Thèse de Doctorat

Mention Biologie Santé

Spécialité Virologie

présentée à *l'Ecole Doctorale en Sciences Technologie et Santé (ED 585)*

de l'Université de Picardie Jules Verne

par

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pour obtenir le grade de Docteur de l'Université de Picardie Jules Verne

Apolipoprotein(a) Inhibits Hepatitis C Virus Through Interaction With Infectious Particles

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To my family and friends

"To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science."
Albert Einstein

"Your theory is crazy, but it's not crazy enough to be true."
Niels Bohr

ACKNOWLEDGMENTS

First of all, I would like to thank all the members of the jury: doctors **Karin Seron** and **Jean-Christophe Meunier**, you give me the honor of accepting to judge this thesis; professor **Stéphane Chevaliez**, professor **Gilles Duverlie** and my supervisors professor **Sandrine Castelain** and doctor **François Helle**. Thank you for agreeing to judge my work and to have brought your expertise.

My sincere thanks to professor **Gilles Duverlie**, for providing me an opportunity to join his team, and for giving me access to the laboratory and research facilities. Without his precious support it would not be possible to conduct this research.

I would like to express my sincere gratitude to my advisor professor **Sandrine Castelain**, for the opportunity to integrate a great project, *ONCOVIR*, coordinated by her, that permitted me to expand my knowledge about the Hepatitis C Virus. Thank you for your trust, support and guidance throughout my work. Thank you as well for revising my thesis manuscript.

I would also like to pay special thanks to my co-advisor Dr. **François Helle**, who has been a tremendous mentor for me. Thank you for encouraging my research so carefully and with such optimism, allowing me to grow as a research scientist. Your advice on both my research and career have been priceless. Thank you for the continuous support, patience (I know, it has been immense!), great humor, motivation, and for sharing your vast knowledge. Your guidance helped me through all the time of research and writing of this thesis. I cannot imagine having had a better advisor and mentor for my PhD study.

I sincerely thank **Agnès Boullier**, **Paulo Marcelo** and **Jean-Marc Domon** for their specialized and experimented help, and to **Corey Scipione** and **Marlys L. Koschinsky** for their precious variants of apolipoprotein(a), without which my work would not be possible.

Thank you **Catherine François**, **Etienne Brochot** and **Abderrahmane Bengrine** for your brilliant comments and suggestions, availability and kindness. Thank you also for the difficult questions which led me to widen my research from various perspectives.

Thank you so much **Véronique Descampes**, **Virginie Morel** and **Carole Fournier** for sharing your wide experience with me, enriching my technical knowledge. Thank you for your patience and kindness.

Thank you **Barbara Duclercq** for being so kind and helpful. Thank you so much **Catherine Moriset** for each conversation, laugh and hug. These three years in the lab. were much more cheerful in your company.

Elodie Martin, thank you for all shared moments in the lab and outside. Thank you for promptly contributing to my comfort and integration in Amiens as well as for all the smiles, help, friendship, comprehension, PATIENCE and much more. My PhD experience without you would not have been the same. **Lynda Handala**, always discreet and mysterious, thank you for each "*royal*" moment shared with you. Your originality, art, ideas, kindness and friendship enriched my life.

I thank all the members of the virology laboratory for their warm welcome and conviviality.

A special thanks to my great and beautiful family. Words cannot express how grateful I am to my **mother** and **father** for all the sacrifices that they have made on my behalf. Your prayer for me was what sustained me this far. Thank you for supporting me in all my decisions. Thanks also to my **grandparents** for the enormous heart and love. My little **sister**, thank you for the friendship and all the beautiful moments shared our entire life.

I would also like to thank all of my friends who supported and encouraged me to strive towards my goal, through these three years and more than 2000 km. Without your strength, all of this time would be more difficult. Each one has a place in my heart. **Sara** and **Pedro**, the discovery of Amiens with you was unforgettable. Thank you for your unconditional support.

At the end, I would like to warmly thank my beloved **Zé** that was always my key support in the moments when there was no one to answer my queries. Thank you for being with me every day, despite the distance, for being attentive and caring. Thank you for turning my flaws into qualities, my sad moments into happy ones, and my tears into beautiful smiles. OBRIGADA!

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A

Aa	Amino acids
ACSL3	Acyl-CoA synthetase long-chain 3
ALT	Alanine transaminase
AP2	Adaptor protein complex 2
Apo	Apolipoprotein
ARFP	Alternative reading frame protein
AST	Aspartate transaminase

C

CARD	Caspase recruitment domains
CD81	Cluster of Differentiation 81
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CETP	Cholesteryl ester transfer protein
CLDN1	Claudin-1
CNV	Copy number variation
CRP	C reactive protein
CsA	Cyclosporine A
CVD	Cardiovascular disease
Cyp	Cyclophilin

D

DAA	Direct-acting antiviral
DC	Dendritic cells
DGAT1	Diacylglycerol acyltransferase-1
DMV	Double-membrane vesicles
DNA	Deoxyribonucleic acid

E

EGFR	Epidermal growth factor receptor
eIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERC	Endocytic recycling compartment
ERK	Extracellular signal-regulated kinases

ESCRT	Endosomal-sorting complex required for transport

F

FASN	Fatty acid synthase
FDA	Food and Drug Administration

G

GAG	Glycosaminoglycans
GAS	Gamma activated sequence
GFP	Green fluorescent protein
GlcNAc	N-acetyl-D-glucosamine
GTP	Guanosine-5'-triphosphate

H

HAV	Hepatitis A virus
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell culture-derived HCV
HCVpp	HCV pseudoparticles
HDL	High density lipoprotein
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HMGCoA	Hydroxy-3-methylglutaryl-CoA
HNF4α	Hepatocyte nuclear factor 4α
HPgV	Human Pegivirus
HSCs	Hematopoietic stem cells
HSPG	Heparan sulfate proteoglycan
HTA	Host-targeting agent
hVAP-A	Human vesicle-associated membrane protein-associated protein A
HVR	Hypervariable region

I

ICAM	Intracellular adhesion molecule
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
IKK-α	IκB kinase-α
IL	Interleukin
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor

ISG	Interferon-stimulated gene
ISGF	Interferon-stimulated gene factor
ITGB1	Integrin beta 1

K

K	Kringle

L

LBS	Lysine-binding site
LD	Lipid droplets
LDL	Low density lipoprotein
LDL-C	LDL cholesterol
LDL-r	LDL receptor
LEL	Large extracellular loops
LGP2	Laboratory of genetics and physiology 2
Lp	Lipoprotein
LPA	apo(a) gene
Luc	Luciferase
LVP	Lipoviroparticle
Lys	Lysine

M

MAM	Mitochondria-associated membranes
MAPK	Mitogen-activated protein kinases
MAVS	Mitochondrial antiviral-signalling proteins
MBL	Mannose-binding lectin
MCP-1	Monocyte chemoattractant protein 1
MDA	Melanoma differentiation-associated protein
MEK	MAPK/ERK kinase
MHC	Major histocompatibility complex
MI	Myocardial infarction
miR	MicroRNA
MKNK1	MAPK interacting serine/threonine kinase 1
MLV	Murine Leukemia Virus
mRNA	Messenger RNA
MSR1	Macrophage scavenger receptor type 1
MTP	Microsomal triglyceride transfer protein

N

nABs	Neutralizing antibodies
NANA	N-acetyl-D-neuraminic acid

NANBH	Non-A non-B hepatitis
NAT	Nucleic acid amplification tests
NF-kB	Nuclear Factor kappa B
NK	Natural killer
NPC1L1	Niemann-Pick C1-like 1
NS	Non-structural
NTP	Nucleoside triphosphate
NTPase	Nucleoside triphosphatase
NTR	Non-translated regions

O

OCLN	Occludin
ORF	Open reading frame
OxLDL	Oxidized LDL
OxPL	Oxidized phospholipids

P

PAMP	Pathogen-associated molecular patterns
PC	Phosphocholine
PCSK9	Pro-protein convertase subtilisin/kexin type 9
PEG	Polyethylene glycol
PI4KIIIα	Phosphatidylinositol 4-kinase IIIα
PKR	Protein kinase R
PLA2	Phospholipase A2
PLA2GXIIB	Phospholipase A2 GXIIB
PLG	Plasminogen gene
PRR	Pattern recognition receptors
PSTPIP2	Proline-serine-threonine phosphatase interacting protein 2

R

RdRp	RNA-dependent RNA polymerase
RDT	Rapid diagnostic tests
RIG-I	Retinoic acid inducible gene-I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction

S

SAA	Serum amyloid A
SDC	Syndecan

SEL	Small extracellular loops
SGIG	Self-generated iodixanol gradient
SNPs	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signaling
SPCS1	signal peptidase complex subunit 1
SPgV	Simian Pegivirus
SPP	Signal peptide peptidase
SR-BI	Scavenger receptor-BI
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
SVR	Sustained virologic response

T

TBK1	TANK Binding Kinase 1
TEM	Tetraspanin-enriched microdomain
TfR1	Transferrin receptor 1
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TMA	Transcription-mediated amplification
TMD	Transmembrane domain
TNF	Tumor necrosis factor
TRIF	Toll-interleukin 1 domain-containing adaptor inducing IFNβ
TTP	TfR1 Trafficking Protein

V

VCAM	Vascular cell adhesion molecules
VLDL	Very low density lipoprotein

W

WGA	Wheat germ agglutinin

RÉSUMÉ

La recherche sur le VHC a longtemps été freinée par l'absence de modèle d'étude permettant l'amplification de ce virus en culture cellulaire. Plusieurs obstacles ont été surpassés pour établir des modèles de culture du VHC mais il reste toujours impossible de cultiver les isolats cliniques de ce virus. En 2005, le clonage de la souche JFH1 (génotype 2a) a permis la mise en place du premier modèle rendant possible l'étude complète du cycle viral du VHC. De nos jours, d'autres systèmes de culture permettant l'étude d'autres génotypes du VHC ont vu le jour. Toutefois, le développement de nouveaux modèles permettant la culture des isolats cliniques reste un enjeu majeur.

Dans cette étude, nous avons émis l'hypothèse que la difficulté à mettre en culture les isolats cliniques du VHC pouvait être due à la présence de facteurs de restriction dans le sérum des patients. En utilisant des sérums séronégatifs pour le VHC, nous avons confirmé l'existence de facteurs sériques inhibiteurs. En combinant des étapes de précipitation au polyéthylène glycol, de gradient d'iodixanol et de chromatographie d'exclusion stérique, nous avons obtenu une fraction purifiée enrichie en facteurs inhibiteurs, à partir des sérums séronégatifs pour le VHC. L'analyse en spectrométrie de masse a permis d'identifier l'apolipoprotéine(a) (apo(a)) comme un inhibiteur potentiel de l'entrée du VHC. L'apo(a) se compose de dix domaines kringle IV (KIV), un domaine kringle V (KV) et un domaine protéase inactif. Les dix KIV sont présents en une seule copie, à l'exception du KIV de type 2 (KIV2), qui est codé en un nombre variable de copies répétées en tandem, ce qui donne naissance à de nombreuses isoformes de taille de l'apo(a). Cette protéine se lie de façon covalente par un pont disulfure à l'apolipoprotéine B des lipoprotéines de basse densité, pour former des lipoprotéines(a). En utilisant un virus recombinant dérivé de la souche JFH1, nous avons confirmé que la lipoprotéine(a) plasmatique et recombinante étaient capables d'inhiber spécifiquement le VHC en interagissant avec les particules infectieuses. En utilisant la lectine WGA, qui est connue pour interagir spécifiquement avec la Lp(a), nous avons montré qu'il était possible de réduire l'effet inhibiteur de l'apo(a) et de rétablir l'infection par le VHC. De façon intéressante, nous avons aussi observé que la protéine apo(a) seule, sous forme recombinante purifiée, était suffisante pour inhiber le VHC. Nos résultats suggèrent également que les isoformes courtes sont moins inhibitrices que les longues. Nous avons aussi observé que les sites de liaison aux lysines dans KIV7, KIV8 et KIV10 n'étaient pas requis pour l'activité inhibitrice. Finalement, nos résultats suggèrent que la fraction lipoprotéique

des lipoviroparticules du VHC est essentielle pour l'inhibition. En effet, contrairement aux VHCcc qui sont associés aux lipoprotéines, les VHCpp sont peu sensibles à l'inhibition par apo(a). Au final, nos résultats mettent en évidence que l'apo(a) est un nouveau composant du métabolisme lipidique capable de moduler l'infection par le VHC.

ABSTRACT

HCV is not an easy virus to work with and it has been necessary to overcome significant hurdles to establish HCV cell culture models. However, further research is still needed as well as efficient culture models for the study of the virus. The identification of the JFH1 isolate permitted the establishment of the first cell culture model supporting the complete HCV life cycle. Nowadays, other cell culture systems cover some of the genotypes. The development of cell culture systems was essential for the direct-acting antiviral development, but is still very important to promote elementary research. The difficulty in cultivating HCV from clinical isolates led to an effort from many teams to overcome this hurdle.

In this study we hypothesized that this hindrance could be due to the presence of restriction factors in patient serum. Thus, using HCV seronegative sera, we confirmed our hypothesis. Combining polyethylene glycol precipitation, iodixanol gradient and size-exclusion chromatography, we obtained a purified fraction enriched in inhibitory factors from HCV seronegative sera. Mass spectrometry analysis identified apolipoprotein(a) (apo(a)) as a potential inhibitor of HCV entry. Apo(a) consists of ten kringle IV domains (KIVs), one kringle V domain (KV) and an inactive protease domain. The ten KIVs are present in a single copy with the exception of KIV type 2 (KIV_2), which is encoded in a variable number of tandemly repeated copies, giving rise to numerous apo(a) size isoforms. In addition, apo(a) covalently links to the apolipoprotein B component of a low density lipoprotein via a disulfide bridge to form lipoprotein(a). Using a recombinant virus derived from the JFH1 strain we confirmed that plasma-derived and recombinant lipoprotein(a) as well as purified recombinant apo(a) variants were able to specifically inhibit HCV by interacting with infectious particles. Using wheat germ agglutinin (WGA) lectin, that is well known to specifically interact with Lp(a), it was possible to reverse the apo(a) inhibitory effect and restore the HCV infection. Our results also suggest that the lipoprotein moiety of HCV lipoviroparticle (LVP) is essential for the inhibition. Indeed, HCVpp were only slightly sensitive to apo(a) contrary to HCVcc, which are associated to lipoproteins. Our results also suggest that small isoforms are less inhibitory than the large ones. Finally, we observed that the functional lysine-binding sites in KIV_7, KIV_8 and KIV_{10} were not required.

Altogether, our results identify apo(a) as an additional component of the lipid metabolism modulating HCV infection.

INTRODUCTION

I. HCV Generalities

The hepatitis is a liver inflammation caused by infectious or non-infectious agents. There are five major viral hepatitis: A, B, C, D and E, that are caused by distinct viruses. Genetically they present a large variability with different pathologies associated. One of these agents is the Hepatitis C virus (HCV) identified in 1989. Its characterization was difficult for lack of cell culture propagation.

HCV infection often progresses into a chronic one, with high risk of developing a fibrosis process, cirrhosis and ultimately hepatocellular carcinoma (HCC). Moreover, the combination of HCV and hepatitis B virus (HBV) represents 75% of all cases of liver diseases worldwide (Ananthakrishnan et al, 2006).

A. Epidemiology and Transmission

HCV infection still represents a global health problem. The most recent estimates of infection demonstrated an increase in seroprevalence over the last 15 years. Thus, there were estimated about 185 million infected individuals worldwide (Messina et al, 2015). Each year, it is also estimated the occurrence of 3 to 4 millions of new infected cases and 350.000 deaths due to all HCV reported issues (Perz et al, 2006).

Studies of prevalence have been made to compare global levels of HCV infection [Figure 1]. Countries in Africa and Asia presented a high HCV prevalence that may exceed 5%, whereas developed countries in North of America, Western Europe, and Australia presented lower prevalence results, generally less than 2,5% (Messina et al, 2015). In addition, Egypt presents the highest HCV prevalence in the world, nationally estimated at 14.7% (Mohamoud et al, 2013). These infection levels in Egypt have historical dimensions that distinguish it from the other countries. The massive transmission is still ongoing today, being associated with medical exposures and also the use of injected drugs (Mohamoud et al, 2013).

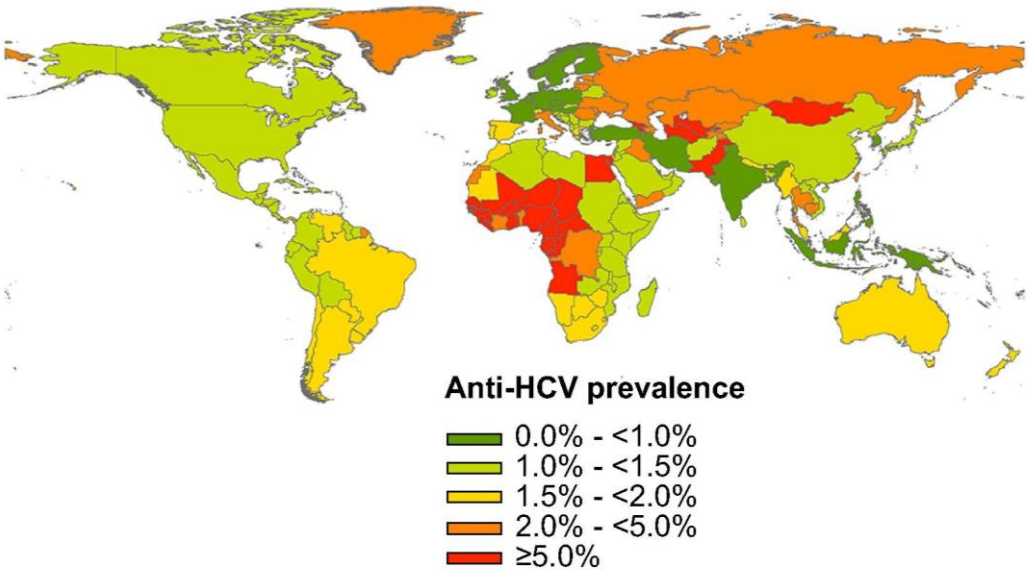

Figure 1: **Worldwide prevalence of HCV**
Global prevalence of anti-HCV antibodies in the adult population.
From (Gower et al, 2014)

The transmission of productive HCV infection is limited to the human population, although the virus has been experimentally transmitted to the primates (Kolykhalov et al, 1997). In the early 90s, the screening of HCV antibodies in the blood and tissues of donors came to prevent these donations as source of HCV transmission (Alter et al, 1990). Until that time the exposure to contaminated blood was the main source of HCV transmission, occurring mostly through transfusions with infected blood and contaminated injections during medical procedures.

Nowadays, although blood transfusions do not represent a significant HCV transmission source in the developed countries, there are other sources that merit attention. The needle sharing between drug users is one of them. Moreover, sexual contact, mainly in homosexual males, becomes a significant problem when the individuals are also (human immunodeficiency virus) HIV co-infected (Lopez-Dieguez et al, 2011). The vertical HCV transmission presents low rates, with favourable spontaneous clearance by the infected children. This route of transmission cannot be prevented due to the lack of vaccine and the impossibility of approved therapy during pregnancy (Arshad et al, 2011; Prasad & Honegger, 2013).

B. Natural History of HCV Infection

On the twentieth century, many individuals who had undergone a blood transfusion went on to develop a liver disease (Alter & Houghton, 2000). Between 1960 and 1970, the scientists developed blood tests to identify HBV and hepatitis A virus (HAV) and after testing the blood samples from post-transfusion illness, the majority of the results were negative for both hepatitis (Dienstag & Purcell, 1977). Later, in the early 1970s, sensitive assays for hepatitis B surface antigen (HBsAg) were developed and confirmed that HBV was responsible for less than 25% of referred cases. Serological tests for HAV also rarely showed its implication. Thus, in 1975, the term non-A non-B hepatitis (NANBH) was adopted for these cases (Feinstone et al, 1975). Despite this discovery, international research efforts to identify the virus failed for the next decade. In 1987, a novel molecular cloning approach to identify the unknown virus appeared and in 1988, it was discovered by isolation of a single cDNA clone that it was a new flavi-like virus (Houghton, 2009a). Thus, in April of 1989, the virus was identified and re-named hepatitis C virus (Choo et al, 1989). Finally, in 1990, the routine screening of blood products for HCV in drug users, patients with tattoos or piercings, healthcare workers, dialysis patients, and those who participate in high risk sexual activities, became ordinary. This was a decision to reduce the number of HCV infections (Westbrook & Dusheiko, 2014).

a. Acute HCV infection

The acute infection is characterized by a short-term illness, occurring within the first 6 months after HCV exposure. Normally, this stage is asymptomatic and consequently difficult to be detected. Sometimes, clinical symptoms are exhibited, such as fatigue, nausea, fever, asthenia, anorexia, digestive disorders and myalgia, but they are not very specific. Jaundice is a more rare symptom but also a relatively specific sign of liver disease (Loomba et al, 2011). Acute HCV infection leads to the increase of transaminase levels, aspartate transaminase (AST) and alanine transaminase (ALT). The spontaneous viral clearance was estimated for around 26% of patients, but an important gender dependent variation was also observed, reaching up to 40% of viral clearance for women (Westbrook & Dusheiko, 2014). In most of cases spontaneous clearance does not occur, so the infection progresses to a chronic infection.

b. Chronic HCV infection

The chronic infection can be defined by the presence of HCV RNA for more than six months following an acute infection. After this period HCV specific antibodies are already detectable. The disease can evolve differently, depending on many factors such as alcohol use; co-infection with other viruses, like HIV or HBV; gender, age and immune status of patients (Westbrook & Dusheiko, 2014). The HCV genotype and viral load can also influence the course of the disease (Santantonio et al, 2008). This stage of infection remains asymptomatic for many years and then, moderate symptoms can emerge associated with transaminase levels floatation and presence of inflammatory syndrome.

Chronic HCV infection can develop into different stages. Firstly, the persistent inflammatory syndrome can lead to a fibrotic process that changes the hepatic metabolism. Consequently, the fibrosis stage may be exacerbated. Thus, studies have shown that 15% to 30% of chronically infected patients develop cirrhosis and 1% to 3% can develop a decompensate disease that can even evolve to a HCC [Figure 2] (Vassilopoulos & Calabrese, 2012). Patients in these last stages need an hepatic transplantation, because their livers lost their architecture and metabolic functions, and consequently, their ability to regenerate (Chen & Morgan, 2006; Westbrook & Dusheiko, 2014). The severity of the disease can be measured by hepatic histopathological analyses and also by fibrosis measurement, with the FibroScan® (hepatic elasticity) or the FibroTest® (several biochemical markers) (Poynard et al, 2012).

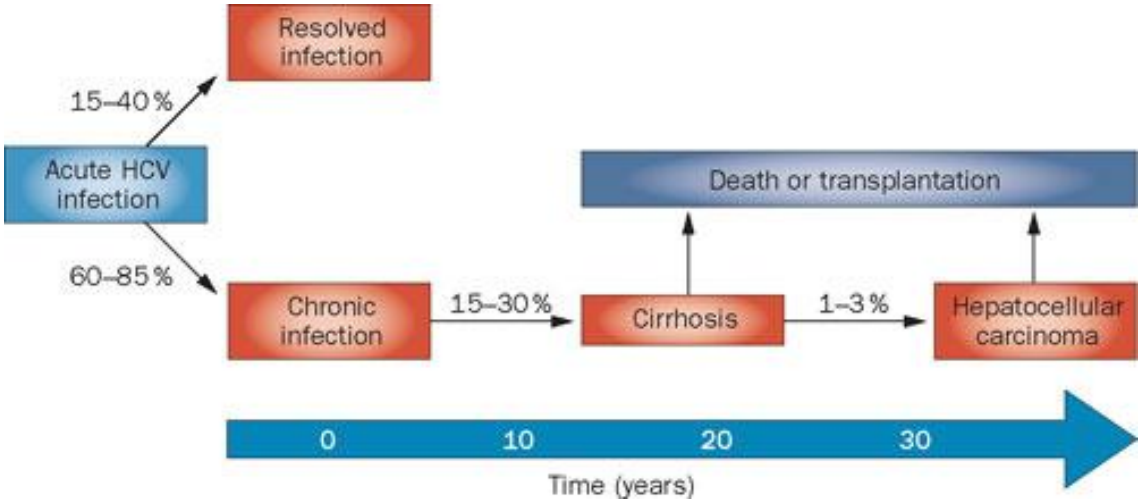

Figure 2: The natural history of HCV infection

The HCV infection frequently develops in a chronic infection, it may also develop in a cirrhosis process or even in a hepatocellular carcinoma, during several years.
From (Vassilopoulos & Calabrese, 2012).

C. Classification and Genetic Variability

After many genomic tests, HCV was classified into the *Flaviviridae* family (Lindenbach & Rice, 2005). The International Committee on Taxonomy of Viruses (ICTV) regroups four genera belonging to this family: Hepacivirus; Flavivirus; Pestivirus and Pegivirus [Figure 3]. HCV was the first virus to be classified into the Hepacivirus genus due to the particular genomic organization of its structural proteins. Until recently, GBV-B was the only other virus classified into this genus. GBV-B was recovered from a laboratory housed tamarind, but its ultimate origin (primate or non-primate) remains unclear (Muerhoff et al, 1995; Simons et al, 1995). Recently, many species belonging to the Hepacivirus genus have been identified, commonly from domestic animals (Scheel et al, 2015). Some species of the Flavivirus genus include the yellow fever, West Nile, dengue, tick-borne encephalitis and Japanese encephalitis viruses. The Pestivirus genus includes the bovine viral diarrhoea virus and the classical swine fever virus, being economically relevant. Members of Pegivirus [e.g. Human Pegivirus (HPgV) and Simian Pegivirus (SPgV)] are widely distributed in a range of

mammalian species, but to date they have not been clearly associated with any disease (www.ictvonline.org).

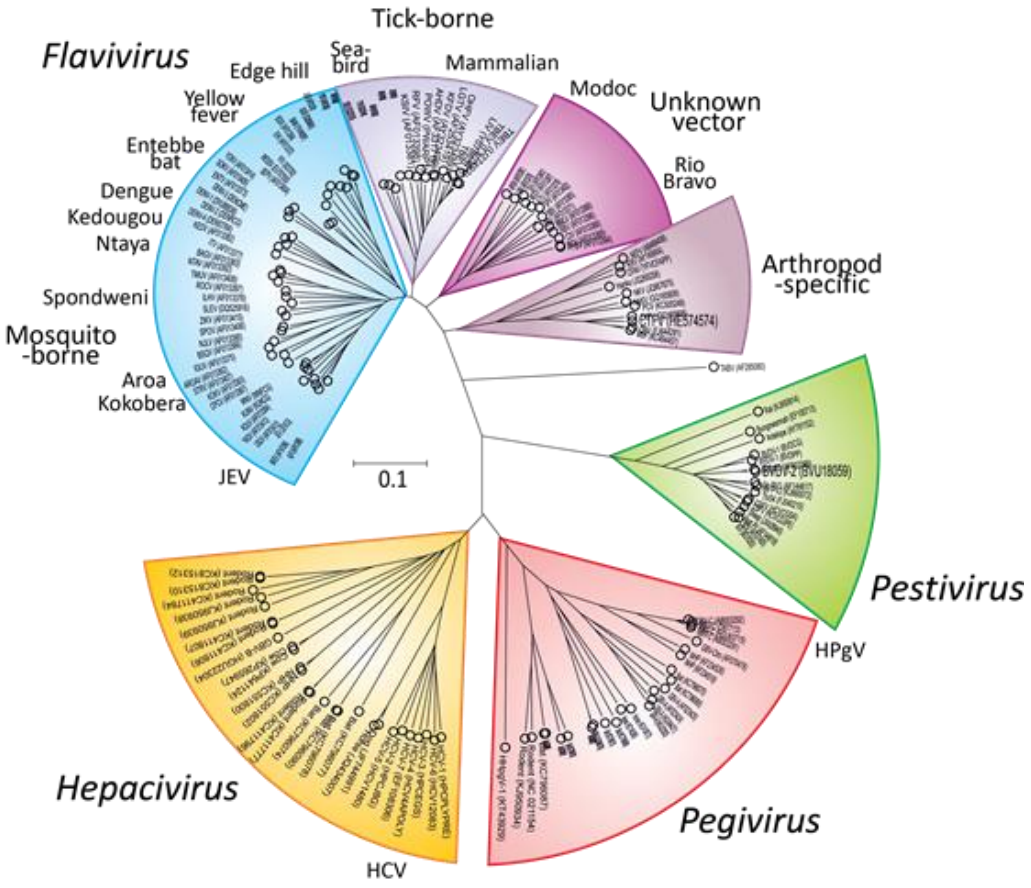

Figure 3: Phylogenetic tree of the *Flaviviridae* family

Phylogeny based on conserved amino acids sequences in the RNA-dependent RNA polymerase of the *Flaviviridae* family. The name of the virus corresponds to the abbreviations listed in the "List of species" of each genus. From (ICTV 2016 - www.ictvonline.org).

The high error rate committed by the HCV RNA-dependent RNA polymerase during the replication step and also the pressure exerted by the host immune system, have led to the evolution of HCV into 7 different genotypes and more than 67 subtypes. These genotypes diverge in 30% of their nucleotide sequences and their subtypes in about 20% between them (Scheel et al, 2015; Smith et al, 2014).

Globally, the genotype 1 is the most prevalent maybe due to the global association of subtypes 1a and 1b with the global dissemination of contaminated blood and blood products during the twentieth century, prior to the HCV discovery. After genotype 1, genotype 3 is the most spread, being found in around 30% of global infections. Genotype 3 is associated with drug users and the migration from some countries, like India or Pakistan, where the subtype 3a is dominant. Although these two genotypes are the most globally prevalent, some lower-income countries have their own HCV prevalence in a large proportion. For example, genotype 2 is most frequent in West Africa and some South America areas (this last one may reflect the movement of populations from the trans-Atlantic slave trade). In its turn, the genotype 4 is commonly found in Central/North Africa, the genotype 5 is particularly common in South Africa and the genotype 6 in East/Southeast Asia. Recently, this last genotype was also found in Germany, Canada and North America. Studies have estimated that about 25% of all HCV cases are caused by genotypes 2, 4, or 6 [Figure 4] (Hajarizadeh et al, 2013; Messina et al, 2015).

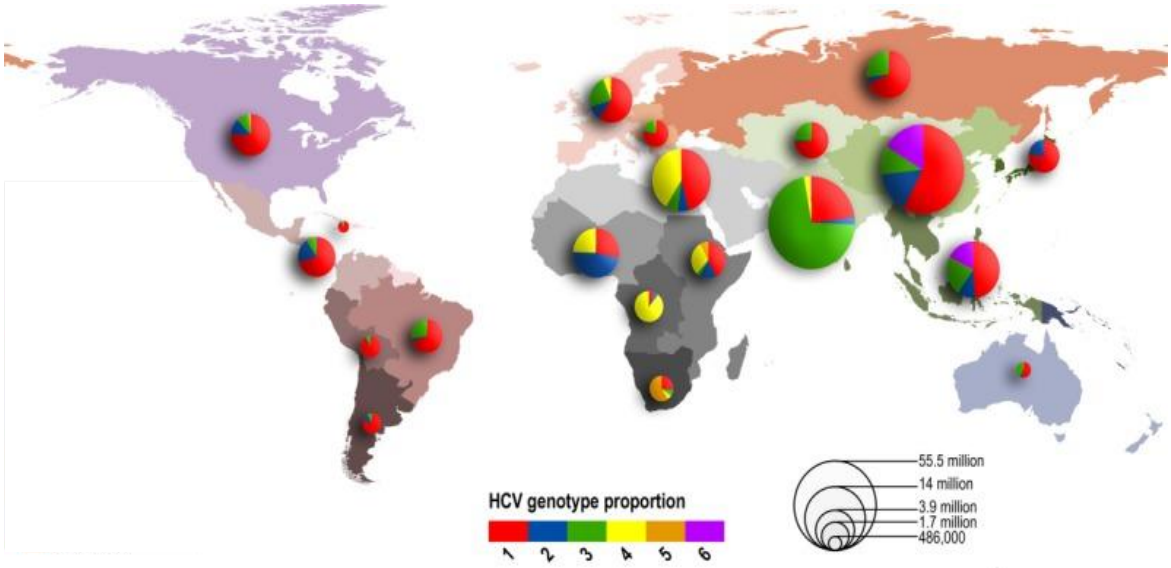

Figure 4: Geographic distribution of HCV genotypes
Prevalence of HCV genotypes that are represented by distinct colors.
The size of pie charts is proportional to the number of seroprevalent cases.
Adapted from: (Messina et al, 2015)

HCV strains, belonging to the rarer genotypes, can quickly increase their prevalence in one area if they appeared associated with efficient transmission routes. Thus, it is widely believed that the high prevalence and dominance of subtype 4a in Egypt may have resulted from its spread through the unsafe injections during public health campaigns. Other subtypes have emerged and have been amplified in other regions by drug users. It is the case of genotype 4d in Europe and the genotype 6a in the Vietnam and Hong Kong (Messina et al, 2015). These remarks suggest that social, behavioral, and demographic factors are very important for the global prevalence of the different genotypes [Figure 4] (Hajarizadeh et al, 2013; Messina et al, 2015).

The genetic variability is essentially due to the HCV RNA-dependent RNA polymerase (RdRp) activity, as referred above. High replication rates increase this variability, contributing to the emergence of mutations. Moreover, the common development to chronicity of the majority of infections and the intra/inter-genotypic recombination phenomena also promote some diversity (Kalinina et al, 2002; Shi et al, 2012). The variability of all genotypes seems to influence the pathogenesis, the immune system escape and the response/resistance to HCV treatment. For example, the genotype 1 is associated with more severe disease and genotype 3 is frequently associated to development of hepatic steatosis (Mirandola et al, 2009; Pawlotsky et al, 1999; Soler et al, 2002). Therefore, studies about HCV genotypes and its diversity are very important to offer relevant information for suitable viral treatment, more specifically contributing to define the treatment duration, which dose to use and which molecule (of direct-acting antiviral drugs) to use.

D. Molecular Biology of HCV

a. HCV genome

HCV genome consists in a 9.6-kb uncapped linear positive single-stranded RNA molecule, composed by two non-translated regions (NTR), 5' and 3', and an open reading frame (ORF). The 5' region includes an internal ribosome entry site (IRES) to initiate the RNA translation (Choo et al, 1991). The ORF encodes a polyprotein precursor of about 3000 amino acids (aa), which is proteolytically processed into 10 mature proteins in a preferential order [Figure 5].

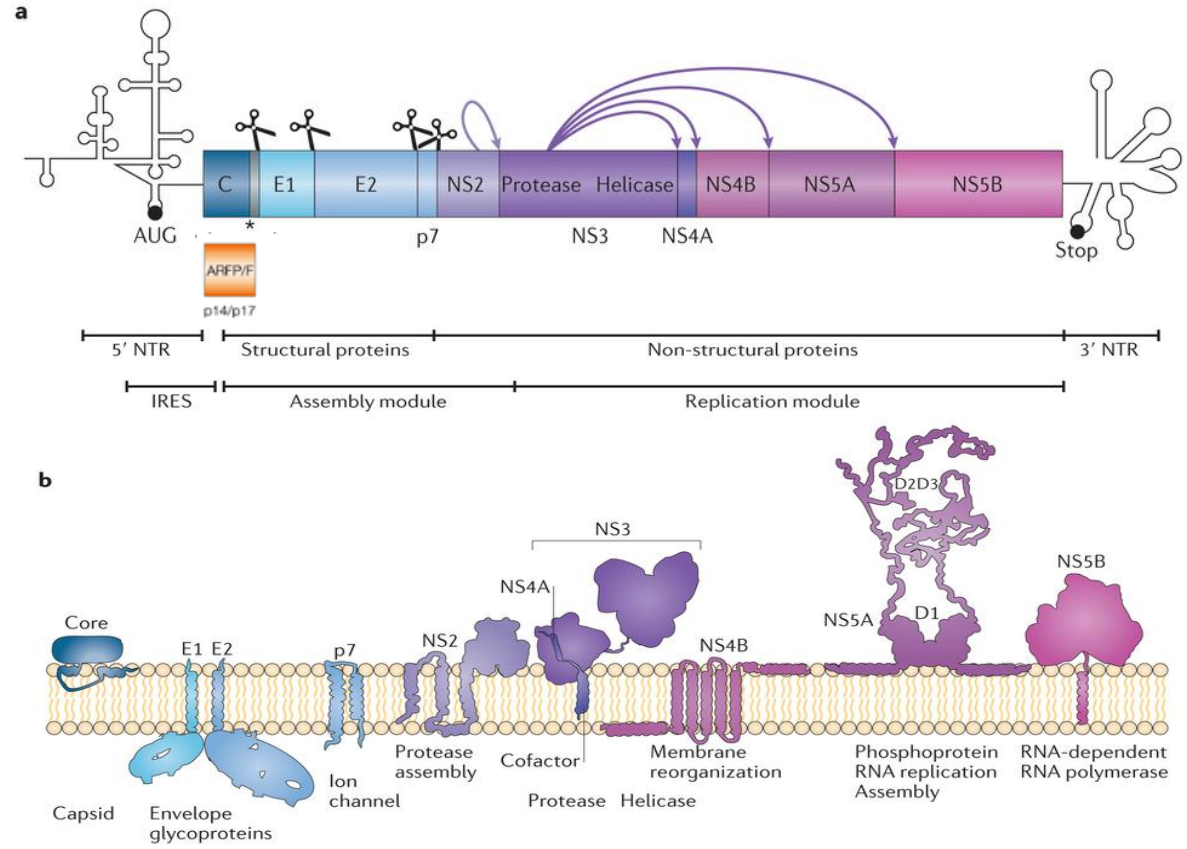

Figure 5: HCV genome and proteins organization

(a) Representation of HCV ORF encoding the polyprotein, flanked by 5′ and 3′ NTRs. The Core, the E1 and E2 glycoproteins, p7 and NS2, are required for virus assembly (called assembly module). The remainder of the non-structural proteins is required for RNA replication (called replication module). In the Core coding sequence may occurs a translational ribosome frame shift yielding to the F protein but its implication in the HCV infection is unclear. (b) Membrane topologies and major functions of the HCV proteins. Adapted from: (Bartenschlager et al, 2013) and (Tan et al, 2002)

On N-terminal are found three structural proteins (Core as well as E1 and E2 envelope glycoproteins) followed by the non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [Figure 5] (Moradpour & Penin, 2013). P7 is the viral protein that separates E2 from NS2 and its classification as non-structural protein has been controversial (Madan & Bartenschlager, 2015). In addition to the major ORF proteins, the HCV core region also encodes an alternative reading frame protein (ARFP) also named protein F - Frameshift, whose function in the viral pathogenesis is not clear (Moradpour & Penin, 2013; Moradpour et al, 2007; Vassilaki & Mavromara, 2009).

i. Non-translated regions

▪ *5' Non-translated region*

The 5' non-translated region is the most conserved of HCV genome, containing 341 nucleotides and located upstream of the ORF. It is composed by four domains (I-IV). Domains II, III and IV are essential to recruit the 40S ribosomal structure without the need of canonical translation initiation factors. These domains constitute the IRES which extends to the first 42 nucleotides of the Core protein coding region (Honda et al, 1996). IRES present all necessary components to initiate the viral RNA translation. Moreover, some subunits of domain III allow the interaction with the eukaryotic initiation factor (eIF) 3 that, in its turn, allows the viral RNA translation (Kieft et al, 2002). Between the domains I and II, 5' NTR presents two target sequences to the microRNA miR-122, separated by 8 or 9 nucleotides. MiR-122 is a cellular microRNA essential to stabilize the HCV RNA and to regulate viral replication, requiring an interaction with 5' NTR (Jopling, 2008). Domains I and II contain regulatory functions of HCV replication with particular relevance in domain I stem-loop structure (Friebe et al, 2001).

▪ *3' Non-translated region*

The 3' non-translated region contains about 225 nucleotides. It is constituted by three regions, one variable sequence of about 40 nucleotides; one long polypyrimidine tract (polyU/UC) and a highly conserved 3'-terminal domain (3'X tail) with 98 nucleotides folded

in three stem-loops. These regions present a role of translation amplifiers, acting closely with the IRES (Kolykhalov et al, 2000). The conserved domains are also implicated in the HCV replication, allowing the interaction between the genomic RNA and the viral replicase complex (Kolykhalov et al, 2000; Yi & Lemon, 2003). Thus, the second stem-loop of 3'X domain interacts with a NS5B coding region, playing a role in the negative stranded-RNA synthesis initiation, during the replication (Friebe et al, 2005). In addition, the third stem-loop of the same domain interacts with NS3 protein, allowing the interaction between NS5A protein and poly U/UC (Huang et al, 2005).

ii. Structural proteins

- *Core*

The Core is the first protein encoded by the HCV ORF and it is located downstream of 5' NTR. An internal signal sequence, located at the C-terminus of the Core coding region and linked with the E1 coding region, traffics the polypeptide to the endoplasmic reticulum (ER). Afterwards, the signal sequence is cleaved by the ER signal peptidase, forming an immature 191-aa Core protein which is then reprocessed to become a mature 21 kDa protein. This newly formed protein joins with the HCV genome to form the nucleocapsid (Kao et al, 2016; Moradpour & Penin, 2013).

Studies identified at least 177 aa in the Core required to produce infectious particles. Core is a dimeric membrane protein stabilized through a disulfide bond and composed by two domains. Domain 1, N-terminal hydrophilic, contains many basic residues and behaves as intrinsically unstructured. These characteristics render the Core able to interact with many cell and viral factors. Thus, the Core is imperative for structural remodeling and packaging of the RNA genome in the viral particles, as well as for the regulation of HCV protein translation. Domain 2, C-terminal hydrophobic, is smaller than domain 1 and associates with the lipid droplets (LDs). Domain 1 interacts with NS5A protein. Both these interactions are crucial to the nucleocapsid assembly process. (Kao et al, 2016; Moradpour & Penin, 2013).

The Core can also be involved in some cellular processes such as regulation of genes associated to the cellular apoptosis; inhibition of Nuclear Factor kappa B (NF-kB) signal

pathway and prevention of tumor necrosis factor (TNF)-α-induced apoptosis pathway (Kao et al, 2016; Moradpour & Penin, 2013).

- *ARFP/F protein*

At the first 11 residues of Core coding sequence may occur a translational ribosome frameshift yielding the ARFP (Kao et al, 2016). The size of this protein varies with the genotype but do not exceed 160 aa (Boulant et al, 2003). Antibodies against ARFP have been detected in HCV chronically infected patients, but its contribution for the infection is unclear. Studies have suggested ARFP implication in the viral replication, pathogenesis processes, immune response modulation, or even in the regulation of protein degradation (McMullan et al, 2007; Vassilaki & Mavromara, 2009; Yuksek et al, 2009). However, more recent studies in cultured cells and in chimpanzee showed that ARFP is not required for detectable HCV replication. Some of ARFP and Core activities seem to be redundant. In addition, translationally-silent mutations created into the ARFP of an infectious HCV RNA and inoculated into chimpanzee, revealed low infectious particles production and hepatic issues (Kao et al, 2016).

- *E1 and E2 envelope glycoproteins*

HCV particles carry two surface glycoproteins, E1 and E2, that form heterodimers. They are type I transmembrane proteins, possessing 190 aa with 31kDa and 365 aa with 70kDa, respectively (Lavie et al, 2007). Each one harbors a large N-terminal ectodomain, exposed into the ER lumen and a short C-terminal transmembrane domain (TMD) with about 30 aa (Helle et al, 2007). After HCV ORF translation, both glycoproteins are processed by an ER signal peptidase, from the N-terminal moiety of the polyprotein. Firstly, the double cleavage at E1 signal peptide from the Core protein and simultaneously at E1 C-terminal sequence leads to the complete processed E1 protein. Then there is a cleavage, this time at the E2/p7 junction, that allows to obtain the complete processed E2 protein [Figure 6].

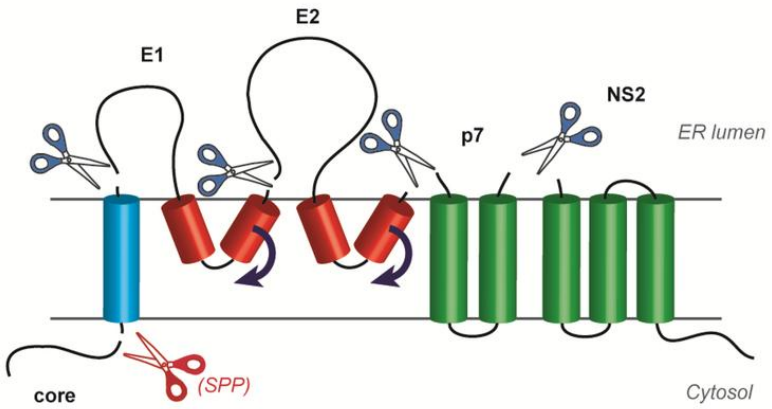

Figure 6: **Cleavage of the polyprotein at the E1 and E2 transmembrane domains**
The blue scissors represent host signal peptidase cleavages and the red scissor represents signal peptide peptidase (SPP) cleavage. Curved arrows indicate the post-cleavage reorientation of the glycoprotein signals of translocation reinitiation. Adapted from: (Vieyres et al, 2014).

The E1 and E2 TMDs consist of two hydrophobic segments, placed into the ER membrane, that are separated by a short polar segment, very conserved and multifunctional. These domains present many functions, such as membrane anchoring, ER retention and they also participate in the non-covalent heterodimerization of E1 and E2. The location and concentration of HCV glycoproteins in the ER compartments seems to be indispensable for the E1E2 heterodimerization and also for the HCV assembly process (Cocquerel et al, 2000; Op De Beeck et al, 2004). For a long time, non-covalent heterodimers were considered as the functional structures for the viral entry. However, more recently it was shown that E1E2 are able to form covalent multimeric complexes, stabilized by disulfide bonds, that are found incorporated on infectious particles (Vieyres et al, 2010).

The E1 and E2 ectodomains are highly N-glycosylated, with up to 6 and 11 glycosylated sites, respectively [Figure 7] (Lavie et al, 2007). This condition is crucial for protein folding, transport through the secretory pathway and escape from the host immune response (Helle et al, 2010). The envelope glycoproteins maturation and folding consist in a complex and interdependent process that involves the ER chaperones, particularly the calnexin, and disulfide bond formation. Accordingly, E1 and E2 ectodomains contain several cysteine residues apt to form the referred disulfide bonds, contributing also to the heterodimer

formation. The cysteine mutations in E1, and mostly in E2, affect the infectivity of viral particles (McCaffrey et al, 2011; Wahid et al, 2013).

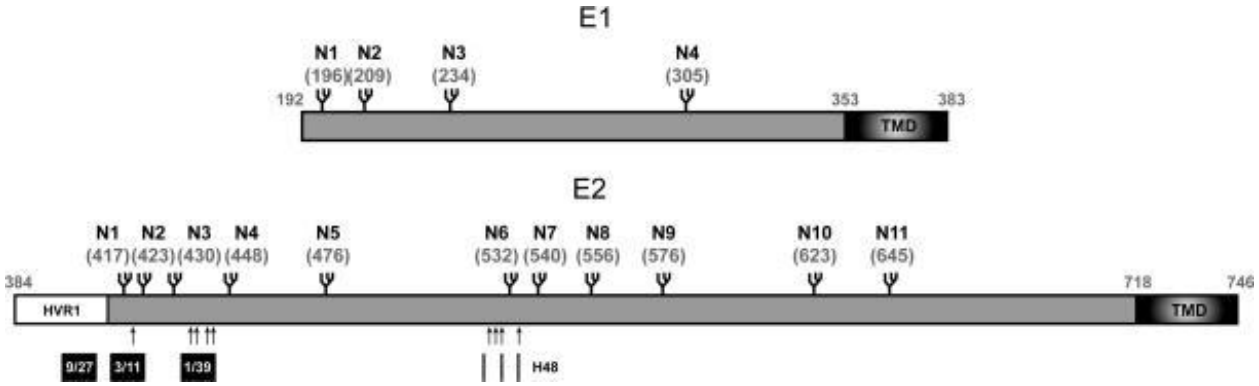

Figure 7: **Representation of N-glycosylation sites in the E1 and E2 glycoproteins of HCV**
N-glycosylation sites (N) are followed by a number that represents its relative position on the sequence. The numbers between parenthesis correspond to the positions of the N-glycosylation sites on the reference polyprotein, the H strain (GenBank AF009606). The epitopes recognized by monoclonal antibodies (9/27, 3/11 and 1/39) are represented by black squares. The black bars indicate the aa 523, 530 and 535, involved in the formation of the conformational epitope of H48. The black arrows indicate the required residues for association with CD81. From (Helle et al, 2007).

Studies have shown that E1 and E2 have not only an important role in HCV particle assembly, but also in viral entry. The hypervariable region (HVR) 1 of E2 is known to interact with host receptors, specific of the viral entry. HVR1 also interacts with apolipoproteins, which is very important to maintain HCV particles with low density, being consequently more infectious. Particularly, the interaction between E2 HVR1 and apoC-I contributes to improve the rates of the viral fusion process. HVR2, much less known, seems to have a role in the heterodimer formation and also in the modulation of the interaction with Cluster of Differentiation (CD) 81 (Albecka et al, 2011; McCaffrey et al, 2011).

iii. Non-structural proteins

- *P7*

P7 is a small protein composed by 63 aa and located between the structural and non-structural proteins of the polyprotein. It is composed of two transmembrane regions that are linked by a basic cytoplasmic loop with both N- and C-terminus facing the ER lumen (Carrère-Kremer et al, 2002). This protein belongs to viroporins, a protein family organized in hexamers or heptamers that presents cation channel activity, facilitating the virus production (Clarke et al, 2006; Luik et al, 2009).

Although p7 is not necessary for HCV RNA replication, it has been shown that it is essential for the assembly and release of HCV infectious particles (Gentzsch et al, 2013; Steinmann & Pietschmann, 2010). Accordingly with a recent study, the absence of p7 leads to the accumulation of capsid assembly intermediates that had not yet completely incorporated viral RNA (Gentzsch et al, 2013). It has also been shown that p7 could protect E2 glycoprotein from a premature degradation caused by the proteasome, during the assembly process. Ion channel activity of p7 seems to be essential but needs to be elucidated. However, a possible role in the prevention of cellular organelle acidification, which may protect the new formed virions from premature acid-induced conformational rearrangements, is not rejected (Atkins et al, 2014; Wozniak et al, 2010).

- *NS2*

The NS2 protein, composed of about 217 aa, is found next to p7 in the C-terminal region of this protein. The junction p7/NS2 is cleaved by the ER signal peptidase, whereas the NS2/NS3 junction is cleaved by the NS2 cysteine protease (Moradpour & Penin, 2013). This protease is essential to release the complete functional NS3 that in its turn is crucial to the HCV viral replication. NS2pro (site catalytic of this protein) is formed by one dimer with two active sites and is located in C-terminal region. The N-terminal region is composed by three putative transmembrane segments (Jirasko et al, 2008; Jirasko et al, 2010). This protein plays an important role in the assembly of HCV infectious particles that is independent of its

protease activity. NS2 interacts with structural and non-structural proteins, leading to the colocalization of all of them at the assembly sites.

- *NS3/4A*

The NS3/4A complex is composed by a non-covalent interaction between NS3 and the cofactor NS4A. NS3 is a multifunctional protein of about 70 kDA. It is composed by one serine protease of about 180 aa, in the N-terminal region and a nucleoside triphosphatase (NTPase)/RNA helicase of about 451 aa, in the C-terminal region [Figure 8] (Yao et al, 1999). The protease domain acquires a chymotrypsin-like fold, constituted by two β-barrel subdomains flanking the enzyme active site. Thus, it is in the N-terminal β-barrel that NS4A is located, assuring the correct conformation of the catalytic site and stabilizing the two proteins (Brass et al, 2008; Schregel et al, 2009). The NS4A cofactor, of about 8 kDa, activates the protease activity that is responsible for cleavage at four sites of the HCV polyprotein, including the autocleavage of NS2/NS3 junction as well as the NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions (Failla et al, 1994). The NS3/4A complex also mediates the cleavage of some host substrates, inactivating them, as it happens with the mitochondrial antiviral-signalling proteins (MAVS) and toll-interleukin 1 domain-containing adaptor inducing IFNβ (TRIF), essential proteins for the host immune response induction. Thus, this complex is a very good target of DAA drugs, as described later (Foy et al, 2005; Li et al, 2005).

The C-terminal acidic portion of NS4A interacts with the NS3 helicase and other HCV proteins, contributing to the RNA replication as well as viral particles assembly. The helicase function is ATP dependent and allows the opening of RNA dimers. This enzyme can also mediate the unfolding of RNA secondary structures during the initiation of replication (Lindenbach et al, 2007; Pang et al, 2002).

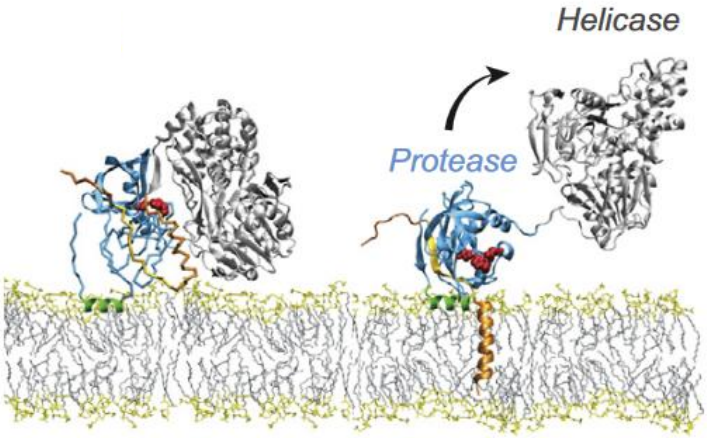

Figure 8: **Hypothetical 3D model of NS3/4A structure**

Adapted from (Paul et al, 2014)

- *NS4B*

NS4B is a hydrophobic protein, of about 27 kDa, that is completely integrated in the ER membrane. It is composed of a central portion harboring four transmembrane domains and two terminal portions at the N- and C-terminal (Gouttenoire et al, 2010). Studies have shown the implication of the N-terminal portion in the assembly of a functional replication complex. It is also suggested that the membrane topology of the NS4B N-terminal may be dynamic and modulated by interactions between proteins within the HCV replication complex (Gouttenoire et al, 2009; Lundin et al, 2006).

The location of NS4B in the ER membranes induces an alteration in these membranes, forming the "membranous web" (described in the viral cycle chapter), which is the base structure for the formation of the replication complexes that contain all the non-structural proteins (Egger et al, 2002). It was also observed that NS4B is able to form oligomers, and its absence leads to the disruption of membranous web formation and consequently HCV RNA replication, suggesting that NS4B oligomerization is required for the establishment of a functional replication complex (Gouttenoire et al, 2010). The lipid modification that occurs in the C-terminal portion, named palmitoylation, was also implicated in the formation of these replication complexes (Yu et al, 2006). NS4B possesses a binding site to the guanosine-5'-triphosphate (GTP), which is important for the viral replication (Thompson et al, 2009).

- *NS5A*

NS5A is a phosphoprotein of about 56-58 kDa, composed by three functional domains (D1–D3) (Penin et al, 2004). D1 is the most important domain, being required to HCV replication. D2 is also required for the same step of HCV life cycle, but just a small segment is truly relevant. Both domains promote NS5A dimerization when they contact with HCV RNA. This phenomenon promotes the development of long chains that attach viral RNA genome to intracellular membranes and may protect it from degradation as well as from detection by pattern recognition receptors (Moradpour & Penin, 2013). The organization of D2 and D3 monomers allows to generate a flexible structure that may explain the promiscuous interaction properties of NS5A with many cellular proteins (Moradpour & Penin, 2013). The cyclophilin (Cyp) A and phosphatidylinositol 4-kinase IIIα (PI4KIIIα) are good examples of NS5A-interecting proteins that are essential for HCV replication (Berger et al, 2011; Kaul et al, 2009; Lim & Hwang, 2011). D3 is not required for the RNA replication, but for the assembly of viral particles, maybe by interacting with the Core protein on lipid droplets and by recruiting apolipoprotein E (apoE) (Appel et al, 2005; Appel et al, 2008; Masaki et al, 2008).

NS5A may present two forms of phosphorylation: p56 of 56 kDa or p58 of 58kDa. Some adaptive mutations reducing the hyperphosphorylation of the protein and consequently improving the RNA replication have been observed in the HCVcc model. On the other hand, NS5A hyperphosphorylated form positively regulates the virion assembly. Thus, the phosphorylation status of NS5A has been associated with the replication and assembly processes regulation (Evans et al, 2004; Neddermann et al, 2004). NS5A is also able to interact with NS5B, regulating its polymerase activity, as well as with many host factors involved in the regulation of transcription, apoptosis or cell cycle control. Therefore, the NS5A interaction with p53 inhibits its apoptosis inducer function and consequently promotes the progression of HCC (Lan et al, 2002). All these NS5A features and interactions make it a good target of the DAAs (later described).

- *NS5B*

NS5B is a RdRp, so being the key enzyme responsible for HCV RNA synthesis (Behrens et al, 1996; Lohmann et al, 1997). Consequently, NS5B is the major target of the DAAs. This RdRp differs from the others by the C-terminal transmembrane sequence that anchors NS5B to the ER membrane. NS5B is a protein of 68 kDa, presenting a catalytic site at the N-terminal, including three subdomains similar to a right hand, with fingers, palm and thumb [Figure 9].

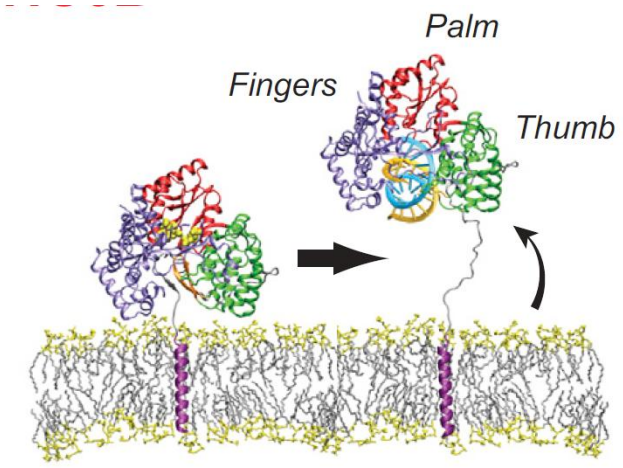

Figure 9: Hypothetical 3D model of NS5B structure

Adapted from (Paul et al, 2014)

It also has a C-terminal membrane insertion sequence that contacts with the active site. The catalytic site is located in the palm subdomain. The single-strand RNA template binds in a groove between the fingers and thumb domains that leads directly to the active site. NTPs access this site through a specific passage beginning at the backside periphery and extending into the active site (located in the palm subdomain). During the initiation of RNA synthesis process the thumb subdomain extensively contacts with the finger subdomain and encircles the active site, resulting in the enzyme close conformation. Thus, the catalytic site provides space for the single-stranded RNA template. High concentrations of GTP can also stimulate

the initiation of RNA synthesis process. Then, the enzyme changes its conformation to an open template-primer form able to bind with double-stranded RNA, during the elongation process (Moradpour & Penin, 2013).

The C-terminal transmembrane domain is essential to anchor NS5B to the ER membrane. This is crucial for the replication process and also for the assembly of functional replicase complexes (Schmidt-Mende et al, 2001). Moreover, the mechanism of RNA replication used by NS5B does not allow to edit genetic errors, so the occurrence of mutations is very frequent, leading to the viral quasi-species emergence.

E. HCV Model Systems

Since HCV discovery in 1989, its study *in vitro* has been very difficult due to the lack of an established HCV-based self-replication system (Choo et al, 1989). There was no sustainable cell model and the replication of HCV contained in the serum of infected patients was very low, in culture cells. Several small animal models also failed, restricting functional studies to humans and chimpanzees. Many research teams tried to cultivate the virus and with the development of new molecular cloning technics, many cell culture systems have been developed (Meuleman & Leroux-Roels, 2009).

a. Replicon system

The first demonstration of HCV replication, *in vitro*, was a viral infection in primary hepatocytes that allowed to detect low HCV RNA levels, being even further reduced by treatment with Interferon (IFN) -α (Lanford et al, 1994). Later, with emergence of cloning methods, the HCV genome transfection became possible. Thus, the numerous copies of HCV RNA obtained, theoretically identical to the viral RNA contained in virions, were transfected in permissive cells, but there was not detection of viral infection. Two reasons for the failure of this transfection system were identified: the lack of an X-tail in the 3'NTR of HCV sequence and/or the presence of inactivating mutations in viral genome (Kolykhalov et al, 1996; Tanaka et al, 1995). After resolution of these handicaps, a fully functional HCV genome from a genotype 1a isolate H77 was created. This strain was able to induce viremia in experimentally inoculated chimpanzees, but the viral replication, *in vitro*, was not detected (Blight et al, 2000; Kolykhalov et al, 1997; Yanagi et al, 1997). Simultaneously to this experiment, other strains were used to produce *in vitro* transcripts, as well as the Con1 strain derived from an HCV genotype 1b isolate.

Short time later, in 1999 the first HCV subgenomic replicon system emerged, after demonstration that HCV structural proteins were not essential for the viral RNA replication (Behrens et al, 1998; Khromykh & Westaway, 1997; Lohmann et al, 1999). This system was derived from the Con1 strain, containing a firefly luciferase (Luc) reporter gene or a selection marker (neomycin phosphotransferase). In this construct, the HCV Core to NS2 region was replaced by two non-HCV sequences, the gene encoding the reporter protein or the selection marker and an additional IRES derived from the encephalomyocarditis virus. This IRES leads

to the synthesis of HCV replicase proteins NS3 to NS5B, whereas the HCV IRES serves to mediate the translation of the selection marker [Figure 10] (Lohmann et al, 1999). Other replicons encoding NS2 to NS5B regions were constructed and tested in several cell lines, but consistent replication levels were obtained only in HuH-7. The selection of Con1 replicating HuH-7 cells enabled for the first time to obtain of a robust cell-based replication system for HCV (Lohmann et al, 1999).

Interestingly, the efficiency of replicon amplification increased significantly during cell culture due to the selection of replication enhancing mutations, generically called cell culture adaptive mutations. The most common mutations were found in the center of NS5A, in NS3 and in very distinct positions of NS4B (Blight et al, 2000; Lohmann et al, 2001). Most of the replication enhancing mutations reduced the phosphorylation status of NS5A (Blight et al, 2000; Lohmann et al, 2003). Studies suggested that reducing NS5A phosphorylation to optimal levels could improve HCV replication, through the interaction of this viral protein with host cell factors, like the human vesicle-associated membrane protein-associated protein A (hVAP-A) or PI4KIIIα (Evans et al, 2004; Reiss et al, 2013).

The HCV permissiveness of the host cells is essential for the efficient *in vitro* viral replication. Thus, some mutations seem to contribute to improve HCV replication, for example in retinoic acid inducible gene-I (RIG-I). This protein recognizes HCV RNA and activates the interferon response, so a mutation in this protein is linked to high permissiveness of HuH-7.5 cells for HCV (Sumpter et al, 2005). On the other hand, cells like HepG2 and Hep3B present lack of permissiveness, which could be explained in part by the low levels of miRNA-122, an essential host cell factor important for HCV replication (Helle et al, 2013; Jopling et al, 2005).

Initially, it was rare to find HCV isolates that could be propagated in cell culture, like Con1, HCV-N or H77 isolates from genotype 1. Sometimes, the introduction of replication enhancing mutations to obtain a viral propagation in cell culture was needed (Blight et al, 2003). Other barriers were the divergence between functionality *in vivo* and complete lack of replication in cell culture as observed for HCV-BK and J6 isolates, but nowadays it is believed that NS3 helicase presents a determinant role in the lack of replication (Grobler et al, 2003; Murayama et al, 2007). Despite these difficulties, the first and unique isolate able to highly replicate in cell lines without mutations was identified. JFH1 is derived from a genotype 2a isolate from a serum of a Japanese patient with fulminant hepatitis (Kato et al, 2003). This isolate is also able to replicate in low permissiveness cell lines such as HepG2,

HeLa, or 293T cells and its helicase, polymerase and 3'NTR are the primary determinants for efficient replication (Lohmann & Bartenschlager, 2014; Murayama et al, 2007).

As final considerations, replicons with efficient reporter systems containing the luciferase gene and the green fluorescent protein (GFP) frame inserted in a site of NS5A allowed the first life-cell imaging studies of viral replication (Lohmann et al, 2003; Moradpour et al, 2004; Wolk et al, 2008). Therefore, they allowed the study of the genome organization and the biochemical characterization of HCV proteins.

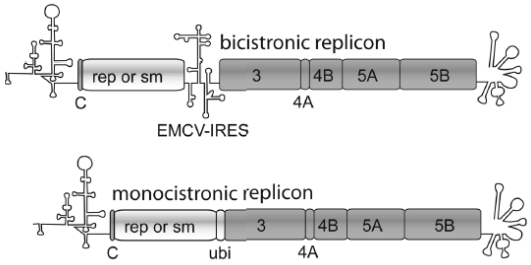

Figure 10: **Schematic representation of the subgenomic replicon structure**
(a) bicistronic replicon presenting a reporter gene (rep) or a selection marker (sm) translated by mediation of the HCV IRES. The EMCV-IRES mediate the translation of HCV proteins.
(b) monocistronic replicon presenting only the HCV IRES that mediate the translation of both rep/sm and HCV proteins. Adapted from: (Lohmann & Bartenschlager, 2014)

b. Pseudoparticules system (HCVpp)

Since the replicon system only allowed the study of HCV replication, complementary models were needed to permit the research of the HCV entry steps. In 2003 the HCVpp system was developed, which consisted in an unmodified HCV envelope glycoproteins assembled onto retroviral core particles (Bartosch et al, 2003b; Drummer et al, 2003; Hsu et al, 2003). These viral particles were generated by transfection of human embryonic kidney (HEK) 293T cells with three plasmids: a construct expressing HCV E1E2, a provirus construct expressing the reporter gene of Luciferase or GFP, and a construct encoding for gag and pol proteins of a retrovirus, such as HIV or Murine Leukemia Virus (MLV) [Figure 11] (Bartosch et al, 2003b; Hsu et al, 2003).

HCVpp system allowed the infectivity evaluation in cell culture through the quantification of Luciferase activity or fluorescent cells. This system has been a valuable tool to identify host cell receptors, like CD81, scavenger receptor-BI (SR-BI), Claudin-1 (CLDN1) or Occludin (OCLN) involved in the viral entry process, including cell binding, endocytosis and fusion (Bartosch et al, 2003c; Flint et al, 2004; Op De Beeck et al, 2004; Ploss & Evans, 2012). In addition, HCVpp system has also been widely used to evaluate potential entry inhibitors and neutralizing antibodies (nAbs) from HCV infected patients or experimentally infected chimpanzees (Bartosch et al, 2003a; Logvinoff et al, 2004; von Hahn et al, 2007). Therefore, HCVpp have been very useful mimicking the early steps of the HCV life cycle and evidencing the favorable tropism of HCV for the hepatic cells (Bartosch et al, 2003b; Hsu et al, 2003; Op De Beeck et al, 2004). Despite all advantages of this system, it was still necessary to create a more complete system, allowing the study of all HCV life cycle steps, including the production of infectious particles.

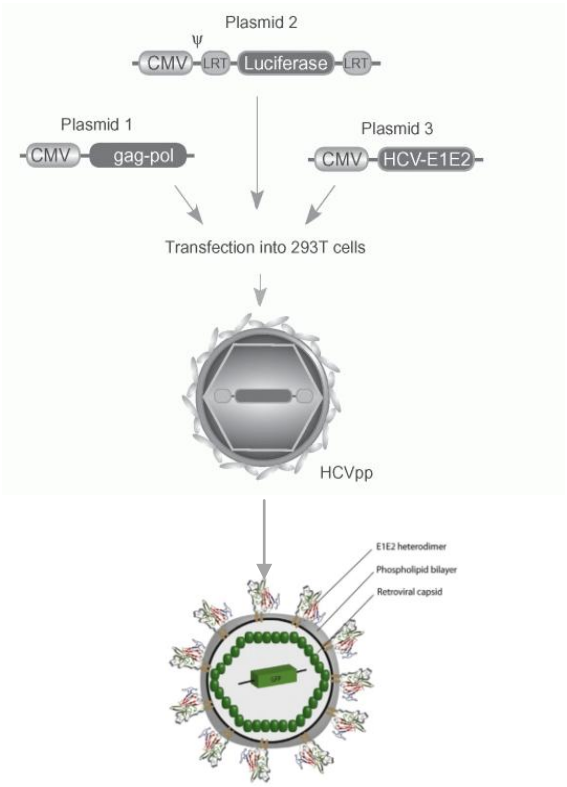

Figure 11: Production of HCV pseudoparticules

For the production of HCVpp, 293T cells are transfected with three expression vectors. One that encodes Gag and Pol retroviral proteins. A second that harbors a sequence for encapsidation and encodes a reporter gene. This vector also contains retroviral sequences that are necessary for the reverse transcription of HCV RNA into a proviral DNA. The third vector encodes HCV E1 and E2 glycoproteins. Adapted from (Lavie et al, 2006a and Douam et al, 2015).

c. Cell culture-derived HCV (HCVcc)

A major breakthrough concerning HCV *in vitro* models was the development of an infection and replication competent HCVcc (Lindenbach & Rice, 2005; Wakita et al, 2005; Zhong et al, 2005). This system, which allows the complete study of HCV life cycle, was developed from the JFH1 full-length genome and produces infectious viral particles without requiring adaptive mutations (Wakita et al, 2005). Studies showed that HCVcc is a fully permissive cell culture system. Secreted HCV particles are able to infect naive HuH-7 cells and chimpanzees and the infection was blocked by CD81 or E2 specific antibodies and by immunoglobulins from chronically HCV-infected patients (Wakita et al, 2005). Other interesting feature was the production of HCV particles with very heterogeneous density, in HuH-7 cells, due to the association with lipoproteins that are host cell components. Since these initial reports, virus titer enhancing mutations that improve the HCVcc system have been identified. Some examples are the mutations in the replicase proteins, NS3 and NS5A (Kaul et al, 2007; Yi et al, 2007), as well as in p7 and NS2 proteins, which showed a relevant role in the virus assembly (Kaul et al, 2007; Russell et al, 2008; Zhong et al, 2006). More recently, the identification of mutations that allow the adaptation of HCV full-length genomes in cell culture, has led to the development of robust and highly infectious *in vitro* systems [Figure 12]. Some examples of these HCV genomes include the TN (genotype 1a), the first effective cell culture system for genotype 1(Li et al, 2012b); the J6 (genotype 2a) (Li et al, 2012a); the J8, DH8 and DH10 (genotype 2b) (Li et al, 2012a; Ramirez et al, 2014) and the DBN3a (genotype 3a) (Ramirez et al, 2016) strains. Infectious culture systems were reported for only a few other genotype 1 and 2 strains (Date et al, 2012; Lu et al, 2014; Pietschmann et al, 2009; Yi et al, 2006). More recently, two other HCV full-length genomes efficiently adapted in cell culture emerged, the HCV-1 and H77C, both derived from a genotype 1 strain. These, together with other infectious full-length HCV genomes, permit functional studies of the HCV life cycle, the viral proteins and their interactions with cellular components (Li et al, 2015).

Progresses in the study of HCV have allowed the creation of HCVcc mimicking all genotypes, through the construction of intergenotypic chimeric genomes (Gottwein et al, 2009; Li et al, 2011; Pietschmann et al, 2006; Scheel et al, 2008). In 2010, Bukh *et al*. developed titrated challenge pools of genotypes 1–6 of HCV, which contributed to better study the immunity against HCV, through controlled studies both in vitro and in vivo. These

systems demonstrated that infection in chimpanzees with all HCV genotypes resulted in relatively high HCV titers and liver inflammation, and that viruses from the challenge genotype pools readily infect human liver–chimeric mice, which permitted further studies with both animal models of HCV infection, so being very useful for the scientific community (Bukh et al, 2010).

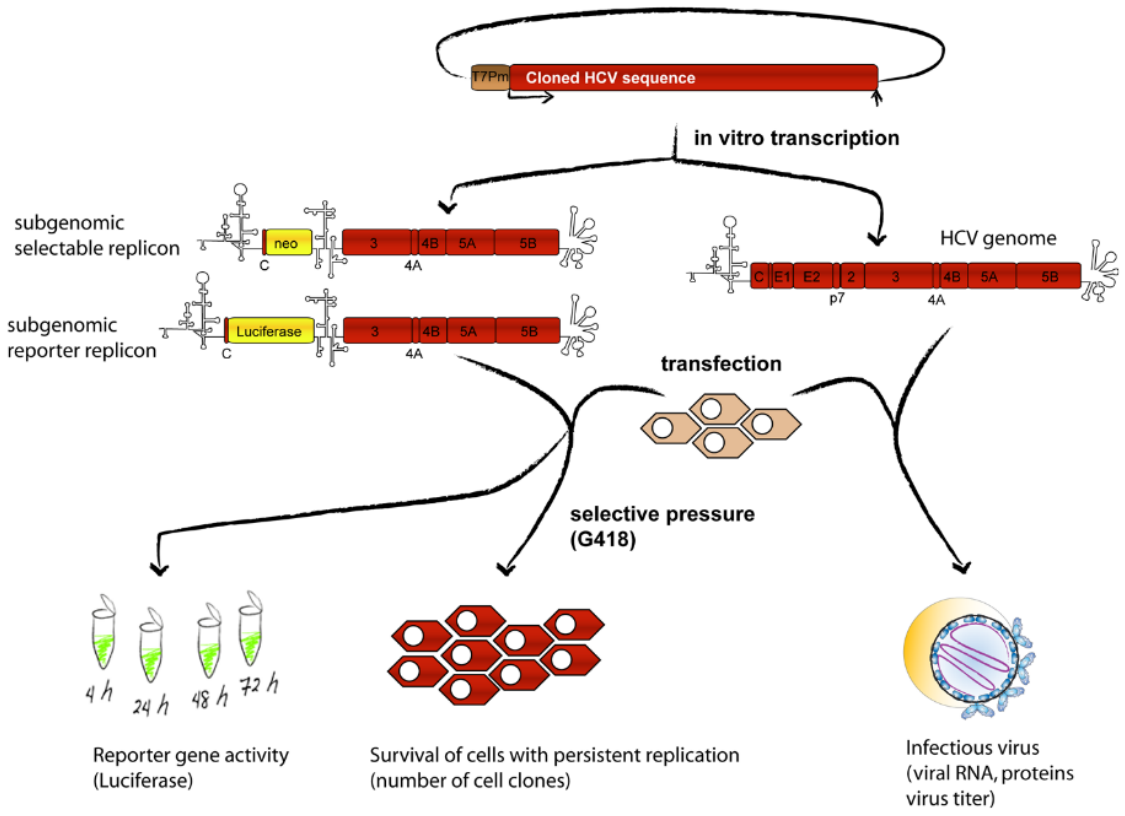

Figure 12: Experimental strategies to create sustainable cell culture models for HCV replication

Viral genome sequences are inserted in a plasmid used to generate *in vitro* transcripts corresponding to RNA copies of HCV sequence. Left half: Subgenomic replicons allow viral replication detection by luciferase assays or selection for drug-resistant cell clones. The number of cell clones obtained reflects the replication capacity. Right half: The HCVcc system, derived from full-length HCV genomes, gives rise to infectious viral particles secretion. From: (Lohmann & Bartenschlager, 2014).

d. Primary human hepatocytes

The research for a more relevant physiological system led to the development of primary cell cultures. Primary human hepatocytes (PHH) are a better alternative and remain the in vitro reference model for the study of the antiviral molecules metabolism and liver pathologies. These differentiated mature polarized cells allow the productive HCV replication but with lower efficacy than HuH-7 cells and with large variations according to the studies. Unlike HuH-7, PHH can be infected with genotypes 1, 2, 3 and 4 of HCV isolated from the infected patients' sera. This culture system produces infectious viral particles, which can infect naive human hepatocytes. These hepatocytes also have many limitations. They are isolated from hepatic resections most frequently from patients with severe pathologies, so their availability is low. The intra- and interindividual hepatocytes variability leads to the lack of reproducibility. Moreover, these PHH do not proliferate and lose their phenotype in culture (Guillouzo et al., 2007). On the other hand, their cryopreservation has been well-standardized through efficient protocols (Terry et al., 2007). Finally, the study of HCV is limited because chronic infection is not evident due to the induction of the innate immune response. Immune response mediated by IFN leads to decreased viral replication and inhibition of HCV infection in PHH (Helle et al., 2013; Liu et al., 2015).

e. Animal models

Despite several landmark advances in the HCV study, including the replicon system in 1999, the HCVpp system in 2003 or the HCVcc in 2005, there are still some weaknesses when compared with *in vivo* models. The complexity of the host immune responses and clinically relevant disease phenotypes are not reproducible in these referred models. Therefore, the development of suitable HCV animal models has proved to be truly necessary.

i. Chimpanzees

Chimpanzees (*Pan troglodytes*) are the closest being relative to humans, sharing more than 98% of nuclear genome so, beyond humans, they are the only ones that can be stably and reproducibly infected by HCV (Goodman et al, 2005; Houghton, 2009b).

This animal model has provided a relevant knowledge about the mechanisms of HCV infection, as well as the antiviral immune responses. Thus, it has been shown that HCV infection is not totally immune preventive for the subsequent virus exposures (Farci et al, 1992). Acute infected chimpanzees, like humans, report significant inflammatory alterations with immune responses detected in the peripheral blood and the liver. There are aminotransferases elevations and inflammatory infiltrates determined by liver biopsies (Bigger et al, 2001; Major et al, 2004; Thimme et al, 2002). However, the infection evolution in chimpanzees is milder than humans, without development of cirrhosis or fibrosis, and just one chimpanzee reported to have developed HCC.

The infection can be induced in chimpanzees by intravenous inoculation with clinical isolates, tissue culture derived virus, or intrahepatic injection of *in vitro* transcribed HCV RNA (Alter et al, 1978; Kolykhalov et al, 1997; Lindenbach et al, 2006; Wakita et al, 2005). Chimpanzees showed to be slightly susceptible to IFN treatment. In contrast, the treatment with the first DAAs was efficient (Bukh, 2004; Choo et al, 1994; Folgori et al, 2006; Olsen et al, 2011). This model presents some disadvantages as HCV study models, including limited availability, ethic issues and elevated cost, which have led to development of suitable alternatives.

ii. Mouse

Mice are naturally non permissive to HCV infection with limited replication in murine cells. However this animal model is privileged because it is easily available and cheaper than other models (Billerbeck et al, 2013; Bukh, 2012; Ploss & Rice, 2009; von Schaewen et al, 2014). Thus, strategies were implemented to adapt HCV to the mouse organism, through the knock down of murine factors that hamper productive HCV infection (Vercauteren et al, 2014a). Also, the introduction of human factors was essential for this adaptation (Anggakusuma et al, 2014; Dorner et al, 2011; Giang et al, 2012).

An important advance was the introduction of essential human co-factors in murine hepatocytes like CD81 and OCLN, essential to the viral entry. Thus, the expression of these human factors by adenoviral gene delivery or by transgenic expression showed to support HCV entry step, allowing the study of potential entry inhibitors and vaccine candidates (Anggakusuma et al, 2014; Dorner et al, 2013; Dorner et al, 2011; Giang et al, 2012). Despite these progresses, the viral replication is still hampered by murine innate immune pathways. However, if the immune signaling is suppressed, the entire HCV life cycle is achieved with production of infectious particles (Chen et al, 2014; Dorner et al, 2013).

The xenograft of mouse liver with human hepatocytes, creating a human-liver chimeric mouse was other significant progress leading to the extensive use of this model for HCV study. Normally, several weeks after transplantation, the hepatic parenchyma is predominantly constituted by human hepatocytes developing the normal architecture of the liver and secreting human proteins (Azuma et al, 2007; Bissig et al, 2010; Mercer et al, 2001; Meuleman et al, 2005). One example was the SCID/Beige model that was transplanted with HuH-7 cells resulting in high levels of viremia with good toleration. This model showed not to be permissive to primary human hepatocytes (Zhu et al, 2006). However, at this date it has already been shown the Trimera mouse model ability to ensure a detectable viremia during 1 month with a xenograft of primary human liver tissue (Ilan et al, 2002).

Fully permissive murine models supporting high production of infectious particles also emerged. There are two murine models predominantly used: the urokinase-type plasminogen activator transgenic severe combined immunodeficiency (uPa-SCID) and the mutated mouse in the Fah, Rag2 and Il2rg genes (FRG). In the first model, the urokinase-type plasminogen activator protein is overexpressed, inducing hepatotoxicity. In the FRG model, the knock-down of important enzymes (consequence of the gene mutations referred above) leads to the

accumulation of catabolites, which are toxic for hepatocytes (Azuma et al, 2007; Bissig et al, 2007; Bissig et al, 2010; Mercer et al, 2001; Meuleman et al, 2005). Therefore, these modifications on murine models lead primarily to the induction of mouse hepatocyte death, which allows the repopulation with human hepatocytes. These models present robustness and reproducibility to HCVcc and high infection levels from HCV clinical isolates, allowing the study of different antiviral strategies (Bukh et al, 2010; Lindenbach et al, 2006).

The immunodeficiency is required to support the hepatocellular engraftment, in order to prevent the rejection of engrafted human hepatocytes. However, the creation of a humanized immunity system has been necessary to the HCV immunopathogenesis and vaccine efficacy studies. Therefore, to achieve this goal, the murine models received human CD34+ hematopoietic stem cells (HSCs) from different human donors that promoted the development and function of human immune cells, as well as myeloid cells and NK cells (Gutti et al, 2014; Rongvaux et al, 2014; Washburn et al, 2011; Wilson et al, 2014). These humanized models may be used to investigate the contribution of these immune cells in HCV infection, as well as other immune pathologies.

F. Immune response against HCV

The immune system plays an important role in the development of chronic hepatitis C, as well as in the progression of liver damage. Indeed, the rate of chronic HCV infection development and severity of liver disease are higher in immunocompromised patients.

a. Innate immune response

i. HCV detection and induction of IFNs response

The innate immune response is the first line of body defense against viral infections. In the early stage of HCV infection the viral titer increases quickly, then slowing down to a plateau level of viremia that corresponds to the introduction of intrahepatic IFN responses. The pattern recognition receptors (PRRs) present in infected cells sense the HCV as non-self-body and trigger multiple signals to activate downstream signaling pathways to clear the virus [Figure 14] (Yang & Zhu, 2015).

The IFNs are the principal cytokines that introduce an antiviral state in infected cells. IFNs also activate and regulate cellular components of innate immunity such as natural killer (NK) cells (Stetson & Medzhitov, 2006). There are three types of IFNs involved in immune responses: the type I including many IFN-α and one IFN-β; the type II including IFN-γ; and type III including IFN-λ (IFN-λ1, IFN-λ2, IFN-λ3 and the newly discovered IFN-λ4) [Figure 13] (Ray, 2013). The types I and III are produced by virus-infected cells and by sentinel cells as well as macrophages and dendritic cells (DCs). These sentinel cells do not have to be infected by viruses to produce IFNs. The type II IFNs are produced by NK cells (Heim, 2013).

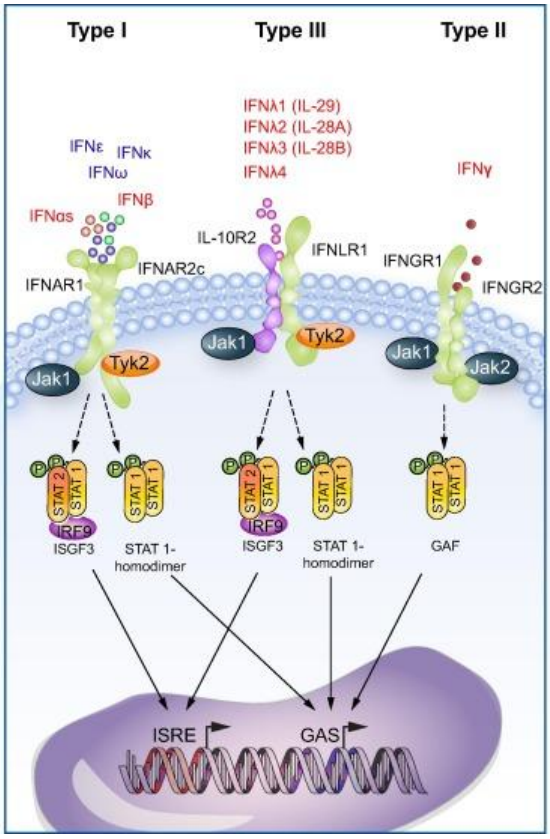

Figure 13: Types of IFN and their receptors

The type I IFN includes the IFN-α and IFN-β, ε, κ, ω and the type III IFN includes IFN-λ. Both types of IFN present an interaction with different receptors, although leading to the same downstream signaling events through the activation of IFN-stimulated gene factor (ISGF) 3 and STAT1 homodimers. The type II IFN includes IFN-γ and activates STAT1 without effect in ISGF3. From: (Heim & Thimme, 2014).

There are two important pathways to sense HCV invasions that converge in the activation of key transcription factors NF-kB and interferon regulatory factors (IRF) 3 and 7. Activated IRF3 and NF-kB lead to the promotion of type I and III IFN genes. One of these pathways is cytosolic, in which different PRRs recognize viral RNA. The most important receptor is the RIG-I that is encoded by the DDX58 gene and belongs to the RIG-I-like receptor (RLR) family, which also includes melanoma differentiation-associated protein (MDA) 5 and laboratory of genetics and physiology 2 (LGP2) (Rehwinkel & Reis e Sousa, 2010; Yoneyama et al, 2004). RIG-I is also a sensor for different members of the Flaviviridae

family, including dengue virus, West Nile virus, and Japanese encephalitis virus (Loo & Gale, 2011). In the early stage of infection the polyuridine motif of the 3′UTR HCV genome is sensed by RIG-I, leading to the activation of two amino terminal caspase recruitment domains (CARD) that produce a downstream signaling through the activation of MAVS (Kawai et al, 2005; Meylan et al, 2005; Seth et al, 2005). MAVS, an essential downstream adaptor in this pathway, propagates the signal to the TANK Binding Kinase 1 (TBK1) and IKKε kinases, which phosphorylate downstream IRF3 and IRF7. Also, MAVS activates NF-kB through the activation of the IKK complex. MDA5, especially when combined with RIG-I, has been shown to activate host responses to infections by the same viruses detected with RIG-I, although its role as a PRR for HCV infection remains unclear. Even if MDA5 ectopic expression in HuH-7 cells confers an antiviral activity against HCVcc, MDA5-deficient cells are still competent to trigger the IFN-β promoter in response to HCV RNA stimulation (Saito et al, 2008; Schoggins et al, 2011). Thus, these studies suggest that MDA5 may be involved in the regulation of HCV innate immune responses, but it is the RIG-I receptor that seems to be the key for HCV recognition.

The other pathways involve the toll-like receptor (TLR) as host PRR recognizing HCV pathogen-associated molecular patterns (PAMPs) and activating the expression of variety of overlapping and unique genes characteristic of immune responses (Akira et al, 2006). There are three TLRs involved in the recognition of viral nucleic acids: TLR3, TLR7, and TLR9, which are expressed in different immune cells. These three receptors are localized in intracellular compartments such as endosomes, contrary to other TLRs. For example, TLR3 is a type I transmembrane protein that recognize HCV dsRNA in the endosome and activate a downstream signal dependent of endosome acidification (Alexopoulou et al, 2001). In order to be recognized by that TLR, the HCV dsRNA is transported to the endosome. The class A scavenger receptor type 1 (MSR1) is expressed on the plasma membrane of infected and adjacent uninfected cells. After the HCV dsRNA is released by infected cells, it can bind to the MSR1 that carries it to the endosomes where the dsRNA is sensed by TLR3. The MSR1-mediated binding, transport, and release of dsRNA to the acidified endosome requires a stretch of conserved basic residues of MSR1 that acts as a key element for the TLR3-mediated PRR, thereby rendering hepatocytes refractory to HCV replication (Dansako et al, 2013). The role of TLR3 in innate immune responses to HCV has shown to be sufficient to trigger antiviral signaling, demonstrating a RIG-I-independent requirement. This receptor induces the activation of IRF3 to increase IFN-β production which signals other cells to

increase their antiviral defenses, being also able to regulate the cell line permissiveness to HCV replication (Binder et al, 2007; Eksioglu et al, 2011).

The TLR7, in turns, is found in plasmacytoid DCs and recognize HCV ssRNA in the endosome. After activation, TLR7 leads to the production of high levels of IFN (Diebold et al, 2004; Kawai & Akira, 2011; Liu, 2005).

All types of IFNs induce the transcriptional activation of hundreds of genes that consequently initiate an antiviral state. These genes are specific of each IFN and target cell type. Usually, IFNs-α and IFNs-λ induce similar sets of genes in cells that express receptors for both, unlike IFN-γ that induces a more distinct set of genes (Der et al, 1998; Dill et al, 2012; Marcello et al, 2006). Also, IFNs-λ present a more specific role in viral defense at epithelial surfaces in the respiratory and gastro-intestinal tract (Mordstein et al, 2010). Type I IFN receptors are constituted by two subunits, Interferon Associated Receptor (IFNAR) 1 and IFNAR2. Type III IFN receptors are constituted by one IFNλ-R1 specific chain (IFNLR1) and other IL-10 receptor (IL-10R2) chain. Finally, the receptors for type II IFNs are composed by two subunits, the IFNγ-R1 (IFNGR1) and IFNγ-R2 (IFNGR2) (Heim & Thimme, 2014).

After each connection between IFN and its receptor, members of the Janus kinase family are activated. These receptors are thus phosphorylated and establish very specific and selective interactions with signal transducer and activator of transcription (STAT) proteins. The specificity of the Jak-STAT signaling is determinant to send the signal from the cell surface to the nucleus. (Darnell, 1997; Heim et al, 1995). Generally, type I and type III IFNs activate STAT1 and STAT2, which leads to the formation of the transcriptionally active ISGF3 complex that consists in three proteins, STAT1, STAT2, and IRF9 then leading to the transcription of multiple ISGs (Darnell et al, 1994; Stark & Darnell, 2012; Zhou et al, 2007). However, type II IFN only activates STAT1. This STAT1 induced by all IFNs binds to gamma activated sequence (GAS) elements in ISGs (Darnell et al, 1994; Zhou et al, 2007). Together, these genes promote a virus clearance from infected cells and a protection of their neighbors.

There are also suppressor of cytokine signaling (SOCS) proteins, which are important negative regulators of Jak-STAT signaling that quickly reduce IFN levels. So, a failure in the SOCS induces the improvement of type I IFN signaling and consequently a development of severe inflammatory disease but with high resistance to viral infections (Alexander et al, 1999; Fenner et al, 2006; Krebs & Hilton, 2001).

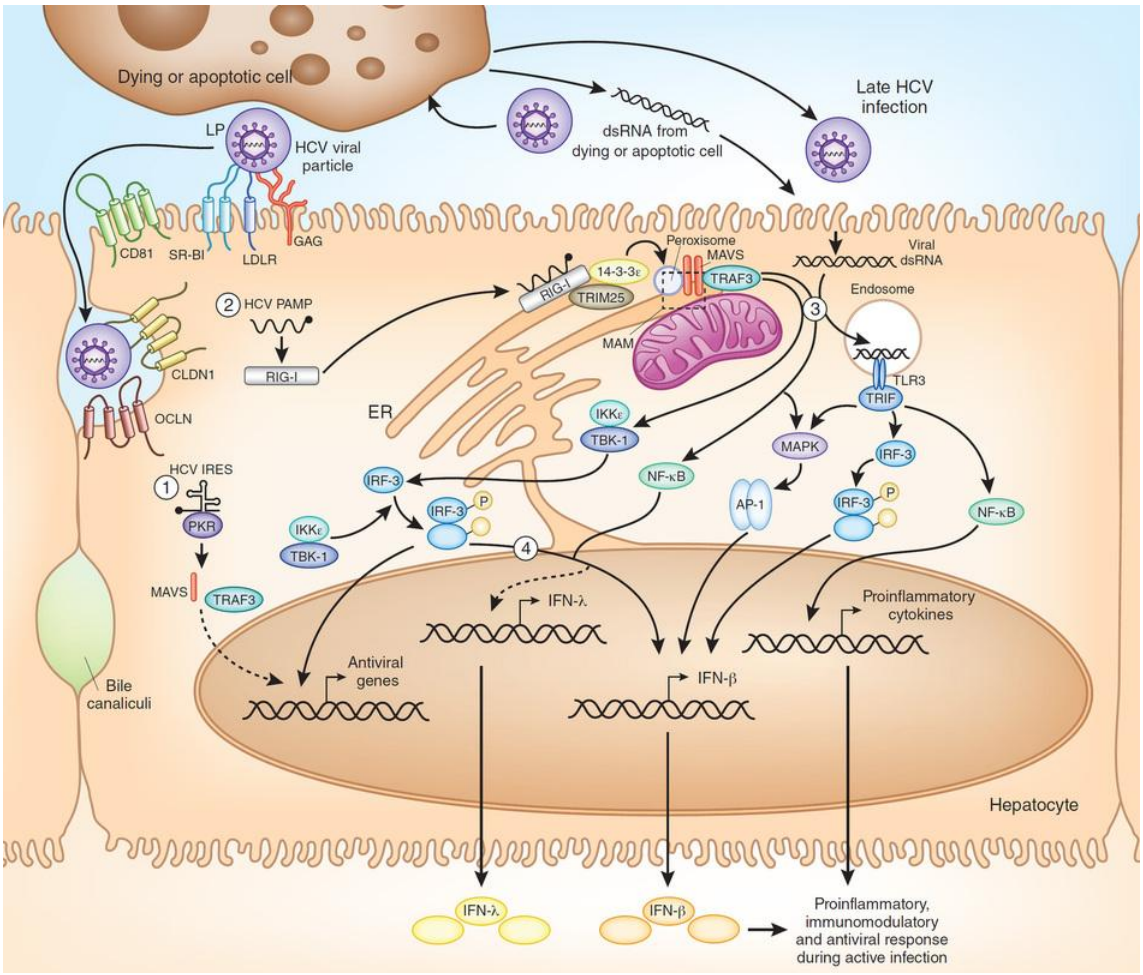

Figure 14: HCV signaling pathways induce innate immune responses through IFN induction in hepatocytes

HCV is firstly sensed by the combined actions of (1) PKR (2) RIG-I, and (3) TLR3 proteins. They recognize dsRNA in the HCV IRES and poly U/UC PAMP during early infection, then detecting dsRNA that accumulates following HCV infection or by uptake of HCV dsRNA from dying cells. Consequently, immunomodulatory genes are induced, as well as IFN-β and other proinflammatory cytokines. (4) The hepatic activation of IFN-λ during HCV infection needs to be completely characterized. From: (Horner & Gale, 2013)

- *HCV escape strategies to host innate immune response*

Like many viruses, HCV has developed different strategies to evade cellular innate immune response. There are many infected individuals that are not able to clear the virus and that develop a chronic infection, maybe due to different mechanisms used by the virus to escape the immune system (Horner & Gale, 2013).

HCV NS3/4A complex is able to block RIG-I signaling via MAVS cleavage, releasing MAVS from the membranes where it is anchored, thereby preventing dimerization and downstream signaling of innate immunity (Foy et al, 2003). These membranes can be: mitochondrial outer membrane, peroxisomal membrane and mitochondria-associated endoplasmic reticulum membranes (MAMs). In addition, MAVS cleavage also contributes to the reduction of IFN pathway induction. Clinical experiments have shown that NS3/4A inhibitors restore antiviral signaling in HCV infected cells by preventing MAVS cleavage (Johnson et al, 2007; Liang et al, 2008). NS3/4A is able to cleavage the TLR3 signaling-adaptor protein –TRIF, which presents a sequence very close to the NS4B/5A cleavage site, reducing TRIF protein abundance and leading to the transduction suppression of TLR3 signaling with consequent HCV persistence (Ferreon et al, 2005; Li et al, 2005)

Protein kinase R (PKR) presents multiple functions of pathogen sensing in cells, being also involved in escape strategies. The interaction between PKR and HCV dsRNA induces some early ISGs with high levels of IFN-β through MAVS. However, there are several PKR-inactivation strategies adopted by HCV, at the PKR/PRR signaling with direct action on PKR by NS5A/E2 or MAVS cleavage by NS3/4A and at the PKR-regulated translational inhibition by NS5A/E2. These actions thus contribute to the viral persistence. HCV interaction with PKR also activates the kinase domain to phosphorylate eIF2α that inhibits the translation of important messenger RNAs (mRNAs), leading to the suppression of IFN and ISGs and to the persistence of the virus (Arnaud et al, 2011; Arnaud et al, 2010; Shimoike et al, 2009).

Other HCV proteins have been implicated in immune processes and collectively, have shown to interfere with IFN signaling or downstream IFN effector functions. In particular, the HCV Core seems to interfere with the JAK/STAT pathway and ISG expression, whereas E2 and NS5A that inhibit the ISGs function (Rehermann, 2009). NS4B also plays an important role blocking the IFN-β production signaling mediated by RIG-I. The stimulator of interferon genes (STING), an activator of RIG-I signaling that presents a structural homology domain with NS4B, is involved in this mechanism. Thus, NS4B suppresses RIG-I-mediated IFN-β

production signaling by direct interaction with STING (Nitta et al, 2013; Yi et al, 2016). It was also shown this same interaction NS4B/STING may suppress the interferon signaling by disruption of the interaction between STING and TBK1 (Ding et al, 2013).

ii. Natural killer cells

The Natural Killer (NK) cells, large granular lymphocytes, are also involved in the innate immune response with an important role in the control of viral infections. They present a direct and regulatory antiviral effect mediated by cytotoxic and non-cytotoxic effectors (Rehermann, 2013; Rosen, 2013). NK cells activate dendritic cells and T cells and also produce type II IFN (IFN-γ) with a smaller impact in HCV immunity than other IFNs that were referred above (Ishii & Koziel, 2008; Zhang et al, 2013). In the liver, NK cells respond to hepatotropic infections. For example, HCV early infection induces an augmentation of NKp46 ligands expression that is an activator receptor in cytotoxic responses (Crispe, 2009; Doherty & O'Farrelly, 2000). NK cells with high levels of this receptor lead to a strong inhibition of HCV, *in vitro*, and to a low level of viral RNA, *in vivo* (Golden-Mason et al, 2012; Kramer et al, 2012).

There are some studies that propose a modification of NK cells activity as a mechanism involved in the HCV persistence. In a fundamental context, the E2 protein interacts with CD81 receptor, which can affect NK cell functions (Crotta et al, 2002; Tseng & Klimpel, 2002). On the other hand, in a clinical context an overexpression of receptors in the NK cells surface could lead to a change in the cytotoxic activity of these cells (Nattermann et al, 2006).

b. Adaptive immune response

The definitive barrier to control HCV infection is the adaptive immune response. This immunity stage is mediated by humoral and cellular responses and begins late, about 6 to 8 weeks after HCV invasion (Shin et al, 2011). In some cases there is a spontaneous viral clearance that is associated with an early neutralizing antibody (nAb) response as well as sustained HCV-specific CD4+ and CD8+ T cell responses (Guidotti & Chisari, 2006). However these components can fail due to different viral evasion strategies.

i. Humoral immunity response

After host evasion by HCV, the B cell receptors, present in the lymphoid organs, recognize HCV antigens. Consequently, these cells promote the secretion of HCV-specific antibodies that are detected in the circulation from around 1 to 2 months after infection (Dahari et al, 2005; Sagnelli et al, 2005; Thimme et al, 2001). Many antibodies are produced but only a few of them are able to inhibit HCV binding, entry, or uncoating. These antibodies are called neutralizing antibodies (Logvinoff et al, 2004).

Neutralizing antibodies can prevent the virus attachment/binding and consequently hamper viral infection and spread. They target epitopes located within the E1E2 envelope glycoproteins, as well as the heterodimer formed by both glycoproteins, preventing viral entry step. Studies have shown that HVR1 is the most relevant epitope target (Kato et al, 1993; van Doorn et al, 1995). The C-terminus region of this HVR1 interacts with SR-BI and represents an effective epitope recognized by specific antibodies. CD81 is also an essential receptor for HCV entry that interacts with E2 and this interaction site is a very good target for the antibodies. Despite the fact that several studies indicate a neutralizing activity of these antibodies *in vitro*, their efficiency *in vivo* is less well understood (Sabo et al, 2011). There are few antibodies identified that specifically target E1, maybe due to its poor immunogenicity. Also, it was reported that infected individuals develop much more antibodies against E2 than E1, which contributes to a lower neutralisation through E1-specific antibodies (Beaumont et al, 2016; Meunier et al, 2008b; Wang et al, 2011). More recently, a study showed that highly potent nAbs against E2 are able to prevent HCV infection and also to eliminate established infection in experimental animal models (de Jong et al, 2014).

Some evidence support a role of nAbs in the control of HCV infection and in the prevention of reinfection. A preliminary induction of cross-reactive nAbs during acute infection is broadly correlated with the spontaneous clearance of HCV (Dowd et al, 2009; Osburn et al, 2010; Osburn et al, 2014; Pestka et al, 2007; Raghuraman et al, 2012; von Hahn et al, 2007). Furthermore, the persistence of infection is associated with the delay of nAbs induction (Pestka et al, 2007). Moreover, studies have shown that the presence of nAbs against E1E2 in the chronically infected patients serum seems to play a role controlling HCV during chronic infection (Ball et al, 2014).

- *HCV escape strategies to humoral immunity response*

The evolution of viral quasi-species may change their target epitopes for neutralization by nAbs (von Hahn et al, 2007). It was proposed that E1E2 glycoprotein interactions with SR-BI may protect from nAbs neutralisation as well as specific glycans on E2 (E2N1, E2N6, and E2N11) (Falkowska et al, 2007; Helle et al, 2007). The E2 HVR1 was pointed to protect the virus from nAbs neutralisation. Studies identified an E2 motif of HVR1 as non-essential for in vivo infectivity and in its absence the virus greatly increased its susceptibility to neutralisation by chronic-phase HCV serum antibodies. Other studies showed that HVR1 protects cross-genotype conserved neutralisation epitopes. However, the mechanism remains to be determined, although it is suggested that HVR1 may block the accessibility of neutralisation epitopes (Prentoe et al, 2011; Prentoe et al, 2015).

Studies have shown an association between viral particle density and sensitivity to nAbs, suggesting that their association to lipoproteins reduces the sensitivity of particles to these antibodies. It was also demonstrated that a mutation at position 451 of E2 alters the relationship between particle density and infectivity, reduces SR-BI dependence and increases virion sensitivity to CD81 and nAbs. Thus, a balanced interaction between HCV particles, lipoprotein components, and viral receptors allows the escape from host immune responses (Grove et al, 2008).

HCV-associated apoE was implicated in the viral escape of neutralization by nAbs isolated from chronically infected patients. This novel strategy may contribute to explain the HCV's remarkable capacity to establish chronic infection. It was observed for different genotypes, so this strategy is revealed pan-genotypic. A recent study demonstrated that the residue 447 of E2 may alter the E2-apoE complex resides conformation on the periphery of an

E2 region. In the case of apoE depletion, the E2 conformational epitopes are exposed, so the level and conformation of virion-associated apoE affect the ability of the virus to escape neutralization by antibodies.

In addition, it was also suggested that HCV may avoid neutralisation by direct HCV cell-to-cell transmission (Brimacombe et al, 2011; Timpe et al, 2008).

ii. Cell-mediated response

Cell-mediated immunity presents an important role in the disease progression, in which self-limited acute HCV infection is associated with sustained HCV-specific CD4+ and CD8+ T cell responses. Referred cells are activated through the recognition between T cell receptors and peptides presented by major histocompatibility complex (MHC) class II molecules, in the lymphoid organs. Thus, T cells target multiple epitopes in structural and non-structural viral proteins (Cooper et al, 1999; Thimme et al, 2002) and remain detectable long time after the infection resolution (Takaki et al, 2000). Many studies sustained the importance of both T cells in the control of HCV infection, proving a clear association between the beginning of virus-specific T cell responses and HCV clearance (Cooper et al, 1999; Diepolder et al, 1997; Lechner et al, 2000; Missale et al, 1996; Takaki et al, 2000; Thimme et al, 2002; Thimme et al, 2001).

The primary antiviral effect of HCV-specific CD8+ T cells involves the production of pro-inflammatory cytokines as well as IFN-γ and TNF-α (non-cytolytic pathway) (Jo et al, 2012). These cells also exhibit, with lower extent, cytolytic effector functions, involving a perforin and/or Fas/Fas-L systems and inducing the lysis of infected cells (Tsai et al, 1997). CD4+ T cells recognize viral antigens and present an essential role as helper to CD8+ T cells, in the control of HCV infection. Therefore, CD4+ T cells behave like central regulators and virus-specific CD8+ T cells like key effectors [Figure 15] (Klenerman & Thimme, 2012).

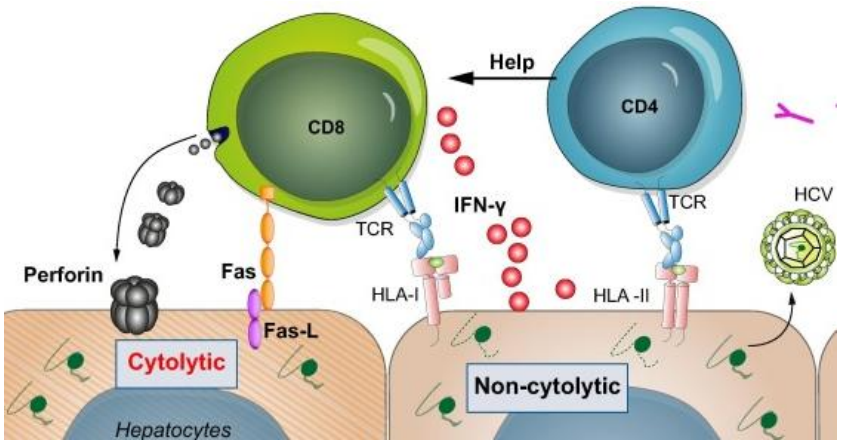

Figure 15: Virus-specific CD8+ T cell responses

The activation of CD8+ T cells can leads to the inhibition of viral replication through the cytolytic (perforin) or non-cytolytic (IFN-γ) effector mechanisms. Help by CD4+ T cells is required. Adapted from: (Heim & Thimme, 2014).

During acute infection, CD4+ T cells are detectable at 3 to 4 weeks after viral invasion, and their immune responses target some HCV proteins like the Core, NS3 and NS4A (Diepolder et al, 1997; Hoffmann et al, 1995). Studies have shown that CD4+ T helper type 1 (TH1) interact with CD8+ T cells, contributing to viral clearance (Klenerman & Thimme, 2012), while CD4+ T helper type 2 (TH2) are implicated in the evolution of chronic infection (Tsai et al, 1997). Thus, the cytokines secreted by TH1 (IL-2 and IFN-γ) lead to the virus elimination, while the cytokines associated to TH2 (IL-4, IL-5 and IL-10) remain during chronic infection (Tsai et al, 1997). The viral clearance through the HCV-specific CD4+ T cells is also associated with some HLA class II alleles that have shown to be protective (Day et al, 2002; Diepolder et al, 1997; Schmidt et al, 2011; Schulze zur Wiesch et al, 2005). An immunodominant CD4+ T-cell epitope was identified, which is recognized by the majority of NS3-specific CD4+ T-cell clones and can be presented to these cells by different HLA alleles (Diepolder et al, 1997). Also, predominant CD4+ T cell epitopes in resolved HCV infection individuals are preferentially located in non-structural proteins and are immunogenic in the context of HLA class II molecules (Schulze zur Wiesch et al, 2005).

In the absence of CD4+ T cells, titers of viremia can be controlled, indicating an initial control of infection by CD8+T cells. However, viral titers begin to increase shortly after, which can be associated to the evolution of viral escape mutations in the epitopes recognized

by CD8+ T cells (Grakoui et al, 2003). These data support the concept that HCV-specific CD8+ T cells are the main antiviral effector cells, with a significant help by HCV-specific CD4+ T cells regarding the viral escape from its response. Also, the loss of CD4+ T cell helper can explain the CD8+ T cell failure and the viral persistence, with rare detection of HCV-specific CD4+ T cell responses in the chronic infection (Lucas et al, 2007).

CD8+ T cells usually emerge about 6 to 8 weeks after infection, followed by an increase of liver enzymes, clinical symptoms, and a decrease of viral load (Gruener et al, 2000; Thimme et al, 2001). Some HLA class I alleles show viral antigens to CD8+ T cell that is associated with spontaneous resolution of acute HCV infection and also supports the importance of these cells in viral clearance (Schmidt et al, 2011). Their cytotoxic effect is expressed in infected target cells that present viral antigens in their surface, through the receptors such as FAS and its ligands or the paracrine secretory factors. More recently, HCV-specific CD8+ T cells showed to produce IL-17 and to induce high expression of CD161 with a possible protective effect in chronic HCV infection (Billerbeck et al, 2010; Northfield et al, 2008). These cells also produce IFN-γ that is decisive to the HCV elimination. Despite its production already in the innate immunity, it is not sufficient, this being the reason why the immune system needs a cellular stimulation response to provide an additional antiviral effect.

- *HCV escape strategies to cell-mediated response*

HCV presents elevated levels of replication that promote the appearance of numerous mutations, which can lead to the failure of the adaptive immune responses with viral escape. These mutations seem to be associated with the development of chronic infection, and their absence with the viral clearance (Cox et al, 2005).

The T cell exhaustion is a demonstration of adaptive immune failure, because with chronic stimulation these cells decrease the secretion of IL-2, TNF-α and IFN-γ as well as their cytotoxic effect (Wherry et al, 2003). The impaired activation of Lck and Akt proteins mediated through the binding of HCV core and gC1qR on T cells has been suggested to help explaining this loss of function (Yao et al, 2004). Other failures in adaptive immune responses can be due to a CD4+ T cell insufficient help, a limited T cell receptor diversity or a viral fitness cost (Neumann-Haefelin & Thimme, 2013).

G. Diagnosis and Treatment of HCV Infection

a. Diagnosis

HCV infection is considered as a silent epidemic. Being asymptomatic, many infected people are not conscious of their infection and consequently, symptoms cannot be used as specific indicators of it (Ascione et al, 2007). However, one can suspect a viral infection through blood analysis showing high levels of transaminases, liver palpation or hepatic biopsy later confirmed by automatic tests. Also, the lack of a preventive or therapeutic vaccine forces the development of higher performance diagnostic methods in order to prevent hepatic issue like fibrosis or HCC.

The HCV diagnosis is based in two types of virological tests: indirect and direct. Indirect tests, namely serologic assays, detect HCV-specific antibodies in the serum, while direct tests detect, quantify and characterize components of the viral particles, particularly HCV RNA. These diagnosis assays are essential to detect the infection, being also crucial to make the best therapeutic decision with an optimal duration and to monitor treatment responses (Ghany et al, 2011; Ghany et al, 2009).

To firstly identify a current HCV infection, the Centers for Disease Control and Prevention (CDC) recommends a sequence of procedures [Figure 16]. This test sequence begins with a rapid assay to detect HCV antibodies in blood. A nonreactive HCV antibody result indicates that no antibody was detected. In this case, no further action is required, except if the person is suspected to have been recently exposed, and so an HCV RNA measure is recommended. A reactive result may indicate a current HCV infection, a past HCV infection that has been resolved or a false positivity. Thus, if HCV RNA is detected (by a direct test), it means the presence of a current infection and the individual is indicated for treatment. If HCV RNA is not detected, this indicates a past resolved infection or false HCV antibody positivity. In this case, to discern both hypotheses, it is recommended to repeat the test with another HCV antibody assay. An HCV RNA assay repetition is also recommended if the person is suspected to have had HCV exposure within 6 months or has clinical evidence of HCV disease, or if there is a suspicion of incorrect handling/storage of the test specimen [Centers for Disease Control and Prevention (www.cdc.gov/mmwr/)].

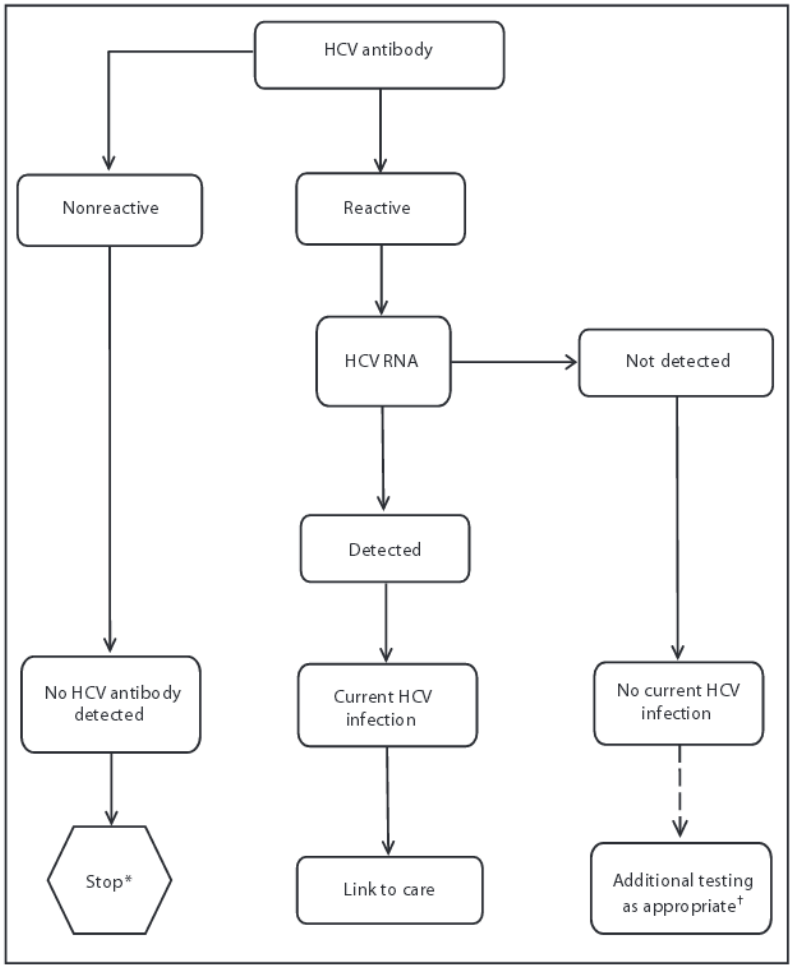

Figure 16: Recommended testing sequence for identifying current HCV infection.
From: Centers for Disease Control and Prevention (www.cdc.gov/mmwr/).

The diagnosis is recommended for all persons with increased risk of HCV infection, in order to avoid infection complications inherent to a late treatment. The risk groups include: persons who have ever injected illegal drugs, including those who injected only once many years ago; recipients of clotting factor concentrates before 1987; recipients of blood transfusions or solid organ transplants before July 1992; patients who have ever received long-term hemodialysis treatment; persons with known exposures to HCV (such as health care workers after needle sticks involving HCV-positive blood, recipients of blood or organs from a donor who later tested HCV-positive); all persons with HIV infection; patients with signs or symptoms of liver disease; children born to HCV-positive mothers (to avoid detecting maternal antibody, these children should not be tested before the age of 18 months) [Centers for Disease Control and Prevention (www.cdc.gov/mmwr/)].

Serologic assays include screening and confirmation of specific antibodies and are preferentially used due to the fact that they are cheaper and easier to perform. The "serologic window" between HCV infection and the specific antibodies detection varies with the patient, thus the seroconversion occurs normally from 6 to 8 weeks after the beginning of infection. In patients that spontaneously resolve their infection, anti-HCV antibodies may decrease slowly over several years. In chronic infection, these antibodies persist indefinitely, except in cases of haemodialysis or immunosuppression in which they become undetectable. Enzyme-linked immunosorbent assay (ELISA) is currently used to detect HCV-specific antibodies in the serum of patients, through the interaction with specific recombinant antigens like Core, NS3, NS4 and NS5, with high specificity and sensibility (Colin et al, 2001; Morishima & Gretch, 1999). However, the large-scale of HCV screening led to the development of rapid diagnostic tests (RDTs), an attractive alternative to the ELISA assays. The OraQuick®HCV Rapid Antibody Test emerged as a fast (20 min to read) and simple test to detect specific antibodies against HCV. It can be performed with oral fluid, whole blood, serum or plasma in very small quantities (20-40 µL), showing to be very specific and sensible. The test uses synthetic peptides and recombinant antigens from the Core, NS3, and NS4 regions of the HCV genome to be recognized by specific antibodies (Chevaliez et al, 2016). There are other RDTs with small variations between them (necessary sample volume, time to read, specificity and sensibility) that were validated using whole venous or capillary blood, but some of them can accept other matrices including oral fluid, serum or plasma (Chevaliez et al, 2016; Poiteau et al, 2016).

In the infected patient serum, HCV RNA is probably the earliest detectable marker of acute infection, preceding the appearance of HCV-specific antibodies by several weeks (Chevaliez & Pawlotsky, 2009). Thus, the nucleic acid amplification tests (NAT) were approved for detection/quantification of HCV RNA in serum or plasma, normally after a reactive result to HCV antibody analysis. Nowadays, the quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) is the most used NAT, presenting high specificity and sensitivity, with detection limit of 10-15 UI/mL. It is also almost completely automatized (Castelain et al, 2004; Chevaliez, 2011). Transcription-mediated amplification (TMA) is also a NAT much less used today because it does not reach the sensitivity levels of real time RT-PCR. The NATs are not only useful for diagnosis but also to monitor viral load during HCV treatment.

The HCV core antigen detection and quantification test was proposed to be used instead of NAT for HCV diagnosis and therapy monitoring. It is performed by a chemiluminescent microparticle immunoassay (Architect HCV Core antigen test) on the Abbott Laboratories device. The advantages when comparing with NAT are that it is cheaper and easier to perform and also that core antigen levels are strongly correlated with HCV RNA levels for different genotypes (Chevaliez et al, 2014; Tillmann, 2014). However HCV core Ag assay presents a major limitation, its lower sensitivity that limits the utility.

Due to disparities in the treatment responses, specific of each genotype, the genotyping is essential to the diagnosis. In a clinical approach, genotyping assays are useful to guide therapy decisions based on each genotype, leading to an adapted treatment (Chevaliez & Pawlotsky, 2009). Thus, different methods are possible like the genome sequencing of HCV regions (e.g. C/E1, NS5A, NS5B) and subsequent phylogenetic analysis (Murphy et al, 2007; Pawlotsky et al, 1997). Among other similar test that can be used, Abbott RealTime HCV Genotype II, approved by FDA, is an in vitro RT-PCR assay for HCV genotype determination in plasma and serum samples. This test detects the genotypes 1-6 and the subtypes 1a and 1b through the use of genotype-specific fluorescent-labeled oligonucleotide probes (Shinol et al, 2012)(www.abbottmolecular.com).

b. Treatment

The evaluation of treatment is based on different criteria: virological, with viral load quantification; biochemical, with normalization of transaminase levels; and histological, with improvement of liver injury. Nevertheless, the virological measure is the most relevant, being represented by the sustained virologic response (SVR), which is defined as the absence of detectable HCV RNA in the serum of patients, 24 weeks after treatment completion.

i. Interferon-α/Ribavirin therapy

From 1986, even before HCV discovery, the non-A, non-B hepatitis was already treated in a small trial with recombinant IFN-α. This drug used to be administrated in monotherapy during 24 weeks, but the rate of SVR to this treatment was very low, ranging from 15 to 20% (Hoofnagle et al, 1986; Poynard et al, 1995). The IFN-α is described as a

modulator of host immunity pathways that induces an antiviral state in implicated cells through the induction of ISG expression. Despite the striking effect of IFN-α on serum aminotransferase levels, this drug presents low efficacy levels, not presenting a specific mechanism of action.

In an attempt to improve the efficacy of treatment, a new molecule emerged: the ribavirin. The ribavirin is a nucleoside analogue that was discovered and developed in 1970, and two years later its activity against different DNA and RNA viruses in culture systems and animals was reported, but its mechanism of action is complex and not well understood yet. It is thought that ribavirin can inhibit HCV through: direct replication inhibition; the increase of mutagenesis rate, resulting in genome errors; the inhibition of inosine-monophosphate dehydrogenase enzyme; or through the modulation of immune response (Feld & Hoofnagle, 2005; Thomas et al, 2011). In 1990 ribavirin was firstly used in monotherapy to treat HCV patients and the results observed were similar to those obtained with IFN-α in monotherapy. Consequently, in 1998, the combination of ribavirin and IFN-α was approved for the chronic hepatitis C treatment and this represented the next breakthrough in combating the referred disease. Both drugs acting in synergy improved the rates of SVR to 38% (McHutchison et al, 1998; Poynard et al, 1998).

In 2001 emerged the third significant improvement in chronic hepatitis C therapy: the recombinant IFN-α with a polyethylene glycol (PEG) group (PEG-IFN-α). This progression allowed great pharmacokinetic changes, like higher and longer-lasting serum concentrations, and dose reduction from three to only one administration per week (Lindsay et al, 2001). This new therapy combining PEG-IFN-α with ribavirin brought a new increase in the rate of SVR to around 55% in general (Fried et al, 2002; Zeuzem et al, 2000). However, this rate is not similar for all HCV genotypes, ranging from 20 to 80%, depending also on the disease stage and genetic polymorphisms of the host, like interleukin 28B gene. This therapy during 24 weeks leads to about 40 to 50% SVR for all genotype 1 infected individuals, while infected people with genotypes 2 and 3 present an infection resolution capacity of about 85% (Hadziyannis et al, 2004).

Despite all progressions, this treatment has many side effects such as nausea, myalgia, headache, anorexia, haemolytic anaemia, severe depression and Influenza-like illness. Thus, new antiviral therapies, PEG-IFN-α independent, were necessary.

ii. HCV-specific direct-acting antiviral agents (DAAs)

In 2011, DAAs emerged acting directly against the virus and targeting key enzymes of HCV life cycle (De Clercq, 2015; Poordad et al, 2011). From cell-based HCV replication systems, and inspired by the success of protease inhibitors for the HIV treatment, the research focused on the NS3 serine protease (and its cofactor, NS4A) as a potential target. Later, after other studies using replicon system, other HCV replication complex proteins as well as NS5A and NS5B emerged as new DAAs potential targets (Fusco & Chung, 2012). DAAs are advantageous because they can be orally administered, they act on different genotypes and they can also be combined, producing a synergic effect.

- *Inhibitors of protease NS3/4A*

The first compound belonging to the NS3/4A inhibitors class was ciluprevir (BILN-2061) that was also the first HCV DAA. This compound was orally bioavailable, peptidomimetic and macrocyclic. Ciluprevir binds to the active site of NS3/4A serine protease, blocking the viral replication. It was administrated to HCV genotype 1 patients resulting in a relevant and rapid reduction of HCV RNA, which proved the ability of DAAs to inhibit HCV in infected people. However, this drug was stopped because of cardiotoxicity (Lamarre et al, 2003).

In May 2011, the first HCV DAAs for viral treatment were approved: boceprevir (Victrelis®) and telaprevir (Incivek®) (Jacobson et al, 2011; Poordad et al, 2011). This first generation of NS3/4A was developed in an attempt to improve the SVR rate for genotype 1 infected individuals. Thus, it was achieved a SVR rate of about 75%. These drugs are linear peptidomimetics that form covalent but reversible adducts with the catalytic site of the enzyme, blocking the processing and maturation of the viral polyprotein. It is known that protease inhibitors have low genetic barrier to resistance mutants, so they are always used in combination with other HCV inhibitors in order to minimize the emergence of resistance (Bartenschlager et al, 2013). Telaprevir and boceprevir were approved in combination with PEG-IFN/RBV for adult patients chronically infected with HCV genotype 1, presenting compensated liver disease. However, Merck decided to stop selling Incivek® in August 2014 and later also to discontinue Victrelis®, 200mg capsules in January 2015. These decisions were due to the surging of new and better generations of DAAs (http://www.fda.gov/).

Afterwards, the second wave of first generation NS3/4A inhibitors emerged with better physicochemical properties and consequently lower duration of treatment, decreasing from 48 to 12 or 24 weeks; lower pill burden; better tolerability with less severe side effects; and higher genetic barrier. Despite these improvements, these drugs always need to be administrated in combination with PEG-IFN/RBV or other DAAs. They can be linear peptidomimetics or macrocyclic inhibitors but this time forming non-covalent adducts with the target. Some examples include the simeprevir and paritaprevir, that are already approved, and others like asunaprevir, danoprevir and vaniprevir that are still in phase II and III clinical trials. Faldaprevir is a good example of the quick and successful development of these molecules, having become obsolete even before its approval, never having been commercialized (Bartenschlager et al, 2013; Gutierrez et al, 2015)

Simeprevir (Olysio®) was approved in November 2013 for the same indications than telaprevir and boceprevir. One year later, it was approved in combination with other DAA (NS5B inhibitor), expanding its indications. In clinical trials, this agent presented pan-genotypic antiviral activity, except against the genotype 3, and it was highly specific and extensively distributed in the liver with high bioavailability after a single oral administration. Studies showed that Simeprevir was safe and well tolerated, reaching to 79%–81% of SVR (Moreno et al, 2012; Rosenquist et al, 2014)

Grazoprevir (MK-5172) was recently approved as a combination drug (Zepatier®), associated with other DAA, against genotypes 1 and 4. This molecule represents a second generation of NS3/4A inhibitor, showing efficacy against all HCV genotypes and presenting an even higher genetic barrier, with more than 90 % of SVR against genotype 1, when in combination (Clark et al, 2013; Summa et al, 2012).

Protease inhibitors are potent antiviral agents with high and fast decrease of serum HCV RNA, so they have become fundamental components of all-oral regimens in late-stage development for genotype 1 HCV [Table 1]. However, the active site of NS3 is not highly conserved among all genotypes, so it was necessary to search other HCV targets that could be good DAA candidates (Clark et al, 2013).

Generation			Active against HCV genotype	Genetic barriers			Resistant association variants			
1st, 1st-wave	VX-950	Telaprevir	1a/1b, 2	Low	V36A/M		R155K/T/Q	A156S/D/T/V		
1st, 1st-wave		Boceprevir	1a/1b, 2	Low	V36A/M		R155K/T/Q	A156S/D/T/V		
1st, 2nd-wave	BI 201335	Faldaprevir	1a/1b, 2	Moderate			R155K/T/Q		D168A/V/T/H	V170A/T
1st, 2nd-wave	TMC-435	Simeprevir	1, 2, and 4–6	Moderate		Q80R/K			D168A/V/T/H	
1st, 2nd-wave	MK-7009	Vaniprevir	1a/1b	Moderate			R155K/T/Q	A156S/D/T/V	D168A/V/T/H	
1st, 2nd-wave	BMS-650032	Asunaprevir	1, 4	Moderate		Q80R/K	R155K/T/Q		D168A/V/T/H	
1st, 2nd-wave	ABT-450	Paritaprevir	1	Moderate			R155K/T/Q		D168A/V/T/H	
2nd	GS-9857		1–4				R155K/T/Q	A156S/D/T/V	D168A/V/T/H	
2nd	ACH-1625	Sovaprevir								
2nd	MK-5172	Grazoprevir	1a/1b, 2, and 4–6	High			R155K/T/Q	A156S/D/T/V	D168A/V/T/H	

Table 1: **Profile of NS3/4A protease inhibitors**

Resistance-associated variants were detected and associated to the first- and second-wave of NS3/4A serine protease inhibitors. They are mainly active against the genotype 1, also presenting some activity against the other genotypes. The genetic barrier varies from low to moderate, except grazoprevir that presents high genetic barrier.

From (Tamori et al, 2016)

- *Inhibitors of NS5A*

The NS5A inhibitors bind to the domain I of this viral protein, blocking its activity in the replication, assembly and secretion of virus. It is believed that the formation of replication complex can be blocked by disruption of multimeric array of NS5A dimers (Fridell et al, 2011).

The first generation inhibitors were efficient against the HCV genotypes 1 and 4 whereas their efficacy against genotypes 2 and 3 was variable (Pawlotsky, 2014). They also showed higher antiviral activity (high SVR24) and a higher barrier of resistance against the HCV genotype 1b, when comparing with genotype 1a [Table 2] (Wang et al, 2014a). Daclatasvir (Daklinza®) was the first NS5A inhibitor to be approved, in August 2014, being indicated against HCV genotype 3. It should be taken once daily for 12 weeks in combination with other DAA. In February 2016, its approval was expanded to use against the genotype 1, with or without Ribavirin. *In vitro* and *in vivo* studies showed that daclatasvir is very potent, with low doses of the drug allowing to eliminate almost all HCV genotypes (http://www.fda.gov/;Wang et al, 2014a).

Generation			Active against HCV genotype	Genetic barriers	Resistant association variants			
1st	BMS-790052	Daclatasvir	1b > 2a > 1a	Moderate			L31F/M/V	Y93C/H/N
1st	GS-5885	Ledipasvir	1a, 1b	Moderate			L31F/M/V	Y93C/H/N
1st	ABT-267	Ombitasvir	1 > 2–6	Moderate	M28T	Q30E/R		Y93C/H/N
Broad activity	MK-8742	Elbasvir	1–4	Unavailable	M28T	Q30L/R	L31<	Y93H/N
2nd	GS-5816	Velpatasvir	1–6	Unavailable				
2nd	ACH-3102		1–5	High				Y93H

Table 2: **Profile of NS5A inhibitors**

Resistance-associated variants were detected and associated to the first- and second-wave of NS5A inhibitors, except for velpatasvir. First-wave inhibitors are mainly active against the genotype 1 and present moderate genetic barrier. Second-wave inhibitors have broad activity against the other genotypes and their genetic barrier is difficult to evaluate.

From: (Tamori et al, 2016)

Ledipasvir binds to NS5A with high affinity and specificity (Link et al, 2014). *In vitro* tests showed that ledipasvir is active against the genotypes 1a, 1b, 4a and 5a, having a reduced effect against genotypes 2 and 3. In clinical trials, it was used to treat HCV genotype 1 infections (Gentile et al, 2014). Thus, in October 2014 ledipasvir was firstly approved in a

combined drug (Harvoni®) with another DAA, for the treatment of chronic hepatitis C genotype 1 infection. One year later, the indication was expanded to the genotypes 4, 5 and 6 and to HIV co-infected individuals (http://www.fda.gov/). Shortly after, in December 2014, ombitasvir was also approved by FDA, in a combined four molecules drug (Viekira Pak®). One month later ombitasvir was approved in Europe with the name Viekirax®. This drug is indicated for the treatment of genotype 1 chronic hepatitis patients and, in combination with RBV, is also indicated to genotype 4. In clinical trials, ombitasvir presented a pan-genotypic activity and a good tolerability (http://www.fda.gov/; DeGoey et al, 2014; Lam & Salazar, 2016).

The second-generation of NS5A inhibitors emerged, exhibiting enhanced and powerful antiviral activity against all HCV genotypes, as well as retaining activity against the viral variants that are resistant to first-generation drugs [Table 2]. One example is elbasvir that was approved in January 2016 in a combined drug (Zepatier®) against the genotype 1 and 4 chronic hepatitis C, with or without compensated cirrhosis. Other molecule is velpatasvir that was approved in June 2016, in another combined drug (Epclusa®), showing activity against genotype 1-6 HCV replicons (http://www.fda.gov/). There are other molecules in phase II and III clinical trials with the goal to target all genotypes and to reduce resistance phenomena. Samatasvir is an example of a pan-genotypic NS5A inhibitor with demonstrated activity against genotypes 1, 2, 3 and 4 (Vince et al, 2014).

NS5A inhibitors showed to be more efficient against a broader set of HCV genotypes than the protease inhibitors, having also a good safety profile. Comparatively with other groups of inhibitors, NS5A inhibitors are used in very low concentrations to obtain same levels of inhibition.

- *Inhibitors of RNA-dependent RNA polymerase NS5B*

The RdRp NS5B inhibitors are grouped in two classes based on their mechanism of action: nucleosides analogues that mimic natural polymerase substrates and act at the active site of the enzyme, and non-nucleosides that bind to the enzyme allosteric sites inducing conformational changes that inhibit it [Table 3] (Haudecoeur et al, 2013; Koch & Narjes, 2007).

The nucleoside inhibitors are highly efficient against a broader range of HCV genotypes because the active site of the RdRp is highly conserved among all genotypes,

keeping around 65% of homology. They also present high barrier to resistance because amino acid substitutions in the active site are usually poorly tolerated and detrimental to the viral replication, resulting in a marked loss in viral fitness (Pawlotsky et al, 2012). These characteristics provide some advantages to nucleoside inhibitors in detriment of non-nucleosides, making them promising drugs among the oral therapeutic options that are available (Koch & Narjes, 2007). However, a drawback of these drugs is their close interference with human mitochondrial RNA polymerase, promoting severe toxicity. This can explain why many compounds did not follow through clinical trials, as happened with valopicitabine and balapiravir (Arnold et al, 2012; Delang et al, 2013). Nevertheless, many other molecules proceeded in assays and others are already approved. One example is mericitabine that shows activity mainly against genotypes 1 and 4 and that proceeds in clinical trials with a good SVR (http://www.fda.gov/; Pawlotsky et al, 2012).

Sofosbuvir is a monophosphorylated pyrimidine nucleotide prodrug that undergoes an intracellular metabolism to become a uridine triphosphate analogue. This analogue is in turn incorporated into the viral RNA, inducing early stop of replication. Sofosbuvir has shown to be very powerful, achieving more than 90% of SVR against different genotypes and states of disease and allowing the reduction of the treatment duration (Lawitz et al, 2013). It also presents high genetic barrier and pan-genotypic activity. Therefore, sofosbuvir revolutionized the HCV treatment, being part of the composition of several drugs recently approved. In clinical trials, sofosbuvir was very effective against genotypes 1 and 4 when combined with PEG-IFN and RBV, also showing some efficacy against genotypes 2 and 3 when combined with RBV alone. Thus, in December 2013, it was firstly approved under the commercial name Sovaldi®, in combination with PEG-IFN and RBV, or RBV alone. This drug was the first interferon-free drug to be approved against the genotypes 2 and 3 (Lawitz et al, 2013). In October 2014, another drug composed by sofosbuvir emerged (Harvoni®), with approval against HCV genotype 1, which was expanded one year later to also include genotypes 4, 5 and 6. In June 2016, this molecule has once again appeared in the composition of a new drug (Epclusa®), which is effective against all HCV genotypes (http://www.fda.gov/).

The binding of the non-nucleoside inhibitors can happen in one of four allosteric sites of NS5B, inducing RdRp conformational changes that block elongation of the nascent RNA chain. Despite their more restricted activity (because binding sites are less conserved than active site) and major risks regarding the formation of resistance mutations, several molecules are valuable components of oral combination regimens in development of HCV treatment.

For example, tegobuvir and beclabuvir are in current clinical trials (Everson et al, 2016; Gentile et al, 2015; Mo et al, 2016). In January 2015, dasabuvir (Exvira®) was approved in combination with other oral DAAs against HCV genotype 1 chronic hepatitis, resulting in a very high SVR, of about 95%, with good tolerability. The treatment with these combined drugs plus ribavirin was also approved for HCV genotype 4 (http://www.fda.gov/).

		Binding site	Active against HCV genotype	Genetic barriers	Resistant association variants							
Nucleotide												
GS-7977	Sofosbuvir		1a, 1b, and 2–6	High	S282T							
Nonnucleoside												
BMS-791325	Beclabuvir	Site I		Moderate	A421V	P495S/Q/L/A/T						
ABT-333	Dasabuvir	Site III		Moderate	C316Y/N	S368T	M414T/I/V/L	Y448C/H	G554D/S	S556G	D559G	
GS9669		Site II		Moderate	L419S	R422K	M423T/I/V/T	I482L/V/T	A486/V/I/T/M	V494A		
MK-3682				Moderate								

Table 3: Profile of NS5B inhibitors

Resistance-associated variants were detected and associated to the nucleotide and non-nucleoside inhibitors.

The nucleotide inhibitor is active against the genotype 1a, 1b and 2-6 and presents high genetic barrier. Non-nucleoside inhibitors present moderate genetic barrier.

From (Tamori et al, 2016)

iii. IFN-free regimens from the past to the present

Infected hepatocytes are quickly cleared, so with efficient suppression of viral replication all infected cells are cleared and there is total eradication of infection. The cure is the principal goal of clinical treatment and SVR is one of the first markers. Nowadays, the novel regimens of HCV DAAs almost allow to achieve the cure with only twelve weeks of treatment. These regimens present high barriers to resistance, very high SVR even in patients traditionally "treatment resistant", pan-genotypic activity and very high tolerability. The multiple new drugs/regimens represent a welcome advance for the HCV field, and particularly for patients who have not responded to standard care [Table 4] (http://www.fda.gov/).

Table 4: **HCV DAAs IFN-free regimens**

Brand Name	**Generic Name**	**Pharmaceutical Company**	**First Approval by FDA**	**Information**
Epclusa®	sofosbuvir 400mg velpatasvir 100mg	Gilead Sciences	June 2016	Fixed dose combination targeting all six HCV genotypes. Approved in a single tablet regimen for patients without cirrhosis or with compensated cirrhosis (SVR12 = 95%–99%) and in combination with RBV for patients with decompensated cirrhosis (SVR12 = 94%)
Zepatier®	elbasvir 50mg grazoprevir 100mg	Merck	January 2016	Approved for: G1a: Treatment-naïve or PEGIFN/RBV-experienced (without baseline NS5A polymorphisms) – 12

				weeks – single regimen (with baseline NS5A polymorphisms) – 16 weeks – Zepatier + RBV G1b: Treatment-naïve or PEG-IFN/RBV-experienced - 12 weeks - single regimen G1a or 1b: PEG-IFN/RBV/PI - Experienced - 12 weeks – Zepatier + RBV G4: Treatment-naïve – 12 weeks PEG-IFN/RBV-experienced - 16 weeks – Zepatier + RBV
Daklinza®	daclatasvir 60 mg	Bristol-Myers Squibb	July 2015	Approved WITH sofosbuvir for: G1: Without or compensated cirrhosis – 12 weeks Decompensated cirrhosis and post-transplant – 12 weeks - with RBV G3: Without cirrhosis – 12 weeks Compensated or decompensated cirrhosis and post-transplant – 12 weeks - with RBV
Viekirax® + Exviera® (EU)	ombitasvir 12.5 mg paritaprevir 75 mg ritonavir 50 mg + dasabuvir	AbbVie	January 2015	Approved for: G1a: Patients without cirrhosis – 12 weeks – Viekirax® + Exviera® + RBV Patients with compensated cirrhosis – 24 weeks – Viekirax® + Exviera® + RBV G1b: Patients with or without compensated cirrhosis – 12

	250 mg			weeks - Viekirax® + Exviera® G4: Patients without cirrhosis – 12 weeks – Viekirax® + RBV Patients with compensated cirrhosis – 24 weeks – Viekirax® + RBV
Viekira Pak® (USA)	ombitasvir 12.5 mg paritaprevir 75 mg ritonavir 50 mg + dasabuvir 250 mg	AbbVie	December 2014	Approved for: G1a: Patients without cirrhosis – 12 weeks – with RBV Patients with compensated cirrhosis – 24 weeks – with RBV G1b: Patients with or without compensated cirrhosis – 12 weeks - single regimen Not recommended for use in patients with decompensated liver disease
Harvoni®	ledipasvir 90 mg sofosbuvir 400 mg	Gilead Sciences	October 2014	Approved for: G1: Treatment-naïve without cirrhosis or with compensated cirrhosis and Treatment-experienced without cirrhosis – 12 weeks Treatment - experienced with compensated – 24 weeks Treatment-naïve and treatment-experienced with decompensated cirrhosis – 12 weeks – with RBV G1 or 4: Treatment-naïve and treatment-experienced liver transplant recipients without cirrhosis, or with compensated cirrhosis – 12 weeks – with RBV G4, 5, or 6: Treatment-naïve and

				treatment-experienced without cirrhosis or with compensated cirrhosis – 12 weeks
Sovaldi®	sofosbuvir 400 mg	Gilead Sciences	December 2013	Approved for: G2: **S**ovaldi + RBV - 12 weeks G3: **S**ovaldi + RBV - 24 weeks G1 or 4: Triple therapy (Sovaldi + PEG-IFN-α + RBV - 12 weeks Gilead conditions: G1 patients who are IFN ineligible – 24 weeks - Sovaldi + RBV HCC awaiting liver transplantation -- 48 weeks or until liver transplantation to prevent post-transplant hepatitis C infection - Sovaldi + RBV Approved WITH simeprevir 150 mg (Olysio®) for: G1: patients without cirrhosis – 12 weeks and patients with cirrhosis – 24 weeks

G – genotype; PI – HCV NS3/4A protease inhibitor

iv. Host-target antiviral agents (HTAs)

The HTAs are the other class of HCV antiviral agents. They exhibit pan-genotypic activity and high barrier to resistance because host factors are genetically stable (von Hahn et al, 2011). Nowadays, this class of agents has been losing impactful in a community where DAAs are very well established and present all advantages discussed above. However, it is believed that HTAs can be useful, acting in synergy with DAAs to reduce viral loads, further expanding the anti-HCV arsenal to address the most difficult cases to treat. The research of HTAs was based in the study of cellular host factors required by HCV in different life cycle stages, such as SR-BI, hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase, CypA, fatty

acid synthase (FASN) and miR-122. Some HTAs act to increase the anti-viral state by triggering components of innate immune responses, like IFNs and also TLR agonists, especially TLR7 and TLR9 (Baugh et al, 2013; Boonstra et al, 2012; Savva & Roger, 2013).

- *Cyclophilin inhibitors*

CypA is a host protein that interacts with NS5B and also presents an essential role in the viral replication through the NS5A regulation (Fernandes et al, 2007; Foster et al, 2011). In addition to CypA, several other Cyps have been reported to be implicated in HCV replication (Gaither et al, 2010; Hanoulle et al, 2009). Cyp inhibitors were the first class of HTAs against HCV reaching clinical development. Nowadays, there are some relevant CypA inhibitors in clinical trials, showing a potent anti-HCV activity. Cyclosporine A (CsA) is a common drug used in transplantation due to its immunosuppressive properties and it was the first Cyp inhibitor to show anti-HCV activity *in vitro* (Hopkins et al, 2012; Paeshuyse et al, 2006). The immunosuppressive property precluded its use as antiviral, but over the years many CsA derivates have emerged that were non immunosuppressive. These showed to disrupt/prevent the formation of CypA-NS5A complexes and thereby inhibit HCV replication and assembly (Naoumov, 2014). They are also able to reestablish the host innate immune response to HCV (Hopkins et al, 2012). Alisporivir is a CsA derivate and the CypA inhibitor that is most advanced in clinical trials, showing efficient reduction of viral load in genotype 2 and 3 chronic hepatitis C patients in combination with RBV (Gallay & Lin, 2013). There are also other Cyp inhibitors in study, as SCY-635 and NIM-811.

- *miR-122 targeting therapy*

MiR are small non-coding RNAs, that regulate gene expression (Ambros, 2004). The miR-122 is one of the most abundant miRs in the liver and beyond its typical function, it binds on two sites at the 5' end of viral RNA, stabilizing the transcript and acting as a cap, thereby enhancing viral translation and replication (Henke et al, 2008; Jopling et al, 2005). It also shields viral RNA from Xrn1 degradation, increasing RNA stability (Li et al, 2013b). HCV cannot replicate in cells lacking miR-122 or in which miR-122 has been sequestered, so molecules targeting it represent very attractive solutions for antiviral therapy (Bandiera et al,

2015). Miravirsen is a locked nucleic acid-modified oligonucleotide antagonizing miR-122, which is also the first molecule to target a miR in a clinical trials phase. In phase II of clinical trials, miravirsen sustained reduced viremia levels without evidence of long-term safety issues in chronic hepatitis C patients (Janssen et al, 2013). It was also efficient in monotherapy for genotype 1 infected patients. Miravirsen may act by two complementary mechanisms: hybridizing to mature miR-122, which blocks the interaction with HCV RNA; and binding to the stem-loop structure of miR-122, which inhibits processing of it precursors (Gebert et al, 2014).

- *Other inhibitors*

There are many host factors and receptors essential for the viral entry, which would make promising targets of HTAs [Table 5]. The receptor SR-BI presents an extremely relevant role to HCV infection, so it was hardly investigated as a potential target of HTAs. Studies have shown that nAbs against SR-BI are able to inhibit the infection in vitro and also viral intrahepatic diffusion in vivo (Lacek et al, 2012; Meuleman et al, 2012). A small molecule antagonist of SR-BI (ITX 5061) also allowed to inhibit HCV entry and is already in clinical trials (Meuleman et al, 2012). *In vitro* tests showed the synergic effect of this molecule with protease and polymerase DAAs.

CD81 and CLDN1 are also potential therapeutic targets that can be neutralized by antibodies. Thus, anti-CD81 monoclonal antibodies were able to avoid the cell to cell transmission, consequently preventing the dissemination of infection (Fofana et al, 2010; Fofana et al, 2013; Fukasawa et al, 2015). The association between CD81 and CLDN1 seems to be relevant for the HCV entry step and it is promoted by epidermal growth factor receptor (EGFR). Thus, erlotinib, an EGFR inhibitor, is already in clinical trials showing anti viral effect by preventing CD81/CLDN1complex formation (Lupberger et al, 2011; Zona et al, 2014).

Factors involved in the lipid metabolism are closely linked to the HCV infection (Felmlee et al, 2013; Popescu et al, 2014). The apolipoproteins which comprise LVPs are the same that are involved in the very low density lipoproteins (VLDL) synthesis, so the VLDL assembly pathway is closely linked to that of HCV (Chang et al, 2007,Jiang, 2009 #2621). Microsomal triglyceride transfer protein (MTP) is a protein involved in the VLDL biogenesis, so the amiodarone, an inhibitor of MTP activity, is able to reduce HCV assembly and release

in vitro (Cheng et al, 2013). The most abundant transcription factor in the liver, the hepatocyte nuclear factor 4α (HNF4α) regulates VLDL secretion but also the expression of phospholipase A2 GXIIB (PLA2GXIIB), which is also a crucial host factor for HCV production. Thus, HNF4α down-regulation by bezafibrate reduced intensively HCV secretion (Li et al, 2014). Statistically, bezafibrate is also associated to the reduction of viral load in chronically HCV-infected patients (Grammatikos et al, 2014).

Category	Target	Compounds	Stage of Development
Entry inhibitors	CD81	mAbs	Mouse model
	SR-BI	mAbs	Mouse model
		ITX-5061	Phase 1
	CLDN1	mAbs	Mouse model
	EGFR	Erlotinib	Phase 1/2
	NPC1L1	Ezetimide	Mouse model
	Endocytosis/fusion	Silymarin/silibinin	Phase 2/3
		Chloroquine	Phase 4
Translation inhibitors	miR-122	Miravirsen	Phase 2
Replication inhibitors	miR-122	Miravirsen	Phase 2
	HMGCoA reductase	Statins	Phase 3
	Cyclophilin	Alisporivir	Phase 2/3
		SCY-635	Phase 2
		NIM811	Phase 2
Assembly inhibitors	α-glucosidase 1	Celgosivir	Phase 2
	DGAT-1	LCQ908	Phase 2
	Cyclophilin	NIM811	Phase 2
	PPARα	Naringenin	Phase 1
	HNF4α	Bezafibrate	Phase 4
Biological response modifiers	Immune responses	IFN-α	FDA-approved
		IFN-λ	Phase 3
		TLR7 agonist	Phase 1
		TLR9 agonist	Phase 3
		Thymosin α1	Phase 3
		Nitazoxanide	Phase 2

Table 5: **List of HTAs for prevention/treatment of HCV infection**
From: (Zeisel et al, 2015)

v. Vaccine advances

Despite an important reduction in HCV transmission due to improved prevention strategies and introduction of new powerful DAA therapies, HCV remains a relevant health problem and there are many reasons to continue trying to develop a vaccine. This would be the best strategy to completely eradicate HCV infections. An optimal vaccine should induce strong neutralizing antibodies and cellular immune responses, providing a global protection from infection and recognizing almost all HCV genotypes with inhibition of viral cell-to-cell transmission (Castelli et al, 2014; Chmielewska et al, 2014).

In contrast to HBV, a vaccine against HCV represents a difficult challenge. There are many viral and non-viral factors that can explain why it is so difficult to develop an HCV vaccine. For example, the high genetic variability (seven genotypes that differ by at least 70%), the high mutation rate, the several evasion mechanisms used by HCV to escape from host innate and adaptive immune system or even the lack of available, fully immunocompetent small animal models (Smith et al, 2014). In an attempt to overcome these hurdles, several types of vaccines were tested, including synthetic peptides, recombinant proteins, plasmids DNA, virus-like particles and even inactivated HCV particles (Ghasemi et al, 2015).

Despite all efforts, only a few of developed vaccines are now in clinical trials [Table 6].

Investigator	Vaccine type	Immunogen	Stage	Application
Chiron/Novartis [46,62,63]	Recombinant protein	Recombinant gpE1/gpE2 in oil/water adjuvants	Phase I [46,63]	Prophylactic vaccine
Okairos [64]	Adenoviral vector vaccine	Adenovirus vectors expressing HCV NS 3, 4 and 5	Phase 2	Prophylactic/Immunotherapeutic vaccine
Innogenetics/ GenImmune [65]	Recombinant protein	Alum-adjuvanted gpE1 glycoprotein	Phase 2 [66] Programme stopped as no effects on viraemia or fibrosis progression	Immunotherapeutic vaccine
Intercell AG (IC41) [67,68]	Peptide	HCV peptide cocktail with polyarginine	Phase 2 in combination with pegylated interferon [69,70]	Immunotherapeutic vaccine
Globe Immune (GI-5005)	Recombinant yeast cells	Heat-killed yeast expressing Core-NS3 fusion protein [71]	Phase 2 in combination with pegylated interferon [72,73]	Immunotherapeutic vaccine
Tripep (ChronVac-C)	Plasmid DNA	DNA-based vaccination with NS3/4A-expressing plasmid	Phase 1b	Immunotherapeutic vaccine
CIGB-230 [74]	Plasmid DNA and recombinant protein	Mixture of plasmid-expressing HCV structural antigens with a recombinant HCV core protein	Phase 1b [75]	Immunotherapeutic vaccine
Transgene (TG4040)	Adenoviral vector vaccine	Modified vaccinia Ankara virus expressing NS proteins (NS3-NS5B)	Phase I	Immunotherapeutic vaccine

Table 6: **Clinical-stage of vaccine candidates for HCV**

From (Zingaretti et al, 2014)

The majority of these are therapeutic vaccines such as ChronVac-C (plasmid DNA encoding NS3/4A), TG4040 (viral vector expressing NS3 to NS5B proteins) and GI-5005 (whole yeast-based expressing Core-NS3 fusion protein). They are promising to be approved in combination with other therapy to treat chronic HCV infection (Habersetzer et al, 2011; Roohvand & Kossari, 2012; Zingaretti et al, 2014).

Prophylactic vaccines to achieve the protection from persistent HCV infection are more ambitious. For now, only two vaccines advanced to clinical trials. The first that appeared is composed by recombinant E1 and E2 glycoproteins (genotype 1a) and was developed by Chiron (now Novartis). This vaccine was able to stimulate high levels of anti-E1E2 nAbs cross-neutralizing the majority of HCV genotypes, as well as to induce very strong HCV specific CD4+ T cell response. Despite these results, the capacity of these antibodies to efficiently protect from persistent infection is not clear (Houghton, 2011; Walker & Grakoui, 2015). The second vaccine is a recombinant virus vector that expresses HCV genotype 1b non-structural proteins (NS3-NS5B) and was developed by Okairos (now GlaxoSmithKline). Unlike Chiron vaccine, this one does not induce nAbs, but long-term memory cells. Indeed, CD4+ and CD8+ T cells, targeting multiple epitopes and recognizing different HCV genotypes, are broadly and quickly stimulated to act by secreting multiple cytokines. In trials, the presence of this long-term memory population was observed more than one year after the inoculation. Despite all successes, there are some failures that are not yet well understood. Thus, the adaptive immunity fails, noting a weak detection of CD8+ T cells expression, a deregulation of factors involved in the memory cell homeostasis and also a silencing of CD4+ T cells by HCV (Folgori et al, 2006; Walker & Grakoui, 2015). In addition, the combination of this genetic vaccine with adjuvant subunit glycoproteins was recently tested, generating humoral and cellular immunity (Chmielewska et al, 2014).

A vaccine to prevent reinfection of chronically infected patients after DAA cure may be the most reasonable and useful aim for a prophylactic HCV vaccine. However, nowadays it is unknown if the vaccine will be efficient to restore the immunity of individuals successfully cured by DAA, comparatively to preventing an infection in naive individuals. This may depend on the ability to restore exhausted CD8+ T cell responses (Walker & Grakoui, 2015).aa

II. HCV life cycle and lipid metabolism

A. Organization of viral particle

Several progresses have been made in the HCV study, but its structure remains poorly characterized. HCV is a small enveloped, single-stranded, positive-sense RNA virus with an icosahedral capsid. The viral particles are small, with about 50 to 80 nm of diameter (Catanese et al, 2013b) and their density is largely heterogenic, ranging between 1.03 and 1.20 g/cm^3. This density variation is due to the association with lipoproteins, constituting the LVPs. HCV was proposed as being a hybrid particle consisting of one virion moiety and one lipoprotein moiety which is composed by cholesteryl esters. The lower density particles have a density similar to that of VLDL and low-density lipoproteins (LDL) and are the most infectious particles (Andre et al, 2002; Gastaminza et al, 2010; Nielsen et al, 2006; Thomssen et al, 1992; Thomssen et al, 1993). *In vivo* and *in vitro*, HCV is found as a combination of very infectious and noninfectious particles, being their infectivity related with their density (Gastaminza et al, 2010; Nielsen et al, 2006). The HCV genome interacts with the Core protein to form the nucleocapsid that is surrounded by a lipid membrane, called viral envelope, in which the envelope glycoproteins (E1 and E2) are anchored. These two proteins are assembled into covalent complexes stabilized by disulfide bridges at the virions surface and form noncovalent heterodimers within infected cells (Vieyres et al, 2010). Also, due to the association with lipoproteins, some apolipoproteins were detected in association with HCV particles such as apoE, apoB, apoA-I, apoC-I, apoC-II, and apoC-III. This interaction with lipoproteins could explain the poor detection or availability of HCV glycoproteins at the virion surface and also play a critical role in HCV entry [Figure 17] (Douam et al, 2015).

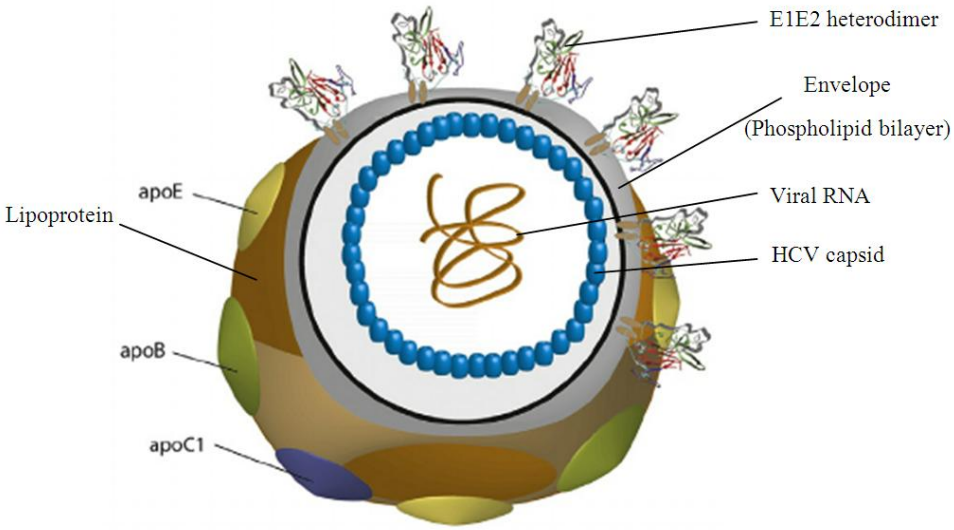

Figure 17: Organization of HCV particle
Adapted from: (Douam et al, 2015)

B. HCV life cycle

In the primary infection, HCV particles circulate in the bloodstream, cross the fenestrated endothelium of liver sinusoids and enter in contact with the hepatocyte surface. Here begins the viral entry, involving multiple interactions between many viral and cellular factors. Firstly, viral particles attach to the non-specific factors on the hepatocytes surface. Heparan sulfate proteoglycan (HSPG) and SR-BI seem to be the first mediators of HCV attachment, involving interactions with HCV lipid moity. HCV glycoproteins were firstly proposed as responsible for virion binding to these receptors, but recent data suggested that apoE could be involved in this first contact, prevailing to the glycoproteins. LDL receptor (LDL-r) was also proposed to interact with HCV in this early entry step. After attachment, the entry process involves a series of specific cellular factors: the tetraspanin CD81, the scavenger receptor SR-BI and then the tight-junction proteins CLDN1 and OCLN. The protein kinase A and the EGFR also seem to have a role in the viral entry [Figure 18] (Dubuisson & Cosset, 2014; Vieyres et al, 2014).

The HCV internalization occurs via clathrin-mediated endocytosis, which drives the virion towards early endosomes. After endosomes acidification, the viral envelope fuse with the cell membrane, releasing the nucleocapsid in the cytoplasm. Uncoating of HCV genome in the cytosol leads to its recognition by cellular ribosomes that use it as messenger RNA for

the translation of viral proteins. Afterwards, this viral RNA is used as a template for the genomic RNA replication by the RNA-dependent RNA polymerase. Finally, HCV RNA is packaged into new viral particles close to the ER and in association with lipid droplets (LD). New viral particles are then secreted by exocytosis, ready to infect naïve cells [Figure 19] (Vieyres et al, 2014).

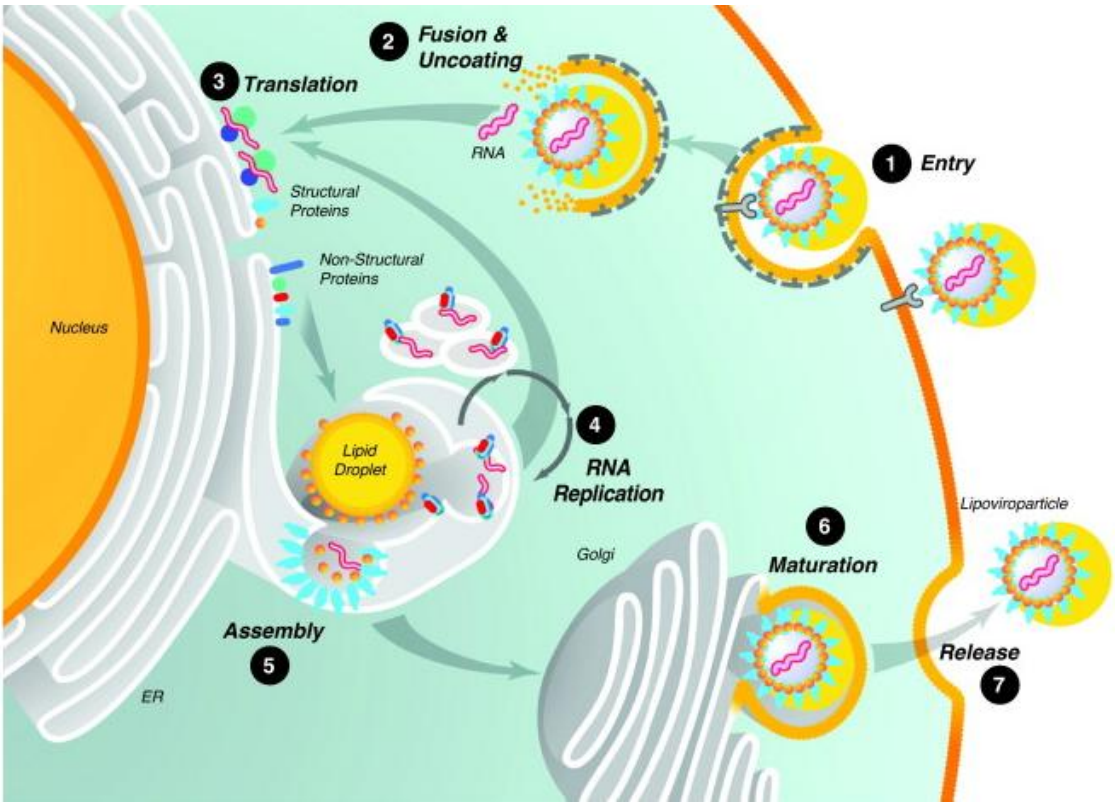

Figure 18: HCV life cycle

(1) LVPs enter in the target cell via receptor-mediated endocytosis. (2 and 3) Viral RNA is released and then translated at the ER, producing a polyprotein precursor. (4) Replication of HCV positive-strand RNA is mediated by the viral RdRp NS5B. (5) Newly synthesized RNA is encapsidated by the viral nucleocapsid core, close to the lipid droplets, and E1E2 glycoproteins are acquired through budding into the ER lumen. (6) Maturation of LVPs in the ER through the interactions with lipoproteins. (7) LVPs leave the cell via cellular Golgi apparatus. From: (Herker & Ott, 2011)

a. Entry

i. Attachment of viral particle

- *Heparan sulfate proteoglycans*

HSPGs are glycosaminoglycans (GAGs) present at the hepatocytes surface, with very early implication on viral entry. Firstly, the HVR1 domain of E2 glycoprotein was proposed to mediate the interaction with HSPG, but this hypothesis could not be confirmed in the context of infectious viral particles (Barth et al, 2003). In contrast, recent data suggests that the apolipoproteins harbored by LVPs, particularly apoE, are the most probable mediators of the interaction (Jiang et al, 2013). The Heparan sulfates attached to syndecan-1 (SDC1) and syndecan-4 (SDC4) were identified as the major receptors for HCV attachment (Lefevre et al, 2014; Shi et al, 2013).

- *LDL-r*

Studies have shown that LDL-r is important for HCV attachment. It is expressed in several tissues, but presents major functions in the liver (Hussain et al, 1999). LDL is captured and cleared from the blood circulation and then it is internalized through the clathrin-mediated endocytosis. LDL-r presents high affinity for different lipoproteins, through the interaction with their apolipoproteins. Thus, it was expected that HCV particles from low-density fraction would interact with LDL-r, allowing their internalization (Agnello et al, 1999). Studies showed clear implication of apoE as HCV attachment mediator on LDL-r, but it seems to contribute for a nonproductive entry pathway, leading to degradation of viral particles (Albecka et al, 2012).

- *SR-BI*

SR-BI is a cell surface receptor highly expressed in the liver and steroidogenic tissues. It is highly glycosylated, composed by two cytoplasmic domains (C- and N-terminal), and one extracellular domain forming a large loop in which reside its physiological functions

[Figure 19] (Dao Thi et al, 2011). It takes part in the lipid metabolism through the interaction with different classes of lipoproteins such as VLDL, LDL and high density lipoproteins (HDL) (Van Eck et al, 2008). The interaction with HDL allows the transfer of cholesteryl esters, leading to the cholesterol enrichment of the plasma membrane (Krieger, 1999). Unlike LDL-r, SR-BI presents an active role in the HCV infection, not only participating in the HCV attachment to the cell, but also playing a role as a specific entry factor. Studies have shown that HCV may interact with SR-BI, not through an E1E2 dependent way, but rather through HCV-associated lipoprotein, especially involving apoE (Dao Thi et al, 2012).

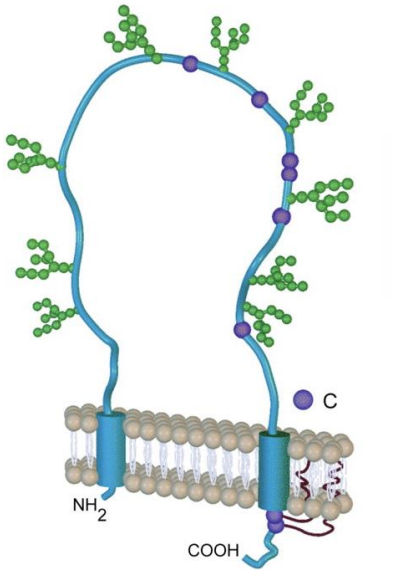

Figure 19: Schematic representation of SR-BI protein

SR-BI present two transmembrane domains (blue cylinders), a large extracellular loop and two short N- and C-terminal cytoplasmic segments. The extracellular loop contains nine sites of N-glycosylation (green dots) and six cysteine residues (purple dots). From: (Cocquerel et al, 2006)

ii. Specific binding

- *SR-BI*

After initial attachment, SR-BI is also suggested as the first specific entry factor. Studies have identified the glycoprotein E2 and HCV associated lipoproteins as the viral

mediators of the interaction with SR-BI (Dao Thi et al, 2012; Maillard et al, 2006; Scarselli et al, 2002). In general, these lipoprotein components make the CD81 binding site on the E2 glycoprotein, less accessible. However, this E2 site can be uncovered by lipoprotein rearrangements, modifications of virion composition or E1E2 conformational changes, during early entry steps. Thus, SR-BI was identified to contribute for these rearrangements, facilitating the interaction of the virion with tetraspanin CD81. Studies have shown a modification of the virion lipid composition by the SR-BI (with its lipid transfer activity), leading to a better exposure of the CD81 binding site (Dao Thi et al, 2012; Zahid et al, 2013). Otherwise, SR-BI can directly interact with HVR1 leading to the unmasking of the CD81 binding site (Bankwitz et al, 2010; Dreux et al, 2009; Prentoe et al, 2011). In addition, it was proposed that the transfer of apoC-I from HDL to LVPs by SR-BI may promote the viral fusion (Dreux et al, 2007; Meunier et al, 2008a; Zahid et al, 2013).

- *CD81*

The tetraspanin CD81 was the first molecule to be described as a specific factor, playing a crucial role in the viral entry (Feneant et al, 2014; Pileri et al, 1998). It is an adaptor cell surface molecule expressed on several cell lines. This molecule is composed by two extracellular loops, one small (SEL) and one large (LEL), fixed to the cell membrane through four transmembrane domains [Figure 20] (Seigneuret, 2006). Studies have shown that HCV entry is dependent of CD81, because if it is blocked or silenced the entry process is strongly inhibited. CD81 was the first reported cellular receptor interacting with a soluble form of the HCV E2 glycoprotein. Specific regions and residues of CD81 involved in the direct interaction with HCV E2 have been identified in the LEL region. CD81 SEL plays an indirect role by mediating the optimal expression of LEL at the cell surface. Thus, CD81 LEL seems to be the key determinant of the viral entry and the other CD81 additional regions only enhance the process (Kong et al, 2013; Zona et al, 2014).

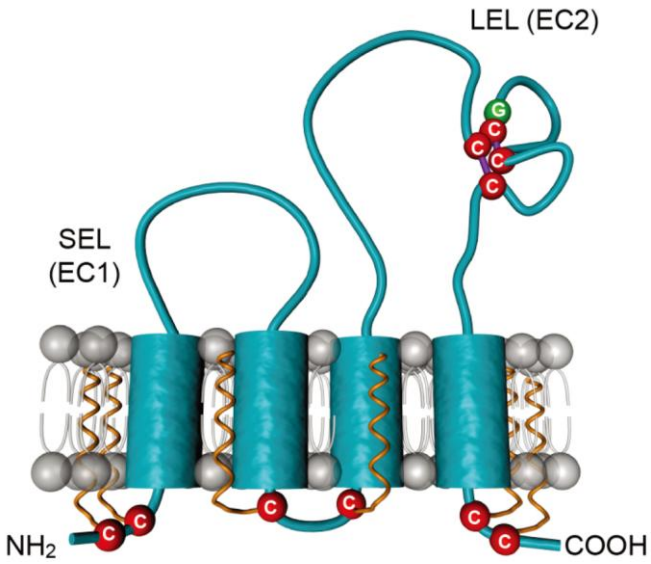

Figure 20: Schematic representation of CD81 tetraspanin

CD81 is composed by four transmembrane domains (blue cylinders), and two extracellular loops, a small (SEL or EC1) and a large (LEL or EC2). The cysteine residues are represented by red balls and constitute palmitoylation sites (orange lines). From: (Feneant et al, 2014)

CD81 can also interact with CLDN1 creating a co-receptor complex that is involved in the virus diffusion toward the internalization site (Harris et al, 2010; Harris et al, 2008). This complex is regulated by different signaling pathways, especially by EGFR and potentially by EphA2, another member of the receptor tyrosine kinase (RTK) superfamily (Lupberger et al, 2011). The stimulation of EGFR pathway is followed by Ras/MEK/ERK pathway activation that could lead to the activation of MAPK interacting serine/threonine kinase 1 (MKNK1), a kinase which facilitates HCV entry downstream of EGFR activation. The EGFR pathway also involves the Rho GTPase family members, like the GTPase HRas. This signaling leads to an actin-dependent delocalization of the virus/CD81/CLDN1 complexes toward apical membrane of hepatocytes, where viral particles are internalized. Accordingly, inhibition of Rho GTPases is able to block HCV entry [Figure 21] (Dubuisson & Cosset, 2014).

Studies about dynamics of tetraspanins showed that CD81 forms dynamic clusters with several membrane proteins at the cell surface, called tetraspanin-enriched microdomains (TEMs). CD81 is found in permanent exchanges with the membrane, which seems to be essential for HCV entry (Harris et al, 2013; Potel et al, 2013). Nowadays, it remains controversial if HCV-CD81 interaction and consequently the viral entry process are TEMs-

dependent (Potel et al, 2013; Rocha-Perugini et al, 2009; Rocha-Perugini et al, 2008; Zhu et al, 2012). For example, a study showed that EWI-2wint interacts with CD81 and restricts it into the TEMs, thus inhibiting HCV entry. This interaction also contributes to impair the complex formation between CD81 and CLDN1 (Montpellier et al, 2011; Potel et al, 2013; Rocha-Perugini et al, 2008).

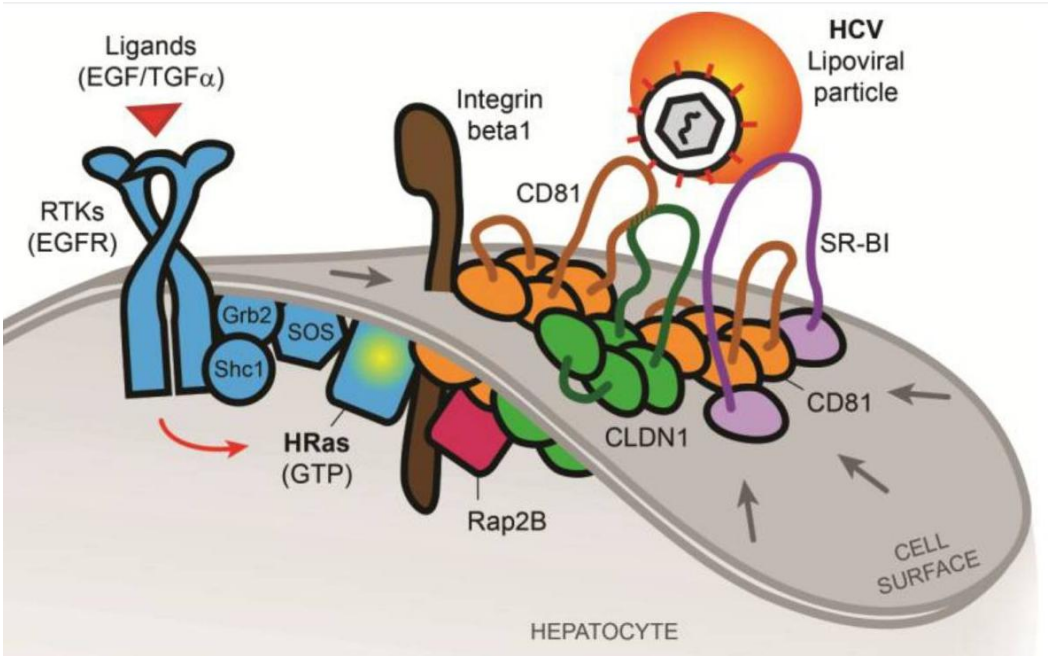

Figure 21: Model of tetraspanin co-receptor formation followed by HCV binding/entry
EGRF activates HRas that act as a key host signaling transducer for HCV entry, promoting the CD81/CLDN1 complex formation. HRas is associated with TEMs containing host receptors including CD81, CLDN1, SR-BI, integrin beta 1 (ITGB1) and Rap2B. From: (Zona et al, 2014)

- *Tight junction proteins*

The tight junction proteins (CLDN1 and OCLN) are also implicated in HCV entry. They are important mediators in the inter-hepatocytes junctions, essential for the maintenance of the large cellular plate constituting the liver tissue.

In 2007, CLDN1 was discovered as an important entry factor for HCV (Evans et al, 2007). It is composed by two extracellular domains (EL1 and EL2) anchored to the cell membrane through four transmembrane domains [Figure 22] (Gunzel & Fromm, 2012).

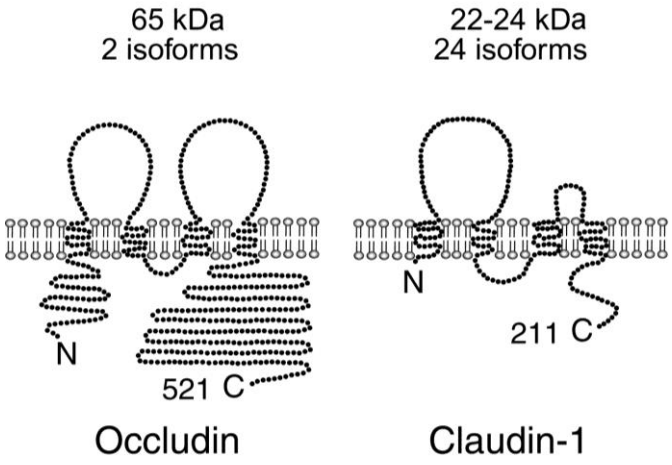

Figure 22: Occludin and Claudin-1 structures

Occludin consists in four transmembrane domains, two extracellular loops, a short intracytoplasmic loop and N- and C-terminal cytoplasmic sequences. Claudin-1 consists in four transmembrane domains, two extracellular loops (one small and one large), a short intracytoplasmic loop, a short N-terminal sequence, and a cytoplasmic C-terminal sequence. From: (Schneeberger & Lynch, 2004)

As referred above, CLDN1 interacts with CD81 forming a complex. In the hepatocytes, CLDN1 is expressed at the basal and lateral membranes as well as in tight junctions. Studies have shown that the localization of CLDN1 in the tight junctions is important for HCV infection, suggesting that viral particle migration to these junctions may be relevant for the virus entry (Liu et al, 2009; Yang et al, 2008). However, CD81/CLDN1 complexes were only detected at the basal membranes (not in tight junctions) during HCVcc infection and the viral entry from these complexes was already proved. Notably, HCV induces the internalization of CD81/CLDN1 complexes simultaneously with its internalization, so demonstrating the relevance of these complexes in viral entry (Douam et al, 2015). Mutagenesis studies of this protein have revealed that its intracellular domains, including the C-terminal domain that is important for the transport of CLDN1 to tight junctions, are dispensable for infection. In contrast, domains within the first extracellular loop EL1 are crucial (Cukierman et al, 2009; Evans et al, 2007).

In 2009, OCLN was described as an important HCV entry factor that determines its tropism for human cells (Ploss et al, 2009). It is another tight junction protein, composed of two extracellular domains (EL1 and EL2), larger than those in CLDN1 and it is also anchored through four transmembrane domains [Figure 22] (Liu et al, 2010; Liu et al, 2009). Studies in

HCVcc model showed the OCLN involvement at a late entry step, its activity being subsequent to that of CD81 and CLDN1 during HCV entry (Benedicto et al, 2009; Douam et al, 2015; Liu et al, 2009). OCLN downregulation decreases both HCV entry and glycoprotein-mediated cell fusion (Douam et al, 2015). Thus, it was demonstrated that this tight junction protein act as a late mediator of virus entry and is crucial for virus internalization as well as membrane fusion (Benedicto et al, 2009; Sourisseau et al, 2013). However, whether OCLN is a true receptor that binds the HCV envelope or is an indirect, though indispensable, entry co-factor required for post-binding steps still remains unclear. In addition, HCV infection seems to modulate localization and expression level of CLDN1 and OCLN in the tight junctions (Benedicto et al, 2009; Liu et al, 2009).

- *Other entry factors*

The cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) was identified as another HCV entry factor. It is expressed at the apical membrane of the human enterocytes and hepatocytes and composed by thirteen transmembrane domains. This receptor is responsible for cholesterol cellular uptake and regulates the homeostasis. Studies associated NPC1L1 with HCV infection after the observation that its downregulation inhibited the viral infection. This inhibition occurred in a late step of viral entry, which is dependent of the virion cholesterol composition. Maybe, NPC1L1 lipid transfer activity could rearrange lipid-associated viral particles (Sainz et al, 2012).

Transferrin receptor 1 (TfR1) has also been described as being involved in HCV entry, but the precise role played by this additional factor remains to be determined (Martin & Uprichard, 2013). TfR1 is expressed in all tissues, constituting the major blood transporter of iron to intracellular compartment. Interestingly, HCV infected patients often present high levels of iron, which is consistent with a recent study that showed a modification of TfR1 expression by HCV, within infected cells, consequently deregulating iron homeostasis. Studies have shown that HCV infection is inhibited by downregulation of TfR1, which seems to act on a step after HCV docking on CD81. Moreover, the downregulation of TfR1 Trafficking Protein (TTP), a protein required for TfR1 internalization, also inhibits the infection, suggesting a potential role in the virus internalization (Martin & Uprichard, 2013).

A model where TfR1 would act concomitantly with tight junction proteins during receptor clustering and virus internalization remains to be defined.

iii. Cell-to-cell transmission

In the hepatoma cells, HCV presents an alternative way to the classical entry to infect naive cells. It spreads from infected to neighboring cells in a process named cell-to-cell transmission (Brimacombe et al, 2011; Catanese et al, 2013a; Timpe et al, 2008; Witteveldt et al, 2009). This efficient process allows HCV to escape from the host nAbs and it is main route for transmission and dissemination of DAA-resistant viruses (Xiao et al, 2014). Many host factors involved in the classical entry are also implicated in this process, such as SR-BI, CLDN1, OCLN, EGFR and EphA2. The role of SR-BI in the cell-to-cell transmission seems to be independent of the interaction with E2, suggesting an implication through its lipid transfer capacity. Moreover, mutations in E1 and E2 glycoproteins allowed to increase the cell-to-cell spread by reduction of SR-BI usage (Catanese et al, 2013a). The TfR1 appears to be less important and the involvement of HCV-associated ApoE is controversial (Barretto et al, 2014; Hueging et al, 2014; Martin & Uprichard, 2013). The NPC1L1 revealed to be essential for this process, in contrast to the VLDL secretory pathway that seems not to be involved in the secretion of viral particles (Barretto et al, 2014; Sainz et al, 2012).

The role of CD81 in this process is controversial. Despite some studies that claimed a dispensable implication of CD81, it was recently shown that expression of the receptor may restore the HCV transmission in cells unable to transmit the virus (Brimacombe et al, 2011; Potel et al, 2013). Moreover, the blockage of E2 and its interaction with the CD81 may abolish this transmission. Thus, two cell-to-cell transmission pathways were suggested, one dependent and another independent of CD81. Recently, a mechanism of HCV transmission mediated by the exosomes was also suggested (Bukong et al, 2014; Ramakrishnaiah et al, 2013).

iv. Endocytosis and virus fusion

The endocytosis is frequently used by viruses to invade the cell and release their genetic material. HCV enters in the cell through clathrin-dependent endocytosis. Interestingly, the viral particles seem to be internalized in a clathrin- and dynamin-dependent manner and associated with CD81-CLDN1 complexes [Figure 23] (Blanchard et al, 2006; Harris et al, 2008; Krieger et al, 2010; Meertens et al, 2006). Other entry factors, including TfR1, could also be involved in this process. Following that, HCV-receptor complex are transported to RAB5A-containing endosomal compartments by a mechanism dependent on actin retrograde transport and along the actin stress fibers (Coller et al, 2009; Farquhar et al, 2012; Meertens et al, 2006).

After endocytosis, the viral envelope merges with endosomal membrane. This fusion process depends on low pH, ranging between 6.3 and 4 in the endosomal compartments (Haid et al, 2009; Lavillette et al, 2006). It involves conformational rearrangements of E1E2 glycoproteins, inclusion of a fusion peptide into the cell membrane, and mixing of the two hemimembranes' lipids, leading to the complete fusion (Helle & Dubuisson, 2008; Weissenhorn et al, 2007). In addition, the fusion step would be facilitated by the presence of cholesterol on the membranes of both the host cell and the viral particle. Thus, the cholesterol transporter, NPC1L1, may contribute to the fusion process, by enriching the membranes in cholesterol (Sainz et al, 2012). Interestingly, this process is improved by apoC-I that has been shown to enhance the HCV infectivity by direct interaction with E1E2. ApoC-I activity does not depend on SR-BI or CD81, so it does not affect the HCV binding or the internalization steps (Dreux et al, 2007).

After fusion, HCV genome is released into the cytosol, where it is directly translated to produce viral proteins and serve as matrix for viral replication.

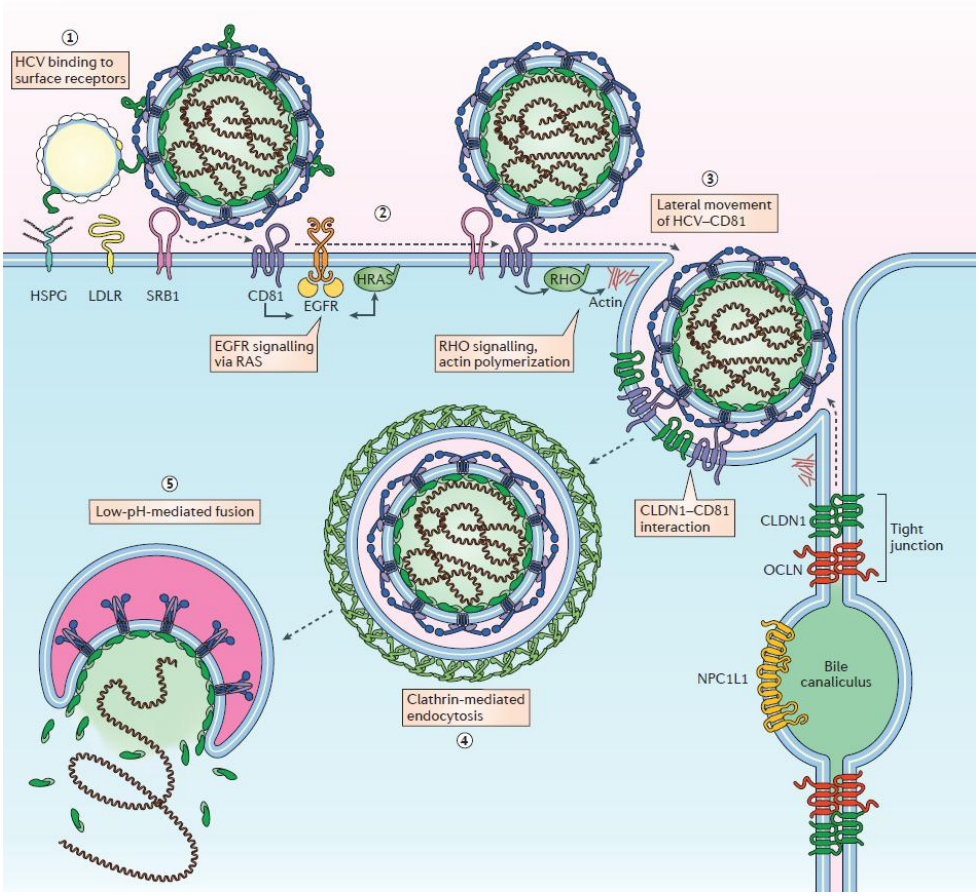

Figure 23: Entry of HCV
From: (Lindenbach & Rice, 2013)

b. Translation and replication

Many viral and cellular factors are involved in the HCV replication, but the viral proteins (NS3/4A, NS4B, NS5A and NS5B) as well as the 5' and 3' untranslated regions are of utmost importance. The HCV genome translation begins with the ribosome binding to the IRES, followed by the recruitment of eIF2, eIF3 and eIF5 factors, resulting in the formation of the ribosomal complex (Kieft et al, 2001; Pestova et al, 1998). This translation results in the production of a polyprotein precursor that is then processed in 10 mature proteins

(referred in the first chapter). This viral genome also serves as template for the synthesis of new RNA positive strands. In its turn, these new nascent RNAs serve as new/additional RNA templates to generate other RNA strands, to produce new viral proteins or to be incorporated into new infectious virions (Dubuisson & Cosset, 2014). This process occurs in the ‘‘membranous web’’, a dense cluster created by HCV-induced rearrangements of intracellular membranes (Lohmann, 2013). Here, double-membrane vesicles (DMVs) are found, most likely derived from the ER and accumulated during the RNA replication (Romero-Brey et al, 2012). Moreover, viral proteins can induce membrane reorganization, but only NS5A is able to induce DMVs (Egger et al, 2002; Reiss et al, 2011; Romero-Brey et al, 2012). Host proteins that interact with NS5A seem to contribute to the formation of these DMVs, like CypA and proline-serine-threonine phosphatase interacting protein 2 (PSTPIP2). In addition, miR-122 can recruit Argonaute 2 to the 5' terminal, stabilizing it and slowing its degradation by the 5' exonuclease Xrn1, also contributing to the virus replication (Jopling et al, 2005; Li et al, 2013b; Shimakami et al, 2012).

HCV replication is closely linked to the host lipid metabolism, occurring in cholesterol and sphingolipids enriched membranes. However, these lipids are not found abundantly in the ER membrane, so HCV hijacks them away and induces their transport to the membranous web. Studies have shown that HCV is able to change the lipid composition of membranes, leading to the different localization of some proteins, like the PI4KIIIα. This protein thus distributes differently its product PI4P in the cell compartments (Bianco et al, 2012; Reiss et al, 2011). HCV benefits of this redistribution to change the lipid composition of the membranous web, attracting sphingolipids and cholesterol through the recruitment of implicated proteins (Khan et al, 2014; Wang et al, 2014b). HCV is also able to induce membrane biosynthesis, modulating the expression of genes involved in lipid metabolism.

LDL-r seems to be necessary for HCV replication, mostly by helping the virus to preserve the adequate amount and variety of lipids at the membranous web. The virus is able to modulate LDL-r expression through the increase of its gene transcription [Figure 24] (Albecka et al, 2012).

c. Assembly and secretion

The viral structural proteins and the genomic RNA are required for the assembly of HCV particles (Lindenbach, 2013). Viral assembly and secretion are difficult of detect and one of the reasons can be these two processes occur very fast. As mentioned previously, this life cycle step is closely linked with the lipid metabolism.

After the synthesis of new viral proteins, the Core protein undergoes a homodimerization and it is transported close to the cytosolic LDs. The Core interaction with LDs seems to lead to the recruitment of the other viral factors involved in the assembly (Barba et al, 1997; Boulant et al, 2005; Moradpour et al, 1996). The Core accumulates near the LDs, leading to the change of their intracellular distribution. Accordingly, in the non-infected cells, the LDs are distributed in the cytoplasm, but after infection they accumulate in the perinuclear region (Boulant et al, 2008). It is important to note that an high Core-LD association is necessary for an efficient production of infectious particles, confirming the Core passage through the LDs before it is transported to ER-derived assembly sites (Boson et al, 2011; Shavinskaya et al, 2007). This Core-LDs association can be influenced by cellular enzymes involved in lipid homeostasis, such as diacylglycerol acyltransferase-1 (DGAT1), IκB kinase-α (IKK-α) and MAPK-regulated cytosolic phospholipase A2 (PLA2) (Herker et al, 2010; Li et al, 2013a; Menzel et al, 2012). After that, the Core is recruited from the LDs to the site of virus budding with the possible help of clathrin adaptor protein complex 2 (AP2) (Neveu et al, 2012).

The recruitment of other replication complex proteins close to the LDs is the next step of HCV assembly. NS5A, more specifically the C-terminal domain, is a central player in the viral assembly. The hyperphosphorylation status of this protein seems to support its recruitment to low-density membrane fractions around LDs, promoting the interaction with LD-Core complex (Lindenbach, 2013; Miyanari et al, 2007). Moreover, host factors like DGAT1 and Rab18 can also recruit NS5A around LDs, promoting the interaction with the Core (Camus et al, 2013; Salloum et al, 2013). Other major components of the viral particle are the E1 and E2 glycoproteins. They form noncovalent heterodimers that are firstly retained in the ER and then transported to close the LDs by NS2, that simultaneously interacts with p7, forming the E1/E2/p7 functional complex. NS2 is a key player in this stage, interacting with the referred complex but also most prominently with the NS3/4A complex. The cellular factor signal peptidase complex subunit 1 (SPCS1) seems to facilitate the formation of NS2-E2

complex associated membrane. In addition to their role in helping the HCV glycoproteins transport to the assembly site, p7 and NS2 seem to play additional functions, for example, p7 is required during the final steps of capsid assembly and envelopment (Suzuki et al, 2013). The NS3/4A enzyme complex is involved, particularly NS3 that acts during an intermediate step of infectious particle assembly, in the packaging of HCV RNA. The interaction between the NS3 helicase and the Core protein is essential during viral assembly, as well as the functional associations with NS2 / E1E2 / p7. The host factor Y-box-binding protein 1 (YB-1) are recruited to LDs, presenting an important role as a hub for HCV assembly (Chatel-Chaix et al, 2011). Finally, NS4B and NS5B are also implicated but their role remains to be determined. The Core association with LDs seems to be temporary because different studies have shown the Core accumulation around LDs in the assembly deficient mutants.

The HCV assembly pathway is closely linked to that of VLDL, so inhibitors of MTP block the production of viral particles (Gastaminza et al, 2008; Huang et al, 2007; Nahmias et al, 2008). Additionally, the acyl-CoA synthetase long-chain 3 (ACSL3), another enzyme involved in VLDL assembly and the HNF4α, a transcription factor that regulates the VLDL secretion, also modulate the production of infectious viral particles (Li et al, 2014; Yao & Ye, 2008). As already mentioned, the HCV particles resemble to VLDL and LDL in terms of associated apolipoproteins and cholesteryl esters. Studies have shown that ApoE is necessary to produce infectious HCV particles, whereas nowadays it is believed that apoB may not be essential for this process (Da Costa et al, 2012; Hueging et al, 2014; Jiang & Luo, 2009). In addition, ApoE was found to interact with NS5A and viral envelope glycoproteins and its role is supposed to be in a post-envelopment step of HCV particle production.

After assembly and budding in the ER lumen, the new formed virions are transported to the Golgi apparatus, where the E1E2 glycoproteins undergo some modifications, particularly in the associated glycans. Then, the viral particles are released from the cell by a clathrin dependent secretory pathway (Benedicto et al, 2015). The endosomal-sorting complex required for transport (ESCRT) pathway is involved in the biogenesis of infectious particles that are then released from the cell (Ariumi et al, 2011; Corless et al, 2010). In addition, ESCRT function might indirectly promote HCV particle release by modulating the endocytic recycling compartment (ERC). During the secretory process, HCV virions acquire their characteristic low buoyant density (Gastaminza et al, 2008; Gastaminza et al, 2006). Finally, it was suggested that p7 protein may neutralize the low pH of compartments within

the secretory pathway, preventing the conformational changes induced by the low pH [Figure 24] (Wozniak et al, 2010).

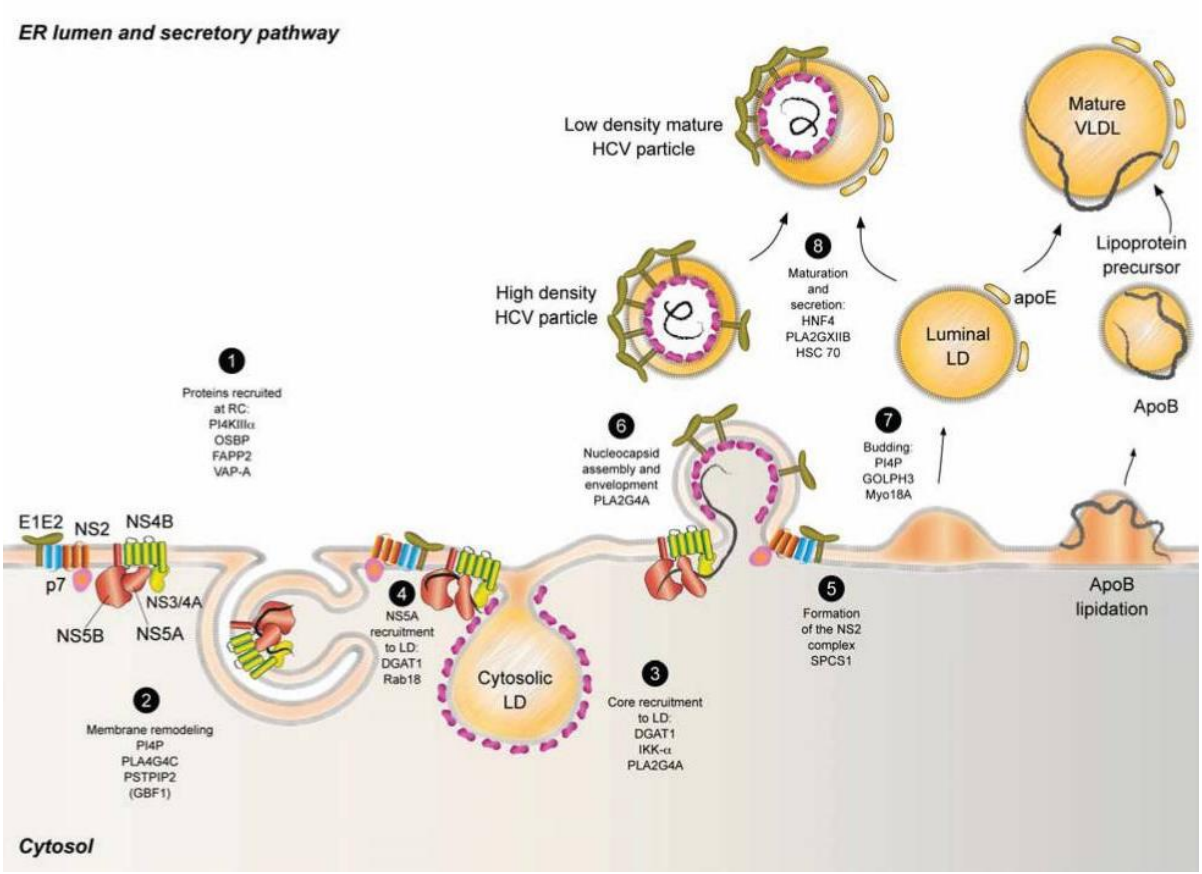

Figure 24: Model of HCV replication and infectious particles assembly
From (Popescu et al, 2014)

d. Hepatocytes tropism

The restricted tropism of HCV to the hepatocytes is still not well understood. The tetraspanin CD81 is expressed in several cell lines, in particular in B cells, acting as a co-stimulatory molecule. However, its association with EWI-2wint may determine the restricted tropism of HCV, since EWI-2wint is broadly expressed in many cell lines but not in the hepatocytes (Rocha-Perugini et al, 2008). SR-B1 and tight junction proteins are detected in diverse cell lines, although they are highly expressed in the hepatocytes. The NPC1L1 is expressed on the apical surface of human hepatocytes and also expressed in enterocytes. Thus, the collective presence of these receptors and entry factors in the hepatocytes, their expression rates and their facility to interact, may explain the HCV restricted tropism to the liver (Dubuisson & Cosset, 2014). In addition, the restricted expression of miR-122 in the hepatocytes also favors the replication of HCV genome.

e. HCV hijacks lipid metabolism

The lipid transport through the bloodstream is done via lipoproteins. Absorbed dietary lipids are re-esterified into triglycerides (TG), cholesteryl esters and phospholipids and then secreted as TG- and apoB-48-rich chylomicrons. On the other hand, the lipids that are synthesized and secreted by the liver are transported in VLDL particles. Both lipoproteins (VLDL and chylomicrons) are involved in the lipid distribution to peripheral and specific target tissues. These processes are controlled by their lipoprotein-associated apolipoproteins, apoC-II, apoB and apoE. LDL is generally recycled by the liver, but can also be used by peripheral cells through the LDL-r. However, high levels of LDL and chylomicron remnants may invade the arterial wall, leading to the development of atherosclerosis. The presence of cholesterol in the plasma membrane is essential to maintain the barrier function between intra- and extracellular environment. Its return to the liver occurs through a process called reverse cholesterol transport, involving HDL and its associated apoA-I. HDL is able to capture free cholesterol *via* interactions with SR-BI, preventing cholesterol accumulation in the artery wall and consequently preventing atherosclerotic disease. After hydrolysis, free cholesterol can be metabolized and excreted into the digestive tract *via* biliary secretion (Vercauteren et al, 2014b).

HCV infection has been associated to the lipid metabolism (Serfaty et al, 2001). The virus is able to alter the lipid homeostasis and high viral concentration may cause hepatic disorders, particularly the HCV genotype 3 that is associated to the development of hepatic steatosis and hypocholesterolemia (Siagris et al, 2006). Studies have shown that HCV infection may regulate the hepatic expression of genes involved in the lipid metabolism, which seems to be relevant for the HCV life cycle and viral clearance, also influencing the anti-HCV treatment efficacy (Walters et al, 2006).

The interaction of HCV particles with apolipoproteins varies depending on whether the viral particles derive from an *in vitro* or *in vivo* system or even from hepatic cancer cells. In this last case, the virus is not associated to the apoA or apoC, because their expression is suppressed in hepatic cancer cell lines. In addition to their association with HCV, the apolipoproteins also play an important role by modulating the HCV infection (Bartenschlager et al, 2011; Douam et al, 2015; Lindenbach, 2013). Many reports have suggested that HCV-associated apoE is an essential mediator of the entry step. It interacts with HCV receptors such as HSPGs, LDL-r and SR-BI, so being important for the binding of viral particles to the cell surface. Moreover, one study showed the importance of apoE for the viral cell-to-cell transmission (Hueging et al, 2014). In addition to its binding with HDL, VLDL, LDL and oxidized LDL (OxLDL), SR-BI also interacts with HCV-associated apoE and E2 glycoprotein and its lipid transfer function seems to be relevant during the HCV post-attachment step (Dao Thi et al, 2012; Zeisel et al, 2007). HDL may change cholesterol content with LVPs or contribute to the host membrane cholesterol enrichment resulting in facilitation of post-attachment entry events, because HCV entry is dependent on the host membrane cholesterol content. Thus, HDL accelerates HCV endocytosis, enhancing the viral entry (Vercauteren et al, 2014b).

LDL-r is also proposed as a mediator of HCV attachment/entry through the interaction with apoE that may compete with LDL particles for the same receptor (Andre et al, 2002; Molina et al, 2007; Monazahian et al, 1999). HCV seems to benefit from LDL-r ability to capture lipoproteins from the circulation, to enter in target cells. However, the implication of this receptor in the HCV infection is controversial, being associated to a non-productive HCV entry (Albecka et al, 2012). NPC1L1, described as being involved in HCV entry, is also crucial for cholesterol absorption and regulation of biliary cholesterol concentration, suggesting that these two involvements may be associated (Altmann et al, 2004; Sainz et al, 2012; Temel et al, 2007). Lipoprotein lipase (LPL) is a triglyceride lipase family protein with

triacylglycerol hydrolase activity, targeting lipoproteins such as chylomicrons and VLDL through their ApoC-II moiety. The triacylglycerol hydrolysis originates fatty acids, used in peripheral tissues, so LPL plays an important role in the globality of lipid metabolism. Moreover this protein has other function based on its heparin- and lipoprotein-binding domains, enabling an indirect interaction between lipoproteins and cell surface HSPG. Thus, the HCV interaction with HSPG may be mediated by LPL. However, some studies have shown a decrease in the HCV infectivity associated to the LPL (Albecka et al, 2012; Andreo et al, 2007; Maillard et al, 2011).

After viral endocytosis, HCV envelope fuses with the endosomal membrane in a process enhanced by the presence of cholesterol and sphingomyelin in the target membrane as well as ApoC-I in the viral membrane (Dreux et al, 2007; Haid et al, 2009). In addition, the HCV RNA replication is also associated to the lipid metabolism. For instance, the decrease of phospholipids levels may affect the ER membrane integrity which is the site of HCV replication. In addition, NS5B contains a sphingolipid binding motif and a reduction of sphingolipid levels has been associated to an HCV replication blockage (Sakamoto et al, 2005). PI4KIIIα is also an essential cofactor for efficient HCV replication (Berger et al, 2009).

The LD is an organelle consisting in triglycerides and cholesteryl esters, surrounded by a phospholipid monolayer and proteins. It maintains the intracellular lipid homeostasis it may interact with the ER, facilitating lipid and protein exchanges with other organelles. LD association with HCV is essential for viral infectious particle production (Miyanari et al, 2007). Moreover, the chronic HCV infection can lead to the LD overproduction that may be linked with steatosis and abnormal lipid metabolism. LDs are also involved in the lipoprotein secretory pathway, which is associated to HCV assembly step (Huang et al, 2007; Olofsson et al, 2009). The assembly/release processes of HCV and VLDL occur simultaneously, leading them to circulate together in the bloodstream. The VLDL assembly includes processes as the lipid transfer from LDs into the ER lumen and its association with apolipoproteins such as apoB and apoE (Vercauteren et al, 2014b).

Apolipoproteins have also an important role in the assembly and production of HCV infectious particles. Thus, once again, studies demonstrated the relevant role of apoE in this cycle step. Recent data demonstrated a direct interaction between apoE and the E2 transmembrane domain, as well as with NS5A, which is important for the maturation of HCV infectious particles (Lee et al, 2014). On the other hand, the role of apoB is still controversial.

Other apolipoproteins are also involved in the formation of HCV particles, like apoA and apoC. Indeed, apoC can enhance HCV infectivity, by promoting membrane fusion (apoC-I) and by reversing lipoprotein lipase-mediated inhibition (apoC-III) (Dreux et al, 2007; Meunier et al, 2005; Sun et al, 2013). The apolipoproteins contain tandem repeats of amphipathic α-helices that interact with the lipoproteins' membrane. Thus, apoA1, apoC1, and apoE present several of these repeats that are similar between them. Recently, a participation of these amphipathic α-helices in the formation of infectious HCV particles through the interaction with them was identified (Fukuhara et al, 2014).

Studies have shown that the density of extracellular infectious HCV particles is much lower than that of intracellular infectious particles, suggesting that infectious HCV particles are assembled in the intracellular space, as high-density precursors and then they acquire components that confer a low-density conformation along their output (Gastaminza et al, 2006).

Some studies have shown inhibitory effects of human plasma compounds such as VLDL, OxLDL, serum amyloid-A (SAA) and lipoprotein lipase (LPL). OxLDL seem to have two roles in the HCV infection, affecting the biophysical properties of the viral particle and disturbing the interaction between HCV and SR-BI, in a non-competitive manner. In chronically HCV-infected individuals, OxLDL levels did not correlate with viral load, but in HCV-negative sera, high levels of OxLDL had a negative effect on HCV infectivity *in vitro*. In addition, OxLDL present pangenotype activity, in which the genotype 4 is the most OxLDL sensitive (von Hahn et al, 2006; Westhaus et al, 2013). VLDL was also associated to the restriction of HCV infection. They seem to block the virus attachment to the cell via a competitive binding to the same cell surface receptors. It is known that LVP-associated apoE mediates the HCV interaction with HSPG, which is also a VLDL receptor. The plasma VLDL levels are beneficial to infected patients by suppressing the transmission and spread of HCV in the liver (Tao et al, 2015).

LPL is synthesized by muscle cells and macrophages, becoming functional at the surface of vascular endothelium. It is a member of the lipase gene family and acts as key enzyme in the metabolism of lipoproteins. LPL hydrolyses triglycerides in chylomicrons and VLDL and is able to form a bridge between these lipoproteins and the HSPG receptor, allowing the lipoproteins internalization in hepatic cells. Thus, studies have shown that LPL is also able to promote the cellular uptake of HCV in an HSPG-dependent manner. LPL seems to present an HCV inhibitory effect, modulating the infectivity *in vivo*. However, the exact

mechanisms by which LPL acts on virus entry and its impact on HCV cell infection require further studies (Andreo et al, 2007). In its turn, SAA seem also to inhibit the viral infection but through of direct interaction with the viral particle (Cai et al, 2007; Lavie et al, 2006b). They are highly conserved acute phase proteins, quickly produced by the liver following a trauma or infection, and are stimulated by the inflammatory cytokines and TNFα. SAA seem to intervene in the cholesterol transportation mediated by SR-BI and to prevent HCV infection, inhibiting viral entry. It was suggested that SAA binds to HCV LVP, blocking the interaction between them and their receptor, SR-BI. In this model, HDL is able to dissociate the interaction between SAA and virus particles, thus enabling an efficient viral entry into hepatocytes. Studies of chronic infection revealed no differences in the SAA concentrations, contrasting with its high production in the acute infection. This is consistent with the observation that SAA is produced only in the early stages of acute infection (Cai et al, 2007; Lavie et al, 2006b).

III. Lipoprotein(a)

A. Main characteristics

Lipoprotein (a) (Lp(a)) was discovered in human serum by Kåre Berg in 1963, during a study of immunological variations in human sera (Berg, 1963). A new antigen associated with LDL was discovered, named Lp referring to the lipoprotein and the small "a" for antigens in human immunogenetics, a terminology accepted at that time. This lipoprotein is a spherical macromolecular complex with a diameter of about 25nm and a density ranging from 1.05 to 1.12 g/mL. Thus, Lp(a) is found in human serum very close to the LDL that presents a density range of 1.019 to 1.063 g/mL (Reblin et al, 1992; Zhang et al, 2007).

Lp(a) has restricted species distribution being present only in primates (humans, Old World monkeys and great apes) and hedgehogs (Boffa et al, 2004; Lawn, 1996; McCormick, 2004). In humans studies have shown that Lp(a) serum concentrations greatly differ from those of other lipids, widely ranging from less than 0.2 mg/dL to more than 200 mg/dL. However, high concentrations are rare with about 90% of population presenting less than 30 mg/dL (Utermann, 2001). To this time, the only major influence on Lp(a) levels is the size polymorphism in the apolipoprotein a [apo(a)] gene (LPA). Apo(a) is the protein component of Lp(a) and it was firstly sequenced and cloned in 1987 (McLean et al, 1987). Studies of Lp(a) global distribution have revealed 2- to 3-fold higher Lp(a) plasma concentrations in Africans than in Europeans and most of Asian populations. Elevated levels of Lp(a) in serum have been positively associated with increased risk for cardiovascular disease (CVD), particularly of myocardial infarction (MI) and stroke (Erqou et al, 2009; Tsimikas & Hall, 2012).

B. Composition

Lp(a) consists of a LDL particle associated to a glycoprotein, apo(a), both in a molar ratio of 1:1. Both are covalently linked via a single disulfide bridge at the C-terminal portion of each protein, apo(a) and apoB-100 from LDL moiety. ApoB-100 is largely hydrophobic,

and portions of the protein are embedded in the Lp(a) lipid moiety. In contrast, apo(a) is hydrophilic, carbohydrate-rich, highly charged, and contains repeated loop structures, called kringles (K) [Figure 25] (Koschinsky & Marcovina, 1997).

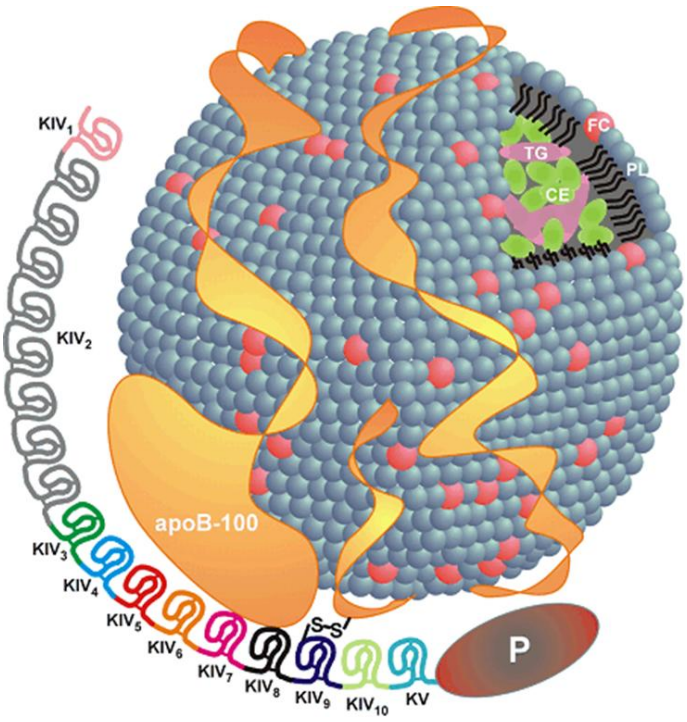

Figure 25: Structure of Lipoprotein(a)
The lipoprotein(a) particle is composed by an LDL-like particle, apoB-100, and the apo(a).
FC: free cholesterol; PL: phospholipids TG: Triglycerides; CE: cholesterol esters
From: (Koschinsky & Marcovina, 2004)

The gene encoding apo(a) was found on chromosome 6q26 and presents high homology with plasminogen (PLG) gene [Figure 26]. Plasminogen is a zymogen that is activated to plasmin and contains five kringles (kringles I-V) and a protease domain. In contrast, apo(a) has various KIV, a KV and an inactive protease domain. More specifically, apo(a) contains 10 distinct tandem repeats, the KIV, that are classified into (KIV1–10) (Koschinsky & Marcovina, 1997). The KIV2 exists in a copy number variation (CNV), in contrast to the other KIV that exist only as single copies (van der Hoek et al, 1993). Some of these KIV domains present lysine-binding sites (LBS), which exhibit weak affinity, such as the ones found in kringle IV types 5–8 (KIV5–8) or strong affinity like in apo(a) kringle IV

type 10 (KIV10). These LBS are involved in the Lp(a) assembly process, in which non-covalent interactions between apo(a) and apoB-100 precede the covalent formation with single disulfide bond (Ernst et al, 1995; Gabel et al, 1996; Trieu & McConathy, 1995).

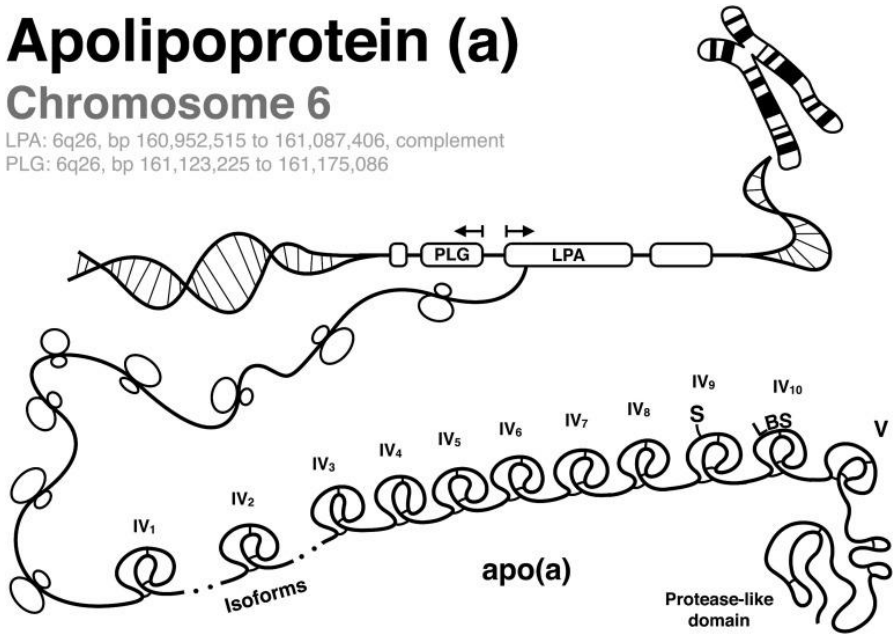

Figure 26: Genetic architecture of PLG and apo(a)

The chromosome 6q26 contains the PLG and the LPA genes. LPA is transcribed into the apo(a) protein, containing KIV1-10, a KV and an inactive protease domain. From: (Leibundgut et al, 2013)

Studies have demonstrated that the initial non-covalent binding involves a highly specific interaction between two lysine residues (Lys680 and Lys690) in apoB-100 N-terminal and two weak LBS in apo(a) KIV7–8 (Becker et al, 2004b). As expected, the lysine and its analogues are potent inhibitors of the non-covalent interaction between apo(a) and apoB-100, and they also reduce the efficiency of covalent Lp(a) formation. Electron microscopy studies showed apo(a) being wrapped around LDL particles, suggesting several contacts between the apo(a) and apoB-100 proteins (Weisel et al, 2001). Other studies have identified multiple apo(a) sequences that interact non-covalently with apoB-100 and vice-versa. These interactions not only anchor apo(a) to apoB-100, but also play important roles in the positioning of these two proteins in a manner that facilitates more non-covalent

interactions and/or disulfide bond formation (Becker et al, 2004b). In addition, it is further speculated that strong LBS of apo(a) KIV10 mediate binding of apo(a)/Lp(a) to other physiological ligands such as fibrin and/or extracellular matrix proteins (Cho et al, 2008).

After non-covalent interactions, apo(a) is covalently bound to the LDL particle through a disulfide bond between apoB-100 Cys4326 and Cys1568 (old nomenclature of first cloned apo(a) cDNA: Cys4057) of apo(a) KIV9, completing the efficient assembly of Lp(a) particle (Dube et al, 2012; Hoover-Plow & Huang, 2013; Koschinsky et al, 1993; Kronenberg & Utermann, 2013). Studies have demonstrated that the conformational status of apo(a) is determinant for the efficiency of covalent Lp(a) particle formation (Becker et al, 2003). Free apo(a) exists in a "closed" conformation that is stabilized by intramolecular interactions between sequences within the N- and C-terminal halves of the molecule. In addition, KIV10 seems to be an important site involved in maintaining the closed conformation of apo(a) (Becker et al, 2004a). Nevertheless, the addition of some lysine analogues induce a substantial conformational change to an "open" apo(a) structure. This protein in the open conformation forms more efficient covalent Lp(a) particles than in the closed form. However, lysine analogues present dual effect, because low concentrations promote Lp(a) assembly due to favorable conformational alterations in apo(a). On the other hand, high concentrations of these analogues reduce the process, inhibiting non-covalent interactions between apo(a) and apoB-100, as described above. So, the efficiency of covalent Lp(a) formation can be regulated by sequences that do not directly participate in the non-covalent process but rather maintain or change the closed conformation of apo(a) (Becker et al, 2003).

Another feature that shows a large influence on the covalent step efficiency of Lp(a) assembly is the copy number variation of KIV2 domains in apo(a). This may represent a new mechanism, which explains the inverse correlation that has been reported between apo(a) isoform size and plasmatic Lp(a) concentrations in the human population (Kronenberg & Utermann, 2013; Schmidt et al, 2016).

a) Polymorphisms within LPA

Lp(a) levels are determined by genetic variants in the LPA locus, being highly heritable, and varying significantly among individuals. Due to the extensive apo(a) gene size polymorphism, homozygous for apo(a) size are rare, so the Caucasian population is characterized by a large amount of heterozygous (Marcovina et al, 1996; Trommsdorff et al,

1995). Single nucleotide polymorphisms (SNPs) have also been described to modulate Lp(a) levels, increasing or decreasing them. Many studies have identified several SNPs that can explain ethnic differences. For example, G-21A is a Lp(a)-increasing SNP more common in African Americans, whereas T3888P and G+1/inKIV8A are two Lp(a)-lowering SNPs more common in Caucasians (Chretien et al, 2006). The 5′pentanucleotide repeat polymorphism (5′PNRP) is found in the promoter region of LPA and four to twelve alleles repeats have been reported, being eight repeats the most frequent in all populations. In the European population high Lp(a) levels associated to a low number of 5'pentanucleotide repeats were reported, in contrast to the Africans in which the association was not found. Accordingly, the alleles with 10 or 11 5'pentanucleotide repeats were associated with short KIV2 CNV alleles and lower Lp(a) levels, in the Europeans (Mooser et al, 1995; Trommsdorff et al, 1995).

Isoform sizes of apo(a) affect the rate of its processing in the ER, so larger isoform sizes have a longer ER retention time and are therefore more susceptible to degradation. This fact can explain, at least in part, the inverse correlation between Lp(a) levels and apo(a) isoform size (White et al, 1997). *In vitro* studies also suggested that smaller apo(a) isoform sizes are associated more readily with LDL to form Lp(a) particles (Becker et al, 2004a). Accordingly, clinical studies have shown that inter-individual differences in Lp(a) levels reflect size-dependent synthesis rates of apo(a) isoforms rather than the rate of Lp(a) catabolism (Krempler et al, 1980; Rader et al, 1994). RNA stability is another factor that influences Lp(a) levels in plasma. However, the efficiency of different apo(a) size isoforms translation was not studied, to date.

The KIV2 CNV is a multiallelic CNV harbored by LPA. These variants range from 3 to more than 40 copies, which gives rise to Lp(a) isoform size heterogeneity, ranging from 300 kDa to 800 kDa (Koschinsky & Marcovina, 1997; Kronenberg & Utermann, 2013; Utermann, 1989). The apo(a) isoform size affects the conformational status of the protein, influencing the efficiency of covalent Lp(a) assembly. The KIV2 repeats affect the final size, conformation, folding, transport and secretion of apo(a), which contributes to 30-70% of Lp(a) variation levels in different ethnic groups (Gaw et al, 1994). These inter-individual differences were particularly found in African descent, including African Americans, who have higher Lp(a) levels comparing with most of the other ethnic groups for a range of apo(a) allele sizes.

b) Oxidized phospholipids and apo(a)

Oxidized phospholipids (OxPL) present an important role mediating several immune, pro-inflammatory and plaque destabilizing processes that accelerate atherogenesis, being also a circulating biomarker associated to CVD. The process that leads to the atherosclerosis is initiated by lipoprotein oxidation, particularly LDL. These OxLDL are present, *in vivo*, within atherosclerotic lesions of humans and animals (Glass & Witztum, 2001). OxLDL are the resulting products from modifications on their lipid and protein moieties, generating "oxidation-specific epitopes". These epitopes are not only immunogenic leading to adaptive humoral responses, but are also a prominent target of several agents of innate immunity (Chou et al, 2008,Glass, 2001 #2353). OxLDL and OxPL exist in the vessel wall and in the plasma. In humans, they are intimately involved in the vulnerability and destabilization of atherosclerotic plaques (Nishi et al, 2002). These evidence were studied through nuclear and magnetic resonance techniques, using murine and human OxPL specific antibodies, such as MDA2, E06, and IK17 (Shaw et al, 2001; Tsimikas et al, 1999).

Following an immunoassay that measures the content of OxPL per apoB-100 (OxPL-apoB-100), with experimental and clinical approaches, it was found that Lp(a) is a preferential lipoprotein carrier of OxPL when compared to LDL [Figure 27] (Bergmark et al, 2008). The phosphocholine (PC) is a headgroup of OxPLs. Thus, using the monoclonal E06 antibody (very specific of OxPL PC group), it was possible to account 85% or more of all PC-containing OxPL found on plasma lipoproteins, present in Lp(a) (Bergmark et al, 2008). It is therefore speculated if Lp(a) may become atherogenic, because it has improved connection to arterial intimal proteoglycans, and thus increases local concentrations of proinflammatory OxPL. OxPL-containing Lp(a) and apo(a) are able to induce apoptosis in ER-stressed macrophages, through CD36-, TLR2/6-, ROS-, and sn2 fatty acid-dependent pathway that activates extracellular signal-regulated kinases (ERK). This pathway may underlie, in part, the ability of Lp(a) to promote the development of atherosclerotic plaques, vulnerable to rupture, and subsequent atherothrombotic events (Seimon et al, 2010).

In addition to be the preferential carrier of OxPL, Lp(a) seems to mediate some potential pro-inflammatory effects, by expressing intracellular adhesion molecules (ICAM), E-selectin, and vascular cell adhesion molecules (VCAM). Lp(a)/apo(a) also promotes the assembly of actin stress fibers, induces gene expression in THP-1 macrophages, including the upregulation of IL-8 expression and it is implicated in pro-inflammatory processes through

the ability to bind to the integrin, Mac-1, on leukocytes. However, a conclusive identification of apo(a) sequences, involved in these processes, requires an elucidation of the underlying mechanisms, which has not been accomplished to date (Scipione et al, 2015).

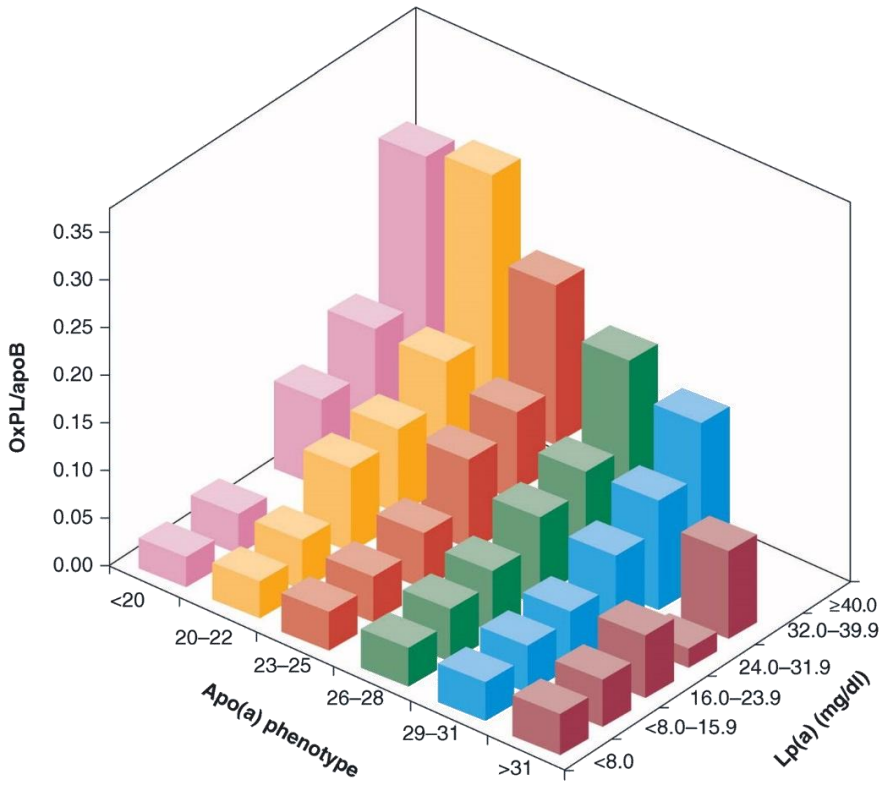

Figure 27: 3D graphic of oxidized phospolipid/apoB-100 levels according to Lp(a) mass and apo(a) phenotypes expressed by the number of KIV2 repeats

The OxPL/apoB levels presented are geometric means (taken as the anti-log of the mean of log-transformed OxPL/apoB values). From: (Taleb et al, 2011)

C. Functions

Until now, Lp(a) functions are not well known. Many studies have emerged proposing some associations and functions for this lipoprotein. For example, the extracellular matrix protein, DANCE/FIBULIN 5 and β-2-glycoprotein I have been identified as Lp(a) (from KIV2) ligands. However, the *in vivo* relevance and the functional implications of these interactions remain unclear.

Ample studies developed in the Austrian and Finnish populations lead to conclude for general population that Lp(a) has no functional importance. These studies detected an LPA site variant that is present with a frequency of about 5% and leads to a fast degradation in plasma of the truncated free apo(a). The homozygotes for the variant did not present any clinical signs or recognizable deficits as well as heterozygotes, with any indication for an increased mortality or morbidity (Lim et al, 2014; Ogorelkova et al, 1999).

D. Metabolism

After several years of research, the exact mechanism of synthesis and catabolism sites of Lp(a) are still unclear. However, it is known that LPA is mainly transcribed in the liver and apo(a) on Lp(a) derives exclusively from the liver, changing its isoform sizes after liver transplantations, according with donor genotype (Kraft et al, 1989).

The site of Lp(a) assembly with apo(a)-apoB-100 interaction also remains controversial. Some studies in isolated hepatocytes or HepG2 cells support an intracellular assembly (Frischmann et al, 2012; Su et al, 1998). Others support an extracellular assembly or at the hepatocyte surface, by non-detection of apo(a)/apoB-100 complexes neither in ER nor in Golgi apparatus in human LPA constructs transfected cells, even blocking the exit of proteins from the Golgi. Accordingly with this hypothesis, these complexes were found in the supernatant of the used cells (Brunner et al, 1996; Demant et al, 2001; Jenner et al, 2005; White & Lanford, 1994).

Little is known about the mode and site of Lp(a) catabolism. It is also unclear if LDL-r plays a role in the removal of Lp(a) from plasma. ApoB-100 on Lp(a) does not readily interact with the LDL receptor. However, the interaction of Lp(a) with LDL-r and other members of the same family has been shown (Schmidt et al, 2016). For example, Lp(a) binds to megalin/glycoprotein 330, a member of the LDL receptor family highly expressed in the kidney. Accordingly, patients with advanced chronic kidney disease presented high plasmatic levels of Lp(a). It was also observed that hemodialysis patients produce similar rates of apo(a) and apoB-100 (on Lp(a)), but show lower catabolic rates when compared to healthy controls. These evidence support an important role of the kidney in the clearance of plasmatic Lp(a) (Lamon-Fava et al, 2014). Recently, SR-BI was identified as a receptor involved in Lp(a) catabolism, but this does not seem to be the main Lp(a) clearance pathway (Yang et al, 2013).

Independent studies have associated the differences in Lp(a) plasma concentrations to differences in its production instead of differences in the catabolism rates (Krempler et al, 1980; Lamon-Fava et al, 2014; Perombelon et al, 1994; Rader et al, 1994). Moreover, short and long isoforms of Lp(a) did not present significantly differences in the fractional catabolic rates (Rader et al, 1994).

In hepatoma cells, the synthesis and secretion of apo(a) are associated with the secretion of triglycerides, suggesting that apoB100–lipid assembly is also a determinant of Lp(a) production (Lamon-Fava et al, 2014). MTP is a protein required for the assembly of apoB-100-containing lipoproteins, in the ER. It was shown that lomitapide, a MTP inhibitor, reduces significantly Lp(a) levels. These reduced levels of Lp(a) were found in individuals with abetalipoproteinemia, in which a MTP gene mutation causes protein loss-of-function. Hepatic apoB-100 synthesis and secretion are also modulated by pro-protein convertase subtilisin/kexin type 9 (PCSK9) independently of the LDL receptor. An overexpression of PCSK9 was able to increase the synthesis of hepatic apoB-100 and subsequently prevent the apoB-100 degradation. In support of these findings, AMG-145, a monoclonal antibody specific to recognize PCSK9, reduced plasma Lp(a) levels significantly. Taken together, these studies support the concept that both apo(a) and apoB-100 synthesis are important regulators of Lp(a) particle assembly and secretion (Lamon-Fava et al, 2014).

E. Association to cardiovascular disease

CVD mediated by atherosclerosis, begins very early in the human life [Figure 28]. However, CVD only clinically manifests after a long latent phase of integrative and cumulative injury to the vessel wall by genetic, environmental, behavioral, and dietary risk factors. Lp(a) elevated levels are defined at birth and potentially contribute to the early risk for CVD, behaving as another genetic risk factor (Tsimikas & Hall, 2012). Lp(a) still represents an important challenge to fundamental researchers who strive to understand the contribution of this lipoprotein to CVD process. To the clinicians, it is also a constant challenge to know when they should measure Lp(a) as a component of risk assessment, and how to best manage high-risk patients with elevated Lp(a) levels (Boffa et al, 2004; Tsimikas & Hall, 2012).

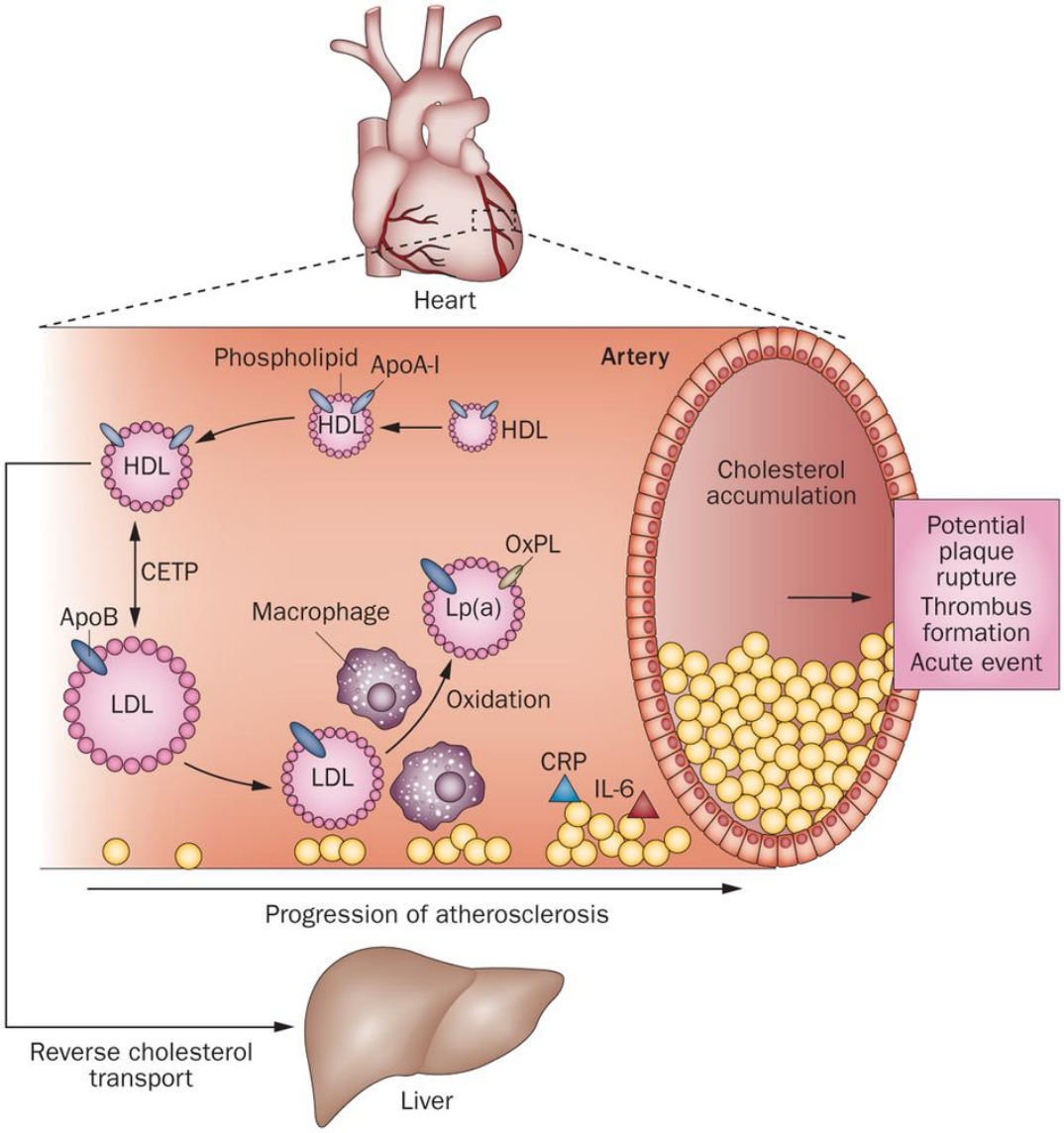

Figure 28: Atherosclerosis process involving cholesterol and lipoproteins

The process involves cholesterol deposition in the artery wall, LDL oxidation, inflammation (mediated by IL-6 and CRP), attraction of monocytes/macrophages and development of atherosclerotic plaques. The plaque rupture can lead to thrombosis. HDL is able to take up some amounts of cholesterol from macrophages and transport back to the liver (reverse cholesterol transport). CETP- cholesteryl ester transfer protein; CRP- C-reactive protein. From: (Jensen et al, 2014)

Unlike other lipoproteins, Lp(a) levels are mainly genetically determined, with a little influence of diet or environment (Faghihnia et al, 2010). High levels of Lp(a) could lead to tissue injury, which can be explained by the association between this lipoprotein and inflammatory cytokines (Maeda et al, 1989; Stenvinkel et al, 1998). Some examples are the

interleukin 6 (IL-6), the TNF-α, the transforming growth factor-β (TGF-β), and the monocyte chemoattractant protein 1 (MCP-1) (Stenvinkel et al, 1998; Szalai et al, 2001). On the other hand, it has also been shown that apo(a) gene expression is up-regulated by IL-6, leading to Lp(a) particles accumulation in inflamed tissues and a possible involvement in the wound healing process. This Lp(a) accumulation, associated to Lp(a) affinity for extracellular matrix proteins, can explain its presence in the early phases of atherosclerosis (Ramharack et al, 1998; Yano et al, 1997).

F. Strategies to lower Lp(a) concentrations

Despite resistance to lower Lp(a) levels with diet changes, several studies have shown that saturated fat enriched diets can significantly decrease Lp(a) levels, as well as polyunsaturated fats (Ginsberg et al, 1998). This last observation is supported by a significant inverse relationship between plasma omega-3 polyunsaturated fatty acids and Lp(a) levels (Kiortsis et al, 2001). Alcohol also seems to have an effect decreasing Lp(a) levels, due to its lipid-lowering action (Paassilta et al, 1998).

More recent studies identified some agents that lower Lp(a) concentrations and also have beneficial effects on the lipid profile, in general. These include inhibitors of the microsomal triglyceride transfer protein, the PCSK9, the CETP, as well as thyroid hormone mimetics, IL-6 receptor blockers and hormone replacement therapies (Godsland, 2001; Kolski & Tsimikas, 2012). Niacin was also identified, long time ago, as effectively lowering Lp(a) levels by reducing its production rates (Boden et al, 2011; Crouse, 1996). However, recent results showed that there are no difference in cardiovascular event risk, associated to high Lp(a) levels, with or without niacin treatment (Albers et al, 2013).

Two other promising approaches to lower Lp(a) are the apheresis and antisense oligonucleotide (ASO). The first, removes almost all apoB-100-containing lipoproteins and it was originally conceived for patients with severe elevations of LDL (Thompson et al, 2010). Apheresis has shown a great reduction of Lp(a) levels, but it also reduces levels of OxPL and lipoprotein-associated phospholipase A2, consequently reducing the incidence rate of major coronary events (Kiechl et al, 2007). Two weeks after the process, although no changes occur in triglycerides, LDL cholesterol (LDL-C) or HDL-cholesterol levels, is generally observed a rebound of the lipoprotein levels, back to high levels (Safarova et al, 2013).

ASO binds to complementary mRNA targets via Watson-Crick base pairing, so an isoform-specific targeting is possible. In the case of Lp(a), this very important feature allows binding to mRNA encoding apo(a), in the region of the KIV2 repeats without altering plasminogen region (Merki et al, 2011). It was also shown that ASO inhibits apoB-100 synthesis in the liver, thus lowering plasma levels of all apoB-100-containing lipoproteins, including Lp(a) (Kolski & Tsimikas, 2012). Later, a second generation of ASO, the Mipomersen (Kynamro®), has emerged with direct action into apoB-100 and it was approved for clinical use to lower LDL-C in patients with homozygous familial hypercholesterolemia. A study realized in Lp(a)-transgenic mice suggested that apoB-100 synthesis is the limiting factor for the Lp(a) particle generation in this LPA transgenic model. This was determined because Mipomersen severely reduced hepatic apoB-100 production and plasma levels and also reduced LDL-C and Lp(a) levels, maintaining apo(a) mRNA and apo(a) levels (Merki et al, 2008).

Optimization of such apo(a) specific ASOs and transposition for clinical application will represent the first therapy specifically directed to elevated Lp(a) levels.

OBJECTIVES

HCV infection still represents a global health problem. The most recent estimates of infection demonstrated an increase in seroprevalence over the last 15 years (Messina et al, 2015). HCV is not an easy virus to work with and it has been necessary to overcome significant hurdles to establish HCV cell culture models. However, further research is still needed as well as efficient culture models, especially of HCV clinical isolates that continue to be very inefficient. This hindrance that hampers the HCV infection from infected human serum, in culture cells could be due to the presence of restriction factors in patient serum. Thus, theses progresses would have impact in the study of the entire HCV life cycle and also in clinical research, allowing the personalization of treatment. In this study we intend to establish efficient cell culture models of HCV clinical isolates, through the identification and ulterior elimination of restriction factors present in the human serum.

Many factors have been identified as interfering with HCV infection. In particular, several innate immune proteins contribute to the virus neutralization and clearance of infected cells. In addition, the pattern recognition molecules (e.g. MBL, serum ficolins and lipoproteins) contribute to the viral recognition, consequently leading to the activation of complement pathway and the direct neutralization of viral particles (Tarr et al, 2012). However, little is still known about the implication of the complement cascade in HCV infection and many studies consider only the specific nAbs as the sole mediators of HCV neutralization (Ball et al, 2014; Tarr et al, 2012). Other factors, not directly implicated in immune processes, were also identified as being involved in the restriction of HCV infection. For example the serum amyloid A, that binds and is internalized by the SR-BI, has a role in the inhibition of infection, being mainly produced by the liver immediately post infection (Lavie et al, 2006b). In addition, the lipoprotein lipase (Andreo et al, 2007), VLDLs (Tao et al, 2015) and OxLDL, have been also identified as restriction factors of HCV infection (Andreo et al, 2007; Flint et al, 2006; Tao et al, 2015; Westhaus et al, 2013). It was suggested that oxLDLs act by perturbing interaction between HCV and SR-BI thus having a potent pangenotype activity (von Hahn et al, 2006; Westhaus et al, 2013).

The specific objectives of my project were:

1. To develop an efficient culture model enabling the dissemination of HCV clinical isolates, *in vitro*.

    a) We firstly search for restriction factors of the virus in the human sera that are the probable reason of the difficulty in cultivate clinical isolates;

    b) We verified their presence and serum fractionation assays followed by mass spectrometry analysis to identify/purify and concentrate the virus inhibitor were performed;

    c) We established protocols to well characterize the identified inhibitor (apo(a)) and searched for a mechanism of action that could explain the inhibition of HCV.

2. It would be interesting to test if the removal of the apo(a) from the human serum, would allow to cultivate the virus from primary isolates, in a highly sustainable culture system.

The results of this work have been the subject of an article, which is under review for publication:

Catarina Oliveira, Carole Fournier, Véronique Descamps, Virginie Morel, Corey A. Scipione, Rocco Romagnuolo, Marlys L. Koschinsky, Agnès Boullier, Paulo Marcelo, Jean-Marc Domon, Etienne Brochot, Gilles Duverlie, Catherine Francois, Sandrine Castelain, and Francois Helle

Apolipoprotein(a) Inhibits Hepatitis C Virus Through Interaction With Infectious Particles. Under review

This article is available in the Results chapter.

RESULTS

ARTICLE

Apolipoprotein(a) Inhibits Hepatitis C Virus Through Interaction With Infectious Particles

Apolipoprotein(a) Inhibits Hepatitis C Virus Through Interaction With Infectious Particles

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Keywords: lipoproteins, lipoprotein(a), lipid metabolism, viral entry, HCV

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List of abbreviations :

HCV, Hepatitis C virus; HCVpp, hepatitis C virus pseudoparticle; HCVcc, hepatitis C virus produced in cell culture; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; apo, apolipoprotein; KIV, kringle IV-like domain; KV, kringle V-like domain; Lp(a), lipoprotein(a); WGA, wheat germ agglutinin; VSVpp, Retroviral particle pseudotyped with the vesicular stomatitis virus glycoprotein G; PEG, polyethylene glycol; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; SGIG, self-generated iodixanol gradient; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; rpm, rotation per minute; RT-qPCR, quantitative reverse transcription polymerase chain reaction; LBS, lysine-binding site

Financial support:

This study was funded by the "Conseil Regional de Picardie" (grant no. : REG13047 "Oncovir"). The funder had no role in study design, data collection and analysis, decision to publish and preparation of the manuscript. Catarina Oliveira was supported by a fellowship from the "Conseil Regional de Picardie". Marlys Koschinsky was supported by a grant from the Canadian Institutes for Health Research.

Abstract

The development of different cell-based models has greatly contributed to improve the knowledge of hepatitis C virus (HCV) life cycle. However, it is still challenging to grow HCV clinical isolates in cell culture. This would open new perspectives to study HCV biology, including drug resistant variants emerging with the new antiviral therapies. In this study we hypothesized that this hurdle could be due to the presence of inhibitory factors in patient serum. Combining polyethylene glycol precipitation, iodixanol gradient and size-exclusion chromatography, we obtained a purified fraction enriched in inhibitory factors from HCV seronegative sera. Mass spectrometry analysis identified apolipoprotein(a) (apo(a)) as a potential inhibitor of HCV entry. Apo(a) consists of ten kringle IV domains (KIVs), one kringle V domain (KV) and an inactive protease domain. The ten KIVs are present in a single copy with the exception of KIV type 2 (KIV_2), which is encoded in a variable number of tandemly repeated copies, giving rise to numerous apo(a) size isoforms. In addition, apo(a) covalently links to the apolipoprotein B component of a low density lipoprotein via a disulfide bridge to form lipoprotein(a). Using a recombinant virus derived from the JFH1 strain we confirmed that plasma-derived and recombinant lipoprotein(a) as well as purified recombinant apo(a) variants were able to specifically inhibit HCV by interacting with infectious particles. Our results also suggest that small isoforms are less inhibitory than the large ones. Finally, we observed that the lipoprotein moiety of HCV lipoviroparticles was essential for the inhibition whereas functional lysine-binding sites in KIV_7, KIV_8 and KIV_{10} were not required. *Conclusions:* Altogether, our results identify apo(a) as an additional component of the lipid metabolism modulating HCV infection.

Introduction

Hepatitis C virus (HCV) is a small positive single-stranded RNA virus that belongs to the Hepacivirus genus in the Flaviviridae family and causes serious liver diseases in humans (1). In the last two decades, the development of different cell-based models such as replicons, retroviral particles pseudotyped with HCV E1E2 envelope proteins (HCVpps) and recombinant viruses derived from the JFH1 strain (HCVccs), has greatly contributed to increase the knowledge about HCV life cycle (2, 3). However, it is still challenging to amplify HCV clinical isolates across different genotypes in tissue culture. This would open new perspectives to investigate viral determinants responsible for the evolution and treatment outcome of hepatitis C. This would also help the study of the resistance to new direct acting antivirals and the development of a vaccine.

A striking feature of HCV particles is the association with lipoproteins (4-6). The nature of the association between HCV virions and the lipoprotein remains undetermined. However, the characterization of HCVcc indicates that their lipid composition resemble to very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) (7) and it has been suggested that HCV particles could be an hybrid structure composed of a virion moiety and a lipoprotein moiety so called lipoviroparticles (4). Nevertheless, alternative models have also suggested that lipoproteins could associate with viral particles through the interaction between apolipoproteins and HCV envelope lipids or proteins (5). In line with this association, several studies have shown that apolipoproteins such as apoE, apoB, apoA1, apoC1, apoC2 and apoC3 are associated with HCV particles and play a critical role in HCV entry (for review, see (6)).

Apolipoprotein(a) (apo(a)) is an apolipoprotein synthetized by the liver, which is found only in humans, nonhuman primates, and old world monkeys (8). In humans, apo(a)

consists of ten types of kringle IV-like domains (KIV), one kringle V-like domain (KV) and an inactive protease-like domain that are highly homologous to plasminogen domains (8). Each of the ten apo(a) KIV domains is present in a single copy with the exception of KIV type 2 (KIV2), which is encoded in a variable number of tandemly repeated copies by the apo(a) gene, ranging from 3 to >40 and giving rise to several apo(a) size isoforms with molecular masses between ~200 and ~800 kDa (8). In human serum, apo(a) covalently links to the apoB component of LDL through a disulfide bridge to form lipoprotein(a) (Lp(a)) (9). An inverse correlation between the size of apo(a) isoforms and the serum levels of Lp(a) (ranging from <1 to >100 mg/dL) has been described (10) and elevated plasma concentrations of Lp(a) have been identified as a risk factor for vascular diseases such as peripheral arterial disease, ischemic stroke, and coronary heart disease (9). It has also been shown that Lp(a) is a preferential carrier of oxidized phospholipids in plasma (11).

In this study, we demonstrated that human serum contains restriction factors that inhibit HCVcc infection, which could explain the difficulty to grow HCV clinical isolates in culture. Performing serum fractionation and mass spectrometry analyses, we identified apo(a) as a potential inhibitor of HCV infection. Using plasma-derived and recombinant Lp(a), we confirmed that Lp(a) inhibits HCVcc infection by interacting with infectious particles. We also used purified recombinant apo(a) variants to show that free apo(a) was sufficient for the inhibitory activity and to decipher the mechanism of inhibition.

Materials and methods

Cell culture. HuH-7-RFP-NLS-IPS were described previously (12) and were obtained by transduction of HuH-7 cells (RCB1366) (13) with Lentivirus pseudoparticles encoding the reporter protein RFP-NLS-IPS (14). These cells were grown at 37°C, 5% CO_2 in Dulbecco's Modified Essential Medium (DMEM; Gibco, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum.

Human plasmas and sera, purified recombinant apo(a) proteins, anti-apo(a) antibody and lectin. Human plasmas and sera were obtained from the ''Biobanque de Picardie''. This certified Biological Resource Center (ISO 9001 and NF S 96–900) obtained the authorization N° AC-2013-1827 from the French Ministry of Research and Higher Education to collect serum samples for scientific usage. All samples were tested negative for HCV, HBV and HIV. The different variants of recombinant apo(a) (6K, 12K, 17K, 17KΔLBS$_{10}$, 17KΔLBS$_{7-8}$, 23K, 27K, 33K, KIV$_{5-9}$ and KIV$_{10}$-P) were expressed and purified as previously described (15, 16). Wheat germ agglutinin (WGA) was purchased from vector laboratories (Burlingame, CA). For immunoprecipitation experiments, we used an in house mouse monoclonal anti-apo(a) antibody ("anti-1-4") which is specific to an N-terminal epitope of apo(a).

HCVcc production and purification. The plasmid encoding the JFH1-derived recombinant virus expressing a luciferase reporter gene (JFH1-CS-A4-RLuc-TM) has been described previously (12). This plasmid was used to produce HCV RNAs by *in vitro* transcription which were electroporated into HuH-7-RFP-NLS-IPS cells, as previously described (12). Supernatants of electroporated cells were recovered and filtered through a 0.45-µm-pore-sized membrane, aliquoted and stored until use at -80°C. For immunoprecipitation experiments, we

used highly infectious viral supernatants purified by iodixanol gradient. Five mL of virus were layered on the top of a 10 to 40% continuous iodixanol gradient. Gradients were ultracentrifuged overnight at 32000 rotation per minute (rpm) and 4°C in a SW32.1 rotor. Fifteen fractions of 1 ml each were collected. The infectivity of each fraction was determined and the seven most infectious fractions were pooled (fractions 5 to 11, with infectivity between 10^6 and 10^7 relative light units).

HCVpps production. HCVpps were produced using a plasmid encoding genotype 2a HCV envelope glycoproteins (JFH1 strain) as previously described (17). Retroviral particles pseudotyped with the vesicular stomatitis virus glycoprotein G (VSVpps) were used as control. Supernatants containing the pseudotyped particles were harvested 48 h and 72 h after transfection, filtered through 0.45-µm pore-sized membranes and conserved at -80°C. Luciferase assays were performed at 72 h post-infection, as indicated by the manufacturer (Promega, Madison, WI).

Polyethylene glycol precipitation. One volume of a polyethylene glycol (PEG)-6000 solution (35% in phosphate-buffered saline (PBS)) was mixed to 4 volumes of pooled sera. The mixtures were incubated overnight à 4°C and centrifuged at 1500 g, 4°C for 30 minutes. The pellets were resuspended in a volume of DMEM equivalent to the pooled sera starting volume. When we combined PEG precipitation with self-generated iodixanol gradient and size-exclusion chromatography, the pellets were resuspended in a volume of DMEM equivalent to one fourth of the pooled sera starting volume in order to concentrate the inhibitors.

Serum fractionation using self-generated iodixanol gradient (SGIG). Serum/iodixanol mixtures (12% (w/v)) were obtained by mixing 18.4 mL of pooled sera with 4.6 mL of iodixanol solution (60% (w/v) Optiprep, Sigma, Oakville, ON). The mixtures were transferred to Beckman Polycarbonate Aluminum Bottles and 3 mL of PBS were carefully layered on top to fill the tube. The bottles were capped with Cap Assembly and centrifuged at 70000 rpm, for 5 h at 4°C using a 70Ti rotor and a Beckman OPTIMA L-100 K BioSafe ultracentrifuge, set at slow acceleration and deceleration. Fractions (1 mL) were collected from the top to the bottom. The lipoprotein content of some fractions was evaluated using "Hydragel 7 Lipo + Lp(a)" (Sebia, Issy les Moulineaux, France), as recommended by the manufacturer.

Size-exclusion chromatography. The experimental condition of the high performance liquid chromatography method was performed according to the study of Tao *et al.* (18). The protein extracts from serum samples were applied onto a calibrated Superose 6 increase 10/300 GL column (AKTA pure) after passage through a 0.22 µm-pore filter unit. The purified fraction was eluted into 80 fractions (0.250 mL/fraction) with PBS 50 mM pH 7.2 at a flow rate of 0.3 mL/min to cover the range of molecules from the void volume to 1 kDa. Elution times of the protein peaks were determined using Unicorn software (version 6.3, GE Healthcare, Uppsala, Sweden).

NanoLC–ESI–MS/MS analysis. Samples were loaded on 12% Mini-PROTEAN® TGX™ Precast Protein Gels and short electrophoresis was used to concentrate proteins in one band. Each sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) band was manually excised from the gels to be hydrolyzed according to Shevchenko *et al.* (19). All digested peptide mixtures were separated by on-line nanoLC and analyzed by nano-electrospray tandem mass spectrometry. The experiments were performed on an Ultimate

3000 RSLC system coupled with an LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific, Waltham, MA). The peptide mixtures were injected onto a nano trap column (Acclaim C18, 100 µm i.d. x 2 cm length) with a flow of 5 $\mu L.min^{-1}$ and subsequently gradient eluted with a flow of 300 $nL.min^{-1}$ from 2-55% acetonitrile / 0.1% formic acid during 35 minutes. Mass data acquisitions were performed using Xcalibur 2.3 software. The mass spectrometer was operated in positive mode in a data-dependent mode as described previously (20). Proteins were identified by automated database searching (Mascot 2.4, Matrix Science) against Swissprot from UniProtKB release 2015-09 non indexed, for homo sapiens taxonomy, for tryptic peptides with up to 2 miscleavages, and carbamidomethylation of cysteins (+57.022 uma) and methionin oxidation (+15.995 uma) variable modifications. Protein identifications were validated only if at least 2 different sequences (in doubly and/or triply charge state) were identified as first candidates using the Percolator node with decoy database searching. Mass accuracy tolerance was set to 10ppm in MS mode, to 0.8 Da in MS/MS mode. Strict false discovery rate was set to 0.01, relaxed false discovery rate was set to 0.05 and validation was based on q-Value.

Plasma Lp(a) isolation. Lp(a) (density 1.06-1.11g/mL) was isolated from normolipemic human plasmas by sequential ultracentrifugation. Potassium bromide (KBr) solutions were made up to the following densities : 1.06, 1.11 and 1.34 g/mL. Densities were adjusted and verified by measurement on a portable digital density meter DMA35 (Anton Paar, Graz, Austria). To isolate Lp(a), we used a Beckman MLA.55 fixed angle rotor in a Beckman optima max-XP ultracentrifuge. Plasma was adjusted to the density 1.06 and centrifuged for 20h (55000 rpm, 4°C). After centrifugation, the supernatant was discarded and the infranatant fluid washed by addition of KBr at a density 1.06. After another centrifugation (20h, 55000 rpm, 4°C), the supernatant was removed and the infranatant was adjusted to the density 1.11

by addition of KBr then centrifuged for 20h (55000 rpm, 4°C). After centrifugation, the supernatant containing Lp(a) was recovered. The isolated Lp(a) was then extensively dialyzed against phosphate-buffered saline containing 0.3 mmol/L ethylenediaminetetraacetic acid. Ethylenediaminetetraacetic acid (0.1 mmol/L, Sigma) was added to prevent further oxidation. To avoid lipoprotein degradation, the Lp(a) preparation was sterile filtered on 0.22µm filter and stocked at 4°C for further utilization. Lp(a) concentration was assessed by apo(a) measurement by immunoturbidimetry on a chemistry automate (Dimension Vista® 500 system, Siemens, Erlangen, Germany).

Recombinant Lp(a) production. To produce recombinant Lp(a), we used the plasmids pCMV-A14, pCMV-A18 and pCMV-A22 encoding apo(a) with thirteen (13K), seventeen (17K) or twenty-one (21K) KIV domains that were previously described (21) and kindly provided by H. Dieplinger (Medical University of Innsbruck, Innsbruck, Austria). A plasmid encoding green fluorescent protein was used as negative control. The plasmids were transfected in HuH-7-RFP-NLS-IPS cells using "GenJet™ In Vitro DNA Tranfection Reagent for HuH-7 Cells" (SignaGen Laboratories). Supernatants were recovered 72 hours post-transfection. Lp(a) concentration was assessed by apo(a) measurement by immunoturbidimetry on a chemistry automate (Dimension Vista® 500 system, Siemens, Erlangen, Germany).

Immunoprecipitation. Protein G Plus Agarose (Pierce) was incubated with the "anti-1-4" mouse monoclonal anti-apo(a) antibody for 2 h at 4°C and then washed with PBS. Anti-apo(a) antibody-coated beads were then incubated for 4 h with the supernatant of HuH-7 cells containing 200 µg/mL of recombinant 21K-Lp(a). Supernatant of non-transfected HuH-7 cells was used as negative control. Alternatively, free 17K-apo(a) variant was also tested, using

PBS as negative control. After washing, the beads were finally incubated with purified HCVcc. The mixtures were incubated overnight at 4°C. The beads were washed 5 times with PBS, and RNAs were extracted by using the QIAamp viral RNA kit (Qiagen, Valencia, CA). HCV genomes were quantified by quantitative reverse transcription polymerase chain reaction (RT-qPCR) as previously described (22).

Infectivity assay. HCVccs, HCVpps or VSVpps were put in contact with HuH-7-RFP-NLS-IPS target cells at 37°C in the presence or absence of the different samples. After 3 h of contact, the medium was changed and cells were further incubated for 48 h (for HCVccs) or 72 h (for HCVpps and VSVpps) with complete medium before luciferase activities were measured as indicated by the manufacturer (Promega, Madison, WI).

Results

Human serum inhibits HCV infection. Despite decades of research, it is still impossible to grow primary HCV isolates in cell culture. To test whether this hurdle could be due to the presence of inhibitory factors in patient serum, we sought to determine if HCV infection is inhibited by human serum. To this end, we inoculated HuH-7-RFP-NLS-IPS cells with a recombinant HCV expressing a Luciferase reporter protein, in the presence of increasing concentrations of human serum derived from four HCV seronegative individuals. Our results revealed that all the tested sera dose-dependently inhibit HCV infection, resulting in a 90% decrease when human serum was added at a final concentration of 50% during inoculation (Fig. 1). These results confirmed our hypothesis that human serum contains HCV inhibitory factors.

HCV serum inhibitors are pelleted by PEG precipitation. PEG precipitation is commonly used for the recovery and concentration of viruses. In search for a protocol which would allow the removal of inhibitory factors from HCV RNA positive sample, we tested whether serum inhibitors are also precipitated by PEG. HCV negative human serum was thus subjected to PEG precipitation and the pellet obtained after centrifugation was resuspended in a volume equivalent to that of the supernatant. The inhibitory activity of various concentrations of the supernatant and the resuspended pellet was then evaluated and compared to that of the starting serum. As shown in Fig. 2A, we observed that the starting serum and the resuspended pellet dose-dependently inhibited HCV infection whereas the supernatant obtained after centrifugation did not have any inhibitory activity. We therefore concluded that PEG precipitation could not be used to separate infectious virions and inhibitory factors from an HCV positive sample. However, when we analyzed the protein content of each sample using Coomassie Blue Staining after SDS-PAGE, we observed that PEG precipitation allowed to partially purify the inhibitory fraction as compared to the starting serum and that several serum proteins, in particular albumin, were efficiently eliminated in the supernatant after precipitation (Fig. 2B).

HCV serum inhibitors are present in low density fractions after SGIG. As mentioned previously, a striking feature of HCV particles is the association with lipoproteins. For this reason, we also wondered whether density gradient would enable to recover an infectious fraction from an HCV positive sample. To test this, we fractionated HCV negative serum samples using SGIG and assessed the inhibitory activity of the different fractions obtained at the end of the experiment. This procedure allow to rapidly separate very-low, low and high density fractions as confirmed by the presence of VLDLs in the fractions 1 to 3 and LDLs in

the fractions 6 to 7, identified by the whitish and the brownish colors, respectively (data not shown). As shown in Fig. 3A, we often observed an inhibitory activity of very low density fractions (fractions 1 to 4). The inhibitory activity of these fractions was highly variable from one serum to another and may correspond to the inhibition raised by VLDLs as recently demonstrated by Tao *et al*. (18). In contrast, with all the sera tested, we observed a higher level of inhibition with the fractions 7 to 11 which present a density slightly higher than LDLs that peaked in fraction 6 (Fig. 3B). Fractions with higher density (12 to 22) did not show any effect on HCV infection. Since HCV is expected to be recovered in low and very-low density fractions after density gradient experiment, we concluded that SGIG could not be used to recover an infectious fraction from an HCV positive sample. However, as for PEG precipitation, we also observed that SGIG procedure permits to partially purify an inhibitory fraction and remove several serum proteins as observed after SDS-PAGE and Coomassie Blue Staining (Fig. 3C).

Purification and identification of HCV inhibitors present in human serum. In attempt to purify and identify the inhibitory factors, we carried out serum PEG precipitation and fractionated the resuspended pellet by SGIG. We then analyzed the protein content of the most inhibitory fraction using mass spectrometry but we identified around one hundred proteins (data not shown). Consequently, we decided to concentrate 4 fold the inhibitory factors using serum PEG precipitation and to use SGIG as an intermediate purification step. In these conditions, we observed that low density inhibitory factors were concentrated in fractions 6 to 13 with a maximal inhibition obtained using fractions 8 and 9 (88% and 90%, respectively; Fig. 4A). We then mixed fractions 8-9 (f8-9) and 10-11 (f10-11) and performed size-exclusion chromatography as a final purification step, as recently described by Tao *et al.*

(18). For both samples, 80 fractions were obtained and we observed that the inhibitory factors concentrated in fractions 45 to 55 (Fig. 4B,C). About thirty proteins were revealed by mass spectrometry in the inhibitory fractions obtained from f8-9. In contrast, we only identified two proteins using f10-11, apoB and apo(a). Of note, apo(a) is known to covalently link to the apoB component of a LDL through a disulfide bridge to form Lp(a) (9), which concurs with an inhibitor that precipitates with PEG and that has a density slightly higher than that of LDL.

Lp(a) inhibits HCV infection. To investigate whether Lp(a) inhibits HCV, we first purified it from two different human plasmas using sequential ultracentrifugation. As shown in Fig. 5A, we observed that both samples dose-dependently inhibit HCVcc infection, even if the efficiency differs between samples. At 500 µg/mL, Lp(a) recovered from plasma 1 and 2 achieved 72% and 44% inhibition, respectively. Importantly, the presence of Lp(a) only during the 3 hours inoculation was sufficient to inhibit HCV suggesting that the entry step was inhibited. Since the apo(a) component of Lp(a) varies widely from one individual to another, we also tested the effect of recombinant Lp(a). To this end, we transfected HuH-7-RFP-NLS-IPS cells with plasmids expressing three isoforms of apo(a) containing 13, 17 or 21 KIV domains (13K, 17K or 21K, respectively). Cell supernatants containing recombinant Lp(a) were recovered 72 hours post-transfection and mixed with HCVccs during inoculation of naïve HuH-7-RFP-NLS-IPS. As shown in Fig. 5B, a dose-dependent inhibition was observed with each form of recombinant Lp(a). Importantly, no inhibition was observed when using supernatants of cells transfected with a plasmid encoding green fluorescent protein as negative control (data not shown). Our results also suggested that the size of the apo(a) component correlates with the inhibition potency. For instance, at 25 µg/mL, 15%, 25% and 46% inhibitions were observed with 13K, 17K and 21K recombinant Lp(a), respectively, and

24%, 43%, 46% inhibition were achieved, at 50 µg/mL. Of note, WGA, a lectin which was demonstrated to have high specificity for Lp(a), restored HCVcc infectivity in a concentration-dependent manner (Fig. 5C). Thus, 100 µg/mL of WGA completely counteracted the inhibitory activity caused by 100 µg/mL of 21K recombinant Lp(a). In absence of Lp(a), WGA had no effect on HCVcc infectivity (Fig. 5C).

Lp(a) interacts with infectious HCV particles. Since WGA is known to specifically interact with apo(a), we sought to determine whether it could interfere with critical interactions between Lp(a) and infectious HCV particles. Recombinant 21K-Lp(a) contained in the supernatant of transfected HuH-7-RFP-NLS-IPS cells was immunoprecipitated using anti-apo(a) antibody-coated beads. Supernatants of non-transfected HuH-7-RFP-NLS-IPS cells were used as negative control. The mixtures were then incubated overnight with purified HCVcc and the amount of captured HCV RNAs was evaluated by RT-qPCR. As represented in Fig. 5D, we observed that 33.6 % of HCV RNAs were fixed to the beads in the presence of r-21K-Lp(a) whereas almost all HCV RNAs (99.2 %) were eliminated in the supernatant of immunoprecipitation in absence of Lp(a). In addition, we also observed that WGA prevents Lp(a) from interacting with HCV particles since only 3.8 % of HCV RNAs were co-immunoprecipitated in the presence of this compound. Altogether, these results suggest that Lp(a) decreases HCV infection by interacting with infectious viral particles.

Apo(a) inhibits HCV infection. In contrast to hepatoma cell lines, HEK293 cells do not synthesize apoB and produce free apo(a) (23). To investigate whether the apo(a) component alone was sufficient for the inhibitory activity, we thus tested the effect of several

recombinant apo(a) variants purified from the conditioned medium of stably expressing HEK293 cells by affinity chromatography (Fig. 6A). As shown in Fig. 6B, we observed that the different apo(a) isoform size variants were able to dose dependently inhibit HCVcc infectivity. In accordance with the results presented in Fig. 5B, we also observed that small isoforms were less inhibitory than large isoforms, with a plateau for isoforms larger than 23K. Importantly, when replacing r-21K-Lp(a) by free 17K-apo(a) variant in the immunoprecipitation assay described above, 39.9 % of HCV RNAs were co-immunoprecipitated, demonstrating that apo(a) alone is sufficient to interact with infectious viral particles (data not shown). Interestingly, we also observed that the inhibition triggered by the 6K, KIV_{5-9} and KIV_{10}-P truncated variants was very weak as compared to that of the 17K isoform (Fig. 6C), suggesting that a substantial numbers of KIV_2 repeats is required for maximal inhibition. However, results obtained with these three variants must be interpreted with caution since they likely adopt a very different conformation as compared compared to full-length apo(a) isoforms (24). Several of the apo(a) kringle domains contain lysine-binding sites (LBSs) that play a key role in the pathogenic activity of Lp(a). Weak LBS in KIV_7 and KIV_8 mediate noncovalent interactions with apoB-100 prior to the disulfide bond formation. A strong LBS in KIV_{10} enables the interaction of apo(a) with several biological substrates including fibrin. Of note, when comparing the inhibition potency of 17K, 17KΔ$LBS_{7,8}$ and 17KΔLBS_{10} apo(a) variants, we observed that point mutations which affect the functionality of the $LBS_{7,8}$ and LBS_{10} did not have any effect on the inhibitory activity (Fig. 6D). Of note, since they are produced from 293T cells, HCVpps are not associated with lipoproteins, which allowed us to investigate the importance of HCV association with lipoproteins for the apo(a) induced inhibition. Using 17K, 23K and 27K apo(a) variants, we showed that 100-fold higher concentrations were required to inhibit HCVpps as compared to HCVccs, suggesting that the lipoprotein moiety of HCV lipoviroparticles is essential for the inhibitory activity of apo(a).

Importantly, we also demonstrated that recombinant apo(a) did not inhibit VSVpps demonstrating that the inhibition is specific to HCV.

Discussion

In this study, we aimed at evaluating the presence of HCV restriction factors in human serum and we observed that large serum particles (precipitated by PEG) with a density slightly higher than LDLs were able to strongly inhibit HCVcc infection. Mass spectrometry analysis identified apo(a) as a potential candidate responsible for this inhibition and our results demonstrated for the first time that apo(a) decreases HCV entry, potentially through an interaction with infectious viral particles. A striking feature of HCV particles is the association with lipoproteins and several studies have already demonstrated that apolipoproteins are associated with HCV particles and modulate HCV infection (4-6). Indeed, it is now well established that apoE is required for HCV infectivity (25-27) and that apoC-I enhances HCV infectivity by promoting membrane fusion (28, 29). In addition, it has also been suggested that apoC-III could increase HCV infectivity by reversing lipoprotein lipase-mediated inhibition (30) whereas apoH could inhibit HCV (31), potentially through interaction with the viral particles (32).

Our results suggest that apo(a) inhibits HCV through an interaction with infectious particles. In addition, in contrast to HCVccs, we observed that HCVpps are only weakly sensitive to apo(a) suggesting that the lipoprotein moiety of HCV lipoviroparticles is essential for the inhibition. In human serum, apo(a) interact non-covalently with the apoB component of a LDL through LBS_7 and LBS_8 before the formation of a disulfide bond leading to the formation of Lp(a). However, our data suggest that not only apo(a) interacts with HCV

infectious particles but also Lp(a). Additionally, we observed that mutation of LBS_7 and LBS_8 did not have any effect on HCVcc inhibition by apo(a). Thus, the interaction of apo(a) with HCV does not seem to involve apoB but potentially another component of the lipoprotein moiety of HCV lipoviroparticles. It is now well established that large amounts of apoE is associated to infectious HCVcc and that the level of apoE correlates very well with infectivity (25). In addition, Boyer *et al.* recently demonstrated that apoE and HCV glycoproteins forms a protein complex associated with HCVcc and also proposed that small amount of apoE is associated with E1E2 on HCVpps (33). Thus, it would be interesting to test whether apoE is a requisite for apo(a) inhibition which could explain the relative resistance of HCVpps to inhibition as compared to HCVcc. Alternatively, the involvement of other lipoprotein components cannot be excluded. Results obtained using isoform size variants suggest that the larger apo(a), the stronger the inhibition is. A substantial numbers of KIV_2 repeats may thus be required for maximal inhibition. Whether these domains permit the interaction of apo(a) with HCV infectious particles will have to be investigated. On the other hand, this result may simply be explained by an increase of the steric hindrance. Little is known about the function of apo(a) but it is now known that Lp(a) is a preferential carrier of oxidized phospholipids in plasma (9). However, the mutation of the LBS_{10}, which is essential for the capture of oxidized phospholipids did not have any effect on the inhibitory activity of apo(a). It is thus unlikely that oxidized phospholipids play a role in the apo(a) mediated HCV inhibition and further studies will be needed to decipher the exact mechanism of HCV inhibition by apo(a).

The development of a culture system enabling the amplification of primary HCV isolates would open new perspectives for the investigation of viral determinants responsible for the evolution and treatment outcome of hepatitis C as well as for the development of a vaccine. Recently Saeed *et al.* elegantly demonstrated that SEC14L2 expression in hepatoma

cell lines promotes HCV RNA replication following inoculation with patient sera, through enhancement of vitamin E-mediated protection against lipid peroxidation (34). We also contributed to show that highly infectious sera displayed a specific profile with low levels of several cytokines/growth factors that could impact hepatocyte biology and infection efficiency (35). Finally, in addition to innate and adaptive humoral immunity components (36, 37), several factors in human serum, such as serum amyloid A (38), lipoprotein lipase (39), VLDLs (18) or oxidized LDLs (40, 41), have been shown to inhibit HCV infection. In this study, we identified apo(a) as an additional inhibitory factor present in human serum. It would be interesting to test whether the removal of some of these factors before inoculation could increase the efficiency of the infection and facilitate the amplification of primary HCV isolates. However, this will be complicated for components which are associated to the viral particles, such as apo(a).

In conclusion, we identified apo(a) as an additional component of the lipid metabolism modulating HCV infection through interaction with lipoviroparticles. Our results bring new insight into the tight link between HCV and lipoproteins. Our study could also open new perspectives for the study of apo(a) function.

Acknowledgments

We thank Elodie Martin, Abderrahmane Bengrine, Catherine Moriset for assistance. We also thank T. Wakita, and C.M. Rice for providing us with reagents and Jean Dubuisson for critical reading of the manuscript.

Competing interests

The authors have declared that no competing interests exist.

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Figure Legends

Fig. 1. Human serum inhibits HCV infection. HuH-7-RFP-NLS-IPS cells were inoculated with HCVcc for three hours in the presence of various concentrations of human serum (5%, 10%, 25% or 50%) derived from four HCV seronegative individuals (S1, S2, S3 and S4). Luciferase assays were performed on infected cells at 48 h post-infection. Results are expressed as percentages of infectivity compared to infection in absence of human serum and are reported as the means ± S.D. of three independent experiments.

Fig. 2. Human serum inhibitors are precipitated with PEG. Human serum was subjected to PEG precipitation. After precipitation, the supernatant was recovered and the pellet was resuspended in an equivalent volume of DMEM. (A) HuH-7-RFP-NLS-IPS cells were then inoculated with HCVcc for three hours in the presence of various concentrations (5%, 10%, 20% or 25%) of the starting serum, the supernatant or the resuspended pellet. Luciferase assays were performed on infected cells at 48 h post-infection. Results are expressed as percentages of infectivity compared to infection in absence of sample and are reported as the means ± S.D. of a duplicate from a representative experiment. (B) The protein content of two sera (A and B) was evaluated by Coomassie Blue Staining after SDS-PAGE and compared to that of the supernatants and the resuspended pellets obtained after PEG precipitation. MW, molecular weight.

Fig. 3. Human serum inhibitors are concentrated in low density fractions after SGIG. Human serum was subjected to SGIG. (A) After ultracentrifugation, twenty-two fractions were collected from the top to the bottom. The inhibitory activity of each fraction was evaluated by

inoculating HuH-7-RFP-NLS-IPS cells with HCVcc for three hours in the presence of the fraction (concentration of 50 %). Luciferase assays were performed on infected cells at 48 h post-infection. Results obtained with two different sera are shown. Results are expressed as percentages of infectivity compared to infection in absence of sample and are reported as the means ± S.D. of a duplicate. (B) The lipoprotein content of fractions 6 and 8 obtained with "serum 1" was evaluated after electrophoresis on a buffered agarose gel and staining with a lipid specific Sudan black stain (Hydragel 7 Lipo + Lp(a)). (C) The protein content of fractions 6 to 11 obtained with "serum 1" was evaluated by Coomassie Blue Staining after SDS-PAGE. MW, molecular weight.

Fig. 4. Identification of inhibitory fractions obtained after PEG precipitation, SGIG and size-exclusion chromatography. Serum inhibitory factors were concentrated 4 fold using PEG precipitation. The resuspended pellet was fractionated in 24 fractions using SGIG. Fractions 8-9 (f8-9) and 10-11 (f10-11) obtained after SGIG were subjected to size-exclusion chromatography as a final purification step. For both samples, 80 fractions were obtained. The inhibitory activity of the fractions obtained after SGIG (A) as well as size-exclusion chromatography on f8-9 (B) and f10-11 (C), was evaluated by inoculating HuH-7-RFP-NLS-IPS cells with HCVcc for three hours in the presence of each fraction (concentration of 50 %). Luciferase assays were performed on infected cells at 48 h post-infection. Results are expressed as percentages of infectivity compared to infection in absence of sample and are reported as the means ± S.D. of a duplicate. The relative protein content of the fractions obtained after size-exclusion chromatography are illustrated by the curves.

Fig. 5. Lp(a) inhibits HCV infection by interacting with infectious particles. Inhibition assays were performed by inoculating HuH-7-RFP-NLS-IPS cells, for three hours, with HCVcc and various concentrations of Lp(a) purified from two different human plasmas (A) or recombinant Lp(a) present in the supernatant of HuH-7 cells transfected with plasmids expressing 13K, 17K or 21K apo(a) (B). Results are expressed as percentages of infectivity compared to infection in absence of sample and are reported as the means ± S.D. of a duplicate from a representative experiment (A) and of three independent experiments (B). (C) RFP-NLS-IPS cells were inoculated during three hours with HCVccs, in the presence or absence of 100 µg/mL of recombinant Lp(a) (21K-r-Lp(a), see Fig. 5B) and various concentrations of WGA. Luciferase assays were performed on infected cells at 48 h post-infection. Results are expressed as percentages of infectivity compared to infection in absence of sample and are reported as the means ± S.D. of three independent experiments. (D) Recombinant 21K-Lp(a) contained in the supernatant of transfected HuH-7-RFP-NLS-IPS cells (200 µg/mL) was immunoprecipitated using anti-apo(a) antibody-coated beads. The supernatant of non-transfected HuH-7-RFP-NLS-IPS cells was used as negative control. The mixtures were incubated overnight with purified HCVcc in the absence or presence of 100 µg/mL of WGA and the amount of captured HCV RNAs was evaluated by RT-qPCR. Results are expressed as the titer of HCV RNA in the immunoprecipitate (percentage of total HCV RNAs in the immunoprecipitate plus supernatant) and are reported as the means ± S.D. of three independent experiments.

Fig. 6. HCV is inhibited by different recombinant apo(a) variants. (A) The schematic diagram shows the topology of the recombinant apo(a) variants that were purified from the conditioned medium of stably expressing HEK293 cells for this study. The reference 17K

apo(a) variant corresponds to a physiological apo(a) isoform which includes all 10 types of KIV sequences present in apo(a) isoforms, as well as the KV and protease-like (P) domains. The black dots denote the presence of amino acid substitutions that inactivate LBS. The bar above KIV_9 denotes the unpaired cysteine residue that mediates covalent attachment to apoB-100. (B, C, D, E) Inhibition assays were performed by inoculating HuH-7-RFP-NLS-IPS cells, for three hours, with HCVccs, HCVpps or VSVpps and various concentrations (0.1, 1, 10 and 100 µg/mL) of the apo(a) variants. Luciferase assays were performed on infected cells at 48 h (for HCVccs) or 72 h (for HCVpps and VSVpps) post-infection. Results are expressed as percentages of infectivity compared to infection in absence of sample and are reported as the means ± S.D. of at least two independent experiments.

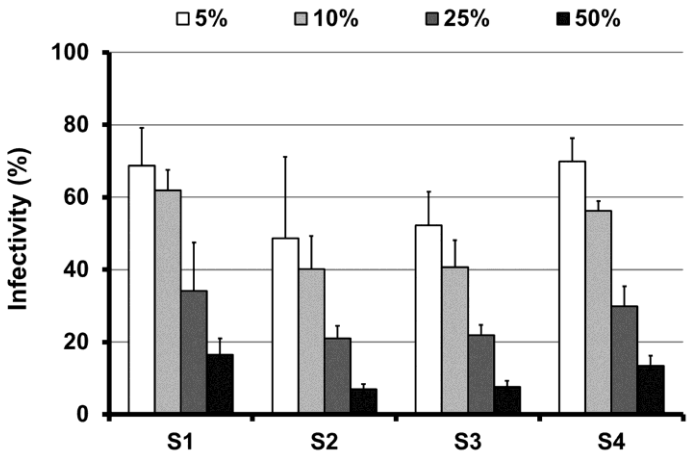


Figure 1

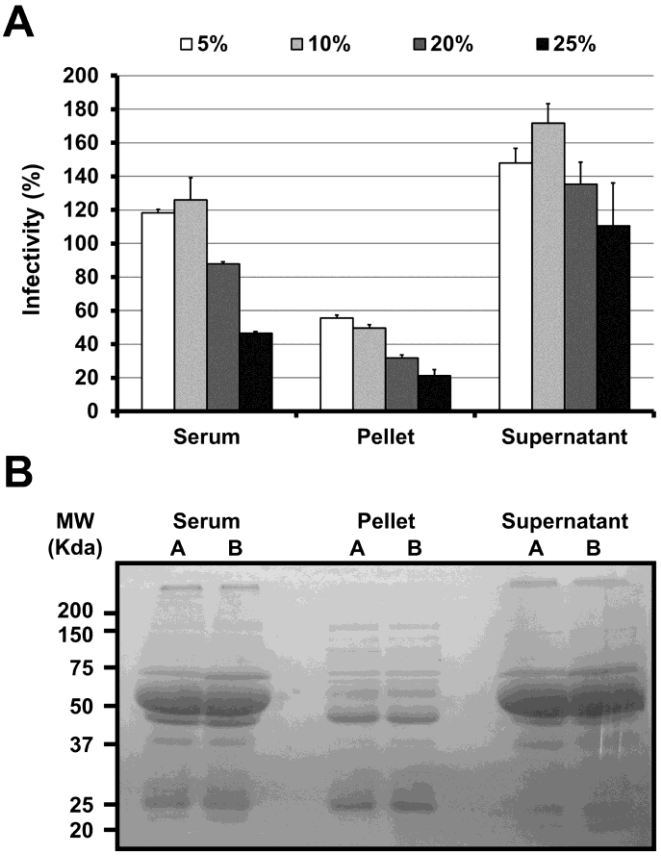

A
5%
10%
20%
25%
Infectivity (%)
200
180
160
140
120
100
80
60
40
20
0
Serum
Pellet
Supernatant
B
MW
(Kda)
Serum
A B
Pellet
A B
Supernatant
A B
200
150
75
50
37
25
20

Figure 2

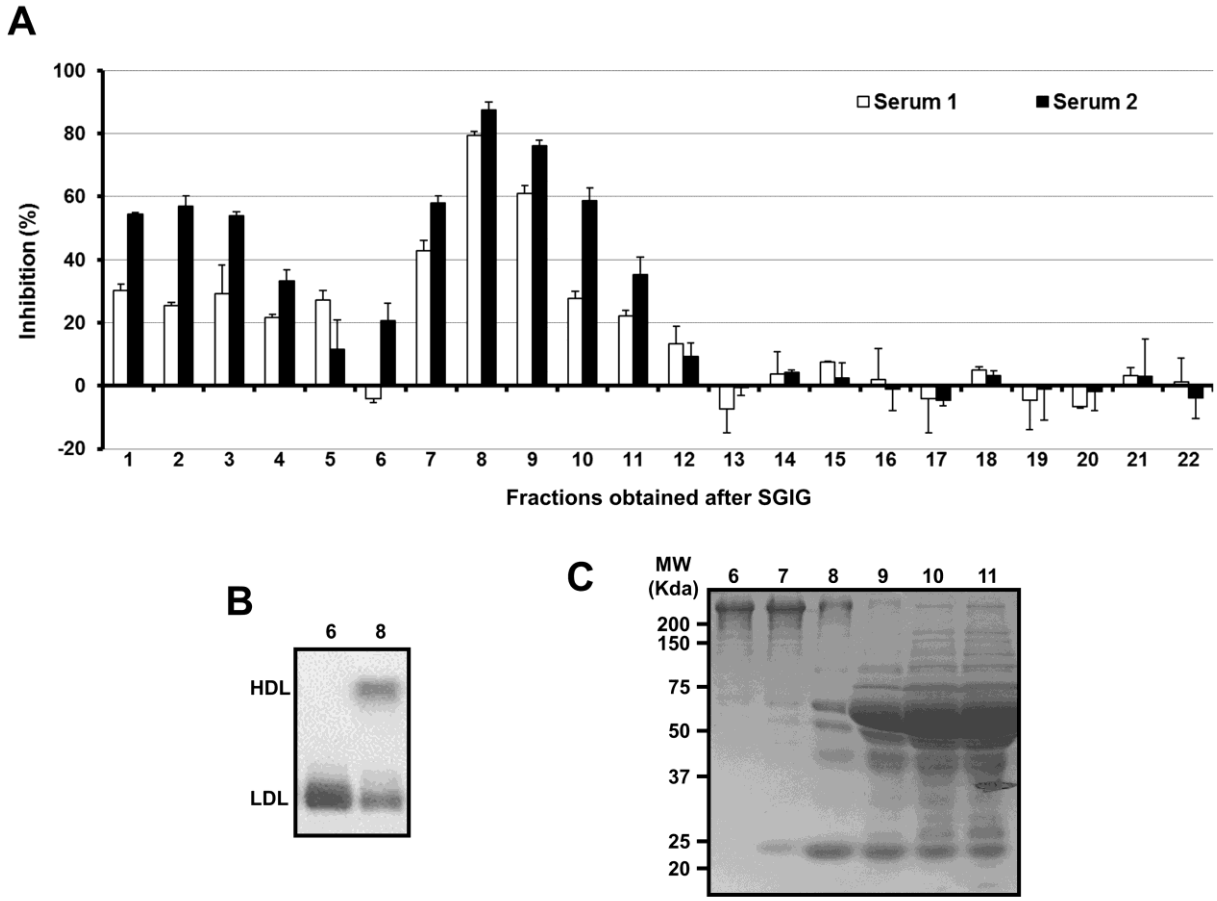

A
Serum 1
Serum 2
Inhibition (%)
100
80
60
40
20
0
-20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Fractions obtained after SGIG
B
6 8
HDL
LDL
C
MW (Kda)
6 7 8 9 10 11
200
150
75
50
37
25
20

Figure 3

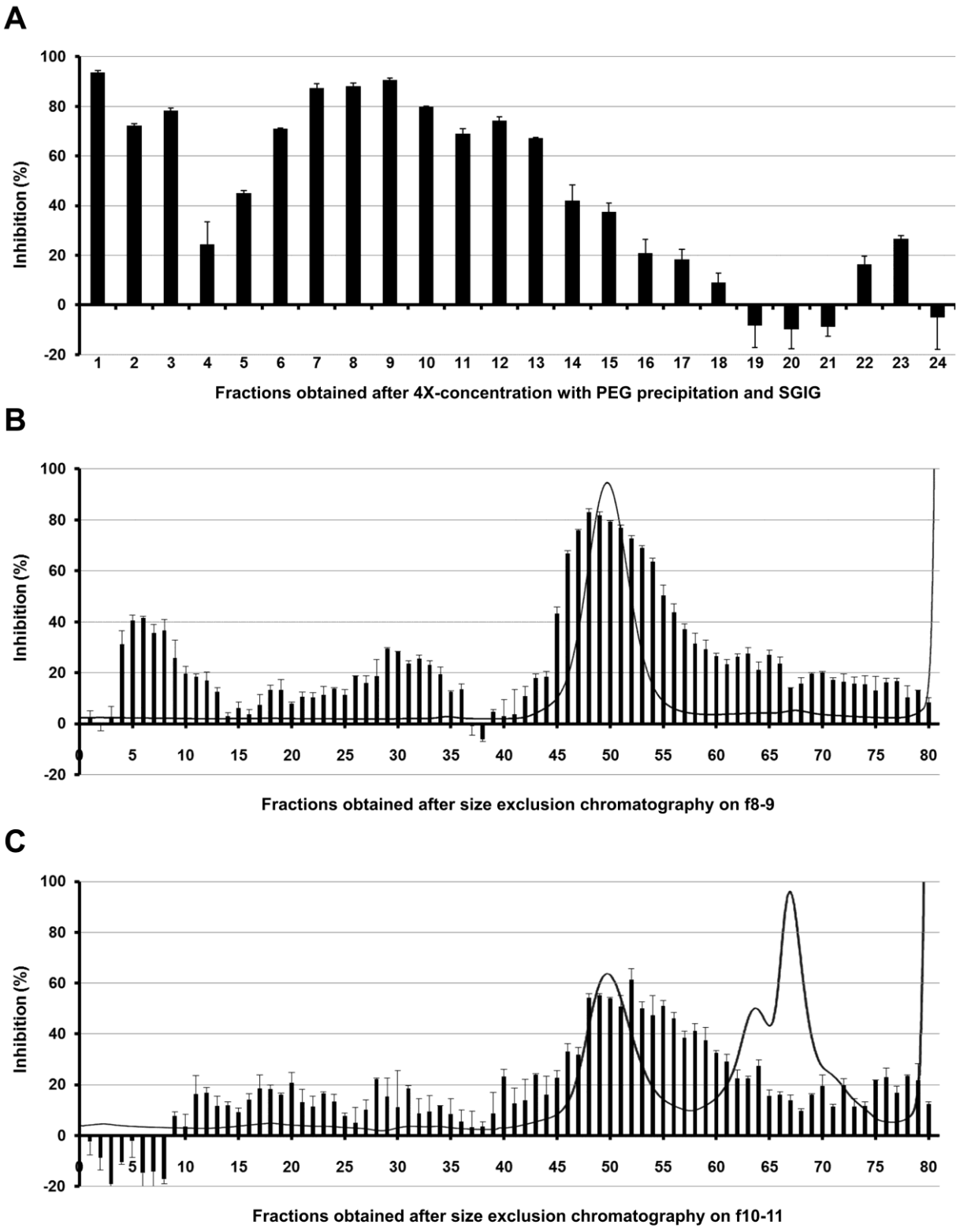

A
Inhibition (%)
100
80
60
40
20
0
-20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Fractions obtained after 4X-concentration with PEG precipitation and SGIG
B
Inhibition (%)
100
80
60
40
20
0
-20
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
Fractions obtained after size exclusion chromatography on f8-9
C
Inhibition (%)
100
80
60
40
20
0
-20
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
Fractions obtained after size exclusion chromatography on f10-11

Figure 4

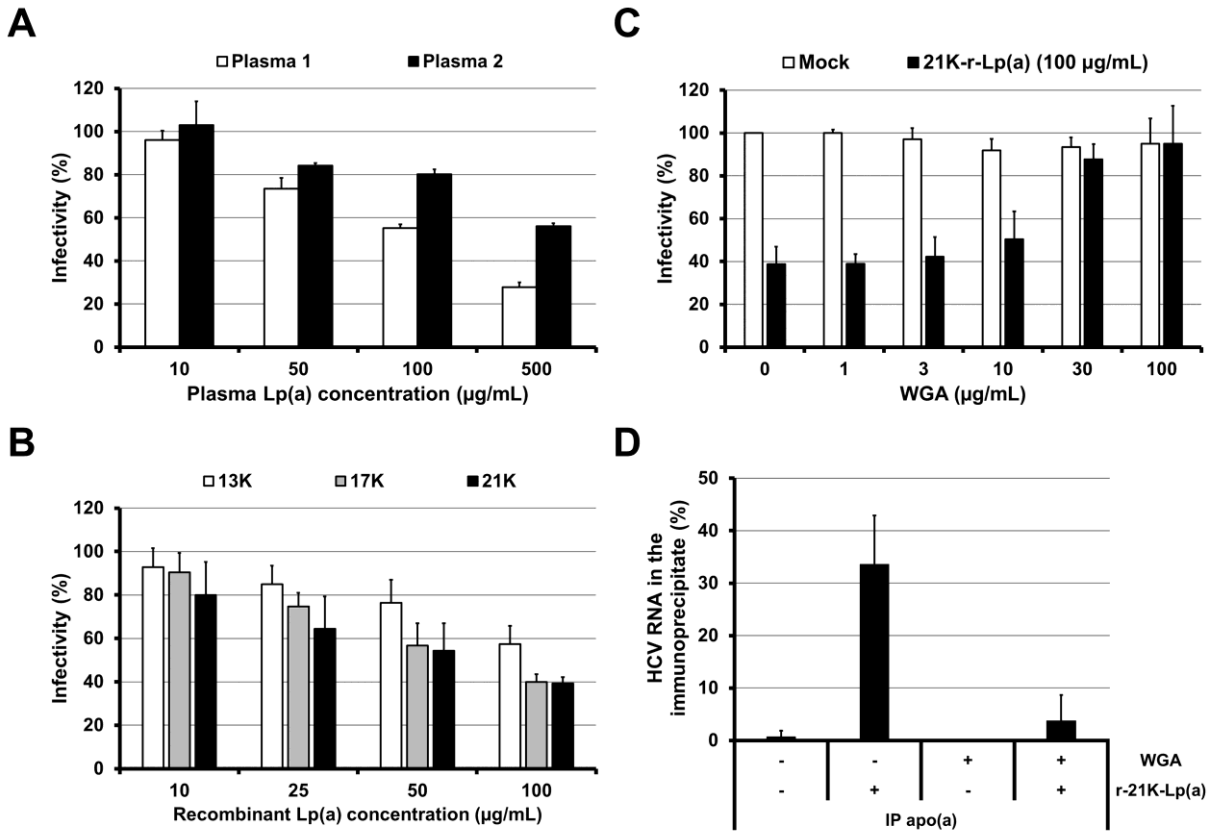

A
Plasma 1
Plasma 2
Infectivity (%)
120
100
80
60
40
20
0
10
50
100
500
Plasma Lp(a) concentration (µg/mL)
C
Mock
21K-r-Lp(a) (100 µg/mL)
Infectivity (%)
0
1
3
10
30
100
WGA (µg/mL)
B
13K
17K
21K
Infectivity (%)
10
25
50
100
Recombinant Lp(a) concentration (µg/mL)
D
HCV RNA in the immunoprecipitate (%)
50
40
30
20
10
0
WGA
r-21K-Lp(a)
IP apo(a)

Figure 5

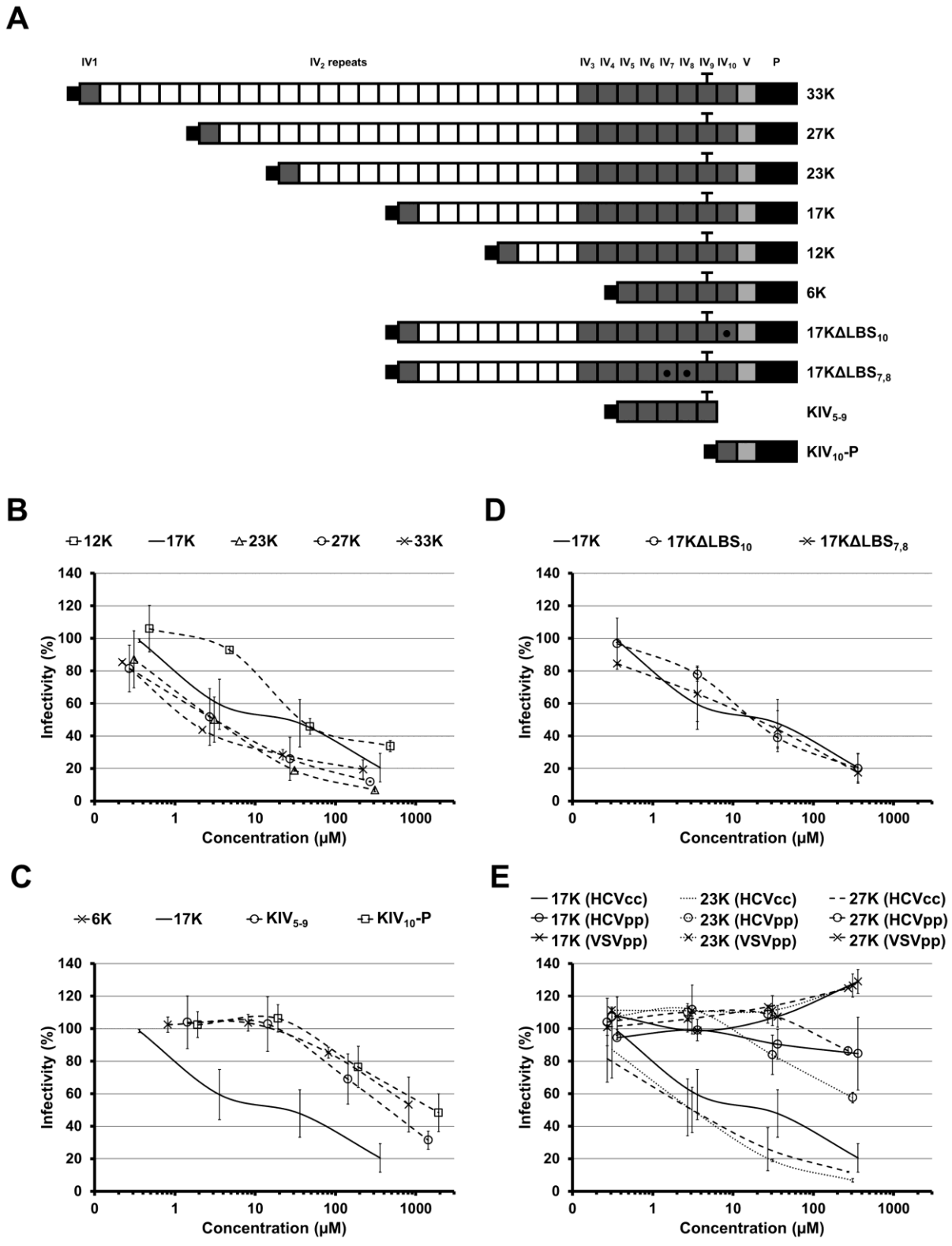

A
IV1
IV2 repeats
IV3 IV4 IV5 IV6 IV7 IV8 IV9 IV10 V P
33K
27K
23K
17K
12K
6K
17KΔLBS10
17KΔLBS7,8
KIV5-9
KIV10-P
B
12K
17K
23K
27K
33K
Infectivity (%)
Concentration (μM)
D
17K
17KΔLBS10
17KΔLBS7,8
Infectivity (%)
Concentration (μM)
C
6K
17K
KIV5-9
KIV10-P
Infectivity (%)
Concentration (μM)
E
17K (HCVcc)
23K (HCVcc)
27K (HCVcc)
17K (HCVpp)
23K (HCVpp)
27K (HCVpp)
17K (VSVpp)
23K (VSVpp)
27K (VSVpp)
Infectivity (%)
Concentration (μM)

Figure 6

DISCUSSION AND PERSPECTIVES

Scientific approach

HCV infection still represents a global health problem. The most recent estimates of infection demonstrated an increase in seroprevalence over the last 15 years (Messina et al, 2015). Despite the new available drugs for HCV treatment, they are still very expensive and sometimes present strong side effects, including skin disorders and anemia, requiring administration of erythropoietin. The HCC and cirrhosis are major complications of chronic hepatitis C. Moreover, HCC is the fifth most common cancer worldwide and its incidence has significantly increased in the last years (Mittal & El-Serag, 2013). The HCC associated to the chronic viral hepatitis is currently one of the first causes of hepatic transplant and the lack of donors is also a recurrent issue.

The research on HCV has been based on *in vitro* studies, for many years. However, infection of cultured cell lines with HCV clinical isolates has not been easy. The first reports of HCV replication in cell culture showed variable and very low levels of replication, thus requiring highly sensitive detection methods. Over the years, the cloning of HCV genomes became possible as well as the development of transfection-based assays that allow the production of large amounts of viral RNA. Thus, the first attempt to establish a complete HCV culture model emerged with the H77 strain, a fully functional HCV genome from a genotype 1a isolate. This strain was able to induce viremia in experimentally inoculated chimpanzees, but the viral replication, *in vitro*, was not detected (Blight et al, 2000; Kolykhalov et al, 1997; Yanagi et al, 1997). Simultaneously to this experiment, other strains were used to produce *in vitro* transcripts, such as the Con1 strain derived from an HCV genotype 1b isolate. Short time later, in 1999 the first HCV subgenomic replicon system emerged, after demonstration that HCV structural proteins were not essential for the viral RNA replication (Behrens et al, 1998; Khromykh & Westaway, 1997; Lohmann et al, 1999). This system was derived from the Con1 strain, containing a firefly luciferase reporter gene or a selection marker (neomycin phosphotransferase). Other replicon systems derived from the H77 (1a), HCV-N (genotype 1b), HCV-BK (genotype 1b) and J6 (genotype 2a) strains emerged. However, they require replication enhancing mutations to robustly propagate in cell culture. The first and, so far, only isolate providing high levels of replication in cell culture without requiring adaptive mutations was developed from the JFH1 isolate (Kato et al, 2001).

JFH1 efficiently replicate in HuH7-derived hepatocytes and this cell culture *model* allows the study of all stages of the HCV life cycle (Wakita et al, 2005). However, it presents some limitations, including a defective IFN system and, due to their tumoral origin, the cells present an abnormal proliferation, modifications in cell signaling pathways by genetic deregulation and a low polarization (Durantel & Zoulim, 2007). Moreover, another genotype 2a isolate derived from the serum of a patient with fulminant hepatitis (JFH2), did not have the same success as JFH1, contrary to what was expected. Its replication was inefficient and required replication enhancing mutations. Thus, despite the success of JFH1, it is still necessary to search for other and more efficient models.

Several teams tried to establish other fully HCV permissive cell culture systems but in all attempts complex cell culture adaptation schemes were required. The mutations allowed the adaptation of HCV full-length genomes in cell culture, leading to the development of robust and highly infectious *in vitro* systems. Some examples of these HCV genomes include the TN (genotype 1a), the first effective cell culture system for genotype 1(Li et al, 2012b); the J6 (genotype 2a) (Li et al, 2012a); the J8, DH8 and DH10 (genotype 2b) (Li et al, 2012a; Ramirez et al, 2014) and the DBN3a (genotype 3a) (Ramirez et al, 2016) strains. Infectious culture systems were reported for only a few other genotype 1 and 2 strains (Date et al, 2012; Lu et al, 2014; Pietschmann et al, 2009; Yi et al, 2006). More recently, two other HCV full-length genomes efficiently adapted to cell culture emerged, the HCV-1 and H77C, both derived from a genotype 1 strain. These, together with other infectious full-length HCV genomes, permit functional studies of the HCV life cycle, the viral proteins and their interactions with cellular components (Li et al, 2015). The creation of HCVcc mimicking all genotypes has also become possible through the construction of intergenotypic chimeric genomes, derived from the JFH1 isolate. Thus, these constructions were based on the combination of J6/JFH1 structural proteins with NS5A or NS3/4A sequences of different genotypes. Therefore, a panel of different HCV genomes, able to mimic the complete viral cycle in cell culture, has become available.

Recently, Saeed et al. identified SEC14L2 as a factor that facilitates the RNA replication of diverse HCV genotypes in several hepatoma cell lines (Saeed et al, 2015). SEC14L2 is naturally expressed in primary human hepatocytes, both from fetal and adult sources, with readily detectable levels. Moreover, it has been shown that the stable expression of SEC14L2 in Huh-7.5 is essential to obtain high levels of HCV RNA replication from several HCV genotypes. This system permits the infection from HCV isolates, derived

directly from patient serum, through enhancement of vitamin E-mediated protection against lipid peroxidation and without any adaptive mutation. Nevertheless, this system does not allow high levels of HCV replication. Therefore, the emergence of other *in vitro* systems for the culture of HCV clinical isolates would open new perspectives. This major progress would have impact for the study of the entire HCV life cycle as well as for clinical research, allowing the personalization of treatment. The improvement of the patient management/treatment would reduce the costs and the duration of treatment. In addition, the early detection of signaling pathways that are potentially deregulated during the HCV infection may allow the anticipation of these deregulations, maybe preventing complications as HCC. The major impact would be the development of an efficient vaccine, because it would be the best strategy to completely eradicate HCV infections.

Search for HCV inhibitor factors in human serum

The hindrance that hampers HCV infection from an HCV seropositive human serum, in cell culture could be due to the presence of restriction factors in patient serum. Our first results confirmed that the presence of such inhibitors is partly responsible for the absence of infection. Indeed, the use of increasing concentrations of infected patient serum results in a dose-dependent inhibition of HCVcc. Several studies have evidenced the presence of HCV specific nAbs in the serum of chronically infected patients (Bartosch et al, 2003a). However, the use of HCV negative sera also causes a dose-dependent inhibition of HCVcc, suggesting that in addition to nAbs, other serum factors are able to inhibit HCV. Therefore, the identification of these restriction factors could allow an effective infection from HCV clinical isolates.

PEG method is widely used to precipitate, concentrate and purify different viruses (Vajda, 1978). Thus, we used this method to study the relative size of HCV inhibitors, present in the serum. Similarly as the HCV particles, these inhibitors also precipitate, so being large particles. Several data suggest that in infected serum, HCV circulates in association with lipoproteins forming LVPs, which presents heterogeneous density (Felmlee et al, 2013; Popescu et al, 2014). In addition, these lipoproteins are essential for an efficient HCV infection. Interestingly lower density viral particles, presenting a density similar to that of VLDL and LDL, are the most infectious particles (Andre et al, 2002; Gastaminza et al, 2010; Nielsen et al, 2006; Thomssen et al, 1992; Thomssen et al, 1993). Thus, we decided to use SGIG to evaluate the HCV inhibitors density from the serum, separating them from the non-

inhibitor fractions. This method allowed to obtain two regions containing HCV inhibitor fractions at distinct density ranges. One of these regions corresponds to the VLDL density and showed weaker and variable inhibition levels than the other region. In addition, Tao *et al*. had already shown that serum VLDL is an HCV restriction factor (Tao et al, 2015). For all these reasons, we decided to proceed our study analyzing the fractions of the other density range. The other group of fractions presented higher inhibition values and the most inhibitory were found with a very close density to LDL (1.019 to 1.063 g/mL), being slightly denser. Interestingly, the lipoprotein(a) is found in this range of densities, varying between 1.05 and 1.12 g/mL (Reblin et al, 1992; Zhang et al, 2007). Thus, we decided to execute the SGIG together with the PEG method to concentrate the seric inhibitors. After a size exclusion chromatography performed with the most inhibitory fractions and a mass spectrometry analysis, we identified apo(a) as a potential inhibitor of HCV infection. Apo(a) being part of the Lp(a) composition may interfere in different steps of HCV life cycle, preventing the infection.

Apolipoprotein(a) inhibits HCV infection

Our results suggest that apo(a) inhibits HCV through an interaction with infectious particles. As previously mentioned, HCV particles are very different from the other viruses, strongly resembling the VLDL and LDL particles, being the levels of TG-rich lipoproteins correlated with those of HCV RNA in infected patients sera (Felmlee et al, 2010). The lipid composition of LVP is very important to maintain their morphology, density and consequently their infectivity. Thus, in the serum, the LVPs are composed by apoA, apoB, apoC (I, II and III), and apoE, which are able to stabilize the lipoproteins (Douam et al, 2015).

Some differences have been observed between hepatic-cell-line- and patient-sera-derived HCV particles, for example concerning their association with the apolipoproteins. Studies have shown that the density of extracellular infectious HCV particles is much lower than that of intracellular infectious particles, suggesting that the interaction with lipoproteins/apolipoproteins is preferentially made in the extracellular space (Gastaminza et al, 2006).

In addition to their association with HCV, the apolipoproteins also play an important role as modulators HCV infection (Bartenschlager et al, 2011; Douam et al, 2015; Lindenbach, 2013). Many reports have demonstrated that HCV-associated apoE is an

essential mediator of the entry step. It interacts with HCV receptors such as HSPGs, LDL-r and SR-BI, so being important for the binding of viral particles to the cell surface. Moreover, one study showed the importance of apoE for the viral cell-to-cell transmission (Hueging et al, 2014). In addition, apoC can also enhance HCV infectivity, by promoting membrane fusion (apoC-I) and by reversing lipoprotein lipase-mediated inhibition (apoC-III) (Dreux et al, 2007; Meunier et al, 2005; Sun et al, 2013). ApoH, an apolipoprotein that does not belong to the LVPs composition, also presents an effect on HCV infection, but contrary to the others, apoH promotes the inhibition of HCV infection, potentially through the interaction with the viral particles (Stefas et al, 2015; Sultanik et al, 2015).

The apolipoproteins have also an important role in the assembly and production of HCV infectious particles. Once again, studies demonstrated the relevant role of apoE in this step of the viral cycle. Recent data demonstrated that a direct interaction between apoE and the E2 transmembrane domain, as well as with NS5A, is important for the maturation of HCV infectious particles (Lee et al, 2014). On the other hand, the role of apoB in this life cycle step is still controversial. Other apolipoproteins also seem to participate in the formation of HCV particles, like apoA and apoC (Dreux et al, 2007; Meunier et al, 2005; Sun et al, 2013). The apolipoproteins contain tandem repeats of amphipathic α-helices that interact with the lipoproteins' membrane, and apoA1, apoC1, as well as apoE have several of these repeats, similar between them. Recently, it was identified an important role of these amphipathic α-helices in the maturation of infectious HCV particles (Fukuhara et al, 2014). ApoJ is a glucose-upregulated molecular chaperone which is not present in LVPs. It promotes the infectious virion production through the stabilization of the Core and NS5A proteins (Lin et al, 2014). Thus, apolipoproteins play a crucial role in HCV life cycle but further studies are necessary to elucidate their precise roles.

Implication of apolipoproteins in the HCV inhibition by apo(a)

In human serum, apo(a) interacts non-covalently with high specificity to the apoB-100 component of a LDL particle, creating Lp(a). This interaction involves two weak LBS of apo(a) KIV7 and KIV8, establishing several contact points between both proteins before the formation of a disulfide bond leading to the formation of Lp(a) (Becker et al, 2004b; Weisel et al, 2001). Our data suggest that not only apo(a) interacts with HCV infectious particles but also Lp(a). However, in the Lp(a) particle, the binding of apo(a) with apoB-100 is already established, suggesting that the mechanism of HCV inhibition does not involve the LVP-

associated apoB-100. Accordingly with this supposition, our results showed that mutation of LBS_7 and LBS_8 did not affect the HCVcc inhibition by apo(a). Interestingly, in contrast to HCVcc, we observed that HCVpp (poorly associated to lipoproteins) are only slightly sensitive to apo(a) suggesting that the lipoprotein moiety of HCV LVPs is essential for the inhibition. Thus, the interaction of apo(a) with HCV does not seem to involve apoB-100 but probably another component of the lipoprotein moiety of HCV lipoviroparticles.

It is also well established that a great amount of apoE is associated to HCVcc and that the level of apoE correlates very well with infectivity (Chang et al, 2007). It was also recently shown that apoE and HCV glycoproteins form a protein complex associated with HCVcc and it has been proposed that a small amount of apoE is associated with E1E2 on HCVpp (Boyer et al, 2014). Thus, it would be interesting to test if apoE is a requisite for apo(a) inhibition which could explain the relative resistance of HCVpp to inhibition, as compared to HCVcc. However, the involvement of other LVP components in the viral inhibition cannot be excluded.

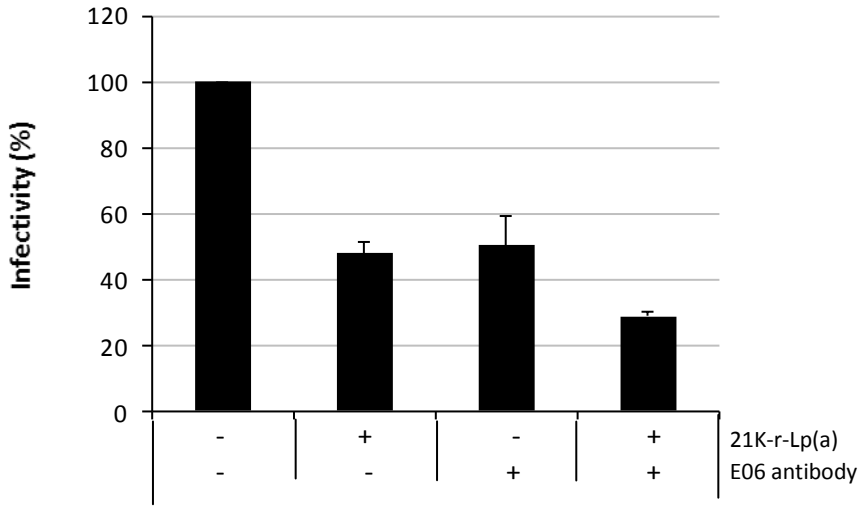
Influence of LPA size polymorphism

Due to its gene size polymorphism, apo(a) may present a large copy number variation of KIV2 domains. Interestingly, our results showed a relation between the apo(a) isoform size and the HCV inhibition strength, so suggesting that the larger the apo(a), the stronger the inhibition is. A substantial number of KIV2 repeats may thus be required for maximal inhibition. Commonly, free apo(a) exists in a "closed" conformation that is stabilized by intramolecular interactions, being the KIV10 an important site involved in the maintenance of apo(a)'s closed conformation. Studies have demonstrated that the conformational status of apo(a) is determinant for the efficiency of covalent Lp(a) particle formation (Becker et al, 2003). Based on these studies, the apo(a) conformation may play a role in the HCV inhibition, by increasing the steric hindrance. Moreover, we tested three apo(a) construction without any repetition of KIV2 and we continued to observe inhibition of HCV infection, even though in a less intense way. Thus, the direct interaction between KIV2 domains and HCV infectious particles does not seem to be the key for the inhibition mechanism.

Influence of oxidized phospholipids

OxLDL are effective inhibitors of HCV-cell entry with pangenotype activity. It was also suggested that OxLDL act through an SR-BI-dependent manner (Westhaus et al, 2013). Interestingly, Lp(a) is known to be the preferential carrier of PC-containing OxPL in human plasma (Bergmark et al, 2008). These OxPL are also detectable in the lipid moiety of Lp(a) and covalently bound to apo(a) (Leibundgut et al, 2013). In addition, the Asp57 -> Ala57 substitution in KIV10 LBS strongly affects the capture of OxPL. Considering all these studies, we wondered whether the OxPL could be implicated in the inhibitory effect of Lp(a)/apo(a) on HCV. To this end, we analyzed the effect of KIV10 LBS mutation on the inhibitory activity. However, in our study we showed that this mutation did not affect the inhibitory activity of apo(a), thus concluding that oxidized phospholipids do not seem to play a role in the apo(a) mediated HCV inhibition.

The E06 monoclonal antibody is able to specifically bind to the PC moiety of OxPL, but not to the PC moiety of non-oxidized phospholipids (Handa et al, 2015). Thus, we also sought to determine whether E06 mAb could decrease the apo(a) mediated inhibition. HCVccs were put in contact with HuH-7-RFP-NLS-IPS target cells at 37°C in the presence or absence of 10µg/mL of E06 antibody and 100 µg/mL of recombinant Lp(a) (as referred in the diagram below - [Graphic 1]).



Graphic 1: Effect of E06 on 21K-r-Lp(a) mediated inhibition.

RFP-NLS-IPS cells were inoculated during three hours with HCVccs, in the presence or absence of 100 µg/mL of recombinant Lp(a) (21K-r-Lp(a)) and in the presence or absence of 10µg/mL of E06 antibody. Luciferase assays were performed on infected cells at 48 h post-infection. Results are expressed as percentages of infectivity compared to infection in absence of sample and are reported as the means ± S.D. of two independent experiments.

After 3 h of contact, the medium was changed and cells were further incubated for 48 h with complete medium before luciferase activities were measured as indicated by the manufacturer (Promega, Madison, WI).

Surprisingly, our results showed that the single presence of E06 in the assay system inhibit 50% of viral infection. Moreover, in the additional presence of 21K-r-Lp(a), E06 was not able to restore the HCV infection. With these results we cannot conclude about the implication of OxPL in the HCV inhibition, being necessary to do further assays. However, the OxPL associated to apo(a) do not seem to be involved in the viral inhibition caused by this protein.

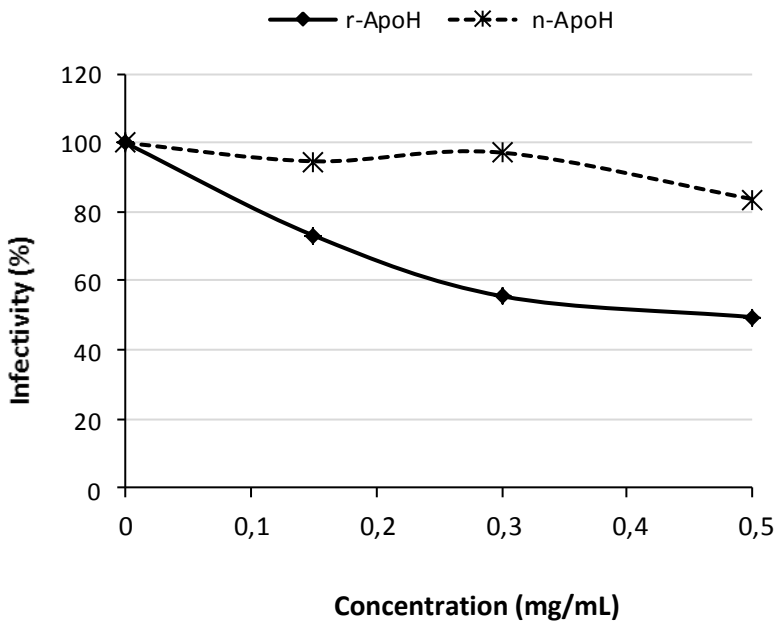
Understanding the apo(a) inhibition mechanism

Until now, Lp(a) functions are not well known. Many studies have emerged proposing some associations and functions for this lipoprotein, but the *in vivo* relevance and the functional implications of these interactions remain unclear. Therefore, it is difficult to decipher the exact mechanism by which apo(a) inhibits HCV infectious particles. SR-BI was recently identified as a receptor involved in the Lp(a) catabolism, even though it is not the main Lp(a) clearance pathway (Yang et al, 2013). Particularly, SR-BI favors the selective lipid uptake of CEs from Lp(a). Thus, SR-BI may have a new physiologic role as receptor for Lp(a). In addition, it is well established that SR-BI is essential for the HCV entry in the hepatic cells. It is one of the most important HCV receptors, on which the virus firstly attaches before establishing a specific interaction. Consequently, the inhibition of HCV infection by apo(a) could be associated with the obstruction of SR-BI, preventing the virus interaction with it.

Coimmunoprecipitation assays showed an association between β-2 glycoprotein I (also named apoH) and the apo(a) kringle IV-domain, in human plasma (Kochl et al, 1997). However, the functional implication of this interaction, *in vivo*, remains unclear. In addition, as referred above, apoH prevents the HCV infection, potentially through the interaction with the viral particles (Stefas et al, 2015; Sultanik et al, 2015). All these features could suggest a potential effect on HCV infectivity mediated by the interaction between apo(a) and apoH.

In order to better understand the potential effect of apoH on the HCV infectivity and potentially its relation with apo(a) inhibitor effect, we used two different forms of apoH, one recombinant protein (r) and a natural protein (n), purified from human plasma. Dose-response assays were performed with both proteins in a concentration range previously used by other

authors when tested the interaction between apoH and the virus [Graphic 2]. Cell viability was monitored simultaneously. We did not observe any toxicity at the tested concentrations. Surprisingly, we observed a relevant HCV inhibition by r-ApoH, contrary to n-ApoH. For this reason, we decided to clarify these results before performing an assay using apoH in the presence of 21K-r-Lp(a), to evaluate if apoH is involved in apo(a) mediated inhibition.



Graphic 2: Dose-response curves of the apoH on the HCV infection.

Inhibition assays were performed by inoculating HuH-7-RFP-NLS-IPS cells, for three hours, with HCVcc, and various concentrations (0.15, 0.30 and 0.5 mg/mL) of r-ApoH or n-ApoH variants. Luciferase assays were performed on infected cells at 48 h post-infection. Results are expressed as percentages of infectivity compared to infection in absence of sample and are reported as the means ± S.D. of at least two independent experiments.

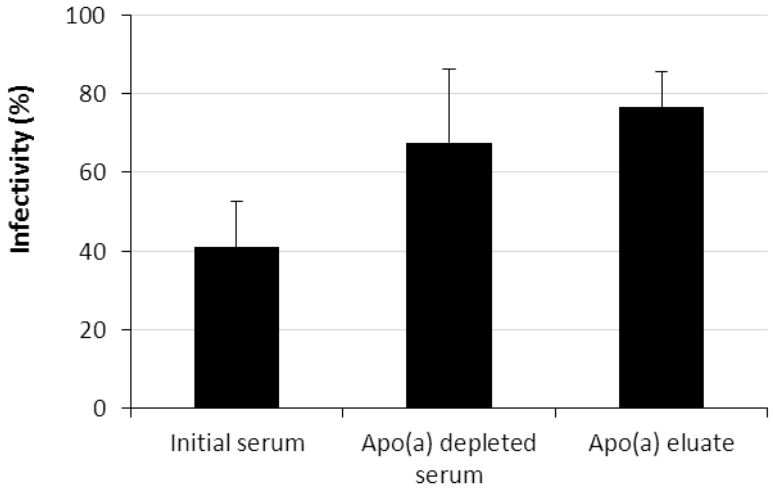
The results presented above showed that the inhibitory effect of apo(a) occurs during the entry step of the viral cycle. To better understand the mechanism of apo(a) inhibition and identify the precise step inhibited, it would be interesting to perform kinetics of HCV entry. To discriminate between virus binding and post-binding events, HuH-7 cells could be inoculated with HCVcc in the presence or absence of specific antibodies of HCV receptors such as SR-BI, CD81 and CLDN1. This could enable to determinate whether apo(a) inhibit early, intermediate or late step of HCV entry (Fofana et al, 2013; Krieger et al, 2010).

Removal of the Lp(a)/apo(a) from the human serum

Lectins are carbohydrate binding proteins that are naturally found in several organisms, such as plants, algae, fungi, invertebrates and vertebrates among others (Vo & Kim, 2010). There is a large diversity of these lectins but they present the common feature to interact specifically and non-covalently with sugars, without changing their composition or structure (Sharon, 2007). WGA is a dimeric carbohydrate-free protein of 36 kDA, which specifically interacts with N-acetyl-D-glucosamine (GlcNAc) (Lienemann et al, 2009). Our results showed that the presence of WGA with HCVcc and apo(a)/Lp(a) allowed to reverse the infection inhibition caused by apo(a)/Lp(a). These results are explained by apo(a) characteristics. It is one of the most heavily glycosylated proteins in plasma, containing numerous O-glycosidic oligosaccharides rich in N-acetyl-D-neuraminic acid (NANA) and G1cNAc, that are targeted by WGA (Seman et al, 1994).

In the line of these results, we wondered whether this lectin could enable to eliminate the apo(a) present in human serum and permit to cultivate the virus from primary isolates, in a highly sustainable culture system. Therefore, we used a WGA agarose conjugate (purchased from Sigma-Aldrich) to capture the apo(a) present in the HCV-negative sera and eliminate the apo(a) from these sera. After that, we tested both of solutions, the initial and the depleted serum. We were able to partially restore the HCVcc infection after apo(a) depletion [Graphic 3]. We also tried to recover the attached apo(a) to test the inhibitory effect in cell culture but elution solution used to recover the apo(a) was toxic.

This method would allow to eliminate apo(a) from HCV-infected sera and maybe to produce an efficient infection from HCV clinical isolates. However, this procedure also presents difficulties because apo(a) is thought to be associated to HCV infectious particles. In addition, other inhibitory proteins are present in the human serum.



Graphic 3: Effect of human negative serum, with or without natural apo(a), in HCV infection

HuH-7-RFP-NLS-IPS cells were inoculated with HCVcc for three hours in the presence of 50% of human serum derived from HCV seronegative individual, 50% of the same human serum but apo(a) depleted or 50% of apo(a) eluate. Luciferase assays were performed on infected cells at 48 h post-infection. Results are expressed as percentages of infectivity compared to infection in absence of all referred solutions and are reported as the means ± S.D. of three independent experiments.

Perspectives and Conclusions

To carry on our project, it would be interesting to better understand the relation between HCV infected individuals and their apo(a) polymorphism. For this, we could try to know if large forms of this protein are more frequently present in HCV infected individual than the standard frequency in the non-infected population. However, this kind of studies is very difficult to perform. It is well known that Lp(a) levels are determined by the LPA size, but due to the variability in the KIV2 number repeats the LPA is highly polymorphic. Thus, there are large interindividual differences in Lp(a) levels associated with any given apo(a) allele size. Consequently, homozygotes for apo(a) size are rare, being reported an heterozygosity index of approximately 94% among Caucasians. In addition, a small apo(a) allele size in a heterozygous individual does not always correspond to the dominant apo(a) protein isoform. Thus, for one genotype, the size and expression of one apo(a) allele influences Lp(a) levels attributable to the other apo(a) allele (Enkhmaa et al, 2011).

The high risk of cardiovascular disease is associated to the small forms of apo(a) that are more concentrated in the blood than the large forms. There are some studies that have

associated HCV to the high risk of cardiovascular disease, but this feature remains unclear (Butt et al, 2009; Roed et al, 2012; Wang & Kao, 2010). Thus, population studies could help to elucidate the phenomenon of HCV inhibition promoted by apo(a).

In conclusion, the presence of many restriction factors of HCV in the human serum is well known. Some examples are IFNs, proteins of complement system, serum amyloid A, mannose binding lectin, defensin, lipoprotein lipase, Hepatic lipase, Heparin, VLDLs or oxidized LDLs (Andreo et al, 2007; Ball et al, 2014; Lavie et al, 2006b; Tao et al, 2015; Tarr et al, 2012; von Hahn et al, 2006; Westhaus et al, 2013). Our study have, therefore, contributed to identify apo(a) as another relevant HCV inhibitor, present in the human serum. Apo(a) is an additional component of the lipid metabolism modulating HCV infection through interaction with LVP. Our results bring new insight into the tight link between HCV and lipoproteins. Our study could also open new perspectives for the study of apo(a) function.

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APPENDIX

A. RÉSUMÉ LONG

L'infection par le virus de l'hépatite C (VHC) est un problème majeur de santé publique qui touche plus de 185 millions de personnes dans le monde (Messina et al, 2015). L'infection par ce virus est généralement chronique (75 à 80 % des cas) et les aspects cliniques peuvent se caractériser par un portage asymptomatique, une hépatite chronique active et une cirrhose qui est fortement associée au développement d'un carcinome hépatocellulaire [Figure 1] (Chen & Morgan, 2006). En général, l'infection par le VHC évolue vers des maladies hépatiques symptomatiques après 20-30 ans. On compte près de 4 millions de nouvelles infections par an et plus de 350 000 personnes meurent de maladies liées au VHC chaque année (Perz et al, 2006). Avec le virus de l'hépatite B, le VHC représente environ 75% de tous les cas de maladie du foie dans le monde et le cancer du foie majoritairement induit par ces infections virales représente le troisième cancer le plus fréquent. La transmission du VHC s'effectue essentiellement par voie parentérale. Avant 1991, la majorité des contaminations était due à la transfusion sanguine. Toutefois, cette voie de contamination a été largement réduite par la mise en place des dépistages sérologiques systématiques chez les donneurs de sang en 1992 puis des tests de dépistage du génome viral en 2001. Aujourd'hui, dans les pays développés, le risque transfusionnel est minime et la toxicomanie par voie intraveineuse représente la principale source de contamination (Alter et al, 1990).

Alors qu'aucun vaccin n'est disponible, le traitement des patients infectés par le VHC a considérablement évolué au cours des 20 dernières années. Initialement basé sur l'utilisation d'interféron-α, il a ensuite évolué vers une bithérapie incluant la ribavirine. Au début des années 2000, la pégylation de l'interféron (ajout de polyéthylèneglycol) a permis d'améliorer les paramètres pharmacocinétiques de cette molécule et d'augmenter encore l'efficacité du traitement (Lindsay et al, 2001). La bithérapie interféron-α pégylé / ribavirine a ainsi été le traitement standard de l'hépatite C chronique pendant une dizaine d'années (Fried et al, 2002; Zeuzem et al, 2000). Le développement récent de plusieurs antiviraux à action directe ciblant les protéines virales non-structurales a finalement conduit à des progrès sérieux dans le traitement des patients. En 2011, l'ajout d'un inhibiteur de la protéase NS3 du VHC (telaprevir ou boceprevir) a tout d'abord permis d'améliorer l'efficacité du traitement pour les

patients infectés par le VHC de génotype 1 (Jacobson et al, 2011; Poordad et al, 2011). Plus récemment, d'autres inhibiteurs spécifiques (nouveaux inhibiteurs de protéases, inhibiteurs de la protéine NS5A et inhibiteurs de la polymérase NS5B) ont montré des résultats cliniques encore plus spectaculaires. Avec les nouvelles combinaisons de drogues sans interféron les nouveaux traitements montrent désormais des efficacités supérieures à 95%. Ces traitements restent cependant extrêmement coûteux et le risque de sélection de souches résistantes doit être pris en compte.

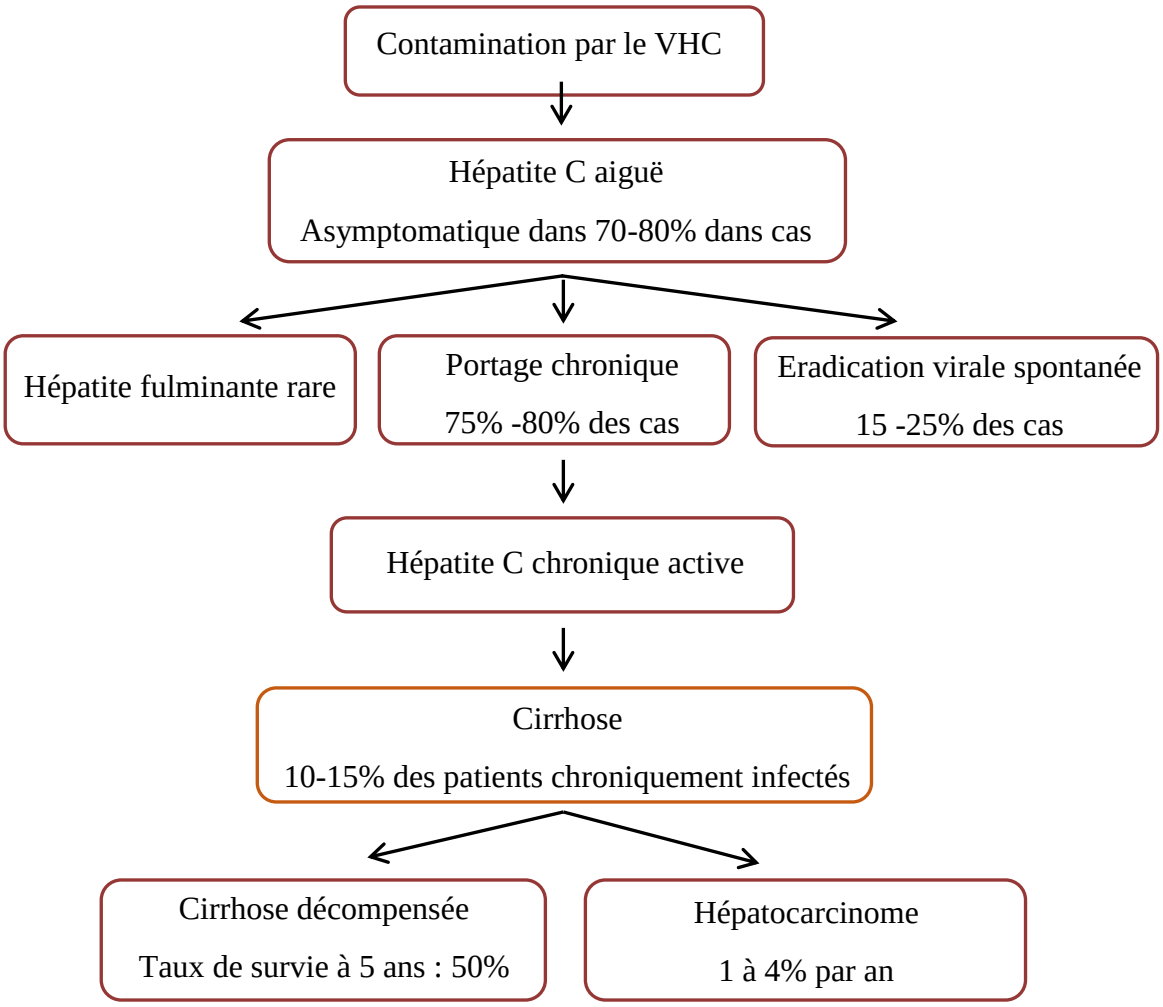

Figure 1 : Histoire naturelle de l'infection par le VHC

L'infection par le VHC débute par une infection aiguë, qui est le plus souvent asymptomatique. Dans la plupart des cas, l'infection évolue vers la chronicité et ainsi peut se compliquer par le développement d'une cirrhose, qui à plus long terme pourra aboutir à une cirrhose décompensée ou à un hépatocarcinome. Adapté de (Chen & Morgan, 2006)

La recherche sur le VHC a longtemps été freinée par le manque de modèle cellulaire permettant l'amplification de ce virus. Différents modèles de substitution ont été développés. Parmi eux, les systèmes de réplicons sous-génomiques et de particules rétrovirales pseudotypées avec les protéines d'enveloppe E1E2 (VHCpp) se sont avérés très utiles pour étudier les étapes de réplication du génome et de l'entrée virale, respectivement. Par ailleurs, en 2005, une avancée majeure a été achevée avec la description d'une souche de génotype 2a isolée à partir d'un patient japonais atteint d'une hépatite fulminante, la souche JFH1 (Wakita et al, 2005). En effet, ce génome se réplique efficacement dans les cellules d'hépatocarcinome HuH-7 et permet de produire des virions (VHCcc pour VHC produit en culture cellulaire) qui sont capables d'infecter des cellules naïves, des chimpanzés ou encore des souris transplantées avec des hépatocytes primaires humains. Pour la première fois, un modèle permettant d'étudier le cycle viral complet du VHC venait d'être développé. Différentes chimères intra- et inter-génotypiques ont aussi été construites à partir de cette souche JFH1, pour permettre de caractériser partiellement les différences entre les différents génotypes et sous-types (Gottwein et al, 2009; Li et al, 2011; Pietschmann et al, 2006; Scheel et al, 2008). Ces différentes constructions sont aujourd'hui couramment utilisées pour l'étude du VHC. Toutefois, plusieurs différences entre le virus produit en culture cellulaire et celui présent dans le plasma des patients ont été observées. Malheureusement, l'amplification de VHC à partir du serum de patients reste encore aujourd'hui très inefficace.

Le VHC est un petit virus enveloppé de 50 à 80 nm de diamètre et à ARN simple brin de polarité positive (Catanese et al, 2013b). Il appartient au genre Hepacivirus dans la famille des *Flaviviridae* (www.ictvonline.org). Le VHC est classé en 7 génotypes (Scheel et al, 2015). Le génome du VHC est un ARN simple brin de polarité positive d'environ 9,6 kb. Il est composé de régions 5' et 3' non-codantes et d'un cadre de lecture ouvert qui code une polyprotéine précurseur d'environ 3000 acides aminés (Choo et al, 1991). Cette polyprotéine est ensuite clivée co- et post-traductionnellement, en une dizaine de protéines virales dont 3 protéines structurales (la protéine de capside ou protéine Core et les protéines d'enveloppe E1 et E2) et 7 protéines non-structurales (p7, NS2, NS3, NS4A, NS4B, NS5A et NS5B) (Moradpour & Penin, 2013). Les protéines non-structurales sont essentielles à la réplication virale mais participent aussi à l'assemblage des nouvelles particules infectieuses. Les protéines structurales du VHC forment les virions. Les protéines d'enveloppe E1 et E2 sont des composants essentiels de l'enveloppe virale. Ces protéines s'assemblent sous forme d'hétérodimères formant de gros complexes covalents stabilisés par des ponts disulfures,

exposés à la surface des virions. Ces protéines jouent un rôle majeur pour l'entrée du VHC dans les hépatocytes (Cocquerel et al, 2000; Op De Beeck et al, 2004 ; Vieyres et al, 2010).

Le cycle viral du VHC commence par l'entrée du virus dans les hépatocytes. Celle-ci se fait par endocytose dépendante de la clathrine et fusion au niveau des endosomes précoces. Après décapsidation, la particule virale libère son ARN génomique dans le cytoplasme. Ce dernier est alors traduit grâce à un site d'entrée ribosomal interne localisé dans la région 5' non-codante du génome. Les protéines non-structurales synthétisées forment alors des complexes de réplication qui permettent la synthèse de nouveaux ARN viraux, en particulier grâce à l'activité ARN-polymérase ARN-dépendante de la protéine NS5B. Les ARN simple brin de polarité positive nouvellement synthétisés peuvent ensuite être encapsidés par la protéine de capside. Les particules virales s'assemblent par recrutement des protéines d'enveloppe E1 et E2 et par bourgeonnement dans la lumière du réticulum endoplasmique, dans un environnement proche des gouttelettes lipidiques. Finalement, les particules virales matures sortent de la cellule infectée après avoir suivi la voie de sécrétion cellulaire [Figure 2] (Dubuisson & Cosset, 2014; Herker & Ott, 2011; Vieyres et al, 2014).

L'étude du cycle viral du VHC a montré que celui-ci était étroitement lié au métabolisme des lipides des cellules hépatiques. Les virions du VHC révèlent une grande hétérogénéité en termes de densité (comprise entre 1,03 et 1,20 g/cm^3) et de composition. Ainsi, les particules virales de faible densité présentent une densité similaire à celle des lipoprotéines de très basse densité (VLDL pour Very Low Density Lipoproteins). Par ailleurs, il est à noter que l'infectiosité des particules virales ayant une faible densité est plus élevée que celle de forte densité (Andre et al, 2002; Gastaminza et al, 2010; Nielsen et al, 2006; Thomssen et al, 1992; Thomssen et al, 1993). Ces différences s'expliquent par l'association des particules virales aux lipoprotéines, donnant naissance à des lipoviroparticules. Ainsi, les apolipoprotéines ApoA-I, ApoB, ApoC-I, C-II, C-III ou encore ApoE, des constituants majeurs des lipoprotéines, sont aussi associées aux particules virales (Douam et al, 2015). Cette interaction des particules virales aux lipoprotéines joue un rôle majeur dans les étapes d'entrée virale mais permet également au virus d'échapper au système immunitaire, en particulier en protégeant contre l'inhibition induite par les anticorps neutralisants. L'association des particules infectieuses du VHC aux lipoprotéines s'explique par le fait que ce virus utilise la machinerie de synthèse des VLDL pour son assemblage et sa sécrétion. En effet, il a été montré que la production du VHC par les hépatocytes peut être réduite grâce à l'utilisation de molécules bloquant l'assemblage des VLDL tels qu'un inhibiteur de la MTP

ou encore des siRNA dirigés contre l'apoB. Cette interconnexion entre la morphogenèse du VHC et la synthèse des VLDL conduit à la production de virions matures enrichis en cholestérol et sphingolipides. De plus, il a été constaté que le cholestérol et les sphingolipides associés aux virions sont importants pour la maturation et l'infectiosité de ces derniers (Dreux et al, 2007; Haid et al, 2009). Etant donné leur hétérogénéité, les particules du VHC ne sont pas parfaitement caractérisées. Il est cependant clairement établi que l'apoE est un élément essentiel présent à la surface des virions. L'analyse des apolipoprotéines associées aux virions produits en culture cellulaire a montré que chacune des particules contient près de 300 molécules d'apoE à sa surface, ce qui suggère un enrichissement remarquable.

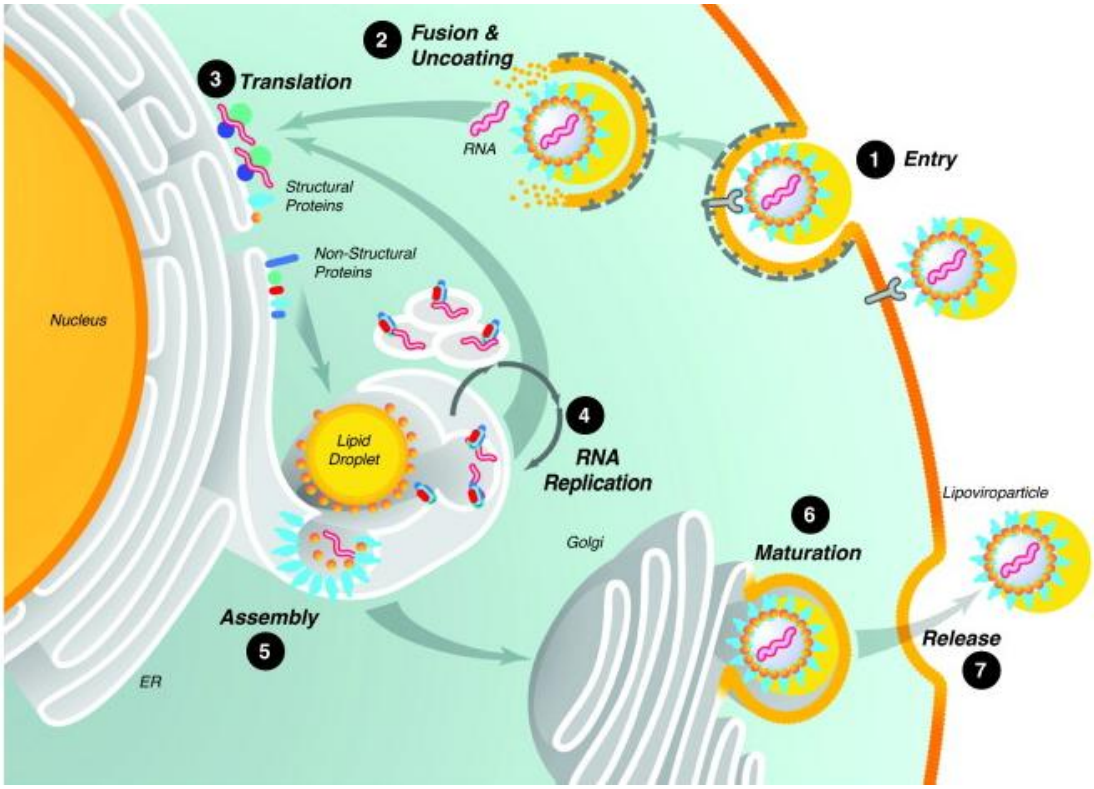

Figure 2: Cycle viral du VHC

(1) Les lipoviroparticules (LVPs) entrent dans la cellule cible en utilisant plusieurs récepteurs spécifiques et une endocytose dépendante de la clathrine. (2 et 3) L'ARN viral est libéré dans le cytoplasme et est traduit en un précurseur polyprotéique au niveau du réticulum endoplasmique (RE). (4) La réplication de l'ARN du VHC est effectuée par l'ARN polymérase ARN dépendante virale, la protéine NS5B. (5) Les ARN viraux néo-synthétisés sont encapsidés dans des capsides virales à proximité des gouttelettes lipidiques et l'enveloppe

virale dans laquelle sont ancrées les glycoprotéines E1 et E2 est acquise par bourgeonnement dans le lumen du RE. (6) La maturation des LVPs se produit au cours du passage dans la voie de sécrétion. (7) Les virions néoformés sont finalement sécrétés hors de la cellule. D'après (Herker & Ott, 2011)

L'entrée du VHC dans les hépatocytes est un évènement multi-étapes complexe impliquant un certain nombre de facteurs cellulaires [Figure 3]. Elle est initiée par la fixation des particules virales à la surface des cellules hôtes. Les protéoglycanes à héparane sulfate (HSPG) et le récepteur des lipoprotéines de faible densité (LDL-r) ont été proposés comme les principaux facteurs d'attachement responsables de l'interaction initiale entre le virion et la surface des hépatocytes. Une série de facteurs cellulaires spécifiques entrent en jeu par la suite : la tétraspanine CD81, le récepteur Scavenger de classe B type 1 (SRB1) et les protéines de jonctions serrées, CLDN1 et OCLN (Dubuisson & Cosset, 2014; Vieyres et al, 2014). La fixation du VHC sur la protéine SRB1 semble importante pour démasquer le site d'interaction à CD81 sur la glycoprotéine d'enveloppe E2 (Dao Thi et al, 2012; Zahid et al, 2013). Une interaction entre CD81 et CLDN1 est également essentielle pour assurer l'entrée du virus (Dubuisson & Cosset, 2014; Harris et al, 2010; Harris et al, 2008). La protéine OCLN semble quant à elle intervenir plus tardivement dans l'entrée virale et son rôle exact reste à préciser (Ploss et al, 2009). Finalement, les particules du VHC entrent dans les hépatocytes par endocytose dépendante de la clathrine (Blanchard et al, 2006; Harris et al, 2008; Krieger et al, 2010; Meertens et al, 2006). Le pH acide des endosomes précoces induit la fusion de l'enveloppe virale avec la membrane de ce compartiment, conduisant à la décapsidation de la particule virale et au transfert de l'ARN viral dans le cytoplasme (Haid et al, 2009; Lavillette et al, 2006). En plus de CD81, SRB1, CLDN1 et OCLN, d'autres facteurs cellulaires impliqués dans l'entrée du VHC ont récemment été identifiés : le récepteur au facteur de croissance épidermique (EGFR) (Lupberger et al, 2011), le récepteur EphA2, le récepteur d'absorption du cholestérol NPC1L1 (Sainz et al, 2012) ou encore le récepteur de la transferrine 1 (TfR1) (Martin & Uprichard, 2013).

Plusieurs études ont montré que les composants du métabolisme lipidique pouvaient réguler l'étape d'entrée du VHC dans les hépatocytes. Par exemple, les lipoprotéines de haute densité (HDL) semblent favoriser cette étape alors que les LDL oxydés et les VLDL

l'inhibent. Plusieurs études ont aussi montré que la lipoprotéine lipase inhibait l'infection par le VHC. Finalement, plusieurs apolipoprotéines sont aussi capables de réguler positivement ou négativement l'infection par le VHC. Il est aujourd'hui clairement établi que l'apoE joue un rôle facilitateur prépondérant. L'apoC-I permet aussi d'augmenter l'infectiosité du VHC en facilitant l'étape de fusion. L'apoC-III pourrait quant à elle augmenter l'infection par le VHC en neutralisant l'inhibition induite par la lipoprotéine lipase. Finalement, l'apoH pourrait inhiber le VHC en interagissant avec les particules virales (Vercauteren et al, 2014b).

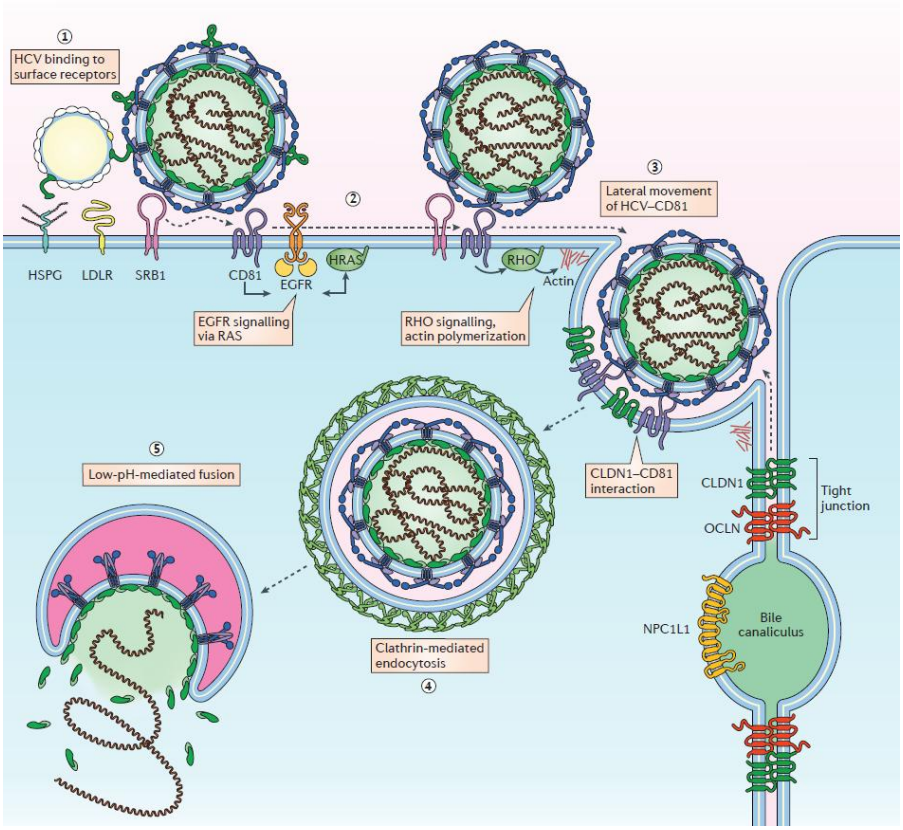

Figure 3 : Entrée du VHC

(1) Les LVPs se lient à la surface des cellules en interagissant avec les HSPG, le LDL-r et le récepteur SRB1. SRB1 peut délipider les lipoprotéines associées aux LVPs et aussi induire des changements de conformation de la glycoprotéine E2, ce qui expose le site de liaison à CD81. (2) L'interaction de E2 avec CD81 entraine l'activation de différentes voies de signalisation. (3) Ces évènements de signalisation favorisent le mouvement latéral des complexes CD81-VHC au niveau des jonctions serrées et l'interaction de CD81 avec CLDN1. (4) L'internalisation du VHC se fait par endocytose dépendante de la clathrine. (5) Le faible

pH du compartiment endosomal induit la fusion de l'enveloppe virale avec la membrane des endosomes précoces. D'après (Lindenbach & Rice, 2013)

Pour de nombreux virus, les interactions virus-récepteurs permettent non seulement de déterminer le tropisme cellulaire, mais également la spécificité d'hôte. Concernant le VHC, il a été montré que l'expression des récepteurs CD81, SRB1, CLDN1 et OCLN dans les hépatocytes de souris rendaient les cellules murines permissives pour l'entrée du VHC. Toutefois, l'origine humaine de ces récepteurs n'est nécessaire que pour CD81 et OCLN. En outre, il est possible d'adapter le VHC à certains facteurs d'entrée murins, comme cela a été montré pour la tétraspanine murine CD81. Les glycoprotéines d'enveloppe du VHC jouent un rôle essentiel dans l'étape d'entrée virale. E1 et E2 interagissent avec les récepteurs cellulaires et permettent la fusion entre l'enveloppe virale et les membranes cellulaires suite à l'internalisation du virion. Le rôle de E1 dans le processus d'entrée du VHC n'est pas encore très clair, mais cette protéine pourrait moduler la liaison aux récepteurs et être impliquée dans processus de fusion. Le rôle de la glycoprotéine E2 est mieux caractérisé. Cette protéine interagit avec les récepteurs CD81 et SRB1.

Objectif du projet de thèse :

Depuis plusieurs années, la recherche sur le virus de l'hépatite C est principalement basée sur l'étude *in vitro* de la souche JFH1, la seule souche se multipliant efficacement en culture cellulaire. Les cellules les plus utilisées sont les cellules HuH-7 et ses dérivées, qui sont les cellules les plus permissives à ce virus. Ce modèle présente toutefois quelques limites. En effet, il présente un système interféron défectueux. Par ailleurs, les études réalisées sur la souche JFH1 ne sont pas systématiquement transposables aux autres génotypes du VHC. La mise en culture d'isolats cliniques du VHC reste quant à elle très inefficace. En condition *in vitro,* seules quelques cellules semblent être sensibles à l'infection sans que l'on puisse observer de propagation de cette infection aux autres cellules. Ainsi, l'amélioration des systèmes d'étude existants et le développement de modèle de culture pour les isolats cliniques seraient un grand progrès. Ceci pourrait permettre de mieux comprendre la physiopathologie de l'hépatite C, de faciliter le développement de nouveaux traitements et d'un vaccin mais également d'accéder à une personnalisation de la médecine.

Des résultats préliminaires ayant permis d'observer que le sérum humain inhibait le VHC, nous avons émis l'hypothèse que la difficulté de mettre en culture les isolats cliniques pouvait provenir de la présence de facteurs inhibiteurs dans le sérum. L'objectif de mon travail de thèse a donc été d'identifier les principaux inhibiteurs du VHC présents dans le sérum humain et de voir si la déplétion de ces facteurs pouvait permettre la mise en culture des isolats cliniques du VHC.

Résultats :

Purification et identification des inhibiteurs du VHC présents dans sérum humain.

La première étape de mon projet a consisté en l'identification des facteurs présents dans le sérum qui influenceraient négativement l'infection par le VHC. Au cours de la première année de ma thèse, j'ai travaillé sur la mise au point d'un protocole permettant d'obtenir une fraction enrichie en inhibiteur(s) du VHC à partir de sérum humain d'individus séronégatifs pour ce virus. Nous avons tout d'abord observé que le(s) inhibiteur(s) présent(s) dans le sérum humain étaient précipités par le polyéthylèneglycol (PEG). Nous avons par ailleurs observé que cette précipitation au PEG permettait de retirer une quantité importante de protéines sériques n'ayant pas d'effet sur l'infection par le VHC, en particulier des protéines majoritaires du sérum comme l'albumine. Dans un second temps, nous avons également observé que les inhibiteurs présents dans le sérum se retrouvaient dans les fractions de faible densité après fractionnement sur gradient d'iodixanol. Deux séries de fractions inhibitrices étaient observées. La première série correspondait aux fractions présentant une densité similaire à celle des VLDL. L'effet inhibiteur observé était toutefois très variable d'un sérum à un autre et nous avons supposé que l'inhibition était induite par les VLDL elles-mêmes, comme suggéré dans l'étude récente de Tao *et al.*. La deuxième série de fractions inhibitrices était quant à elle observée avec tous les sérums testés et correspondait à des fractions présentant une densité légèrement supérieure à celle des lipoprotéines de faible densité (LDL). De façon intéressante, cette technique nous permettait également de retirer des protéines sériques non-relevantes dans le cadre de notre étude. Suite à ces résultats encourageants, nous avons donc décidé de coupler ces deux techniques afin de purifier au maximum le(s) inhibiteur(s) présent(s) dans le sérum. Nous avons ainsi procédé à une précipitation au PEG puis à un fractionnement sur gradient d'iodixanol et nous avons finalement recueilli les fractions présentant un effet inhibiteur sur l'infection par le VHC.

L'analyse en spectrométrie de masse de ces fractions nous a permis d'identifier une quarantaine de protéines. Afin d'améliorer encore notre protocole de purification, nous avons alors décidé d'ajouter une étape de chromatographie d'exclusion stérique. Au final, l'analyse en spectrométrie de masse des fractions inhibitrices obtenues après précipitation au PEG, gradient d'iodixanol par ultracentrifugation, et chromatographie d'exclusion stérique nous a conduit à l'identification de deux protéines, l'apolipoprotéine(a) (apo(a)) et l'apolipoprotéine B (apoB).

Apolipoprotéine(a) et lipoprotéine(a).

L'apo(a) est une apolipoprotéine synthétisée par le foie qui n'est retrouvée que chez l'Homme, les primates non-humains et les singes de l'ancien monde. Chez l'Homme, l'apo(a) est constituée de dix domaines kringle IV (KIV), un domaine Kringle V (KV) et un domaine protéase inactif, tous étant homologues aux domaines du plasminogène (Gabel & Koschinsky, 1995; Koschinsky & Marcovina, 1997 ; McLean et al, 1987). Chacun des dix domaines KIV de l'apo(a) n'est présent qu'en une seule copie excepté le domaine KIV_2 qui lui est présent en un nombre variable de copies répétées en tandem (entre 3 et >40 copies), ce qui donne naissance à plusieurs isoformes présentant des masses moléculaires comprises entre ~200 and ~800 kDa. Dans le sérum humain, l'apo(a) se lie par un pont disulfure à l'apoB des LDL pour former des lipoprotéines(a) (Lp(a)) [Figure 4](Schmidt et al, 2016). En conséquence, la densité des Lp(a) est légèrement augmentée comparé à celle des LDL. Une corrélation inverse entre la taille de l'isoforme apo(a) et la concentration plasmatique (comprise entre <1 et >100 mg/dL) a été décrite. Par ailleurs, plusieurs études ont montré que les personnes présentant une concentration plasmatique élevée en Lp(a) présentaient un risque plus élevé de développer des maladies vasculaires. Les différents domaines kringle de l'apo(a) contiennent des domaines de liaison pour des lysines (LBS pour « Lysine Binding Site ») qui jouent un rôle dans l'activité pathogénique de la Lp(a). Par exemple, les LBS de faible affinité, présents dans les domaines KIV_7 et KIV_8, permettent l'interaction non-covalente avec l'apoB avant la formation du pont disulfure. Le LBS de forte affinité présent dans le domaine KIV_{10} permet quant à lui l'interaction de l'apo(a) avec différents substrats biologiques comme la fibrine et les protéines de la matrice extracellulaire (Becker et al, 2004b; Ernst et al, 1995; Gabel et al, 1996; Trieu & McConathy, 1995). Aussi, la Lp(a) est désormais connue pour jouer le rôle de transporteur privilégié des phospholipides oxydés dans le sang (Bergmark et al, 2008).

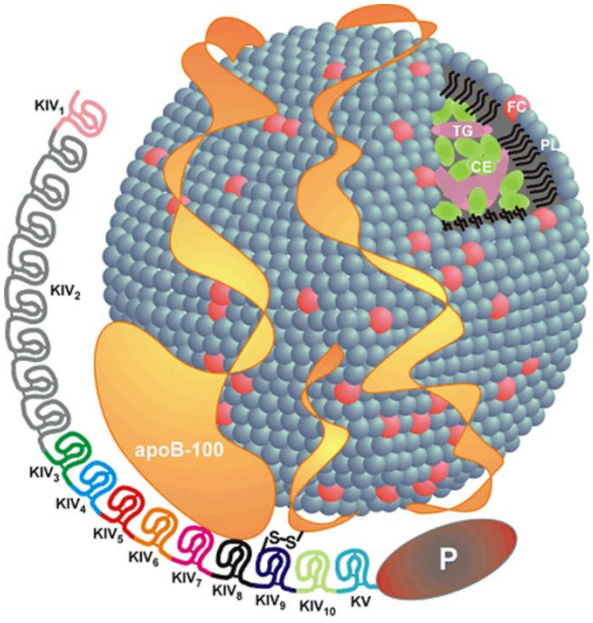

Figure 4: Structure de la Lipoprotéine(a)

D'après (Koschinsky & Marcovina, 2004)

La Lp(a) inhibe l'infection par le VHC en interagissant avec les particules infectieuses.

Afin de tester l'effet de la Lp(a) sur l'infection par le VHC, nous avons tout d'abord purifié cette Lp(a) à partir de différents plasmas humains, en utilisant des ultracentrifugations séquentielles. Nous avons ainsi observé que la Lp(a) dérivée de plasma était capable d'inhiber le VHC de façon dose-dépendante, même si la force d'inhibition variait en fonction des échantillons. De façon importante, la présence de cette Lp(a) durant les 3 heures d'inoculation était suffisante pour réduire l'infection suggérant que l'inhibition concerne les étapes d'entrée virale. Puisque les isoformes d'apo(a) varient fortement d'un individu à un autre, nous avons également testé l'effet de Lp(a) recombinantes produites en culture cellulaire. Pour cela, nous avons transfecté des cellules HuH-7-RFP-NLS-IPS avec des plasmides codant différentes isoformes d'apo(a), contenant 13, 17 ou 21 domaines KIV. Les surnageants des cellules transfectées, contenant la Lp(a) recombinante, ont été récoltés 72 heures après transfection et mélangés avec du VHC recombinant durant l'inoculation de cellules HuH-7-RFP-NLS-IPS naïves. Ces expériences nous ont permis de démontrer que chaque forme de Lp(a)

recombinante inhibait de façon dose-dépendante l'infection par le VHC. Par ailleurs, les résultats obtenus avec ces formes recombinantes suggéraient également qu'il existait une correlation entre la taille de l'apo(a) et la force de l'inhibition. Finalement, nous avons aussi mis en évidence que des concentrations croissantes en « Wheat Germ Agglutinin », une lectine connue pour interagir spécifiquement avec la Lp(a), permettaient de restaurer l'infection par le VHC. Puisque la WGA interagit spécifiquement avec l'apo(a), nous avons souhaité tester si cette lectine pouvait interférer avec une interaction de la Lp(a) avec les particules infectieuses. Pour cela, nous avons immunoprécipité de la Lp(a) recombinante à l'aide de billes couvertes d'anticorps anti-apo(a) et incubé ces billes avec du virus purifié. La quantité de virus fixé aux billes a finalement été évaluée par quantification de l'ARN viral. Cette technique nous a ainsi permis de montrer qu'environ 30 % du virus étaient fixés aux billes en présence de Lp(a) alors que plus de 99% du virus étaient éliminés dans le surnageant de l'immunoprécipitation en absence de Lp(a). Par ailleurs cette technique nous a permis de prouver que la WGA empêchait l'interaction entre la Lp(a) et le VHC puisque seulement 3 % du virus co-immunoprécipitaient en présence de ce composé [Figure 5].

L'apo(a) inhibe l'infection par le VHC en interagissant avec les particules infectieuses.

Contrairement aux cellules d'hépatocarcinome, les cellules HEK293 ne synthétisent pas d'apoB et par conséquent sécrètent de l'apo(a) libre. Pour voir si l'apo(a) seule était suffisante pour inhiber le VHC, nous avons testé l'effet de différentes formes recombinantes de cette protéine, purifiées par chromatographie d'affinité, à partir du surnageant de cellules HEK293 stablement transfectées. Ces expériences nous ont permis de mettre en évidence que l'apo(a) seule était capable d'inhiber de façon dose-dépendante l'infection par le VHC. Par ailleurs, en accord avec les résultats obtenus avec la Lp(a) recombinante, nos résultats suggéraient aussi que les isoformes les plus grandes avaient une force d'inhibition plus importante. En remplaçant la Lp(a) recombinante par de l'apo(a) seule dans le test d'immunoprécipitation décrit précédemment, environ 40 % du virus étaient fixés aux billes couvertes d'anticorps anti-apo(a), suggérant que l'apo(a) seule interagit avec les particules virales du VHC.

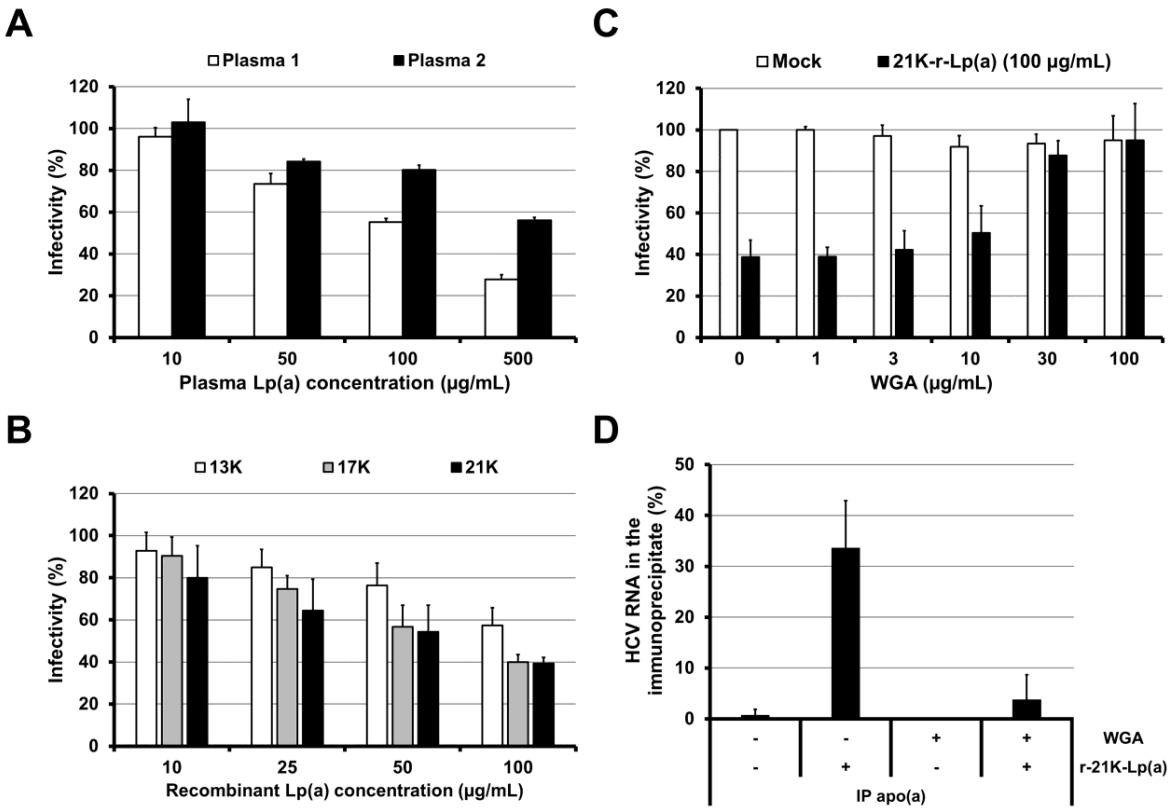

Figure 5

La Lp(a) inhibe l'infection par le VHC en interagissant avec les particules infectieuses. Des cellules HuH-7-RFP-NLS-IPS ont été incubées pendant trois heures avec des HCVcc en présence de concentrations croissantes en Lp(a) purifiée à partir de deux plasmas humains différents (A) ou de Lp(a) recombinante récoltée dans le surnageant de cellules Huh-7 transfectées avec des plasmides exprimant des protéines apo(a) de différentes tailles, 13K, 17K ou 21K (B). Les résultats sont exprimés en pourcentage d'infection par rapport à une infection en absence d'échantillon et correspondent à des moyennes ± écart-type de duplicats d'une expérience représentative (A) et de trois expériences indépendantes (B). (C) Des cellules HuH-7-RFP-NLS-IPS ont été inoculées pendant trois heures avec des HCVcc, en présence ou en absence de 100 µg/mL de Lp(a) recombinante (21K-r-Lp(a), voir Fig. 5B) et de concentrations croissantes en WGA. Les mesures de l'activité luciférase, permettant d'évaluer les niveaux d'infection, ont été réalisées sur les cellules infectées 48h après l'infection. Les résultats sont exprimés en pourcentage d'infection par rapport à l'infection en absence d'échantillon et correspondent à des moyennes ± écart-type de trois expériences indépendantes. (D) De la Lp(a) recombinante (200 µg/mL de 21K-r-Lp(a)) a été

immunoprécipitée en utilisant des anticorps anti-apo(a). Les mélanges ont ensuite été incubés avec des HCVcc purifiés pendant la nuit, en absence ou en présence de 100 µg/mL de WGA. La quantité d'ARN du VHC capturée a finalement été évaluée par RT-PCR quantitative. Le surnageant des cellules HuH-7-RFP-NLS-IPS non-transfectées a été utilisé comme témoin négatif. Les résultats sont exprimés comme le titre d'ARN du VHC co-immunoprécipité (pourcentage par rapport à la quantité d'ARN total du VHC dans le surnageant et l'immunoprécipité) et sont présentés comme des moyennes ± écart-type de trois expériences indépendantes.

L'utilisation de différentes formes tronquées a permis de montrer qu'un nombre substantiel de copies du domaine KIV_2 était nécessaire pour l'activité anti-VHC. Ces résultats sont toutefois à prendre avec précautions car il a été montré que ces formes tronquées adoptent une conformation différente comparé aux formes complètes de l'apo(a). En utilisant des formes recombinantes mutées au niveau des LBS ($\Delta LBS_{7,8}$ et ΔLBS_{10}) nous avons observé que la fonctionnalité de ces LBS n'était pas nécessaire. Finalement, l'utilisation de particules rétrovirales pseudotypées avec les protéines d'enveloppe du VHC, produites en cellules HEK293, nous a permis de montrer que la partie lipoprotéique des lipoviroparticules du VHC était nécessaire pour l'activité inhibitrice de l'apo(a). En effet, ces pseudoparticules, qui ne sont pas associées aux lipoprotéines, sont beaucoup moins sensibles à l'apo(a) que le virus natif produit en culture cellulaire. Au contraire, les particules rétrovirales pseudotypées avec la protéine G du virus de la stomatite vésiculeuse ne sont pas sensibles à l'apo(a), démontrant que l'inhibition du VHC est spécifique [Figure 6]

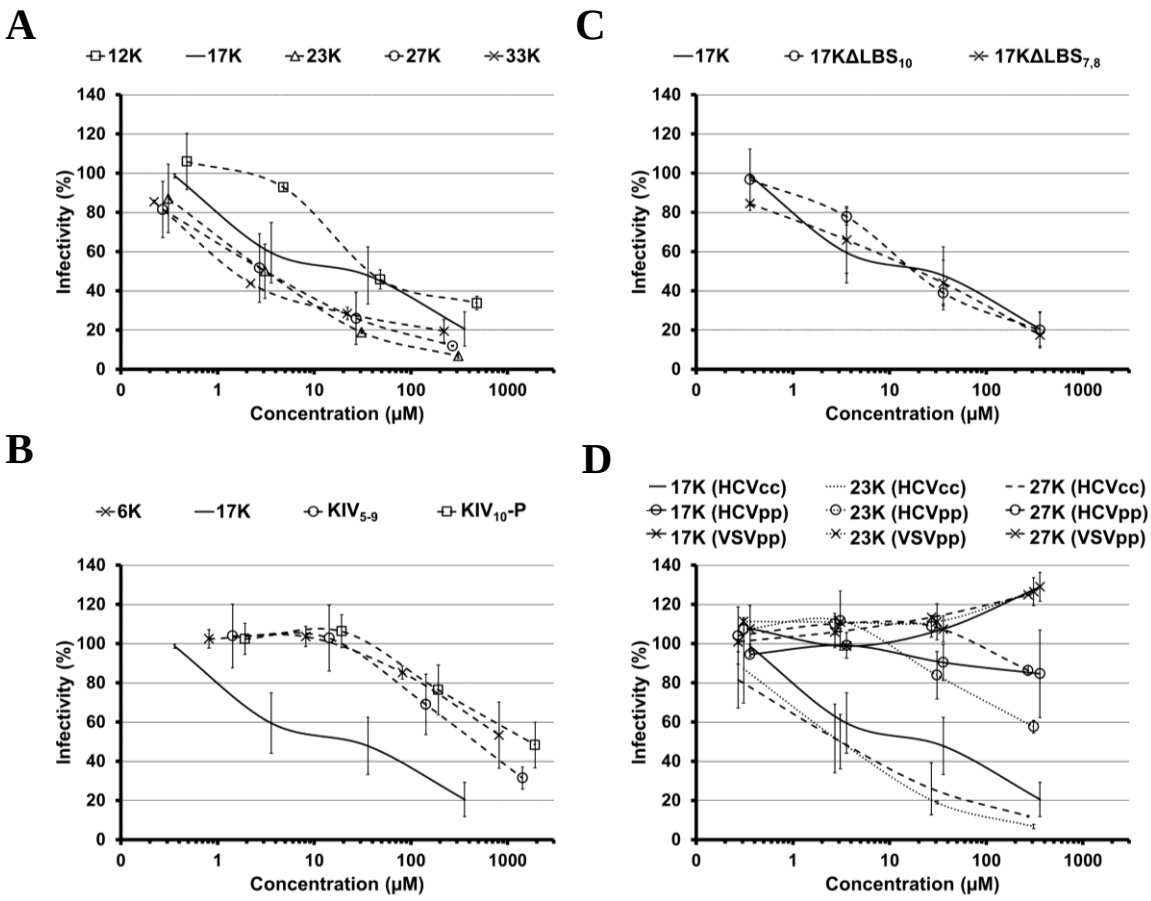

Figure 6

Le VHC est inhibé par différentes formes recombinantes d'apo(a). (A,B,C,D) Les mesures d'infection ont été effectuées après inoculation de cellules HuH-7-RFP-NLS-IPS, pendant trois heures, avec HCVcc, HCVpp ou VSVpp en présence de concentrations croissantes des différentes formes de l'apo(a) (0,1, 1, 10 et 100 µg/mL). Les mesures ont été réalisées sur les cellules infectées, 48h (pour HCVcc) ou 72h (pour HCVpp et VSVpp) post-infection. Les résultats sont exprimés en pourcentages d'infection par rapport à une infection en absence d'échantillon et correspondent à des moyennes ± écart-type d'au moins deux expériences indépendantes.

Conclusion :

En conclusion, nos travaux ont permis d'identifier un nouveau composant du métabolisme lipidique qui régule l'infection par le VHC. Nos résultats suggèrent en effet que l'apo(a) réduit l'infection du VHC en interagissant avec les lipoviroparticules. Cette étude apporte de nouvelles connaissances sur le lien entre le VHC et les lipoprotéines. D'autres travaux seront nécesaires pour mieux comprendre le mécanisme exact de l'inhibition du VHC par l'apo(a). Aussi, nos résultats pourraient ouvrir de nouvelles perspectives pour le développement d'un modèle de mise en culture des isolats cliniques du VHC.

RÉSUMÉ

Dans cette étude, nous avons émis l'hypothèse que la difficulté à mettre en culture les isolats cliniques du VHC pouvait être due à la présence de facteurs de restriction dans le sérum des patients. En utilisant des sérums séronégatifs pour le VHC, nous avons confirmé l'existence de facteurs sériques inhibiteurs. En combinant des étapes de précipitation au polyéthylène glycol, de gradient d'iodixanol et de chromatographie d'exclusion stérique, nous avons obtenu une fraction purifiée enrichie en facteurs inhibiteurs, à partir des sérums séronégatifs pour le VHC. L'analyse en spectrométrie de masse a permis d'identifier l'apolipoprotéine(a) (apo(a)) comme un inhibiteur potentiel de l'entrée du VHC. En utilisant un virus recombinant dérivé de la souche JFH1, nous avons confirmé que la lipoprotéine(a) plasmatique et recombinante étaient capables d'inhiber spécifiquement le VHC en interagissant avec les particules infectieuses. En utilisant la lectine WGA, qui est connue pour interagir spécifiquement avec la Lp(a), nous avons montré qu'il était possible de réduire l'effet inhibiteur de l'apo(a) et de rétablir l'infection par le VHC. De façon intéressante, nous avons aussi observé que la protéine apo(a) seule, sous forme recombinante purifiée, était suffisante pour inhiber le VHC. Nos résultats suggèrent également que les isoformes courtes sont moins inhibitrices que les longues. Nous avons aussi observé que les sites de liaison aux lysines dans KIV7, KIV8 et KIV10 n'étaient pas requis pour l'activité inhibitrice. Finalement, nos résultats suggèrent que la fraction lipoprotéique des lipoviroparticules du VHC est essentielle pour l'inhibition. Au final, nos résultats mettent en évidence que l'apo(a) est un nouveau composant du métabolisme lipidique capable de moduler l'infection par le VHC.

ABSTRACT

In this study we hypothesized that this hindrance could be due to the presence of restriction factors in patient serum. Thus, using HCV seronegative sera, we confirmed our hypothesis. Combining polyethylene glycol precipitation, iodixanol gradient and size-exclusion chromatography, we obtained a purified fraction enriched in inhibitory factors from HCV seronegative sera. Mass spectrometry analysis identified apolipoprotein(a) (apo(a)) as a potential inhibitor of HCV entry. Using a recombinant virus derived from the JFH1 strain we confirmed that plasma-derived and recombinant lipoprotein(a) as well as purified recombinant apo(a) variants were able to specifically inhibit HCV by interacting with infectious particles. Using WGA lectin, that is well known to specifically interact with Lp(a), it was possible to reverse the apo(a) inhibitory effect and restore the HCV infection. Our results also suggest that the lipoprotein moiety of HCV LVP is essential for the inhibition. Indeed, HCVpp were only slightly sensitive to apo(a) contrary to HCVcc, which are associated to lipoproteins. Our results also suggest that small isoforms are less inhibitory than the large ones. Finally, we observed that the functional lysine-binding sites in KIV_7, KIV_8 and KIV_{10} were not required. Altogether, our results identify apo(a) as an additional component of the lipid metabolism modulating HCV infection.