrough signal, especially when this stop codon is Opal (UGA) (30, 39, 41-43). The sequence verification for genotypes 2 and 3 revealed that in genotype 3 the cytosine occurs naturally right after codon 111, where the Opal was detected, while in genotype 2 an adenine is found in the same place. This could explain why readthrough happens in one genotype, but not in the other, and shows that, in genotype 3, the cytosine plays a crucial role on Opal readthrough, once its change for an adenine resulted in the complete replication abolishment.

Other possibility is that some RNA structural characteristics can work as a signal for stop codon readthrough, like stem-loops and pseudoknots (44-46). Once this process is

linked with the interaction among different nucleotides across the same RNA molecule, the occurrence of these secondary structures can vary according to the genome variability. Some predictions for viral RNA folding, for both genotypes, were made in the pursuit of these secondary structures, and we could notice that the occurrence of stem-loops right after Opal position is more likely to happen in genotype 3 than genotype 2. Despite this is just a prediction of RNA secondary structure, the high affinity interactions shows that this is an interesting possibility. Our results suggest that Opal readthrough could happen as an association of both factors, the subsequent cytosine and the downstream stem-loop presence.

The replication results clearly shows that genotype 3 Opal mutants can replicate, but in a lower level when compared to wild-type replicons. In a host panorama, where immune system and antiviral treatment impose a selective pressure (47, 48), this poor replication fitness could lead to the Opal mutant vanishing. However, the NS5A variability studies showed that Opal maintenance happens even under Interferon administration (27-29, 38). Our hypothesis was that the Opal persistence in this population could be enabled by the transcomplementation with wild-type viral proteins. It is described in the literature that HCV defective genomes can be maintained in the host with the support of viral proteins derived from other variants (49-51), but our assays showed no signal of transcomplementation. The detection of the stop codon in the early mentioned study could only be done by NS5A cloning and sequencing due to its low frequency (28). Perhaps the lower replication level of the Opal mutant is sufficient to maintain it in a minor frequency in the quasispecies population.

In this study we observed that the presence of Opal stop codon, in the position 111 of NS5A, is not determinant for the premature translation interruption in genotype 3. Some RNA signals for stop codon readthrough were detected only in genotype 3 NS5A, reinforcing that Opal readthrough at this site could be specific for this genotype. Since transcomplementation was not observed in our assays, the Opal maintenance mechanism is still unclear, and new studies are necessary to explain the reason why Opal is persistent during an Interferon treatment regimen.

AUTHOR CONTRIBUTIONS: GRFC and JW performed the experiments. CB, PR and MH supervised the study. GRFC, JW, CB, PR and MH wrote the manuscript.

CONFLICTS OF INTEREST: The author(s) declare that there are no conflicts of interest.

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FIGURE SUBTITLES

Figure 1: *In vitro* Opal replication phenotype for: A) Genotype 3 subgenomic replicon SGR-luc-S52; B) Genotype 2 subgenomic replicon SGR-feo-JFH-1; C) Genotype 3 subgenomic replicon SGR-luc-DBN3a.

Figure 2: Impact of subsequent cytosine on Opal readthrough. A) Genotypes 2 (GT2) and 3 (GT3) sequences, highlighting in red the nucleotide right after the stop codon. B) Opal replication in GT3 after changing the cytosine (C) for an adenine (A). B) Opal replication in GT2 after changing the adenine (A) for a cytosine (C).

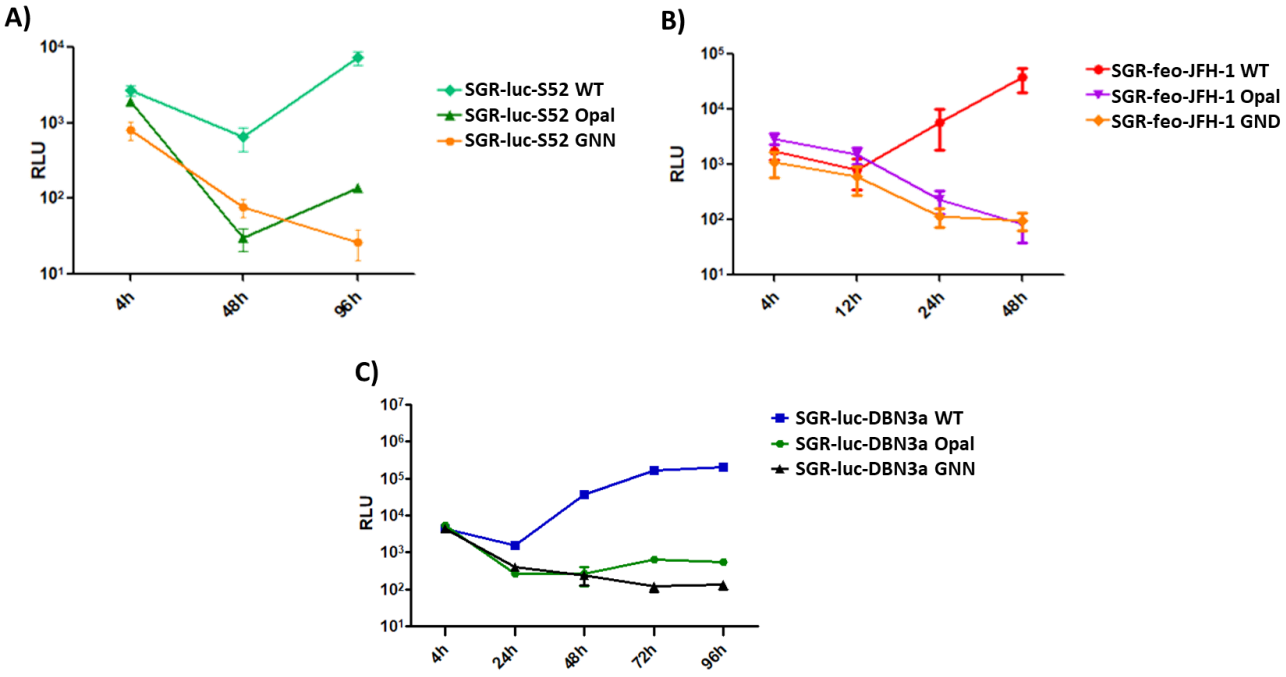
Figure 3: Viral RNA secondary structure for: A) Genotype 2 (SGR-feo-JFH-1); B) Genotype 3 (SGR-luc-S52).

Figure 4: Transcomplementation assay. A) Electroporation in Huh 7.5 cells; B) Electroporation in Huh 7.5 cells stably expressing the DBN3a-neo subgenomic replicon. RLU = Relative Luminescence Units;

Figure 5: Transcomplementation assay by lipid transfection, inserting wild-type (WT) pSGR-DBN3a-GFP concomitantly with Opal and GNN mutants, in the same concentration (1.5 µg), into Huh 7.5 cells.

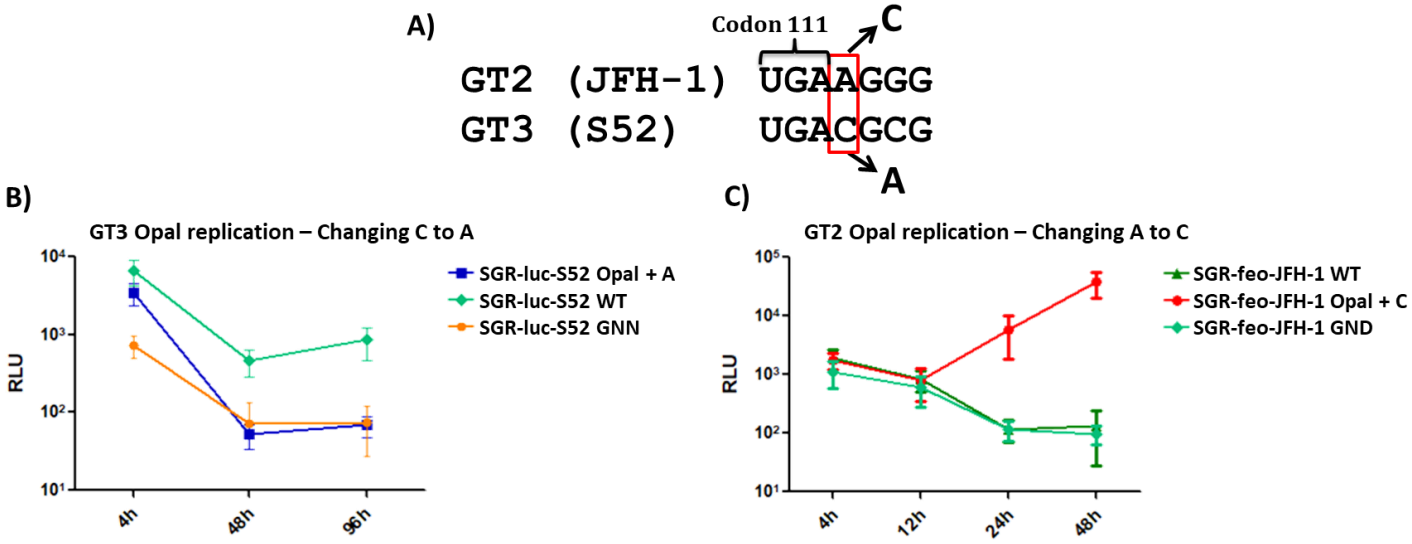
FIGURES

Figure 1



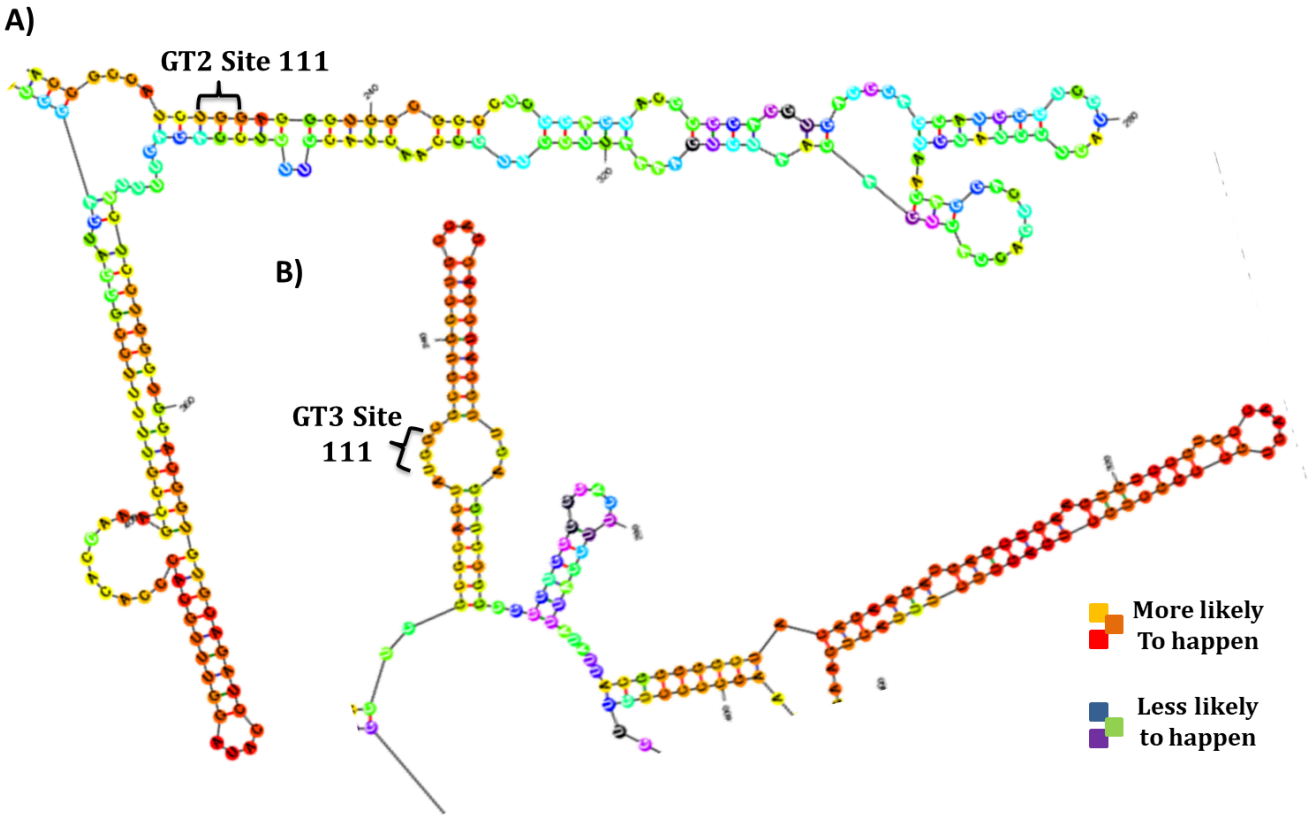
Fonte: Própria, 2020

Figure 2



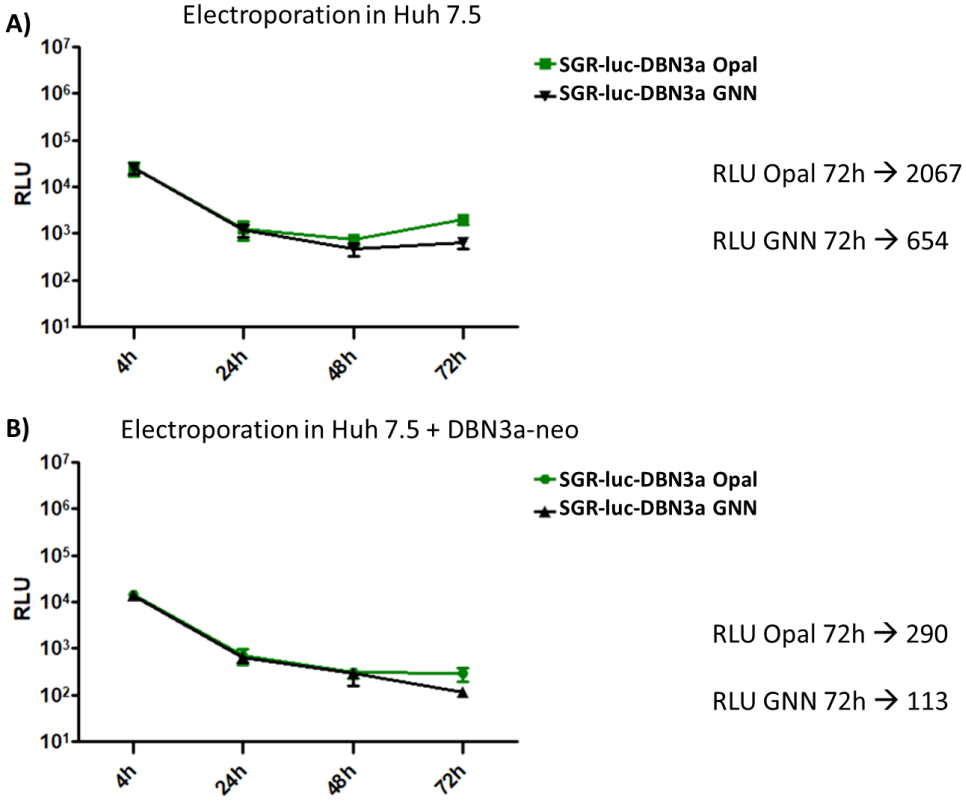
Fonte: Própria, 2020

Figure 3



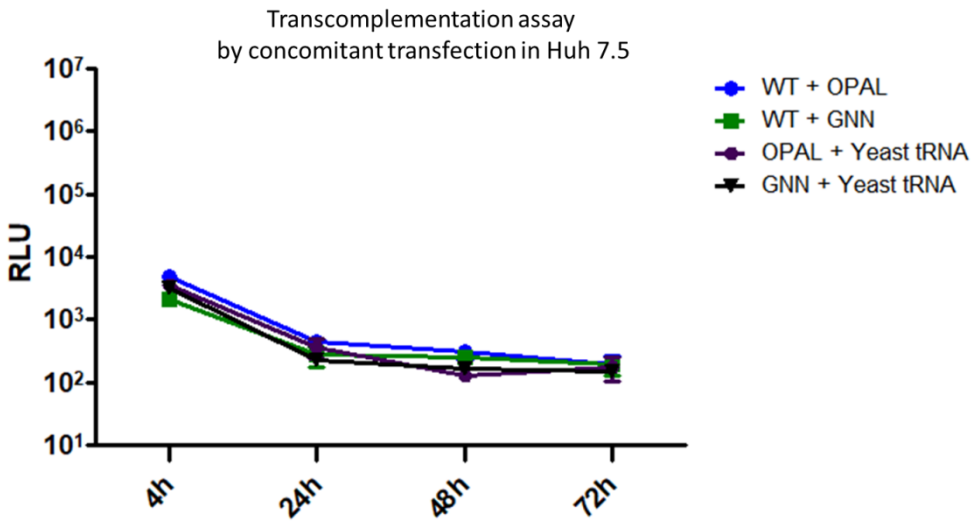
Fonte: Própria, 2020

Figure 4



Fonte: Própria, 2020

Figure 5



Fonte: Própria, 2020

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Anexo B

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ORIGINAL ARTICLE

Baseline resistance associated substitutions in HCV genotype 1 infected cohort treated with Simeprevir, Daclatasvir and Sofosbuvir in Brazil

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Summary

Background: The World Health Organization estimates that 1% of the world population (71 million) is infected with hepatitis C virus (HCV). In 2015, three direct-acting antivirals (DAAs), simeprevir (SMV), sofosbuvir (SOF) and daclatasvir (DCV) were included in the Brazilian protocol for the treatment of chronic hepatitis C. Despite the fact that the use of these drugs is associated with higher treatment response rates and with lower incidence of side effects, studies have shown the association between the presence of viral resistance mutations and the failure of pharmacological treatment.

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Aim: This way, this study aimed to evaluate the safety and effectiveness of treatment for HCV genotypes 1a and 1b infected patients with these DAAs, also analyzing the occurrence and prevalence of baseline resistance associated substitutions (RAS), observing the impact of these mutations into the treatment success.

Methods: Clinical data were collected from all the 262 HCV infected patients included for comparative analysis, while serum samples collected from 144 of these individuals, before treatment, were submitted to molecular biology approaches for mutation analysis into NS3, NS5A and NS5B regions.

Results: Regarding the treatment regimens, 49.6% of the patients received SOF + DCV ± ribavirin and 50.4% used SOF + SMV ± ribavirin. The sustained virological response at 12 weeks post-treatment (SVR12) rate was 92.7% (93.9% for SOF plus DCV and 91.7% for SOF plus SMV). No clinical or laboratorial factor was statistically associated with SVR. The most common adverse reactions were haematological events, nausea/vomiting, headache and asthenia. Out of 144 blood samples, 70 (48.6%) had detected RAS, 34.8% treated with SOF + DCV ± ribavirin and 61.3% SOF + SMV ± ribavirin. The resistance mutations against SMV were detected into NS3: substitutions G122S (28%), I170V (22.7%), Y56F (17.3%) and V132I (14.7%). The mutations against DCV R30Q (9.1%), P58H (6.1%) and Q62E (6.1%) were observed into NS5A, and for SOF the mutations A421V (10.6%), L159F (6.4%) and C316N (6.4%) were present inside NS5B viral protein. Four patients did not reach SVR, three of them presented viruses carrying RAS (1 treated with SOF + DCV and 2 with SOF + SMV). Some of these mutations, like R30Q (present in relapsing samples) and L159F, are well known by their influence on antiviral resistance, while others, like C316N, have a compensatory effect on viral fitness, maintaining these baseline RAS. *Conclusion:* The use of treatment regimens composed of SOF and DCV or SOF and SMV showed a high SVR rate, despite of a high rate of RAS, and a good tolerability profile in patients with HCV genotype 1. However, the high occurrence of baseline RAS observed in this casuistic is still a concern and studies like this show the necessity to understand how they are maintained in the population and to direct more efficiently the use of DAAs.

Introduction

Chronic infection of hepatitis C virus (HCV) affects about 71 million people worldwide, causing approximately 400,000 deaths every year due to complications like cirrhosis, liver failure and hepatocellular carcinoma [1]. In Brazil, the number of infected people is around 1.5 million, being the major cause of death among all viral hepatitis [2].

Until 2010, the pharmacological therapy for chronic hepatitis C (CHC) was based in the use of interferon what was associated with low effectiveness rates and significant side effects [3]. Advances on the virology field provided a better understanding on HCV life cycle and viral proteins function, allowing the development of specific drugs that could directly target these viral components, the direct acting antivirals (DAAs), with improved therapy success (response rates over 90%) and lower incidence of side effects [4—7].

The main objective of CHC treatment is to cure the infection or to achieve sustained virological response (SVR). SVR is defined as the non-detection of HCV RNA in the blood 12 (SVR12) or 24 weeks (SVR24) after the end of treatment [8]. The Brazilian guidelines has recommended the use of Sofosbuvir (SOF - NS5B polymerase inhibitor) plus Simeprevir (SMV - NS3/4A protease inhibitor) or Daclatasvir (DCV - NS5A inhibitor) for the treatment of HCV genotype 1 since 2015. These therapeutic regimens can also be associated with ribavirin (RBV) in some cases [2,9,10].

HCV is known to replicate in a high rate and this fact, associated with the lack of proofreading activity of the viral RNA polymerase NS5B, provides a high mutation rate that confers to HCV a high genetic variability. This variability can be observed inside the same host, where HCV can circulate as a pool of genetic related variants, with small differences among them, called quasispecies [11—13]. This characteristic, associated with the selective pressure exerted by the DAAs during treatment, can work as an important tool for viral resistance acquisition, selecting mutations that can lead to changes in the amino acid chain, modifying the viral protein and, consequently, preventing the interaction and inhibition by the DAAs. In some cases, these resistant variants can occur naturally, before the treatment with the antivirals [14—16].

Despite all the issues involving treatment and resistance acquisition and considering the incorporation of new antiviral treatments for CHC in Brazil, there are scarcity of studies that evaluate the success and safety of DAAs usage in this country. This way, this study aimed to evaluate the safety and effectiveness of treatment for HCV genotypes 1a and 1b infected patients with these DAAs, also analyzing the occurrence and prevalence of baseline resistance associated substitutions (RAS), observing the impact of these mutations into the treatment success.

Materials and methods

Study setting and ethical aspects

A prospective cohort study was performed at the University Hospital, Ribeirão Preto School of Medicine, University of

São Paulo, Brazil (HCFMRP-USP), a tertiary teaching institution linked to the Brazilian Public Health System. All the molecular experiments and analysis were performed at Genomic Studies Laboratory of the São Paulo State University (UNESP). This study was approved by the Research Ethics Committee of HCFMRP-USP with the approval number 1.398.168.

Patients

Patients older than 18 years chronically infected with HCV genotype 1 who started treatment from December 2015 to June 2017 were included. Following the Brazilian protocol for HCV treatment at the time the study was conducted, patients with advanced fibrosis (F3 or F4) or diagnosis of F2 for more than 3 years were included. In addition, patients with mild/moderate fibrosis could be treated when they had cryoglobulinemia, porphyria cutanea tarda, chronic kidney disease, post-liver transplant setting or non-hepatic solid organ transplant [17].
Patients with decompensated cirrhosis and/or prior non-responders to first generation of protease inhibitors (PI) based treatment received SOF (400 mg daily) plus DCV (60 mg daily) with or without RBV for 24 weeks. The remaining genotype 1 patients were treated with SOF plus DCV or SMV (150 mg daily) with or without RBV for 12 weeks [2]. Patients coinfected with Human Immunodeficiency Virus and/or hepatitis B virus were excluded. All the individuals included signed the Informed Consent Form.

Clinical data collection

Clinical and laboratory data were collected on three moments (before, during and after treatment) through access to the hospital computerized system. Regarding characterization of study population in the pretreatment period, the following information were collected: sociodemographic data (age, sex, race), anthropometric data (weight and height), besides clinical information concerning hypertension, diabetes mellitus, obesity, liver fibrosis staging, hepatic encephalopathy, ascites, esophageal varices, prior CHC treatment history (treatment-naïve patients treatment-experienced patients), prior CHC treatment response (relapsers non-responders), HCV subgenotype, RASs, length treatment, use of RBV. The results of the following laboratory tests performed before treatment were recorded: alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total serum bilirubin (TB), international normalized ratio (INR), creatinine and HCV viral load. Cirrhosis staging was performed according to Child-Turcotte-Pugh score and Model for End-Stage Liver Disease (MELD) score was also calculated.
The liver fibrosis stage was determined by biopsy (METAVIR score) or through non-invasive exams, point shear wave elastrography (pSWE) or transient elastography, considering the most recent result when two exams were available. Patients with esophageal varices, as cites and/or splenomegaly were considered as F4. The Body Mass Index (BMI) over 30 Kg/m^2 were considered obese [18].
During treatment, the safety of the therapy was analyzed by the incidence of adverse events. Anemia, neutropenia and thrombocytopenia where considered using the respective thresholds: serum hemoglobin levels < 13.9 g/dL (men) and < 12 g/dL (women), neutrophils < 1700/mm^3 and platelets < 130,000/mm^3.
To verify the therapy response, the HCV viral load was determined at the end of treatment and 12 weeks after the end. The viral load quantification was carried out by real-time polymerase chain reaction-based method, with limit of detection < 12 UI/mL.

Investigation of mutations prevalence

To evaluate the presence and prevalence of resistance associated mutations in the viral genome, serum samples from the patients included in the study were collected before the beginning of the DAAs treatment and viral targets were determined according to the treatment regimen for each individual. For those treated with SMV and SOF, NS3 and NS5B viral proteins were analyzed, while for those treated with DCV and SOF NS5A and NS5B were investigated.

RNA extraction and cDNA synthesis

The viral RNA was extracted from the serum samples using a TRIzol extraction protocol. In 1.5 ml tubes, for each sample, 250 µl of serum and 750 µl of TRIzol (Thermo Fisher Scientific) were added and homogenized, followed by 5 min incubation at room temperature. After that, the RNA separation, precipitation and elution were performed according to manufacturer's protocol. For the cDNA synthesis, the High Capacity cDNA Archive Kit (Life Technologies) was used following the manufacturer's guidance protocol.

Viral targets amplification

The viral genomic regions, correspondent to the DAAs' targeted viral proteins NS3, NS5A and NS5B were amplified by Nested PCR using the Long PCR Enzyme Mix (Thermo Fisher Scientific) following the manufacturer's instructions, with focus on the regions where the resistance associated mutations occur. Due to the genetic variability between subtypes 1a and 1b, specific primers were designed for each subtype (Supplementary Table 1). Since the mutations in NS5B are distributed within the entire protein, it was necessary to amplify the whole NS5B genomic region. For this, we performed two different Nested-PCR reactions, dividing NS5B into two different amplicons (NS5B-I and NS5B-II), corresponding to each half of the whole viral gene.

Sequencing analysis for resistance associated mutations

Once amplified, the PCR products were submitted to the sequencing reaction using the BigDye Terminator v3.1 (Life Technologies) and the internal primers of the Nested-PCR reaction. The sequences were obtained using the ABI 3130 XL Genetic Analyzer (Life Technologies) and then were aligned by the Clustal W method using the BioEdit 7.1.11 software [19,20], where resistance associated mutations were detected according to the comparison with reference

Table 1 Clinical and demographical characteristic of studied population according to the treatment scheme.

Variables	SOF + DCV ± RBV	SOF + SMV ± RBV	All treated
	(*n* = 130)	(*n* = 132)	(*n* = 262)
Age (years), mean ± SD	56.7 ± 9.3	56.6 ± 10.8	55.0 ± 10.0
Male sex, (%) *n*	83 (63.8)	69 (52.3)	152 (58.0)
Obesity, *n* (%)			
BMI < 30.0 Kg/m^2	65 (50.0)	46 (34.8)	111 (42.4)
BMI ≥ 30.0 Kg/m^2	30 (23.1)	26 (19.7)	56 (21.4)
Missing data	35 (26.9)	60 (45.5)	95 (36.3)
Diabetes mellitus, *n* (%)	41 (31.5)	43 (32.6)	84 (32.1)
Hypertension, *n* (%)	72 (55.4)	61 (46.2)	133 (50.8)
AST[a] (≥ 3 ULN), *n* (%)	18 (13.8)	23 (17.4)	41 (15.6)
ALT[b] (≥ 3 ULN), *n* (%)	20 (15.4)	28 (21.2)	48 (18.3)
Liver fibrosis stage, *n* (%)			
F0/F1/F2	20 (15.4)	33 (25.0)	53 (20.2)
F3	15 (11.5)	32 (24.2)	47 (17.9)
F4	95 (73.1)	67 (50.8)	162 (61.8)
Cirrhosis stage, *n* (%)			
Child-Pugh A	65 (68.4)	58 (86.6)	123 (75.9)
Child-Pugh B	26 (27.4)	3 (4.5)	29 (17.9)
Child-Pugh C	2 (2.1)	0 (0.0)	2 (1.2)
Missing data	2 (2.1)	6 (8.9)	8 (4.9)
MELD score, mean ± SD	10.1 ± 4.1	8.1 ± 2.4	9.1 ± 3.4
HCC, (%) *n*	10 (7.7)	7 (5.3)	17 (6.5)
Liver transplantation, *n* (%)	2 (1.5)	1 (0.8%)	3 (1.1)
Previous treatment history, *n* (%)			
Naïve patients	42 (32.3)	72 (54.5)	114 (43.5)
Experienced patients	88 (67.7)	60 (45.5)	148 (56.5)
Previous treatment response[c], *n* (%)			
Relapsers	35 (39.8)	24 (40.0)	59 (39.9)
Non-responders	53 (60.2)	34 (56.7)	87 (58.8)
Missing data	0 (0.0)	2 (3.3)	2 (1.4)
HCV subgenotype, *n* (%)			
1a	70 (53.8)	65 (49.2)	135 (51.5)
1b	51 (39.2)	53 (40.1)	104 (39.7)
Non-subgenotyped	9 (6.9)	14 (10.6)	23 (8.8)
HCV-RNA level (UI/mL)[d], *n* (%)			
< 800,000	72 (55.4)	64 (48.5)	136 (51.9)
≥ 800,000	58 (44.6)	68 (51.5)	126 (48.1)
RASs[e], *n* (%)	24 (34.8)	46 (61.3)	70 (48.6)
Treatment duration, *n* (%)			
12 weeks	63 (48.5)	132 (100.0)	195 (74.4)
24 weeks	67 (51.5)	0 (0.0)	67 (25.6)
Use of RBV, *n* (%)	109 (83.8)	62 (46.9)	171 (65.3)

ALT: alanine aminotransferase. AST: aspartate aminotransferase. BMI: body mass index. DCV: daclatasvir. HCC: hepatocellular carcinoma. HCV: hepatitis C vírus. MELD: Model for End-stage Liver Disease. RASs: Resistance-associated substitutions. RBV: ribavirin. SD: standard deviation. SMV: simeprevir. SOF: sofosbuvir. ULN: upper limit of normal.

[a] Number of patients that presented the ratio between the last AST value before treatment and the value of ULN 3. ≥

[b] Number of patients that presented the ratio between the last ALT value before treatment and the value of ULN 3. ≥

[c] For the percentage calculation, the total number of patients refers to the total of experimented patients (*n* = 88, *n* = 60, *n* = 148, respectively).

[d] Values refer to the last viral load performed before starting treatment.

[e] Percentages calculated in relation to the total of clinical samples which viral genome regions were amplified and analyzed (*n* = 69, *n* = 75, *n* = 144, respectively).

sequences H77 for genotype 1a (GenBank Accession number AF011753.1) and HCV-K1-R2 for genotype 1b (GenBank accession number D50481.1) [19].

Statistical analysis

The quantitative variables were shown as mean and standard deviation (SD) or median and interquartile range when the distribution was not normal. The qualitative variables were categorized and the absolute and relative frequencies were described. The chi-square test and the Odds Ratio (OR) calculation were proposed to verify the association between SVR and the categorized variables, the association between drug adverse reactions described with the used therapeutic regimen and to investigate the relation between before treatment mutations and variables related to the patient. To proceed with the analysis, global SVR results were considered and they were performed by the intention to treat. For all the association analysis, a 5% of significance level (α) was established and the tests were performed using the Statistical Package for Social Sciences software (SPSS Inc., Version 17.1.0).

Results

Relation among clinical variables, treatment response and RASs presence

Two hundred and sixty two patients were included, with predominance of male (58%) and Caucasian/white (79.4%). The main clinical and demographical characteristics of the studied population, according to the treatment regimen, are described in Table 1. Considering all patients enrolled in this study, the overall SVR rate was 94.3% (Fig. 1).

Regarding the endpoints cited in Fig. 1, SVR rates may range according to treatment responses in patients with missing data for the worst-case and best-case scenarios about SVR status. Considering patients treated with DCV+ SOF RBV, the study showed a SVR rate of 95.4% (worst-case scenario) that could achieve 98.5% (all patients with missing SVR data would have achieved SVR). In relation to the group treated with DCV + SMV RBV, our results indicated SVR rate of 93.2% (worst-case scenario) that could achieve 97.7% (best-case scenario). All relapsing patients (two who used DCV + SOF + RBV and three who used SMV + SOF + RBV) had liver cirrhosis. Of all the relapsing patients, three of them completed the prescribed therapy, being treated for 12 weeks, and the other two patients (one which used SMV and SOF and one which used DCV and SOF) had discontinued treatment due to adverse event. All patients that died during the treatment already presented decompensated cirrhosis before starting therapy. Concerning patients with hepatocellular carcinoma history before the beginning of treatment, 14 (82.4%) achieved SVR12. The other three had no treatment response data. All individuals that had liver transplantation history achieved SVR12. The SVR12 rates, according to selected clinical variables associated with therapy response and treatment regimen are described in Table 2.

Regarding the safety of the interferon-free therapies evaluated, we performed an association analysis between the occurrence of most common side effects and the different antiviral treatment regimens (Table 4).

When analyzing the possible association between SVR and baseline clinical data by the chi-square test, we did not identify a relation among all the analyzed variables and the SVR12 endpoint (Table 3). The same side effects listed on Table 4 were analyzed for their association with the use of RBV (regimens with RBV vs regimens with- out RBV). We detected evidences of the relation between RBV administration and incidence of anemia (P = 0.001), nausea/vomiting (P = 0.014), appetite loss (P = 0.028), asthenia/fatigue (P = 0.001) and skin reactions (P = 0.015).

Amplification efficiency

Since different genomic regions, from different subtypes, were amplified by Nested-PCR, different amplification efficiency was observed. For genotype 1a we had 100% of efficiency for all regions (NS3, NS5A and NS5B). Meanwhile, genotype 1b had different efficiency rates for each region, where amplification was efficient in 90.9% of NS3 samples and 84.6% of NS5A samples. For NS5B, due to the partitioned amplification, the observed efficiencies were 90.14% and 74.65% for first fragment (NS5B-I) and second fragment (NS5B-II), respectively.

Resistance associated substitutions prevalence in the population

Investigation of substitutions related to DAAs resistance acquisition was performed in samples from 144 patients included in this study. Seventy patients (48.6%) presented variants carrying mutations related to antiviral treatment failure. When comparing the data between the treatment groups, 24 patients (34.8%) who were treated with DCV + SOF presented variants previously associated with therapy evasion before starting treatment, while 46 patients (61.3%) of those receiving SMV + SOF were carrying resistant mutants (Table 5).

Seventy-six patients were infected with subtype 1a and 63 patients with subtype 1b. However, in five patients it was not possible to determine the subtype. Twenty five (32.9%) with genotype 1a presented viral variants carrying resistance associated substitutions. Mutations were present in NS3 region in 14.5% (11/25), 2.6% (2/25) on NS5A and 19.7% (15/25) on NS5B. Forty-one out 63 (65.1%) subtype 1b presented resistance mutations detected in viral variants, 26/63 (41.3%) presented NS3 substitutions, 3/63 (4.8%) in NS5A and 17/63 (27%) showed NS5B mutations related to therapy escape.

The most prevalent mutation was against SMV with the substitution G122S observed in 28% of the cases, followed by I170V (22.7%), Y56F (17.3%) and V132I (14.7%). For mutations against DCV, R30Q (9.1%) was the leading one and followed by P58H (6.1%) and Q62E (6.1%), while resistance mutations for SOF were headed by A421V (10.6%), the most recurrent one, L159F (6.4%) and C316N (6.4%).

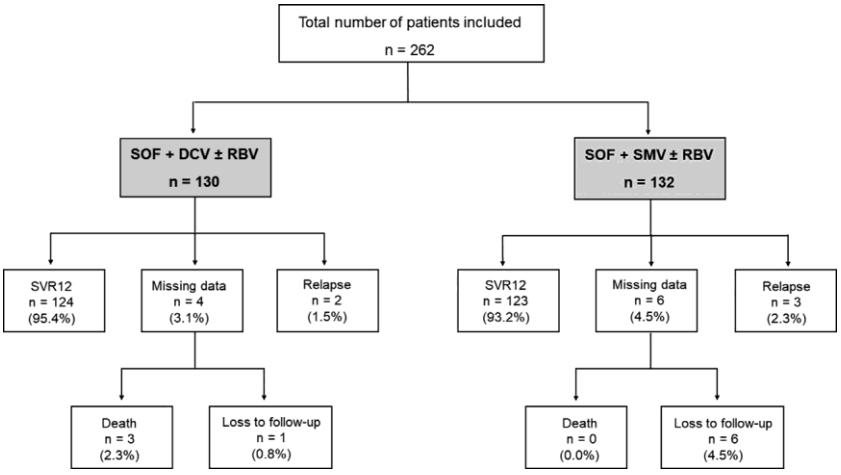

Figure 1 Clinical samples description and work flow. Treatment outcome for all included patients according to treatment scheme.

Relation between previous treatment, viral subtype and mutations occurrence

To evaluate the possible relation between viral subtype, previous treatment and occurrence of mutations, an association analysis was performed. According to the data, virus circulating in patients previously treated with first generation protease inhibitors were 20% more inclined to carry second generation DAAs RAS in NS3 than those who were not treated before (Table 6).

Regarding the relationship between viral subtype and the occurrence of mutations against SMV, DCV and SOF, HCV genotype 1b presented 20% more chances to carry RAS than genotype 1a, independent of previously treatment regimens (Table 6).

Discussion

Therapies based on DAAs usage are well known for their high SVR rate and low side effects occurrence, especially when compared to interferon based schemes, and here we describe a treatment effectiveness rate higher than 90%, corroborating to the observed in other genotype 1 studies with administration of SOF associated with DCV or SMV, with and without the addition of RBV [21—27]. The majority of the patients included had advanced liver fibrosis or even CHC complications as decompensated cirrhosis. Advanced liver fibrosis is considered the most important factor associated with treatment failure in the era of DAAs [26,28—30]. The characteristics of the population and the small number of non-responder/relapsers could explain the fact we did not find association between clinical and demographic variables and the response to treatment.

Regarding the therapy safety, some side effects were highly prevalent in both treatment groups, like nausea/vomiting, headache, asthenia/fatigue and skin reactions. The usage of RBV, according to our data, is possibly related with higher incidence of anemia, nausea/vomiting, appetite loss, asthenia/fatigue and skin reactions. These results consistently agree with data from literature and support the caution when prescribing RBV to patients with anemia or skin diseases history [25,26,30]. Two relapsing patients, from different therapy groups, did not complete the treatment due to adverse events. Both were treated with RBV and presented common side effects. The one treated with SOF + DCV +RBV presented asthenia, appetite loss and cough, while the other, treated with SOF + RMV + RBV presented asthenia and headache, with posterior anemia confirmation. Also, our data suggests that the combination SOF + DCV would be associated with nephrotoxicity and thrombocytopenia, but this result does not corroborate with published studies [26,27,31]. Additional studies evaluating the safety of DCV, SOF and SMV administration are necessary to confirm these results.

As observed in our results, for both subtypes, NS5A presented the low resistance mutation frequency when compared to other DAAs targets here investigated. This data differs from those published by Malta et al., where they found mutations inside NS5A in 14.6% of genotype 1a and 6% of genotype 1b infected patients. In our study, the frequency was 2.6% for 1a and 4.8% for 1b. The mutations that occurred in each case are also different, where for genotype 1a the substitutions Y93H and Q30H/R were present in only one sample (2.6%) while R30Q (15.15%) was the predominant in our genotype 1b samples. The most prevalent changes in their study were M28V and Q30H/R (14.6%) for genotype 1a and L31F/V and Y93H (6%) for 1b [32].

Our results regarding mutations inside NS5B are similar to another study from the literature, especially for genotype 1a. We observed the presence of resistance mutations in 19.7% of genotype 1a samples, close to the 20.1% described in literature. For genotype 1b, this number is a little more discrepant, 27% against 16.3% from the published work. The resistance associated mutations L159F, C316N and A421V were detected in both studies [33].

Since the aim of this study was to evaluate the frequency and prevalence of resistance associated mutations to the DAAs distributed by the Brazilian health system, it was important to understand how these mutations contribute to the antiviral escape. According to the literature, the most important NS3 mutations related to SMV failure are V36M, R155K/G/T, Q80K/R, S122R and D168A/E/H/V [34]. In our data, none of these mutations were identified. The most

Table 2 SVR12 rates according to demographic and clinical variables.

Variables	SOF + DCV ± RBV (*n* = 130)		SOF + SMV ± RBV (*n* = 132)	
	n (%)	SVR12, *n* (%)	*n* (%)	SVR12, *n* (%)
Age (years)				
< 55 years	59 (45.4)	56 (94.9)	57 (43.2)	54 (94.7)
≥ 55 years	71 (54.6)	66 (93.0)	75 (56.8)	69 (92.0)
Sex				
Male	83 (63.8)	78 (94.0)	69 (52.3)	65 (94.2)
Female	47 (36.2)	46 (98.0)	63 (47.7)	58 (92.1)
AST[a]				
< 3x ULN	112 (86.2)	106 (94.6)	109 (82.6)	103 (94.5)
≥ 3x ULN	18 (13.8)	18 (100.0)	23 (17.4)	20 (87.0)
ALT[b]				
< 3x ULN	110 (84.6)	104 (94.5)	104 (78.8)	96 (92.3)
≥ 3x ULN	20 (15.4)	20 (100.0)	28 (21.2)	27 (96.4)
Liver fibrosis				
F0/F1/F2/F3	35 (26.9)	35 (100.0)	65 (49.2)	63 (96.9)
F4 or cirrhosis	95 (73.1)	89 (93.7)	67 (50.8)	60 (89.6)
Previous treatment history				
Naïve patients	42 (32.3)	41 (97.6)	72 (54.5)	67 (93.1)
Experienced patients	88 (67.7)	83 (94.3)	60 (45.5)	56 (93.3)
Previous treatment response[c]				
Relapsers	35 (39.8)	33 (94.3)	24 (40.0)	24 (100.0)
Non-responders	53 (60.2)	50 (94.3)	34 (56.7)	30 (88.2)
Missing data	0 (0.0)	—	2 (3.3)	2 (100.0)
HCV subgenotype				
1a	70 (53.8)	64 (91.4)	65 (49.2)	60 (92.3)
1b	51 (39.2)	51 (100.0)	53 (40.2)	51 (96.2)
Non-subgenotyped	9 (6.9)	9 (100.0)	14 (10.6)	12 (85.7)
HCV-RNA level (UI/mL)				
< 800,000	72 (55.4)	69 (95.8)	64 (48.5)	60 (93.8)
≥ 800,000	58 (44.6)	55 (94.8)	68 (51.5)	63 (92.6)
RASs[d]				
Yes	24 (34.8)	23 (95.8)	46 (61.3)	44 (95.7)
No	45 (65.2)	44 (97.8)	29 (38.7)	29 (100.0)
Treatment duration				
24 weeks	66 (50.8)	65 (98.5)	0 (0.0)	—
12 weeks	59 (45.4)	58 (98.3)	131 (99.2)	123 (93.9)
Others[e]	5 (3.8)	1 (20.0)	1 (0.8)	0 (0.0)
Use of RBV				
With RBV	109 (83.8)	104 (95.4)	60 (45.5)	55 (91.7)
Without RBV	21 (16.2)	20 (95.2)	72 (54.5)	68 (94.4)

ALT: alanine aminotransferase. AST: aspartate aminotransferase. DCV: daclatasvir. HCV: hepatitis C vírus. RASs: Resistance-associated substitutions. RBV: ribavirin. SMV: simeprevir. SOF: sofosbuvir. SVR12: undetectable HCV-RNA 12 weeks after treatment completion. ULN: upper limit of normal.

[a] Values referred to ratio between the last AST before treatment value and ULN.

[b] Values referred to ratio between the last ALT before treatment value and ULN.

[c] For the percentage calculation, the total numbers of patients are referred to the total of experimented patients (*n* = 88 and *n* = 60, respectively).

[d] Percentages calculated in relation to the total of clinical samples where the viral genome regions were amplified and analyzed after blood collection (*n* = 69, *n* = 75, respectively).

[e] Patients that were treated in a shorter time in relation to the prescribed therapy according to the occurrence of adverse event, death or follow-up loss.

prevalent was G122S, followed by I170V, Y56F and V132I. This substitution in the position 122 from a Glycine (G) to a Serine (S) in our samples probably has no impact on treatment failure, once the Serine amino acid is considered the wild type in the work of Sorbo et al., [34]. Since the occurrence of a substitution is a random event and its fixation depends on the evolutionary forces acting on the genome, virus from different geographic regions present differences in positions that are not subjected to negative selection conferring population characteristics according

Table 3 Analysis of the association of demographic and clinical variables withSVR12.

Variable	OR (CI 95%)	*P*
Treatment regimen[a]	1.512 (0.248; 9.208)	0.654
Age	1.058 (0.968; 1.157)	0.215
Sex	0.917 (0.150; 5.584)	0.925
AST[b]	1.375 (0.150; 12.640)	0.778
ALT[c]	0.870 (0.099; 7.622)	0.900
Liver cirrhosis	0.254 (0.030; 2.145)	0.208
Previous treatment history	0.322 (0.035; 2.920)	0.314
Previous treatment response[d]	2.137 (0.217; 21.077)	0.515
HCV subgenotype	0.405 (0.042; 3.955)	0.437
HCV-RNA level (UI/mL)[e]	4.373 (0.482; 39.682)	0.190
RASs[f]	0.452 (0.040; 5.101)	0.607
Use of RBV	0.319 (0.038; 2.697)	0.294
Treatment duration[g]	0.943 (0.096; 9.226)	0.960

ALT: alanine aminotransferase. AST: aspartate aminotransferase. CI: confidence interval. DCV: daclatasvir. HCV: hepatitis C vírus. OR: odds ratio. RASs: resistance-associated substitutions. RBV: ribavirin. SVR12: undetectable HCV-RNA 12 weeks after treatment completion.

[a] DCV+SOF ± RBV vs SMV+SOF ± RBV.
[b] Ratio between the last value of before treatment AST exam and the upper limit of normality (<3.0 x ≥ 3.0).
[c] Ratio between the last value of before treatment ALT exam and the upper limit of normality (<3.0 x ≥ 3.0).
[d] Relapsing patients vs non-responder patients.
[e] <800,000 x ≥ 800,000 UI/mL.
[f] Presence of at least one RAS.
[g] 12 weeks treatment vs 24 weeks treatment.

Table 4 Analysis of the association between the most common adverse events according to treatment regimens adjusted by the use of RBV.

AE	SOF + DCV (*n* = 130)	SOF + SMV (*n* = 132)	OR (CI 95%)	*P*
Anemia	83 (63.8%)	51 (38.6%)	1.708 (0.984; 2.965)	0.060
Neutropenia	25 (19.2%)	13 (9.8%)	1.963 (0.916; 4.206)	0.080
Thrombocytopaenia[a]	50 (38.5%)	26 (19.7%)	2.164 (1.199; 3.906)	0.010
Abdominal pain	9 (6.9%)	6 (4.5%)	0.967 (0.322; 2.903)	0.950
Nausea/vomiting	24 (18.5%)	14 (10.6%)	1.319 (0.622; 2.797)	0.470
Diarrhea	14 (10.8%)	4 (3.0%)	2.818 (0.858; 9.257)	0.090
Appetite loss	15 (11.5%)	8 (6.1%)	1.311 (0.515; 3.338)	0.570
Headache	33 (25.4%)	29 (21.9%)	1.011 (0.547; 1.870)	0.970
Dizziness	7 (5.4%)	5 (3.7%)	1.081 (0.313; 3.727)	0.900
Asthenia/fadigue	41 (31.5%)	32 (24.2%)	0.857 (0.472; 1.558)	0.610
Psychiatric disorders[b]	7 (5.4%)	2 (1.5%)	2.716 (0.517; 14.278)	0.240
Myalgia	10 (7.7%)	7 (5.3%)	1.273 (0.438; 3.702)	0.660
Skin reactions	10 (7.7%)	15 (11.4%)	0.430 (0.178; 1.04)	0.060
Nephrotoxicity[c]	20 (15.4%)	7 (5.3%)	4.374 (1.621; 11.803)	< 0.001
Dry cough	10 (7.7%)	3 (2.3%)	3.465 (0.851; 14.109)	0.080
Xerostomia/disgeusia	7 (5.4%)	1 (0.8%)	6.025 (0.674; 53.862)	0.110

AE: adverse events. CI: confidence interval. DCV: daclatasvir. OR: odds ratio. RBV: ribavirin. SMV: simeprevir. SOF: sofosbuvir.

[a] For those patients who already showed thrombocytopenia before treatment, any decrease into platelets count compared to before treatment values were considered.

to sampling location, not necessarily related to therapy outcome [35]. Probably the substitution for Arginine (R) instead of Serine (S) or Glycine (G) is the responsible for resistance acquisition. The mutation Q80K is one of the most described but presents low resistance levels against SMV. In our results, this mutation was observed in only two clinical samples, from responding patients, corroborating with the low prevalence described for Q80K in HCV genotype 1 clinical samples from Brazil [36].

The substitutions M28A/T, Q30E/H/K/R, L31I/M/V, H58D and Y93C/H/N are the most described associated with resistance to DCV [34]. We were able to identify mutations like

Table 5 Prevalence of resistance associated mutations prevalence in genotype 1 (subtypes a and b) infected patients against Simeprevir, Daclatasvir and Sofosbuvir. Mutations are presented according to the antiviral regimen and viral proteins targeted by the DAAs (NS3, NS5A and NS5B).

	SOF+DCV (*n*=69)		SOF+SMV (*n*=75)	
	n	%	*n*	%
NS3 RAS				
T54I	—	—	1	1.3
T54S	—	—	2	2.7
V55A	—	—	2	2.7
V55I	—	—	2	2.7
Y56F	—	—	13	17.3
Q80K	—	—	2	2.7
Q80L	—	—	1	1.3
S122G	—	—	5	6.7
S122N[a]	—	—	2	2.7
G122S	—	—	21	28.0
G122T	—	—	2	2.7
G122N	—	—	2	2.7
V132I	—	—	11	14.7
V132V	—	—	1	1.3
I170V	—	—	17	22.7
NS5A RAS				
L28V	1	1.4	—	—
L28M	3	4.3	—	—
R30Q[a]	6	8.7	—	—
L31M	1	1.4	—	—
Q30L	1	1.4	—	—
H58P	1	1.4	—	—
P58H	4	5.8	—	—
P58S	1	1.4	—	—
P58T	1	1.4	—	—
Q62E	4	5.8	—	—
Y93H	2	2.9	—	—
NS5B RAS				
L159F	4	5.8	5	6.7
C316N	4	5.8	5	6.7
V321F	0	0.0	2	2.7
V321N	0	0.0	1	1.3
A421V[a]	5	7.2	10	13.3
Y448H	1	1.4	0	0.0
A553G	0	0.0	1	1.3
A553S	0	0.0	1	1.3
A553V	0	0.0	1	1.3
S556G	4	5.8	1	1.3
S556T	0	0.0	1	1.3

SOF: sofosbuvir; DCV: daclatasvir; SMV: simeprevir; RAS: Resistance associated substitution.

[a] RAS detected in viruses circulating into relapsing patients.

Table 6 Association analysis between previously treatment regimens, HCV viral subtype and presence of resistance asso- ciated mutations.

	OR	CI 95%	*P*
Previous TT with dual therapy	4.375	0.487; 39.265	0.219
Previous TT with PI[a]	0.973	0.059; 16.153	0.747
Viral subtype[b]	0.228	0.112; 0.466	0.001

OR: odds ratio; CI: confidence interval; TT: treatment; PI: protease inhibitor.

[a] For this analysis, only resistance-associated substitutions

L31M, H58P instead of H58D and Y93H, corroborating to the literature. However, the most prevalent ones were R30Q, P58H and Q62E. R30Q is just the opposite way from mutations at position 30 showed in the study from Sorbo et al. [34]. However, according to previous studies, in genotype 1b infected patients, R30Q confers resistance to DCV, and was detected together with P58S, a modification that could be acting as a compensatory mutation, reestablishing the viral fitness that could be altered when R30Q is present and enabling, its persistence in the population [37,38]. Interestingly, our results showed that R30Q was present in one of the genotype 1b relapsing patients, and was present together with P58H. These data could indicate that R30Q had an impact on antiviral treatment failure for this patient and that substitutions on site 58, regardless of the inserted amino acid, are possibly important for the resistance associated mutation persistence.

Considering the NS5A mutation L31M, Pawlotsky showed that, when tested *in vitro*, this mutation conferred high resistance rates in subgenomic replicon systems for all HCV genotypes, while Zhou et al. confirmed the relation with drug resistance and also described a compensatory effect of this mutation on viral fitness loss associated with substitutions at position 30 [39,40]. Despite the already described high resistance rates conferred against many NS5A inhibitors, the mutation Y93H was detected in low frequency in our study, only two patients, and both of them presented SVR [38].

In the case of SOF, the literature cites the mutations S282T, L159F and V321A as the most common in NS5B RNA polymerase [34]. In our results, the observed mutations, in order of recurrence, were A421V, L159F, C316N and V321N. The substitution S282T is known to confer, *in vitro*, high resistance rates against SOF due to the fact that this position is situated near the catalytic site [41—45]. In our study we did not find this mutation, and according to the literature it is really difficult to detect it in non-treated clinical samples, a situation that can be explained by the fact that this mutation leads to low viral fitness [46].

The mutation L159F is described to be structurally near to amino acid S282 (4 angstroms) of NS5B. Once S282 is near to the catalytic site and has influence on antiviral escape, L159F could interact with it as well, contributing to therapy failure [47]. An *in vitro* study showed that L159F confers high resistance to SOF, but is also associated with viral fitness decay when detected alone. However, when this mutation is followed by C316N, the viral fitness is recovered [48]. In our study, L159F and C316N were detected only in patients infected with subtype 1b and always occurred together in the same clinical sample, reinforcing this compensatory relation between them. Our data also corroborates with

other studies showing that this double mutation is highly frequent, especially in Brazilian samples (25% against 3.78%, 6.05% and 1.16% for North America, Europe and Asia, respectively) [33,49].

Many clinical aspects are important to determine the success or failure of the treatment, while other characteristics turn these associations more difficult to be done. We screened here personal and clinical aspects of the individuals included in the study willing to determine the relation among them and SVR. The majority of patients presented advanced fibrosis during treatment and, with the low number of relapsing or non-responding individuals, the association among some clinical aspects and SVR was not possible to be made. Despite these limitations, with our data we can observe that therapies based on the usage of SMV, DCV and SOF, for genotype 1, are more effective and safe than the previously treatments offered in Brazil, presenting high SVR rate and high tolerability by the patients.

Our main focus was the observation of mutations related to antiviral resistance and how they were circulating in the studied population prior to treatment. We detected a high occurrence of baseline RAS that are well known to confer high resistance rates, like R30Q in NS5A and L159F into NS5B. We also observed the high frequency of compensatory mutations that enable the maintenance of these RAS, like L31 M and C316N into NS5A and NS5B, respectively. The combination of more than one DAA during therapy, targeting more than one viral protein, showed to be an efficient way to avoid resistance provided by these baseline RAS. However, this simultaneous circulation could imply in an important impact on SVR rates in the future, especially due to the continuous use of these DAAs, acting as a selective pressure. More studies are necessary to evaluate the impact of baseline RAS in the failure of treatment and its consequences. This could also allow the improvement of antiviral treatment by choosing the more adequate therapy scheme.

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Disclosure of interest

The authors declare that they have no competing interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.dx.org/10.1016/j.clinre.2019.07.015.

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