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Restoration of systemic redox balance and mitochondrial function by directly acting antivirals in peripheral blood mononuclear cells of chronic HCV patients

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1. INTRODUCTION

Hepatitis C is an infectious disease caused by Hepatitis C virus (HCV) primarily affecting the liver. HCV infection is a global health concern affecting more than 71 million people around the world and nearly 400,000 people die every year with this viral related cirrhosis and hepatocellular carcinoma (HCC) [1].

The advent of new direct antiviral drugs (Direct-Acting Antivirals or DAAs) has substantially changed the therapeutic management of patients with chronic HCV-related hepatitis. For many years, in fact, the only possibility of recovery from the chronic infection was by treatment with Intereferon (IFN) in combination with ribavirin given over a period of time between six and eighteen months. The main limitations of the aforementioned therapy were essentially represented by the manifestation of numerous side effects during treatment, low response (30-70%) to overall treatment and contraindication to administer for patients with advanced liver disease [2,3].

On the contrary, the use of the new direct antivirals guarantees the decrease in viral load in a few weeks of treatment, and ultimately the sustained viral response (SVR). The effectiveness and extreme manageability of these treatments has allowed the usage of these drugs in patients with hepatic cirrhosis and in patients for whom antiviral therapy was contraindicated or difficult to manage such as transplanted patients, psychiatric patients or simply in elderly subjects. Patients who achieved SVR after DAA treatment showed less cirrhosis-related complications, as well as reduced risk for mortality and hepatocellular carcinoma [4,5]. At the same time, clearance of the infection does not reduce risks to develop cirrhosis and cancer to the initial level, as observed in the uninfected

population. One of the factors that contributes to liver injury is alteration of the redox balance in the host cell, due to increased production of reactive oxygen species (ROS) and altered expression of scavenging enzymes [6].

Oxidative stress is defined as the production of reactive species which overwhelms the antioxidant defences, is a process deeply involved in the progression of liver damage during chronic hepatitis C [7]. Indeed, patients affected by chronic hepatitis C present with altered pro and antioxidant markers in both body fluids and liver specimens [8,9]. Excessive oxidative markers may be the result of chronic inflammation, iron overload, along with HCV encoded proteins [10]. The non-structural protein 5A affects the intracellular Ca2+ signalling, which in turn triggers production of reactive species by mitochondria [11], and the HCV core protein may cause oxidative damage exerting a direct effect on mitochondria [12]. In addition, chronic HCV infection is associated with decreased circulating antioxidant defences, such as reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) [9,13]. Recent evidence also suggests that the disruption of redox balance observed in infected patients is irrespective of HCV genotype [14]. However, much less is known about redox biology of viral infections since studies so far have been focused on the mechanism by which HCV augments ROS production and limits antioxidant defence. Several studies have suggested that HCV replication and production of infectious virions are suppressed by lipid peroxides whose production is augmented by the infection [15]. By removing the noxious stimulus of the virus and inflammation, rapid and complete response observed early after starting DAA therapy might normalise redox homeostasis in chronically infected HCV patients. The response to a traditional antiviral regimen (PEGylated IFN α-2b and oral ribavirin) is accompanied by an improvement in circulating

redox balance, while failure is characterised by persistent oxidative stress [16].

It is well known that HCV proteins may interfere with host antiviral signalling, shutting down the cascade of innate immune response [17]. Several other mechanisms exist, but in essence, once HCV enters the cell, the virus behaves to undermine innate immune response using viral enzymes, core and envelope proteins. However, in chronic HCV infection another specific phenomenon occurs, namely T-Cell exhaustion, a dysfunction or physical elimination of antigen-specific T cells [18]. Exhausted T cells show altered proliferative abilities, sustained up-regulation of a wide array of co-inhibitory receptors, altered metabolic fitness and failure for transition to quiescence [19]. DAAs rapidly inhibit viral replication and results in rapid elimination of those viral products active in evading the immune response allowing for restoration of immune activity against HCV.

Owing to the rapid cessation of HCV replication and the consequent clearance of viral antigens with an efficacy near to 100%, DAAs may be beneficial to the systemic redox balance and play a role in limiting liver damage and starting fibrosis regression. The present study was designed to investigate the impact of DAA treatment on circulating markers of oxidative damage and antioxidant defence in a cohort of patients affected by chronic hepatitis C. To know the overall repercussions occurred on immune cells we analysed the gene expression profile change in the peripheral blood mononuclear cells (PBMCs) of chronic HCV patients treated with a combination of IFN-free DAA therapy.

1.1. Epidemiology

HCV belongs to the genus Hepacivirus of the family Flaviviridae. It is approximately 9.6 kb with a single stranded positive sense Ribonucleic acid (RNA) genome [20]. There are seven major genotypes 1, 2, 3, 4, 5, 6 and 7 and >84 subtypes to date with distinct global distribution. Genotype (GT) 1a and 1b prevail (about 50%) in Europe and North America followed by GT3 (about 25%) and GT4 (about 20%). GT2, GT5 and GT6 are rare in western countries [21]. Infection by all genotypes depends on the interaction of structural and non-structural viral proteins with host proteins. Genotype variability is important to dictate response to the therapy and to develop effective HCV vaccines with potential use. HCV mainly replicates in the cytoplasm of hepatocyte and causes acute or chronic hepatitis C [22]. About 50-70% of the patients with acute HCV are developing chronic disease showing liver inflammation. Among chronic patients, 20-30% of them further develop hepatic fibrosis and liver cirrhosis after 30 years. HCV induced Cirrhotic patients have a 3-5% risk of developing liver cancer [23].

HCV is mainly transmitted by blood (medical or surgical procedures, blood transfusion and intravenous drug use). Acute infection caused by HCV is self-resolving in 20-50% of cases but does not confer permanent immunity. In most cases (50-80%), HCV infection becomes chronic leading to cirrhosis (10-20% of cases) in 10-20 years and hepatocellular carcinoma (1-4% per year in patients with HCV related cirrhosis). Chronic HCV infection is asymptomatic for a prolonged period of time, so many people are unaware of the infection and have not been tested. In Europe data suggests that only 10-40% of HCV positive individuals are aware of the infection status of HCV [24].

1.2. HCV life cycle

Life cycle of HCV is a multistep process that includes attachment, entry, endocytosis, un coating of genetic material and replication. Upon entering the blood circulation, HCV interacts with lipid molecules like Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) to become lipoviral particles (LVPs). Liver cells are the main target of HCV within the host, as it contains all the entry receptors for HCV infection. Viral genome enters into the cytosol upon fusion of viral and host membrane that facilitates replication of viral RNA and translation [25].

1.2.1. Entry of HCV into hepatocytes

HCV has a positive single stranded RNA genome with an icosahedral protein coat encapsulated by an outer lipid membrane with two viral glycoproteins E1 and E2 embedded to it (Figure 1). E1 and E2 envelope proteins act as complexes of disulphide-bound heterodimers and play a crucial role in virus entry. E2 is involved in receptor binding whereas E1 is likely to be involved in viral fusion phase [26]. Thus, once the HCV particle reaches the hepatocyte surface, it interacts first with glycosaminoglycans and syndecans followed by binding to more specific receptors including scavenger receptor B1 and the tetraspanin CD81. The viral particle complexed with these entry factors reaches tight junctions and engages in further interactions with claudin-1 (CLDN1) and occludin. These factors set an essential entry receptor for the virus entry primed by the E1-E2 complex [27].

The viral particle subsequently enters the cell via receptor and clathrin mediated endocytosis. After its release into the cytosol the clathrin coated vesicle interacts with the motor protein dynein. Dynein transports the vesicle by walking along microtubules to reach the Endoplasmic reticulum (ER) and acidification of the endosome

lumen induces conformational changes of the viral envelope glycoproteins which in turn interact with the endosomal membrane leading to fusion of viral and endosomal membranes. Membrane fusion is followed by uncoating of the nucleocapsid and the release of viral RNA genome into the cytosol. Similar mechanism could be involved in cell to cell spread in the host organism [28,29].

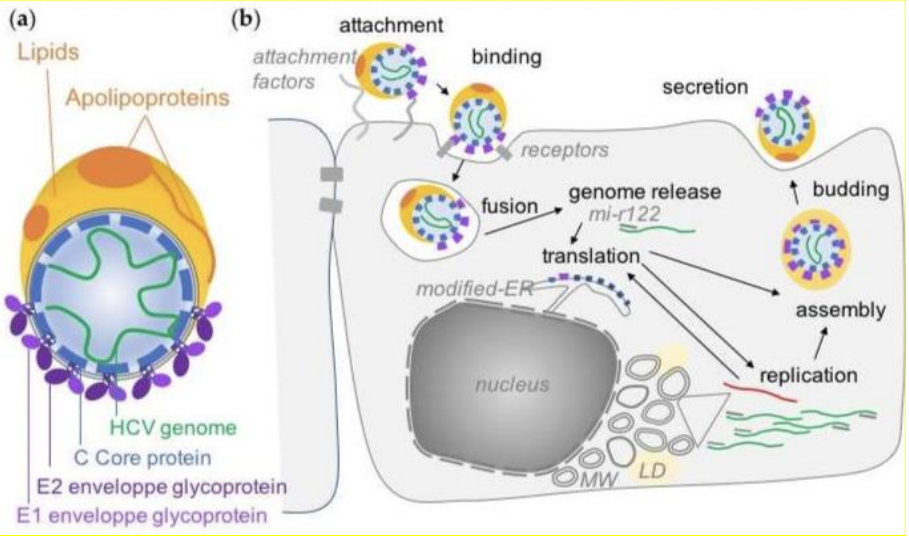

Figure 1. Life cycle of HCV virus (a) HCV particle with RNA genome tightly associated with E1, E2 glycoproteins, lipids and apolipoproteins. (**b**) Overview of HCV entry into the cell. (Alazard-Dany N et al., 2019).

1.2.2. HCV RNA translation and replication

The viral RNA is around 9.6 kb in length with a single open reading frame with highly structured non-translated regions [30]. Binding and assembly of ribosome subunits on the viral RNA starts the translation of HCV polyprotein. A signal sequence located in the beginning of the translated polyprotein allows the ribosome to be targeted to the translocon on the ER membrane. Translated single polyprotein is co and post-translationally modified by both viral and host proteases giving rise to 10 mature proteins (core, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The core, structural

proteins E1 and E2 which makeup the viral particle is produced from the N-terminal end and other nonstructural proteins are produced from the C-terminus end of polyprotein. The non-translated region of RNA contains cis-acting elements which are important for genome translation, replication and internal ribosomal entry site at 5'end [31,32].

The replicase components NS3-4A, NS4B, NS5A and NS5B are sufficient to support viral RNA replication. HCV replicase proteins, in concert with host factors, induce rearrangements of the ER membrane, including the formation of double membrane vesicles [33]. These vesicles cluster to form the membranous web, which represents the site of HCV RNA replication. Viral RNA synthesis is catalysed by the RNA dependent RNA polymerase activity of NS5B, which acts in concert with other viral non-structural proteins (NS3/4A, NS4B, NS5A) as well as several host factors [34]. After synthesis of a negative strand RNA intermediate, multiple positive strand progeny RNAs are generated from this template and either used for translation and replication or packaged into nucleocapsid particles.

NS2 viral protease, is involved in maturation of viral polyprotein and assembly of viral particles. NS3 viral protease is a helicase and also involved in proteolysis of downstream parts of polyprotein. Acting as a cofactor NS4A interacts with NS3 and anchors it to the membrane. P7 is a viroporin involved in assembly and secretion of virions [35]. Most of the viral membrane proteins are involved in the reorganisation of the cellular membranes required for HCV genome replication and viral assembly. In early HCV infection, the massive rearrangement of cellular membranes forms a membranous web by double membrane vesicles originating from ER [36]. Along with other replication complex proteins, NS4B and NS5A play a key role

in the formation and maintenance of membranous webs containing replication factories of the virus [37]. Viral proteins also recruit several cellular factors like cyclophilins, cellular peptidyl-propyl cis-trans isomerase. Cyclophilin A interacts with NS5A in the formation of membranous webs.

1.2.3. HCV Assembly, Budding and Secretion

HCV Viral assembly occurs at assembly sites of replication complexes associated with ER derived membranes [38]. NS5A plays a key role in viral replication, assembly and delivery of RNA genome to the core proteins for assembly into nucelocapsids [39,40]. P7 viroporin acts in concert with NS2 in the assembly process as well as at a later stage of viral envelopment [41]. They coordinate the recruitment of core and envelope glycoproteins E1, E2 as well as NS3, NS5A to the assembly sites. Along with viral proteins, several cellular proteins apolipoprotein E, DGAT1 and ESCRT act as key factors for HCV assembly [42].

The viral envelope glycoproteins are acquired by budding at the ER, a process which appears to be linked to the VLDL machinery. Maturation of lipoviro particles may require unconventional passage through Golgi apparatus and a trans-endosomal secretary route. Newly synthesized virus particles are thought to be transported to the cell surface in export vesicles via the cellular secretory pathway. Finally, these are released from the cell by exocytosis to reach the bloodstream [43]. In HCV life cycle, viral proteins are multipurpose and are involved in several functions of viral replication reflecting the genetic economy. As these viral proteins are involved in one or more steps of the viral cycle, targeting these proteins is a potent antiviral target in the fight of HCV infection.

1.3. Pathophysiology of HCV infection

HCV infection is associated with chronic hepatic inflammation due to oxidative stress and immune response of the infected cells [44]. Infected hepatocytes recruit immune cells by production of cytokines, chemokines and numerous growth factors that perpetuate the local inflammatory response and activate myofibroblasts and stellate cells [45]. Sustained inflammation observed during chronic HCV infection may be due to response of T helper 2 cells and CD8+ T cells that induce apoptosis [46,47]. Activation of hepatic stellate cells causes fibrogenesis-characterised by excess production of extracellular matrix which leads to liver fibrosis and ultimately developing to cirrhosis [48]. The changes in hepatocyte proliferation during progression of HCV infection could be due to direct interaction of viral proteins with proteins involved in the regulation of cell cycle and loss of cell cycle checkpoints due to Deoxyribonucleic acid (DNA) damage induced by oxidative stress [6].

Both innate and adaptive immune responses are activated in response to HCV. Viral RNA in the host activates pattern recognition receptors present on the cell surface, endoplasm and endosomes to initiate an immunogenic response via pathogen associated molecular pattern (PAMP) [49]. With an aim to create an antiviral state, IFNs are secreted in response to activation of Nuclear factor kappa B (NF-κB) and the JNK pathway. Wide variety of immune cells secrete type 1 IFN-α, IFN-β, IFN-ω which bind to their specific receptors and activate a subset of genes called IFN sensitive genes (ISGs). ISGs may include protein kinase R and 2',5'-oligoadenylate synthase that induce shutdown of mRNA translation and help to cleave viral and host single stranded RNA respectively. T-lymphocytes, macrophages and dendritic cells secrete type II -IFN-γ to clear acute

and chronic HCV infection. Similar to type I IFNs, type III IFN-λ bind to hepatocyte receptors and activate many ISGs. IFN-β is observed in liver macrophages and kupffer cells of HCV infected patient's liver biopsy [50]. Patients with chronic HCV infection and HCV infected chimpanzees have been detected with high concentrations of IFN-γ. In HCV infected patients, IFNs including IFN- γ are produced by non-parenchymal cells, they escape from HCV induced attenuation of type I IFN production. Despite immune response, HCV still persists by inhibiting ISGs [1].

The majority of HCV infected patients develop a chronic hepatitis C infection associated with inflammation, steatohepatitis, progressive fibrosis, and finally leading to cirrhosis [51]. Several risk factors enhancing HCV-related HCC progression are hepatitis B virus or Human immunodeficiency virus (HIV) coinfection, obesity, insulin resistance, and non-alcoholic steatohepatitis [52]. HCV may directly induce HCC by altering energy metabolism, apoptosis, angiogenesis, epithelial mesenchymal transition (EMT), and oxidative stress [53]. HCV infection has incompletely understood apoptosis process that plays an important role in tumorigenesis. Inhibition of apoptosis escapes from crucial checkpoints during the life cycle of transformed cells. Moreover, HCV proteins Core, NS2, NS3, and NS5A interact and suppress the transcriptional activity of p53, a critical tumour suppressor protein [54-56]. In addition, defective autophagy in mice results in spontaneous development of HCC due to the accumulation of autophagy cargo p62 [57]. Autophagy deficiency in hepatocytes favour EMT and autophagy stimulation may promote early pre-neoplastic liver nodules in a Resistant-Hepatocyte rat model [58].

1.4. Structure and function of Mitochondria

Mitochondria are involved in significant number of human diseases such as cardiovascular disorders, neurodegenerative diseases, diabetes, and also plays a key role in ageing. Mitochondria are also one of the favorite target for HCV that impacts its functions and affecting cellular growth, proliferation, and differentiation. Viral proteins modify mitochondrial functions by binding to outer membrane of mitochondria and getting imported to intermembrane space and matrix [59]. Clinical observations revealed the existence of hepatic mitochondrial dysfunction and oxidative stress in HCV infected patients. Analysis of human liver samples displayed ultrastructural changes in mitochondria, lipid peroxidation products, DNA adducts, and GSH depletion. These effects on mitochondria alter its functions significantly resulting in the pathogenesis of the disease [60].

Mitochondrial structure comprises outer mitochondrial membrane (OMM), inter membrane space (IMS), inner mitochondrial membrane (IMM), and matrix (Figure 2). The OMM is freely permeable to nutrients, ions, energy molecules like Adenosine diphosphate (ADP), Adenosine triphosphate (ATP), and other low-molecular weight solutes. The IMM contains many folds into the matrix known as cristae. IMM is strictly permeable to oxygen and impermeable except through specific transporters [61]. The mitochondrial respiratory chain complexes are multi-subunit structures embedded in IMM that help in the electron transport chain (ETC) for ATP production. Mitochondrial DNA (mtDNA) is circular with about 16,500 base pairs containing 37 genes. mtDNA encodes for 13 proteins of ETC complexes and ATP synthase, 22 transfer RNAs and 2 ribosomal RNAs [62]**.** Nuclear DNA encodes the other proteins of mitochondria in the cytoplasm and then import

into the mitochondria.

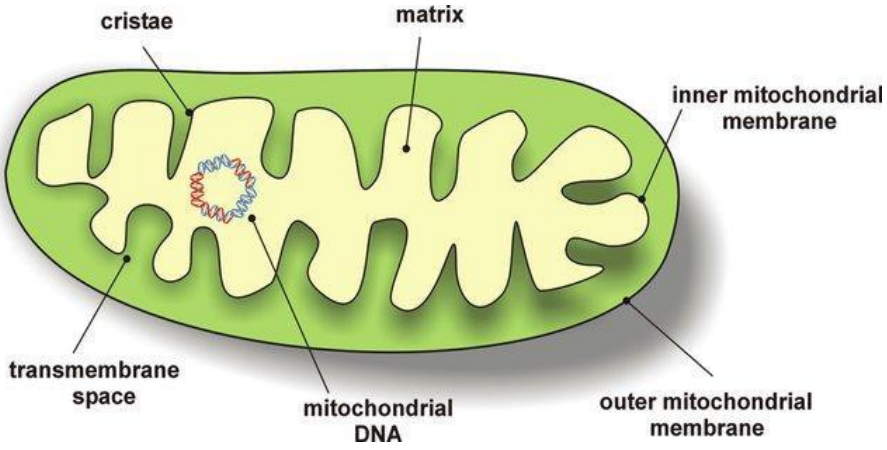

Figure 2: Mitochondria structure and components (Labieniec-Watala M. etal., 2012).

The ETC contains a series of four proteins (complex I-IV) with mobile carriers ubiquinone and cytochrome c (Figure 3). These components together with ATP synthase (complex V) produce ATP through oxidative phosphorylation (OXPHOS). Nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) generated by the Tricarboxylic acid cycle act as substrates for complex I and complex II respectively. During ETC, electrons are transferred from NADH to the complex I then passes them to ubiquinone (CoQ), inducing the pumping of protons from matrix to IMS through complex I. Electrons from FADH2 are accepted by complex II and subsequently transferred to CoQ, which is not accompanied by proton translocation unlike complex I. CoQ then passes electrons to complex III which passes them to cytochrome c coupled with pumping of protons from matrix to IMS. Cytochrome C passes electrons to Complex IV (cytochrome *c* oxidase) where the molecular oxygen is reduced to water. Complex V is a multi-subunit containing extra membranous (F1) and transmembrane (F0) domains functioning under rotational motor mechanism allowing ATP

production [63].

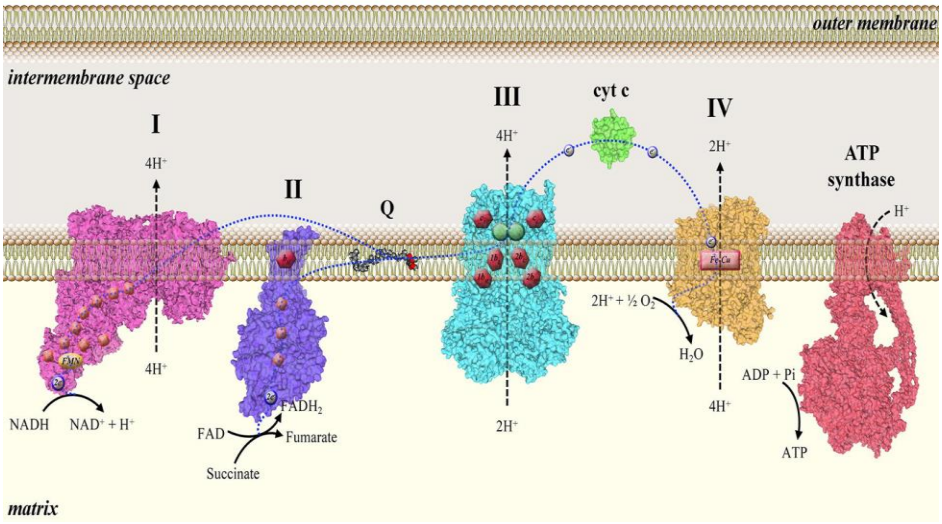

Figure 3: Schematic representation of OXPHOS in mitochondria. The process of OXPHOS is the main pathway in the cell to produce adenosine triphosphate (ATP). Mitochondrial electron transport chain consists of four complexes: Complex I (NADH-coenzyme Q reductase), Complex II (succinate dehydrogenase), Complex III (coenzyme Q-cytochrome c reductase), and Complex IV (cytochrome c oxidase). Complexes I, III, and IV establish the proton gradient across the IMM for ATP synthesis by ATP synthase (Branca JVV et al., 2020).

Mitochondria are the main sources of ROS in the cell. During ETC, though most of the electrons donated are migrated to complex IV are accepted by protons that react with oxygen to form water, few electrons react directly with oxygen and form superoxide anion radical [64]. Manganese superoxide dismutase localised in the mitochondria, spontaneously reacts with superoxides and dismutates into oxygen and Hydrogen peroxide (H2O2). Mitochondrial GSH peroxidase and peroxiredoxins detoxify H2O2 into water. Under physiological conditions, ROS act as signalling molecules depending on their intensity and oxidative stress [65,66]. ROS at lower concentrations are important for metabolic adaptation, while moderate levels of ROS involves in regulation of inflammatory mediators. ROS at higher

concentrations, activates apoptotic and autophagic pathways [65-67].

1.5. Oxidative stress in HCV infection

Metabolic dysregulation such as insulin resistance, steatosis and iron overload has been observed in chronic HCV patients. Evidence suggests that oxidative stress could be one of the underlying mechanisms of these pathologies. Mitochondrial respiratory chain enzymes are modulated and inactivated by HCV proteins including core, E1 and NS3 that cause disruption of mitochondrial transmembrane potential and leakage of electrons, that induce rise of intracellular ROS levels [68,69]. All these molecular events together impair mitochondrial and cellular signalling pathways. Excess generation of ROS and decreased capacity of the antioxidant system leads to oxidative stress that can cause cellular damage [70]. ROS either directly interact with biological molecules including lipids, proteins and nucleic acids or activate classical signalling cascades like protein kinases, cytokines and transcription factors which regulate stress responses that in turn stimulate inflammatory response [71].

In the mid-1900s oxidative stress occurrences were detected in chronic HCV patients. Expression of HCV proteins in an infected cell activates several pathways including mitogen activated protein kinase (MAPK), ER response, NF-κB and calcium signalling [72]. HCV proteins such as E1, E2, NS3/4A, NS4B and NS5A are involved in induction of oxidative stress in hepatocytes [7]. Replication of HCV or expression of core proteins lead to mitochondrial alterations accompanied by massive ROS production due to inhibition of complex 1 activity of ETC [73]. Mitochondrial dysfunction could be due to core induced increase of prohibitin expression, a mitochondrial chaperone that regulates expression of complex IV and possibly complex I of ETC (2013_Alexander V.

Ivanov et al., 54). In chronic HCV patients, mitochondrial dysfunction was observed in hepatocytes and other cell types including lymphoma cells, lymphocytes (PBMCs) [74].

ROS induction by HCV has been evidenced by measurement of ROS, antioxidant levels, measurement of expression and activity levels of antioxidant defence enzymes and interaction products of ROS with biological molecules in liver biopsies, as well as blood samples or blood cells of chronic HCV patients. ROS concentrations were increased two to five fold in liver tissues and lymphocytes of chronic HCV patients [68,75]. In addition, elevated levels of Pro-oxidant capacity or clastogenic score i.e., ability to modify DNA in plasma were reported in patients [8,76]. Decreased levels of antioxidant defence enzymes such as manganese or Cu/Zn SOD, CAT and GSH reductase / peroxidase (GSH-Px) were reported in PBMCs in chronic HCV patients, though increased levels are also reported. However, no changes were observed in the expression of the same enzymes in the liver of the same patients, suggesting that alterations in PBMCs could be a secondary event [77]. HCV patients have displayed significant higher values of lipid peroxidation, advanced oxidation protein products and 8-hydroxyguanosine (8-OHdG) levels in PBMC compared to healthy controls [78,79]. Higher 8-OHdG was also detected in liver samples of chronic HCV patients [80].

ERis actively involved in induction of ROS in cytoplasm as excess production of viral proteins in the infected cell induces unfolded protein response (UPR) accompanied by release of calcium to cytosol [81]. Released calcium is quickly absorbed by the mitochondria resulting in elevation of ROS. Normally, cells have sufficient capacity to combat ROS using antioxidant systems thus preventing tissue damage [72]. However, during an infection there

is a disruption in the balance that can induce accumulation of ROS and oxidative stress. Induction of ROS production by HCV is activated through calcium redistribution between ER, cytoplasm and mitochondria. Evidence reports that HCV core protein increases mitochondrial Ca^{2+} uniporter activity. Furthermore, core protein and NS5A expressing cells deplete ER Ca^{2+} reserves causing rise of cytoplasmic Ca^{2+} concentration by inhibition of SERCA and induction of passive leak of calcium ions respectively [82,83]. Several antioxidant agents (GSH, S-adenosil-methionine and thioredoxin) and antioxidant enzymes (SOD, CAT, GSH-Px and heme oxygenase-1) are involved in the protection of cell against oxidative stress damage [84,85].

In HCV, the infected cell family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are an additional source of ROS. Calcium signalling activates several NADPH oxidases [86]. Family of NADPH oxidase contains seven transmembrane enzymes such as NADPH-oxidase 1-5 (NOX1-5), Dual oxidase 1,2 (DUOX1,2) involved in ETC and thus produce superoxide anion or H2O2 [87]. Evidence suggests that NOX 1 and 4 may act as primary source of superoxide anion and contribute to producing H2O2 in HCV infected cells. It was shown that the HCV core is the main regulator of NOX4 expression via transforming growth factor beta-1. In HCV infected cells special emphasis on NOX in induction of oxidative stress is given due to their ability to produce ROS in the nucleus, which leads to the formation of DNA damage. Increased expression of NOX4 and elevated levels of superoxide levels in nucleus are reported in HCV infected hepatocytes [88,89].

Another potential contributor of ROS in HCV infected cells is ER-residing Cytochrome P450 Family 2 Subfamily E Member 1 (CYP2E1) [90,91]. It was reported that expression of CYP2E1 is

enhanced in the livers of chronic HCV patients with early stage fibrosis [92]. In addition, HCV infection or expression of the individual core proteins E1, E2 and NS4B increase ROS production by induction of ER Stress and UPR [93].

Data reports that in order to promote replication, HCV proteins can alter several intra cellular signalling pathways by finely regulating the oxido-reductive state of the host cell. HCV replication is critically controlled by MAPK and phosphoinotsitide-3 kinase (PI3K) signalling pathways that in turn are modulated by phosphorylation cascades and oxidative stress. Oxidative stress plays a central role in HCV induced liver damage by ROS production from HCV infected cells and infiltrating immune cells. In addition, HCV core protein directly interacts with mitochondria and reduces the complex 1 activity of ETC by decreasing the mitochondrial NADPH levels. Overexpression of HCV core protein diminished the levels of GSH and induced the levels of Glutathione disulphide [12,94]**.**

1.6. Immune cell response in HCV infection

Immune system is a complex network that helps the body to fight infections and other diseases. It can be divided into innate and adaptive immunity based on their response to the pathogen. Adaptive immunity is mediated by B and T lymphocytes. Both the immune components act together and in parallel with regulatory signalling molecules known as cytokines and chemokines to eradicate the pathogen. Innate immunity is the first line of defence against invading pathogens. Innate immunity is mediated by macrophages, natural killer (NK) cells, dendritic cells and granulocytes (Figure 4). NK cells act by perforin secretion and cell lysis of infected cells whereas Macrophages, granulocytes, and dendritic cells act as antifungal, anti-parasitic and antimicrobial by their endocytic and

phagocytic activity [95,96]. Further, by their antigen presenting property they uptake, process and present the antigens to major histocompatibility complex class II to CD4+ T cells. They stimulate the adaptive immune response by secretion of proinflammatory cytokines such as interleukin (IL)1, IL6, IL12, IFNs α/β and chemokines such as IL8. CD4+ T lymphocytes up on activation differentiate into T helper (Th)-1 and Th-2 cells and T cytotoxic cells and B lymphocytes into plasma cells (antibody- specific cells). These important cellular events are mediated by signals delivered by toll-like receptors through recognition of microbial or viral proteins, nucleic acids and mediated by release of different cytokines such as IFN- γ, Tumour necrosis factor-α (TNF-α), IL-10 [95-97].

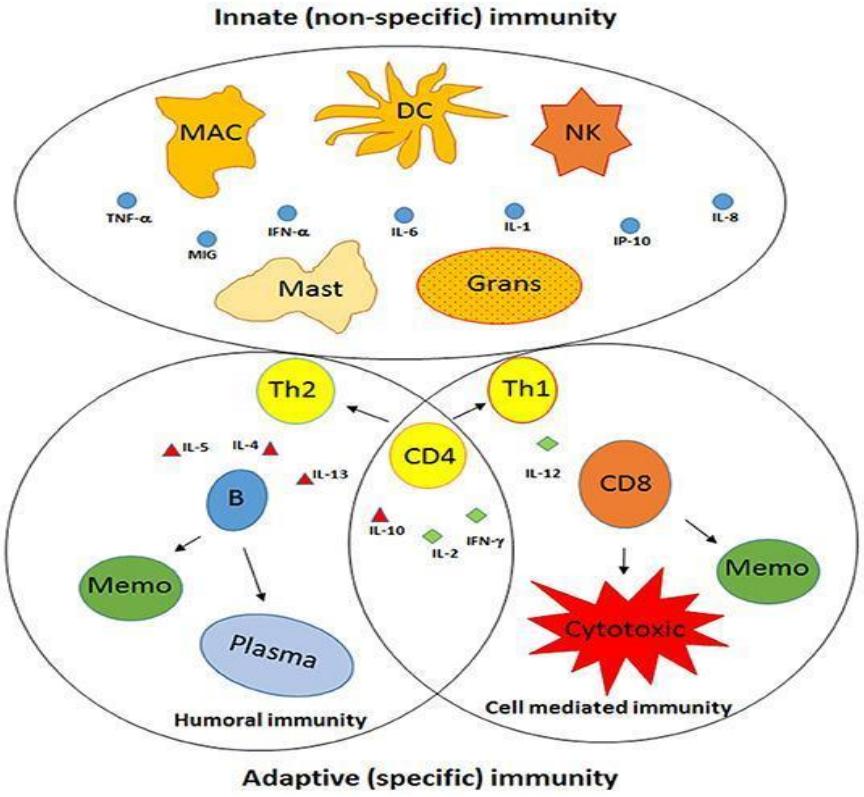

Figure 4. Components of innate and adaptive immunity (Alhetheel AF. etal., 2022).

Cytokines are a large group of signalling molecules that act through cell surface receptors produced in response to innate and adaptive immunity. Cytokines are produced by a vast variety of cells like macrophages, B lymphocytes, T lymphocytes mast cells, as well as endothelial cells, fibroblasts and stromal cells [96]. Initially defined lymphokines or monokines were later referred to as cytokines to more correctly indicate that all nucleated cells are able to produce and respond to them. They are not hormones or growth factors because unlike hormones they are not secreted by one specific cell type and above all they are secreted only in response to precise cellular signals but not in a constitutive way. Cytokines include chemokines, IFNs, ILs, lymphokines and tumour necrosis factors. They are divided into pro-inflammatory and anti-inflammatory cytokines depending on their response activation function such as inflammatory or control of the inflammatory response. Pro Inflammatory cytokines are involved in upregulation of inflammatory reactions that include IL-1α and 1β, IL-2, IL-6, IL8, TNF-α, IFN γ. while IL belong to the group of anti-inflammatory cytokines such as IL-4, IL-10 control the proinflammatory response [98].

Chemokines play an important role in the pathogenesis of chronic HCV related liver diseases. HCV infection causes inflammation through binding and interaction of HCV RNA and protein components with pattern recognition receptors (PRRs) or other cellular structures of the host. Immune or inflammatory response is activated upon interaction of viral proteins with PRRs such as toll-like receptors (TLRs)1-10. Proinflammatory cytokines are produced via several viruses derived PAMPs, HCV RNA and viral proteins. For instance, MAPK TLR3 signalling by HCV double stranded RNA during HCV replication releases high amounts of IL8 [99]. TLR7

mediated signalling induces pro-IL1b mRNA expression [100], TLR2 mediated inflammatory pathway is triggered by conformation mediated interaction with HCV TLR core or NS3 proteins [101]. TLR4 mediated signalling by NS5A protein activates signal transduction cascades and stimulates secretion of IFN-β and IL6 through activation of NF-κB in both hepatocytes and B cells [102]. In addition to PRRs, HCV protein E2 binds with other cellular structures such as CD81 and activates MAPK to secrete Chemokine (C-C motif) ligand 5 (CCL-5) [103]. HCV NS5B protein activates immune signalling via TANK binding kinase and NF-κB to produce IFNs and inflammatory cytokines [104]. In short, uncontrolled and excessive inflammatory response is triggered by interaction of HCV components with host cells and exacerbates the progression of disease.

PBMC plays an important role in clearing pathogens that include monocytes (precursor of macrophages) and lymphocytes (NK cells, CD4+ cells, CD8+ cells, B cells). Evidence shows that HCV causes infection in hepatocytes and other cell types such as bone marrow cells and PBMCs [105,106] through interaction with CD81 molecules on the cell surface [107]. In sustained responders viral RNA is detectable in PBMCs for upto 5 years despite the disappearance in serum. PBMCs are important sites of HCV replication and are proposed to be main extrahepatic sources of reinfection [108,109]. Persistence of HCV infection in PBMCs results in chronic immune activation and immune system exhaustion [110,111]. PBMCs play a crucial role in elimination of pathogen by phagocytic and cytolytic activities through regulation of different signalling pathways such as janus kinase/ signal transducer and activator of transcription (JAK-STAT), PI3K and MAPK pathways in hepatocytes and macrophages [112,113].

1.7. Current antiviral targets

Earlier, HCV was treated with immunomodulatory agents such as IFN and pegylated IFN (pegIFN) to attain SVR which is defined as undetectable levels of HCV RNA at least twelve (SVR12) or 24 weeks (SVR24) after the cessation of the treatment [114]. Guanosine nucleoside analogue, Ribavarin was also used along with IFN therapy for HCV treatment [2]. However, these therapies have severe side effects and SVR is maintained in only 40-50% of the patients [115]. Lack of efficacy, genotype resistance to treatment, and side-effects of pegIFN/RBV prompted the search for new HCV drugs and the development of DAA agents. With the development of DAAs, treatment of HCV has evolved to target specific components of HCV that are involved in replication and processing to achieve SVR [116].

1.7.1. NS3/4A Inhibitors:

These are the first DAAs introduced into the market targeting the protease domain of NS3. The protease activity of NS3 plays a crucial role in maturation of viral polyprotein by cleaving peptide junctions into non-structural proteins together with NS4A cofactor. NS4A acts as an important cofactor for NS3 enzymatic activities by localising NS3 in the membrane and helps in holding the HCV replication complex at the cellular membrane [117,118]. These inhibitors can affect several functions of complexes such as NS3, NS4 and different domains of NS3, allowing inhibition of multiple steps by targeting a single molecule [119,120]**. T**hese molecules are considered as protease inhibitors and are suffixed with –previr. In the beginning of the DAA era, the first antivirals that came into the market are boceprevir and telaprevir followed by simeprevir. These were peptidomimetic linear ketoamides that bind to the active site of protease domain of NS3. However, due to their severe side effects, low genetic barrier and restriction to genotype 1 resulted in

discontinuation of their use in most countries [121]. Further research in the development of new protease inhibitors resulted in increased potency and improved genetic resistance barriers such as Glecaprevir, paritaprevir, grazoprevir and voxilaprevir Improved efficacy shown by the combination therapy of these molecules with other viral protein targeting DAAs allowed the development and approval of IFN free regimen treatment with lower side effects and high SVR [122].

1.7.2. NS5B inhibitors

Two types of NS5B inhibitors were developed that target the catalytic or non-catalytic sites of the protein. The first molecules developed against NS5B targets belong to a class of nucleo(s)tide inhibitors that bind to the catalytic site of the enzyme [42]. These inhibitors terminate RNA synthesis by mimicking the natural substrate of the enzyme that are incorporated into the nascent RNA chains. These molecules are suffixed with –buvir. In 2013, FDA approved the first NI Sofosbuvir, a uridine analogue followed by dasabuvir. Sofosbuvir is involved in the catalytic site of HCV RNA polymerase which is highly conserved between genotypes, suggesting the pan genotypic potential of the drug with a minimal risk of emergence of viral resistance. Sofosbuvir is the commonly used antiviral agent in most HCV treatment combinations due to its favourable safety-tolerability profile, high genetic barrier resistance and its activity against most genotypes [123].

The second class of NS5B inhibitors include non-nucleoside inhibitors that do not bind to the catalytic site of the enzyme [124]**.** They exert their effect by inhibiting the conformational changes required for the polymerase activity. The only FDA approved NNI till date is Dasabuvir that targets NS5B and is recommended only for

HCV genotype 1**.** NIIs show lower barriers to resistance and poor pangenotypic activity as they target poorly conserved sequences [125]. However, when used in combination with more potent drugs it improves the efficacy and reduces the duration of the treatment.

1.7.3. NS5A inhibitors

NS5A is a potential antiviral target essential for viral RNA replication and viral assembly and genome transfer to assembly sites. NS5A interacts with many viral and cellular proteins including itself to form dimers or oligomers by existing in different phosphorylated forms. NS5A inhibitors are suffixed with –asvir [126]. Daclatasvir, ledipasvir and ombitasvir are first-generation drugs. Declatasvir inhibits HCV replication probably by inhibiting the formation of double membrane vesicles essential for viral genome replication or acting on assembly [127,128] or by preventing the delivery of RNA to the core protein. Though the exact mechanism of inhibition of NS5A is still not known, it seems to act on multiple steps of the viral life cycle, augmenting its effects. The second generation NS5A inhibitors include elbasvir, velpatasvir and pibrentasvir. These molecules have lower toxicity than first generation drugs and are optimised to be pan-genotypic [42].

2. AIM OF THE STUDY

The main objective of the study was to investigate the impact of DAAs treatment on circulating redox markers of oxidative damage and antioxidant defense and restoration of mitochondrial function and energy homeostasis in peripheral blood mononuclear cells of chronic HCV patients.

3. MATERIALS AND METHODS

3.1. Study population and design

We conducted an observational study on 196 HCV+ and HIV- patients who started therapy with DAAs as standard-of-care treatment for HCV-related hepatitis. All patients were referred to the C.U.R.E. (Centro per la Ricerca e la Cura delle Epatopatie) at the University of Foggia, Italy. Patients with concurrent active cancer and other chronic diseases or taking antioxidant supplementation were excluded. Fifty-seven percent of patients were females and the median age was 63 years (range 35–90 yrs).

Peripheral whole blood samples were collected from patients at multiple time points, including at baseline on the day of starting therapy (T0), after 4 weeks of drug administration (4wks), on the day of the final treatment (EoT), and at 12 weeks post-treatment (sustained virological response, SVR12). Patients with all the samples collected at baseline, during (4wks) and at the end of treatment (EoT), and after the treatment (SVR12) were considered for the final analysis. Liver fibrosis was assessed by transient elastography, performed with the Fibroscan® (Echosense, Paris, France) medical device, using the M or the XL probe after overnight fasting following standard requirements of the Echosense [129]. Stiffness cut-off values reported by Arena et al. were used for staging liver fibrosis during transient elastography [130]. 7.8 KPa, 10.8 KPa and 14.8 KPa were used as cut-off points for mild fibrosis, significant fibrosis and cirrhosis, respectively. The Authors' institutional review board approved the clinical investigation, which was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all patients to participate in the study. Hepatitis C treatment

antiviral therapy and treatment duration (12 or 24 weeks) were indicated for each patient according to the viral genotype/ subtype and the severity of liver disease, according to European Medicine Agency and National Drug Agency reimbursement restrictions.

Patients received one of the following regimens: (for observational study)

1. sofosbuvir + simeprevir;

2. sofosbuvir/ledipasvir;

3. sofosbuvir + daclatasvir;

4. ombitasvir/paritaprevir/ritonavir ± dasabuvir (3D);

5. glecaprevir/pibrentasvir

Real-time polymerase chain reaction was used for HCV-RNA quantification (Abbott RealT*ime* HCV Amplification Reagent Kit on Abbott Automatic m2000 system), with a lower detection of 12 IU/mL. Patients were followed up monthly with clinical and laboratory evaluations during antiviral therapy. Virologic response was assessed at week 4, at the end of treatment, and at 12 weeks after the end of treatment to determine the SVR. A rapid virologic response (RVR) was defined as undetectable HCV-RNA after 4 weeks of therapy. SVR12 was defined as undetectable HCV-RNA 12 weeks after the treatment completion. All patients included in this study successfully cleared the virus at the end of therapy with subsequent attainment of SVR. The serum levels of alanine aminotransferase and aspartate aminotransferase were also normalised in most of these patients following HCV clearance.

3.2. Laboratory Measurements

3.2.1. Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood samples were obtained from patients with an

overnight fasting at all the study points. After collection within 1-2 h, the whole fraction of intact PBMCs were isolated from Ethylenediamine tetraacetic acid **(**EDTA)-blood by density medium centrifugation at 25 °C, using HistopaqueR1077 Hybri-Max TM (Sigma) following a standard protocol. Briefly, 6 ml of blood was layered on top of a 3 ml of Histopaque and centrifuged at 800 × g for 30 min at 25 °C. The separated mononuclear band was carefully collected (~1 ml), resuspended in 10 ml PBS and centrifuged again at 800 × g for 20 min. The pellet was washed twice in the same conditions. Finally, the dry pellet was stored at −80 °C until use.

For respirometry, enzymatic and protein analyses samples from identical isolation of cells were used.

3.2.2. Measurement of cytokines

Cytokine measurement was performed using Biochip Array Technology (BAT) by Randox Laboratories. The array was used to measure serum levels of IL-2, IL-6, IL-8, IL-10 and TNF-α. The assay was performed using Randox apparatus according to manufacturer's recommendations. Samples were tested in duplicate, and expressed as the mean of two measurements.

3.2.3. Measurement of serum levels of HNE and MDA adducts

Serum fluorescent adducts formed between peroxidation-derived aldehydes and proteins such as 4-hydroxynonenal (**HNE**) and malondialdehyde (MDA) were measured by spectro-fluorimetry as previously reported [131].

3.2.4. Measurement of serum levels of 8-OHDG

The concentration of 8-OHdG in serum was analysed using a highly sensitive 8-OHdG enzyme-linked immunosorbent assay (ELISA) monitoring kit (Jaica, Fukuroi, Japan), according to the manufacturer's instructions [132].

3.2.5. Measurement of Antioxidant levels in PBMC

SOD, CAT and GSH-Px activities were analysed in PBMC by spectrophotometry method following the respective Cayman Assay kits procedures: SOD (n° 706002), CAT (n° 707002), GSH-Px (n° 703102).

3.2.6. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated from PBMC samples using the RNAeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. Samples were quantified by absorption spectrophotometry, and RNA integrity was confirmed using nondenaturing agarose gel electrophoresis. cDNA was obtained using a random hexamer primer and a SuperScript III Reverse Transcriptase kit as described by the manufacturer (Invitrogen, Frederick, MD, USA). A PCR master mix containing the specific primers (SOD1): forward, TGT GGG GAA GCA TTA AAG G; reverse, CCG TGT TTT CTG GAT AGA GG; (CAT) : forward, GCC ATT GCC ACA GGA AAG TA; reverse, CCA ACT GGG ATG AGA GGG TA; (GSH-Px) : forward, GGA GAC CTC ACC CTG TAC C; reverse, GTC ATT CAC CAT GTC CAC C; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, CAA GGC TGA GAA CGG GAA; reverse: 59-GCA TCG CCC CAC TTG ATT TT-39) was added, along with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). Real-time quantification of mRNA was performed with a SYBR Green I assay and evaluated using an iCycler detection system (Bio-Rad Laboratories). The threshold cycle (CT) was determined, and the relative genes expression was subsequently calculated as follows: fold change = $2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT_{target} - CT_{housekeeping}$ and $\Delta(\Delta CT) = \Delta CT_{treated} - \Delta CT_{control}$.

3.2.7. Mitochondrial respiration and glycolytic activity quantification

In order to quantify mitochondrial respiration and glycolysis, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) respectively were measured, using a Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Biosciences, Billerica, MA) according to the manufacturer's instructions. Experimental setup for PBMCs was based on a previous work of Jones et al (10), with minor adjustments. Cells were plated at $0.1x10^6$ cells/well of a 96-well Seahorse plate. A utility plate containing sterile water (200 μl/well) together with the cartridge was placed in a CO2-free incubator at 37°C overnight. The day after, the water was replaced with a calibrant solution and, after 1 h incubation at 37° C in a CO2-free incubator, the utility plate with the cartridge containing injector ports and sensors was run on the Seahorse for calibration. Cells were sequentially treated with inhibitors: 0.5 μM oligomycin, 1 mM carbonyl-cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), 0.5 μM rotenone/antimycin A, and 50 mM 2- deoxyglucose. Analysis was performed using Wave software.

3.2.8. DNA isolation, DNA integrity and mtDNA copy number

Total DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's protocols with minor modifications. mtDNA and nDNA damage was determined using the DNA damage assay described by Wang et al (11). Briefly, a qPCR was performed, using 6 and 30 ng DNA for mtDNA and nDNA analysis, respectively. Primers for MT-RNR1 and NDUFA9 were used to represent mtDNA and Ndna respectively (MT-RNR1: forward, AAA CTG CTC GCC AGA ACA CT, reverse, CAT GGG CTA CAC CTT GAC CT; NDUFA9: forward, GCA AGG GTC CCT ATG AGA GAA, reverse, CAA GAA CGA GGG GAA AAG TG). A total volume of 20μl containing 50 ng of total DNA, 5%

DMSO, 1 × GeneAmp® PCR Buffer II, 1.25 mM MgCl2, 2.5 mM dNTP, 0.5 μM forward and reverse primers and 0.075 U/μl AmpliTaq Gold® DNA polymerase and the following program was used: 94 °C for 5 minutes then for 40 cycles, 94 °C for 30 seconds, 70 °C for 30 seconds, 72 °C for 30 seconds and finally 72 °C for 3 minutes. mtDNA copy number was determined by real- time qPCR quantification of the nuclear (HBB) and mitochondrial (MT-RNR1) templates.

3.2.10. Western blot Analysis

PBMC pellets (about 20 μl) were re-suspended and incubated on ice in 2.5 × their volume of Radioimmunoprecipitation assay buffer **(**RIPA) buffer (50 mm Tris/HCl (pH 7.4), 150 mm NaCl, 1 mm PMSF, 1 mm EDTA, 1% Triton X-100, 1% Sodium Dodecyl Sulfate **(**SDS) (v/v) and 1% (v/v) Protease Inhibitor Cocktail (Sigma-Aldrich) for 20 minutes, vortexed every 5 minutes and then centrifuged for 20 min at 51,000 × g (4 °C). The protein concentration in supernatant was determined and proteins (20 μg per well) and a molecular weight marker (See Blue Plus2 Prestained Standard, Invitrogen) were separated by 12% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)54 (Mini-Protean System (Bio-Rad)). Protein was mixed with loading buffer (50 mm Tris/HCl (pH 6.8), 12% (v/v) glycerol, 4% SDS, 2% (v/v) 2-mercaptoethanol and 0.01% (w/v) Bromphenol Blue) for 30 minutes at 37 °C. Proteins were subsequently semidry electroblotted onto polyvinylidene difluoride **(**PVDF) membranes (Merck), membranes air-dried overnight, rinsed with 100% methanol (v/v) and blocked in TBS (Tris-Buffered Saline) with 5% non-fat dried milk for 1 hour. Membranes were incubated with primary antibodies (see below) in TBS containing 0.1% (v/v) Tween-20 and 2% non-fat dried milk overnight at 4 °C. Secondary detection was carried out with peroxidase conjugated secondary antibodies (Sigma-Aldrich) in

TBS containing 0.1% (v/v) Tween-20 and 2% non-fat dried milk for 1 hour. The proteins were then visualized with the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) using Syngene Imaging System and the intensity of signal was quantified with the Quantity One 1-D Analysis Software (Bio-Rad) for further analysis.

Primary antibodies: Complex III subunit Core 2 monoclonal antibody MS304/D1129 (1:10000); Complex II subunit 30 kDa Ip monoclonal antibody MS203/D1205 (1:2000); Complex I subunit NDUFB6 monoclonal antibody MS108 (1:2000), all Mitosciences; Anti-ATPB antibody (3D5) - Mitochondrial Marker (Complex V beta subunit) ab14730 (1:1000); Ms mAb to MTCO2 (12C4F12, Mitochondrially Encoded Cytochrome c Oxidase subunit 2) ab10258 (1:10000); Anti-beta-actin antibody ab8227 (1:3333); Anti-Citrate synthetase antibody [2H8BB6] ab128564 (1:2500) - all Abcam. Secondary antibodies: Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in goat A8924 (1:2500); Anti-Rabbit IgG (whole molecule)-Peroxidase antibody produced in goat A0545 (1:2500), Sigma-Aldrich.

3.2.11. Respiratory chain complexes activity

Dry pellets of isolated PBMCs were resuspended in PBS to a protein concentration of approximately 3-4 mg/ml, determined by bradford assay. The specific activity of NADH-decylubiquinone oxidoreductase (NQR) (complex I), succinate decylubiquinone DCPIP (2,6-dichlorophenolindophenol) reductase (SQR) (complex II) and cytochrome c oxidase (complex IV) were measured using a Beckman Coulter Spectrophotometer. Briefly, to disrupt the mitochondrial membranes for complex I measurement 100 µg of the cell suspension was first lysed by 3 min incubation in distilled water.

Rotenone- sensitive complex I activity was measured in assay medium with final volume of 1 ml (50 mM TRIS, pH 8.1, 2.5 mg/ml Bovine serum albumin (BSA), 0.3 mM Potassium cyanide **(**KCN), 0.1 mM NADH, 50 μM decylubiquinone without and with 3 μM rotenone) and followed the decrease in absorbance at 340 nm due to the NADH oxidation (ε = 6.22 mM−1 cm−1).

Complex II activity was measured in 1 ml of assay medium containing 10 mM potassium phosphate pH 7.8, 2 mM EDTA, 1 mg/ml BSA, 200 μg of cell protein 0.3 mM KCN, 10 mM succinate, 3 μM rotenone, 0.2 mM ATP, 80uM DCPIP , 1 μM Antimycin, 50 μM decylubiquinone. The decrease in absorbance at 600 nm due to the oxidation of DCPIP at 600 nm (ε = 20.1 mM−1 cm−1) was recorded.

For determination of complex IV activity, the cell suspension was solubilized by incubation with 1.5% n-dodecyl-β-D-maltoside for 15 minutes at 4 °C, then centrifuged for 10 minutes at 10,000 g and resulting supernatant was used for assay activity. Complex IV activity was measured by incubating of 100 μg of cell supernatant protein in 1 ml of assay medium (40 mM potassium phosphate, pH 7.0; 1 mg/ml BSA; 25 μM reduced cytochrome c) by following the oxidation of cytochrome c (II) at 550 nm (ε = 19.6 mM−1 cm−1). All assays were performed at 37 °C.

3.3. Statistical analysis

Data were expressed as count and percentages for qualitative values, and as mean ± standard deviation of the mean (SDM) for quantitative variables. Gaussian distribution of the samples was evaluated by Kolgomorov–Smirnov test. Temporal trends of laboratory markers were studied using the one-way analysis of variance (ANOVA). Each analysis was conducted for the whole study population. Further

analyses were performed comparing patients with no or mild fibrosis (F0/F1) vs significant fibrosis (F3/F4), according to the METAVIR scoring system [133], genotype 1 vs genotype non-1, sofosbuvir-based vs sofosbuvir-free treatment, RVR vs non-RVR protease-inhibitor (PI)-based vs PI-free treatment. The significance of differences between different groups at each time studied was assessed by the two-way analysis of variance (ANOVA), to test the main effects of time and fibrosis/genotype/treatment/response as between-subject factor; the interaction time × fibrosis/genotype/treatment/response was studied, and a Sidak test was applied as post hoc test for multiple comparisons. A linear regression model was used to analyze the association between changes in serum HCV-RNA or TNF-α and circulating markers of oxidative stress (HNE- and MDA-protein adducts, 8-OHdG). All tests were 2-sided, and P values < 0.05 were considered statistically significant. Statistical analysis was performed with the Statistical Package for Social Sciences version 23.0 (SPSS, Inc., Chicago, IL) and the package Graph- Pad Prism 7.0 for Windows (GraphPad Software, Inc., San Diego, CA). Microarray data statistical analysis was performed using the GeneSpringGX14.9 Software. Lowly expressed genes were filtered out using a 10-percentile cut-off. Paired T test was carried out to identify differentially expressed genes between conditions (FDR=0.1; FC>2). To assess the biological relationships among the top selected transcripts, we used the IPA software (http://www.ingenuity.com) as previously described (12, 13).

4. RESULTS

4.1. Baseline patient characteristics

Baseline characteristics of patients are shown in Table 1. Nearly half of the study population (45%) had baseline ALT levels ≤ 1.5 x ULN. The median baseline log10 HCV RNA level was 5.58 IU/mL (range 2.3–6.9 IU/mL) and genotype 1 was the most frequently detected (61%). 96 patients (49%) were treated with sofosbuvir-based regimens while 100 patients (51%) were treated with sofosbuvir-free regimen. All study participants reached SVR at the end of the treatment and 6 relapses were observed after treatment. RVR was observed in 61 (31%) patients.

HCV viremia during antiviral treatment and after the end of treatment is shown in Figure 5.

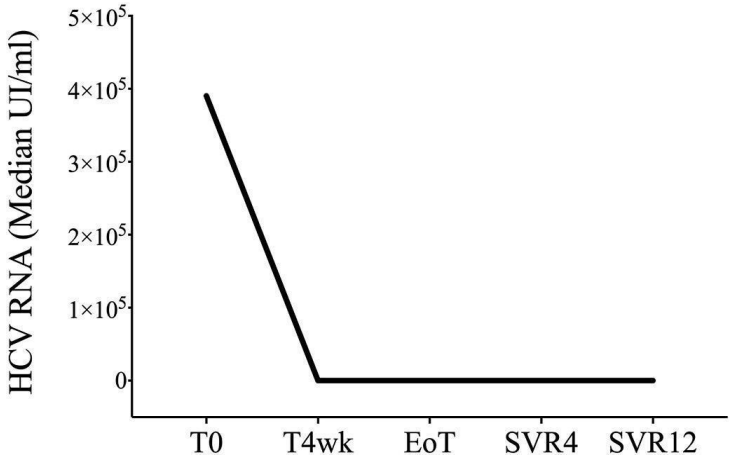

Figure 5. HCV viremia during treatment and after the end of treatment. At week 4 DAAs induce a rapid decrease in median HCV RNA levels

Table 1: Graphic and clinical characteristics of the study population.

variable	N = 196	
Age (years)	63 (35-90)	
Gender (M/F)	112 (84%) / 57 (43%)	
Cirrhosis	94 (48%)	
	Child-A	76 (81%)
	Child-B	18 (19%)
HCV genotype	1a/1b	120 (61%)
	2	38 (20%)
	3	20 (10%)
	4	18 (9%)
DAAs treatment	Sofosbuvir-based regimen	96 (49%)
	Sofosbuvir-free regimen	100 (51%)
DAAs treatment	Sofosbuvir + simeprevir	19
	Sofosbuvir/ledipasvir	13
	Sofosbuvir + daclatasvir	21
	Sofosbuvir + ribavirin	29
	Sofosbuvir + velpatasvir	14
	Ombitasvir/paritaprevir/ritonavir ± dasabuvir	42
	Glecaprevir/pibrentasvir	58
Treatment duration	24 weeks	59 (30%)
	< 24 weeks	137 (70%)

Liver function test	ALT ≤ ULN	88 (45%)
	ALT > 2 ULN	45 (23%)
Log10 HCV-RNA (UI/mL)	5.58 (2.3–6.9)	
Previous treatment	PegIFN + ribavirin	53 (27%)
	DAAs (1st or 2nd generation) Naïve	10 (5%)
	Naïve	133 (68%)
Cryoglobulinemia	10 (5%)	
Diabetes	47 (24%)	
RVR	61 (31%)	
HCV-RNA undetectable at the EoT	196 (100%)	
Sustained Virological Response (SVR)	190 (97%)	

DAA, direct antiviral agent; ALT, alanine aminotransferase; ULN, upper limit of normal; PegIFN, pegylated interferon.

4.2. Effect of antiviral treatment on circulating redox balance

To assess the impact of DAA treatment on systemic redox balance, we measured circulating markers of oxidative stress (such as serum proteins oxidatively modified by lipoperoxidative reactions, as well as oxidatively damaged DNA), and the serum antioxidant capacity. We observed a significant decrease of both HNE- and MDA-protein adducts in the serum of patients at the end of treatment, which persisted 12 weeks after treatment discontinuation (Figure 6A and 6B). Serum levels of 8-OHdG were reduced at 4 weeks after

initiation of therapy and persisted low at the end of treatment and at SVR12 (Figure 6C). On the other side, we did not notice any significant change in the serum trolox equivalent antioxidant capacity at any time of the study. Taken together, these results suggest that the improvement in circulating redox balance was observed in HCV patients treated with DAAs is associated with a reduction in the oxidative injury to proteins and DNA, even though we did not describe an increased systemic reactive species scavenging ability.

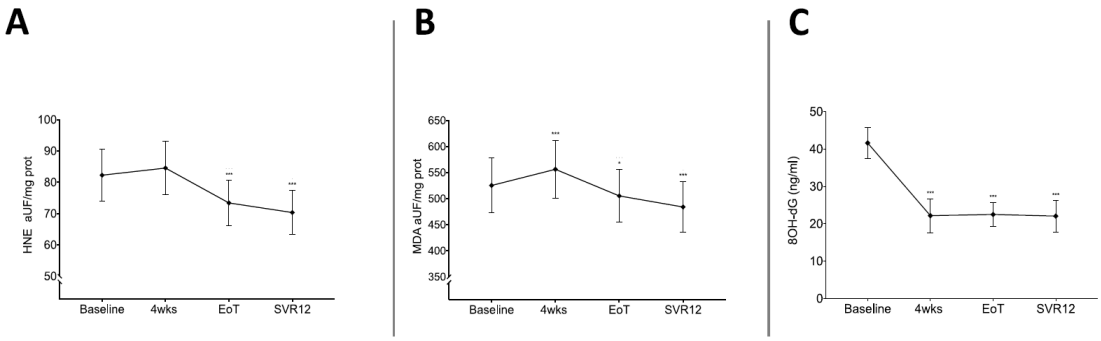

Figure 6. Hydroxynonenal (HNE) and malondialdehyde (MDA) – protein adducts (panels A and B), and 8-hydroxydeoxyguanosine (8-OHdG, panel C) serum levels at baseline (T0), during antiviral treatment (4wks and EoT) and after the end of therapy (SVR12). Data are expressed as mean standard error of the mean. Statistical differences were assessed by the one-way analysis of variance for repeated measures and the Tukey as post hoc test. * = $p < 0.05$ vs T0; *** = $p < 0.001$ vs T0; ▪ = $p < 0.05$ vs 4wks; T0, baseline; 4wks, after 4 weeks of therapy; EoT, end of treatment; SVR12, 12 weeks after the end of treatment.

4.3. Effect of antiviral treatment on circulating inflammation cytokines

Cytokine profile during and after treatment is reported in Figure 7. IL-6 and IL-8 did not change across time points (Figure 7A and 7B) as compared to baseline whereas IL-10 and TNF-α normalised at the end of treatment onwards (Figure 7C and 7D). At the same time IL-10 significantly decreased from 1.53 pg/ml to 0.2 pg/ml ($p < 0.05$) and TNF-α from 4.24 pg/mL to 2.82 pg/mL ($p < 0.01$) and this was confirmed at SVR (IL-10 0.1 pg/ml ad TNF-

α 0.8 pg/ml, respectively). The same findings were confirmed in a subgroup analysis by treatment regimen used.

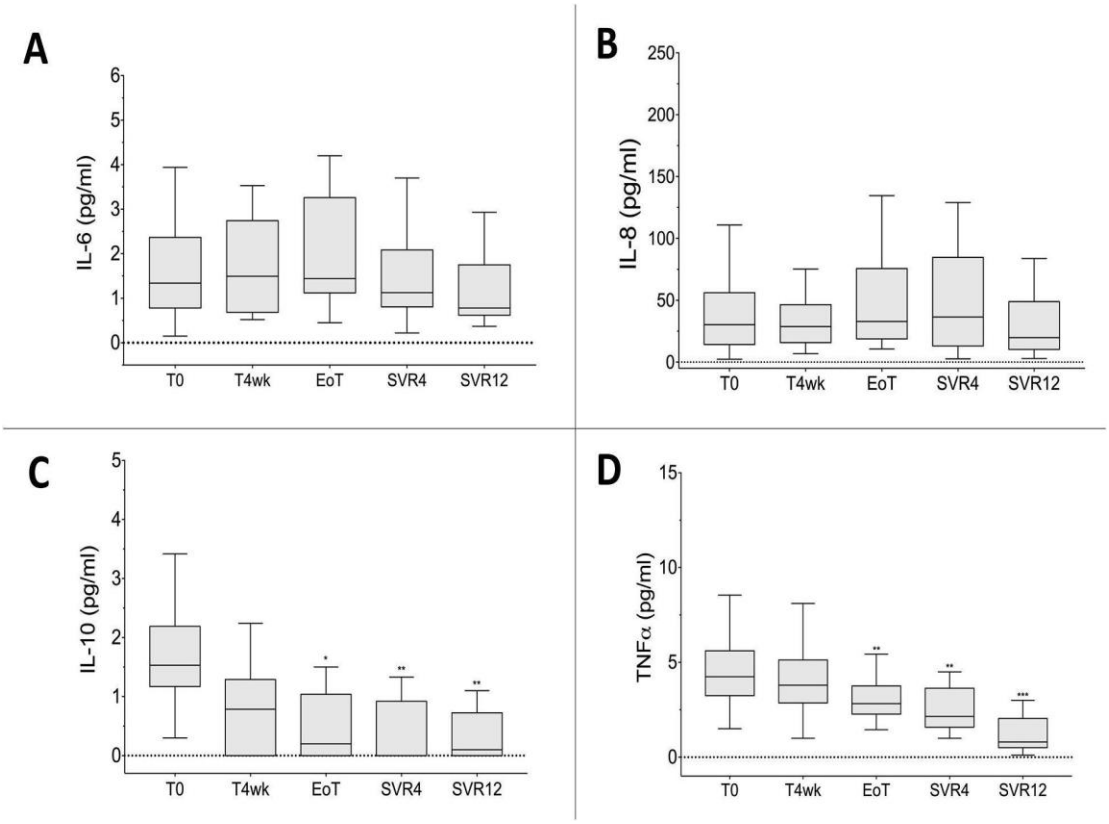

Figure 7. Cytokine levels at baseline, during antiviral treatment, and after the end of therapy. Interleukin-6 (A) and interleukin-8 (B) serum levels did not change over time. IL-10 (C) and TNF-α (D) concentration decreased progressively during treatment and reached statistical significance at the end of treatment. Values are shown as the median and interquartile range (IQR). Outliers are not reported. T0: baseline; T4wk: after 4 weeks of therapy; EoT: end of treatment; SVR4: 4 weeks after the end of treatment; SVR12:12 weeks after the end of treatment. *=p < 0.05vsT0;**=p < 0.01vsT0;***=p < 0.001vsT0.

4.4. DAA treatment restores redox balance by improving antioxidant enzymes activity

To better analyse the impact of DAA treatment on the circulating

redox balance, and to clarify the possible mechanism of reduced oxidative damage, the enzymatic activity of several antioxidant enzymes (such as SOD, CAT, and GSH-Px) was performed in PBMC extracted from patients at each time point. Of note, the SOD activity was reduced at 4 weeks after initiation of therapy, but it increased with respect to baseline at the end of treatment and persisted higher at SVR12 (Figure 8A). As compared to baseline, we observed an increase in both the CAT and the GSH-Px activity at 4 weeks after initiation of therapy, which continued to rise until the end of the study period (Figure 8B and 8C). We also evaluated the mRNA expression of antioxidant genes in PBMC. We noticed that the SOD gene expression followed a similar trend to its enzymatic activity during and after the DAA treatment (Figure 8D). On the contrary, gene expression of CAT and GSH-Px was in- creased after 4 weeks of DAA therapy and at the EoT with respect to baseline, but it decreased to baseline level at SVR 12 (Figure 8E and 8F).

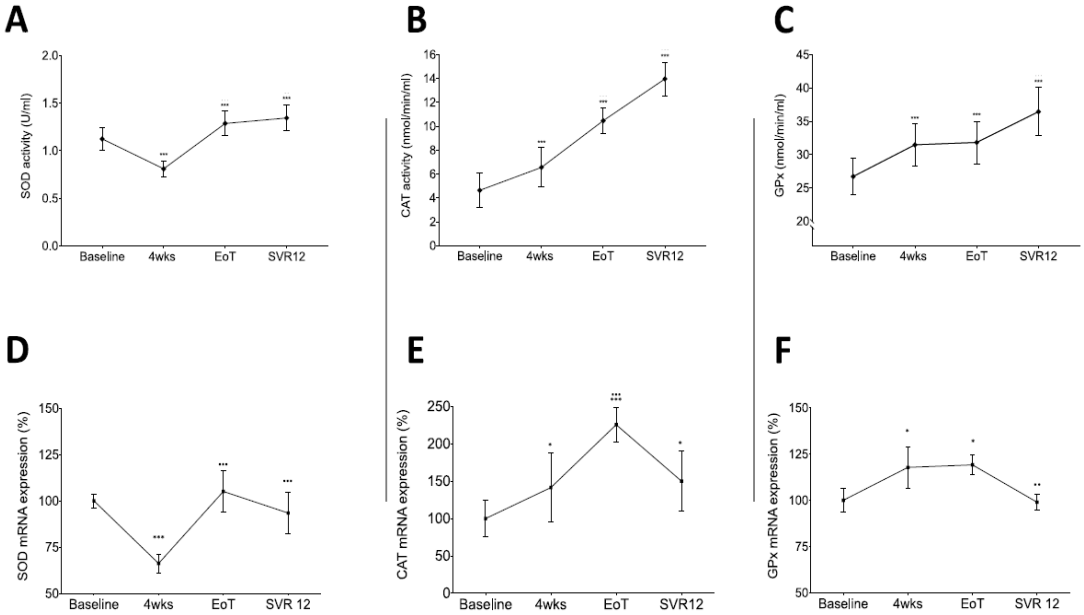

Figure 8. Superoxide dismutase (SOD, panel A), catalase (CAT, panel B) and glutathione peroxidase (GSH-Px, panel C) activities, and mRNA expression

(panels D–F) in peripheral blood mononuclear cells (PBMC) isolated from patients at baseline (T0), during antiviral treatment (4wks and EoT) and after the end of therapy (SVR12). Data are expressed as mean ± standard error of the mean. Statistical differences were assessed by the one-way analysis of variance for repeated measures and the Tukeyasposthoctest.*=p < 0.05vsT0;***=p < 0.001vsT0;·=p < 0.05vs4wks;··=p < 0.01vs4wks;···=p < 0.001vs4wks.T0,baseline;4wks, after 4 weeks of therapy; EoT, end of treatment; SVR12, 12 weeks after the end of treatment.

4.5 Association between HCV genotype, cirrhosis and treatment regimen on circulating redox markers

We then compared the impact of DAA treatment on markers of redox balance and antioxidant enzyme activities in patients affected by HCV genotype 1 versus genotype non-1, sofosbuvir-based versus sofosbuvir-free treatment, RVR versus non-RVR, and no/mild fibrosis (F0/F1) versus significant fibrosis (F3/F4). We did not find any significant difference between subgroups related to genotype, treatment regimen, or RVR. On the contrary, when the two-way ANOVA analysis was performed in subgroups of patients with different fibrosis stage, a significant effect of fibrosis resulted for the serum 8-OHdG levels ($F = 80$, $p < 0.0001$) and CAT activity ($F = 1162$, $p < 0.0001$), while an interaction effect (time × fibrosis) was observed for serum 8- OHdG ($F = 274.2$, $p < 0.0001$), SOD activity ($F = 139.2$, $p < 0.0001$), and CAT activity ($F = 602.4$, $p < 0.0001$). Of interest, we observed that the serum 8-OHdG level was markedly reduced in the F3/F4 group as compared with F0/F1 patients at the end of treatment and at SVR12 (Figure 9A). No differences were described for the circulating levels of both the HNE- and the MDA-protein adducts. We also observed that the SOD activity was higher in the PBMC from F3/F4 patients rather than F0/F1 ones at SVR12 (Figure 9B), but the CAT activity was higher in the F0/F1 group with respect to F3/F4 at baseline, at 4 weeks after initiation of therapy, and at SVR12 (Figure 9C).

A subgroup analysis including patients previously treated with

pegIFN was performed and we did not observe any significant differences in comparison to overall data.

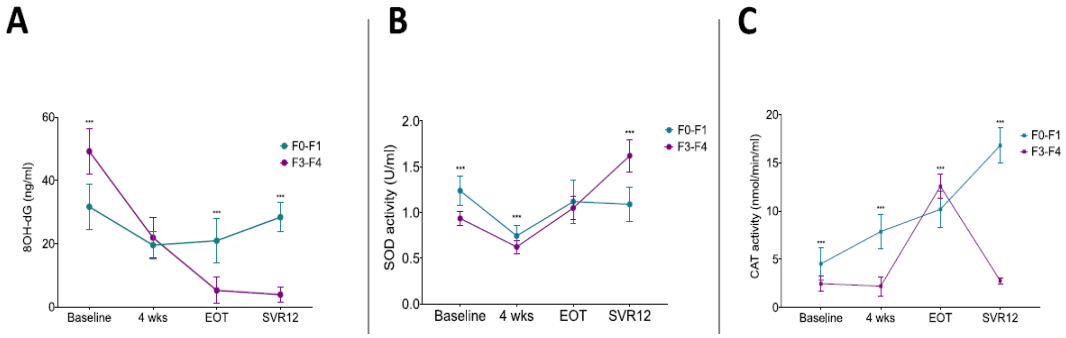

Figure. 9. Serum 8-hydroxydeoxyguanosine level (8OH-dG, panel A), superoxide dismutase activity (SOD, panel B), and catalase activity (CAT, panel C) in peripheral blood mononuclear cells, in patients at baseline (T0), during antiviral treatment (4wks and EoT) and after the end of therapy (SVR12), according to mild (F0–F1) or severe (F3–F4) liver fibrosis. Data are expressed as mean ± standard error of the mean. Statistical differences were assessed by the two-way analysis of variance for repeated measures, and the Sidak as post hoc test. *** = $p < 0.001$ F0–F1 vs F3–F4. T0, baseline; 4wks, after 4 weeks of therapy; EoT, end of treatment; SVR12, 12 weeks after the end of treatment.

4.6. Relationship among viremia, cytokine levels and circulating markers of oxidative stress

To assess the relationship between changes in viremia and redox balance during and after the DAA treatment, a linear regression model was designed which correlated the variation of HCV-RNA (independent variable) and the variation of serum HNE- and MDA-protein adducts, and 8-OHdG. A significant positive relationship was observed among changes registered in serum HCV-RNA and 8-OHdG between 4wks and baseline (Figure 10A). We further designed a linear regression model to study the relationship between the variation of circulating TNF-α and serum markers of oxidative stress. Interestingly, a positive association was found among changes in serum TNF-α and HNE-protein adducts between SVR12 and baseline (Figure 10B).

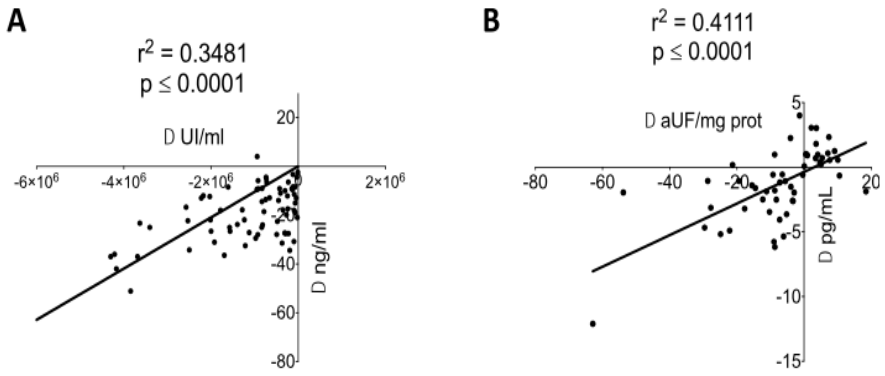

Figure 10. Linear regression analysis between the variation of serum HCV-RNA and 8-OHdG from baseline to 4wks (panel A), and between the variation of serum HNE- protein adducts and TNF-α levels from baseline to SVR12 (panel B).

4.7. Effect of antiviral treatment on mitochondrial energy homeostasis in PBMCs

We investigated the influence of DAAs on PBMCs cellular bioenergetics by measuring OXPHOS with OCR and glycolysis with ECAR. The antiviral therapy did not influence neither basal nor ATP-linked respiration at any time of the study. However, both the maximal respiratory capacity and the reserve capacity significantly increased 4 weeks after starting therapy and persisted higher at EoT and three months later (SVR12) (Figure 11A, 11B). On the other hand, cellular glycolysis measured in terms of ECAR did not change along the time (Figure 11C). Mitochondrial complexes from PBMC were run on Western Blot to quantify respiratory complexes subunits and showed that DAAs treatment was associated with the significant increase in the level of complex I, complex IV, complex V subunits, and citrate synthase, from 4wks to SVR12. Subunits of the complex III notably increased at EoT and persisted until SVR12 (Figure 11D). Enzymatic activities of the ETC complexes were thus measured to verify whether the modifications observed in energy availability were related to recovery of specific complexes. Interestingly,

complex I and complex IV activity significantly increased in PBMCs at EoT, and persisted high at SVR12 (Figure 11E). Overall, these data suggest that viral eradication with DAAs therapy induced a prompt adaptation in immune cells which in turn improved mitochondrial metabolism.

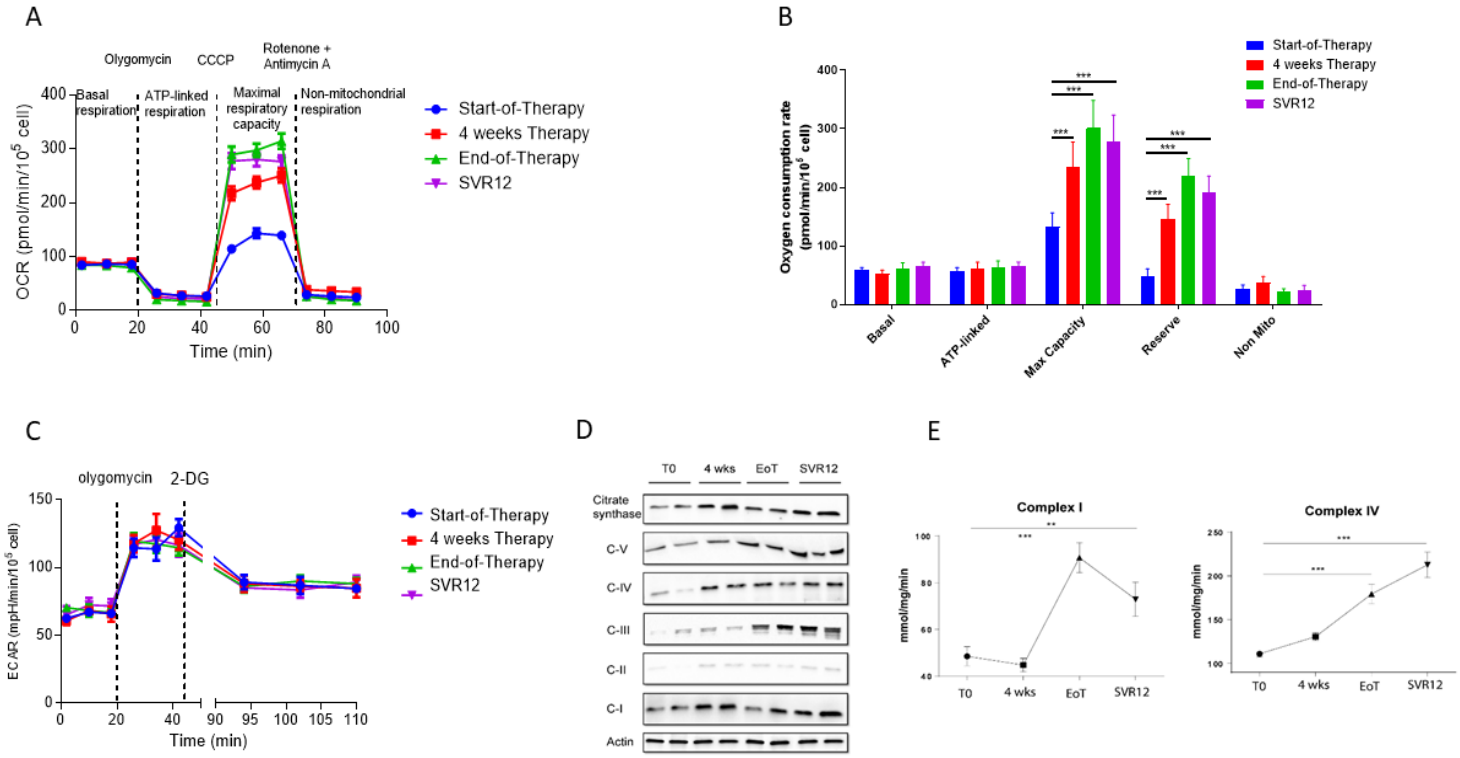

Figure 11. Viral eradication with DAAs restores mitochondrial function in PBMCs. A, B. Oxygen consumption rate of patients at T0, 4wks, EoT and SVR12 (n=12 for each time-point; each sample was run in triplicate). C. Extracellular acidification rate (ECAR) of patients at T0, 4wks, EoT and SVR12 (n=12 for each time-point; each sample was run in triplicate). D. Protein levels of ETC complex I, II, III, IV and V subunits, and citrate synthase determined by western blot (representative pictures). E. Complex I and IV enzymatic activity at T0, 4wks, EoT, SVR12 (n=12 for each timepoint). Data shown as mean ± SEM; **: $p < 0.01$; ***: $p < 0.001$. OCR, oxygen consumption rate, 4wks, 4 weeks of therapy, Eot, end of therapy, SVR12, 12 weeks of sustained virological response; ETC, electron transport chain.

4.8. mtDNA damage in PBMCs and effects of DAAs

Subunits of mitochondrial complexes are partially encoded by nuclear DNA (nDNA) and in part by mitochondrial DNA (mtDNA). To address the hypothesis that HCV eradication could depend on the DNA encoding and since it is well known that DNA copy number varies upon bioenergetic stimuli and oxidative stress [134-136], we isolated nuclear and mitochondrial DNA from PBMCs. We did not observe any difference in terms of mtDNA copy number along the observation time (Figure 12A). As mtDNA reacts to alterations in mitochondrial metabolism and DNA repair factors influence the modification of mtDNA upon metabolic manipulations [137], we assessed mitochondrial DNA integrity using a qPCR-based method, which detects any modification that inhibits the TaqαI restriction enzyme [138]. In line with the results of mitochondrial activity, mtDNA damage in PBMCs was reduced after 4 weeks of treatment, an effect that persisted until the end of observation, at SVR12 (Figure 12B). The lower mtDNA damage level was not likely to be due to mitophagic removal of damaged molecules, as the copy number of mtDNA was similar at all the time points of the study and no changes were observed in terms of nDNA damage (Figure 12A, 12C).

These results suggest that HCV chronic infection selectively induces mtDNA damage that is completely reversed by HCV eradication, and this is already evident 4 weeks after starting DAAs therapy.

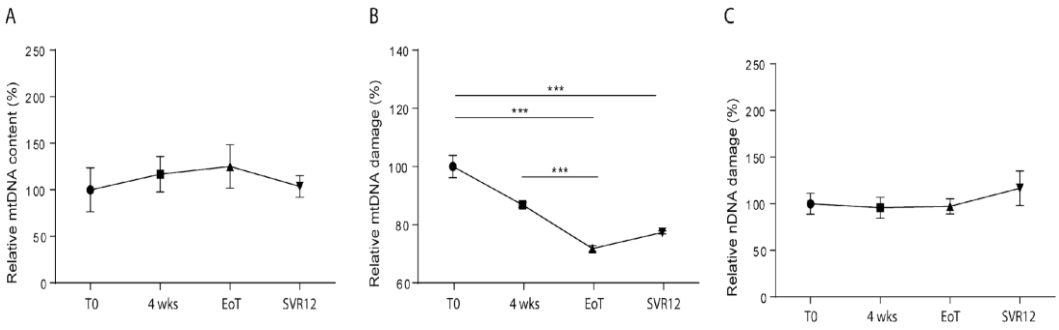

Figure 12. Treatment with DAAs recovered mtDNA damage. A. mtDNA content in PBMCs at T0, 4wks, EoT and SVR12, normalized to T0. B. mtDNA damage at T0, 4wks, EoT and SVR12, normalized to T0 determined by qPCR. C. mtDNA damage at T0, 4wks, EoT and SVR12, normalized to T0, determined by qPCR. (n=12 for each time point). Data shown as mean ± SEM; ***: $p < 0.001$ mtDNA, mitochondrial DNA; nDNA, nuclear DNA; 4wks, 4 weeks of therapy, EoT, end of therapy, SVR12, 12 weeks of sustained virological response.

4.9. Different DAAs regimens differently affect PBMCs rewiring

We investigated whether different therapy regimens could elicit different immune responses. To do this, we differentiated patients treated with Sofosbuvir-based therapy from patients receiving Sofosbuvir-free regimens. Most of Sofosbuvir-free regimens are protease inhibitors (PI) and the increase of Complex-I and Complex IV activity over time was significantly higher in PI treated subjects compared to non-PI treated patients (Figure 13A, 13B). Moreover, the beneficial effect of HCV eradication in terms of mtDNA damage was more prominent in PI patients (Figure 13C). Therefore, different DAAs displayed different cellular immune rewiring and PI were the ones which more potently changed PBMCs metabolic adaptation.

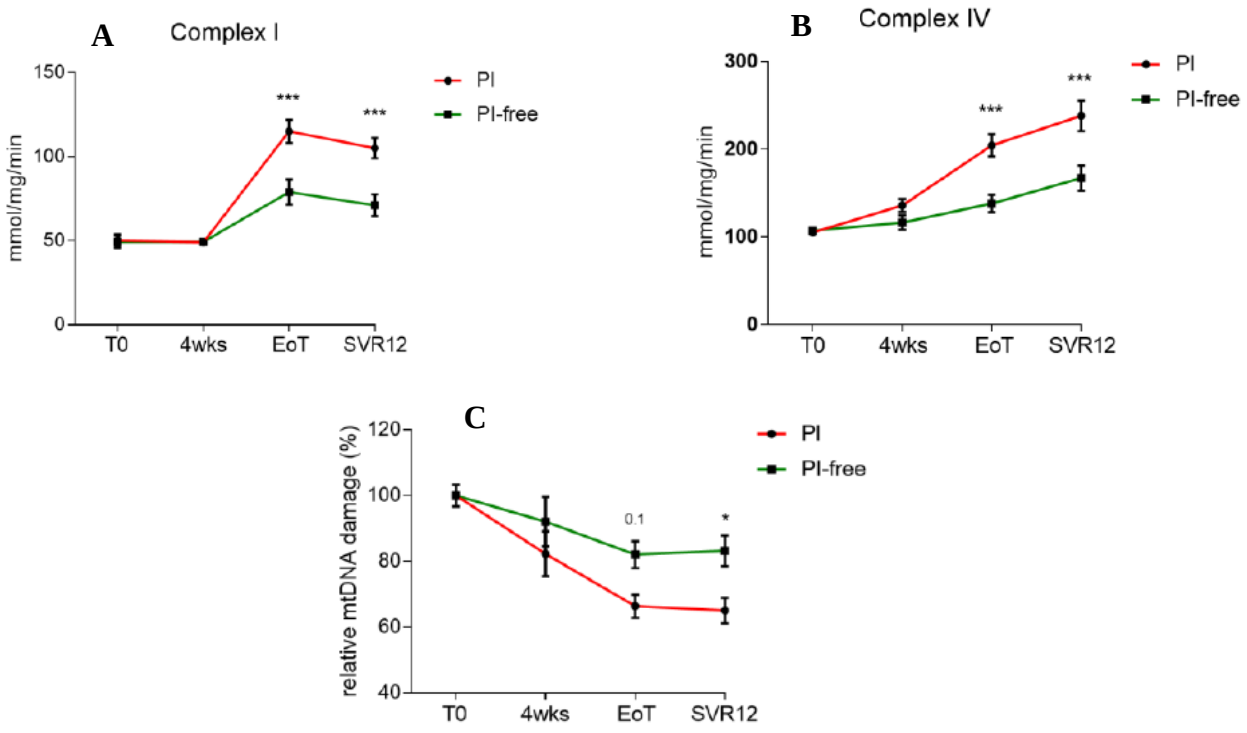

Figure 13. A, B. Enzymatic activity of complex I and IV at at T0, 4wks, EoT and SVR12, of patients treated with protease inhibitors containing or protease inhibitors free therapy regimens (n=5 for each group). C. mtDNA damage at T0, 4wks, EoT and SVR12, of patients treated with protease inhibitors containing or protease inhibitors free therapy regimens. PI, protease inhibitors (n=6 for each group). Data shown as mean ± SEM; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. 4wks, 4 weeks of therapy, EoT, end of therapy, SVR12, 12 weeks of sustained virological response; mtDNA, mitochondrial DNA; nDNA, nuclear DNA.

5. DISCUSSION

The present study demonstrates for the first time that DAA treatment restores circulating redox homeostasis in patients affected by chronic hepatitis C. This beneficial effect occurs irrespective of the HCV genotype and of antiviral regimen.

Liver injury associated with chronic HCV infection is caused by the specific immune response and a direct impairment in infected cells [139,140]. Among the cellular mechanisms by which HCV causes liver injury, oxidative stress plays a determinant role [7,141]. Indeed,

oxidative stress has been long considered a hallmark of chronic hepatitis C [10]. Oxidative stress is determined by an extensive production of reactive species, which damage biological macromolecules such as lipids, DNA and proteins. Lipid peroxidation produces high levels of reactive aldehydes, such as HNE and MDA, which in turn form adducts with proteins including enzymes and signaling molecules – with consequent structural and functional alterations [131]. On the other side, high reactive species can oxidise DNA bases or induce single and double-stranded DNA breaks, with consequent modifications that contribute to carcinogenesis. Oxidative damage to DNA increases the risk for patients affected by chronic hepatitis C to develop hepatocellular carcinoma [142].

Our data show that 4 weeks after starting DAA therapy, HCV replication is completely suppressed and antioxidant enzymes SOD and GSH-Px are over-expressed both in terms of mRNA and enzymatic activity. Very interestingly, a clear reduction in the level of 8-OHdG, a fine marker of oxidative DNA damage, was observed that persisted several weeks after treatment discontinuation. We also analysed typical markers of lipid oxidation by measuring HNE and MDA adducts to serum proteins and observed a significant reduction similar to 8-OHdG but starting later.

CAT and GSH-Px expression and activity seem to recover early after starting DAAs therapy whereas lipid peroxidation reduction becomes evident later. Very interestingly, SOD showed a reduction in terms of mRNA expression and in terms of activity 4 weeks after starting DAAs with a significant increase at the end of therapy that persisted three months later. It is worth noting that HCV may directly induce the oxidation of DNA and the impairment of DNA repair enzymes [143]. This evidence strengthens the hypothesis that DAA

therapy reverses this harmful effect in part by reducing the HCV-mediated production of reactive species. On the other side, HCV is sensitive to lipid peroxidation since the oxidative membrane damage induces modifications in the membrane-bound replicase components [15]. This could partially explain the observed latency in the reduction of circulating HNE- and MDA-protein adducts, with respect to 8-OHdG, in our patients.

Further to be a direct consequence of viral replication, oxidative stress in chronic HCV infection is also mediated by pro-inflammatory mediators produced by lymphoid cells, since the virus may cause cytopathic effects to circulating lymphocytes and monocytes [144]. PBMC in chronically infected HCV patients present with increased lipid peroxidation products and SOD activity, while no modification of CAT and GSH-Px activity [145]. Our data show that, with respect to baseline, SOD activity and gene expression decreased after 4 weeks of DAA treatment, to raise up at the end of treatment. Conversely, we observed a constant increase of both CAT and GSH-Px activity and gene expression after the initiation of DAA treatment. SOD dismutates superoxide anion to H2O2, which in turn is catabolized by CAT and GSH-Px. The balance between antioxidant enzymes is crucial to prevent and counteract oxidative stress. An augmented activity of CAT or GSH-Px with respect to SOD would be protective against oxidant-induced damage [146,147].

Oxidative stress induces the proliferation of hepatic stellate cells promoting collagen synthesis and the consequent development of fibrosis in chronic hepatitis C [10]. Indeed, the level of oxidative markers increased parallel to the fibrosis stage, suggesting that their detection is important for monitoring the progression of HCV-related hepatitis [148]. Our data show that, at baseline, high fibrosis

stage CHC patients showed higher oxidative DNA damage and lower SOD and CAT activities as compared to low fibrosis stages. Very interestingly, the effect of DAA treatment on circulating redox balance was significantly higher in F3/F4 as compared to F0/F1 patients. These data confirm that HCV- infected patients with high fibrosis stage get the most favorable treatment efficiency with DAA. Further basic research is required to completely elucidate the bio molecular mechanisms underlying such observations.

HCV adopts several mechanisms to evade innate and adaptive immune response, provoking a high rate (about 80%) of chronic infections. The interference with antigen presenting and processing, and the IFN signalling disturbance are only some of the wide plethora of escape strategies [149]. T-cell exhaustion, another effect of HCV chronic infection, entails the sequential loss of T cell effector functions such as altered proliferative abilities, up-regulation of many co-inhibitory receptors, altered metabolic fitness, failure for transition to quiescence, and acquisition of antigen-independent memory T cell responsiveness [19]. It was described that the exhausted HCV-specific T cells are unable to switch to OXPHOS, show mega-mitochondria and lower membrane potential, hence mitochondrial dysfunction [150].

Recent reports indicate that adequate nutrient supply and energy generation are main determinants of immune function [150,151]. Moreover, metabolic deficiency or alteration in the nutrient sensing signalling contribute to T-cell exhaustion during chronic viral infections [152]. Since the considerable chronic immune rearrangement provoked by HCV infection, we asked how the DAAs-induced rapid viral clearance could affect immune cell function. So we dissected PBMCs cellular bioenergetics, demonstrating that HCV chronic infection was associated with the

inhibition of OXPHOS that limits mitochondrial complexes activity through protein subunit depletion. Mitochondrial dysfunction induces mtDNA response to support respiration [134,135]. However, in chronic HCV infection, PMBCs mtDNA was damaged and hence not able to completely restore the loss of proteins. Therapy with DAAs and the eradication of the infection drastically reduced mtDNA damage, supporting the ETC complexes synthesis and quickly restoring mitochondrial activity and function.

As HCV is not the only pathogen that targets mitochondria to evade the host immune system, it is important to look at immune response beyond SVR to understand the long-term impact of HCV eradication. Moreover, it is important to verify if the same mitochondrial rearrangement occurs in liver leukocytes and if this is implicated in cancer development.

In conclusion, treatment with DAAs improved the circulating redox status of patients affected by chronic hepatitis C. In addition, the most benefit is reported in patients with high fibrosis stage and is independent of the treatment regimen used. By modulating the antioxidant status DAA therapy may prevent morbidity and liver complication in chronic HCV patients. Defining the contribution of mitochondrial dysfunction in immune cells during chronic viral infections is of crucial importance to understand the virus-host relationship and find new potential therapeutic targets. In particular, the study of the mechanisms underlying the immunometabolism modulation vigorously states a putative therapeutic approach either in chronic liver diseases or in incurable viral infections.

ABBREVIATIONS

8-OHdG	8-hydroxyguanosine
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAT	Biochip Array Technology
BSA	Bovine serum albumin
CAT	Catalase
CCl5	Chemokine (C-C motif) ligand 5
CLDN	Claudin
CoQ	Ubiquinone
CT	Threshold cycle
CYP2E1	Cytochrome P450 Family 2 Subfamily E Member 1
DAAs	Direct-Acting Antivirals
DCPIP	2,6-dichlorophenolindophenol
DNA	Deoxyribonucleic acid
DUOX	Dual oxidase
ECAR	Extracellular acidification rate
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
EoT	End of treatment
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GT	Genotype

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HNE	4-hydroxynonenal
IMM	Inner mitochondrial membrane
IMS	Inter membrane space
ISGs	Interferon sensitive genes
JAK-STAT	Janus kinase/ signal transducer and activator of transcription
KCN	Potassium cyanide
LDL	Low-density lipoprotein
LVPs	Lipoviral particles
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamde adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NK	Natural killer
NOX	NADPH oxidase
NS	Non-structural
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
pegIFN	Pegylated Interferons
PI	Protease-inhibitor

PMSF	Phenylmethylsulfonyl fluoride
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative Real-time polymerase chain reaction
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
ROS	Reactive oxygen species
RVR	Rapid virologic response
SDM	Standard deviation of the mean
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SOD	Superoxide dismutase
SVR	Sustained viral response
TBS	Tris-Buffered Saline
Th	T helper
TLR	Toll-like receptors
UPR	Unfolded protein response
VLDL	Very low-density lipoprotein

REFERENCES

1. Bhattacharjee, C.; Singh, M.; Das, D.; Chaudhuri, S.; Mukhopadhyay, A. Current therapeutics against HCV. *Virusdisease* **2021**, *32*, 228-243, doi:10.1007/s13337-021-00697-0.
2. Chung, R.T.; Gale, M., Jr.; Polyak, S.J.; Lemon, S.M.; Liang, T.J.; Hoofnagle, J.H. Mechanisms of action of interferon and ribavirin in chronic hepatitis C: Summary of a workshop. *Hepatology* **2008**, *47*, 306-320, doi:10.1002/hep.22070.
3. Compagnoni, S.; Bruno, E.M.; Madonia, G.; Cannizzaro, M.; Madonia, S. Direct antiviral agents in hepatitis C virus related liver disease: Don't count the chickens before they're hatched. *World journal of gastroenterology* **2021**, *27*, 2771-2783, doi:10.3748/wjg.v27.i21.2771.
4. Carrat, F.; Fontaine, H.; Dorival, C.; Simony, M.; Diallo, A.; Hezode, C.; De Ledinghen, V.; Larrey, D.; Haour, G.; Bronowicki, J.P., et al. Clinical outcomes in patients with chronic hepatitis C after direct-acting antiviral treatment: a prospective cohort study. *Lancet* **2019**, *393*, 1453-1464, doi:10.1016/S0140-6736(18)32111-1.
5. van der Meer, A.J.; Feld, J.J.; Hofer, H.; Almasio, P.L.; Calvaruso, V.; Fernandez-Rodriguez, C.M.; Aleman, S.; Ganne-Carrie, N.; D'Ambrosio, R.; Pol, S., et al. Risk of cirrhosis-related complications in patients with advanced fibrosis following hepatitis C virus eradication. *Journal of hepatology* **2017**, *66*, 485-493, doi:10.1016/j.jhep.2016.10.017.
6. Ivanov, A.V.; Valuev-Elliston, V.T.; Tyurina, D.A.; Ivanova, O.N.; Kochetkov, S.N.; Bartosch, B.; Isaguliants, M.G. Oxidative stress, a trigger of hepatitis C and B virus-induced liver carcinogenesis. *Oncotarget* **2017**, *8*, 3895-3932, doi:10.18632/oncotarget.13904.
7. Ivanov, A.V.; Bartosch, B.; Smirnova, O.A.; Isaguliants, M.G.; Kochetkov, S.N. HCV and oxidative stress in the liver. *Viruses* **2013**, *5*, 439-469, doi:10.3390/v5020439.
8. Emerit, I.; Serejo, F.; Filipe, P.; Alaoui Youssefi, A.; Fernandes, A.; Costa, A.; Freitas, J.; Ramalho, F.; Baptista, A.; Carneiro de Moura, M. Clastogenic factors as biomarkers of oxidative stress in chronic hepatitis C. *Digestion* **2000**, *62*, 200-207, doi:10.1159/000007814.

9. Jain, S.K.; Pemberton, P.W.; Smith, A.; McMahon, R.F.; Burrows, P.C.; Aboutwerat, A.; Warnes, T.W. Oxidative stress in chronic hepatitis C: not just a feature of late stage disease. *Journal of hepatology* **2002**, *36*, 805-811, doi:10.1016/s0168-8278(02)00060-0.
10. Choi, J.; Ou, J.H. Mechanisms of liver injury. III. Oxidative stress in the pathogenesis of hepatitis C virus. *American journal of physiology. Gastrointestinal and liver physiology* **2006**, *290*, G847-851, doi:10.1152/ajpgi.00522.2005.
11. Gong, G.Z.; Siddiqui, A.; Su, X.S. [The effect of Hepatitis C virus nonstructural protein 5A on cell cycling]. *Zhonghua gan zang bing za zhi = Zhonghua ganzangbing zazhi = Chinese journal of hepatology* **2004**, *12*, 47-48.
12. Okuda, M.; Li, K.; Beard, M.R.; Showalter, L.A.; Scholle, F.; Lemon, S.M.; Weinman, S.A. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* **2002**, *122*, 366-375, doi:10.1053/gast.2002.30983.
13. Melhem, A.; Stern, M.; Shibolet, O.; Israeli, E.; Ackerman, Z.; Pappo, O.; Hemed, N.; Rowe, M.; Ohana, H.; Zabrecky, G., et al. Treatment of chronic hepatitis C virus infection via antioxidants: results of a phase I clinical trial. *Journal of clinical gastroenterology* **2005**, *39*, 737-742, doi:10.1097/01.mcg.0000174023.73472.29.
14. Limongi, D.; Baldelli, S.; Santi, F.; D'Agostini, C.; Palamara, A.T.; Nencioni, L.; Ciotti, M. Redox alteration in patients infected by different HCV genotypes. *Le infezioni in medicina* **2018**, *26*, 249-254.
15. Yamane, D.; McGivern, D.R.; Wauthier, E.; Yi, M.; Madden, V.J.; Welsch, C.; Antes, I.; Wen, Y.; Chugh, P.E.; McGee, C.E., et al. Regulation of the hepatitis C virus RNA replicase by endogenous lipid peroxidation. *Nature medicine* **2014**, *20*, 927-935, doi:10.1038/nm.3610.
16. El-Kannishy, G.; Arafa, M.; Abdelaal, I.; Elarman, M.; El-Mahdy, R. Persistent oxidative stress in patients with chronic active hepatitis-C infection after antiviral therapy failure. *Saudi journal of gastroenterology : official journal of the Saudi Gastroenterology Association* **2012**, *18*, 375-379, doi:10.4103/1319-3767.103429.
17. Liu, H.M.; Gale, M. Hepatitis C Virus Evasion from RIG-I-Dependent Hepatic Innate Immunity. *Gastroenterology research and practice* **2010**, *2010*, 548390, doi:10.1155/2010/548390.

18. Wherry, E.J.; Ha, S.J.; Kaech, S.M.; Haining, W.N.; Sarkar, S.; Kalia, V.; Subramaniam, S.; Blattman, J.N.; Barber, D.L.; Ahmed, R. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* **2007**, *27*, 670-684, doi:10.1016/j.immuni.2007.09.006.
19. Saeidi, A.; Zandi, K.; Cheok, Y.Y.; Saeidi, H.; Wong, W.F.; Lee, C.Y.Q.; Cheong, H.C.; Yong, Y.K.; Larsson, M.; Shankar, E.M. T-Cell Exhaustion in Chronic Infections: Reversing the State of Exhaustion and Reinvigorating Optimal Protective Immune Responses. *Frontiers in immunology* **2018**, *9*, 2569, doi:10.3389/fimmu.2018.02569.
20. Rebbani, K.; Tsukiyama-Kohara, K. HCV-Induced Oxidative Stress: Battlefield-Winning Strategy. *Oxidative medicine and cellular longevity* **2016**, *2016*, 7425628, doi:10.1155/2016/7425628.
21. Spengler, U. Direct antiviral agents (DAAs) - A new age in the treatment of hepatitis C virus infection. *Pharmacology & therapeutics* **2018**, *183*, 118-126, doi:10.1016/j.pharmthera.2017.10.009.
22. Li, H.; Huang, M.H.; Jiang, J.D.; Peng, Z.G. Hepatitis C: From inflammatory pathogenesis to anti-inflammatory/hepatoprotective therapy. *World journal of gastroenterology* **2018**, *24*, 5297-5311, doi:10.3748/wjg.v24.i47.5297.
23. Webster, D.P.; Klenerman, P.; Dusheiko, G.M. Hepatitis C. *Lancet* **2015**, *385*, 1124-1135, doi:10.1016/S0140-6736(14)62401-6.
24. Rabaan, A.A.; Al-Ahmed, S.H.; Bazzi, A.M.; Alfouzan, W.A.; Alsuliman, S.A.; Aldrazi, F.A.; Haque, S. Overview of hepatitis C infection, molecular biology, and new treatment. *Journal of infection and public health* **2020**, *13*, 773-783, doi:10.1016/j.jiph.2019.11.015.
25. Dustin, L.B.; Bartolini, B.; Capobianchi, M.R.; Pistello, M. Hepatitis C virus: life cycle in cells, infection and host response, and analysis of molecular markers influencing the outcome of infection and response to therapy. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **2016**, *22*, 826-832, doi:10.1016/j.cmi.2016.08.025.
26. Tong, Y.; Lavillette, D.; Li, Q.; Zhong, J. Role of Hepatitis C Virus Envelope Glycoprotein E1 in Virus Entry and Assembly. *Frontiers in immunology* **2018**, *9*, 1411, doi:10.3389/fimmu.2018.01411.

27. Dubuisson, J.; Cosset, F.L. Virology and cell biology of the hepatitis C virus life cycle: an update. *Journal of hepatology* **2014**, *61*, S3-S13, doi:10.1016/j.jhep.2014.06.031.
28. Fan, H.; Qiao, L.; Kang, K.D.; Fan, J.; Wei, W.; Luo, G. Attachment and Postattachment Receptors Important for Hepatitis C Virus Infection and Cell-to-Cell Transmission. *Journal of virology* **2017**, *91*, doi:10.1128/JVI.00280-17.
29. Ramakrishnaiah, V.; Thumann, C.; Fofana, I.; Habersetzer, F.; Pan, Q.; de Ruiter, P.E.; Willemsen, R.; Demmers, J.A.; Stalin Raj, V.; Jenster, G., et al. Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. *Proceedings of the National Academy of Sciences of the United States of America* **2013**, *110*, 13109-13113, doi:10.1073/pnas.1221899110.
30. Choo, Q.L.; Kuo, G.; Weiner, A.J.; Overby, L.R.; Bradley, D.W.; Houghton, M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **1989**, *244*, 359-362, doi:10.1126/science.2523562.
31. Niepmann, M.; Shalamova, L.A.; Gerresheim, G.K.; Rossbach, O. Signals Involved in Regulation of Hepatitis C Virus RNA Genome Translation and Replication. *Frontiers in microbiology* **2018**, *9*, 395, doi:10.3389/fmicb.2018.00395.
32. Tsukiyama-Kohara, K.; Iizuka, N.; Kohara, M.; Nomoto, A. Internal ribosome entry site within hepatitis C virus RNA. *Journal of virology* **1992**, *66*, 1476-1483, doi:10.1128/JVI.66.3.1476-1483.1992.
33. Biswas, A.; Treadaway, J.; Tellinghuisen, T.L. Interaction between Nonstructural Proteins NS4B and NS5A Is Essential for Proper NS5A Localization and Hepatitis C Virus RNA Replication. *Journal of virology* **2016**, *90*, 7205-7218, doi:10.1128/JVI.00037-16.
34. Lohmann, V.; Korner, F.; Koch, J.; Herian, U.; Theilmann, L.; Bartenschlager, R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **1999**, *285*, 110-113, doi:10.1126/science.285.5424.110.
35. Lohmann, V. Hepatitis C virus RNA replication. *Current topics in microbiology and immunology* **2013**, *369*, 167-198, doi:10.1007/978-3-642-27340-7_7.
36. Romero-Brey, I.; Merz, A.; Chiramel, A.; Lee, J.Y.; Chlanda, P.; Haselman, U.; Santarella-Mellwig, R.; Habermann, A.; Hoppe, S.; Kallis, S., et al. Three-dimensional architecture and biogenesis of membrane structures associated with hepatitis C virus replication. *PLoS pathogens* **2012**, *8*, e1003056, doi:10.1371/journal.ppat.1003056.

37. Egger, D.; Wolk, B.; Gosert, R.; Bianchi, L.; Blum, H.E.; Moradpour, D.; Bienz, K. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *Journal of virology* **2002**, *76*, 5974-5984, doi:10.1128/jvi.76.12.5974-5984.2002.

38. Miyanari, Y.; Atsuzawa, K.; Usuda, N.; Watashi, K.; Hishiki, T.; Zayas, M.; Bartenschlager, R.; Wakita, T.; Hijikata, M.; Shimotohno, K. The lipid droplet is an important organelle for hepatitis C virus production. *Nature cell biology* **2007**, *9*, 1089-1097, doi:10.1038/ncb1631.

39. Boson, B.; Denolly, S.; Turlure, F.; Chamot, C.; Dreux, M.; Cosset, F.L. Daclatasvir Prevents Hepatitis C Virus Infectivity by Blocking Transfer of the Viral Genome to Assembly Sites. *Gastroenterology* **2017**, *152*, 895-907 e814, doi:10.1053/j.gastro.2016.11.047.

40. Zayas, M.; Long, G.; Madan, V.; Bartenschlager, R. Coordination of Hepatitis C Virus Assembly by Distinct Regulatory Regions in Nonstructural Protein 5A. *PLoS pathogens* **2016**, *12*, e1005376, doi:10.1371/journal.ppat.1005376.

41. Denolly, S.; Mialon, C.; Bourlet, T.; Amirache, F.; Penin, F.; Lindenbach, B.; Boson, B.; Cosset, F.L. The amino-terminus of the hepatitis C virus (HCV) p7 viroporin and its cleavage from glycoprotein E2-p7 precursor determine specific infectivity and secretion levels of HCV particle types. *PLoS pathogens* **2017**, *13*, e1006774, doi:10.1371/journal.ppat.1006774.

42. Alazard-Dany, N.; Denolly, S.; Boson, B.; Cosset, F.L. Overview of HCV Life Cycle with a Special Focus on Current and Possible Future Antiviral Targets. *Viruses* **2019**, *11*, doi:10.3390/v11010030.

43. Falcon, V.; Acosta-Rivero, N.; Gonzalez, S.; Duenas-Carrera, S.; Martinez-Donato, G.; Menendez, I.; Garateix, R.; Silva, J.A.; Acosta, E.; Kouri, J. Ultrastructural and biochemical basis for hepatitis C virus morphogenesis. *Virus genes* **2017**, *53*, 151-164, doi:10.1007/s11262-017-1426-2.

44. Neumann-Haefelin, C.; Thimme, R. Adaptive immune responses in hepatitis C virus infection. *Current topics in microbiology and immunology* **2013**, *369*, 243-262, doi:10.1007/978-3-642-27340-7_10.

45. Nishitsuji, H.; Funami, K.; Shimizu, Y.; Ujino, S.; Sugiyama, K.; Seya, T.; Takaku, H.; Shimotohno, K. Hepatitis C virus infection induces inflammatory cytokines and chemokines mediated by the cross talk between hepatocytes and stellate cells. *Journal of virology* **2013**, *87*, 8169-8178, doi:10.1128/JVI.00974-13.
46. Bonilla, N.; Barget, N.; Andrieu, M.; Roulot, D.; Letoumelin, P.; Grando, V.; Trinchet, J.C.; Ganne-Carrie, N.; Beaugrand, M.; Deny, P., et al. Interferon gamma-secreting HCV-specific CD8+ T cells in the liver of patients with chronic C hepatitis: relation to liver fibrosis--ANRS HC EP07 study. *Journal of viral hepatitis* **2006**, *13*, 474-481, doi:10.1111/j.1365-2893.2005.00711.x.
47. Franceschini, D.; Del Porto, P.; Piconese, S.; Trella, E.; Accapezzato, D.; Paroli, M.; Morrone, S.; Piccolella, E.; Spada, E.; Mele, A., et al. Polyfunctional type-1, -2, and -17 CD8(+) T cell responses to apoptotic self-antigens correlate with the chronic evolution of hepatitis C virus infection. *PLoS pathogens* **2012**, *8*, e1002759, doi:10.1371/journal.ppat.1002759.
48. Yamane, D.; McGivern, D.R.; Masaki, T.; Lemon, S.M. Liver injury and disease pathogenesis in chronic hepatitis C. *Current topics in microbiology and immunology* **2013**, *369*, 263-288, doi:10.1007/978-3-642-27340-7_11.
49. Dustin, L.B. Innate and Adaptive Immune Responses in Chronic HCV Infection. *Current drug targets* **2017**, *18*, 826-843, doi:10.2174/1389450116666150825110532.
50. Lau, D.T.; Fish, P.M.; Sinha, M.; Owen, D.M.; Lemon, S.M.; Gale, M., Jr. Interferon regulatory factor-3 activation, hepatic interferon-stimulated gene expression, and immune cell infiltration in hepatitis C virus patients. *Hepatology* **2008**, *47*, 799-809, doi:10.1002/hep.22076.
51. Heim, M.H.; Thimme, R. Innate and adaptive immune responses in HCV infections. *Journal of hepatology* **2014**, *61*, S14-25, doi:10.1016/j.jhep.2014.06.035.
52. El-Serag, H.B. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* **2012**, *142*, 1264-1273 e1261, doi:10.1053/j.gastro.2011.12.061.
53. Bartosch, B.; Thimme, R.; Blum, H.E.; Zoulim, F. Hepatitis C virus-induced hepatocarcinogenesis. *Journal of hepatology* **2009**, *51*, 810-820, doi:10.1016/j.jhep.2009.05.008.

54. Bieging, K.T.; Mello, S.S.; Attardi, L.D. Unravelling mechanisms of p53-mediated tumour suppression. *Nature reviews. Cancer* **2014**, *14*, 359-370, doi:10.1038/nrc3711.
55. Kao, C.F.; Chen, S.Y.; Chen, J.Y.; Wu Lee, Y.H. Modulation of p53 transcription regulatory activity and post-translational modification by hepatitis C virus core protein. *Oncogene* **2004**, *23*, 2472-2483, doi:10.1038/sj.onc.1207368.
56. McGivern, D.R.; Lemon, S.M. Virus-specific mechanisms of carcinogenesis in hepatitis C virus associated liver cancer. *Oncogene* **2011**, *30*, 1969-1983, doi:10.1038/onc.2010.594.
57. Fimia, G.M.; Corazzari, M.; Antonioli, M.; Piacentini, M. Ambra1 at the crossroad between autophagy and cell death. *Oncogene* **2013**, *32*, 3311-3318, doi:10.1038/onc.2012.455.
58. Grassi, G.; Di Caprio, G.; Santangelo, L.; Fimia, G.M.; Cozzolino, A.M.; Komatsu, M.; Ippolito, G.; Tripodi, M.; Alonzi, T. Autophagy regulates hepatocyte identity and epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions promoting Snail degradation. *Cell death & disease* **2015**, *6*, e1880, doi:10.1038/cddis.2015.249.
59. Brault, C.; Levy, P.L.; Bartosch, B. Hepatitis C virus-induced mitochondrial dysfunctions. *Viruses* **2013**, *5*, 954-980, doi:10.3390/v5030954.
60. Zorov, D.B.; Juhaszova, M.; Sollott, S.J. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiological reviews* **2014**, *94*, 909-950, doi:10.1152/physrev.00026.2013.
61. Kuhlbrandt, W. Structure and function of mitochondrial membrane protein complexes. *BMC biology* **2015**, *13*, 89, doi:10.1186/s12915-015-0201-x.
62. Anderson, S.; Bankier, A.T.; Barrell, B.G.; de Bruijn, M.H.; Coulson, A.R.; Drouin, J.; Eperon, I.C.; Nierlich, D.P.; Roe, B.A.; Sanger, F., et al. Sequence and organization of the human mitochondrial genome. *Nature* **1981**, *290*, 457-465, doi:10.1038/290457a0.
63. Yang, D.; Oyaizu, Y.; Oyaizu, H.; Olsen, G.J.; Woese, C.R. Mitochondrial origins. *Proceedings of the National Academy of Sciences of the United States of America* **1985**, *82*, 4443-4447, doi:10.1073/pnas.82.13.4443.
64. Kotiadis, V.N.; Duchen, M.R.; Osellame, L.D. Mitochondrial quality control and communications with the nucleus are important in maintaining mitochondrial function and cell health. *Biochimica et biophysica acta* **2014**, *1840*, 1254-1265, doi:10.1016/j.bbagen.2013.10.041.

65. Finkel, T. Signal transduction by mitochondrial oxidants. *The Journal of biological chemistry* **2012**, *287*, 4434-4440, doi:10.1074/jbc.R111.271999.
66. Win, S.; Than, T.A.; Zhang, J.; Oo, C.; Min, R.W.M.; Kaplowitz, N. New insights into the role and mechanism of c-Jun-N-terminal kinase signaling in the pathobiology of liver diseases. *Hepatology* **2018**, *67*, 2013-2024, doi:10.1002/hep.29689.
67. Meakin, P.J.; Chowdhry, S.; Sharma, R.S.; Ashford, F.B.; Walsh, S.V.; McCrimmon, R.J.; Dinkova-Kostova, A.T.; Dillon, J.F.; Hayes, J.D.; Ashford, M.L. Susceptibility of Nrf2-null mice to steatohepatitis and cirrhosis upon consumption of a high-fat diet is associated with oxidative stress, perturbation of the unfolded protein response, and disturbance in the expression of metabolic enzymes but not with insulin resistance. *Molecular and cellular biology* **2014**, *34*, 3305-3320, doi:10.1128/MCB.00677-14.
68. Bhargava, A.; Raghuram, G.V.; Pathak, N.; Varshney, S.; Jatawa, S.K.; Jain, D.; Mishra, P.K. Occult hepatitis C virus elicits mitochondrial oxidative stress in lymphocytes and triggers PI3-kinase-mediated DNA damage response. *Free radical biology & medicine* **2011**, *51*, 1806-1814, doi:10.1016/j.freeradbiomed.2011.08.009.
69. Machida, K.; McNamara, G.; Cheng, K.T.; Huang, J.; Wang, C.H.; Comai, L.; Ou, J.H.; Lai, M.M. Hepatitis C virus inhibits DNA damage repair through reactive oxygen and nitrogen species and by interfering with the ATM-NBS1/Mre11/Rad50 DNA repair pathway in monocytes and hepatocytes. *Journal of immunology* **2010**, *185*, 6985-6998, doi:10.4049/jimmunol.1000618.
70. Ryter, S.W.; Kim, H.P.; Hoetzel, A.; Park, J.W.; Nakahira, K.; Wang, X.; Choi, A.M. Mechanisms of cell death in oxidative stress. *Antioxidants & redox signaling* **2007**, *9*, 49-89, doi:10.1089/ars.2007.9.49.
71. Mittler, R.; Vanderauwera, S.; Suzuki, N.; Miller, G.; Tognetti, V.B.; Vandepoele, K.; Gollery, M.; Shulaev, V.; Van Breusegem, F. ROS signaling: the new wave? *Trends in plant science* **2011**, *16*, 300-309, doi:10.1016/j.tplants.2011.03.007.
72. Lozano-Sepulveda, S.A.; Bryan-Marrugo, O.L.; Cordova-Fletes, C.; Gutierrez-Ruiz, M.C.; Rivas-Estilla, A.M. Oxidative stress modulation in hepatitis C virus infected cells. *World journal of hepatology* **2015**, *7*, 2880-2889, doi:10.4254/wjh.v7.i29.2880.
73. Korenaga, M.; Wang, T.; Li, Y.; Showalter, L.A.; Chan, T.; Sun, J.; Weinman, S.A. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases

reactive oxygen species (ROS) production. *The Journal of biological chemistry* **2005**, *280*, 37481-37488, doi:10.1074/jbc.M506412200.

74. Bourges, I.; Ramus, C.; Mousson de Camaret, B.; Beugnot, R.; Remacle, C.; Cardol, P.; Hofhaus, G.; Issartel, J.P. Structural organization of mitochondrial human complex I: role of the ND4 and ND5 mitochondria-encoded subunits and interaction with prohibitin. *The Biochemical journal* **2004**, *383*, 491-499, doi:10.1042/BJ20040256.
75. Valgimigli, L.; Valgimigli, M.; Gaiani, S.; Pedulli, G.F.; Bolondi, L. Measurement of oxidative stress in human liver by EPR spin-probe technique. *Free radical research* **2000**, *33*, 167-178, doi:10.1080/10715760000300721.
76. Serejo, F.; Emerit, I.; Filipe, P.M.; Fernandes, A.C.; Costa, M.A.; Freitas, J.P.; de Moura, M.C. Oxidative stress in chronic hepatitis C: the effect of interferon therapy and correlation with pathological features. *Canadian journal of gastroenterology = Journal canadien de gastroenterologie* **2003**, *17*, 644-650, doi:10.1155/2003/710693.
77. Larrea, E.; Beloqui, O.; Munoz-Navas, M.A.; Civeira, M.P.; Prieto, J. Superoxide dismutase in patients with chronic hepatitis C virus infection. *Free radical biology & medicine* **1998**, *24*, 1235-1241, doi:10.1016/s0891-5849(97)00437-1.
78. Cardin, R.; Saccoccio, G.; Masutti, F.; Bellentani, S.; Farinati, F.; Tiribelli, C. DNA oxidative damage in leukocytes correlates with the severity of HCV-related liver disease: validation in an open population study. *Journal of hepatology* **2001**, *34*, 587-592, doi:10.1016/s0168-8278(00)00098-2.
79. Zuwala-Jagiello, J.; Pazgan-Simon, M.; Simon, K.; Warwas, M. Advanced oxidation protein products and inflammatory markers in liver cirrhosis: a comparison between alcohol-related and HCV-related cirrhosis. *Acta biochimica Polonica* **2011**, *58*, 59-65.
80. Kitada, T.; Seki, S.; Iwai, S.; Yamada, T.; Sakaguchi, H.; Wakasa, K. In situ detection of oxidative DNA damage, 8-hydroxydeoxyguanosine, in chronic human liver disease. *Journal of hepatology* **2001**, *35*, 613-618, doi:10.1016/s0168-8278(01)00171-4.
81. Gong, G.; Waris, G.; Tanveer, R.; Siddiqui, A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, *98*, 9599-9604, doi:10.1073/pnas.171311298.

82. Dionisio, N.; Garcia-Mediavilla, M.V.; Sanchez-Campos, S.; Majano, P.L.; Benedicto, I.; Rosado, J.A.; Salido, G.M.; Gonzalez-Gallego, J. Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes. *Journal of hepatology* **2009**, *50*, 872-882, doi:10.1016/j.jhep.2008.12.026.
83. Robinson, L.C.; Marchant, J.S. Enhanced Ca2+ leak from ER Ca2+ stores induced by hepatitis C NS5A protein. *Biochemical and biophysical research communications* **2008**, *368*, 593-599, doi:10.1016/j.bbrc.2008.01.127.
84. Immenschuh, S.; Ramadori, G. Gene regulation of heme oxygenase-1 as a therapeutic target. *Biochemical pharmacology* **2000**, *60*, 1121-1128, doi:10.1016/s0006-2952(00)00443-3.
85. Tsan, M.F. Superoxide dismutase and pulmonary oxygen toxicity: lessons from transgenic and knockout mice (Review). *International journal of molecular medicine* **2001**, *7*, 13-19, doi:10.3892/ijmm.7.1.13.
86. Bedard, K.; Krause, K.H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiological reviews* **2007**, *87*, 245-313, doi:10.1152/physrev.00044.2005.
87. Takac, I.; Schroder, K.; Zhang, L.; Lardy, B.; Anilkumar, N.; Lambeth, J.D.; Shah, A.M.; Morel, F.; Brandes, R.P. The E-loop is involved in hydrogen peroxide formation by the NADPH oxidase Nox4. *The Journal of biological chemistry* **2011**, *286*, 13304-13313, doi:10.1074/jbc.M110.192138.
88. Boudreau, H.E.; Emerson, S.U.; Korzeniowska, A.; Jendrysik, M.A.; Leto, T.L. Hepatitis C virus (HCV) proteins induce NADPH oxidase 4 expression in a transforming growth factor beta-dependent manner: a new contributor to HCV-induced oxidative stress. *Journal of virology* **2009**, *83*, 12934-12946, doi:10.1128/JVI.01059-09.
89. de Mochel, N.S.; Seronello, S.; Wang, S.H.; Ito, C.; Zheng, J.X.; Liang, T.J.; Lambeth, J.D.; Choi, J. Hepatocyte NAD(P)H oxidases as an endogenous source of reactive oxygen species during hepatitis C virus infection. *Hepatology* **2010**, *52*, 47-59, doi:10.1002/hep.23671.
90. Avadhani, N.G.; Sangar, M.C.; Bansal, S.; Bajpai, P. Bimodal targeting of cytochrome P450s to endoplasmic reticulum and mitochondria: the concept of chimeric signals. *The FEBS journal* **2011**, *278*, 4218-4229, doi:10.1111/j.1742-4658.2011.08356.x.

91. Lu, Y.; Cederbaum, A.I. CYP2E1 and oxidative liver injury by alcohol. *Free radical biology & medicine* **2008**, *44*, 723-738, doi:10.1016/j.freeradbiomed.2007.11.004.

92. Nakai, K.; Tanaka, H.; Hanada, K.; Ogata, H.; Suzuki, F.; Kumada, H.; Miyajima, A.; Ishida, S.; Sunouchi, M.; Habano, W., et al. Decreased expression of cytochromes P450 1A2, 2E1, and 3A4 and drug transporters Na+-taurocholate-cotransporting polypeptide, organic cation transporter 1, and organic anion-transporting peptide-C correlates with the progression of liver fibrosis in chronic hepatitis C patients. *Drug metabolism and disposition: the biological fate of chemicals* **2008**, *36*, 1786-1793, doi:10.1124/dmd.107.020073.

93. Cao, S.S.; Kaufman, R.J. Unfolded protein response. *Current biology : CB* **2012**, *22*, R622-626, doi:10.1016/j.cub.2012.07.004.

94. Lieber, C.S. Role of oxidative stress and antioxidant therapy in alcoholic and nonalcoholic liver diseases. *Advances in pharmacology* **1997**, *38*, 601-628, doi:10.1016/s1054-3589(08)61001-7.

95. Chaplin, D.D. 1. Overview of the immune response. *The Journal of allergy and clinical immunology* **2003**, *111*, S442-459, doi:10.1067/mai.2003.125.

96. Medzhitov, R.; Janeway, C., Jr. Innate immunity. *The New England journal of medicine* **2000**, *343*, 338-344, doi:10.1056/NEJM200008033430506.

97. Delves, P.J.; Roitt, I.M. The immune system. First of two parts. *The New England journal of medicine* **2000**, *343*, 37-49, doi:10.1056/NEJM200007063430107.

98. Dinarello, C.A. Proinflammatory cytokines. *Chest* **2000**, *118*, 503-508, doi:10.1378/chest.118.2.503.

99. Li, K.; Li, N.L.; Wei, D.; Pfeffer, S.R.; Fan, M.; Pfeffer, L.M. Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. *Hepatology* **2012**, *55*, 666-675, doi:10.1002/hep.24763.

100. Chattergoon, M.A.; Latanich, R.; Quinn, J.; Winter, M.E.; Buckheit, R.W., 3rd; Blankson, J.N.; Pardoll, D.; Cox, A.L. HIV and HCV activate the inflammasome in monocytes and macrophages via endosomal Toll-like receptors without induction of type 1 interferon. *PLoS pathogens* **2014**, *10*, e1004082, doi:10.1371/journal.ppat.1004082.

101. Dolganiuc, A.; Oak, S.; Kodys, K.; Golenbock, D.T.; Finberg, R.W.; Kurt-Jones, E.; Szabo, G. Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. *Gastroenterology* **2004**, *127*, 1513-1524, doi:10.1053/j.gastro.2004.08.067.

102. Machida, K.; Cheng, K.T.; Sung, V.M.; Levine, A.M.; Foung, S.; Lai, M.M. Hepatitis C virus induces toll-like receptor 4 expression, leading to enhanced production of beta interferon and interleukin-6. *Journal of virology* **2006**, *80*, 866-874, doi:10.1128/JVI.80.2.866-874.2006.

103. Nattermann, J.; Nischalke, H.D.; Feldmann, G.; Ahlenstiel, G.; Sauerbruch, T.; Spengler, U. Binding of HCV E2 to CD81 induces RANTES secretion and internalization of CC chemokine receptor 5. *Journal of viral hepatitis* **2004**, *11*, 519-526, doi:10.1111/j.1365-2893.2004.00545.x.

104. Yu, G.Y.; He, G.; Li, C.Y.; Tang, M.; Grivennikov, S.; Tsai, W.T.; Wu, M.S.; Hsu, C.W.; Tsai, Y.; Wang, L.H., et al. Hepatic expression of HCV RNA-dependent RNA polymerase triggers innate immune signaling and cytokine production. *Molecular cell* **2012**, *48*, 313-321, doi:10.1016/j.molcel.2012.07.032.

105. Alhetheel, A.; Albarrag, A.; Hakami, A.; Shakoor, Z.; Alswat, K.; Abdo, A.; Al-Hamoudi, W.; Alomar, S. In the peripheral blood mononuclear cells (PBMCs) of HCV infected patients the expression of STAT1 and IRF-1 is downregulated while that of caspase-3 upregulated. *Acta virologica* **2020**, *64*, 352-358, doi:10.4149/av_2020_313.

106. Bare, P. Hepatitis C virus and peripheral blood mononuclear cell reservoirs Patricia Bare. *World journal of hepatology* **2009**, *1*, 67-71, doi:10.4254/wjh.v1.i1.67.

107. Kondo, Y.; Ueno, Y.; Kakazu, E.; Kobayashi, K.; Shiina, M.; Tamai, K.; Machida, K.; Inoue, J.; Wakui, Y.; Fukushima, K., et al. Lymphotropic HCV strain can infect human primary naive CD4+ cells and affect their proliferation and IFN-gamma secretion activity. *Journal of gastroenterology* **2011**, *46*, 232-241, doi:10.1007/s00535-010-0297-2.

108. Laskus, T.; Radkowski, M.; Piasek, A.; Nowicki, M.; Horban, A.; Cianciara, J.; Rakela, J. Hepatitis C virus in lymphoid cells of patients coinfected with human immunodeficiency virus type 1: evidence of active replication in monocytes/macrophages and lymphocytes. *The Journal of infectious diseases* **2000**, *181*, 442-448, doi:10.1086/315283.

109. Lerat, H.; Rumin, S.; Habersetzer, F.; Berby, F.; Trabaud, M.A.; Trepo, C.; Inchauspe, G. In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood* **1998**, *91*, 3841-3849.

110. Nguyen, L.N.; Nguyen, L.N.T.; Zhao, J.; Schank, M.; Dang, X.; Cao, D.; Khanal, S.; Thakuri, B.K.C.; Zhang, J.; Lu, Z., et al. Immune Activation Induces Telomeric DNA Damage and Promotes Short-Lived Effector T Cell Differentiation in Chronic HCV Infection. *Hepatology* **2021**, *74*, 2380-2394, doi:10.1002/hep.32008.

111. Radkowski, M.; Opoka-Kegler, J.; Cortes, K.C.; Bukowska-Osko, I.; Perlejewski, K.; Pawelczyk, A.; Laskus, T. Evidence for immune activation in patients with residual hepatitis C virus RNA long after successful treatment with IFN and ribavirin. *The Journal of general virology* **2014**, *95*, 2004-2009, doi:10.1099/vir.0.064709-0.

112. Darnell, J.E., Jr.; Kerr, I.M.; Stark, G.R. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **1994**, *264*, 1415-1421, doi:10.1126/science.8197455.

113. Imada, K.; Leonard, W.J. The Jak-STAT pathway. *Molecular immunology* **2000**, *37*, 1-11, doi:10.1016/s0161-5890(00)00018-3.

114. Laursen, T.L.; Sandahl, T.D.; Kazankov, K.; George, J.; Gronbaek, H. Liver-related effects of chronic hepatitis C antiviral treatment. *World journal of gastroenterology* **2020**, *26*, 2931-2947, doi:10.3748/wjg.v26.i22.2931.

115. Palumbo, E. Pegylated interferon and ribavirin treatment for hepatitis C virus infection. *Therapeutic advances in chronic disease* **2011**, *2*, 39-45, doi:10.1177/2040622310384308.

116. Spearman, C.W.; Dusheiko, G.M.; Hellard, M.; Sonderup, M. Hepatitis C. *Lancet* **2019**, *394*, 1451-1466, doi:10.1016/S0140-6736(19)32320-7.

117. Brass, V.; Berke, J.M.; Montserret, R.; Blum, H.E.; Penin, F.; Moradpour, D. Structural determinants for membrane association and dynamic organization of the hepatitis C virus NS3-4A complex. *Proceedings of the National Academy of Sciences of the United States of America* **2008**, *105*, 14545-14550, doi:10.1073/pnas.0807298105.

118. Pang, P.S.; Jankowsky, E.; Planet, P.J.; Pyle, A.M. The hepatitis C viral NS3 protein is a processive DNA helicase with cofactor enhanced RNA unwinding. *The EMBO journal* **2002**, *21*, 1168-1176, doi:10.1093/emboj/21.5.1168.

119. Beran, R.K.; Serebrov, V.; Pyle, A.M. The serine protease domain of hepatitis C viral NS3 activates RNA helicase activity by promoting the binding of RNA substrate. *The Journal of biological chemistry* **2007**, *282*, 34913-34920, doi:10.1074/jbc.M707165200.

120. McGivern, D.R.; Masaki, T.; Lovell, W.; Hamlett, C.; Saalau-Bethell, S.; Graham, B. Protease Inhibitors Block Multiple Functions of the NS3/4A Protease-Helicase during the Hepatitis C Virus Life Cycle. *Journal of virology* **2015**, *89*, 5362-5370, doi:10.1128/JVI.03188-14.

121. Pan, Q.; Peppelenbosch, M.P.; Janssen, H.L.; de Knegt, R.J. Telaprevir/boceprevir era: from bench to bed and back. *World journal of gastroenterology* **2012**, *18*, 6183-6188, doi:10.3748/wjg.v18.i43.6183.

122. Vermehren, J.; Park, J.S.; Jacobson, I.M.; Zeuzem, S. Challenges and perspectives of direct antivirals for the treatment of hepatitis C virus infection. *Journal of hepatology* **2018**, *69*, 1178-1187, doi:10.1016/j.jhep.2018.07.002.

123. Stanciu, C.; Muzica, C.M.; Girleanu, I.; Cojocariu, C.; Sfarti, C.; Singeap, A.M.; Huiban, L.; Chiriac, S.; Cuciureanu, T.; Trifan, A. An update on direct antiviral agents for the treatment of hepatitis C. *Expert opinion on pharmacotherapy* **2021**, *22*, 1729-1741, doi:10.1080/14656566.2021.1921737.

124. Eltahla, A.A.; Luciani, F.; White, P.A.; Lloyd, A.R.; Bull, R.A. Inhibitors of the Hepatitis C Virus Polymerase; Mode of Action and Resistance. *Viruses* **2015**, *7*, 5206-5224, doi:10.3390/v7102868.

125. El Kassas, M.; Elbaz, T.; Hafez, E.; Wifi, M.N.; Esmat, G. Discovery and preclinical development of dasabuvir for the treatment of hepatitis C infection. *Expert opinion on drug discovery* **2017**, *12*, 635-642, doi:10.1080/17460441.2017.1322955.

126. Berger, C.; Romero-Brey, I.; Radujkovic, D.; Terreux, R.; Zayas, M.; Paul, D.; Harak, C.; Hoppe, S.; Gao, M.; Penin, F., et al. Daclatasvir-like inhibitors of NS5A block early biogenesis of hepatitis C virus-induced membranous replication factories, independent of RNA replication. *Gastroenterology* **2014**, *147*, 1094-1105 e1025, doi:10.1053/j.gastro.2014.07.019.

127. Guedj, J.; Dahari, H.; Rong, L.; Sansone, N.D.; Nettles, R.E.; Cotler, S.J.; Layden, T.J.; Uprichard, S.L.; Perelson, A.S. Modeling shows that the NS5A inhibitor daclatasvir has two modes of action and yields a shorter estimate of the hepatitis C virus half-life.

Proceedings of the National Academy of Sciences of the United States of America **2013**, *110*, 3991-3996, doi:10.1073/pnas.1203110110.

128. McGivern, D.R.; Masaki, T.; Williford, S.; Ingravallo, P.; Feng, Z.; Lahser, F.; Asante-Appiah, E.; Neddermann, P.; De Francesco, R.; Howe, A.Y., et al. Kinetic analyses reveal potent and early blockade of hepatitis C virus assembly by NS5A inhibitors. *Gastroenterology* **2014**, *147*, 453-462 e457, doi:10.1053/j.gastro.2014.04.021.
129. Bellanti, F.; Lauletta, G.; Villani, R.; Lipsi, M.R.; Natalicchio, M.I.; Sansonno, D.; Vendemiale, G.; Serviddio, G. Combined Effects of 2 Interleukin 28B Polymorphisms on the Therapeutic Outcome of Hepatitis C Patients With Circulating Cryoglobulins. *Medicine* **2015**, *94*, e1409, doi:10.1097/MD.0000000000001409.
130. Arena, U.; Vizzutti, F.; Abraldes, J.G.; Corti, G.; Stasi, C.; Moscarella, S.; Milani, S.; Lorefice, E.; Petrarca, A.; Romanelli, R.G., et al. Reliability of transient elastography for the diagnosis of advanced fibrosis in chronic hepatitis C. *Gut* **2008**, *57*, 1288-1293, doi:10.1136/gut.2008.149708.
131. Gegotek, A.; Skrzydlewska, E. Biological effect of protein modifications by lipid peroxidation products. *Chemistry and physics of lipids* **2019**, *221*, 46-52, doi:10.1016/j.chemphyslip.2019.03.011.
132. Ebina, S.; Chiba, T.; Ozaki, T.; Kashiwakura, I. Relationship between 8-hydroxydeoxyguanosine levels in placental/umbilical cord blood and maternal/neonatal obstetric factors. *Experimental and therapeutic medicine* **2012**, *4*, 387-390, doi:10.3892/etm.2012.617.
133. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. The French METAVIR Cooperative Study Group. *Hepatology* **1994**, *20*, 15-20.
134. Lee, H.C.; Wei, Y.H. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *The international journal of biochemistry & cell biology* **2005**, *37*, 822-834, doi:10.1016/j.biocel.2004.09.010.
135. Liu, C.S.; Tsai, C.S.; Kuo, C.L.; Chen, H.W.; Lii, C.K.; Ma, Y.S.; Wei, Y.H. Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes. *Free radical research* **2003**, *37*, 1307-1317, doi:10.1080/10715760310001621342.

136. Medeiros, T.C.; Graef, M. Autophagy determines mtDNA copy number dynamics during starvation. *Autophagy* **2019**, *15*, 178-179, doi:10.1080/15548627.2018.1532263.

137. Pawar, T.; Bjoras, M.; Klungland, A.; Eide, L. Metabolism and DNA repair shape a specific modification pattern in mitochondrial DNA. *Mitochondrion* **2018**, *40*, 16-28, doi:10.1016/j.mito.2017.09.002.

138. Wang, W.; Scheffler, K.; Esbensen, Y.; Eide, L. Quantification of DNA Damage by Real-Time qPCR. *Methods in molecular biology* **2016**, *1351*, 27-32, doi:10.1007/978-1-4939-3040-1_3.

139. Mengshol, J.A.; Golden-Mason, L.; Rosen, H.R. Mechanisms of Disease: HCV-induced liver injury. *Nature clinical practice. Gastroenterology & hepatology* **2007**, *4*, 622-634, doi:10.1038/ncpgasthep0961.

140. Nelson, D.R. The immunopathogenesis of hepatitis C virus infection. *Clinics in liver disease* **2001**, *5*, 931-953, doi:10.1016/s1089-3261(05)70202-6.

141. Paracha, U.Z.; Fatima, K.; Alqahtani, M.; Chaudhary, A.; Abuzenadah, A.; Damanhouri, G.; Qadri, I. Oxidative stress and hepatitis C virus. *Virology journal* **2013**, *10*, 251, doi:10.1186/1743-422X-10-251.

142. Tanaka, H.; Fujita, N.; Sugimoto, R.; Urawa, N.; Horiike, S.; Kobayashi, Y.; Iwasa, M.; Ma, N.; Kawanishi, S.; Watanabe, S., et al. Hepatic oxidative DNA damage is associated with increased risk for hepatocellular carcinoma in chronic hepatitis C. *British journal of cancer* **2008**, *98*, 580-586, doi:10.1038/sj.bjc.6604204.

143. Pal, S.; Polyak, S.J.; Bano, N.; Qiu, W.C.; Carithers, R.L.; Shuhart, M.; Gretch, D.R.; Das, A. Hepatitis C virus induces oxidative stress, DNA damage and modulates the DNA repair enzyme NEIL1. *Journal of gastroenterology and hepatology* **2010**, *25*, 627-634, doi:10.1111/j.1440-1746.2009.06128.x.

144. Qian, C.; Camps, J.; Maluenda, M.D.; Civeira, M.P.; Prieto, J. Replication of hepatitis C virus in peripheral blood mononuclear cells. Effect of alpha-interferon therapy. *Journal of hepatology* **1992**, *16*, 380-383, doi:10.1016/s0168-8278(05)80674-9.

145. Boya, P.; de la Pena, A.; Beloqui, O.; Larrea, E.; Conchillo, M.; Castelruiz, Y.; Civeira, M.P.; Prieto, J. Antioxidant status and glutathione metabolism in peripheral blood mononuclear cells from patients with chronic hepatitis C. *Journal of hepatology* **1999**, *31*, 808-814, doi:10.1016/s0168-8278(99)80281-5.

146. Amstad, P.; Moret, R.; Cerutti, P. Glutathione peroxidase compensates for the hypersensitivity of Cu,Zn-superoxide dismutase overproducers to oxidant stress. *The Journal of biological chemistry* **1994**, *269*, 1606-1609.

147. Amstad, P.; Peskin, A.; Shah, G.; Mirault, M.E.; Moret, R.; Zbinden, I.; Cerutti, P. The balance between Cu,Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. *Biochemistry* **1991**, *30*, 9305-9313, doi:10.1021/bi00102a024.

148. Mahmood, S.; Kawanaka, M.; Kamei, A.; Izumi, A.; Nakata, K.; Niiyama, G.; Ikeda, H.; Hanano, S.; Suehiro, M.; Togawa, K., et al. Immunohistochemical evaluation of oxidative stress markers in chronic hepatitis C. *Antioxidants & redox signaling* **2004**, *6*, 19-24, doi:10.1089/152308604771978318.

149. Gokhale, N.S.; Vazquez, C.; Horner, S.M. Hepatitis C Virus. Strategies to Evade Antiviral Responses. *Future virology* **2014**, *9*, 1061-1075, doi:10.2217/fvl.14.89.

150. Schurich, A.; Pallett, L.J.; Jajbhay, D.; Wijngaarden, J.; Otano, I.; Gill, U.S.; Hansi, N.; Kennedy, P.T.; Nastouli, E.; Gilson, R., et al. Distinct Metabolic Requirements of Exhausted and Functional Virus-Specific CD8 T Cells in the Same Host. *Cell reports* **2016**, *16*, 1243-1252, doi:10.1016/j.celrep.2016.06.078.

151. Pearce, E.L.; Pearce, E.J. Metabolic pathways in immune cell activation and quiescence. *Immunity* **2013**, *38*, 633-643, doi:10.1016/j.immuni.2013.04.005.

152. Assmann, N.; Finlay, D.K. Metabolic regulation of immune responses: therapeutic opportunities. *The Journal of clinical investigation* **2016**, *126*, 2031-2039, doi:10.1172/JCI83005.