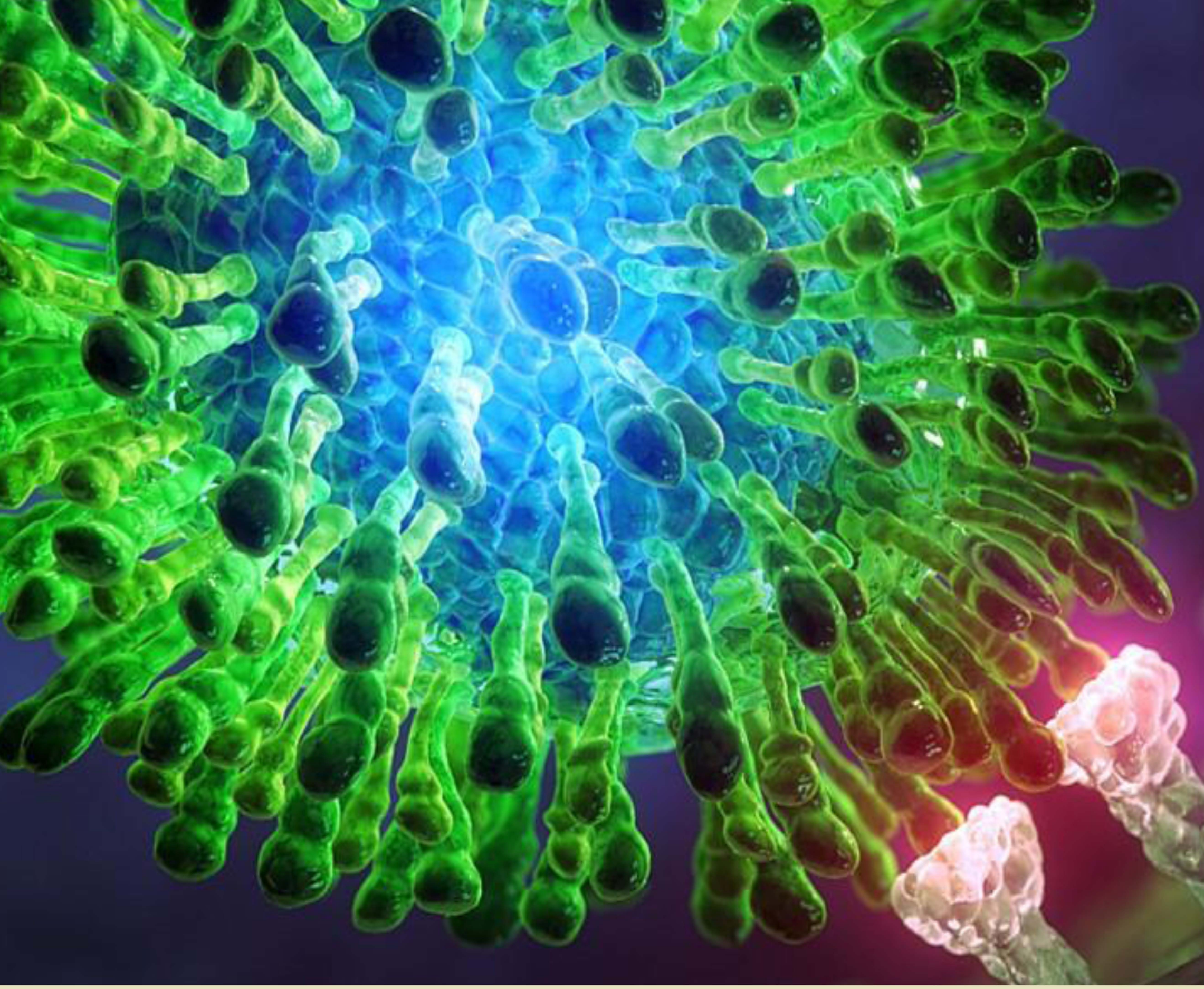

Pedro Miguel Batista Mendes

Hepatitis C Virus (HCV) infection: molecular study of immune response mediators

Master´s Degree in Biochemistry´s dissertation, supervised by António Martinho and Professor Paula Morais and presented to the Department of Life Sciences of University of Coimbra.

July 2016

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Universidade de Coimbra

Hepatitis C Virus (HCV) infection: molecular study of immune response mediators

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requesitos necrssários à obtenção do Grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor António Martinho (Instituto Português do Sangue e Transplantação de Coimbra, IPST, IP) e da Professora Doutora Paula Morais (Universidade de Coimbra)

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"What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on."

Jacques Yves Cousteau (1910-1997)

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General Index

Figures Index:

Tables Index:

Abbreviations:

µl – Microlitter

µM – Mililitter

A – Adenine

aa – Aminoacid

ActB2 – Actin B2

Ala – Alanine

AMV – Avian myeloblastosis virus

APC – Antigen presenting cell

ARFP – Alternative reading frame protein

Asp – Aspartate

BDC2A – Blood dendritic cell antigen2

C – Citosine

cDNA – Complementary DNA

CLDN - Claudine

CT – Cycle threshold

CTL – Citotoxic T lymphocyte

CXCL – Chemokine (C-X-C motif) ligand

Cys - Cysteine

D - Domain

DAA – Direct acting antiviral

dATP – Deoxyadenine triphosphate

DC – Dendritic cell

DCIR – Dendritic cell immunoreceptor

dCTP – Deoxycitosine triphosphate

ddATP – Dideoxyadenine triphosphate

ddCTP – Dideoxycitosine triphosphate

ddGTP – Dideoxyguanine triphosphate

ddNTP – Dideoxynucleotide triphosphate

ddTTP – Dideoxytimine triphosphate

dGTP – Deoxyguanine triphosphate

DNA – Deoxiribonucleic acid

dNTP – Deoxynucleotide triphosphate

dsRNA – Double-strand RNA

dTTP – Deoxytimine triphosphate

eIF – Eukariotic initiation factor

ER – Endoplasmic Rethiculum

FOXP3 – Forkhead box P3

G - Guanine

GAG - Glycosaminoglycan

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GAS – Gamma activated sequence elements

Glu – Glutamic acid

GTP – Guanine triphosphate

HCV – Hepatitis C virus

HDL – High density lipoprotein

Hist - Hystidine

HLA – Human lymphocyte antigen

HSC – Hepatic stellate cell

hVAP – Human vesicle-associated membrane protein-associated protein

HVR – Hyper variable region

I.S. – Internal standard

IFI27 – Interferon alpha induced protein 27

IFIT1 – Interferon induced protein with tetratricopeptide repeats 1

IFN-α – Interferon alpha

IFN-β – Interferon beta

IFN-γ – Interferon gamma

IFN-λ – Interferon lambda

IgG – Immunoglobulin G

IKK-ε – Inhibitor of nuclear factor kappa-B kinase subunit epsilon

IL - Interleukine

IRES – Internal ribosomal entry site

IRF – Interferon regulatory factor

ISDR – Interferon-α sensitive determining region

ISG – Interferon stimulated gene

ISGF – Interferon stimnulator factor

IU – International units

JAK – Janus kinase

K3EDTA – tri-potassium ethylenediaminetetracetic acid

KC – Kupffer cell

kDa - kilodalton

KIR – Killer immunoglobulin-like receptors

LDL – low density lipoprotein

LDL-R – low density lipoprotein receptor

LEL – Large extracellular loop

LSEC – Liver sinusoidal endothelial cell

L-SIGN – Liver/lymph node-specific intercellular adhesion molecule-3-grabing integrin

MAM – Mitochondrial- associated membrane

MAVS – Mitochondrial antiviral signaling protein

MDA – Melanoma differentiation antigen

MHC – Major histocompatibility complex

Min - minute

ml - mililitter

mM - milimolar

MMLV – Moloney murine leukemia virus

mRNA – messenger RNA

MyD88 – Myeloid differentiation primary response gene 88

nAb – Neutralizing antibody

NANBH – Non A and non B hepatitis

NCR – Natural cytotoxicity receptor

NK – Natural killer cell

NKR – Natural killer cell receptor

NKT – Natural killer T cell

NLP – Nucleocapsid like particle

NS – Non-structural

nt - Nucleotide

NTP – Nucleotide triphosphate

NVR – Non-virulogical response

OAS – 2´-5´-oligoadenylate synthetase

OCLN - Ocludin

ORF – Open reading frame

PAMP – Pathogen-associated molecular pattern

PBMC – Peripheral blood mononuclear cell

PCR – Polymerase chain reaction

PD-1 – Proggramed death 1

pDC – Plasmoytoid dendritic cell

pegIFN-α – Pegylated interferon alpha

PePHD – PKR-eIF2a phosphorylation homology domain

PI3K – Phosphoinositide 3-kinase

PIAS – Protein inhibitor of activated STAT1

PKR- RNA-dependent protein kinase

PP2Ac – Protein phosphatase 2A

PRR – Pattern recognition receptor

psk - Pseudoknot

qRT-PCR – Quantitative real-time polymerase chain reaction

RBV – Ribavirin

RdRp – RNA-dependent RNA-polymerase

RIG-I – Retinoic acid inducible gene I

RNA – Ribonucleic acid

rSSO – reverse sequence-specific oligonucleotide

RT – Reverse transcriptase

SAP – Shrimp alcaline phosphatase

SAPE – R-Phycoerythrin-conjugated Streptavidin

SEL – Small extracelular loop

Ser – Serine

SOCS – Suppressor of cytokine signaling

SR-B1 – Rceptor scavenger B type 1

ssRNA – Single strand RNA

STAT – Signal transducer and activator of transcription

SVR – Sustainable virulogical response

T – Timine

TBK-1 – NF receptor-associated factor family member-associated NF-kB activator-binding kinase-1

TGF-β1- Transforming growth factor beta-1

Th – T helper cell

Thr - threonine

TIM-3 – T cell Ig and mucin domain-containing molecule 3

TJ – Tight junction

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor-alpha

TRAF -

TRIF – Toll/interleukin-I receptor domain-containing adapter-inducing IFN-β

tRNA – transference RNA

U – Uracil

UTR – Untranslated region

Abstract:

Hepatitis C is a worldwide disease that targets over 150 million people. The cause of this global issue is the hepatitis C virus (HCV) and once the infection takes place, in most cases, it evolves into a chronic infection which can lead to major liver problems like cihrrosis and hepatocellular carcinoma. The virus main strength lies in its high genetic variability and its ability to trick and avoid the host´s immune response mechanisms. This makes effective treatment very difficult to accomplish. The standard therapy available consistes in a combination of pegylated interferon alpha (pegIFN-α) and ribavirin (RBV) and its success rates are variable. Many stratagies for the development of a vaccine are being studied but a definite and reliable vaccine is yet to be accomplished. Some newly developed strategies consisting in direct acting antivirals have been used with the standard therapy with increased success rates. However, this is still an expensive approach to fight HCV infections. This whole treatment process can be very demanding for the patients, both physicaly and financially, so if the response of the standard treatment could be predicted, then the patients could be given a more adequate and personal treatment with higher chance of success and less stress for both patients and hospitals.

This thesis´ work revolves around some appects of viral and human genetics that can be used for predicting patients´ response to pegIFN-α and RBV. The main objectives focus on (i) studying the genetic expression of some mediators of the human immune response before the beginning of treatment, (ii) studying the viral genetic variability at the 5´UTR and NS3 levels and (iii) analysing the patients´ HLA typing in contest of the treatment outcome to determine possible genetic predisposition.

Regarding the genetic expression, the genetic profiles are variable as some matched what was expected, i.e. the case of IFN stimulated genes being more expressed in patients who did not respond to treatment, before it begins, while others did not. Although some genetic variability was detected among the 5´UTR amplified regions, the lack of data was an impediment for a complete comparision with treatment outcomes. Regarding HLA typing, although interesting, did not allow for a significant correlation with the response to infection.

Keywords: Hepatitis C virus, pegylated interferon-α, ribavirin, immune response, genetic expression, genetic variability, HLA typing.

Resumo:

Hepatite C é uma doença a nível global que afecta mais de 150 milhões de pessoas. A causa para este problema global é o vírus da hepatite C (VHC) e na maior parte dos casos, quando a infecção se desencadeia esta desenvolve-se numa infecção crónica que pode levar a problemas de fígado graves, como cirrose ou carcinoma hepatocelular. A principal vantagem que o vírus apresenta é a sua elevada variabilidade genética e a sua capacidade de evitar os mecanismos de resposta imune do hospedeiro. Isto torna o desenvolvimento de um tratamento eficaz muito difícil de se conseguir. A terapia padrão disponível consiste numa combinação entre interferão alfa peguilado (pegIFN-α) e ribavirina (RBV) e apresenta taxas vaiáveis de sucesso. Têm sido estudadas várias estratégias para o desenvolvimento de uma vacina, contudo ainda se está por conseguir uma vacina eficaz e definitiva. Algumas estratégias mais recentes consistem na utilização de antivirais de acção directa juntamente com o tratamento padrão e que elevam as taxas de sucesso. Contudo, esta é ainda uma alternativa cara de combate à infecção por VHC. Todo este procedimento pode ser muito desgastante, quer fisicamente como financeiramente, para os pacientes, por isso se a sua resposta à terapia padão pudesse ser antecipada, poder-se-ia apresentar aos doentes um programa de tratamento mais adequado, eficaz e com menos desgaste tanto para doentes com hospitais.

O trabalho desta tese revolve em torno de alguns aspectos de genética humana e viral que podem ser predicativos da resposta ao pegIFN-α e RBV. Os princiais objectivos focam-se em (i) estudar a expressão genética de alguns mediadores de resposta imune antes de se iniciar tratamento, (ii) estudar a variabilidade genética do VHC ao nível da 5´ UTR e NS3 e (iii) analisar a tipagem HLA dos pacientes no contexto do tipo de resposta ao tratamento e determinar uma possível predisposição genética.

Na análise da expressão genética, os perfis observados são variáveis uma vez que alguns foram observados de acordo com o esperado, como é o caso dos genes estimulados por interferão que foram expressados num nível mais elevado, antes do tratamento, em pacientes que não responderam ao mesmo, enquanto outros não se

apresentaram da mesma forma. Apesar de terem sido dtectada alguma variabilidade genética entre as regiões amplificadas de 5´UTR, a falta de informação não permitiu uma correlação com o resultado do tratamento da infecção. No que respeita à tipagem HLA, apesar de interessante não permitiu uma correlação significativa com a resposta à infecção.

Palavras-chave: vírus da hepatice C, interferão-α peguilado, ribavirina, resposta imune, expressão genética, variabilidade genética, tipagem HLA.

Chapter 1:

Introduction

A. Hepatitis C:

Hepatitis C is a blood-borne liver disease caused by the hepatitis C virus (HCV)[1]. Previously known as non-A, non-B hepatitis (NANBH), its agent, hepatitis C virus, was first identified in 1989 when its genome was isolated from the plasma of infected chimpamzees[2] and its infectious role in causing non-A, non-B hepatitis was confirmed, also in 1989, in a HCV antiboby assay sensitive and specific for NANBH[3].

Hepatitis C is a worldwide disease. It is estimate that 130-150 million people are chronically infected with HCV, being Africa and Central and East Asia the most affected regions. HCV can inflict both acute and chronic infections. Although its severity can range from mild illness, a few weeks-lasting, to a lifelong problem and in worst cases, infected patients will develop liver cirrhosis and cancer, being that hepatitis C-related liver diseases kill approximately 700 000 people each year. Despite being asymptomatic, acute infections are spontaneously cleared in 15-45% of infected patients in the first 6 months of infection. The chronic form of infection will develop in the remaining 55-85% of patients. Of the later, 15-30% are at risk of developing cirrhosis.[1]

Being a blood-borne virus, HCV is usually transmitted through per-coetaneous exposure to infected blood and/or blood products. The most common risk factors to transmission of HCV are unsafe injection practices using shared injection equipment; transfusions using unscreened infected blood/blood products and reused or inadequately sterilised medical material. Less common, is HCV´s transmission through sexual practices or from mother to child. However, the virus does not spread through food, water, breast feeding or by any casual contact with infected people.[1]

B. Hepatitis C Virus (HCV):

a) The virus:

The Hepatitis C virus is an enveloped *hepacivirus* from the *Flaviviridea* family. It has a positively charged single-stranded RNA chain with approximately 9.6kb[2,4,5],

encoding a polyprotein with about 3011aa[6]. Inside its envelope is the nucleocapsid, enclosuring the RNA genome, with various copies of one small protein[7].

The N-terminal of the open reading frame (ORF) is home to the structural proteins (core, E1 and E2) and in the remaining ORF we find the non-structural proteins (p7, NS2, NS3, NS4A/B, and NS5A/B). The ORF is flanked by 2 highly conservative regions, the 5′-untranslated region (5′-UTR) and 3′-untranslated region (3′-UTR) (Figure 1).[7]

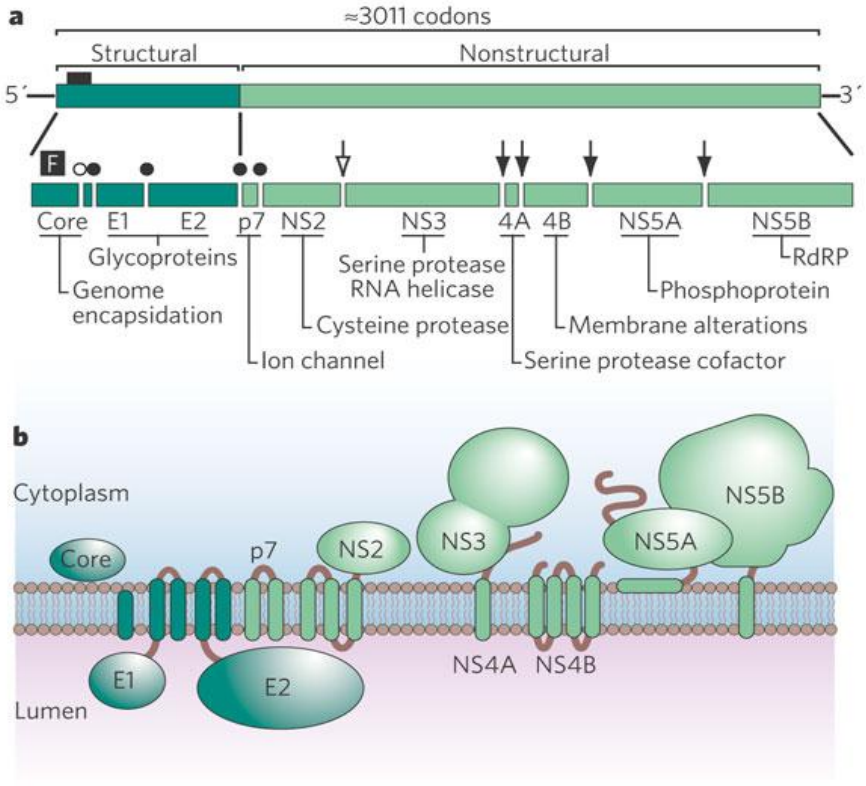


Figure 1: HCV genome and viral proteins. a) HCV viral gemone´s structure with structural and non-structural genes and corresponding proteins constitutive of the viral polyprotein, as well as cleaving sites. White circle indicate the cellular signal peptide peptidase cleaving site, while black circles show the cellular signal peptidase cleaving sites. White arrow refers to NS2/3 protease cleaving site, black arrows show NS3/4A protease cleaving sites; b) HCV protein organization in a cellular membrane.[6]

The HCV population is a genetically heterogeneous one due to its RNA polymerase lack of proof reading ability. This leads to the appearance of mutations which are the source of genotype diversification and the existence of quasispecies. Quasispecies are a mixture of distinct, however genetically related, viral subpopulations within a viral population in a single infected subject[8]. In this way there are 6 major HCV genotypes known worldwide and various subtypes[7]. In Europe, the genotype distribution is similar to the one in Brazil where the 1b genotype is the most frequent of them all[9]. In Portugal, genotypes 1a, 1b and 3a are the most common.[5,10]

b) 5´-UTR and 3´-UTR:

The hepatitis C virus´s genome is flanked by two highly conserved regions at the 5´ and 3´ ends of the genome, 5´-untranslated region and 3´-untranslated region respectively. This conservation, in contrast to the high degree of variability of the remaining genome, is indicative of importance to the viral lifecycle.

i. 5´-UTR:

The 5´-untranslated region (5´-UTR) is an uncapped and highly conserved 341 nucleotides (nts) sequence located at the 5´ end of the HCV´s genome[11]. It is the most conserved region in the entire HCV genome, with over 90% conservation between HCV genotypes[12], and it is composed of 4 domains (I, II, III and IV), with several stem loops and a pseudoknot (Figure 2). Domain I comprehends the fist 40nts of the 5´UTR but although it participates in RNA replication it is not essential for RNA translation[12]. The same cannot be said about the remaining domains. Domain II has several loops within its stem structure. As for domain III, it connects with domain II through the pseudoknot and is base-paired with the sequence immediately upstream of domain IV[11]. Finally, domain IV has a small stem loop where the initiation codon AUG is located[12]. The domains II, III and IV, in addition to the first 12 to 30nts of the core encoding gene, form the internal ribosome entry site (IRES). The IRES binds to the 40S ribosomal subunit, through domain III´s affinity to 40S[11], promoting the 1st step of the polyprotein´s translation, therefore being essential for the viral lifecycle[7].

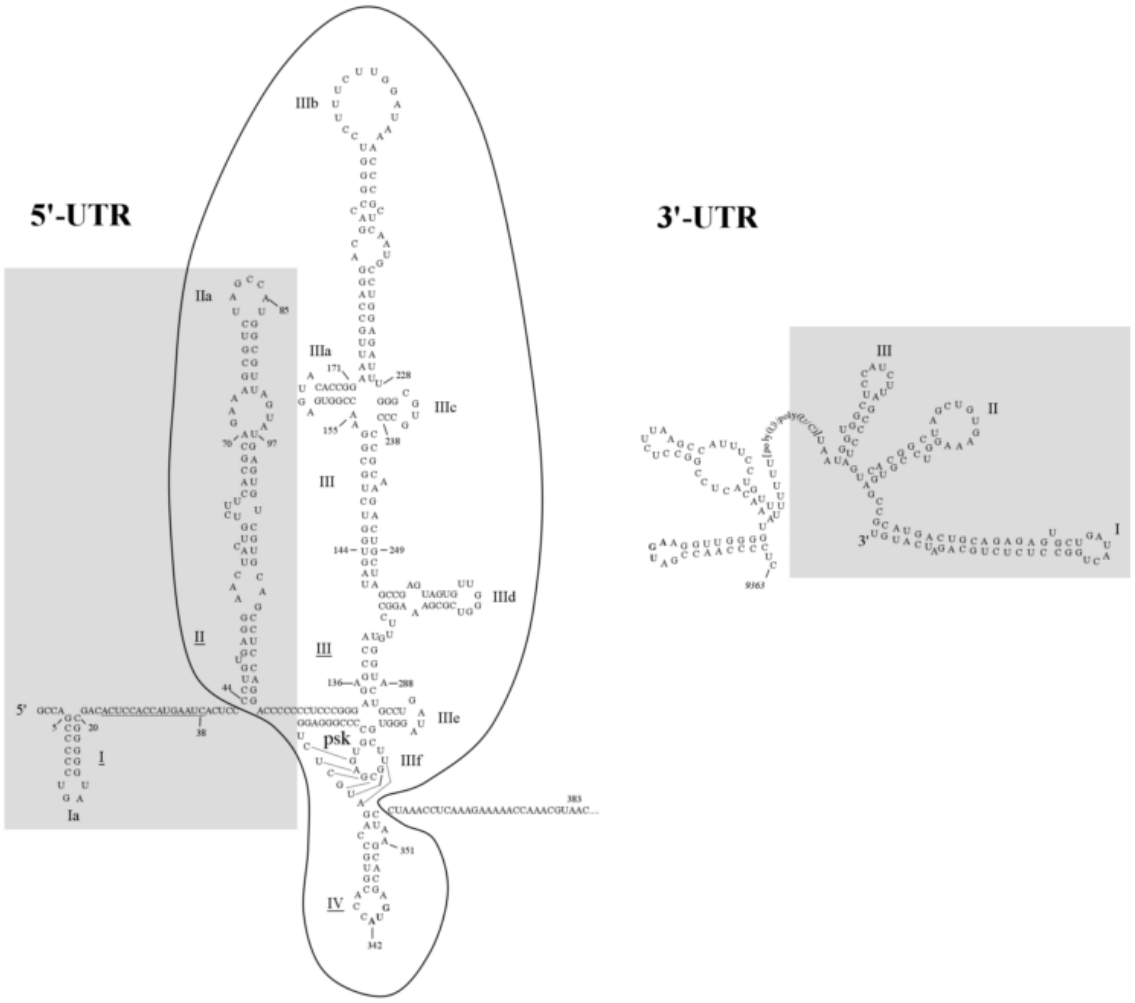


Figure 2: 5´-UTR and 3´-UTR regions of the HCV´s genome, with subregions and domains. In the 5´-UTR image, signaled by the closed line, is the IRES region. The psk designaton represents the pseudoknot. The shaded boxes represent 5´UTR and 3´UTR RNA elements possibly involved in RNA replication. The 342 and 9375 nucleotides, bold characters, represent the start and end codons respectively.[12]

ii. 3´-UTR:

On the other end of the viral genome is the 3´-untranslated region (3´-UTR). The 3´-UTR has a variable length, usually being around 225nts, and can be divided into three regions. First, there is the variable region, with 30-40nts, then, a long poly (U) and poly (UC) track, and finally, a highly conserved 98nts 3´-terminal stretch, also called 3´X region, with 3 stem loops[13] (Fig. 2). The 3´-UTR interacts with the NS5B´s RNA-

dependent RNA-polymerase (RdRp) and the two stem loop structures at the 3´end of the coding sequence. Both the 3´X region and the 52 upstream nucleotides of the poly (U/C) tract are essential for RNA replication. As for the remaining of the 3´-UTR sequence, it has the purpose of enhancing the viral replication.[7]

c) Structural proteins:

As said before, the N-terminal of the ORF is composed by the genetic sequences of the structural proteins core, E1 and E2. These proteins will be the structure of the viral nucleocapsid and envelope.

i. Core:

At the beginning of the polyprotein is the core protein. This RNA binding protein is responsible for constructing the viral nucleocapsid.[14] Its mature form is a 21kDa (P21) protein and has three distinct domains.[7] The domain 1 (D1) is located at the hydrophilic core´s N-terminal, about 120aa, and is responsible for RNA binding and nuclear localization. Next is domain 2 (D2), located at the hydrophobic C-terminal, around 50aa, and its function is to mediate core´s association with the lipid droplets, endoplasmic reticulum´s (ER) and outer mitochondria´s membranes. Around the very last 20aa is domain 3 (D3), which forms the signal peptide for envelope protein E1.[7,14] The core protein exists as a dimeric or multimeric forms and has a tryptophan rich sequence at D1´s 82-102aa which is suggested to allow P21 to interact with itself creating, *in vitro*, nucleocapsid like particles (NLPs), with a critical role in capsid assembly, in a cell free system. In a bacterial system, the 75 N-terminal residues are sufficient to NLP assembly. The core protein is suspected of interacting with host´s cellular pathways. Its influence affects pro- and anti-apoptotic functions, hepatocyte growth in Huh-7 cell lines, progression of tissue injury and fibrosis, regulation of c-myc and c-fos activity, alteration of viral promoters´ transcription, induction of hepatocellular carcinoma and lipid droplets´ formation and steatosis formation.[7]

In the core´s N-terminal encoding region it is possible to find a -2/+1 ribosomal frameshift, called frameshift protein (F or ARFP), which can be produced during infection and have an influence in viral persistence.[7]

ii. E1:

Envelope glycoprotein 1 (E1) is one of the two proteins constitutive of the virion envelope and responsible for viral entry and fusion. E1 is a 33-35kDa type I transmembrane glycoprotein with a non-covalent heterodimer conformation. It has a 160aa N-terminal ectodomain composed of several proline and cysteine residues but no intramolecular disulfide bonds. At the C-terminal it has the transmembrane domain, 30aa, with 2 streches of hydrophobic aa set apart by a polar region of conserved charged aa. The C-terminal is responsible for membrane anchoring, ER localization, and heterodimer assembly. This protein is highly glycosylated, with 5 glycosylation sites. And it is also suggested to intervene in intra-cytoplasmic virus-membrane fusion.[7]

iii. E2:

Envelope glycoprotein 2 (E2) is the other type I transmembrane glycoprotein that works to form the HCV virion and enable viral entry and fusion. E2 is a 70-72kDa glycoprotein, which like E1, is formed by non-covalent heterodimers. It also has an N-terminal ectodomain, of 334aa, with several cysteine and proline residues, zero intramolecular disulphide bonds and a 30aa C-terminal transmembrane domain with a conserved charged aa polar region between 2 streches of hydrophobic aa. E2´s C-terminal also participates in membrane anchoring, ER localization and heterodimer assembly.[7] E2 has a higher level of glycosylation than E1, with 11 glycosylation sites. It is the glycans, from E1 and E2, which promote E1E2 complex formation and viral envelope glycoprotein folding.[14] E2 is also characterized by the presence of two hyper-variable regions (HVR1 and HVR2), which differ in 80% between HCV genotypes and subtypes within a single genotype. HVR1 is a 27aa neutralizing epitope with conserved physiochemical properties of its residues and overall conformation, suggesting an important role in HCV´s lifecycle. This protein is also important for viral attachment and infection through its positively charged residues that interact with the negatively

charged molecules of the cell surface. HVR1 also interplays with HDL and scavenger receptor B type 1 (SR-B1), facilitating infection of Huh7 cells by HCV pseudoparticles.[7]

d) Non-structural proteins:

i. p7:

Protein p7 is a 63aa integral membrane polypeptide with 2 transmembrane domains of α-helices connected by a cytoplasmic loop and located in the ER. Its cleavage from the polyprotein is of the responsibility of the host cell´s ER signal peptidases.[14] Deletions or mutations in the cytoplasmic loop result in suppressed infectivity of intra-liver HCV´s cDNA transfection in chimpanzees studies. This backs the idea of p7´s importance to HCV´s lifecycle. *In vitro*, p7 has shown to be of the viroporin family and act as a calcium ion channel.[7]

ii. NS2:

The non-structural protein 2 (NS2) is a 21-23kDa non-glycosylated transmembrane protein. This protein is responsible for ER membrane association through its 2 internal signal sequences.[7] Together with NS3´s N-terminal domain, its C-terminal forms the NS2/3 auto protease, which is a zinc-dependent metalloprotease responsible for cleaving the NS2-NS3 site of the polyprotein, therefore being essential for viral replication[14,15]. From this, NS2 loses its protease activity and suffers a phosphorylation-dependent degradation by the proteasome through the protein kinase casein kinase 2. NS2 can also interact with the host´s cell proteins affecting, liver and non-liver-specific promoters´ and enhancers´ controlled, reporter genes. Despite these interactions, its influence on the viral lifecycle is not clear.[7]

iii. NS3:

The nonstructural protein 3 (NS3) is divided into 2 domains and has a multifunctional activity. At the N-terminal (1st third of the protein) it is the serine

protease domain and at remaining two thirds (C-terminal) it is the helicase/NTPase domain.[7,14]

It forms the NS3/4A protease, alongside NS4A protein, which has the function of cleaving the polyprotein at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions. This cleavage happens at the NS3 serine protease domain, in its catalytic triad formed by the Hist57 (1083 in the polyprotein), Asp81 (1107 in the polyprotein) and Ser139 (1165 in the polyprotein) residues.[7,14,16] This matches the fact that the first 211aa of NS3 are sufficient for NS4A-NS5B processing[17]. The substitution of His and Ser by alanine will cancel its cleaving activity without compromising NS3´s structure[14]. NS3 is also known to interfere with the host´s immune response. To this end, NS3 acts to antagonize of the double-stranded RNA-dependent IRF3 signaling pathway and to prevent the dsRNA signaling through TLR3. With this it is possible to say that NS3 blocks the RIG-I signaling pathway.[7,18]

As for the helicase/NTPase domain of NS3, it has 442aa, is located at the NS3´s C-terminal and is part of the helicase superfamily-2. Its functions vary from RNA-stimulated NTPase activity to unwinding of RNA regions. In the latter, the NS3 helicase/NTPase acts over RNA regions with extensive secondary structure through coupling unwinding and NTP hydrolysis. Its activity is controlled by the previously mentioned protease domain and by the NS5B RdRp.[7]

iv. NS4A:

The nonstructural protein 4A (NS4A), 54aa protein, is a cofactor of the NS3 protein. Its central amino acids (21-30aa) allow the NS3/4A protease stabilization, ER membrane localization and cleavage-dependent activation, especially for the NS4B/5A junction.[7] Its highly hydrophobic N-terminal is involved in the ER targeting by NS3. And the very last amino acids of the C-terminal form the transmembrane helix that anchors the NS3/4A complex to the ER membrane.[14]

v. NS4B:

This 27kDa integral membrane protein, co-localized alongside other NS proteins in the ER membrane, has 4 transmembrane domains and an N-terminal amphipathic helix for membrane association. The NS4B has several functions and properties. It is responsible for being a membrane anchor for the replication complex, inducing the formation of the membranous web where the replication complex is formed, through inducing the morphological change of ER. It also inhibits cell synthesis, modulates NS5B RdRp activity, amongst others.[7,14]

vi. NS5A:

NS5A is a phosphorylated 56-58kDa zinc metalloprotein. Its N-terminal, composed of the 1st 30aa, is an amphipathic α-helix responsible for membrane localization in perinuclear membranes and for assembly of the replication complex.[7] Its importance is backed up by the fact that mutation in this helix will interrupt membrane association and disable the formation of replicon-harbouring cells.[14] The remaining protein is comprised of 3 domains, I, II and III, being the domain I the most noteworthy. The Dominion I, located downstream of the amphipathic α-helix, has a zinc-coordination motif, composed of four cysteine residues, important for structural integrity. The inhibition and abolishment of the HCV´s replicon RNA replication, due to mutations in NS5A or alterations of its zinc-binding site, respectively, is proof of the NS5A´s importance to HCV´s lifecycle.[7,14]

The NS5A protein participates in the formation of the replication complex in connection with lipid rafts from intracellular membrane, by binding to the C-terminal region of a vesicle-associated membrane associated protein (33kDa), hVAP33. The phosphorylation level of NS5A is also important for the viral lifecycle as its hyperphosphorylation results in an assembly incompetent state of the replication complex. NS5A also interacts with NS5B, being a modulator of RdRp, in a non-clear way, and with geranylgeranyated cellular protein, which is required for the assembly of the replication complex.[7]

NS5A also interacts with IFN-α´s response by means of its IFN-α sensitivity-determining region (ISDR). This region interacts with PKR protein kinase, an antiviral effector of IFN-α, inhibiting it by binding to it.

vii. NS5B:

The last of the non-structural proteins is the NS5B. This 65kDa protein is a membrane protein, being a tail-anchored protein with a RNA-dependent RNA polymerase function. The α-helical transmembrane domain of the C-terminal, composed of 21 residues, is responsible for the pos-translational targeting of the cytosolic side of the ER. The N-terminal of NS5B, 530aa, has a "right hand" polymerase shape with a "finger, palm and thumb" structure and it is the finger/thumb interaction which completely encircles the catalytic site of NS5B where the synthesis of HCV´s positive and negative strand RNAs takes place.[7]

The NS5B protein can also interact with cellular components like the hVAP-33 protein, where the C-terminus of the 1st interacts with N-terminus of the 2nd to take part of the HCV´s replication complex´s formation. NS5B also binds to cyclophilin B, a cellular peptidyl-prolyl cys-trans isomerase that modulates the RNA binding ability of NS5B, regulating HCV´s replication.[7]

e) HCV´s viral lifecycle:

The HCV life cycle begins with its attachment to the target cells and entry through endocytosis. Upon entry the virus undergoes replication and translation followed by post-translation processings and viral assembly. When the newly assembled virus is ready, it is released to the extracellular environment. This is illustrated as a hypothetical HCV life cycle in Figure 3.[7]

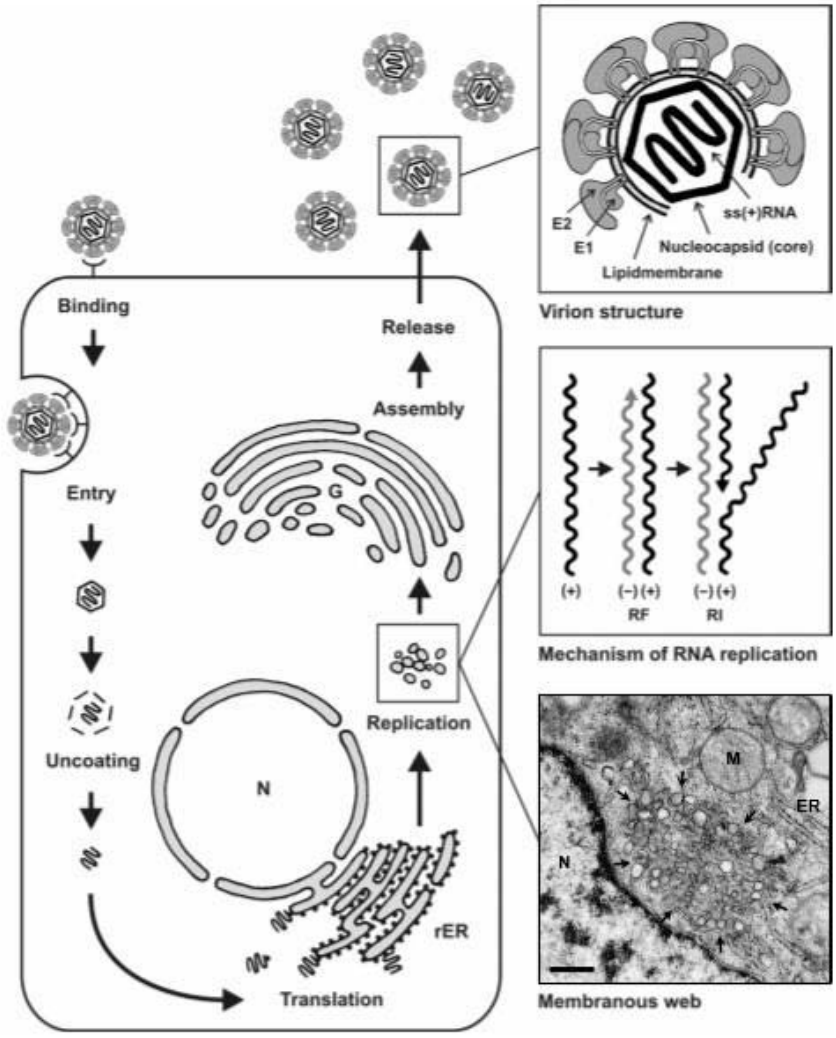


Figure 3: Suggested HCV life cycle. The virus stars by attaching to the target cell and through several interactions with cellular surface receptors, like CD81, SR-B1, LDL-R, OCLD and CLDN1, it manages to enter the cell. Once inside its nucleocapsid is disintegrated and the genomic RNA exposed to the cytoplasm. Next, it is directed to the ER where it undergoes translation and replication. New viral particles are assembled and released to the extracellular environment.[7]

Cell attachment and entry occur due to viral interaction with several putative receptor molecules located at cell surface such as glycosaminoglycans (GAGs), tetraspanin CD81, scavenger receptor class B type 1 (SR-B1), claudin1 (CLDN1), ocludin (OCLN), low-density lipoprotein (LDL) receptor (LDL-R) and others.[5,7,14,19–22]

First, there are interactions between cell glycosaminoglyans and viral lipoproteins as well as between viral particles associated with LDLs and LDL-Rs,[5,19,20] which facilitates viral attachment to the target cells[14]. Following the initial attachment, the HCV interacts with both CD81 and SR-B1 through the envelope glycoprotein E2. From CD81´s 4 hydrophobic transmembrane regions and 2 extracellular loop domains, small extracellular loop (SEL) and large extracellular loop (LEL), it is the LEL domain that is crucial to interact with HCV´s E2.[5,7,21–23] As for SR-B1 it has a large, highly glycosylated extracellular loop, 2 short cytoplasmic domains and 2 transmembrane domains. SR-B1 is also a low-density lipoprotein receptor due to its multiligand receptor characteristic[5,20,24] and it is suggested that is the extracellular loop that interacts with the HVR1 of E2[7]. Later in this process, the attached virus is led to the *tight junctions* (TJ), where it comes in contact with claudin 1 (CLDN1), where residues responsible for HCV entry were detected, and ocludin (OCLN)[5,25–27]. The process of cell entry by the HCV is pH- and endocytosis-dependent[7], being mediated by clatrin with membrane fusion occurring in the endosomes[5,20,28]. The acidic environment of the endosomes leads to conformational changes of the E proteins and consequently membrane fusion[5,20,29].

Once the virus is inside the cell the genetic material must be released from the nucleocapsid. After decapsidation the genomic HCV RNA (+ sense), released into the cytoplasm, is used for polyprotein translation and replication.

Starting with translation, being of positive sense, the RNA can act like mRNA and be immediately translated in a cap-independent process.[14] The viral polyprotein translation occurs in the rough endoplasmic reticulum and is mediated by the IRES, which recruits cell proteins like eukaryotic initiation factors eIF2 and eIF3 as well as viral proteins. The IRES is also involved in the formation of translation initiation complexes 40S, 48S and 80S. In the beginning it binds directly to ribosomal subunit 40S to form a stable pre-initiation complex, through 5´UTR´s domain II and basal part of III (except stem-loop IIIb)[12], which with the assembly of eIF3, eIF2, GTP and the initiator tRNA forms the 48S complex. The initiator tRNA is set at the position P of the 40S complex in order to base-pair with the mRNA´s start codon. The GTP suffers hydrolysis leading the eIF2 to dissociate from the complex after releasing the initiator tRNA. The 2nd GTP hydrolysis plus the interaction with eIF5B brings into association

the ribosomal 60S subunit forming the functional 80S translational complex. Viral protein synthesis proceeds from here.[5,7,30–37]

With the HCV precursor polyprotein synthesized, it undertakes post-translational processing in order to produce structural and non-structural viral proteins.[14] Between the core and E1 sequences there is an internal signal sequence, which targets the polyprotein to the ER membrane where the E1´s ectodomain is translocated to the ER lumen. Here, the cellular signal peptidase cleaves the signal sequence releasing core P23 (immature form). Core´s immature form is further processed by the cellular signal peptide peptidase, being cleaved into core P21 (mature form). The signal peptidase is also responsible for cleaving the E1-E2, E2-P7 and P7-NS2 junctions. As for E1 and E2, their maturation involves N-glycosilation and alteration of conformation with formation of E1E2 heterodimers. There is also the NS2-NS3 *cis*-cleavage by the zinc-dependent NS2/3 auto-protease. Then, NS3 plus the NS4A as a cofactor assemble the NS3/4A protease. This enzyme recognizes the Asp/GluXXXXCys/Thr-Ser/Ala sequence and cleaves NS3-NS4A in a *cis*-conformation, downstream of the threonine residue. It also performs the *trans*-cleavage of NS4A-NS4B, NS4B-NS5A and NS5A-NS5B by cleaving downstream of the cysteine residue.[5–7,38,39]

The next stage is viral replication. This step occurs in the membranous web, results from NS4´s mediated rearrangement of ER membranes[14], is fatty acids and cholesterol rich and it is overall a complex of membrane-associated multiproteins containing all viral NS proteins, except NS2[12,17]. It is in this structure that the replication complex is formed by the association of viral proteins, cellular components and emerging RNA strands. Its formation also has the involvement of lipid rafts through protein/protein interactions of hVAP-33 and both NS5 proteins.[7] HCV replication *per se* is a 2 step semi-conservative and asymmetric process mediated by the NS5B RNA-dependant RNA polymerase. In the 1st step, we have the synthesis of a (-) RNA strand from the template HCV´s (+) RNA strand. This new strand will act as a template for the 2nd step where (+) RNA strands are synthesized[7,14] in a 5-10 fold way per template[12]. The new strands will be sent to polyprotein synthesis, where they will serve as intermediates for more RNA replication, or will be packaged into new viral particles.

Finally, the last stage in HCV´s lifecycle is the assembly of new virions and their release into the pericellular space via exocytosis.[14] It is reported that the two clusters of

basic residues from core´s N-terminus and genomic viral RNA are sufficient for self-assembly of the capsid and that are the interactions between the RNA and core´s nucleotides 23-41 and domains I and III that initiate particle formation. Viral assembly takes place in the ER through association between E1 and E2 transmembrane domains and ER membranes.[7] The presence of structural proteins in ER and Golgi apparatus indicate a role in particle envelopment and maturation before the new virions are released by the secretory pathway.[7,14]

C. Immune response to HCV:

a) Innate immune response:

After the viral entrance in the hepatocytes, the 1st line of defence is the innate immunity (Figure 4) and consists in a cascade of intracellular events leading to an antiviral state of the infected cell.[5,40] There are conserved and specific HCV regions in the viral genome, called pathogen-associated molecular patterns (PAMP), that are recognised by germline-encoded pattern-recognition receptors (PRRs) such as the retinoic acid inducible gene I (RIG-I), melanoma differentiation antigen 5 (MDA5) and the toll-like receptor 3 (TLR3), triggering a response by the host. RIG-I and MDA5 recognise the polyuridine motifs of the 3´UTR region of the viral genome within the cytoplasm, acting like cytoplasmic viral sensors, cytosolic pathway.[41,42] This PAMP/PRR binding recruits MAVS protein located in the mitochondria and the peroxisomes. This protein binds to the endoplasmic reticulum through the mithochondrial-associated membrane (MAM).

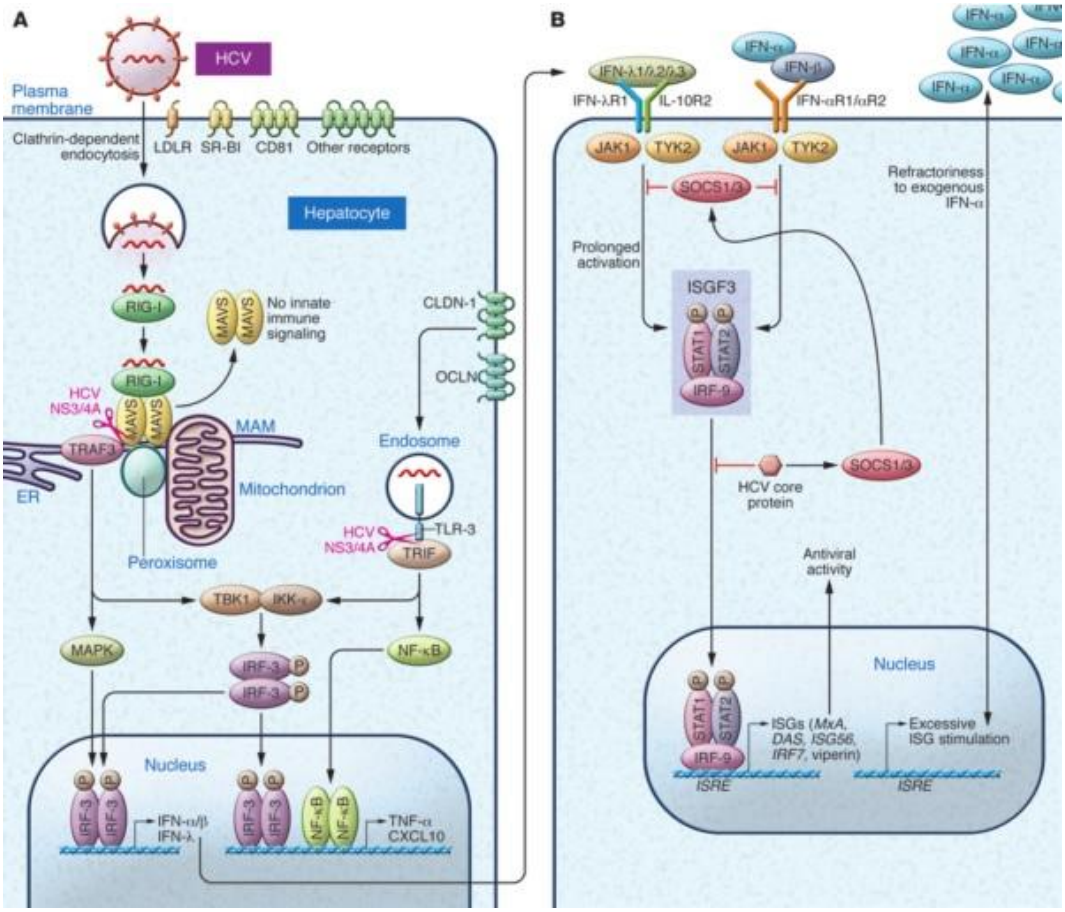


Figure 4: Anti-HCV innate immune response signaling pathways´ activation and progression as well as the role of HCV´s proteins in inhibiting the host´s immune response. Upon infection the viral dsRNA activates RIG-I and TLR3 signaling pathways resulting in the production of type I and III IFNs as well as cytokines and kemokines. The IFNs produced therefore activate the Jak/STAT pathway leading to the formation of ISGF3 and consequently ISG expression which have an antiviral function. Viral proteins like NS3/4A and core can act over these mechanisms, RIG-I and TLR-3 (NS3/4A) and Jak/STAT and ISGF3 (core), inhibiting the host´s immune response.[4]

On the other hand, TLR3, a transmembrane PRR located within intracellular compartments like endosomes, recognises the stem loop double stranded RNA (dsRNA) structures within the viral genome[41,42,45] Its targets are virus released to the extracellular environment by infected cells and incorporated into endosomes. The activated receptor, triggers the TLR signaling pathway, recruits the Toll/interleukin-I receptor domain-

containing adapter-inducing IFN-β (TRIF) and follow a similar signalling pathway as the one triggered by MAVS.[4,5,46]

Recent studies show that the HCV is also recognised by HCV antiviral protein kinase PKR, another dsRNA binding protein. This kinase, when activated by HCV dsRNA phosphorylates the eIF2α suppressing host mRNA cap-dependent translation, therefore inhibiting local protein synthesis.[40,43] Yet, it does not have any effect over viral translation as HCV uses the IRES cap-independent translation mechanism. Nonetheless, activated PKR also triggers a kinase-independent transduction cascade through MAVS, TRAF3 signalling pathways, inducing ISGs and IFN-β production.[44] However, PKR can induce the translation of IFN-stimulated genes (ISGs) like ISG15, an ubiquitin-like modifier that inhibits RIG-I´s ability to recruit MAVS and TRAF3 therefore triggering a response, which leads to a net proviral effect.[4] So, PKR´s role in determining an antiviral or pro-viral state lies in the balance between its different functions.[43]

Both MAVS and TRIF signalling pathways activate IKK-ε and TBK-1 kinases, which phosphorylate the interferon regulatory factor 3 (IRF-3) leading to its dimerization and retention in nucleus[43]. TRIF also summons the nuclear factor-kappa B (NF-kB). These activated intermediates follow cell´s PAMP-responsive signalling pathways and are translocated to the nucleus[40] where they trigger the transcription of type I and III IFNs, IFN-α/β and IFN-λ respectively, chemokines and proinflammatory cytokines. Through an autocrine and paracrine signalling, by IFNs, of neighbouring cells, an amplification loop of anti-HCV response is activated.[4,41] These IFNs will then trigger the JAK/STAT cascade, phosphorylating STAT-1 and STAT-2 in order to form STAT-1/STAT-2 heterodimmers.[4,5,47] These heterodimmers bind to IRF-9 creating the ISGF3 transcription factor, which is transferred to the nucleus where it will bind the IFN-stimulated response element (ISRE) region of the DNA[43]. This results in the production of interferon-stimulated genes, such as ISG56, IFITM1, OAS 1, viperin, IRF-7 and PKR, with antiviral activity of degradation and translation inhibition of RNA.[4]

IFN-α can have another role besides amplifying cellular response and increasing the duration of IFN production. It also activates the maturation of immune effector cells and antigen presenting cells like dendritic cells, potentiating in this way the indirect

modulation of adaptive immune responses and cell-mediated defences by leading to the production of proinflammatory cytokines by hepatic cells.[5,40,48]

However, the innate immune defences do not only comprise the intracellular events of infected hepatocytes. In this matter it is of notice the role of the natural killer cells (NK), the natural killer T cells (NKT), the kupffer cells and the dendritic cells (DC). These multicellular responses (Figure 5) may indicate new targets for future studies.

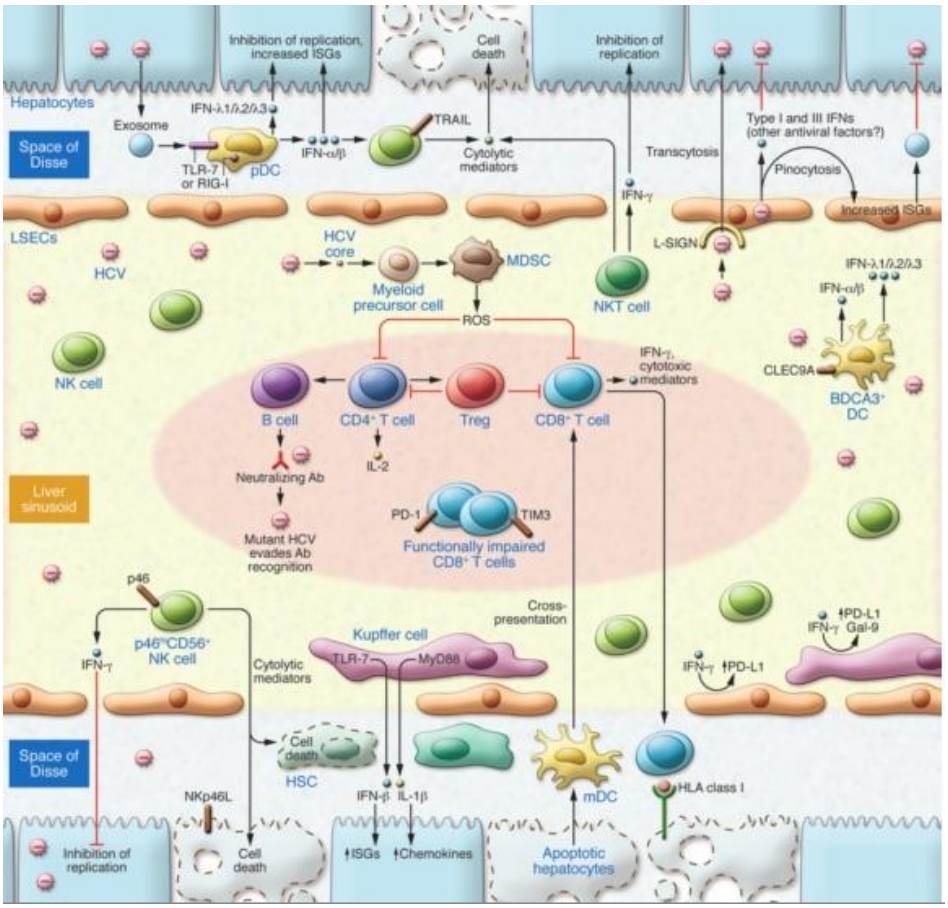


Figure 5: Multicellular immune response to HCV in the liver. Upon viral infection, viral RNA can be transferred into pDCs, leading to inhibition of viral replication through strong IFN production, mainly type I IFNs. On the other end, BDCA3+ DCs have a higher production of type III IFNs without direct cell-to-cell contact. As for NK and NKT cells, their antiviral roles are built upon the production of type II IFNs and cytolytic functions. IFN-α induces NK´s TRAIL, being associated with viral control while $NKp46^{hi}$NK cells inhibits HCV replication and mitigates liver fibrosis by destroying HSCs. Also, KCs instigate innate immune responses as well as inflammatory ones upon HCV phagocytosis. LSECs are able to pinocytose HCV particles and express a wide range of IFNs. KCs can be induced to up-regulate promoters of T cell dysfunction, by IFN-γ. Viral control through clonal expansion of B cells and CTLs is mediated by multispecific and polyfunctional CD4+ T cells, especially if it results in an early induction of nAbs and HCV-specific CTLs. Attenuation of the T cell responses and immune mediated liver damage can be characterized by the presence of PD-1 and TIM-3 in functionally impaired CTLs and the activation of FOXP3+ Tregs and CD33+ myeloid-derived suppressor cells On the viral end, HCV´s core protein in known to inhibit type I IFN based responses as well as promoting liver fibrosis.[4]

First of all, DCs play a pivotal role in innate and adaptive immunities. Among the dendritic cells we have plasmocytoid DCs (pDCs) which are the major source of type I IFN production. However this production is mediated by cell-to-cell contact and TLR-7 activation, independent to intra pDCs HCV RNA replication. Moreover, it is proportional to the number of hepatocytes infected with the virus. In the latter case infected hepatocytes release exosomes containing viral RNA which is transferred to pDCs inducing the IFN-α secretion. It is also shown that pDCs have a sentinel role through an intracellular response to HCV-PAMP recognition by RIG-I, potentiating a strong type I and type III IFN production. In comparison to pDCs there are blood DC antigen 3 expressing DCs (BDC3A+ DCs) that express the C-type lectin CLEC9A and in which the expressed type III IFNs levels are higher but the type I IFN levels are lower.[4]

Another major intervenient in innate immune responses are the natural killer cells (NKs). These cells play a central role during early response against HCV infection by eliminating HCV infected cells. This may occur directly, by cytolitic mechanisms, or indirectly, by cytokine production (IFN-γ). Their activity is tightly regulated by natural killer cell receptors (NKRs), which can be inhibitory, such as killer Ig-like receptors (KIRs) and some members of the NKG2 family (NKG2A), and can also be activatory, like natural cytotoxicity receptors (NCR), NKp30 (NCR3), NKp44 (NCR2) or NKp46 (NCR1), and other members of the NKG2 family (NKG2C/D). Taking NKp46 for example, it is the major human NCR, being present in NK-mediated cell death and highly expressed in patients who show higher levels of spontaneous clearance of HCV infections. Its stimulation, $NKp46^{hi}$ by TLR signalling, which increases cytotoxicity related gene expression, leads to high IFN-γ-mediated anti-HCV activity *in vitro*. As for intrahepatic $NKp46^{hi}$ it inversely correlates with HCV RNA titers. Intrahepatic $NKp46^{hi}$ also presents anti-fibrotic role as the blockage of this receptor reduces NK-mediated hepatic stellate cell (HSCs) death. It is also reviewed that NK cells can also be indicative of the outcome of the HCV treatment as they are activated by cytokine stimulation. One example is the expansion of $CD56^{-}$ NK cells in pre-treatment, which produce MIP-1β are predicative of failure of pegIFN-α/Ribavirin therapy. Additionally, NK´s regulatory and reciprocal interaction with B and T cells, DCs, endothelial cells as well as macrophages is responsible for attenuating or amplifying the immune response.[4]

It is the hepatic reticuloendothelial system, composed of Kupffer cells (KCs) and liver sinusoidal ECs (LSECs), the first in contact with pathogens from the gastrointestinal tract. Contrary to the fact that ISG expression in hepatocytes predicts a non-response to pegIFN-α/Ribavirin therapy, the upregulation of ISGs in KCs predict an antiviral response in a positive and independent way. KCs proceed to capture HCV through phagocytic uptake, which triggers the RIG-I/MAVS pathway inducing an innate immune response dependent of IFN-β. An inflammatory IL-1β dependent signalling is also induced through TLR-7/MyD88 dependent pathway and NLRP3 inflammasome pathway. This induction of pro-inflammatory genes and consequent immune cell recruitment results in liver fibrosis and cirrhosis. During a chronic HCV infection, the activated macrophages/monocytes lack tolerance, a mechanism that gives protection against excessive inflammation, towards TLR ligands responsible for inducing pro-inflammatory cytokines. As for liver sinusoidal endothelial cells (LSECs), they are responsible for eliminating circulating waste molecule, CTL tolerance and apoptosis induction and clearing the bulk of blood-borne human adenovirus. There is also, C-type lectin L-SIGN expression, by LSECs, which mediates HCV particles´ capture and transcytosis through the endothelial barrier, therefore facilitating the direct contact between HCV and hepatocytes.[4]

b) Adaptive immune response:

The adaptive immune response can be divided into two responses. First there is the humoral immunity lead by the B cells and respective antibody production. The second comprises the cellular immune response featuring the CD4+ T helper (Th) cells and CD8+ cytotoxic T lynphocytes (CTLs).[14]

i. Humoral Immune Response:

Starting with the humoral immune response, it is mediated by B lymphocytes, producers of, but not only, neutralizing antibodies (nAbs), detectable 7-8 weeks after infection[14], and has a role of controversial importance in dealing with HCV infections. Firstly, studies in hypogammaglobulinemic humans that cleared the virus spontaneously suggest that the nAbs´ response is expendable due to a weak nAbs/viral clearing

association and a major role of cell-mediated immunity.[4,49,50] However in this study the population was heterogenic and the viral inoculate ill defined. In a different study, now with a homogenous women population and a consistent inoculate from a sole source, the results show that in case of spontaneous eradication of infection an early nAbs response is induced and directed at HCV E1 and E2 glycoproteins.[4,43] Consistent with other studies regarding nAb epitopes[49,51]. Yet the range of epitopes studied is not restricted to E1 and E2 but also to HVR-1, CD81, SR-B1, OCLD and CLDN1, with mixed results[49]. On the other hand, the development of chronic infection besides coming with a late and inefficient nAbs´ response, it also shows an augmented viral envelope sequence evolution due to selective pressure.[4,43,50]

ii. Cellular Immune Response:

Here the main viral eradication mediators are the CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ T helper cells (Th). While CD4+ T cells recognize the antigens presented by antigen presenting cells through means of the major histocompatibility complex (MHC) class II, CD8+ T cells are brought into action by recognising the antigens presented by infected cells via the MHC class I. When activated, CD4+ T cells are responsible for direct activation of B cells and macrophages amongst other effector functions.[5,14,52] In fact, CD4+ T cells are shown to prime effector memory CTLs and limit immune evasion.[4] CD4+ T cells, when activated, differentiate into several subpopulations like CD4+ T helper 1 (Th1), responsible for CTL activation and over inflammatory promotion, and CD4+ T helper 2 (Th2) cells, connected to proliferation of B-lymphocytes and consequent production of antibodies (humoral response). Th1 are also characterized by their production of IL2, IFN-γ and TNF-α, while Th2 secrete IL4, IL5, IL6 and IL10. Also of notice is the fact that Th1 and Th2 downregulate one another.[5,53]

On the other hand, CD8´s effector functions include the death of infected cells, via granzyme and perforin, amongst others, as well as the inhibition of viral replication by secreting IFN-γ, TNF-α and other cytokines, avoiding cell death.[5,14,52] But CTLs can also have a damaging effect on the liver. In case of a viral persistance, like chronic HCV, the continuous CTL presence followed by inflammatory and pro-fibrotic cytokines production by stellate cells is responsible for immune mediated and

progressive liver damage.[4] Of notice is also CTL´s functional avidity, which refers to its response´s overall sensibility to antigen density. In fact, CTLs with high avidity have a substantial secretion of IFN-γ even when presented with low peptide concentrations by APCs. CTLs also have the ability to cross-recognise mutated epitopes which is prominently important against infections with quick development of escape mutations.[4] Therefore, CTLs with higher functional avidity and broader cross-recognition are optimal for HCV infection control[50]. Moreover, human T cells become responsive to HCV peptide-loaded hepatocytes upon HCV TCR transduction. The result is an *in vitro* inhibition of viral replication.[4]

iii. Major Histocompability Complex:

The major histocompatibility complex (MHC) is known in humans as the "human leukocyte antigen" (HLA) system. This large DNA region, which contains over 200 coding loci, is located at the short arm of the 6th chromosome (6p21).[54,55] This complex has a very important role in immune response mediation as it is involved in self and foreign antigen recognition and presentation to T cells.[56] This system is inherited in a Mendelian way as a HLA haplotype, one from each parent with genetic recombination possibly leading to new allelic combinations.[57] These new allelic combinations increase the HLA disparities between donor and receptor reducing the chances of a successful blood transfusion or organ transplant as the probability of identical HLA genes greatly decreases. In this way, HLA is a major immunological barrier.[56]

The MHC can be devided into 3 regions, encoding 3 classes of antigens, Class I, II and III. However, the MHC Class III region does not take part in the HLA system.[58] The HLA Class I antigens can be found in the surface of most nucleated cells, being responsible for peptide presentation to circulating CD8+ T cells. In the Class I region we can find the genes encoding the heavy chains (α) of Class I molecules, the classical HLA-A, HLA-B and HLA-C genes.[59,60] On the other hand, the HLA Class II consists of the HLA-DQ, HLA-DR and HLA-DP loci and is responsible for peptide presentation to CD4+ T helper cells. These antigens can only be found in antigen presenting cells, dendritic cells, B-lynphocytes, endothelial cells and macrophages.[61] Yet, after stimulus they can also be expressed in other cells. The molecules encoded by HLA Class II genes

are heterodimmers of α and β glycosilated peptide chains associated in a non-covalent way.[62]

HLA Class I and II present a high level of polymorphisms, in the α1 and α2 domains in HLA class I and in the 1st amino terminal β1 domain of DRB1, DQB1 and DPB1; determining the HLA Class I moleules´ specificity or in case of HLA Class II, the efficiency of the adaptive immune response as it increases the diversity of antigens recognized and presented to T cells.[63,64]

c) Innate and adaptive immune responses´ cross-talk:

The production of IFNs during the innate immune response indirectly modulates the adaptive immune response, as said before. The IFN-α accomplishes this by inducing the maturation of antigen presenting cells, which lead to resident hepatic cell production of proinflammatory cytokines.[5,40,48]

Starting with its effect over NK cells, IFN-α promotes the activation and proliferation of NK cells as well as the production of IL-15, which supports their survival. In this way, NK are mediated towards the lysis of infected liver cells.[48,65] NK cells also produce IFN-γ[66], proven to limit HCV replication *in vitro*, with direct antiviral effects and associated with viral clearance when produced by immune effector cells in chimpanzee models. IFN-γ activates the JAK/STAT pathway resulting in homodimers of STAT-1.[41,67] Once inside the nucleus, these homodimers promote the expression of genes from the gamma-activated sequence elements (GAS), which overlap considerably with the expressed genes from the ISRE and genes whose products, are directly involved in antigen presenting and processing.[5,40]

As for the effect over DCs, IFN-α promotes their maturation and modulates their viral antigen presentation. This modulation, via IFN-α´s influence over DCs´ ability for antigen presentation, contributes to CD4+ T cells´ differentiation into T helper-1 cells (Th1) which response is intertwined with clearance of viral infection. DCs are also present in the CD8+ T cells cross priming and their production of IFN-γ. In this way, co-stimulatory signals during cross presentation of antigen to CD8+ T cells can determine if the cells are cross-tolerized for anergy or cross primed for a cytotoxic response. IFN-α production can also be seen in DCs through the TLR-3 pathway. This

leads to the induction of cross priming of CD8+ T cells by expressing co-stimulatory molecules and cytokines. Therefore, this links the induction of the adaptive immune response to the signalling processes of host response.[5,40]

d) HCV´s escape mechanisms:

The most problematic aspect of HCV is its ability to avoid and escape the host´s immune defences. Either from genetic mutations or by inhibiting immune pathways, the HCV can usually find a way to avoid immune responses and escalate the infection to chronic status and even result in aggravated liver damage like hepatocellular carcinoma and cirrhosis. To effectively control the HCV infection there is need for an effective innate and adaptive immune responses as well as a viral elimination during the acute state of infection. If the immune response reveals to be insufficient and inadequate, then the virus will develop persistence and a chronic stage of infection.

i. Evading the innate immune response

HCV´s impact over host´s innate immune response starts with its induction. To this end the virus makes use of its NS3/4A protease to block RIG-I and TLR-3 signalling pathways, impairing the production of type I IFNs. This control happens at the MAVS and TRIF level (Figure 4). The NS3/4A targets MAVS molecules cleaving the MAM anchorage synapse, severing the MAVS/MAM connection and inhibiting the downstream signalling of IRF3, whose inhibition is essential for a robust viral replication.[43,44] Also of notice is the mention by Thimme and colleagues[43] that because HCV RNA genome is a weak RIG-I inducer, the NS3/4A has a "head-start" to inhibit the pathway before it is activated by dsRNA. As for TRIF it is directly targeted by the viral protease, which in conjunction with degradation and destabilization post-cleavage, results in a reduction in relative abundance levels. With targeting of TRIF, HCV might diminish viral suppression and CTL response by avoiding an excess inflammation and chemokine induction respectively.[44] Nonetheless, it is conceivable that the bulk of hepatic IFN is not only produced by hepatocytes, but rather by infiltrating lymphoid cells which are not productively infected by HCV and consequently not inhibited by the NS3/4A protease.[43] As for the core, this protein has 2 targets. Firstly, the core protein

targets the JAK/STAT signalling pathway by inducing the SOCS1/3 expression, which inhibits the formation of the ISGF3, by inhibiting STAT1 activation (figure 4), through phosphorylation by JAK[43], and translocation[14]. Secondly, it acts directly over the ISGF3/ISRE interaction, therefore preventing the expression of ISGs.[4] Besides these actions of core it is also reported that there is no decrease in phosphorylated STAT-1 but an upregulation of protein phosphatase PP2Ac and indirect hypomethylation of STAT-1. This encourages the STAT-1/PIAS1 association, which inhibits ISG transcription and impairs ISG expression when the patient is treated with IFN-α. This fact may lead to some controversy over core impact in host´s defences.[43] The E2 and NS5 can also affect the immune response, E2 by inhibiting the PKR receptor, throught its PKR-eIF2a phosphosrylation homology domain (PePHD), and NS5´s ISDR region[68–70], by binding to 2-5 OAS and inhibiting the 2-5 OAS/RNaseL pathway. NS5 also binds to PKR and downregulates its expression and inhibits IFN based antiviral functions through the induction of IL8.[14]

The HCV can also affect DCs´ response to infection, through its E2 glycoprotein. E2 acts by triggering the binding, to pDCs, of type C lectin immunoreceptors, blood DC antigen 2 (BDC2A), and DC immunoreceptor (DCIR), which antagonises IFN production and affecting pDC´s ability to respond to HCV´s infection inside the hepatic microenvironment.[4] Moreover, the HCV core protein has an interfering role over KCs. Has it acts onto TLR-2 the core protein induces IL-1β, IL-10 and TNF-α secretion by KCs, as well as inhibits TLR-3-mediated response to type I IFNs. The core also attenuates TNF-related apoptosis-inducing ligand (TRAIL) expression by KCs and upregulates programmed death ligand 1 (PD-1) expression, promoting T cell dysfunction and viral persistence. Both effects are mediated by phosphoinositide 3-kinase (PI3K) which indicates a MyD88 and TRIF- dependent TLR signalling pathway shared regulation.[4] Regarding E2 and NK cells, E2 inhibits NK´s cytotoxicity and IFN production by crosslinking HCV receptor CD81.[14]

HCV might also exploit the host´s autophagic processes to suppress type I IFN production. This way, HCV reduces immune-recognition therefore preventing a rapid antiviral cytokine production and being able to settle its strong replication machinery before being detected. Also, in the early infection, very low cytokine levels can delay the activation of immune system´s defences such as natural killer cells and cellular and humoral responses.[43]

There is also the hypothesis that the HCV can infect dendritic cells and replicate inside them.[71] This is backed by some studies where HCV RNA sequences, like negative chain intermediates, are detected inside dendritic cells isolated from chronically infected patients.[5,72–74]

ii. Evading the adaptive immune response

Due to HCV RNA dependent RNA polymerase´s high replication rate and lack of proofreading capability, the virus presents a high rate of error insertion, brought upon by selective pressure and viral fitness costs[4], which translates into HCV´s mutational escape, its primary form of immune response evasion. For example, mutations at E2 glycoprotein's HVR result in viral avoidance of B cell-mediated response.[14] In chronically infected patients, viral amino acid changes lead to inhibition in HCV-specific CD8+ T cell responses.[75,76] These mutations affect the HCV-specific T cell recognition by diminishing the epitope-MHC binding affinity and compromising HCV antigens proteossomal processing.[5,14]

As seen before, the development of an acute HCV infection is characterised with a powerful and sustainable CD4+ T response and a strong HCV-specific T cell and multi-specific CD8+ T cell mediated response are observed alongside a constrain in viral escape mutations, achieving viral eradication.[4,77–79] As for the patients who develop a chronic state of infection, they show a weak and/or short lived T cell response with possible functional defects in HCV-specific effector T cells resulting in poor viral control and consequent viral persistence.[71,78] While in an efficient response there is present a Th1 response, with a Th2 downregulation, in a chronic infection it is present a Th2 response with Th1 downregulated and high IL-4 and IL-10 levels.[51,80] During chronic infections HCV-specific CD8+ T cells may not fully contain viral replication allowing disease progression. Alongside this, CD4+ T cell- and CD8+ CTLs- mediated cellular immune responses result in extensive liver damage, cirrhosis and HCC, due to lasting inflammatory responses.[81] Meanwhile, in acute HCV infections, a period of CD8+ T cell dysfunction is observed[78,82], suggesting that a CD8+ T cell downregulation occurs in order to limit tissue damage during acute infection, where viral replication is at its highest.[5,71]

It is also of notice that a weak CTL response joined with a debilitated CD4+ T response leads to viral persistence but viral mutations are not visible. On the other hand, viral persistence with development of viral mutations is present in cases of strong oligospecific CTL response combined with flawed CD4+ T response. T cell "malfunction" can have multiple causes such as anergy, exhaustion or high inhibitory receptors´ expression.[4] By inducing HCV-specific T cells´ anergy, they lose their functionality, by being improperly primed and due to the absence of inflammatory and/or co-stimulatory signals; therefore, the virus can also evade the immune response mechanisms. In this phenomenon, T-cells can be affected in their cytotoxicity, production of TNF-α and IFN-γ, and proliferative capability.[14,83] It is reported that fully differentiated CD8+ T cells, essential for the successful control of HCV infections, appear faster and with higher frequency in SVR patients. This fact allowed the proposition that the virus might block or redirect CD8+ T cell differentiation.[5,84] As for T cell exhaustion, there is co-stimulation and inflammation followed by the priming of T cells to antigen resulting in excess of stimulation and long term loss of function.[4] The third case involves the presence of inhibitory receptors such as PD-1 (Programmed cell death protein 1) and TIM-3 (T cell Ig and mucin domain-containing molecule 3). PD-1, an immunoreceptor tyrosine–based inhibition motif-containing (ITIM-containing) receptor, is expressed on activated T cells and is a hyporesponsiveness mediator. It is upregulated during the chronic infection inside the intrahepatic compartment and PD-L1, its ligand, is expressed by non-parenchymal cells (KCs, LSECs or DCs) and by IFN-exposed hepatocytes. In HCV-specific CTLs, a high expression of PD-1 is characteristic of a late and unsustainable response to IFN-based therapy and an impaired *in vitro* replication control. If PD-1 is stimulated alongside multiple inhibitory receptors, such as 2B4, CD160, KLRG1 or TIM-3, it is possible to identify HCV-specific CTLs presenting an impaired cytokine production and proliferation. As said before, PD-1 can relate to the outcome of the standard treatment. $TIM-3^{pos}PD-1^{pos}$ HCV-specific CTLs are predicative of viral persistence in acutely infected patients due to decreased IFN-γ, TNF-α and CD107a production (CTL dysfunction) in the presence of high levels of TIM-3 and PD-1. To reinforce this statement, a blockage of different co-inhibitory pathways improves CTL´s effector function.[4]

D. Therapy

a) Treatment and response

Due to the asymptomatic nature of acute hepatitis C the disease tends to not be detected at this early stage therefore the treatments are limited. As for the chronic infected patients, the standard treatment available is a pegylated IFN-α and ribavirin (RBV) combination, pegIFN-α/RBV. IFN-α is the base of the therapy due to its importance to potentiate pre-existing antiviral responses and its diminished production or absence makes for an insufficient viral eradication[85]. The fact that it is pegylated provides an increase in its half-life in sera, resulting in a more consistence of IFN levels in blood despite using lesser quantities of pegIFN-α. As for the RBV it is an antiviral, ribonucleoside analogue with a broad-spectrum that inhibits ribonucleoprotein synthesis and decreases viral infectivity by modulating IFN signalling and having a direct antiviral effect over HCV RNA-dependent RNA polymerase, interfering in viral transcription.[5,85,86] This treatment inhibits/reduces the cytolitic inflammatory response through down-regulation of IL-2 and IL-12 and up-regulation of IL-10 restores the Th1 and Th2 population balance and increases cytolytic T-cell response´s efficiency in HCV-infected cells. Also, in comparison to the unpegylated version of IFN-α, this therapy comes to improve HCV antiviral effectiveness, Th2 down-regulation, and CD4/CD8 activation post viral challenge down-regulation and macrophage activity.[85]

Once the virus stops being detected in the blood, the patient is said to be cured. Overall, HCV infected patients have two kinds of answers towards the standard pegIFN-α/RBV therapy. They can either develop a sustained virulogical response (SVR) or a non-sustained virulogical response (NVR). If the response to the treatment is successful, then the HCV RNA remains undetectable after 6 months of post-treatment and the patient achieve SVR. However, because the duration of treatment can be variable, if the patients can clear the virus prior to the 6-month mark, then they can shorten its duration (e.g. from a year to 6 months).[5,87] An important factor is that the pegIFN-α/RBV has variable HCV eradication rates, from 75-90%, for genotypes 2 and 3, to 45-52%, for genotypes 1 and 4.[5,88] As the designation suggests, for the patients with NVR the standard therapy did not work. In these cases, the patient might or might not get to a point where the viral levels are undetected but if they do, then they relapse

returning to higher levels. Here, the patients might retake the treatment but the possibility for a successful response is very low.[5,89–91]

Due to the low clearing rate of the standard therapy towards the genotype 1, its side effects and the crescent knowledge of HCV´s proteins and lifecycle, new "weapons" against the virus, like direct acting antivirus (DAA), have been in development.[5,92] DAA, like bocepravir and telaprevir, are inhibitors of HCV NS3/4A protease and when combined with pegIFN-α/RBV have been proven to increase SVR rate to 75% in patients with no therapy record.[5,93,94] The most recent treatments focus on the DAA sofosbuvir, which targets the viral RNA polymerase NS5B. These increase the rates of a successful therapy to over 95% and have the advantage of being effective against all HCV genotypes. Although all HCV are comparticipated by the government, they are not available for everyone.

Despite the existence of DAA that improve therapy success rates, the development of an affordable preventive vaccine would bring the world closer to the epidemic control. Chmielewska and colleagues have studied the combination of adevoviral vectors capable of expressing full-length and truncated E1E2 glycoproteins boosted with protein antigens, MF59-adjuvanted recombinant E1E2 glycoproteins, in mice and guinea pigs. Their results show a strong and broad T cell, HCV-specific CD4+ and CD8+, and cross-neutralizing antibody, Th1-type IgG, response, even greater then with the vaccine components acting separately. Yet more studies are required, specially a further combination with a HCV NS vaccine.[95]

b) Influence over the response outcome

An important factor for reducing the stress, to which the chronically infected patients are subjected to, both financially (high therapy costs) and physically (associated side effects), is to be able to predict what kind of response the patients show by the end of the standard pegIFN-α/RBV treatment. There are several factors that can influence patients´ response towards the standard therapy, like viral and intrinsic factors, and even anticipate what kind of response to expect, such as genetic factors.[5,96]

Starting with viral factors, the main influence aspects are viral genotypes and the presence of quasispecies. As mentioned before, different genotypes show different SVR

rates, with genotype 2 or 3 infected patients showing higher SVR ratings than genotype 1 or 4 infected patients.[88] Despite that, viral ability for high replication with no proofreading leads to its high capability to produce adaptive mutations that will decrease response mechanism´s success[14], e.g. mutations at NS5´s ISDR region can be predicative of IFN-α treatment outcome.[97] Of notice is the suggestion that RBV might be a HCV mutagen, increasing viral mutations.[85,98] Even higher pre-treatment viral charges (800 000 UI/ml) are associated with lower treatment sensibility.[5,99,100]

Talking about other factors, intrinsic factors like age, sex, ethnicity, obesity, cirrhosis, and steatosis can be indicative of lower response rates.[101,102] Co-infection with HIV or HBV can also indicate a negative response.[103] And even side effects can have a disruptive effect over the treatment (20% of patients)[14], as IFN-α can induce leucopoenia, thrombocytopenia, neutropenia, exhaustion or depression, and RBV can cause haemolytic anemia.[5,14,104]

For genetic factors, different ISG and cytokine expression profiles[105–107] and the presence of single nucleotide polymorphisms (SNPs) in the human genome[108,109] can be important predictors of response outcome. Talking about the genetic expression of mediators of immune response, like IFN stimulated genes, this deregulation has been observed in studies like from Bieche, Asselah and colleagues[105,106] and Helbig and colleagues[110]. Two examples, of the distinct expression profiles between SVR and NVR patients, detected are IFI27 and CXCL9 for treatment response in evaluated patients was 79.3%, to which the predicative accuracy for SVR and NVR was respectively 70% and 100%.[106] These evaluations have shown that the basal levels of ISG expression are higher in NVR patients[111] and that this over-expression leads to ISG saturation levels that cannot be further increased, significantly, during treatment which results in treatment inefficiency[112] (Figure 6).[5] The genetic profile study has also been performed in peripheral blood mononuclear cells (PBMCs), which allows for a less invasive approach, but with lesser extent. Younossi and colleagues have compared several human RNA transcripts´ genetic profiles, from PBMCs, with the response patterns of standardly treated patients and observed that an SVR response could be predicted with STAT-6 expression profile and that the IGS´s SVR predictability could be extended to 24 hours after the beginning of the treatment.[107]

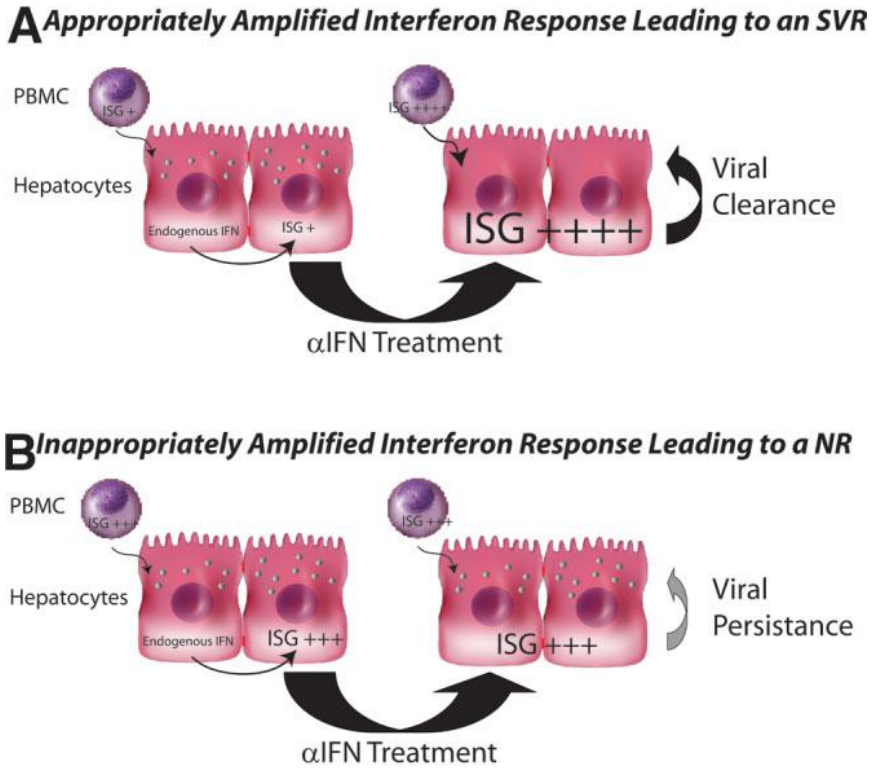


Figure 6: ISG mediated immune response against HCV infection.[112] During the Standard pegIFN-α/RBV treatment, the supply of IFN-α stimulates the expression and production of ISGs which are vital for viral clearance. For a SVR, there must be a very significantly high increase in ISG stimulation or else it will not impact the virus and the infection will endure.

There are even cases where a correlation between HLA typing and HCV response can be deduced. HLA typing targets the region that encodes the human major histocompatibility complex genes that are extremely polymorphic, which help limit the propagation of pandemic pathogens.[64] In literature, a relationship between the outcome of HCV infection and the HLA, polymorphisms and haplotypes, are reported in several studies.[64,113–116] For example, Cangussu and colleagues (2011)[64] reports a protection against chronic HCV infection related to the DRB1*1101 and DQB1*0301 antigens; McKiernan *et al.* (2004)[116] reports a link between viral clearance and the alleles A*03, B*27, DRB1*0101, DRB1*0401 and DRB1*15 and the halpotypes A*03-B*07-DRB1*15-DQB1*0602 or A*02-B*27-Cw*01-DRB1*0101-DQB1*0501; between B*08 and chronic HCV infection; There is also between HCV spontaneous clearance

and DRB1*04, DQB1*0301, DQA1*03, as well as among DQB1*0302 and protection from HCV infection[117].

E. Objectives:

With the introductory portion of this thesis complete its time to clarify the objectives of this work. The overall objectives of the thesis were:

- To get new insights on HCV infection and immune response
- To evaluate immunologic markers with predictive potential for a sustained immune response (SVR)

There are important mechanisms that could be predicative of patients´ response to the standard pegIFN-α/RBV therapy. The parcial objectives of this thesis were:

- To perform a comparative analysis of the HCV 5´UTR genetic sequences to determine possible mutations indicative of a determined response to the standard therapy;
- To optimize the NS3 PCR amplification program used by Henriques (2012) and to obtain NS3 sequences in order to create a genetic library capable of determining, through a comparative analysis, putative genetic mutations that might be indicative of what kind of response the patients will develop when subject to the pegIFN-α/RBV treatment;
- To evaluate the genetic expression of some immunologic mediators of the response to HCV, connected to the presence and activity of dendritic cells, in order to see if they are descriptive of the response given by the patients to the pegIFN-α/RBV treatment;
- To investigate if there is genetic predisposition of the patients to develop SVR or NVR in response to the pegIFN-α/RBV treatment, by means of HLA typing of the infected patients;

Hopefully, this will allow doctors to anticipate and adjust the HCV treatment accordingly, in order to treat the viral infection more effectively and with less physical and financial stress for the patients.

Chapter 2:

Methodologies

A. Study population and samples:

For this thesis there were used blood samples collected from HCV chronically infected patients who were looking to begin the standard PegIFN-α/RBV therapy. Their progress was followed by clinical consultation and evaluated 1, 6 and 12 months after the start of the treatment. However, this research will focus on blood collections from before the beginning of the therapy.

The viral mutagenic profiling of the 5´UTR and NS3 genes could be studied through the analysis of viral RNA extracted from serum samples previously collected from HCV infected patients (Table I).

Table I: Samples of the chronically HCV-infected patients involved in the 5´UTR and NS3 genetic variability study, with respective HCV genotypes.

Sample	605336	410125	314215	415465	LMDCR	RLHJS	SPDMR	315941	235647	243802
HCV Genotype	1g	1a; 1b	UND[a]	1g; 1b	1g	UND[a]	1a; 1b	1a	1g	1g
Age	UND	61	63	79	29	42	39	36	41	40
Gender	UND	M	F	M	M	M	M	UND	M	M

[a] Sequence too short to be determined

For the genetic expression study of immune response mediators, peripheral blood samples collected in PAXgene® tubes (PreAnalytiX®) were used, which allows for a higher protection of the genomic RNA intended to be extracted, against RNases and other enzymes as well as its immediate stabilisation by minimizing the ex-vivo chances of genetic expression modifications.[118] These samples were evaluated upon the genetic expression of the following genes: IFN-α, IL10, TGF-β1, IFIT1, IFI27, OAS3, IRF-2, IRF-8, FOX-P3 and IL4. From the peripheral blood samples available it was possible to find 55 that matched the desired testing conditions (Table II). The details about the study population are shown in Table XXX of the annexes. There were also tested 6 samples of healthy people to act as negative controls.

Table II: Samples of the patients involved in the evaluation of the genetic expression of the immunologic mediators, with gender, age, response to pegIFN-α treatment (SVR or NVR), HCV genotype and viral load.

Response	Gender			Age		Genotype						Viral load (IU/ml)	Patients
	M	F	UND	≤41	>41	1	1-a	1-b	3-a	4-a	UND	Mean	N
SVR	30	11	1	22	20	1	16	8	13	1	3	5 646 004	42
NVR	13	0	0	7	6	0	4	5	2	1	1	6 953 662	13

Finally, peripheral blood samples were also collected in collection tubes coated with K3EDTA (Greiner Bio-One VACUETTE®), which blocks the coagulation cascade by calcium ion binding, in order to extract the genomic DNA and perform the HLA genotyping of the chronically infected patients.

The response towards the treatment, whether it is a sustained virological response (SVR) or a non-sustained virological response (NVR), is known and it will be essential for determining a correlation between genetic expression, mutagenic profile or HLA typing and the response observed in the patients.

B. Genetic variability of the HCV´s 5´UTR determined by sequencing:

a) Viral RNA extraction:

In order to extract HCV´s RNA the QIAamp® MinElute® Virus Spin Kit from QIAGEN® was utilized. Before starting, the serum samples were subjected to a 2 minute centrifugation at maximum speed so the serum would be cleaner. Then, retrieving 400µl of serum´s supernatant or in case of lower sample volume, 200µl, was necessary. This volume was transferred to a 2ml eppendorf. In the low serum volume case, 200µl of

0.9% NaCl solution were added to the serum, so that the 400µl needed to perform the protocol was matched.

Next, it was the preparation of the reagents, QIAGEN® protease, AVE buffer and the mixture carrier RNA and AVE buffer into 2ml tubes designated A, B and C respectively, with volumes varying dependently on the number of samples intended for extracting the viral RNA. The remaining consumables were set and the rotor adaptors equipped with a QIAamp® MinElute® column and a 1.5ml collection tube, accordingly to the QIAamp® MinElute® Kit protocol sheet. Finally, the QIAcube® was started with the standard large body-fluid samples´ QIAamp® MinElute® Kit protocol. When the protocol was finished the extracted RNA was put on ice for immediate use or stored at -25ºC for later use.

b) Research and quantification of HCV using the HCV GeneProof kit

This step used the GeneProof´s Hepatitis C virus (HCV) PCR kit which allowed detecting the presence of HCV´s RNA, therefore evaluating the efficiency of the extraction process. The kit amplifies the 5´UTR RNA sequence by using a reverse transcriptase present in the master mix. The amplification product growth is detected via a fluorescence marked probe in a real time PCR, the FAM fluorophore for the virus and the JOE/VIC reporter for the internal standard (I.S.). The Master Mix used contains an internal standard, GAPDH mRNA, in order to control sample quality as well as the diagnostic process. The kit also provides 4 standards, HCV 10^1; 10^2; 10^3 and 10^4 IU/µl.[119] These standards work as positive controls and can also be used to create a calibration curve to quantify the viral load.

Each reaction contains 20µl of Master Mix and 5µl of RNA, RNase free water (negative control) or HCV standard (positive control)[119]. The reactions were prepared in 96 well plates and placed in the LightCycler® 480 (Roche) to undergo the real-time PCR. The amplification program ran as follow (Table III). As for the results they were interpreted according to the schematics in Table IV.

Table III: qRT-PCR for the GeneProof HCV diagnostic using the LightCycler® 480 (Roche).

	Temperature (ºC)	Time (min)	Cycles
Reverse transcription	42	5:00	1
Initial denaturation	95	0:10	1
Denaturation	95	0:05	50
Annealing	58	0:40	
Extension	72	0:10	

Table IV: Diagram for the analysis of the GeneProof´s qRT-PCR results.

Results	HCV FAM (+)	HCV FAM (-)
I.C. JOE/VIC (+)	(+) Valid	(-) Valid
I.C. JOE/VIC (-)	(+) Valid*	(-) Invalid**
*	High HCV concentration can compete with the I.S. for the limited reagents, reducing and even nullifying I.S. signal	
**	Unsuccessful viral RNA extraction.	

c) cDNA synthesis of 5´UTR for sequencing:

With the RNA extracted it is time to synthesise the complementary DNA (cDNA). To do that, it is used the reverse transcriptase of the aviarian mieloblastose virus (AMV-RT). The AMV-RT has a high processing capability, uses DNA, RNA and RNA:DNA hybrid templates and is active for temperatures of over 50ºC. However it has the particularity of having an intrinsic RNase H activity *in vitro*, which can result in the

destruction of the RNA template before the beginning of the reverse transcriptase therefore reducing the cDNA production.[5,120]

As for the cDNA synthesis methodology *per se*, first there is need to prepare the reaction master mix following the reagents and volumes indicated in Table V. In this step the reverse primer 1CH was utilized in order to produce the cDNA sequence relative to the desired amplification product, the 5´UTR RNA sequence. In order to nullify the RNase H activity of the AMV-RT, it was added RNAsin, an inhibitor of the ribonuclease activity, to the master mix, protecting, in this way, the RNA template.

Table V: Components of the AMV-RT reverse transcription reaction master mix with respective volumes and final concentration per reaction.

Reagents	Volume (µl)/reaction	Final concentration
5x RT Buffer[a]	5	1x
2mM dNTPs	2.5	0.2mM
5µM Primer 1CH	5	1µM
20U/µl RNAsin[b]	0.5	10U/µl
10U/µl AMV-RT[a]	0.5	5U/µl
RNase free water	1	-
Total	14.5	-

[a]Promega, Madison, USA; [b]Nuclear Laser Medicine

The reactions were prepared in 500µl eppendorf tubes. There 10.5µl of RNA were added to 14.5µl of reaction master mix. After mixing, the reaction was subjected to incubation in the Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®) following the program on Table VI. After incubation, the cDNA samples were put on ice if needed right away. If not they must be stored at -25ºC.

Table VI: Incubation program for the cDNA synthesis in the Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®).

	Temperature (ºC)	Time (min)
Reverse transcription	42	50:00
Enzyme inactivation	95	10:00
	4	∞

d) 5´UTR nested PCR amplification:

Having the cDNA synthesised, the protocol proceeded to amplifying the sequence of interest. For that purpose, it was opted to perform a nested PCR, which uses 2 sets of primers instead of just one and consists in 2 regular PCRs performed one after the other. In the 1st PCR, it is amplified a wider region of the genome than that of interest with the help of the 1st set of primers. The region of the genome that is intended to study, which is within the region amplified in the 1st PCR round, is targeted and amplified in the 2nd round of the nested PCR, where the 2nd pair of primers is used. This results in more specific and reliable amplifications.

Because the reverse primer 1CH was already used to synthesise the cDNA, in the 1st PCR only use the respective forward primer, the 2CH primer, was necessary. As for the 2nd PCR the Bky80 (forward) and Bky78 (reverse) primers (Table VII) were the ones chosen to amplify the desired 5´UTR sequence.

Table VII: Primers used in the nested PCR for the 5´UTR amplification.[5]

	Primer designation	Sequence (5´→3´)	Location in HCV genome (nucleotides)
Primers used in the 1st round PCR	2CH (forward)	AAC TAC TGT CTT CAC GCA GAA	53-73
	1CH (reverse)	GGT GCA CGG TCT ACG AGA CCT C	341-321
Primers used in the 2nd round PCR	Bky80 (forward)	GCA GAA AGC GTC TAG CCA TGG CGT	68-91
	Bky78 (reverse)	CTC GCA AGC ACC CTA TCA GGC GT	311-288

The 1st PCR starts by preparing the reaction master mix with the reagents and respective volumes as presented in the following table.

Table VIII: Reaction mix components and respective volumes and concentrations for the 1st round of the nested PCR for 5´UTR amplification.

Reagents	Volume (µl)/reaction	Final concentration
5x GoTaq® Flexi Buffer[a]	10	1x
2mM dNTPs	5	0.2mM
25mM $MgCl_2$[a]	6	1.5mM
5µM Primer 2CH	5	0.25µM
5U/µl GoTaq® G2 Flexi DNA Polimerase[a]	0.5	2.5U/µl
RNase free water	18.5	-
Total	45	

[a]Promega, Madison, USA

For each reaction, 45µl of reaction mix were transferred to either 500µl tubes or 200µl 8tube-stripes where it was mixed with 5µl of cDNA, making for a total volume of 50 µl. For the negative control, 5µl of RNase free water were used in place of the cDNA. The reactions were then incubated in a Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®) accordingly to the program shown in Table IX. If the products are needed right after amplification, they are put on ice when the nested is complete. If not, they are stored at 4ºC.

Table IX: Incubation program for the 1st round of the nested PCR for 5´UTR amplification.

	Temperature (ºC)	Time (min)	Cycles
Initial Denaturation	94	4:00	1
Initial annealing	50	1:00	1
Initial extension	72	1:00	1
Denaturation	94	0:30	30
Annealing	60	0:30	
Extension	72	0:30	
Final Extension	72	10:00	1
	4	∞	

As for the second round PCR, the procedure was similar but instead of using primer 2CH, the primers Bky80 and Bky78, which are more specific for the region intended to be amplified, were the ones chosen. So, for each sample, a reaction mix was prepared using the reagents as shown in Table X.

Table X: Reaction mix components and respective volumes and concentrations for the 2nd round of the nested PCR for 5´UTR amplification.

Reagents	Volume (µl)/reaction	Final concentration
5x GoTaq® Flexi Buffer[a]	10	1x
2mM dNTPs	5	0.2mM
25mM $MgCl_2$[a]	6	1.5mM
6.67µM Primer Bky80	5	0.33µM
6.67µM Primer Bky78	5	0.33µM
5U/µl GoTaq® G2 Flexi DNA Polimerase[a]	0.5	2.5U/µl
RNase free water	13.5	-
Total	45	

[a]Promega, Madison, USA

Then for each reaction I mixed 45µl of reaction mix and 5µl of amplified product from the first PCR round, completing the final volume of 50 µl. In the negative control case, 5µl of RNase free water were used instead of amplification product. After this step the reactions were incubated in a Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®) according to the following program (Table XI). If the products are needed right after amplification, they are put on ice when the nested is complete. If not, they are stored at 4ºC.

Table XI: Incubation program for the 2nd round of the nested PCR for 5´UTR amplification.

	Temperature (ºC)	Time (min)	Cycles
Initial Denaturation	94	2:00	1
Denaturation	94	0:30	35
Annealing	60	0:30	
Extension	72	0:30	
Final Extension	72	10:00	1
	4	∞	

e) Agarose gel electrophoresis

Finalised the amplification, it was time to confirm if the nested protocol was successful. To do that, it is used a 2% agarose (Seakem® ME Agarose, Lonza, Rockland, USA) gel electrophoresis. In this step, the molecules in the amplification product are separated, by the action of an electric field, according to their size, because all of them have a total negative charge and a similar charge/mass ration. The separated molecules are visible due to the ethidium bromide ($EtBr_2$) added to the gel which by binding to the DNA molecules, between adjacent nucleosides, allows them to emit fluorescence when exposed to UV light.

To prepare the gel and run the electrophoresis it was opted for the Morgan™ SSP Maxi Gel Electrophoresis System from Texas BioGene, Inc. The 2% agarose gel was submerged in a TAE(1x) solution and the electrophoresis itself ran for 15-20min at 180Voltz. The final results were visualised in a UV transilluminator.

f) ExoSap purification of 5´UTR PCR products for sequencing

Before proceeding to the sequenciation of the amplified HCV 5´UTR sequences it is necessary to purify the nested PCR products so that the excess reagents are removed. For that intent I used the ExoSap method of purification.

The ExoSap method consists simply in the adding 4µl of ExoSap-IT (Allele SEQR) reagent to each 10µl of amplification product I wanted to purify. This method utilises the endonuclease I and shrimp alkaline phosphatise (SAP) enzymes in order to destroy the primers and dNTPs, respectively, still present from the amplification step. After mixing, the tubes were incubated in the Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®) accordingly to the following program (Table XII). This method has the advantage of being easier to perform than the other available method, the JETQuick® PCR Product Purification Spin Kit (GENOMED), and without the risk of losing the amplified product.

Table XII: Incubation program for the ExoSap purification of the PCR product.

	Temperature (ºC)	Time (min)
Enzyme activation	37	15:00
Enzyme inactivation	80	15:00
	4	∞

After the purification is complete it proceeds to a 2% agarose gel electrophoresis to confirm the presence and evaluate the condition of the purified product. Then, follows to the sequencing step where the product is prepared to sequenciation.

g) Sequencing reaction

The final step of this protocol is to sequence the amplified genetic sequence using the Sanger method. This method consists in an amplification reaction in the presence of fluorescently marked dideoxynucleotide triphosphates (ddNTPs) which act as synthesis terminators of the new DNA sequence. Besides ddNTPs, these reactions need a DNA template, one of the primers used in the PCR process (forward or reverse) dNTPs and a DNA polymerase.

To that intent, it is used the BigDye® Terminator v1.1 Cycle Sequencing Kit from Applied Biosystems™. Two reactions for each sample were prepared, one for each

primer (forward or reversed). For each reaction are mixed 6µl of RNase free water, 1µl of primer, 2µl BigDye® terminator and 1µl of purified PCR product. The reactions were prepared in 200µl 8tube-strips and incubated in a Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®) following the incubation program shown in table XIII.

Table XIII: Incubation program for the sequencing reaction.

	Temperature (ºC)	Time (min)	Cycles
Denaturation	96	0:20	25
Extension	60	2:00	
	4	∞	

After incubation, the sequence must be purified, to remove the excess ddNTPs, before being sent to the 3137 DNA Analyser from Applied Biosystems™, life technologies™, where it is analysed using a capillary electrophoresis in a POP-7 matrix and with a 10x 3730 buffer.

This purification was realised using a Sephadex G50 (Sephadex™ G50, GE Healthcare, Sweden) matrix, prepared by mixing 5g of Sephadex G50 with 60ml of ultra pure water. First, a Pasteur pipette is used to fill in the wells of the filtration plate I would use, with the Sephadex solution. Then, the excess of water is removed with 2 centrifugations of 1200xg for 2 min, performed "back to back". After that, the filtration plate is put over the 96 well sequencing plate and another 2min 1200xg centrifugation is performed. To match the minimum volume of 15µl needed for the reading, were added 5µl of ultra pure water to the wells containing the samples. The plate was ready to run. The plate was inserted in the 3137 DNA Analyser (Applied Biosystems™, life technologies™) and the plate schematic drawn. The sequenciator ran the samples in a POP-7 matrix and with a 10x 3730 buffer. As for the data, it was detected by an internal detector connected to the software 3037 series DataColection™ 4. The data was then extracted and analysed using the SeqScape v2.6 program, which allows for a comparative nucleotidic evaluation between the sequenced samples and a reference sequence.

C. Genetic variability of the HCV´s NS3 through sequencing:

Because the laboratorial procedures for the study of the NS3 region of the HCV genome are similar to the ones used for studying the 5´UTR region. This section will proceed to only describing the cDNA synthesis and the nested PCR amplification methods, which are the steps where these differences are visible.

a) cDNA synthesis of NS3 for sequencing:

With the RNA extracted from the serum, using the QIAamp® MinElute® Virus Spin Kit from QIAGEN®, it proceeds to synthesise the complementary DNA (cDNA). To do that, the Invitrogen™, by life technologies™, SuperScript® VILO™ cDNA Synthesis Kit is used. This kit uses a 5x VILO™ Reaction Mix containing random primers, $MgCl_2$ and dNTPs; as well as a 10x SuperScript® Enzyme Mix which contains SuperScript® III reverse transcriptase (a version of the M-MLV RT, which has increased thermal stability and decreased RNase H activity compared to AMV-RT), RNase OUT™ recombinant ribonuclease inhibitor (protection against RNA degradation by ribonuclease contamination) and proprietary helper protein. This kit allows for full genome synthesis.

For each sample intended to use, it is prepared a reaction mix of 4µl of 5x VILO™ Reaction Mix, 2µl of SuperScript® Enzyme Mix and 6µl of RNase free water.[122] To this mix was added 8µl of RNA. After mixing, the reaction was subjected to incubation in the Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®) following the program on Table XIV. After incubation, the cDNA samples were put on ice if needed right away. If not they were stored at -25ºC.

Table XIV: incubation program for the cDNA synthesis using the VILO™ cDNA synthesis kit.

	Temperature (ºC)	Time (min)
Annealing	25	10:00
Reverse transcription	42	1:00:00
Enzyme inactivation	85	5:00
	4	∞

b) NS3 nested PCR amplification:

With the cDNA synthesised, it is time to proceede to amplifying the HCV NS3 gene. For that purpose a nested PCR, as mentioned in the 5´UTR section, is performed. The primers used in this step were already drawn and used by Henriques (2012)[5] and are listed in Table XV.

Table XV: Primers used in the amplification of the NS3 region of the HCV genome, with sequence and genomic location.[5]

Primer designation	Sequence (5´→3´)	Location in HCV genome (nucleotides)
3NS1BF2	GGG GTC ATT ACG TCC AAA TGG	3118-3138
3NS1BR2	ATC CTG CCC CAC ATG ACC AC	5367-5386
3NS1BF3	CTA TGG AAA CTA CCA TGC GGT	3928-3948
3NS1BR3	CGA GTT GTC CGT AAA GAC CGG	3951-3971
3NS1BF4	CCT TGA TGT GTC CGT ACT ACC	4589-4609
3NS1BR4	ATT AGA GCG TCT GTT GCC ACG	4631-4651

However, due to the size of the NS3 gene, around 1900bp, one nested PCR is not sufficient to construct a full sequence of the NS3, as the optimal size of the amplicon is around 500bp. To that intent it is necessary to use different combinations of primers to amplify different sections of the genetic sequence. Figure 7 shows the representation of the NS3 gene with the binding sites of the primers used and their combinations throughout the amplification process and respective target amplification sections of NS3. However, due to optimizing issues and lack of time only PCR-1.1, PCR-2.1 and PCR-2.2 were performed.

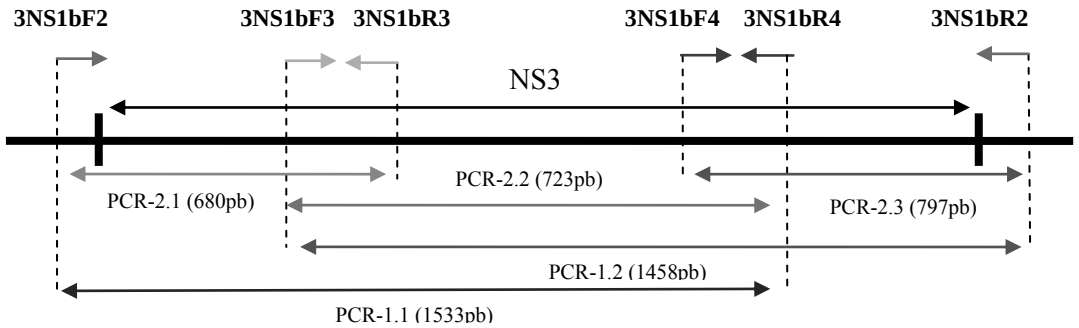


Figure 7: Schematic of the NS3 amplification strategy with the different sets of primers for each individual amplification round. PCR-2.1 and PCR-2.2 will be the 2nd rounds for the PCR-1.1 1st round, while the PCR-2.3 will follow the PCR-1.2.

In the first round of the first nested PCR (PCR-1.1), the primers used, NS31bF2 and NS31bR4, were prepared in a 1/10 dilution. In this step was prepared a reaction master mix with the reagents shown in the following table.

Table XVI: Reagents and respective volumes per reaction for the nested PCR 1st round, PCR-1.1, reaction mix.

Reagents	Volume (µl)/reaction
5x GoTaq® Flexi Buffer[a]	10
2mM dNTPs	5
25mM $MgCl_2$[a]	6
10µM Primer 3NS1bF2	5
10µM Primer 3NS1bR4	5
5U/µl GoTaq® G2 Flexi DNA Polimerase[a]	0.5
RNase free water	13.5
Total	45

[a]Promega, Madison, USA

For each reaction, 45µl of master mix were transferred to 200µl 8tube-strips where it was mixed with 5µl of cDNA, making for a total volume of 50 µl. The reactions were then incubated in a Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®) accordingly to the program shown in Table XVII.

Table XVII: Incubation program for the nested PCR 1st round, PCR-1.1.

	Temperature (ºC)	Time (min)	Cycles
Initial Denaturation	94	2:00	1
Denaturation	94	0:30	30
Annealing	53	1:00	
Extension	72	1:00	
Final Extension	72	10:00	1
	4	∞	

As for the second round PCR (PCR-2.1), the procedure was similar but instead of using primers NS31bF2 and NS31bR4, the primers used were NS31bF2 and NS31bR3 which are more specific for the region intended to amplify. So, for each sample, a master mix using the reagents as shown in Table XVIII was prepared.

Table XVIII: Reagents and respective volumes per reaction for the nested PCR 2nd round, PCR-2.1, reaction mix.

Reagents	Volume (µl)/reaction
5x GoTaq® Flexi Buffer[a]	10
2mM dNTPs	5
25mM $MgCl_2$[a]	6
10µM Primer 3NS1bF2	5
10µM Primer 3NS1bR3	5
5U/µl GoTaq® G2 Flexi DNA Polimerase[a]	0.5
RNase free water	13.5
Total	45

[a]Promega, Madison, USA

Then for each reaction 45µl of master mix and 5µl of amplified product of the first PCR were mixed completing the final volume of 50 µl. After this step the reactions were incubated in a Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®) according to the following program (Table XIX).

Table XIX: Incubation program for the nested PCR 2nd round, PCR-2.1.

	Temperature (ºC)	Time (min)	Cycles
Initial Denaturation	94	2:00	1
Denaturation	94	0:30	35
Annealing	53.7	1:00	
Extension	72	1:00	
Final Extension	72	10:00	1
	4	∞	

The other 2nd round PCR tested with some success was PCR-2.2 which follows the same principle as the above but with different primers (3NS1BF3 and 3NS1BR4) in the preparation of the reaction master mix (Table XX) and a slightly different incubation protocol (Table XXI).

Table XX: Reagents and respective volumes per reaction for the nested PCR 2nd round, PCR-2.2, reaction mix.

Reagents	Volume (µl)/reaction
5x GoTaq® Flexi Buffer[a]	10
2mM dNTPs	5
25mM $MgCl_2$[a]	6
10µM Primer 3NS1bF3	5
10µM Primer 3NS1bR4	5
5U/µl GoTaq® G2 Flexi DNA Polimerase[a]	0.5
RNase free water	13.5
Total	45

[a]Promega, Madison, USA

Table XXI: Incubation program for the nested PCR 2nd round, PCR-2.2.

	Temperature (ºC)	Time (min)	Cycles
Initial Denaturation	94	2:00	1
Denaturation	94	0:30	35
Annealing	51.7	1:00	
Extension	72	1:00	
Final Extension	72	10:00	1
	4	∞	

Regardless of which NS3 segment was amplified, every resulting product was subjected to an electrophoresis evaluation followed by a purification step and finally sequencing reaction as described for the 5´UTR genetic study. The only point of notice is remainder of the fact that the primers used for the sequencing reaction must be from the same set used the amplification process.

D. Genetic expression of immune response mediators:

a) Interest genes:

This part of the study will focus on genetic expression of some genes that have roles and impact in the immune response against the hepatitis C virus. The studied genes are as follow: IFN-α, TGF-β1, IFIT1, IFI27, OAS3, IRF-2, IRF-8, FOXP3, IL-4 and IL-10. IFN-α was chosen for being a main intervenient in the immune response mechanisms and the star feature of the standard pegIFN-α and ribavirin threatment. The IFIT1, IFI27 and OAS3 genes were chosen for being genes that have an expression stimulated by IFN-α. On the other hand, IRF-2 and IRF-8 are regulators of type I IFN´s activity. As for TGF-β1, FOXP3, IL-4 and IL-10, these are genes expressed by several cells and with important impact on T and B cells´ role in the immune response.

b) PAXgene® RNA extraction:

The total RNA was extracted from PAXgene® tubes from the infected patients before they started the pegIFN-α therapy. The extraction was preformed with the QIAsymphony® PAXgene® Blood RNA Kit and with help of the QIAsymphony® SP from QIAGEN®. This kit uses a combination of silica-based nucleic acid purification with magnetic particles. Once inside the extractor, the samples are digested with DNase1 and the extractor uses MagAttract® magnetic particles covered with a silica surface to collect the RNA, in the presence of a chaotropic salt and isopropanol. The RNA is ultimately eluted with BR5 elution buffer.

The first step consists in extracting 1-3ml of blood from the PAXgene® tubes into 5ml polystyrene round-bottom tubes. The tubes were then sealed with parafilm and subject to centrifugation at 2000xg for 10min, in order to pellet the sample content. After centrifugation, the supernatant was discarded and the pellet ressuspended, by vortex, with a mix solution of QSX2 buffer (280µl per sample) and proteinase K (20µl per sample). Then, 200µl of BR2 buffer (contains guanidine thiocyanate) were added to each ressuspended sample. The next step was to load the samples into the QIAsymphony® SP, prepared according to the QIAsymphony® PAXgene® Blood RNA Kit Handbook (2012)[123], where the total RNA is extracted and eluated in 80µl of BR5 into 1,5ml eppendorf tubes. After the extraction was completed, the eluate tubes were incubated for 5min at 65ºC in an eppendorf termomixer.

c) RNA quantification:

After extracting the total RNA from the PAXgene® blood samples, the procedure follows to quantifying the RNA extracted and its purity degree (Table XXXI of the annexes). To that intent the QuaWell Q3000 was used. With just a 2µl sample of RNA it gives the values for Abs260, Abs280, Abs260/Abs280 (purity degree) and concentration (ng/µl). For RNA samples the accepted 260/280 ratio is ~2.0, considered "pure" RNA. Values under that ration may indicate the presence of DNA (which is considered pure at ~1.8), proteins, phenol or other strong 280nm absorbent contaminants.[124]

d) RNA integrity:

In this intermediate step, it was performed a RNA 6000 Nano Assay to evaluate the integrity of the total RNA extracted (Table XXXI of the annexes). This assay uses RNA analysis chips, Figure 8, to analyse RNA fragments, which are driven through the chip´s interconnected set of microchannels, where they are electrophoretically separated according to their fragment size.[125] With this assay it also possible to determine the RNA fragments´ concentration (Table XXXI of the annexes).

Figure 8: RNA Nano LabChip®, Agilent Technologies, Inc. Agilent RNA 6000 Nano Kit Guide, ©Agilent Tachnologies, Inc. 2013.

To do that it was used the RNA 6000 Nano LabChip® kit from Agilent Technologies, Inc. The assay protocol was followed according to the manufacturer's instructions. The 1st step was to set the chip priming station, with a new syringe and the base plate at the correct position, for the correct preparation of the chip. Next, the decontamination of the Agilent 2100 bioanalyzer´s electrodes was necessary. For that, it started by pipetting 350µl of RNaseZAP® into an electrode cleaner. The cleaner was inserted into the Agilent 2100 bioanalyzer, and left there for about 1min. The electrode cleaner was removed and replaced by a new electrode cleaner containing 350µl of

RNase-free water. This 2nd cleaner was inside the bioanalyzer for 10sec before being removed. After that the bioanalyzer´s lid was left open for another 10sec, so the water on the electrodes could evaporate, before being closed once again. The following step was to prepare gel matrix. After equilibration for 30min at room temperature, 550µl of RNA 6000 Nano gel matrix were pipetted into a spin filter and later subjected to a 10min centrifugation at 1500*g. The gel was then divided in 65µl aliquots and stored at 4ºC. To continue with the procedure, the preparation of a gel-dye mixture was in order. One aliquot of prepared gel and the RNA 6000 Nano dye concentrate were left at room temperature to incubate. After that, the dye concentrate was mixed in vortex for 10sec and spin downed. Then, 1µl of dye concentrate was mixed into the 65µl gel aliquot and subjected to a 10min centrifugation at 13000*g.[126]

With a new RNA Nano chip placed on the chip priming station, 9µl of gel-dye mix were transferred to the well marked with a G inside a black circle. The station was closed and the plunger pressed until it was locked. After 30sec, the plunger was released, the station opened and 9µl of gel-dye mix were pipetted into the 2 wells marked with **G**. The following step was to load 5µl of RNA 6000 Nano marker into all the 12 sample wells and the "ladder" well. The RNA 6000 ladder was divided into 2µl aliquots. One of those was heat denaturated for 2min at 70ºC, alongside the 2µl sample aliquots intended for testing. Then, 1µl of RNA ladder was pipette into the corresponding well and the same was done for the samples. The prepared RNA chip was subjected to a 1min vortex at 2400rpm, in the IKA vortexer, and inserted in the Agilent 2100 bioanalyzer where the Eukaryote Total RNA Nano programm was ran. The integrity results (Table XXXI of the annexes) are given in the form of RNA integrity numbers (RIN) which values range from 1 (totaly degraded RNA) to 10 (intact RNA) (Figure 9).[126,127]

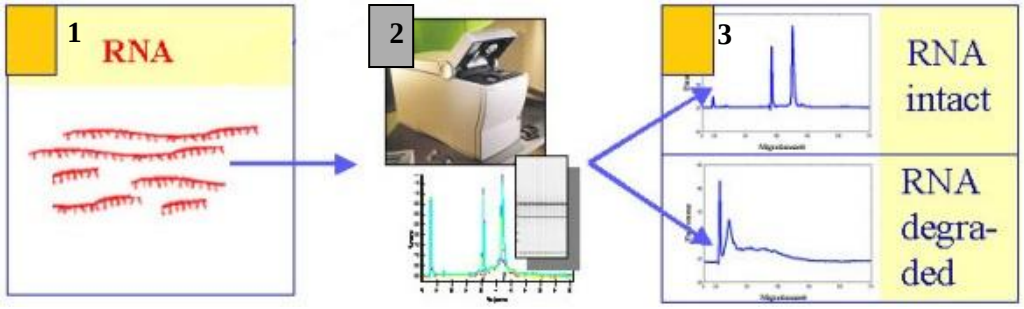


Figure 9: Application of the RNA 6000 Nano LabChip® kit. 1) Different sized RNA fragments, which will be analyzed by the Agilent 2100 bioanalyzer. 2) Agilent 2100 bioanalyzer and corresponding electrophorogram and gel results. 3) Example of intact RNA and degraded RNA electrophorograms. The degradation of the target RNA is given through the RNA integrity number (RIN) which ranges from RIN 10 (intact RNA) to RIN 1 (completely degraded RNA). Adapted from: Schroeder, Mueller *et al.* (2006).[127]

e) cDNA synthesis:

In order to synthesize the cDNA it was used the iScript™ reverse transcriptase (RT) supermix for qRT-PCR (BIO-RAD™). As the name suggests, the iScript reverse transcriptase is a version of the SuperScript™ III reverse transcriptase, which in turn is a version of the MMLV reverse transcriptase (RT) was utilized. This enzyme is a RNA dependent DNA polymerase with the purpose of transcribing long templates. Because it has a weaker RNase H activity than the AMV RT, there is no need to add an RNase H inhibitor to the reaction.

Starting from the total RNA collected previously, each reaction was prepared in 0.5ml tubes according to the following table (Table XXII). After preparing the reaction, it was subject to vortex before incubation in the BIO-RAD™ thermal cycler according to the program shown in Table XXIII.

Table XXII: Components for the cDNA synthesis reaction mix and respective volumes per reaction.

	Volume (µl)
iScript™ RT supermix	4
Total RNA	8
Nuclease-free water	8
Total	20

Table XXIII: Thermal cycler´s incubation program for the cDNA synthesis step.

	Temperature (ºC)	Time (min)
Annealing	25	5:00
Reverse transcription	42	30:00
Enzyme inactivation	85	5:00
	4	∞

After incubation, 10µl of RNase-free water were added to the cDNA samples and put on ice if needed right away. If not they were stored at -25ºC.

f) qRT-PCR amplification and relative quantification:

The final stage of the genetic expression study is the amplification and relative quantification of the synthesised cDNA through qRT-PCR. This step allows for a relative quantification of the expressed genes with lesser risk of PCR contaminaltions[128].

Here the SYBR® Green qRT-PCR method, which uses the SYBR® Green fluorochrome that binds to the amplification product, between the double stands of DNA, and emits fluorescence proportionally to the PCR amplification product, was used. This allows us to follow the kinetics and efficiency of the amplification process.

The process started by preparing the reaction mix, containing 5µl of 2x Quantitect™ SYBR® Green PCR master mix (HotStar® DNA polymerase, Quantitect™ SYBR® Green PCR buffer, dNTP mixture, SYBR® Green I and RNase free water) 1µl of 10x geNorm™ primer mix and 2µl of RNase free water, per reaction. The 8µl reaction mix was transferred to a 96well plate and 2µl of cDNA template was added to it, making for a final volume of 10µl. The plate was then sealed, mixed by vortex and centrifuged at 2000xg for 1min before being incubated in the LightCycler® 480 (Roche). The incubation ran accordingly to the Table XXIV.

Table XXIV: qRT-PCR incubation program in the LightCycler® 480 (Roche).

	Temperature (ºC)	Time (min)	Cycles
Enzyme activation	95	15:00	1
Amplification	94	0:15	50
&	55	0:30	
Signal acquisition	72	0:30	
Melting curve	95	0:15	1
	60	0:15	1
	95	Continuous	1
Cooling	40	0:30	1

The results from the qRT-PCR (Figure 10) were visualized according to two factors, the melting curves and the cycle threshold (Ct). The melting curves tell the aplification´s specificity. As for the relative quantification (RQ), it was obtained using the Pfaffl method which uses the Ct values, the cycle corresponding to the first moment when the amplification is detected, of each samples. The RQ of each gene is given throught the Ct values and the formula $RQ=2^{-\Delta Ct}$.

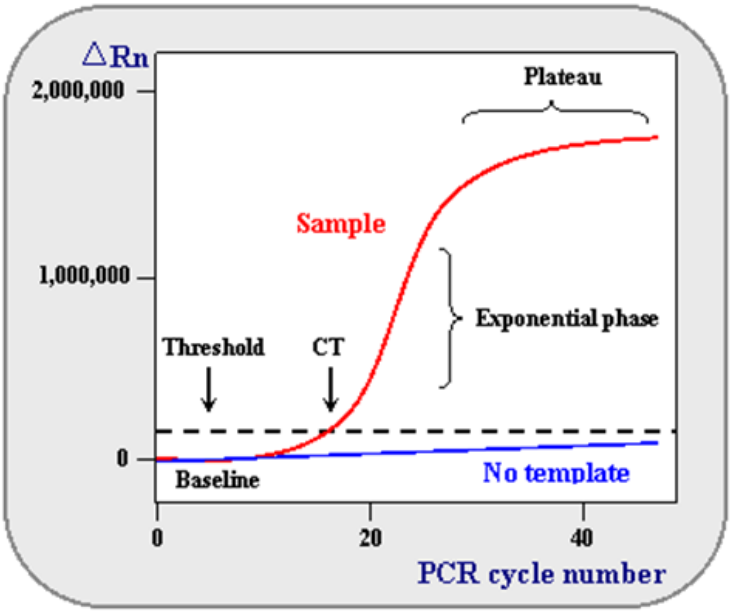


Figure 10: Generic model of the qRT-PCR plot results. The CT value, used in the Pfaffl method, is determined by the amplification cycle in which the signal of the amplification product crosses the threshold line. The earlier the CT value, the higher the genetic expression. If the amplification signal does not cross the threshold the sample is considered negative.[129]

g) Normalization and statistical analysis:

In order to determine the normalized genetic expression (NGE) of the genes in study, it is first necessary to normalize the obtained data in order to compensate for inter- and intra-kinetic RT-PCR variations, from run-to-run and from sample-to-sample. To do that, it is important to determine 2 reference genes, generally *housekeeping genes*. These 2 genes were selected by the geNorm™ program (primer design), from a pool of 5 genes: actin-β2 (ActB2), subunit 1 of splicing factor 3a (SF3A1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cytochrome C1 (CYC1) and Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (YWHAZ), subject to qRT-PCR of 13 random cDNA samples, between controls and test samples.

For this study, the 2 most suitable reference genes regarding the samples being study were ActB2 and GAPDH. Now, that the reference genes are selected, all the

samples in study were put through a qRT-PCR for these genes and the results analyzed once again by the geNorm™ program to acquire the normalization factor of each individual sample. With these values is finally possible to determine the normalized genetic expression (NGE) of the interest genes, as the NGE values are obtained through the quotient between RQ of the gene of interest and the normalization factor of the respective sample.

As for the statistical analysis of the data, it was performed with the IBM SPSS® Statistics 20 and GraphPad Prism 5 programs. The results´ normality was determined by the Kolmogorov-Smirnov test. The genes presenting a non-normal distribution were subject to a Dunn´s test and a Mann-Whitney test while the ones with a normal distributuion were submitted to a Bonferroni´s test and a Tukey´s test. The statistically significant results were considered for a p-value < 0.05.

E. HLA typing study

a) Genomic DNA extraction

This process starts with the extraction of 200µl of blood, from the sample EDTA tubes, into 1,5ml eppendorfs. To facilitate this extraction it is used the automatic dispenser HAMILTON® MICROLAB STARlet. This allowed for a faster and more precise pipeting of the samples.

Next, the eppendorfs containing the blood were set into the BIOROBOT M48 (QIAGEN®) for DNA extraction. The protocol followed was the one from the MagAttract® DNA Blood Mini M48 kit (QIAGEN®, 2012), Figure 11. This kit uses the MagAttract® technology, a silica-based DNA purification with the help of magnetic particles. This protocol uses magnetic particles coated with silica to which the DNA will bind in the presence of a chaotropic salt. The DNA is then washed and ringed to increase its purity. Finally, it is eluted in provided water into 1.5ml eppendorfs. After the extraction, the eppendorfs containing the purified DNA are put in ice for immediate use or stored at -25ºC if not needed right away.[130]

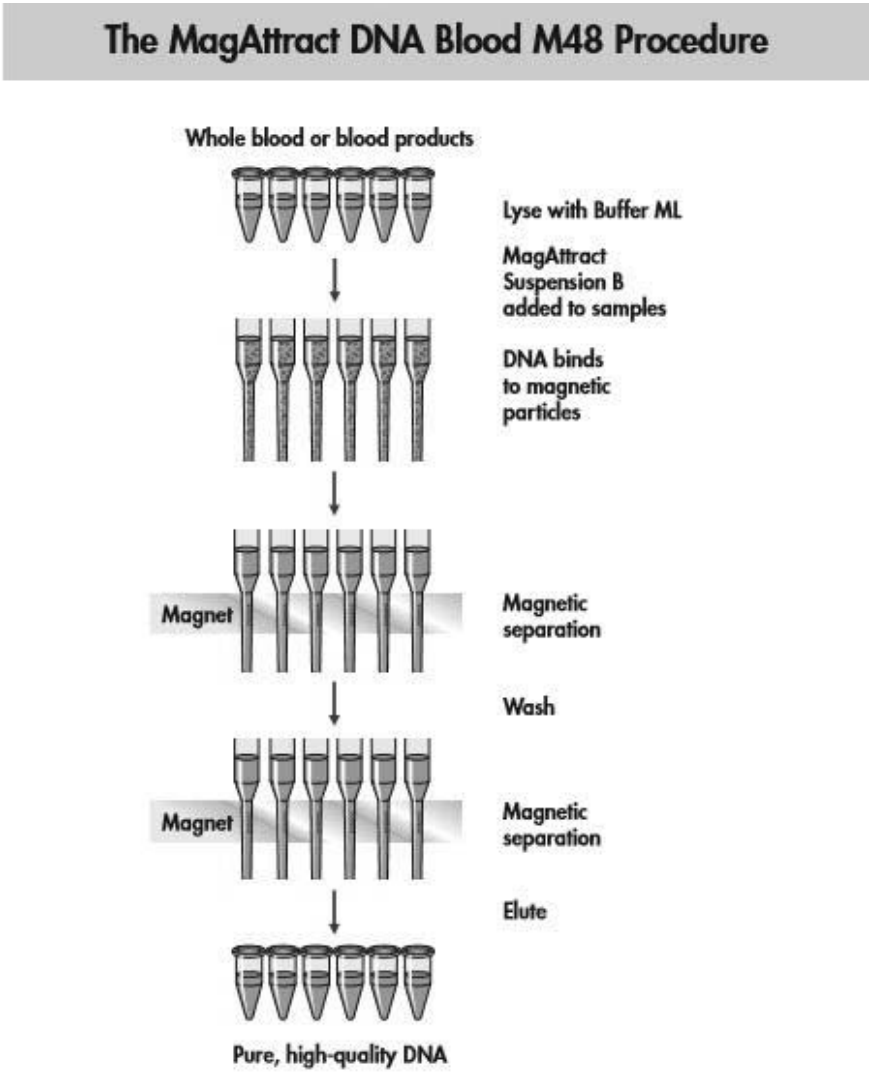


Figure 11: Schematic of the MagAttract® DNA extraction. Steps involved in the DNA extraction protocol, from cell lyses to DNA elution, used through the MagAttract® DNA Blood Mini M48 kit.[130]

b) HLA loci amplification

From now on, the proceedure follows the Labtype® SSO Typing tests. These tests use the reverse SSO DNA typing method with the Luminex® technology applied to it. Here the DNA extracted is amplified through PCR using specific primers. The amplification product is also biotinylated in the process to allow its detection with R-Phycoerythrin-conjugated Streptavidin (SAPE). After amplification the amplification

product undergoes denaturation and rehybridised to complementary DNA probes conjugated to microspheres coded with fluorescence. Then, the LABScan100™ (Luminex® 100/200) identifies the intensity of the individual microsphere´s fluorescence, the reaction pattern, which is compared to published HLA gene sequences´ associated patterns, determining the HLA typing of the samples.[131] The principle and procudure is illustrated in Figure 12.

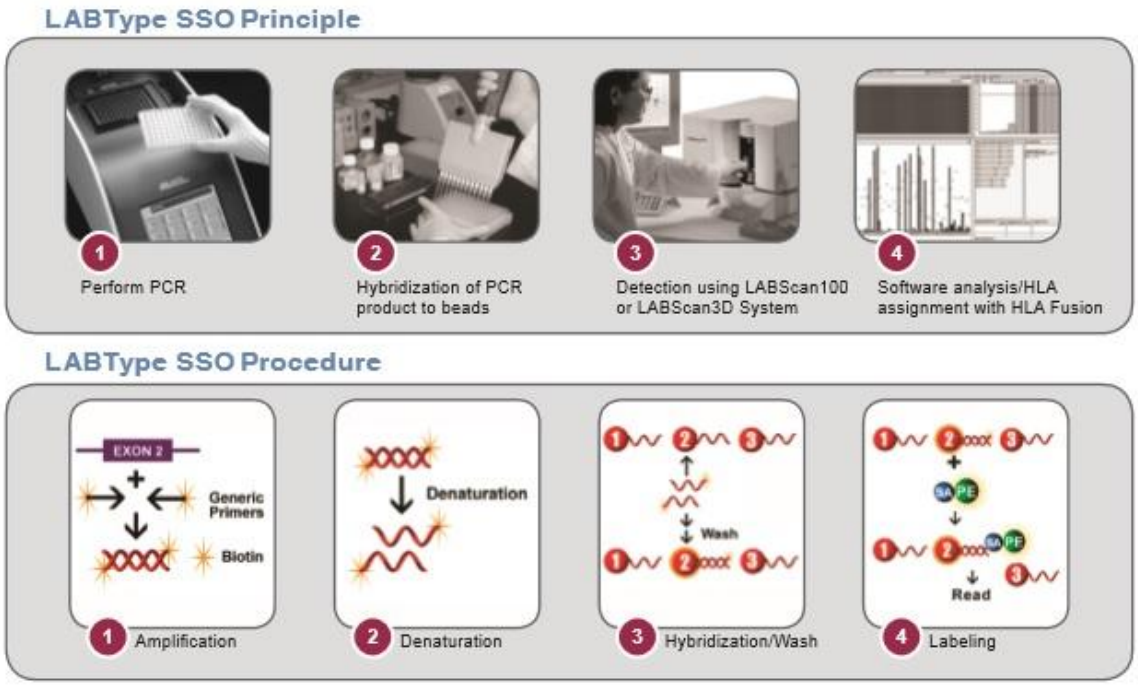


Figure 12: Schematics for the LABType™ SSO DNA typing principle and procedure.[132]

Starting by the amplification of target HLA loci, it is intended to study the class I loci HLA-A; HLA-B and HLA-C; as well as the class II loci HLA-DQ (HLA-DQA1 and HLA-DQB1) and HLA-DR. So, 5 different amplification reactions were prepared, one for each locus, per DNA sample. For each reaction, 1µl of DNA was mixed with 9µl of reaction mix. The different reaction mixes were prepared accordingly to Table XXV. Each mix designation on the mentioned table follow the name in the kit used for the target locus, One Lambda Labtype™ rSSO1A, for locus A, One Lambda Labtype™

rSSO1B, for locus B, One Lambda Labtype™ rSSO1C, for the C locus, One Lambda Labtype™ rSSO2Q for HLA DQ and One Lambda Labtype™ rSSO2B1 for HLA DR.

Table XXV: Reaction mix´s components for the amplification of the different loci, with respective volumes per reaction.

Volumes (µl)	rSSO1A	rSSO1B	rSSO1C	rSSO2Q	rSSO2B1
D mix	6.9	6.9	6.9	6.9	6.9
Amplification Primers	2	2	2	2	2
Amplitaq® DNA polymerase[a]	0.1	0.1	0.1	0.05	0.05
Total volume	9	9	9	8.95	8.95

[a]Invitrogen™, Applied Biosystems® by life technology™

The reactions were prepared in 96well PCR® microplates, AXYGEN® and incubated in the Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®) according to Table XXVI.

Table XXVI: Incubation program for the amplification of the HLA loci.

	Temperature (ºC)	Time (min)	Cycles
Initial denaturation	96	3:00	1
Denaturation	96	0:20	5
Annealing	60	0:20	
Extension	72	0:20	
Denaturation	96	0:10	30
Annealing	60	0:15	
Extension	72	0:20	
Final extension	72	10:00	1
	4	∞	-

c) Hybridization and reading

When the amplification if finished, the samples are denaturated and hybridized with metallic beads associated with complementary DNA speciffic to each locus, as mentioned before. The HLA hybridization protocol starts with the denaturation of the PCR product. For that, 2.5µl of denaturation buffer are added to 5µl of amplification product and the reaction is left to incubate for 10min at room temperature. This preparation and the following steps occur in a clean 96well PCR® microplate, AXYGEN®. After the incubation comes the neutralization of the reaction and hybridization of the DNA with the specific HLA loci spherical particles. In the neutralization step, 5µl of neutralizing buffer is added to each well. For the hybridization step, 17µl of hybridization buffer and 2µl of bead mixture, per reaction, are combined to prepare the hybridization mix which is added to each well. Then, after sealing and a through vortex at low speed, it is incubated for 15min at 60ºC in the Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®). Next come a series of 3 washing steps where 100µl of wash buffer is added and the reaction is subject to a centrifugation of 1300*g for 5min. In the end of each centrifugation, the supernatant must be removed with a swift and steady decantation to avoid loss of the beads.

The following step is a detection one, where 49.5µl of SAPE buffer and 0.5µl of SAPE Stock (100x), i.e. 50µl of 1x SAPE solution, are added to each well containing the hybridized DNA and the plate in put in the Peltier Thermal Cycler, DNA Enginer® (BIO-RAD®), for 5min at 60ºC, for incubation after the plate is sealed and subject to vortex. Following this, another 2 washing steps, as described above, are required.

Now, the samples are ready to be sent to the Luminex LABScan™ 100. Here 70µl of Luminex buffer are used to ressuspend the beads and the content of each well is transferred to an ELISA plate. It is this final plate that is transferred to the LABScan™ for interpretation of patterns of fluorescent signals´ intensity in HLA fusion software from One Lambda®.

d) Statistical analysis

The statistical analysis started with help from the programs HLA Fusion, Arlequin35 and Mycrosoft Excel, for the determination of the relative frequencies of HLA antigens, A, B, C, DRB1, DQA1 and DQB1; and haplotypes, DRB1-DQB1, with the latter being determined considering the linkage disequilibrium between DRB1 DQB1 antigens (annexes´ Table XXXVI). These frequencies were then transfered to the IBM SPSS Statistics 20 program where they were submitted to a Chi-Square test. The results were considered statistically significant for a p-value < 0.05.

Chapter 3:

Results &Discussion

A. Genetic variability of the HCV´s genome

a) 5´-UTR

This region was studied because of its importance for HCV replication (domains I and II) and translation (IRES region). Its high conservation (>90% between genotypes) also allows for an evaluation of the viral presence in the samples besides just evaluating mutagenic profiles.

In this study, the proceedure was unable to amplify the entirety of the 5´UTR sequence. However a considerable fragment, 244 nucleotides (nt67-311), was amplified. In this sequence is comprehended most of the domain II and it follows until the beginning of the subdomain IIIf. Amplification was verified in 10 samples, being that 8 samples amplified both domains while 2 amplified only in domain II. The amplified sequences were compared with the reference sequences of the most common HCV genotypes in Portugal, HCV-1a, HCV-1b and HCV-3a, however in the last case due to the high number of nucleotidic divergencies (over 3x the number of cases of the genotype 1) it is possible to say that the samples were not of the genotype 3 and the data was not considered. When comparing the sequences in study with the HCV reference sequences from HCV database, these were considered to be from genotype 1 (Table I). Four of the sequence put to test in HCV database showed 100% compatibility with genotype 1g, while others showed to be compatible with two genotypes, 1a and 1b. In the second case, the sequences tested are shared between the two genotypes. The samples 314215 and RLHJS did not have their genotype determined in this process due to their amplified sequence being too short which would increase the incertenty of the obtained results.

The summaries of the genetic mutations are represented in Tables XXVII and XXVIII. Do to the high conservation of this region the detected mutations were at a low number, around 4 to 5 per patient. There are several mutations related to the presence of more than one base at the same nt position which might be related to the presence of viral quasispecies. The majority of mutations are present in the domain III, as Henriques (2012) and Simões (2007) also reported[5,133]. In relationship to the reference sequences, the studied samples present 6 and 14, in HCV1a and HCV-1b respectively, mutations in dsRNA regions, as well as 32 changes in ssRNA regions and 2 deletions, in both cases. Comparing the results, the most common mutations occur at the nt positions: nt104

(C104Y), nt107 (G107A/R/-), nt175 (T175C/Y), nt204 (C204A/Y) and nt224 (G224A), with their respective frequencies shown in table XXIX. The only shown divergence from HCV-1a to HCV-1b is in nt243 where the samples match the HCV-1a reference sequence. This might indicate that these HCV samples are closer to being of the 1a genotype than the 1b.

Table XXVII: Summary of genetic mutations present in the HCV-1b 5´UTR amplified sequence, from nt67-311. The dots represent identical nucleotides to the reference HCV-1b sequence, while ? represent the unamplified sequence.

5´UTR	Domain II							Domain III										
Nt	67	76	96	98	104	107	127	145	165	175	199	204	222	224	227	243	248	309
HCV1b Ref.	C	C	A	G	C	G	T	G	A	T	T	C	G	G	T	G	T	G
605336	T	S	.	.	Y	A	.	.	.	C	.	A	.	A	.	A	.	?
410125	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	?
314215	?	.	.	.	Y	A	?	?	?	?	?	?	?	?	?	?	?	?
415465	?	.	.	.	.	R	.	.	M	C	.	Y	.	A	.	A	.	.
LMDCR	?	.	.	.	.	R	.	.	.	C	.	A	.	A	.	A	.	.
RLHJS	?	.	.	S	.	-	.	?	?	?	?	?	?	?	?	?	?	?
SPDMR	?	.	W	.	.	.	.	.	.	.	.	.	-	.	W	A	.	?
315941	?	?	.	.	Y	A	.	.	.	.	.	.	.	.	.	A	.	?
235647	?	.	.	.	.	A	.	.	.	Y	?	?	.	A	.	A	.	R
243802	?	S	.	.	Y	A	.	.	.	Y	.	A	.	A	.	A	.	R

Table XXVIII: Summary of genetic mutations present in the HCV-1a 5´UTR amplified sequence, from nt67-311. The dots represent identical nucleotides to the reference HCV-1a sequence, while ? represent the unamplified sequence.

5´UTR	Domain II							Domain III										
Nt	67	76	96	98	104	107	127	145	165	175	199	204	222	224	227	243	248	309
HCV1a Ref.	**C**	**C**	**A**	**G**	**C**	**G**	**T**	**G**	**A**	**T**	**T**	**A**	**G**	**G**	**T**	**A**	**T**	**G**
605336	T	S	.	.	Y	A	.	.	.	C	.	.	.	A	.	.	.	?
410125	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	?
314215	?	.	.	.	Y	A	?	?	?	?	?	?	?	?	?	?	?	?
415465	?	.	.	.	Y	R	.	.	M	C	.	Y	.	A	.	.	.	.
LMDCR	?	.	.	.	.	R	.	.	.	C	.	.	.	A	.	.	.	.
RLHJS	?	.	.	S	.	-	.	?	?	?	?	?	?	?	?	?	?	?
SPDMR	?	.	W	.	.	.	.	.	.	.	.	C	-	.	W	.	.	?
315941	?	?	.	.	Y	A	.	.	.	.	.	C	.	.	.	.	.	?
235647	?	.	.	.	.	A	.	.	.	Y	?	?	.	A	.	.	.	R
243802	?	S	.	.	Y	A	.	.	.	Y	.	.	.	A	.	.	.	R

Table XXIX: Comparison between genetic mutations present in the HCV-1a and HCV-1b 5´UTR amplified sequence.

Mutation (HCV-1a)	Nr. Of cases	Relative freq. (%)		Mutation (HCV-1b)	Nr. Of cases	Relative freq. (%)	
C67T	2	5,263158		**C67T**	2	4,347826	
C76S	2	5,263158		**C76S**	2	4,347826	
A96W	1	2,631579		**A96W**	1	2,173913	
G98S	1	2,631579		**G98S**	1	2,173913	
C104Y	4	10,52632		**C104Y**	4	8,695652	
G107A	5	13,15789		**G107A**	5	10,86957	
G107R	2	5,263158	21.052627	**G107R**	2	4,347826	17.391309
G107-	1	2,631579		**G107-**	1	2,173913	
A165M	1	2,631579		**A165M**	1	2,173913	
T175C	3	7,894737		**T175C**	3	6,521739	
T175Y	2	5,263158	13.157895	**T175Y**	2	4,347826	10.869565
A204C	3	7,894737		**C204A**	3	6,521739	
A204Y	1	2,631579	10.526316	**C204Y**	1	2,173913	8.695652
G222-	1	2,631579		**G222-**	1	2,173913	
G224A	5	13,15789		**G224A**	5	10,86957	
T227W	1	2,631579		**T227W**	1	2,173913	
T248C	1	2,631579		**G243A**	8	17,3913	
G309R	2	5,263158		**T248C**	1	2,173913	
Total	38	100		**G309R**	2	4,347826	
				Total	46	100	

Although some sequences were amplified, due to not being able to determine what kind of response these patients presented by the time the treatment ended, it was not possible to make a comparison of mutations between the two responses. This way, only an analisys based on wether these mutations alter or not 5´UTR´s function could be made.

Laporte and colleagues (2000)[134] report that IRES´ activity is independent from the number of mutations, that their position on the sequence is more important. The 5´ UTR has a GGG triplet in nt266-268, essencial to IRES and ribosomal 40S subunit interaction[135], as it is responsible for 40S and eIF3 recognition of loop IIIb[136]. In this study this triplet shows no modification, the same was verified by Simões (2007)[133],

which indicates an intact IRES functionality as a G→C substitution would implicate loss of function[137]. Mutations in the loops IIIb, IIId and IIIf can affect 5´UTR´s tridimentional structure as they are essencial for maintenance and conservation of IRES´ activity.

As for domain II, although it has 5 small ORF, their functionality or even if they are translated it is not known. Substitutions in nt85 and nt96 positions are reported[138]. In the 1st case, these mutations strongly inhibit IRES´ activity, but this was not detected in my study. For the 2nd, which this study detected in one sample´s sequence, mutations in this position have a moderate effect over IRES´ efficiency. It is also reported that between nt120-130, essencial for PTB protein binding to IRES, mutations compromise viral protein translation[139].

b) NS3 region

As seen earlier, the NS3 does not only have a fundamental role in the viral life cycle, replication and translation, but it is also present in viral defenses against host´s immune responses. This is an interesting target to study due to its impact in viral pathogenicity and high genetic variability which difficults DAA´s antiviral action. That is why it was proposed that a mutagenic profile was conducted in NS3 genetic sequences of HCV from chronically infected patients and the results compared with existing data and reported results[140].

In the beginning, a touchdown nested PCR program was attempted without results. A different approach was necessary. In order to test and optimize new PCR programs, and by analyzing some existent articles of successful HCV genomic replication using plasmids containing the HCV genome[98,141–145], it was attempted to get in contact with some authors of said articles without a positive response. Without a plasmid for protocol optimization with minimal sample waste it was opted to use some samples with higher viral load, i.e. higher chance of viral genome amplification, and adapted some reported successful PCR programs[16,68,146,147] to the reagents available. This time some success in amplifying the HCV NS3 sequence was observed. However, the program only worked for a very small number of samples, so it was insufficient for a significant mutagenic comparison and analysis.

This unsuccessful amplification of the HCV NS3 region might be related with some inefficiency of the available primers to successfully anneal with the highly variable NS3 gene and the long length of the amplification product, especially in the first stages of the nested PCR. So, in order to overcome this, different primers, which amplify smaller segments of cDNA, should be tested in future studies. Another reason for the unsuccessful amplification might have been the low viral load and consequent viral RNA in some samples.

B. Genetic expression of immune response mediators

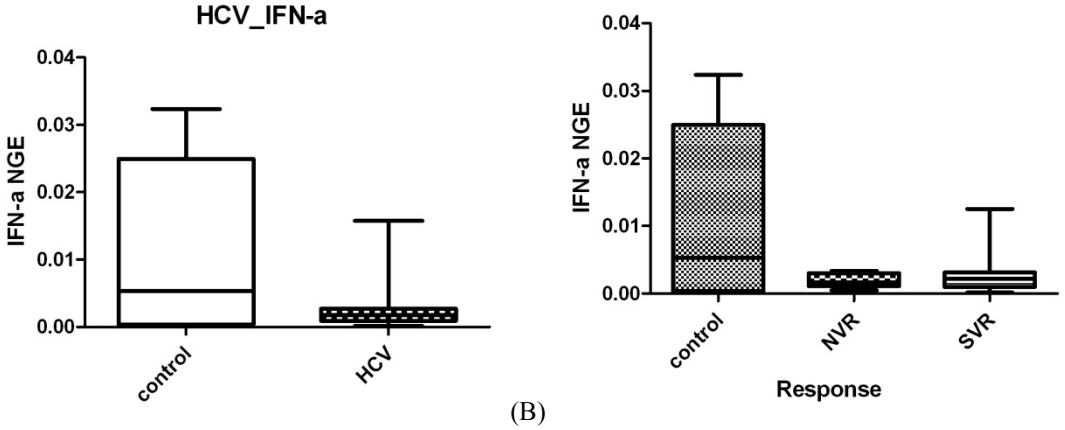
Here are discussed the obtained results for the normalised genetic expression of the following genes: IFN-α, TGF-β1, IFIT1, IFI27, OAS3, IRF2, IRF8, FOXP3 and IL-4, IL-10. The results were obtained through normalisation with the housekeeping genes ActB2 and GAPDH. The figures shown represent the normalized genetic expression of the interest genes with the indication of its median, minimum and maximum values. The genetic expression data can be seen in more detail in Tables XXXII through XXXV in the annexes chapter, for infected patients Vs controls and SVR patients Vs NVR patients Vs Controls respectively.

The RNAs used were tested for its purity, integrity and concentration in order to evaluate its quality (Table XXXI in the annexes). The RNA´s purity was measured with the NanoDrop technology values ranging from 1.51 to 2.16. Its integrity was calculated resorting to a RNA nanochip, here the RIN values ranged from 2.0 to 7.4. As for RNA´s concentration, it could be calculated from both thechniques with the results varying from one to the other, with generally higer concentrations in the nanochip approach. In the NanoDrop thechnique, the RNA´s concentration is calculated in base of the samples´ absorvance which can be influenced by the presence of other species like DNA or impurities. On the other hand, with the nanochip, the RNA fragments are subjected to electrophoresis and it is from the separated bands that the concentration is calculated. Therefore, the latter is more trustworthy. That is why the concentration values illustrated in Table XXXI diverge so much.

a) IFN-α (Interferon-alpha)

The IFN-α, encoded by this intronless gene, is a type I IFN produced by several leukocytes and even more by macrophages. Type I IFNs, alongside the other 2 IFN types are responsible for the induction of a antiviral state in cells. Besides its antiviral activity, IFN-α is also responsible for stimulating the production of a protein kinase and an oligoadenylate synthetase.[148]

When comparing the normalised expression levels of IFN-α between healthy controls and HCV infected patients it is observable that this cytokine is more expressed in the control sample (Figure 13A). However this difference is not significant, p-value > 0.05. A similar case is case is presented when dividing the infected patients according to their reponse to the pegIFN-α and ribavirin treatment (Figure 13B). Here, the expression levels in the two responses are similar but lower then the ones presented by the controls and with no statistically significant difference, p-value > 0.05.



(A) (B)

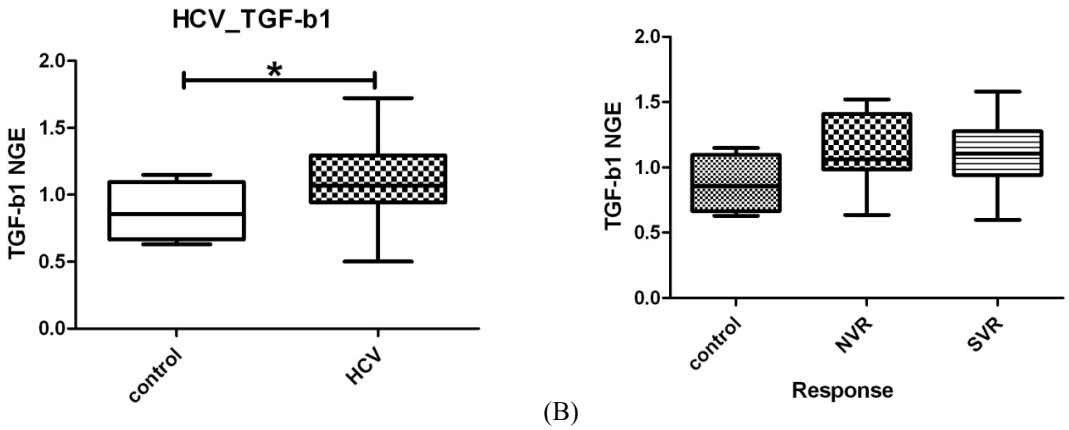
Figure 13: Graphic representation of the normalized expression levels of IFN-α, prior to treatment with pegIFN-α and ribavirin, of HCV infected patients (A) and between patients with virological or non-virological responses (B). In figure 13A there is an increase in expression of the IFN-α gene in the control samples over the HCV infected ones. However, these differences are not significant. When the infected samples are sorted according to their response to pegIFN-α/RBV, figure 13B, the same observation is made.

It is known that IFN-α plays a major role in the innate immune defence against HCV. So it is acceptable that in case of chronic HCV infection, where the host immunity was not effective in clearing the virus, the expression levels of this gene would be significantle lower in chronically infected subjects. Mukerjee and coworkers (2012)[149] have shown this very result in their study. Although my results show a tendency for this same premise, they are not yet significant, most likely due to the high standard deviation values shown by my statistical analysis.

b) TGF-β1 (Transforming growth factor beta1)

The TGF-β1 encoded by this gene is a multifunctional cytokine secreted by many cells and responsible for regulating cell differentiation, proliferation, migration and adhesion, amongst other cell functions. This protein can also regulate other growth factors in both a positive and negative way. TGF-β1 potentiates, in a concentration-dependent way, the lineage differentiation of Treg cells, when in high concentrations, or T-helper 17 (Th17) cells, in case of low TGF-β1 concentrations.[150]

The next analysed gene was the TGF-β1 gene and when comparing with healthy controls, there is a significant increase in expression in case of the infected patients, p-value < 0.05 (Figure 14A). As for the treatment outcome, a slight expression increase in non-responding patients is seen but with no statistical significance (Figure 14B).



(A) (B)

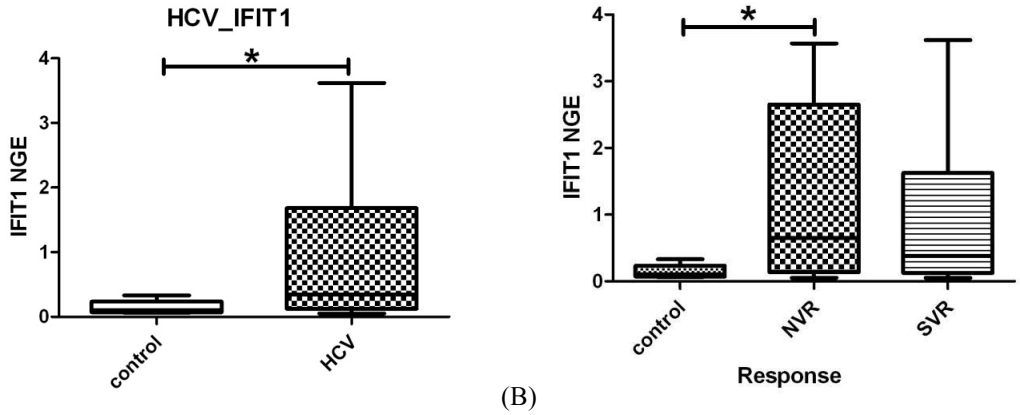
Figure 14: Graphic representation of the normalized expression levels of TGF-β1, before treatment with pegIFN-α and ribavirin, in HCV infected patients (A) and between patients with virological or non-virological responses (B). Although there is a significant higher expression of the TGF-β1 gene in infected patients over the control (A), this significance is lost when the infected samples are sorted by their response to therapy (B).

Amongst its functions, TGF-β1 is a strong profibrinogenic cytokine potentiating an accelerated liver fibrinogenesis through proliferation of hepatic stellate cells in HCV-infected people. The higher TGF-β1 levels observed in figure 14A, are indicative of its role in liver fibrosis and desease progression as HCV infected patients are prone to a HCV-induced, ROS-mediated, increase in TGF-β1 levels.[151] The higher TGF-β1 levels can also be indicative of a higher presence of Treg cells due to TGF-β1 mediated differenciation.

c) IFIT1 (Interferon-induced protein with tetratricopeptide repeats 1)

The translated protein is an interferon-induced antiviral RNA-binding one that presents tetratricopeptide repeates and may inhibit the initiation of viral replication and translation. Because IFIT1 specifically binds to single-stranded 5-triphosphate RNA (PPP-RNAs), specific from viruses, it acts as a sensor of viral RNA and as an inhibitor[152] of viral mRNA expression.

In the case of IFIT1´s expression, there is a statistically significant increase of genetic expression in infected patients (Figure 15A). This significance, p-value < 0.05, is extended to the expression of IFIT1 in non-SVR patients when compared to the controls (Figure 15B). Although there is also a slightly higher expression in responsive patients, in comparison with the controls, this difference is not significant, nor the differences between patients with different therapy outcomes, p-value > 0.05.



(A) (B)

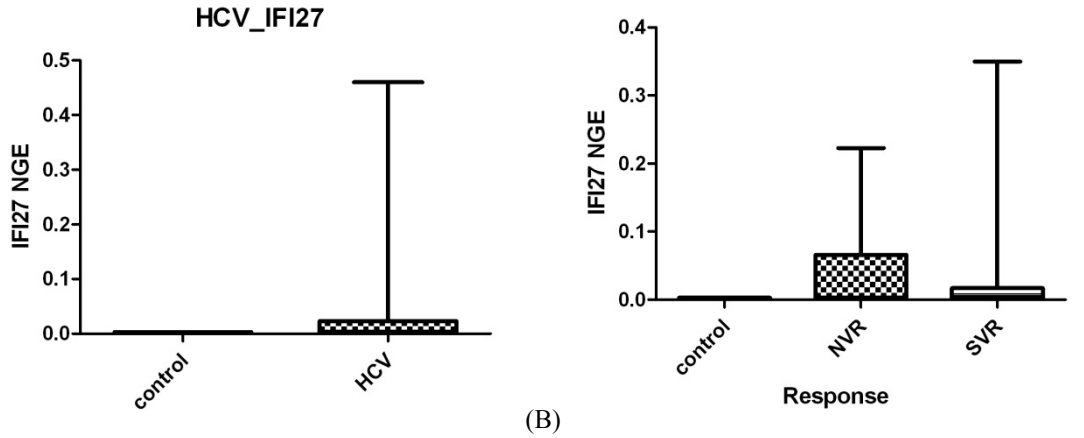
Figure 15: Graphic representation of the normalized expression levels of IFIT1, in HCV infected patients (A) and between patients with virological or non-virological responses (B), before treatment with pegIFN-α and ribavirin. In A, the IFIT1´s expression is significantly higher in infected samples. When the samples are separated by their response the expression levels continue to be significantly higher in NVR patients over the controls.

As an ISG, IFIT1 is expected to be expressed in higher levels in HCV infected patients especially in the ones who do not respond to the IFN-based therapy due to their ISG basal levels being already in nearly full induction.[112] The data is consistant with and supportive of this idea as HCV infected patients have significantly higher levels of IFIT1 expression in comparison to healthy individuals which difference is also observed for NVR patients.

d) IFI27 (Interferon alpha-inducible protein 27)

This gene encodes the protein IFI27 which functions as a cell death promoter by mediating IFN-induced apoptosis. This is characterised by the release of cytochrome C from the mitochondria as well as the activation of BAX and several caspases in a rapid and robust way.[153]

As for the IFI27 gene there are no significant differences, p-value > 0.05, in expression between infected patients and controls not between patients with different treatment outcomes. Nonetheless, there was a slight and non-significant increase of expression in HCV infected patients. (Figure 16)



(A) (B)

Figure 16: Graphic representation of the normalized expression levels of IFI27, in HCV infected patients (A) and between patients with virological or non-virological responses (B), before treatment with pegIFN-α and ribavirin. In this case, in both figures the expression levels do not present significant changes between the tested samples.

This ISG is part of the genetic signature, indicated by Asselah, Bieche and colleagues (2008)[106], predicative of treatment response. With this alongside the data from Shackel and McCaughan (2007)[112] it is espected that the IFI27 expression levels would be significantly higher in HCV infected patients who do not response to therapy.

However, the results in this study despite not baring a significant difference, p-value > 0.05, sugest a tentative inclination to higher levels of IFI27 within the NVR group.

e) OAS3 (2´-5´-Oligoadenylate synthetase 3)

This gene encodes an IFN-induced dsRNA-activated antiviral enzyme that synthesizes the 2-5-oligoadenylate dimmers which bind and activate RNase L. This activation results in the termination of viral replication, through the inhibition of protein synthesis by degradating cellular and viral RNA. Besides its antiviral activity, OAS3 also takes part in cell differentiation, growth, gene regulation and apoptosis.[154]

When analysing the gene expression for OAS-3, was found a lower expression in the control group in comparison to the infected one, p-value < 0.05. Though the normalised gene expression is higher in nonresponder patients in comparison to patients who respond to treatment, this difference is not significant, p-value > 0.05. (Figure 17)

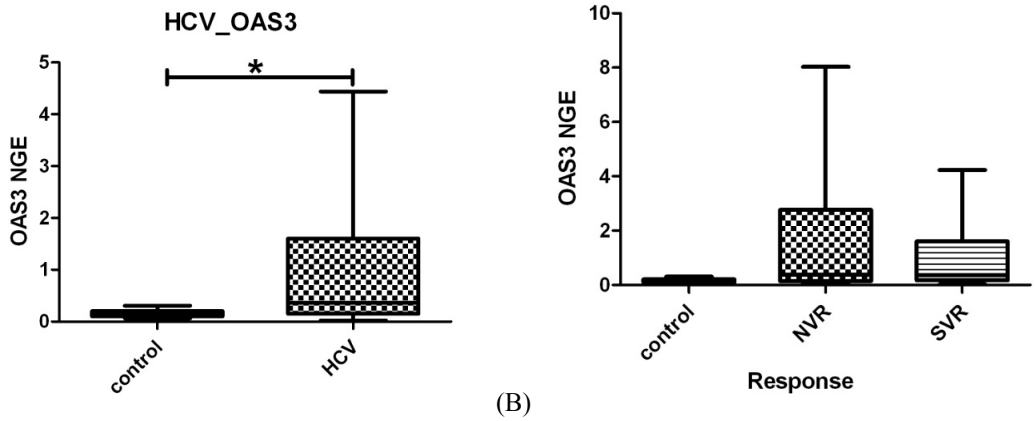


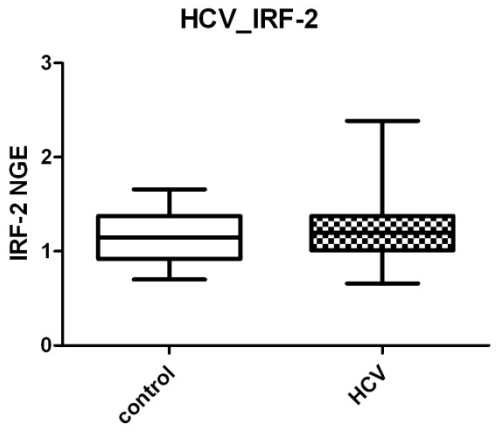
Figure 17: Graphic representation of the normalized expression levels of OAS-3 in HCV infected patients, before treatment with pegIFN-α and ribavirin (A) as well as between patients with virological or non-virological responses (B). As for the OAS3 expression, the HCV infected patients expresses this gene in a higher and significant level than the control. On the other hand, this significance is lost when the infected samples are divided for whether they develop a sustainable or non-sustainable response.

OAS3 is an ISG with an important role in protein synthesis inhibition[154] so it is expected to be strongly expressed in HCV infected individuals, especially in the ones who do not present a sustainable response[112]. Evaluating the data collected in this study it is visible that the OAS3 levels are indeed significantly increased in infected patients and that although it is not significant, there is a tendency for this increase to be translated to patients with NVR.

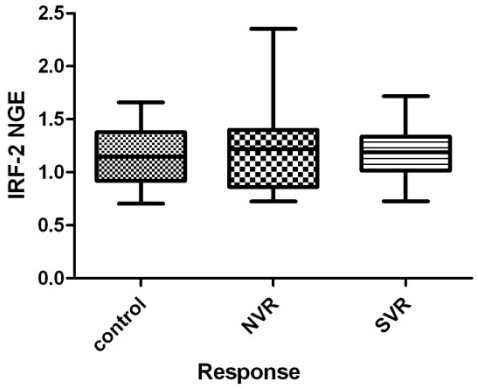
f) IRF-2 (Interferon regulatory factor 2)

This gene translates into an IFN regulatory transcription factor which acts as an antagonizer of IRF1-mediated transcriptional activation of type-I IFNs by binding to the genes in the interferon consensus sequence (ICS), IFN-inducible MHC class I and ustream regulatory region of type I IFNs genes, repressing them. It is also involved in cell cycle regulation through transcriptional activation during cell growth.[155]

Regarding the genetic expression of IRF-2, all the groups present similar levels of gene expression. This is visible for both cases, HCV infected patients Vs control group (Figure 18A) and non-responder patients Vs responder patients (Figure 18B), with no statistically significant difference, p-value > 0.05.



(A)



(B)

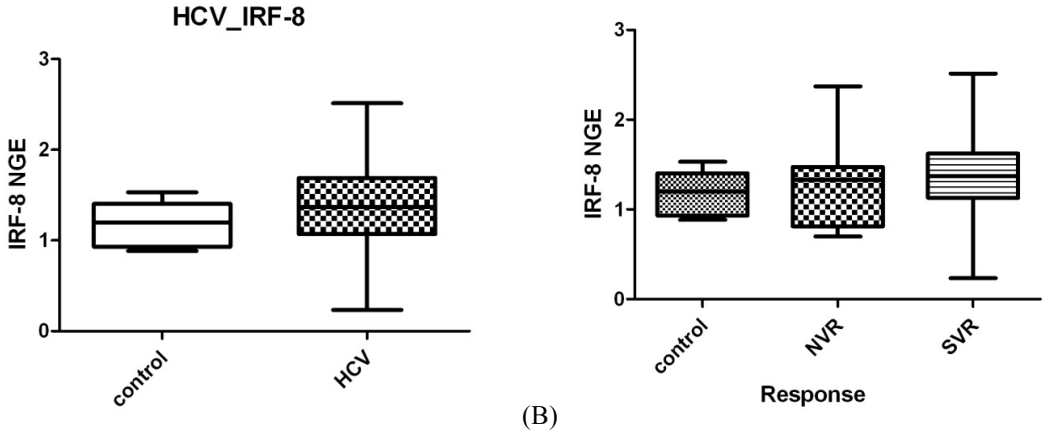
Figure 18: Graphic representation of the normalized expression levels of IRF-2 in HCV infected patients (A) and between patients with virological or non-virological responses (B), before treatment with pegIFN-α and ribavirin. Here, both in A and B the IRF-2 gene is expressed in similar levels between infected and non-infected individuals.

Regarding IRF-2, a negative regulator of type I IFN signalling and mediated gene expression, Mukherjee and colleagues (2012)[149] report higher levels of this protein in HCV chronically infected patients and Younossi and colleagues (2012)[156] defend that lower IRF-2 levels might be related to the achievement of a NVR. In the case of this study, such correlations could not be found as none of the groups in test showed significant differences from each other. Therefore, such assumptions could not be made.

g) IRF-8 (Interferon regulatory factor 8)

This encoded IFN consensus sequence-binding protein (ICSBP) is a regulatory transcription factor, whose family regulates the expression genes stimulated by type I IFNs, induced during viral infection, by binding to the ISRE. IRF-8 binds to the ICS, has a negative regulatory function in immune system´s cells and is involved in the differentiation of CD8+ dendritic cells.[157]

As for the study of IRF-8 expression it is seen a similar case to the IRF-2 expression. Here also, there is no significant differences, p-value > 0.05, between HCV infected and control groups (Figure 19A) nor between patients with different treatment outcomes (Figure 19B).



(A) (B)

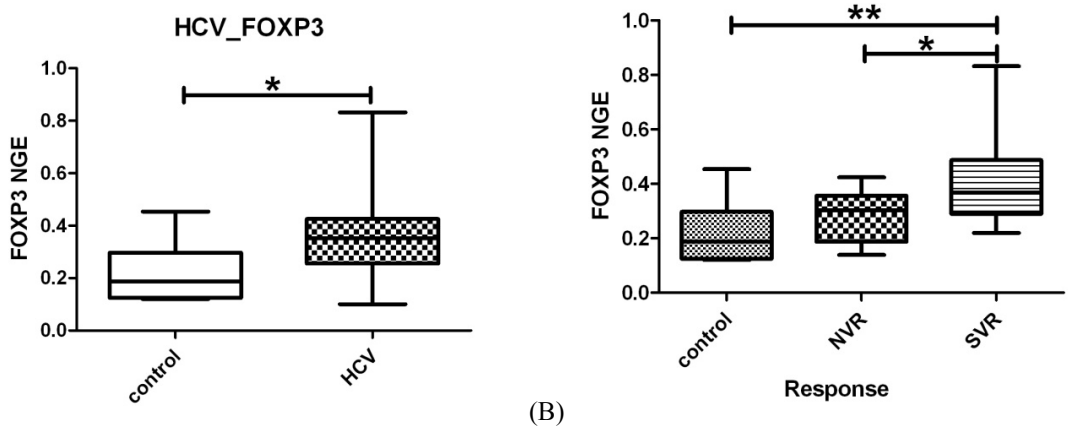
Figure 19: Graphic representation of the normalized expression levels of IRF-8, in HCV infected patients (A) and between patients with virological or non-virological responses (B), prior to therapy begining. The IRF-8 expression levels are expressed in the tested groups in a similar way, not presenting any significant differences.

IRF-8 being a nuclear protein mainly produced by pDCs and an inducer of IL-12 expression and consequently of Th1 immune response, upon infection, it is an enhancer of the immune response against HCV[158]. However the results shown in this study do not present any significant difference between studied groups. These come into accord with the results obtained by Henriques (2012)[5] where the changes in expression levels were only evident for responsive patients around the 3 month mark after the beginning of treatment. This also indicates a similar contribution of IRF-8 to the Th1 response in both groups of infected individuals in a pretreatment setting.

h) FOXP3 (Foxhead box P3)

The encoded protein, FOXP3, is a transcriptional regulator that helps maintaining the immune system´s homeostasis, being essential for the inhibitory function and development of Treg cells and for the modulation of conventional T-cells´ expansion and function. Its role in Treg´s suppressive activity accounts for the activation and repression of different genes. Furthermore, FOXP3 is an inhibitor of cytokine expression (IL2 and IFN-γ) and of T-cell effector function.[159]

In the case of FOXP3´s expression is where the differences are more significant. Here, the overall expression in HCV infected individuals is significantly higher, p-value < 0.05, than the healthy group (Figure 20A). On a more specific note, this significance is extended, to the different outcomes of pegIFN-α plus ribavirin therapy. In this situation, it is visible a statistically significant, p-value < 0.05, increase in SVR patients in contrast to individuals who do not respond to treatment. Also of notice is SVR´s very significant expression of FOXP3 in comparison to the control group, p-value < 0.01. (Figure 20B)



(A) (B)

Figure 20: Graphic representation of the normalized expression levels of FOXP3 in HCV infected patients (A) and between patients with virological or non-virological responses (B), before treatment with pegIFN-α and ribavirin. In the 1st figure, the chronically infected patients present higher expression levels of FOXP3. When, the infected samples are separated by their response to standard therapy, the significance is seen in SVR patients. The SVR patients present significantly higher FOXP3 levels than NVR patients and very significantly higher expression levels than the control samples.

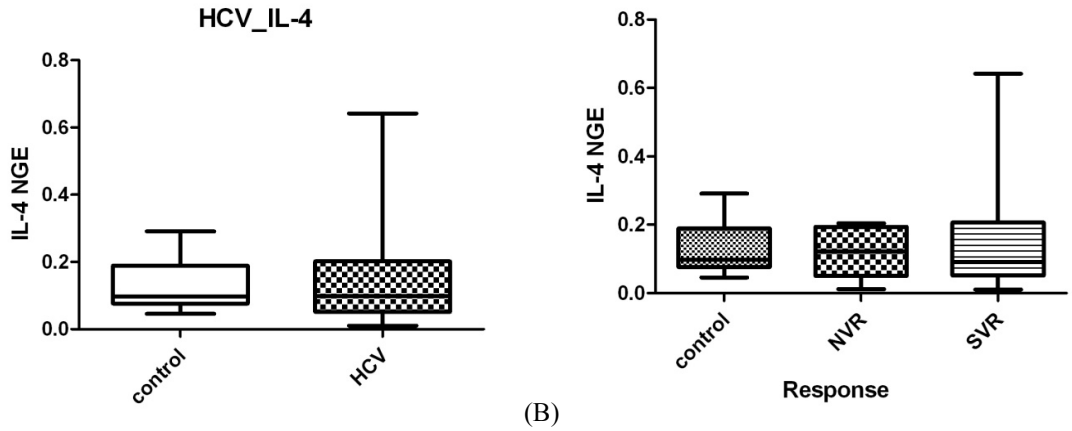
The case of FOXP3 is where most differences are noted and maybe the most controversial. Here, the results reveal significant increase in FOXP3 levels in HCV chronically infected patiens when in comparison to healthy controls. This is in consensus with the literature as the expression of FOXP3 in indicative of the presence of Treg cells which are imunosupressors of proinflammatory activity. With this in mind it is clear that this suppressor activity of efector T cells will result in viral persistence and therefore this protein would be higher expressed in patients who do not respond to treatment.[160–166] However this is not the case observed in this study as SVR patients are the ones with a higher expression of FOXP3. This apperant contradictoring information brings to mind the hypothesis that in SVR patients we might see a reversible loop of suppressive and activating activities. In order to control the imflammatory responses the activation of Treg cells comes forth, as per the presence of FOXP3. However, as this effect potenciates viral persistence an up-regulation of naive T cell activation signals will appear, e.g. through the IL-6 activation of the PI3K/AKT pathway, IL-6 production by DCs or by activation of CD80 and CD86 by APCs. This promotes the activation of T cells and therefore regulation of its suppression by Treg cells.[167] However, Henriques (2012)[5] has reported in her thesis that IL-6 expression levels were increased in NVR patients which excludes it from my hypothesis leaving the activation by APCs. The cycle continues as the persistence of a proimflammatory environment that promotes the expression of FOXP3 and Treg cell activation to counter it. In this way we could see a significantly higher FOXP3 expression in SVR patients.

i) IL-4 (Interleukin 4)

This gene translates into a pleiotropic cytokine produced by activated T-cells. IL-4 takes part in the activation processes of several cell types including B-cells. It is responsible for the induction of MHC class II´s expression on resting B-cells as well as enhancing the expression and secretion of IgE and IgG1.[168]

The next gene in focus was IL-4. Here the genetic expression was not different in either case of study. Both between patients and controls and between individuals with

different therapy outcomes, the levels of IL-4 remained identical and with no significant changes, p-value > 0.05 (Figure 21).



(A) (B)

Figure 21: Graphic representation of the normalized expression levels of IL-4, in HCV infected patients (A) and between patients with virological or non-virological responses (B) also prior to the begining of therapy. The Il-4 expression levels in both A and B do not present significant changes among the tested groups.

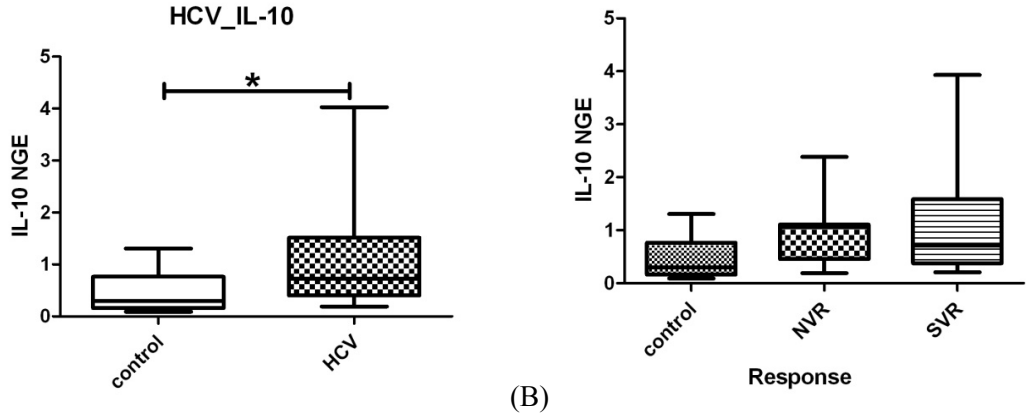
IL-4 is shown to have several effects over the immune response. It can act as an anti-inflammatory cytokine by down-regulating Th1 differenciation and potentiating a Th2 response. And it can have a pro-apoptotic effect over hepatocytes, amongst other cells.[169] Due to the none presence of any significant stimulation of IL-4 in either infected and non-infected patients, in this case it is not possible to determine if there is a relation between the treatment outcome and the activated T cell response.

j) IL-10 (Interleukin 10)

This gene encodes the protein IL-10, a cytokine produced by monocytes and lymphocytes. IL-10 is responsible for the down-regulation of Th1 cytokines, MHC class II antigens and costimulatory molecules expression on macrophages, as well as for the enhancement of B cell proliferation and survival and consequently antibody production. This cytokine is a NF-kB activity blocker and JAK-STAT pathway regulator. It also

costimulates B, T and mast cell differentiation and proliferation and is an inhibitor factor of cytokine (IFN-γ, IL2, IL3, TNF and GM-CSF) synthesis.[170]

Regarding IL-10, there is a significant difference, between HCV infected patients and healthy controls, p-value < 0.05, with the highest normalised genetic expression of IL-10 belonging to the infected patients (Figure 22A). However, this statistically significant difference disappeared when comparing the IL-10 expression with the treatment outcomes (Figure 22B).



(A) (B)

Figure 22: Graphic representation of the normalized expression levels of IL-10, before treatment with pegIFN-α and ribavirin, of HCV infected patients (A) and between patients with virological or non-virological responses (B). Although in B the there are no noteworthy changes in expression levels from any group, in A, the IL-10 expression levels are significantly increased in HCV infected patients.

Being a Th2 cytokine, IL-10 has an immune-regulatoty effect over Th1´s immune response and an anti-inflammatory effect. Therefore, higher levels of IL-10 are characteristic of Th2-mediated immune responses and with protection against immune-mediated liver damage. It is reported that this cytokine is found at high seric levels in chronically HCV infected patients and that these levels are diminished upon treatment[171]. In this way, after treatment, the IL-10 levels would be higher for NVR patients, as the therapy would have no success in clearing the infection[5]. In the results

there are in fact significantly higher levels IL-10 in infected indviduals, as expected due to the presence of a Th2-mediated immune response. However, the absence of significant differences between patients with different treatment outcomes indicates that prior to the pegIFN-α/RBV therapy, both groups present similar responses and that the IL-10 levels will evolve as the disease progresses and the therapy is administrated.

k) Overall discussion of the genetic expression results

These experiments have shown a variety in results. Some results did not show any significant difference, p-value > 0.05, like the case of IFN-α, IFI27, IRF-2, IRF-8 and IL-4. Others showed a statistical significant difference, p-value < 0.05, between the control samples and the infected patients but this difference did not carry through when the infected patients were divided according to their pegIFN-α/RBV therapy outcome, TGF-β1, OAS3 and IL-10. And yet those that showed diffences between infected and non-infected individuals and even when the infected samples were sorted into their treatment outcome, IFIT1 and FOXP3, p-value < 0.05. From the genes that showed any statistical significance most of them appeared as expected, it is the case of TGF-β1, OAS3, IL-10 and IFIT1. However in the FOXP3 case, the results obtained where not.

Combining the results from OAS3 and IFIT1, both ISGs, there is a consistance with the article form Shackel and McCaughan (2007)[112]. These genes are indicative of a non-sustainable response to treatment against HCV due to their higher expression, which rends the IFN stimulation during therapy inconsequent. When combining with tentative results from IFN-α translation it is possible to consider that the ISGs are expressed not so extensively through IFN-α signaling but rather by type III IFNs, as also seen by Thomas *et al.* (2012)[172].

As for FOXP3, these are the most controversial results as the literature shows that this gene is expressed in higher levels in patients who do not respond to treatment while the results of the present study showed otherwise, that FOXP3 is more expressed in SVR´s patients. We believe that this might be due to a suppression of the Treg inhibitory ativity by activation of effector T cells that would diminish Tregs´ role and keep the high levels of FOXP3[167].

Finally, the genes that did not show significant differences, this fact might be due to the small pool of samples, especially from NVR patients, and the obtained high standard deviation values that do not allow for an accurate statistical analysis. So, more samples need to be analysed alongside other genes. The genes studied in this thesis were chosen for being mostly connected to IFN production and modulation of IFN production. So maybe genes connected to other intervenients of the immune response like NK cells should be interesting to study.

C. HLA typing

a) Allelic study

The study of the HLA typing´s impact on the response to HCV infection started by analyzing the different HLA antigens, A, B, C, DQA1, DQB1 and DRB1 in separate and their role in both the development of chronicity and in the achievement of a sustainable virological response to the pegIFN-α and ribavirin threatment. At a second point it was performed the same analysis but with HLA DRB1-DQB1 haplotype.

In both cases the data from the HCV infected patients was assessed as a whole, side by side with a healthy population. After this first analysis the infected samples were sorted according to the response given by the patients to the standard pegIFN-α and ribavirin therapy. The new rearranged data was then placed against the same control population.

i. HLA-A

Starting with the HLA-A, the analysis of the relative frequencies of the antigens present in the HCV infected samples versus the ones in the control population (Figure23) was similar between the two populations, with no statistically significant differences, $\chi^2(18)=26.296$, $p=0.093$. In this way, there is no visible impact of the HLA-A antigens, on their own, over the development of chronic HCV infection.

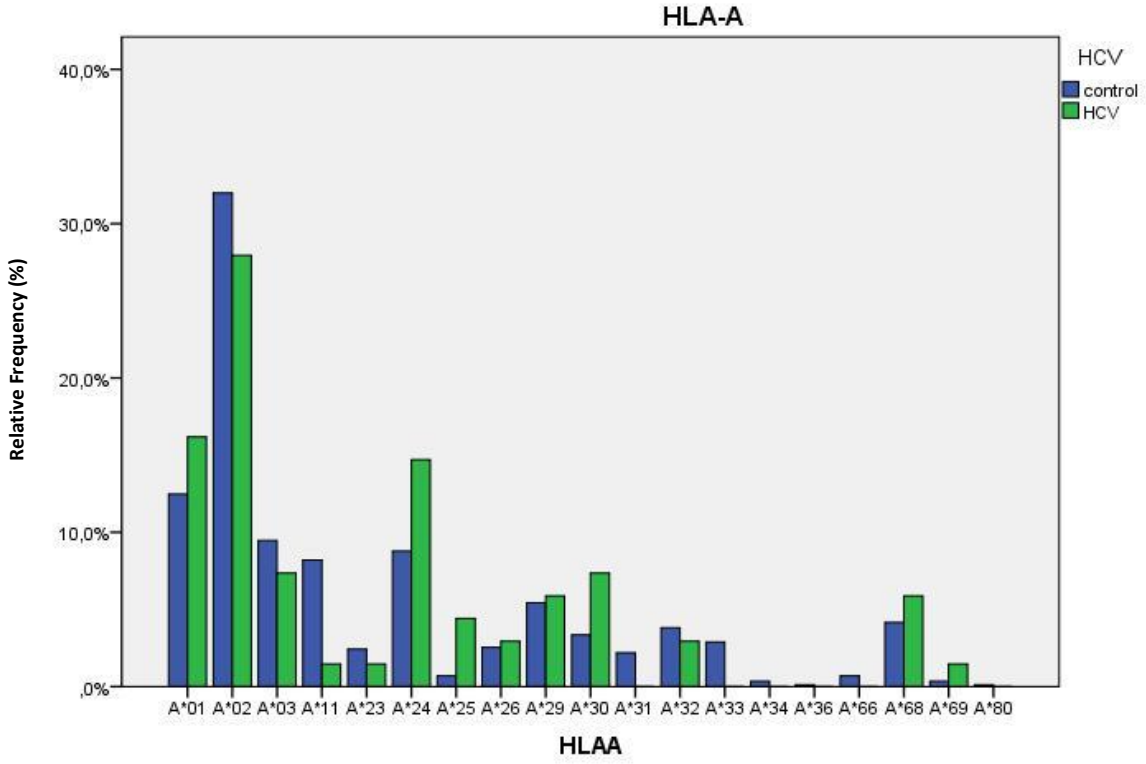


Figure 23: Relative frequencies of the different HLA-A antigens. Frequencies of the HLA-A antigens detected in the test samples versus the frequencies of the same antigens in the blood donor population (control). Here, the allelic frequencies of the HLA-A antigens from HCV infected patients are around the same values presented by the control population. Some present sighly higher frequencies, A*11, but not in a significant way, $p>0.05$.

After the separation of the samples according to their response to therapy and their comparison with each other and the control population (Figure 24), as well as before, a significant correlation between the two responses and the controls was not obtained, $\chi^2(36)=33.074$, $p=0.609$. However, a tendency for patients with HLA-A*02 to not respond to therapy could be observed.

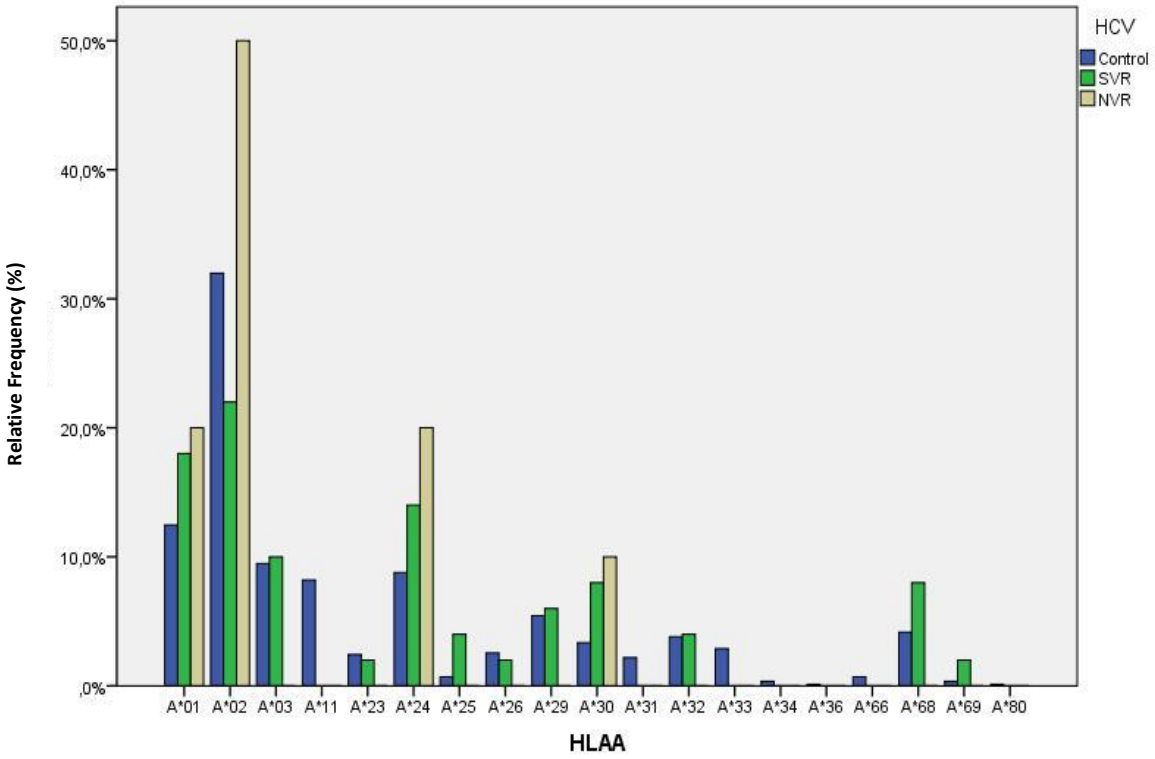


Figure 24: Relative frequencies of the different HLA-A antigens in different therapy outcomes. Frequencies of the HLA-A antigens detected from patients with different treatment responses versus the frequencies of the same antigens in the blood donor population (control). When the scenario showcases the response given by the patients towards the treatment, the biggest change happens in NVR´s A*02 relative frequency which reaches near 50%. However, in addition to the remaining frequencies the observed changes are not statistically significant, $p>0.05$.

ii. HLA-B

As for the HLA-B antigens, it also started with a more wide evaluation by placing the data from the infected patients against the healthy population (Figure 25). Through the analysis of the chi-square a significant correlation was not found, $\chi^2(27)=31.786$, $p=0.24$. Dispite that, the B*44 might be of interest as it shows an inclination for being more present in chronically infected patients.

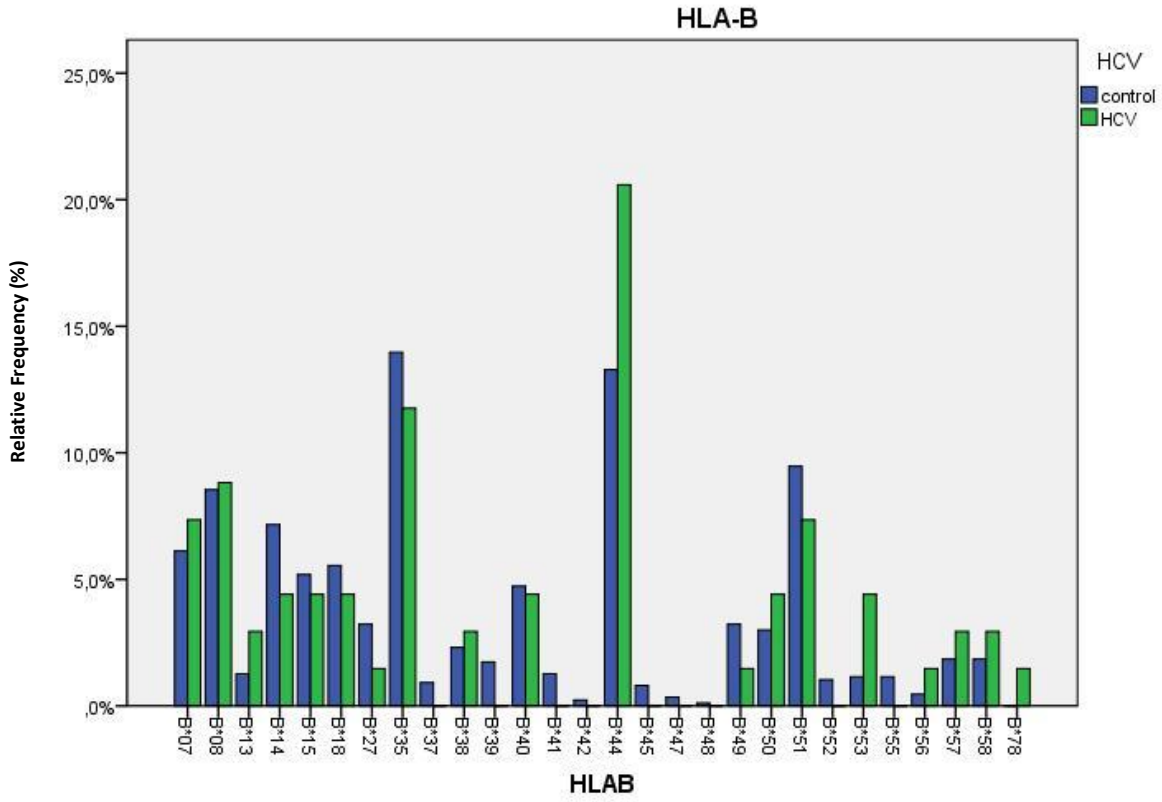


Figure 25: Relative frequencies of the different HLA-B antigens. Frequencies of the HLA-B antigens detected in the test samples versus the frequencies of the same antigens in the blood donor population (control). In this case, the B*44 antigen present a higher frequency in infected individuals. Anyway, the results do not change significantly from controls to infected samples, $p>0.05$.

After this first evaluation it was time to bring the response given by the patients to the standard HCV treatment into consideration (Figure 26). Despite some antigens appearing more frequently in NVR patients, B*13, B*14, B*18, B*40, B*51, this test has shown no statistical significance, $\chi^2(54)=51.564$, $p=0.224$.

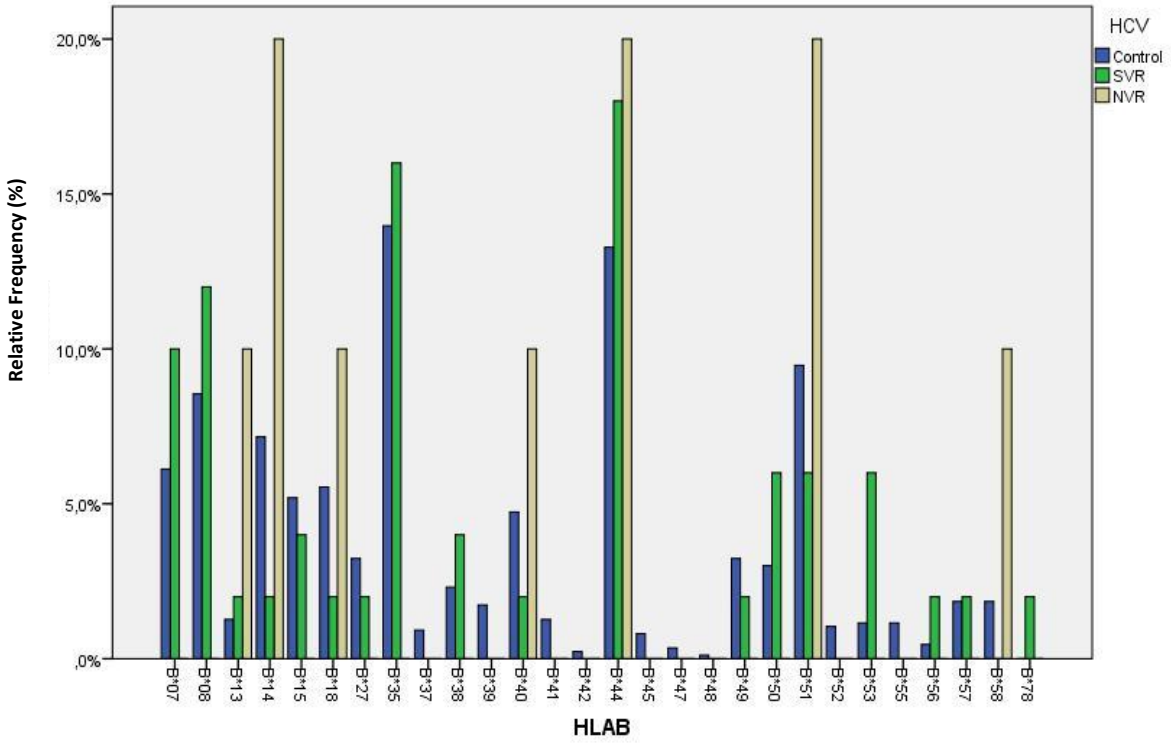


Figure 26: Relative frequencies of the different HLA-B antigens in different therapy outcomes. Frequencies of the HLA-B antigens detected from patients with different treatment responses versus the frequencies of the same antigens in the blood donor population (control). Here, the observed frequencies change a little as NVR frequencies grow higher than the the SVR and control ones. Yet, the observed differences do not provide a significant result, $p>0.05$.

iii. HLA-C

Now, for the study of the HLA-C antigens, the first analysis (Figure 27) did not show any evidence of a relation between these antigens and the devdelopmento of chronic HCV infections, $\chi^2(12)=3.236$, $p=0.994$. As for the SVR and NVR analysis (Figure 28), there were also no significant results $\chi^2(24)=21.301$, $p=0.621$. Yet some could say that the antigens C*05, C*08 and C*14 tend to be more present in NVR patients.

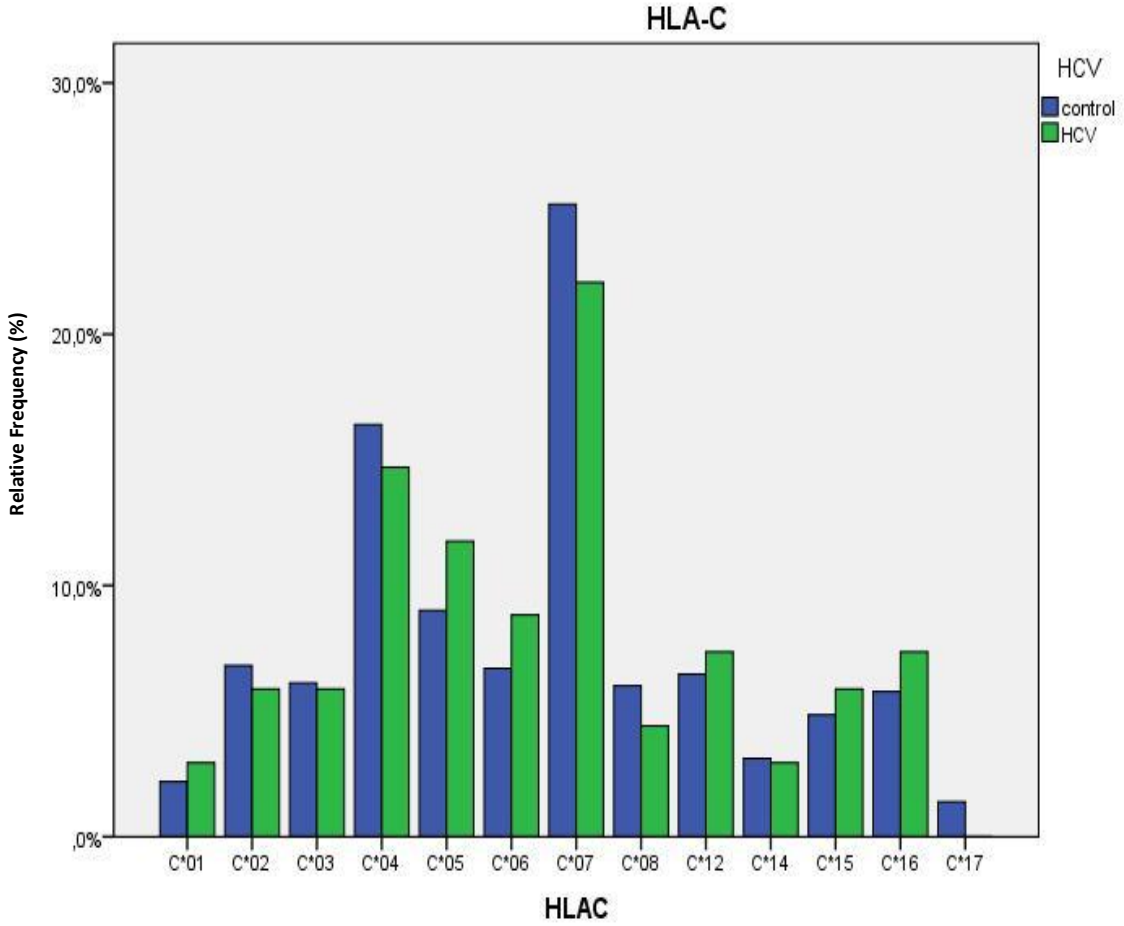


Figure 27: Relative frequencies of the different HLA-C antigens. Frequencies of the HLA-C antigens detected in the test samples versus the frequencies of the same antigens in the blood donor population (control). In this figure, the results illustrated are very similar to each other resulting in a non-significant evaluation, $p>0.05$.

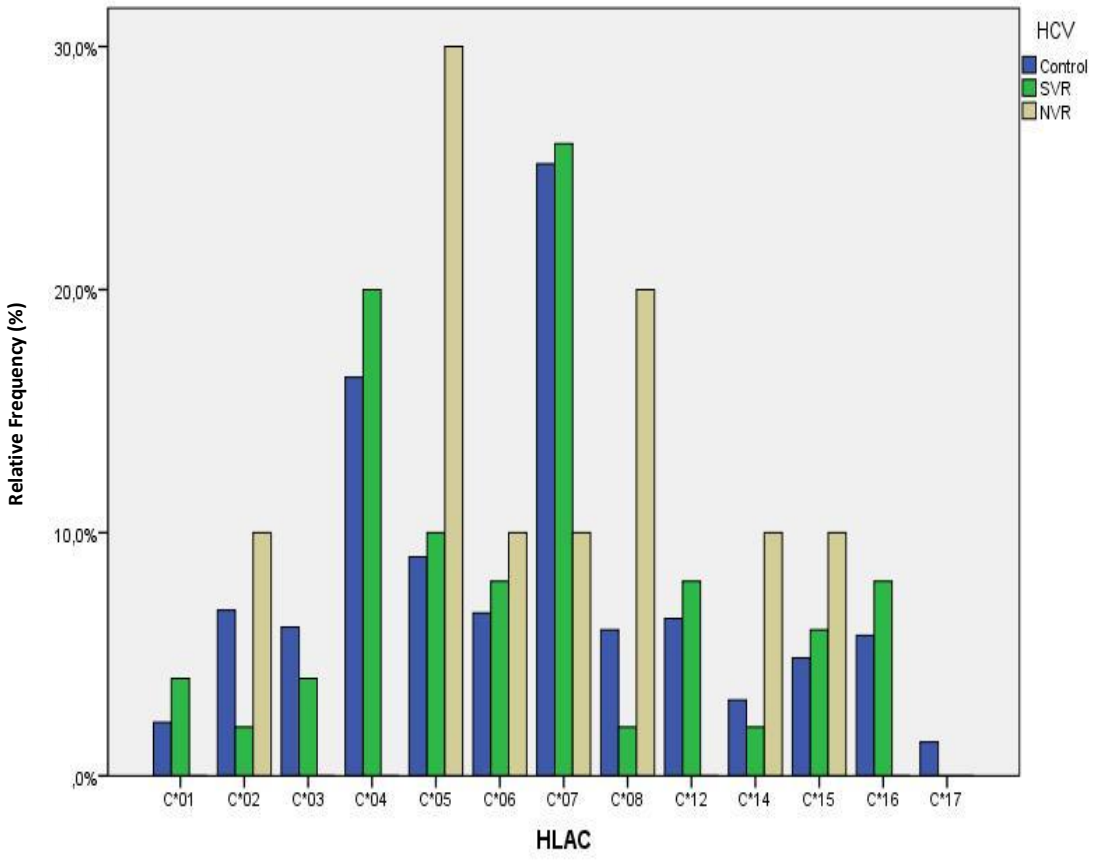


Figure 28: Relative frequencies of the different HLA-C antigens in different therapy outcomes. Frequencies of the HLA-C antigens detected from patients with different treatment responses versus the frequencies of the same antigens in the blood donor population (control). Here the frequencies´ display change with NVR presenting increased frequencies, than SVR and controls, in C*05, C*08 and C*14, and lower in C*07. However, even with these results their disparity is not significant, $p>0.05$.

iv. HLA-DRB1

Following to the HLA class II antigens and beginning with HLA-DRB1, the results show are also unable to determine if there is a genetic predisposition for developing chronic infection (Figure 29), $\chi^2(15)=11.320$, $p=0.73$. The same can be seen in Figure 30, where the infected samples were divided for their response to therapy, $\chi^2(30)=18.891$, $p=0.942$. The only antigens of appearant interest are DR*01, DR*12 and DR*14 for NVR patients and DR*13 for SVR ones.

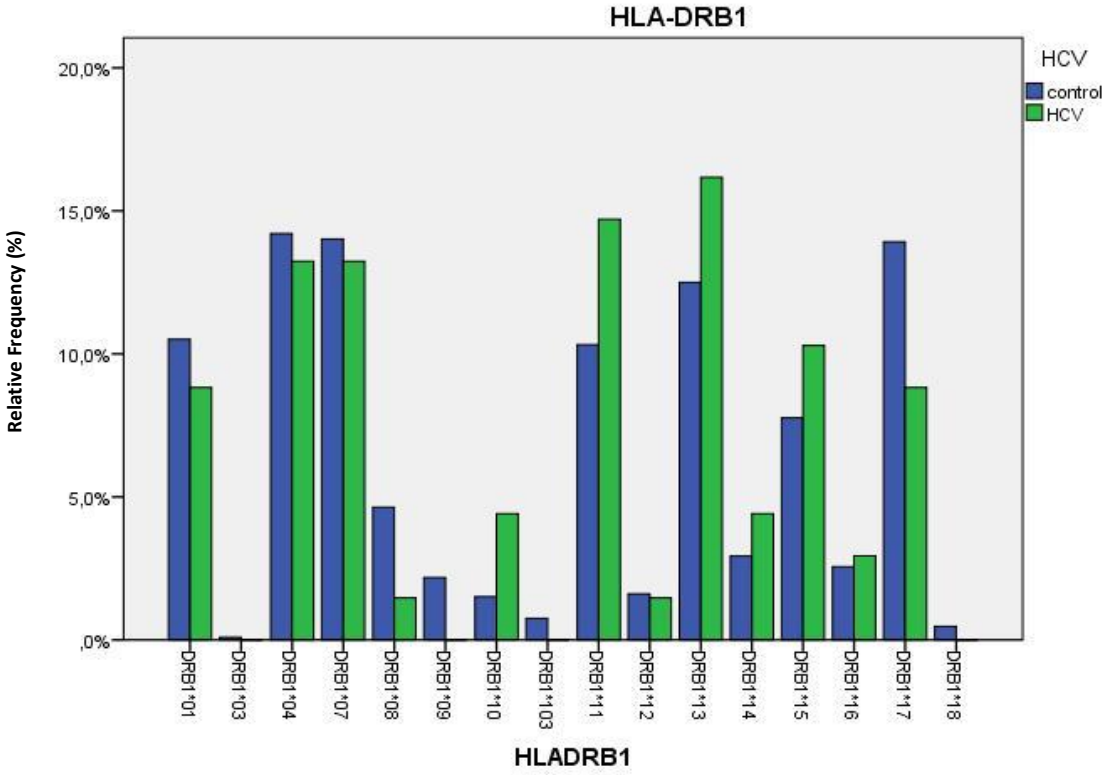


Figure 29: Relative frequencies of the different HLA-DR antigens. Frequencies of the HLA-DR antigens detected in the test samples versus the frequencies of the same antigens in the blood donor population (control). In this case, the results are shown in a similar way as the previous control Vs HCV infected figures. And here also, the differences are nor statistically significant, $p>0.05$.

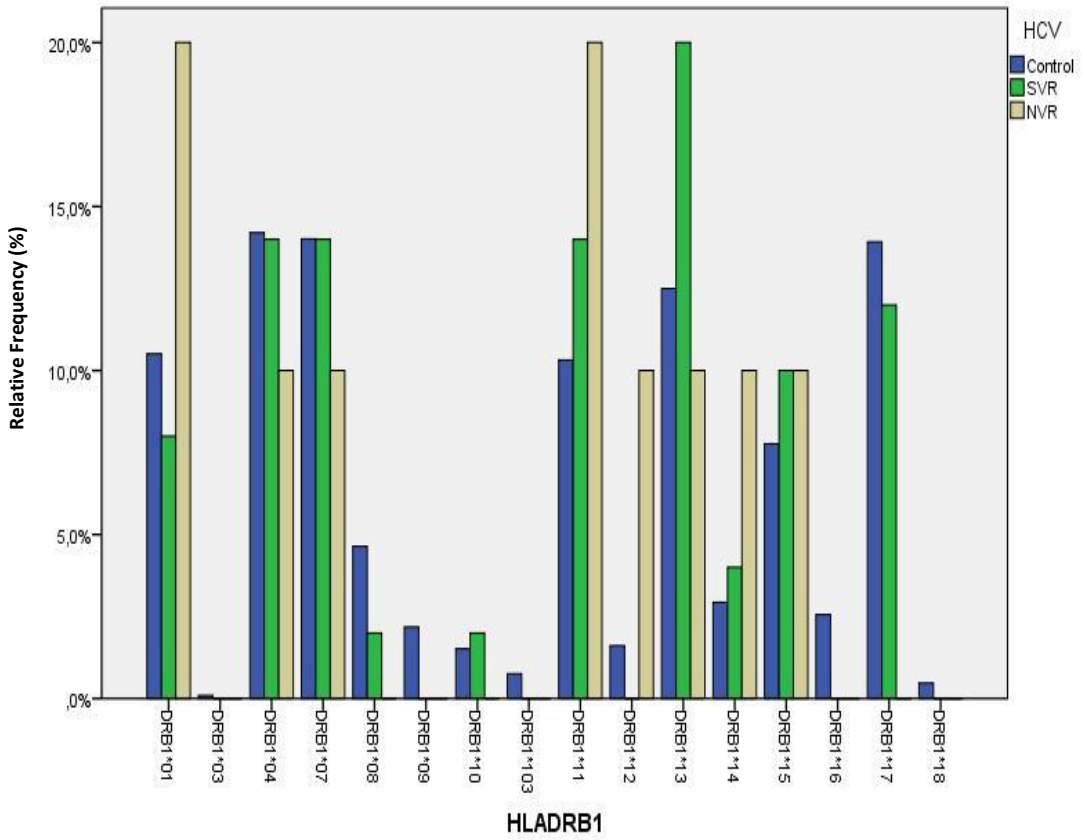


Figure 30: Relative frequencies of the different HLA-DRB1 antigens in different therapy outcomes. Frequencies of the HLA-DRB1 antigens detected from patients with different treatment responses versus the frequencies of the same antigens in the blood donor population (control). DRB1*01, DRB1*11 and DRB1*14 appear to have increased occorence in NVR patients while DRB1*13 is more present in SVR individuals. Anyhow, the illustrated results are not different in a significant way, $p>0.05$.

v. HLA-DQA1

Analyzing both parts of the study of the HLA-DQA1 antigens, infected Vs healthy (Figure 31) and SVR Vs NVR Vs Control (Figure 32), no conclusion could be made. This because in both cases the results did not show any significant differences that would allow for an association between antigens and the infection, $\chi^2(5)=4.259$, $p=0.513$, or the response to therapy, $\chi^2(10)=2.994$, $p=0.982$.

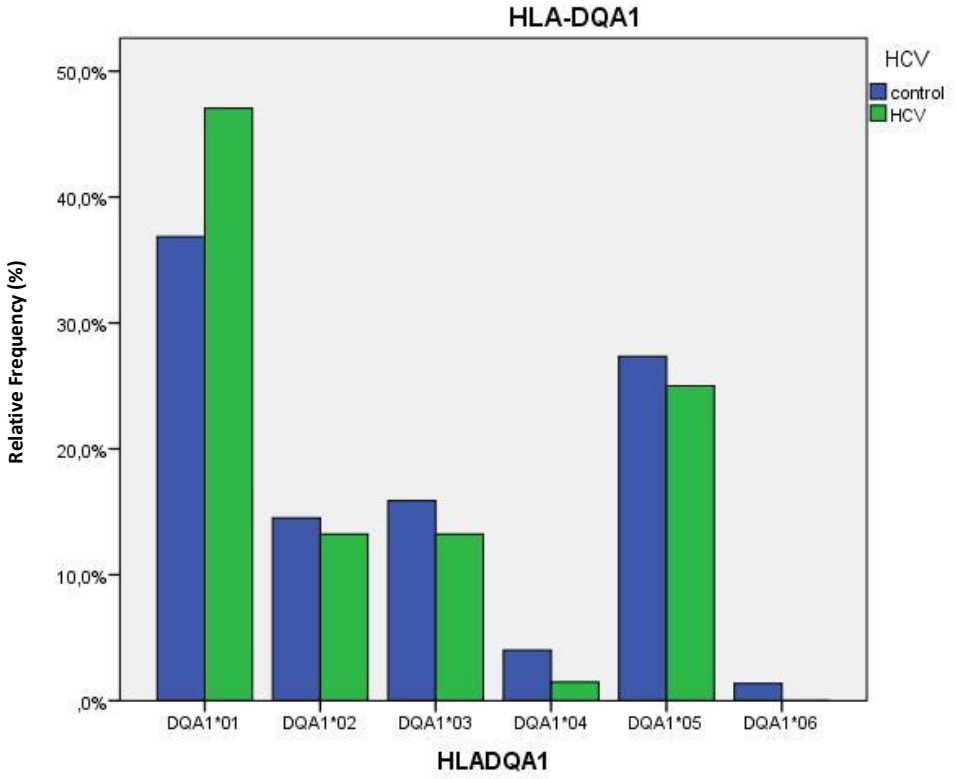


Figure 31: Relative frequencies of the different HLA-DQA1 antigens. Frequencies of the HLA-DQA1 antigens detected in the test samples versus the frequencies of the same antigens in the blood donor population (control). The results shown are very similar to each test group. Therefore no significant changes, $p>0.05$, can be identified.

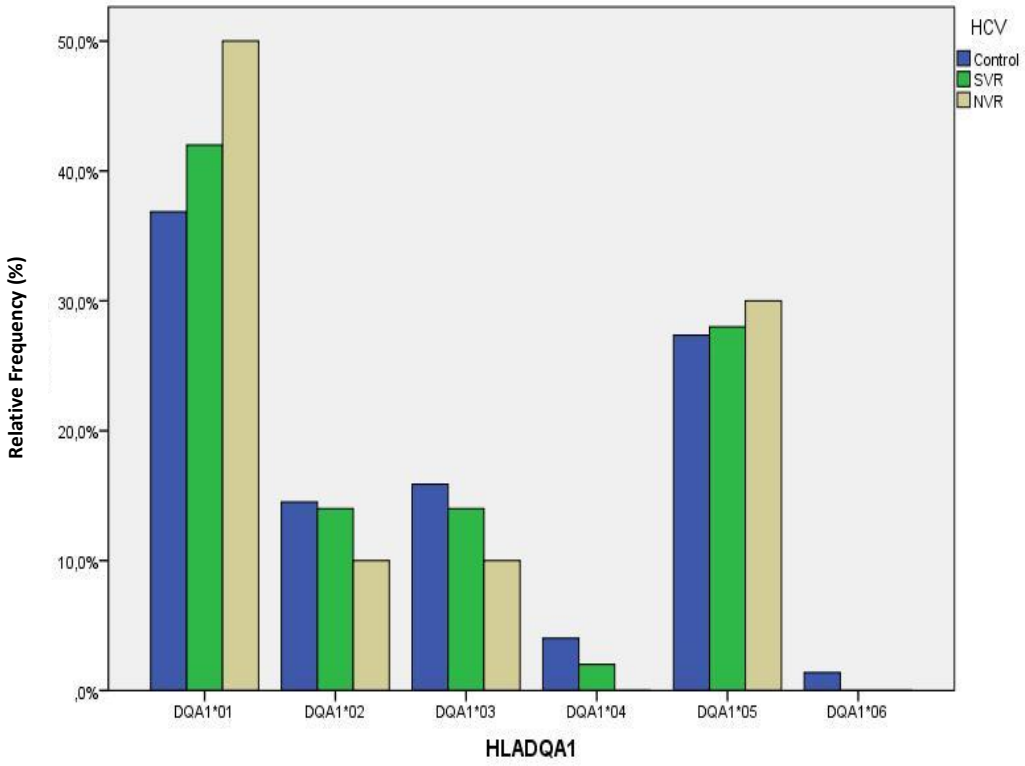


Figure 32: Relative frequencies of the different HLA-DQA1 antigens in different therapy outcomes. Frequencies of the HLA-DQA1 antigens detected from patients with different treatment responses versus the frequencies of the same antigens in the blood donor population (control). Even when the samples infected with HCV are sorted by their response to therapy the allelic frequencies remain very similar between the tested pools. In this way, they are not statistically significant, $p>0.05$.

vi. HLA-DQB1

A similar case can be made for the HLA-DQB1 typing study. In the wider study (Figure 33) no association could be made due to the non-statistically significant data, $\chi^2(6)=4.149$, $p=0.657$. In the response test (Figure 34), apart from some non-significant tendencies, DQB1*05 and DQB1*07 for NVR patients, no statistically significant conclusion could be made, $\chi^2(12)=7.052$, $p=0.854$.

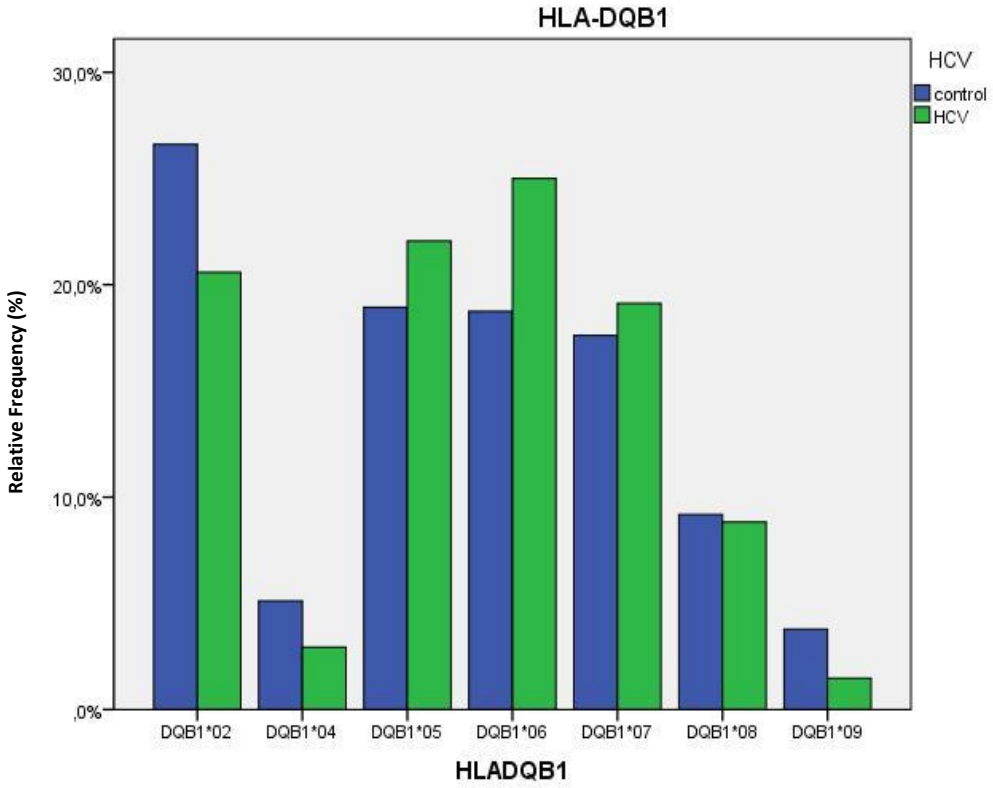


Figure 33: Relative frequencies of the different HLA-DQB1 antigens. Frequencies of the HLA-DQB1 antigens detected in the test samples versus the frequencies of the same antigens in the blood donor population (control). Here, the allelic frequencies between controls and infected patients do not present significant disparities, $p>0.05$.

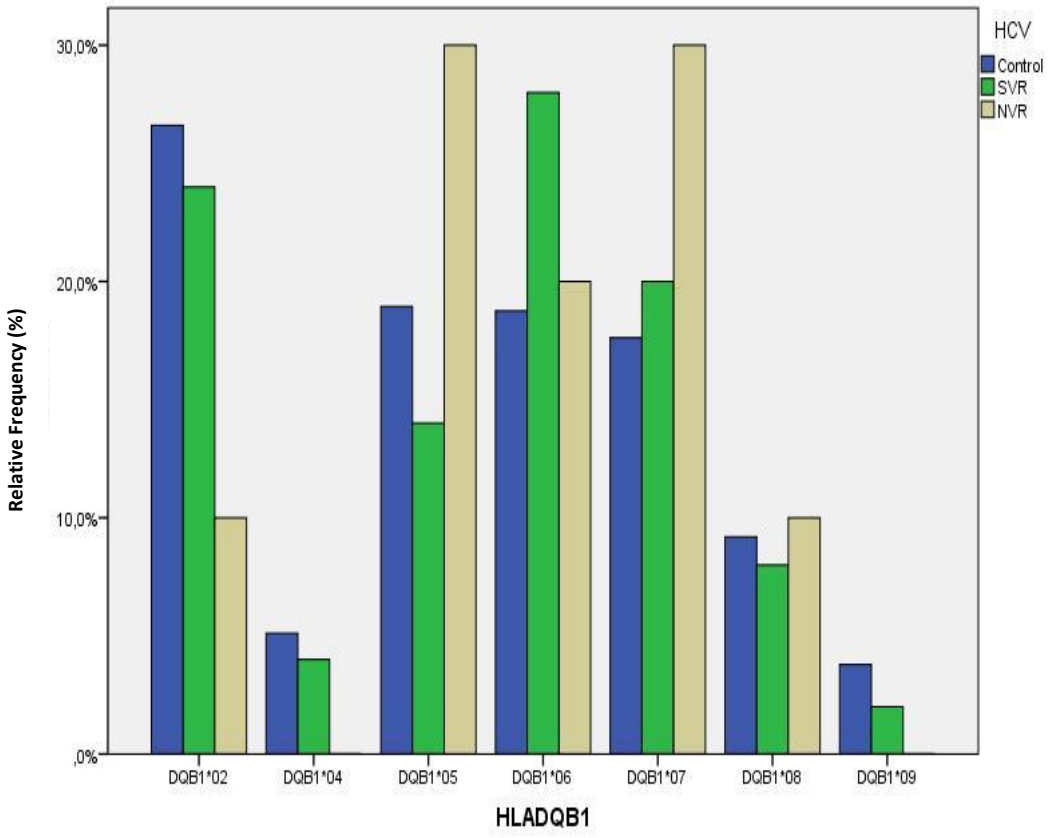


Figure 34: Relative frequencies of the different HLA-DQB1 antigens in different therapy outcomes. Frequencies of the HLA-DQB1 antigens detected from patients with different treatment responses versus the frequencies of the same antigens in the blood donor population (control). Bringing the response outcomes to the table the results shift a little, with NVR patients showing higher frequencies of DQB1*05 and DQB1*07. And yet, the presented disparities are not significant, $p>0.05$.

vii. HLA typing – overall discussion

Analyzing the typing results there was no case where a correlation, between the patients´ genetic information and the development of a HCV chronic infection or the standard pegIFN-α/RBV treatment´s outcome, could be made. Nonetheless, several studies in this field have been made with some interesting results regarding a genetic predisposition from the patients to clear the viral infection or let it evolve into a chronic infection[64,113–116].

The results present in this study were not as expected, as some assoiciations between HLA typing and HCV infection were already reported in the before mentioned literature, especially regarding HLA-DRB1 and –DQB1. To this fact might have contributed the limited sample pool, as especially the number of NVR samples was very small. So, a study with a wider sample pool would have helped shine a better light over these results.

b) Haplotipic study

In this study, it started from the HLA typing results and determined the DRB1-DQB1 haplotypes according to the respective linkage disequilibrium, i.e. the non-random allelic association at different loci[173].

First, the HCV infected patients were campared with the control population (Figure 35). Here, some tendencies, like DRB1*11-DQB1*07 and DRB1*13-DQB1*06, could detected, being higher in infected individuals and DRB1*17-DQB1*02 being increased in healthy people. However, none of the frequencies detected has shown to be of any statistical significance, $\chi^2(36)=18.839$, $p=0.992$. Therefore, it was not possible to affirme that there is a genetic predisposition for developing chronic hepatitis C.

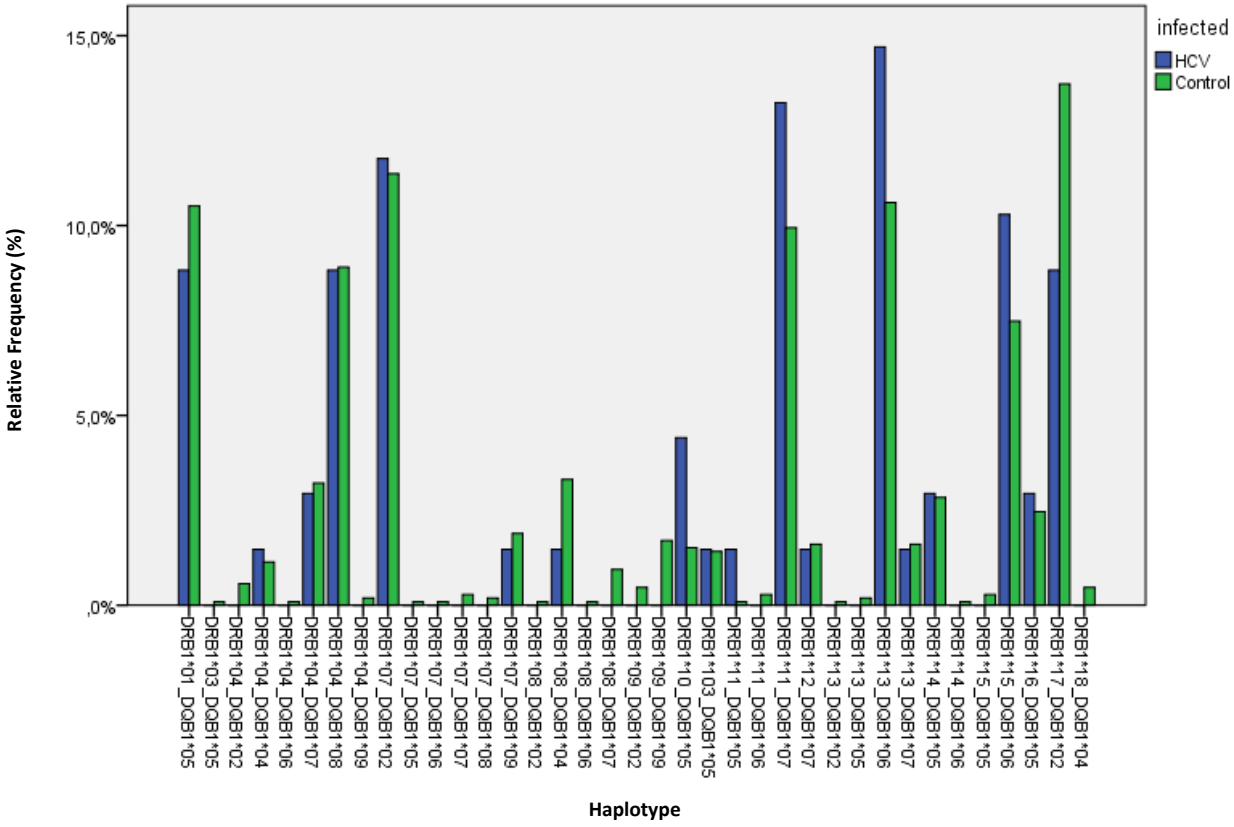


Figure 35: Relative frequencies of the different DRB1-DQB1 haplotypes. Frequencies of the HLA- DRB1-DQB1 haplotypes detected in the test samples versus the frequencies of haplotypes present in the blood donor population (control). These results illustrate that most of these haplotypes have identical relative frequencies. Whlie in some cases, like DRB1*10-DQB1*05 and DRB1*17-DQB1*02, the haplotype is more frequent in HCV infected patients or in the control population, respectively. Still, the observed results do not exhibit significant differences, $p>0.05$.

Meanwhile, the previously analysed samples were divided accordingly to their response to the standard pegIFN-α and Ribavirin treatment (Figure 36 and 37). Here, the frequency distribution changed a little but yet with no statistical significance, $\chi^2(72)=22.624$, $p=1.000$ and $\chi^2(14)=9.989$, $p=0.763$ (respectively for figures 36 and 37). The only notes of mention are speculative as patients with DRB1*01-DQB1*05, DRB1*11-DQB1*07 and DRB1*14-DQB1*05 haplotypes show a tendency for a non-sustainable response while individuals with the DRB1*13-DQB1*06 haplotype tend to

develop a sustained response. Again, no statement about the existence of a genetic predisposition for developing either a SVR or a NVR can be made.

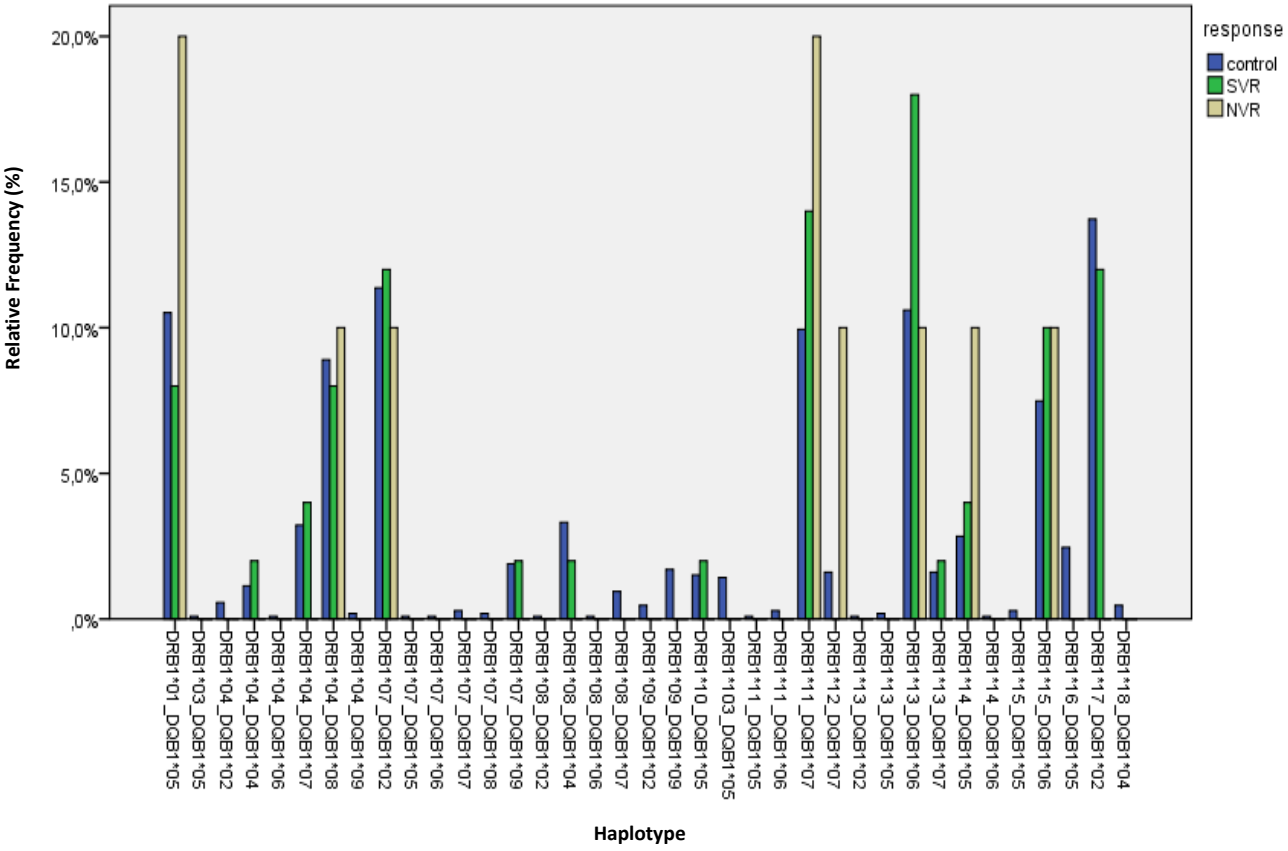


Figure 36: Relative frequencies of the DRB1-DQB1 haplotypes in regard the different therapy outcomes. Frequencies of the HLA-DRB1-DQB1 haplotypes detected in the test samples divided according to the pegIFN-α/RBV treatment versus the frequencies of haplotypes present in the blood donor population (control). The frequencies´ landscape, when the infected samples are sorted by their response to treatment, remains without providing evidence of a significant relation between the haplotypes and the therapy outcome. Some haplotypes are slightly more present in NVR samples, DRB1*01-DQB1*05, DRB1*11-DQB1*07 and DRB1*14-DQB1*05, or in SVR samples, DRB1*13-DQB1*06. But, the results remain without statistical significance, $p>0.05$.

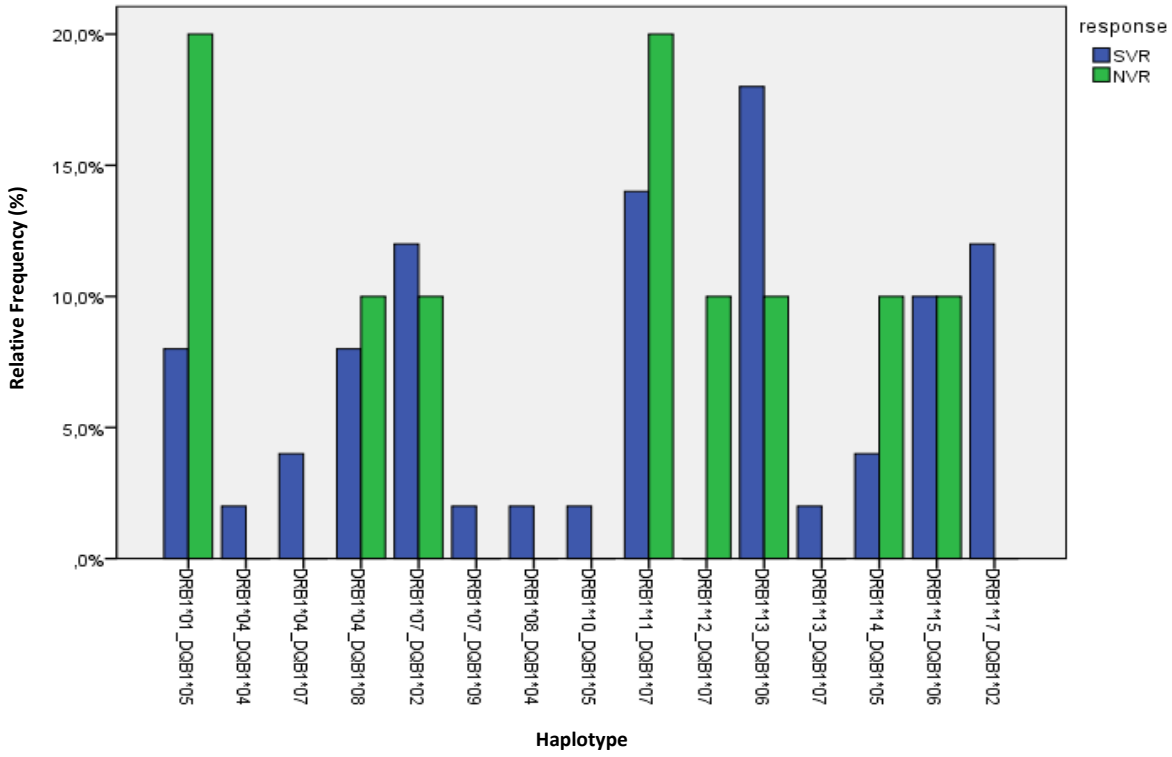


Figure 37: Relative frequencies of the DRB1-DQB1 haplotypes according to the SVR and NVR outcomes. Frequencies of the HLA-DRB1-DQB1 haplotypes detected in the test samples divided according to the pegIFN-α/RBV therapy. This closer look of the haplotypes in patients with different outcomes is a closer look to the results shown in Figure 37. As said before, some haplotypes are slightly more evident in samples from NVR patients, DRB1*01-DQB1*05, DRB1*11-DQB1*07 and DRB1*14-DQB1*05, or from patients who respond to treatment, DRB1*13-DQB1*06. However, these results remain without statistical significance, $p>0.05$.

This lack of ability to interpret a haplotypic relation with the pegIFN-α/RBV therapy outcome might be due to the small sample pool especially in regard to NVR patients. Therefore, further studies are in order with a larger sample pool so that the relations between DRB1-DQB1 haplotypes and treatment outcomes already described as well as possible new ones could be identified and studied.

Chapter 4:

Conclusion

A. Conclusion:

The hepatitis C virus is a very difficult virus to eradicate due to its high genetic variability and ability to avoid the host immune system. This can be very stressfull to the patients who need to undergo therapies with variable success rates and side effects. That is why the ability to predict treatment outcomes allowing for a more personal and efficient therapy is so important for both patients and hospitals. With this thesis it was tried to bring information that would enunciate previously reported results as well as shine new light on less studied topics, contributing to this overall objective.

In the genetic expression the small sample pool was an influencial aspect in the statistical analysis of the results. The expression of genes present in the immune response against the HCV was focused in the time period prior to the beginning of treatment for being when these results are more impactfull. Although not all, some of the tested genes presented significant differences from infected patients and patients with different treatment responses. Some of those were from interferon-stimulated genes, IFIT1 and OAS3, with increased expression in infected patients and patients with NVR. This goes in with the report from Shackel and McCaughan (2007)[112] where it is hypothesized an already maximized response in patients who do not respond to therapy. The results from IL-10, TFG-β1 and FOXP3 sugest a role of Treg cells in patients with a sustainable response. However this comes into conflict with some literature where this Treg up-regulation occurs in NVR patients rather than SVR ones.

The 5´UTR from the viral genome, being one of the most conservative it was expected not to observe many mutations. Yet some variability was noticed, especially on the domain III that could lead to an IRES´ loss of functionallity and affect viral RNA´s stability and viral replication. There were also noticed some mutations that could indicate the presence of viral quasispecies. However, the small number of amplified sequences and the lack of crucial information about the patients´ response to treatment limited their evaluation and no association between both could be made.

As for the study of NS3´s genetic variability study, several attempts were made and several amplification programs tested but with very few to none satisfying results. From the sequence´s genetic variability that would prevent the primers from annealing and the size of the target seuquece to the integrity of the viral RNA, there are many factors that could have influenced the procedure in order to prevent the sequence´s amplification

and sequenciation. Nonetheless, NS3 a very important protein for HCV´s life cycle and immunity evasion mechanisms that should be further studied.

Finally, in the study of the HLA typing of the infected patients was inconclusive as none of the tested antigens/haplotypes and study scenarios showed associations between the genetic profile and the response to standard therapy. Here, a small and unbalanced sample pool was the most likely influence factor for this. This is a very interesting topic because some correlations between HLA antigens and haplotypes and viral clearance or persistence were already reported.

Anyway, the study of possible prediction factors for therapy outcomes is a very important subject. This is an area that still needs studying from which we still have much to learn and understand. The preliminary results may suggest a focal point of interest as well as new directions of investigation.

B. Future perspectives:

Future projects should focus on expanding the sample pool of the work done for this thesis. Then, it should turn to the mutagenic profiling of the HCV NS3 and NS5 due to its importance for viral life cycle. However, if possible turn to new methods. This can be put in two topics.

First, regarding the sequencing analysis of viral genes encoding key proteins like NS3 and NS5 it would be useful and interesting to explore the use of plasmids and new sequencing methods. The use of plasmids would greatly increase the chance of optimizing the nested PCR process without the need to waste precious samples. And the exploration of new sequencing techniques, like next-generation sequencing, which has been shown to being able to near fully reconstruct viral genome and give an improved depth to sequencing analysis[174–176].

Secondly, it would be interesting to perform a genetic expression profiling of the immune mediators involved in the role of other major immune response “players”, such as natural killer cells, in the host´s immune response.

The ideal scenario would be to have patients in beginning of infection and that would naturally clear the infection.

Another limitation of this study is that these results come from peripheral manifestations and it would be fantastic to analize cellular infiltrates in infected patients´ biopsies.

These suggestions might widen our understanding of the world of hepatitis C and the virus itself in Portugal and help build a brighter future for HCV infected patients.

Annexes:

A. Genetic expression of immune response mediators

Table XXX: Samples of the patients involved in the evaluation of the genetic expression of the immunologic mediators, with respective gender, age, response to pegIFN-α treatment (SVR or NVR), HCV genotype and viral load.

Sample	Response	HCV genotype	Viral Load (IU/ml)	Gender	Age
415465	NVR	1-b	384 000	M	79
407375	NVR	3-a	1 657 762	M	49
406466	NVR	3-a	1 987 798	M	37
406228	SVR	1-a	2 818 968	F	65
406226	SVR	1-a	236 158	M	46
405653	SVR	1-b	983 052	M	35
404881	SVR	3-a	207 211	M	44
404056	SVR	1-b	1 287 914	M	51
403479	SVR	1-a	709 024	M	40
403249	SVR	3-a	256 625	M	34
403248	SVR	3-a	7 583 537	M	55
403192	SVR	3-a	7 074 009	M	38
403191	SVR	3-a	10 501 395	M	43
403190	NVR	1-a	4 234 012	M	42
403077	SVR	1-b	473 178	M	39
400512	NVR	1-b	1 183 913	M	42
399790	SVR	1	719 843	M	43
399055	SVR	3-a	7 162 233	M	39
398911[a]	SVR	1-a	4 246 729	F	52
404823	SVR	1-a	3 675 952	F	50
398958	SVR	3-a	1 898 538	M	37
403566	SVR	1-a	2 485 670	M	44
403247	SVR	1-a	3 429 714	F	43
334593	NVR	1-a	2 450 000	M	30
366690	NVR	1-a	238	M	30
332639	SVR	1-a	1 220 000	M	41
399734	SVR	1-b	14 877 243	M	49
328074	SVR	1-a	886 000	M	41
325390	SVR	4-a	3 870	M	36
317841	SVR	-	-	M	25
317845	SVR	-	-	M	41

315309	SVR	1-a	-	M	44
314819	SVR	1-b	841 000	F	63
315942	SVR	1-b	203	F	40
313691	SVR	1-a	61 600	-	56
312724	NVR	-	64 300	M	48
318504	NVR	1-b	-	M	37
326314	SVR	1-a	132 000 000	M	41
309157	SVR	1-a	-	M	41
306589	SVR	1-a	-	M	47
307098	SVR	-	1 740	F	43
248529	NVR	4-a	141	M	49
248530	SVR	3-a	5 328 982	F	36
316338	SVR	1-a	-	F	38
316818	NVR	1-a	-	M	32
315967	SVR	3-a	-	M	41
314017	NVR	1-b	37 500	M	28
317617	SVR	3-a	-	M	35
311769	NVR	1-b	-	M	29
406227	SVR	1-a	11 286 031	M	42
402794	SVR	3-a	7 422 740	M	32
399677	SVR	1-b	305 090	M	25
404055	SVR	1-b	4 123 813	F	31
403478	SVR	3-a	1 738 476	F	41
399427	SVR	3-a	577 965	M	38

[a]Co-infected with HIV

Table XXXI: Characterization of the extracted RNA for the genetic expression study according to RNA integrity, purity and concentration.

Samples	RIN	Concentration (ng/µl) (RNAchip)	$\frac{Abs260}{Abs280}$	Concentration (ng/µl) (nanodrop)
334593	4.7	34	1.71	32.1
366690	3.4	14	1.55	7.7
312724	N/A	6.5	1.7	6.1
318504	5.0	38	1.89	19
248529	4.2	19	1.91	12.3
316818	6.9	50	1.78	44.6
314017	7.1	40	1.86	38.6
311769	6.7	17	1.87	15
332639	7.4	36	1.87	32.2
328074	6.6	29	1.66	31
325390	6.8	13	1.72	11.5
317841	7.2	13	1.78	8.2
317845	N/A	19	1.77	15.2
315309	7.5	11	2.16	7.7
314819	5.2	12	1.64	17.9
315942	6.8	11	1.82	12.8
313691	7.2	15	1.6	11.5
326314	N/A	27	1.82	9.3
309157	5.4	67	1.8	45.5
306589	2.3	13	1.63	10.7
307098	2.4	13	1.74	12.2
316338	5.7	38	1.74	33
315967	6.3	22	1.8	23.7
317617	5.9	23	1.81	26.6
415465	4.6	24	1.91	23.8
407375	3	21	1.82	16.8
406467	4.8	74	1.75	39.2
406466	N/A	14	1.51	8.1
406228	5.3	11	1.58	10.8
406226	4.7	354	1.78	28.7
405653	5.8	148	1.83	17.4
405652	7.0	317	1.81	21.1
404881	5.5	437	1.67	39.4
404056	3.1	326.5	1.76	33.2
403479	7.6	117	1.77	13.8

403249	5.0	450	1.81	36.3
403248	5.7	313.5	1.81	38
403192	5.8	473	1.86	28.7
403191	5.6	297	1.88	20.4
403190	7.3	224	1.86	23.3
403077	4.2	453	1.83	30.5
400512	5.5	18	2.07	13.6
399790	6.5	16	1.99	21.1
399055	6.4	30	1.99	17.6
398911	5.7	44	1.96	20.9
404823	5.6	15	1.93	15.4
398958	7.4	49	1.9	34.1
403566	4.8	41	1.9	12.1
403247	4.0	44	1.81	41.5
351773	6.0	24	1.85	12.6
399734	6.1	25	1.95	22.8
316524	5.9	25	1.9	22.6
253797	2.3	17	1.62	18.9
249543	2.6	69	1.66	37.4
247524	1.8	11	1.8	10.2
248530	N/A	79	1.65	37.3
250144	3.6	38	1.59	16
406227	2.0	20	1.93	5.1
402794	4.3	30	2.07	13.1
399677	4.9	101.5	1.87	55.5
407377	2.6	103	1.64	36.2
404055	2.2	22	1.53	13.1
403478	2.5	75	1.85	31.7
399427	N/A	73	1.9	29.6

Table XXXII: Statistical analysis of the genes tested for the genetic expression study (HCV infected patients Vs Healthy controls).

Samples	Genes			Statistic	Std. Error
control (N=6)	IL10(NGE)	Mean		,4603	,1822
		95% Confidence Interval for Mean	Lower Bound	-,0081	
			Upper Bound	,9288	
		Std. Deviation		,4464	
	IFNa(NGE)	Mean		,0110	,0055
		95% Confidence Interval for Mean	Lower Bound	-,0031	
			Upper Bound	,0252	
		Std. Deviation		,0135	
	TGFb1(NGE)	Mean		,8745	,0854
		95% Confidence Interval for Mean	Lower Bound	,6551	
			Upper Bound	1,0939	
		Std. Deviation		,2091	
	IFIT1(NGE)	Mean		,1474	,0421
		95% Confidence Interval for Mean	Lower Bound	,0392	
			Upper Bound	,2557	
		Std. Deviation		,1032	
	IFI27(NGE)	Mean		,0020	,0004
		95% Confidence Interval for Mean	Lower Bound	,0011	
			Upper Bound	,003	
		Std. Deviation		,0009	
	OAS3(NGE)	Mean		,1586	,0328
		95% Confidence Interval for Mean	Lower Bound	,0742	
			Upper	,2431	

			Bound		
		Std. Deviation		,0804	
	IFR2(NGE)	Mean		1,1547	,1297
		95% Confidence Interval for Mean	Lower Bound	,8212	
			Upper Bound	1,4882	
		Std. Deviation		,3178	
	IRF8(NGE)	Mean		1,1862	,1035
		95% Confidence Interval for Mean	Lower Bound	,9201	
			Upper Bound	1,4523	
		Std. Deviation		,2535	
	FOXP3(NGE)	Mean		,2199	,0504
		95% Confidence Interval for Mean	Lower Bound	,0903	
			Upper Bound	,3495	
		Std. Deviation		,1235	
	IL4(NGE)	Mean		,1289	,0355
		95% Confidence Interval for Mean	Lower Bound	,0376	
			Upper Bound	,2201	
		Std. Deviation		,087	
HCV (N=64)	IL10(NGE)	Mean		1,1229	,122
		95% Confidence Interval for Mean	Lower Bound	,8791	
			Upper Bound	1,3667	
		Std. Deviation		,9759	
	IFNa(NGE)	Mean		,1329	,1228
		95% Confidence Interval for Mean	Lower Bound	-,1125	
			Upper Bound	,3784	
		Std. Deviation		,9825	
	TGFb1(NGE)	Mean		1,1063	,0329

	95% Confidence Interval for Mean	Lower Bound	1,0406	
		Upper Bound	1,1720	
	Std. Deviation		,2630	
IFIT1(NGE)	Mean		1,0961	,2081
	95% Confidence Interval for Mean	Lower Bound	,6802	
		Upper Bound	1,512	
	Std. Deviation		1,6650	
IFI27(NGE)	Mean		,2672	,0916
	95% Confidence Interval for Mean	Lower Bound	,0841	
		Upper Bound	,4503	
	Std. Deviation		,7331	
OAS3(NGE)	Mean		1,0748	,1786
	95% Confidence Interval for Mean	Lower Bound	,7179	
		Upper Bound	1,4316	
	Std. Deviation		1,4285	
IFR2(NGE)	Mean		1,2782	,0566
	95% Confidence Interval for Mean	Lower Bound	1,1652	
		Upper Bound	1,3913	
	Std. Deviation		,4526	
IRF8(NGE)	Mean		1,3728	,0607
	95% Confidence Interval for Mean	Lower Bound	1,2516	
		Upper Bound	1,4941	
	Std. Deviation		,4854	
FOXP3(NGE)	Mean		,4641	,0639
	95% Confidence Interval for Mean	Lower Bound	,3364	
		Upper Bound	,5918	

	Std. Deviation		,5112	
IL4(NGE)	Mean		,1609	,0252
	95% Confidence Interval for Mean	Lower Bound	,1104	
		Upper Bound	,2113	
	Std. Deviation		,2019	

Table XXXIII: Comparison of means between Controls and HCV infected patients

NGE	Control	HCV
IL-10	0.4603±0.4464	1.1229±0.9759*
IFN-α	0.0110±0.0135	0.1329±0.9825
TGF-β1	0.8745±0.2091	1.1063±0.2630*
IFIT1	0.1474±0.1032	1.0961±1.6650*
IFI27	0.0020±0.0009	0.2672±0.7331
OAS3	0.1586±0.0804	1.0748±1.4285*
IRF2	1.1547±0.3178	1.2782±0.4526
IRF8	1.1862±0.2535	1.3728±0.4854
FOXP3	0.2199±0.1235	0.4641±0.5112*
IL-4	0.1289±0.087	0.1609±0.2019

Values are Mean ± SD. n = Control (6); HCV (64). Significant difference from Control indicated by * ($P<0.05$).

Table XXXIV: Statistical analysis of the genes tested for the genetic expression study (Patients w/ SVR Vs Patients w/ NVR Vs Healthy controls).

Samples and response	Genes			Statistic	Std. Error
Control (N=6)	IL10 (NGE)	Mean		,4603	,1822
		95% Confidence Interval for Mean	Lower Bound	-,0081	
			Upper Bound	,9288	
		Std. Deviation		,4464	
	IFN-α (NGE)	Mean		,0110	,0055
		95% Confidence Interval for Mean	Lower Bound	-,0031	
			Upper Bound	,0252	
		Std. Deviation		,0135	
	TGF-β1 (NGE)	Mean		,8745	,0854
		95% Confidence Interval for Mean	Lower Bound	,6551	
			Upper Bound	1,0939	
		Std. Deviation		,2091	
	IFIT1 (NGE)	Mean		,1474	,0421
		95% Confidence Interval for Mean	Lower Bound	,0392	
			Upper Bound	,2557	
		Std. Deviation		,1032	
	IFI27 (NGE)	Mean		,0020	,0004
		95% Confidence Interval for Mean	Lower Bound	,0011	
			Upper Bound	,003	
		Std. Deviation		,0009	
	OAS3 (NGE)	Mean		,1586	,0328
		95% Confidence Interval for Mean	Lower Bound	,0742	
			Upper Bound	,2431	

		Std. Deviation		,0804	
	IFR2 (NGE)	Mean		1,1547	,1297
		95% Confidence Interval for Mean	Lower Bound	,8212	
			Upper Bound	1,4882	
		Std. Deviation		,3178	
	IRF8 (NGE)	Mean		1,1862	,1035
		95% Confidence Interval for Mean	Lower Bound	,9201	
			Upper Bound	1,4523	
		Std. Deviation		,2535	
	FOXP3(NGE)	Mean		,2199	,0504
		95% Confidence Interval for Mean	Lower Bound	,0903	
			Upper Bound	,3495	
		Std. Deviation		,1235	
	IL4(NGE)	Mean		,1289	,0355
		95% Confidence Interval for Mean	Lower Bound	,0376	
			Upper Bound	,2201	
		Std. Deviation		,087	
NVR (N=13)	IL-10 (NGE)	Mean		1,3802	,3511
		95% Confidence Interval for Mean	Lower Bound	,6151	
			Upper Bound	2,1452	
		Std. Deviation		1,2660	
	IFN-α (NGE)	Mean		,0047	,002
		95% Confidence Interval for Mean	Lower Bound	,0004	
			Upper Bound	,0090	
		Std. Deviation		,0072	
	TGF-β1 (NGE)	Mean		1,1201	,0785
		95% Confidence Interval for Mean	Lower Bound	,9491	

		Upper Bound	1,2910	
	Std. Deviation		,2829	
IFIT1 (NGE)	Mean		2,1002	,8498
	95% Confidence Interval for Mean	Lower Bound	,2486	
		Upper Bound	3,9518	
	Std. Deviation		3,0640	
IFI27 (NGE)	Mean		,6708	,3739
	95% Confidence Interval for Mean	Lower Bound	-,1439	
		Upper Bound	1,4855	
	Std. Deviation		1,3482	
OAS3 (NGE)	Mean		1,6055	,6205
	95% Confidence Interval for Mean	Lower Bound	,2534	
		Upper Bound	2,9575	
	Std. Deviation		2,2374	
IFR2 (NGE)	Mean		1,4045	,2149
	95% Confidence Interval for Mean	Lower Bound	,9362	
		Upper Bound	1,8728	
	Std. Deviation		,775	
IRF8 (NGE)	Mean		1,2454	,1321
	95% Confidence Interval for Mean	Lower Bound	,9576	
		Upper Bound	1,5332	
	Std. Deviation		,4763	
FOXP3 (NGE)	Mean		,4709	,1884
	95% Confidence Interval for Mean	Lower Bound	,0604	
		Upper Bound	,8815	
	Std. Deviation		,6794	
IL-4 (NGE)	Mean		,1172	,0202

		95% Confidence Interval for Mean	Lower Bound	,0731	
			Upper Bound	,1612	
		Std. Deviation		,0729	
SVR (N=42)	IL-10 (NGE)	Mean		1,0382	,1343
		95% Confidence Interval for Mean	Lower Bound	,7669	
			Upper Bound	1,3095	
		Std. Deviation		,8706	
	IFN-α (NGE)	Mean		,2005	,1871
		95% Confidence Interval for Mean	Lower Bound	-,1772	
			Upper Bound	,5783	
		Std. Deviation		1,2123	
	TGF-β1 (NGE)	Mean		1,0984	,0334
		95% Confidence Interval for Mean	Lower Bound	1,0310	
			Upper Bound	1,1659	
		Std. Deviation		,2164	
	IFIT1 (NGE)	Mean		,8619	,1424
		95% Confidence Interval for Mean	Lower Bound	,5743	
			Upper Bound	1,1494	
		Std. Deviation		,9227	
	IFI27 (NGE)	Mean		,1876	,073
		95% Confidence Interval for Mean	Lower Bound	,0402	
			Upper Bound	,335	
		Std. Deviation		,473	
	OAS3 (NGE)	Mean		,935	,1656
		95% Confidence Interval for Mean	Lower Bound	,6004	
			Upper	1,2695	

		Bound		
	Std. Deviation		1,0735	
IFR2 (NGE)	Mean		1,2044	,0382
	95% Confidence Interval for Mean	Lower Bound	1,1272	
		Upper Bound	1,2816	
	Std. Deviation		,2476	
IRF8 (NGE)	Mean		1,3839	,0699
	95% Confidence Interval for Mean	Lower Bound	1,2427	
		Upper Bound	1,5251	
	Std. Deviation		,4531	
FOXP3 (NGE)	Mean		,4976	,0776
	95% Confidence Interval for Mean	Lower Bound	,3408	
		Upper Bound	,6543	
	Std. Deviation		,503	
IL-4 (NGE)	Mean		,1708	,0369
	95% Confidence Interval for Mean	Lower Bound	,0962	
		Upper Bound	,2454	
	Std. Deviation		,2394	

Table XXXV: Comparison of means between Controls, NVR patients and SVR patients

NGE	Control	NVR	SVR
IL-10	0.4603±0.4464	1.3802±1.2660	1.0382±0.8706
IFN-α	0.0110±0.0135	0.0047±0.0072	0.2005±1.2123
TGF-β1	0.8745±0.2091	1.1201±0.2829	1.0984±0.2164
IFIT1	0.1474±0.1032	2.1002±3.0640§	0.8619±0.9227
IFI27	0.0020±0.0009	0.6708±1.3482	0.1876±0.473
OAS3	0.1586±0.0804	1.6055±2.2374	0.935±1.0735
IRF2	1.1547±0.3178	1.4045±0.775	1.2044±0.2476
IRF8	1.1862±0.2535	1.2454±0.4763	1.3839±0.4531
FOXP3	0.2199±0.1235**	0.4709±0.6794$^{\delta}$	0.4976±0.503
IL-4	0.1286±0.087	0.1172±0.0729	0.1708±0.2394

Values are Mean ± SD. n = Control (6); NVR (13); SVR (42). Significant difference Control Vs SVR indicated by * ($P<0.05$), ** ($P<0.01$). Significant difference Control Vs NVR indicated by § ($P<0.05$). Significant difference NVR Vs SVR indicated by δ ($P<0.05$).

B. HLA typing

Table XXXVI: Linkage disequilibrium between HLA antigens DRB1 and DQB1.

DRB1			DQB1 Association
DR1			DQ5[a]
DR103			DQ5[a]
DR2	DR15	DR51	DQ6[a]; DQ2
	DR16	DR51	DQ5[a]; DQ7[b]
DR3	DR17	DR52	DQ2
	DR18	DR52	DQ4
DR5	DR11	DR52	DQ7[b]; DQ5[a]; DQ6[a]
	DR12	DR52	DQ7[b]
DR6	DR13	DR52	DQ6[a]; DQ7[b]; DQ2; DQ8[b]
	DR14	DR52	DQ5[a]; DQ7[b]; DQ8[b]
DR7		DR53	DQ2; DQ9[b]
DR4		DR53	DQ7[b]; DQ8[b]; DQ4
DR9		DR53	DQ9[b]; DQ2
DR8			DQ4; DQ7[b]; DQ8[b]
DR10			DQ5

Less Frequent; [a]belongs to DQB1*01; [b]belongs to DQB1*03

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