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LAY SUMMARY

-HCV eradication improves atherosclerosis although without clearly defined mechanisms

-We hypothesized that reduction of oxidative stress, as measured by serum F_2-isoprostanes, may be involved in the improvement of atherosclerosis after HCV eradication

-We demonstrated that the reduction of carotid intima-media thickness after viral clearance is directly and independently associated with the reduction of circulating F_2-isoprostanes

ABSTRACT

Background and aims. HCV eradication improves non-hepatic outcomes such as cardiovascular diseases although without clearly defined mechanisms. In this study we aimed to assess whether improvement of carotid atherosclerosis may be linked to a reduction of systemic oxidative stress after viral clearance.

Methods. We studied a retrospective cohort of 105 patients (age 62.4±11.2 years; 62 males) with F3/F4 fibrosis, characterized by carotid ultrasonography at baseline and at sustained virologic response (SVR) follow-up. Levels of 8-iso-prostaglandin $F_{2\alpha}$ (F_2-isoprostanes) and other oxidative stress markers were measured on frozen sera. Association between change (denoted as Δ) of oxidative stress markers (exposures) and change of carotid intima-media thickness (cIMT) (outcome) was examined using multiple linear regression.

Results. Subclinical atherosclerosis, defined as the presence of carotid plaque and/or cIMT≥0.9, was present in 72% of the cohort. All patients achieved SVR that led to reduction of cIMT (0.92±0.20 vs 0.83±0.21 mm, P<0.001). HCV eradication markedly decreased serum levels of F_2-isoprostanes (620.5 [143.2; 1904.1] vs 119.51 [63.2; 400.6] pg/ml, P<0.0001), lipid hydroperoxides (13.8 [6.3; 20.7] vs 4.9 [2.3; 9.6] nmol/µl, P<0.0001) and 8-hydroxy-2'-deoxyguanosine (558.9 [321.0; 6301.2] vs 294.51 [215.31; 408.95] pg/ml, P<0.0001) whereas increased serum GPx activity (10.44 [4.6; 16.3] vs 13.75 [9.42; 20.63] nmol/min/ml, P=0.001). By multiple linear regression analysis ΔcIMT was independently associated with ΔF_2-isoprostanes (β: 1.746 [0.948; 2.543]; P<0.0001) after adjustment for age, baseline F_2-isoprostanes and baseline IMT.

Conclusions. Besides association of lipid peroxidation with severity of liver disease, the reduction of F_2-isoprostanes may be involved in the improvement of atherosclerosis after HCV eradication.

INTRODUCTION

Hepatitis C virus (HCV) chronic infection affects about 70 million of individuals worldwide with an estimated global prevalence of 1.0% widely changing according to geographical areas [1]. This epidemiological scenario is clinically relevant because chronic hepatitis C (CHC) is associated with liver-related morbidity and mortality, but also with extrahepatic complications such as insulin resistance [2,3] and cardiovascular diseases [4]. The availability of safe and effective direct antiviral agents (DAA) is currently showing us the impact of HCV eradication on hepatic and non-hepatic outcomes [5] although the molecular mechanisms that underlie the protective effects of HCV clearance against cardiovascular events [6] remain to be fully elucidated.

Oxidative stress indicates an imbalance between reactive oxygen species (ROS) production and ability of a biological system to counteract ROS damage to cells and organs [7]. Oxidative stress can be triggered by several *noxae* including viruses and bacteria [8]. It is a common opinion that although increased levels of ROS have a beneficial effect in counteracting infections in the acute phase, establishment of a chronic infection may induce oxidative stress leading to cell damage and organ dysfunction . Increased ROS production targets various intracellular macromolecules, leading to lipid peroxidation, protein oxidation and nucleic acid oxidative damage [9].

Previous reports showed that HCV triggers oxidative stress in *in vitro* models of infection [10] and clinical data have consistently suggested that oxidative stress is a feature of patients with CHC [11]. To this regard, Valgimigli and colleagues measured ROS levels in HCV-infected liver samples demonstrating higher levels as compared to control and showed that ROS correlated with histological disease activity [12,13]. Unfortunately, ROS detection is technically difficult and therefore it is preferable the use of lipid, DNA/RNA and protein oxidation biomarkers in clinical studies [14]. Among markers of oxidative stress, 8-iso-prostaglandin $F_{2\alpha}$ (F_2-isoprostanes) are considered not only the most accurate marker of

lipid peroxidation [15] but signaling molecules that are involved both in liver fibrogenesis [16]

and atherogenesis [17]. For this reason, we hypothesize that the improvement of subclinical

atherosclerosis [18] that follow HCV eradication may be linked to the decrease of systemic

oxidative stress and in particular to the reduction of circulating levels of proatherogenic

mediators such as F_2-isoprostanes.

METHODS

Patients

For sample size calculation we considered our previous study assessing changes in carotid intima-media thickness (cIMT) in patients with advanced liver fibrosis, treated with DAA [18]; based on it, a sample of 105 would be sufficient to detect a decrease in carotid IMT with an α error of 0·05 and a statistical power of 0·9. [18] Frozen sera were available for outpatients recruited between middle 2015 and early 2016 at the Gastroenterology and Hepatology Unit of the University of Palermo, that were characterized for carotid atherosclerosis. At the time of enrollment, the Italian Medicines Agency (Agenzia Italiana del Farmaco, AIFA) did not allow treatment of patients with a milder stage of hepatitis C or advanced cirrhosis (Child-Pugh B and C) and criteria of eligibility for DAA treatment were: F3 fibrosis, diagnosed by histology and/or by liver stiffness measurement (≥10 to ≤12 KPa by FibroScan); compensated Child A cirrhosis, diagnosed by histology and/or by LSM (>12 KPa), and/or by clinical evidence of portal hypertension. Beyond AIFA criteria, additional exclusion criteria for our study were: 1) hepatocellular carcinoma; 2) liver disease of different or mixed etiology (i.e., excessive alcohol consumption, hepatitis B, autoimmune liver disease, Wilson's disease, hemochromatosis, α1-antitrypsin deficiency); 3) HIV infection; 4) treatment with hepatotoxic drugs; 5) active drug addiction. Patients were tested at baseline for HCV-RNA (real-time PCR COBAS TaqMan HCV Test v2.0 and Roche diagnostics, S.p.A Monza, Italy) and HCV genotype (Versant HCV Genotype 2.0 Assay LIPA, Siemens, Erlangen, Germany). HCV-RNA was repeated after 4 weeks of therapy, at the end of therapy, and 12 weeks after stopping treatment. Sustained virologic response (SVR) was defined as HCV-RNA undetectable after 12 weeks from the end of antiviral therapy.

Assessment of carotid atherosclerosis was performed by using a high-resolution B-mode ultrasonography equipped with a multifrequency linear probe. cIMT was measured as the

difference between the first (intima lumen) interface and the second (media adventitia) interface on the far wall of the common carotid artery in a section free of plaque beginning 10 mm below their bifurcations and including the bifurcations for 10 mm. In agreement with joint guidelines from the European Society of Hypertension and the European Society of Cardiology[19], subclinical atherosclerosis was considered as the presence of asymptomatic carotid plaque (focal thickening ≥ 1.5 mm) or cIMT ≥0.9 mm without plaque. IMT measurement was performed by the same operator at baseline and at follow-up. Imaging and clinical data were collected at the time of the enrollment and 9 months after the end of antiviral therapy.

The study was conducted in accordance with the principles of the Declaration of Helsinki and its appendices, and with local and national laws. Approval was obtained from the local Institutional Review Board and Ethics Committee, and written informed consent was obtained from all patients.

Laboratory methods

Measurement of a panel of oxidative stress parameters was performed on -80 °C frozen serum samples that were collected before the beginning of DAA treatment (baseline) and 6 months after the end of DAA (SVR follow-up). Lipid peroxidation *status* was assessed by measuring levels of lipid hydroperoxides (LOOH) through a modified ferrous oxidation/xylenol orange assay, at λ=560 nm, as previously described [20] and by measuring levels of 8-epi-prostaglandin $F_{2\alpha}$ (F_2-isoprostanes), the most represented isomer of the isoprostanes series, through an ELISA kit (#516351 from Cayman Chemical, Ann Arbor, MI), according to manufacturer's instruction. Serum levels of thiol groups, containing predominantly reduced glutathione, were determined spectrophotometrically at λ=412 nm by Ellman's reagent, as previously described (17). As marker to evaluate oxidative damage to nucleic acids we used an ELISA kit (#589320 Cayman Chemical) that detects the three oxidized guanine species: 8-hydroxy-2'-deoxyguanosine from DNA, 8-

hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA; to simplify we indicate this marker as 8-OHdG, that is the most common one [21]. Serum activity of glutathione peroxidase (GPx) was measured through a colorimetric assay (#703102 Cayman Chemical), according to manufacturer's instruction. Protein oxidation/nitrosylation was assessed by measuring the biomarker 3-nitrityrosine (3-NT) [22] through an ELISA kit (#SKT-126 from StressMarq Biosciences, Victoria, Canada).

Statistical analysis

Statistical analysis was performed by GraphPad Prism 8 (GraphPad Software, CA). Continuous variables are presented as mean ± SD or median IQR (interquartile range), based on data distribution, established by Shapiro normality test. Differences from baseline to follow-up were assessed by paired T test or Wilcoxon signed ranks test based on data distribution. Multivariable logistic regression analyses were performed to establish the association between oxidative stress parameters and the presence of esophageal varices, carotid plaque and cIMT≥0.9 as dependent categorical variable. Multiple linear regression analysis was performed to assess the association between change (denoted as Δ) of oxidative stress markers (exposures) and change of cIMT (outcome). If values of exposures showed non-normal distribution, log-transformed values were considered for logistic and linear regression analyses. Calibration and discrimination ability of logistic models were checked using the Hosmer–Lemeshow statistic.

RESULTS

General features of the study population

The main features of the study population are showed in Table 1. Mean age was 62.4±11.2 years, with a 59% of males (62/105). Among 105 patients, 53 had fibrosis F3, 52 had compensated cirrhosis (27 Child A, 25 Child B). Among the 52 patients with cirrhosis, 26 of them had small esophageal varices. The most prevalent genotypes were 1b (79/105) and 1a (12/105). Liver stiffness in the whole cohort was 12 [10.1; 19.4] kPa. As concern metabolic features, 25% of patients (26/105) were overweight/obese; mean serum total cholesterol and triglycerides were in the normal range. Thirty percent of patients had arterial hypertension (32/105), 12% had type 2 diabetes (13/105); 15% were active smokers. Overall, subclinical atherosclerosis was present in 72% (76/105) of the cohort. All patients were treated with DAA as showed in Table 1, according to therapeutic schedules suggested by EASL guidelines available at the time of the enrollment [23].

Effects of HCV eradication on clinical parameters

All patients achieved an SVR following DAA treatment. As showed in Table 2, HCV eradication reduced serum AST (46 [28.7; 74.5] vs 20 [17; 26] U/l, P<0.0001), ALT (58.5 [30; 89] vs 20 [14.7; 26.2] U/l, P<0.0001), and GGT (43.5 [27; 90.2] vs 21 [16.7; 34.7], P<0.0001) whereas increased levels of platelets (139±61 vs 160±72 10^3/µl, P<0.05) and albumin (4.0 [3.8; 4.2] vs 4.1 [3.9; 4.3] mg/dl, P<0.05). Total bilirubin and INR were unchanged. SVR led to a reduction of liver stiffness (12 [10.1; 19.4] vs 10.6 [6.7; 17] kPa, P<0.0001) and IMT (0.92±0.20 vs 0.83±0.21 mm, P<0.001). As concern metabolic parameters, we observed a slight but significant decrease in fasting glucose (98 [88; 108] vs 94 [86.5; 104] mg/dl, P<0.05) despite no change of body weight from baseline to SVR follow-up. HCV clearance also increased serum total cholesterol (156±40 vs 168±44 mg/dl, P<0.05) and triglycerides (85.1±32 vs 94.0±40.5 mg/dl, P<0.05), although values remained in the normal range.

Effects of HCV eradication on oxidative stress parameters

Changes in serum levels of oxidative stress markers are showed in Figure 1. As concern lipid peroxidation parameters, we found that serum F_2-isoprostanes levels were markedly reduced at follow-up after viral treatment (620.5 [143.2; 1904.1] vs 119.51 [63.2; 400.6] pg/ml, P<0.0001). Decrease of serum F_2-isoprostanes was widely observed across sub-groups discriminated according to stage of fibrosis and metabolic parameters. Specifically, F_2-isoprostanes were reduced by HCV eradication in patients with F3 or F4, with normal or impaired fasting glucose and independently of BMI (Suppl. Table 1). In agreement with abatement of F_2-isoprostanes levels, HCV eradication markedly decreased serum LOOH (13.8 [6.3; 20.7] vs 4.9 [2.3; 9.6] nmol/μl, P<0.0001) (Figure 1). For both markers of lipid peroxidation, reduction from baseline to SVR was greater in patients with cirrhosis (Suppl. Table 1). By simple linear regression, F_2-isoprostanes were associated with LOOH at baseline (r=0.670, P<0.0001) and SVR (r=0.490, P<0.0001).

As concern the antioxidant *status*, serum GPx activity was significantly higher at SVR compared to baseline (10.44 [4.6; 16.3] vs 13.75 [9.42; 20.63] nmol/min/ml, P=0.001) (Figure 1). Again, increase of GPx activity after HCV clearance was widely observed in all sub-groups (Suppl. Table 1), and as observed for lipid peroxidation markers, Δ change of GPx activity was greater in patients with cirrhosis compared to F3 (Suppl. Table 1). HCV eradication led also to lower serum levels of total thiols (17.22 [10.66; 28.44] vs 3.34 [2.41; 4.91] nmol/μl, P<0.0001) in the whole cohort (Figure 1) and this result was confirmed in patients with F3 or F4 and in all subgroups (Suppl. Table 1). By simple linear regression, GPx activity was inversely associated with LOOH at baseline (r=-0.24, P<0.05) and SVR (r=-0.29, P<0.01), indicating the existence of a LOOH/GPx co-regulation in health and disease.

As concern nucleic acid oxidative damage, serum 8-OHdG at baseline were lowered following HCV eradication (558.9 [321.0; 6301.2] vs 294.5 [215.3; 408.95] pg/ml,

P<0.0001) (Figure 1). The reduction of 8-OHdG was observed across subgroups with various statistical strength (Suppl. Table 1). We also measured 3-NT levels as markers of protein oxidation/nitrosylation. However, SVR did not significantly change 3-NT levels (799.8 [419.5; 1287.1] vs 821.2 [539.6; 1201.6] nmol/l, P=0.74) neither in the whole cohort (Figure 1) or in the subgroups (Suppl. Table 1), thus showing that the role of protein oxidative/nitrosative stress is less relevant in this context.

Association of oxidative stress with clinical parameters and outcomes

Levels of lipid peroxidation were proportional to the severity of fibrosis, as showed by higher serum F_2-Isoprostanes and LOOH in patients with F4 compared to F3 (1494 [420.2; 2435] vs 428.1 [112.4; 1167] pg/ml, P<0.05) and (17.3 [22.0; 7.94] vs 9.7 [19.9; 5.8] nmol/µl, P<0.05) respectively. In a logistic regression model including all baseline oxidative stress parameters, adjusted for age, log-transformed F_2-isoprostanes were independently associated with the presence of esophageal varices (OR 3.7 [1.42; 9.62]) (Table 3).

In a logistic regression analysis including all oxidative stress parameters at baseline and considering cIMT≥0.9 as categorical variable, log-transformed values of F_2-Isoprostanes were also independently associated with the presence of cIMT≥0.9 (OR 0.32 [0.08; 1.24], P<0.05) (Suppl. Table 2) whereas in a logistic regression model evaluating the risk of carotid plaque, log-transformed values of LOOH were associated with its presence (OR 1.61 [0.75; 3.43], P<0.05) (Suppl. Table 3).

Finally, the most important aim of the study was to evaluate whether changes of any of the assessed oxidative stress parameters was associated with reduction of cIMT. As showed in Table 4, in a multiple linear regression model including changes (Δ) of the six oxidative stress with changes of cIMT, ΔF_2-Isoprostanes was independently and directly associated with ΔcIMT (β: 1.746 [0.948; 2.543], P<0.0001), indicating that the higher is the reduction of circulating F_2-Isoprostanes, the higher is the reduction of cIMT.

DISCUSSION

In this study we aimed to establish whether improvement of cardiovascular outcomes following HCV eradication may be linked to reduction of systemic oxidative stress and demonstrated that the decrease of carotid intima-media thickness after viral clearance is independently associated with the decrease of circulating F_2-isoprostanes.

F_2-isoprostanes are prostaglandin-like compounds that can be formed via a non-enzymatic free radical-initiated peroxidation of arachidonic acid [15]. Besides being considered the most accurate and popular marker of lipid peroxidation [24], F_2-isoprostanes plays a role in several pathological process including liver fibrogenesis. In a rat model of carbon tetrachloride-induced hepatic fibrosis, plasma levels of F_2-isoprostanes progressively increase from fibrosis to cirrhosis and correlate with hepatic collagen content [25]. Furthermore, *in vitro* studies demonstrated that treatment of hepatic stellate cells (HSCs) with F_2-isoprostanes stimulate their activation to myofibroblasts [25] and these effects are mediated by activation of receptors analogous to those for thromboxane A2 expressed on HSCs [26,27].

F_2-isoprostanes show also potent vascular effects including vasoconstriction, monocyte adhesion to the endothelium, platelet aggregation and smooth muscular cells proliferation [17], indicating that they are crucially involved in the atherosclerosis process. In agreement with HSC data, studies in animal models of atherosclerosis demonstrated that F_2-isoprostanes can directly promote atherogenesis by activating the thromboxane A2 receptor [28]; consistently, the regression of atherosclerotic lesions is accompanied by reduction of F_2-isoprostanes [29]. In addition to pre-clinical evidence, clinical data report that F_2-isoprostanes are tightly associated to carotid atherosclerosis [30,31].

Overall, on the basis of our results and data reported in literature, we suggest that circulating F_2-isoprostanes generated by lipid peroxidation in hepatocytes during chronic HCV infection can exert fibrogenic effects in the liver and once released in the

bloodstream can trigger vascular processes associated with onset and progression of atherosclerosis (Figure 2). Activation of thromboxane A2 receptor could be envisioned as the biologic link between the events occurring in liver and vasculature, leading to liver fibrosis and atherogenesis, respectively.

Among other oxidative stress mechanisms described so far in chronic HCV infection, lipid peroxidation has been usually reported in patients by measuring levels of reactive aldehydes as biomarkers [11]. To our knowledge, De Maria et al. and Barbaro et al. were the first that, by biochemical analysis, showed higher malondialdehyde (MDA) respectively in serum and liver of CHC patients compared to healthy subjects [32,33]. However, a first direct demonstration of the link between lipid peroxidation and fibrosis came from the study of Paradis et al. who reported that higher levels of MDA-protein adducts, as assessed semiquantitatively by immunohistochemistry, were associated with higher stages of fibrosis in liver sections of HCV-infected patients [34].

In our study we evaluated changes of LOOH, which are organic hydroperoxides, derived by peroxidation of membrane- and lipoprotein-bound lipids, that may damage other macromolecules, thus inducing cell dysfunction and death [35]. Togashi et al. showed higher hepatic LOOH levels compared to healthy controls, proportionally to histological severity of CHC [36]. Furthermore, LOOH have been demonstrated to promote carcinogenesis in a mouse model of HCV-hepatocellular carcinoma [37]. LOOH are selectively reduced by GPx4, a monomeric protein belonging to the family of selenocysteine peroxidases [38], that is down-regulated by HCV *in vitro* [39]. LOOH are reactive species that if not efficiently reduced by GPx4 may undergo further conversion to reactive aldehydes [35], which have been shown to activate the signaling cascade leading to fibrosis in HCV [40]. Here we showed that the LOOH/GPx axis is clinically evident in CHC. GPx activity has been already demonstrated to be lower in patients with CHC compared to healthy individuals [41,42] and *in vitro* data show that GPx exert protective anti-HCV effects [43-45].

Finally, we would like to point out the importance of a marked reduction of 8-OHdG following HCV-eradication in our cohort. Beside lipid peroxidation, another feature of CHC is the presence of oxidative DNA damage, as assessed by the marker 8-OHdG that previous studies showed to be higher in the liver of patients with CHC compared with controls [46-48]. From a clinical point of view, 8-OHdG expression has been showed to be an independent predictor of HCC development [49]. For this reason, our results of a ten-fold decrease of 8-OHdG after viral clearance can partly explain the lower incidence of HCC following DAA treatment [50].

Our study has some limits. Oxidative stress was not measured on liver samples because it was not ethically possible a follow-up biopsy; however, we used well-characterized biomarkers of lipid, protein, and nucleic acid damage. Furthermore, serum GPx activity was measured without discriminating between the different isoforms; nonetheless, since GPx4 displays a reducing activity specific for LOOH we are confident that our results reflect GPx4. Another limitation lies in the lack of data about the impact of HCV eradication on oxidative stress in patients with HCV infection and milder liver fibrosis. Finally, despite we adjusted for variables that may confound results, we cannot rule out the existence of potential residual confounding.

In conclusion, our study demonstrated an independent and direct association between reduction of subclinical atherosclerosis and reduction of F_2-isoprostanes after viral clearance that may provide a molecular rationale explaining improvement of cardiovascular outcomes following SVR.

Table 1. General features of the study population (n=105)

Parameter (units)	
Age (y)	62.4±11.2
Males (n)	62 (59%)
F3/cirrhosis (n)	53/52
Child-Pugh-Turcotte score A5/A6 (n)	27/25
F1 esophageal varices (n)	26
Genotype 1a/1b/2/3/4 (n)	12/79/5/5/4
OMB/PAR/RIT/DAS±RBV, SOF/LED±RBV, SOF+DAC± RBV,	60, 24, 9, 7, 4, 1
Diabetes (n)	13 (12%)
Arterial hypertension (n)	32 (30%)
Smoking	16 (15%)
Subclinical atherosclerosis	76 (72%)

Table 2. Changes in clinical parameters at SVR after DAA treatment (n=105)

Parameter (units)	Baseline	SVR12	P
Body weight (kg)	74.6±14.2	74.8±13.8	NS
HCV-RNA (U/ml)	950000 (317250; 1740000)	Undetectable	<0.0001
AST (U/l)	46 (28.75; 74.5)	20 (17; 26)	<0.0001
ALT (U/l)	58.5 (30; 89)	20 (14.75; 26.25)	<0.0001
GGT (U/l)	43.5 (27; 90.25)	21 (16.75; 34.75)	<0.0001
Total bilirubin (mg/dl)	0.77 (0.5; 1)	0.7 (0.42; 1)	NS
Direct bilirubin (mg/dl)	0.34 (0.2; 0.44)	0.24 (0.2; 0.35)	0.01
INR	1 (0.96; 1)	1 (1; 1.1)	NS
Albumin (g/dl)	4.0 (3.8; 4.2)	4.1 (3.9; 4.3)	<0.05
Platelet count (10^3/µl)	139±61	160±72	<0.05
Fasting plasma glucose (mg/dl)	98 (88; 108)	94 (86.5; 104)	<0.05
Total cholesterol (mg/dl)	156±40	168±44	<0.05
Triglycerides (mg/dl)	85.1±31.7	94.0±40.5	<0.05
Creatinine (mg/dl)	0.8 (0.7; 0.9)	0.8 (0.7; 0.9)	NS
Liver stiffness (kPa)	12 (10.1; 19.4)	10.6 (6.7; 17)	<0.0001
Intima-media thickness (mm)	0.92±0.20	0.83±0.21	<0.001

Results are expressed as mean ± SD or median IQR based on data distribution and consistently P are calculated by paired T-test or Wilcoxon test, respectively.

Table 3. Multivariable logistic regression analysis, adjusted for age, between baseline oxidative stress parameters (exposure) and the risk of esophageal varices (outcome).

Parameter (log-transformed)	OR (95% CI)	P
F_2-isoprostanes	8.76 (1.8; 42.7)	**0.007**
LOOH	0.27 (0.07; 1.01)	0.05
GPx activity	0.74 (0.31; 1.77)	0.495
Total thiols	0.59 (0.25; 1.4)	0.232
8-OHdG	0.46 (0.17; 1.3)	0.146
3-NT	0.76 (0.34; 1.69)	0.505

*reported per standard deviation increase in oxidative stress parameters. LOOH, lipid hydroperoxides; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; GPx, gluthatione peroxidase; 3-NT, 3-nitrotyrosine

Table 4. Multivariable linear regression analysis, adjusted for age, baseline exposure and baseline outcome, between Δoxidative stress (exposure) and ΔcIMT (outcome)

Δ Parameter	β (95% CI)	P
F_2-isoprostanes	1.746 (0.948; 2.543)	**<0.0001**
LOOH	0.368 (-0.139; 0.874)	0.151
GPx activity	-0.042 (-0.377; 0.294) 0.114 (-0.335; 0.563)	0.805
Total thiols	-0.058 (-1.544; 1.429)	0.938
8-OHdG	-2.22 (-9.453; 5.012)	0.542
3-NT	0.114 (-0.335; 0.563)	0.614

Results are expressed as standardized beta coefficients with 95%CI. LOOH, lipid hydroperoxides; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; GPx, gluthatione peroxidase; 3-NT, 3-nitrotyrosine

Suppl. Table 1. Changes in oxidative stress parameters at sustained virologic response (SVR) after DAA treatment in subgroups discriminated according to fibrosis, blood glucose, and BMI

Parameter (units)	F3 fibrosis (n=53)		
	Baseline	SVR	P
F_2-isoprostanes (pg/ml)	428.1 [112.4; 1167]	144.6 [67.5; 505.2]	<0.05
LOOH (nmol/µl)	9.67 [5.84; 19.9]	5.7 [2.25; 10.9]	<0.01
GPx activity (nmol/min/ml)	10.4 [5.3; 16.3]	12.7 [8.7; 20.6]	<0.05
Thiols (nmol/µl)	18.4 [11.4; 33.4]	3.2 [2.2; 5.1]	<0.0001
8-OHdG (pg/ml)	4018 [343; 7720]	283.8 [183; 403]	<0.0001
3-NT (nmol/l)	863 [432; 1251]	822 [540; 1254]	NS
	Compensated cirrhosis (n=52)		
	Baseline	SVR	P
F_2-isoprostanes (pg/ml)	1494 [420; 2435]	90.0 [53.9; 188]	<0.0001
LOOH (nmol/µl)	17.3 [7.94; 22]	4.8 [2.5; 7.8]	<0.0001
GPx activity (nmol/min/ml)	7.6 [3.57; 15.3]	15.5 [10.1; 22.2]	<0.0001
Thiols (nmol/µl)	16.7 [9.3; 21.7]	3.4 [2.5; 4.96]	<0.0001
8-OHdG (pg/ml)	456.7 [249; 804]	309.5 [240; 438]	<0.05
3-NT (nmol/l)	741 [391; 1580]	801 [495; 1157]	NS

LOOH, lipid hydroperoxides; GPx, glutathione peroxidase; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 3-NT, 3-nitrotyrosine

Parameter (units)	Blood glucose <100 mg/dl (n=70)		
	Baseline	SVR	P
F$_2$-isoprostanes (pg/ml)	596 [114; 1913]	129 [60; 338]	0.0005
LOOH (nmol/µl)	14.1 [6.1; 20.1]	4.8 [2.3; 9.4]	<0.0005
GPx activity (nmol/min/ml)	11.2 [4.6; 16.4]	13.2 [8.5; 19.2]	<0.05
Thiols (nmol/µl)	18.4 [10.7; 31.0]	3.2 [2.5; 4.4]	<0.0001
8-OHdG (pg/ml)	815.4 [381.5; 7888]	319 [218; 449]	<0.0001
3-NT (nmol/l)	892 [432; 1785]	827 [563; 1261]	NS
	Blood glucose ≥100 mg/dl (n=35)		
	Baseline	SVR	P
F$_2$-isoprostanes (pg/ml)	795 [200; 2023]	108 [61; 522]	0.001
LOOH (nmol/µl)	12.5 [6.5; 20.9]	6 [2.3; 9.7]	0.0005
GPx activity (nmol/min/ml)	8.9 [4.1; 14.1]	15.7 [11.9; 21.9]	<0.001
Thiols (nmol/µl)	15.1 [9.9; 24.2]	3.5 [1.8; 5.9]	<0.0001
8-OHdG (pg/ml)	362 [183; 3018]	283 [188; 355]	<0.05
3-NT (nmol/l)	732 [332; 1213]	768 [453; 1140]	NS

Parameter (units)	BMI <25 kg/m^2 (n=79)		
	Baseline	SVR	P
F$_2$-isoprostanes (pg/ml)	636 [148; 1923]	130 [67.6; 496]	<0.0001
LOOH (nmol/µl)	13.9 [6.3; 20.7]	5.3 [2.3; 10.3]	<0.0001
GPx activity (nmol/min/ml)	9.8 [4.0; 16.6]	13.4 [8.9; 20.8]	<0.005
Thiols (nmol/µl)	17.3 [11.3; 30.7]	3.1 [2.1; 4.3]	<0.0001
8-OHdG (pg/ml)	642 [296; 7167]	294.5 [200; 389]	<0.0001
3-NT (nmol/l)	912 [414; 1278]	896 [266; 1233]	NS
	BMI ≥25 kg/m^2 (n=26)		
	Baseline	SVR	P
F$_2$-isoprostanes (pg/ml)	526 [119; 1793]	87 [47.6; 379]	<0.01
LOOH (nmol/µl)	10.2 [5.8; 21.8]	4.6 [2.6; 7.7]	0.005
GPx activity (nmol/min/ml)	10.8 [14.3; 5.0]	16.4 [11.3; 21.1]	<0.05
Thiols (nmol/µl)	15.1 [9.8; 25.6]	4.0 [2.9; 5.2]	<0.0001
8-OHdG (pg/ml)	379 [307; 4239]	304 [224; 511]	<0.05
3-NT (nmol/l)	732 [394; 1911]	663 [421; 887]	NS

Suppl. Table 2. Multivariable logistic regression analysis, adjusted for age, between oxidative stress parameters at baseline (exposure) and the risk of cIMT≥0.9 (outcome)

Parameter (log-transformed)	OR (95% CI)[*]	P
F_2-isoprostanes	0.32 (0.08; 1.24)	**0.047**
LOOH	1 (0.43; 2.33)	0.324
GPx activity	0.63 (0.26; 1.54)	0.798
Total thiols	0.34 (0.12; 1.01)	0.139
8-OHdG	1.17 (0.46; 2.97)	0.783
3-NT	0.71 (0.24; 2.11)	0.784

[*]reported per standard deviation increase in oxidative stress parameter. LOOH, lipid hydroperoxides; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; GPx, gluthatione peroxidase; 3-NT, 3-nitrotyrosine

Suppl. Table 3. Multivariable logistic regression analysis, adjusted for age, between oxidative stress parameters at baseline (exposure) and the risk of carotid plaque (outcome)

Parameter (log-transformed)	OR (95% CI)[*]	P
F_2-isoprostanes	0.75 (0.36; 1.58)	0.10
LOOH	1.61 (0.75; 3.43)	**0.03**
GPx activity	1.06 (0.59; 1.91)	0.50
Total thiols	1.27 (0.72; 2.24)	0.46
8-OHdG	1.51 (0.7; 3.22)	0.90
3-NT	1.14 (0.65; 2.03)	0.14

[*]reported per standard deviation increase in oxidative stress parameter. LOOH, lipid hydroperoxides; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; GPx, gluthatione peroxidase; 3-NT, 3-nitrotyrosine

Figure 1. Effects of HCV eradication following DAA on oxidative stress parameters.

Results are showed as median with 95% CI, n=105. P is calculated by Wilcoxon test because all parameters had a non-normal distribution both at baseline and at follow-up. LOOH, lipid hydroperoxides; GPx, glutathione peroxidase; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 3-NT, 3-nitrotyrosine.
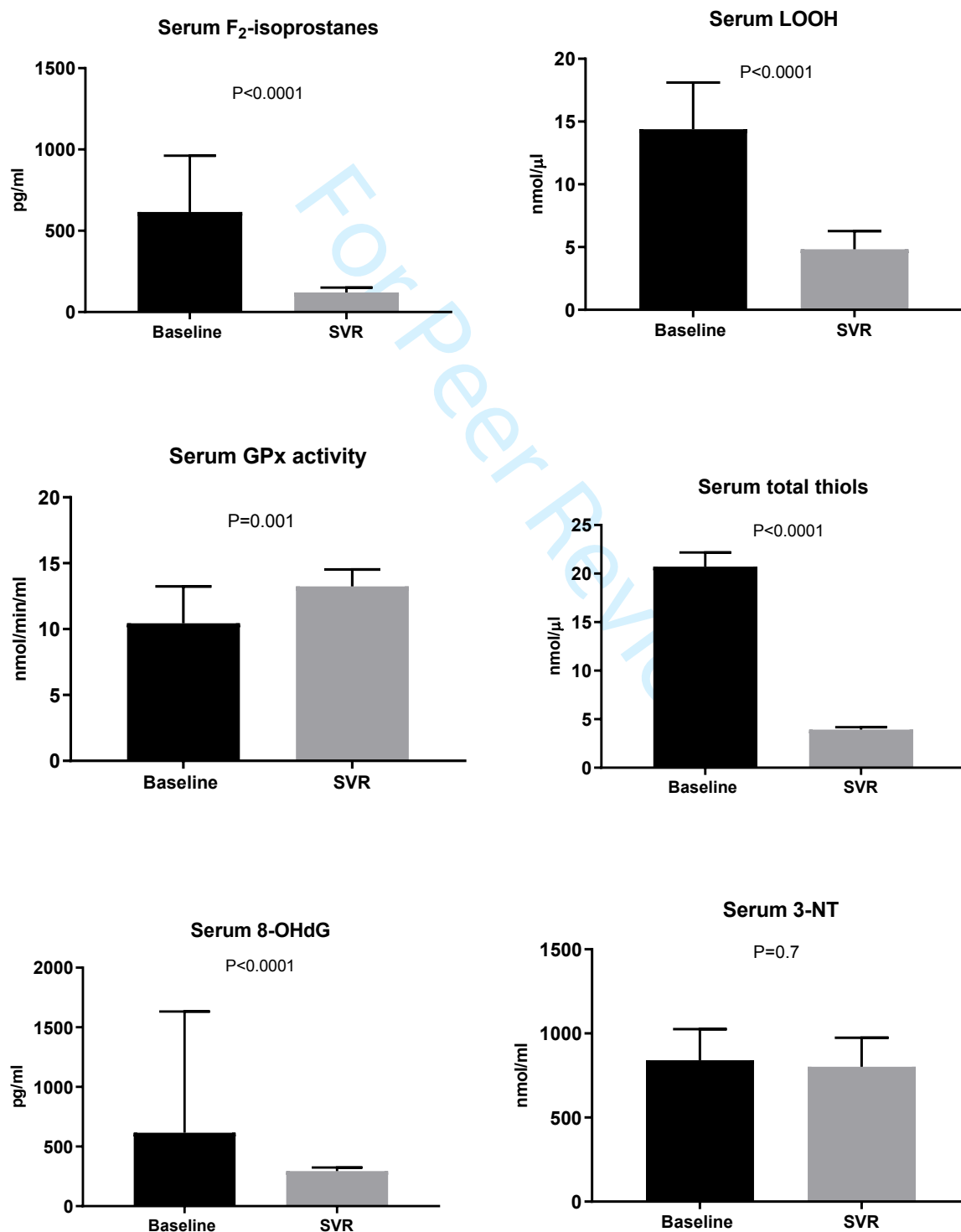
Figure 2. Plausible mechanisms by which oxidative stress produced in the liver during chronic hepatitis C promotes atherosclerosis. HCV infection induces free radical production that leads to peroxidation of lipid macromolecules in the hepatocyte and thus to overproduction of lipid hydroperoxides and in particular of F_2-isoprostanes.

F_2-isoprostanes exert fibrogenic effects locally by stimulating hepatic stellate cells (HSC) and once released in the bloodstream exert atherogenic effects. F_2-isoprostanes bind to the thromboxane A2 receptor on the endothelium promoting vasoconstriction, monocyte aggregation and smooth muscular cells proliferation. HCV eradication interrupts this vicious cycle by reduction of circulating F_2-isoprostanes
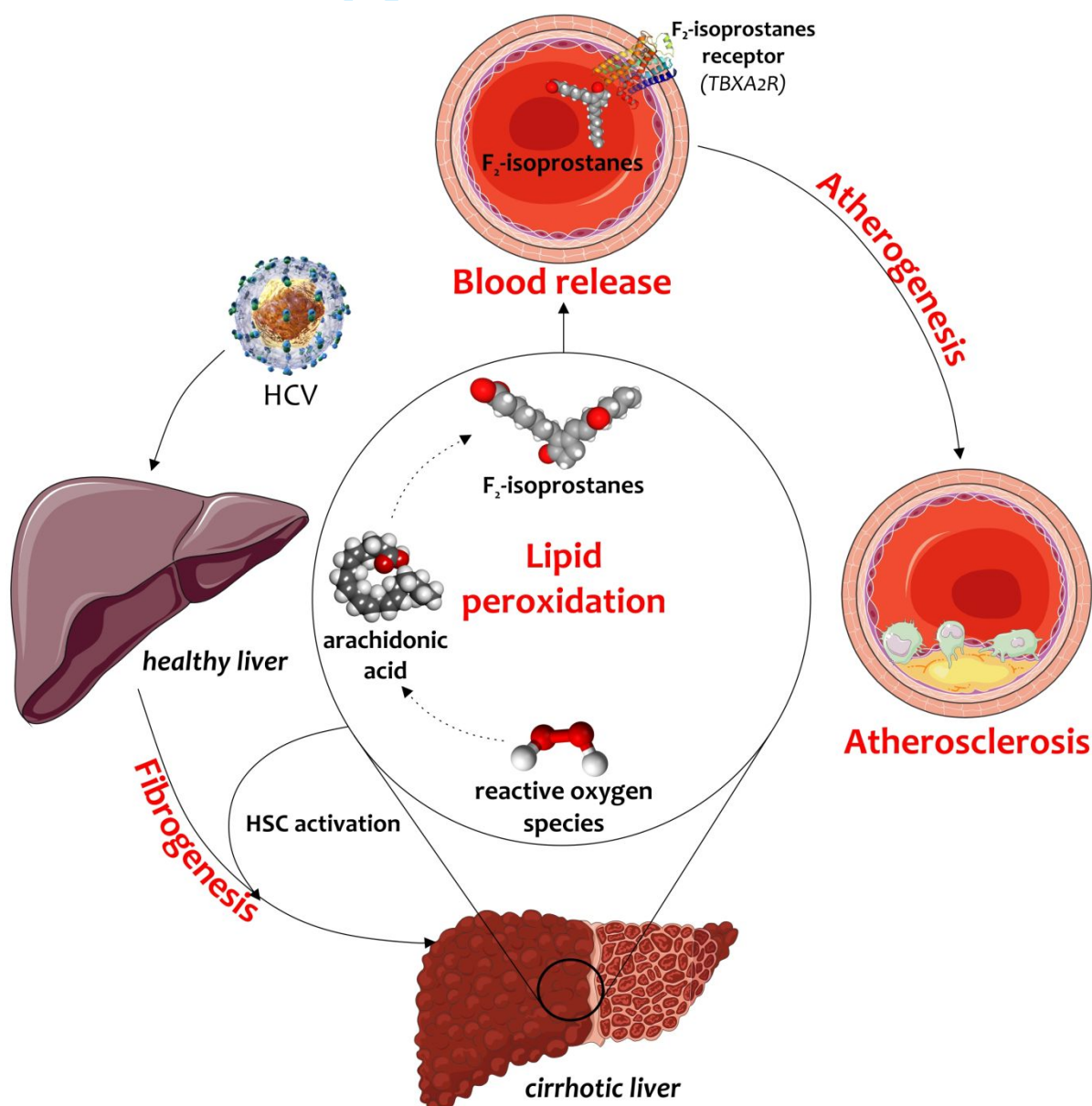
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