

The significance of platelet microparticles in patients with chronic hepatitis C and their association with antiviral treatment and smoking

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Abstract

Background Platelet microparticles (PMPs) are platelet-derived membrane vesicles involved in cardiovascular diseases and atherosclerosis. Chronic hepatitis C (CHC) is associated with increased atherosclerosis, but the effect of therapy on its atherogenic potential has not been adequately studied.

Methods We evaluated PMP levels before and after treatment with pegylated-interferon-alfa and ribavirin in 28 CHC patients compared with 20 non-alcoholic fatty liver disease (NAFLD) patients and 20 healthy volunteers (HV).

Results Twenty-four (86%) CHC patients achieved sustained virological response (SVR). PMP levels were determined at baseline in CHC, NAFLD patients, and HV, and at end-of-treatment (EOT) and 24 weeks post-treatment (SVR24) in CHC patients. PMP levels at baseline were higher in CHC than NAFLD patients ($P < 0.001$) and HV ($P = 0.007$). Higher PMPs at baseline were observed in smokers than non-smokers with CHC ($P = 0.006$). Among smokers from all groups, PMPs at baseline were higher in CHC than NAFLD patients ($P = 0.001$) and HV ($P = 0.006$). In CHC patients, PMPs declined from baseline to both EOT ($P = 0.035$) and SVR24 ($P = 0.006$). Only CHC patients with SVR had a significant decline in PMPs from baseline to SVR24 ($P = 0.018$). PMPs at EOT and SVR24 in all CHC patients were similar to PMPs in NAFLD patients and HV.

Conclusions PMP levels are increased in CHC patients, particularly smokers, which further supports the atherosclerotic potential of CHC and suggests a potentially synergistic effect of smoking and CHC on the atherosclerotic process. Since PMP levels in CHC patients with SVR were similar to NAFLD patients and HV, the atherosclerotic potential of CHC seems to be abolished by effective antiviral treatment.

Keywords Atherosclerosis, hepatitis C, interferon, platelet microparticles, smoking

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Introduction

Microparticles are heterogeneous submicron vesicles released from different cell types. They are divided in exosomes and microvesicles [1]. Platelet-derived microparticles (PMPs) are shed from activated platelet membranes [2] and constitute the most abundant microparticles in the blood circulation. They contain procoagulant proteins from platelets and express various antigens from platelet surface [3,4]. PMPs possess phosphatidylserine, an anionic phospholipid resulting in the assembly of components of the clotting cascade [5]. In addition, PMPs participate in the process of atherosclerosis

by activating the endothelial cells and aggregating leukocytes in the vascular intima [4]. The surface of PMPs is reported to be 100-fold more procoagulant than that of activated platelets [6]. In turn, PMPs activate platelets by transferring proinflammatory lipids [7]. Elevated plasma PMPs levels are associated with atherothrombotic diseases such as coronary artery disease [8], or those predisposing to atherothrombosis such as type 2 diabetes [9], polycystic ovary syndrome [10] etc.

Chronic hepatitis C (CHC) is associated with steatosis and insulin resistance/diabetes [11] and many studies show an association between CHC and cardiovascular events and/or high-risk factors for atherosclerosis [12,13]. However, the mechanisms of CHC leading to atherogenic potential need further investigation.

The aims of the current study were: first, to identify if patients with CHC had higher concentrations of PMPs compared with controls; second, to examine if factors associated with atherosclerosis were related to PMP levels; and third, to test if antiviral treatment had a beneficial effect on PMP levels.

Patients and methods

Patient population

Twenty-eight consecutive patients with CHC (7 with genotype 1; 15 with genotype 3; and 6 with genotype 4) visiting the liver outpatient clinics of the Academic Department of Internal Medicine at Hippokratio Hospital of Athens, Greece, in 2011, who completed a course of antiviral treatment and post-treatment follow up were included in this prospective study. Twenty consecutive patients with known non-alcoholic fatty liver disease (NAFLD) and 20 consecutive healthy volunteer blood donors (HV) were used as control groups. Patients with NAFLD had elevated γ -glutamyl-transpeptidase (GGT) with or without elevated alanine aminotransferase (ALT), ultrasonographic evidence of liver steatosis and no other cause of liver injury. Patients with co-infections, diabetes, cardiovascular diseases, autoimmune or malignant diseases, anticoagulation or antiplatelet therapy were excluded from all study groups. The study was approved by the local Ethical Committee and all patients and controls gave a written informed consent to participate in the study.

Diagnosis of CHC was based on the presence of both anti-hepatitis C virus (HCV) antibodies and HCV RNA in the serum. HCV genotype was also determined. All patients were treated with combination of pegylated interferon- α and ribavirin. Patients infected with HCV genotype 1 and 4 were treated for 48 weeks, whereas patients infected with genotype 3 for 24 weeks. Treatment was discontinued in patients who failed to achieve HCV RNA decline of ≥ 2 logs by week 12 or had detectable HCV RNA at week 24. Sustained virological response (SVR) was defined by undetectable HCV RNA at 24 weeks after the end of treatment (EOT). Relapsers were defined as patients who achieved undetectable HCV RNA at

EOT but had detectable HCV RNA at 24 weeks post-treatment. Non-responders were considered patients with detectable HCV RNA at EOT [14].

Blood collection and laboratory studies

Complete blood count, including white blood cells, hemoglobin, and platelet count, prothrombin time, and international normalized ratio, activated partial thromboplastin time, fibrinogen levels, d-dimers, and liver function tests including ALT, aspartate aminotransferase, alkaline phosphatase, GGT, total bilirubin were performed using commercially available assays.

Anti-HCV antibodies were detected by commercially available enzyme immunoassays and serum HCV RNA by commercially available polymerase chain reaction assays (sensitivity: 50 IU/mL). HCV genotype was determined by a commercially available assay (InnoLipa, Innogenetics, Gent, Belgium).

Determination of PMPs

Blood sample was collected into a 5 mL tube containing 3.2% sodium citrate (BD, Plymouth, UK) and preparation-measurement of PMPs was performed at room temperature within 4 h post-collection. PMPs were distinguished from non-platelets events in platelet poor plasma by their expression of the surface marker Glycoprotein IIIa (CD61) using PE anti-human CD61 monoclonal antibody (BioLegend) and their binding capacity with Annexin-V using FITC conjugated, Annexin-V (BioLegend). They were distinguished from platelets by forward scatter to that of fluorescence-labeled reference beads reagent (Megamix, Biotex) which allowed standardizing the set-up of PMPs analysis region (0.5-1 μ m) and guaranteeing the stability of the settings [15]. The number of CD61 and Annexin-V double-positive events was calculated relative to the number of beads added to samples (Perfect count microspheres, Cytognos) and were expressed as events/ μ L. To avoid unspecific antibody binding, Annexin-V binding buffer (Biolegend) was also used. Antibody solutions were centrifuged prior to FACS to avoid artifacts due to aggregation. Flow cytometric analysis was performed with a FACScan flow cytometer (Coulter).

Statistical analysis

The clinical and laboratory data were collected, categorized and analyzed using Statistical Package for Social Sciences (SPSS) version 21 (Chicago, IL). Data were expressed as median (range). Univariate comparisons were performed using Mann-Whitney *U*-test for continuous variables and Chi-square test for categorical variables. Wilcoxon matched-pair test was used

for the comparison of PMPs at different time-points. P values of <0.05 were considered to be significant.

Results

Characteristics of the patients

Demographic, laboratory and clinical characteristics of patients with CHC at baseline are summarized in Table 1. The main epidemiological characteristics like age and gender as well as the prevalence of smokers were similar among patients with CHC, patients with NAFLD and HV. Body mass index was higher in patients with NAFLD ($P<0.001$) compared to either CHC patients or HV. Two patients with CHC but no patient with NAFLD had a suspicion of compensated cirrhosis according to transient elastography measurements (liver stiffness measurement >12 kPa).

Twenty-four CHC patients (86%) achieved SVR; two (7%) did not respond to treatment; and 2 (7%) relapsed. Both non-

responders and relapsers were considered as non-SVR cases. Three patients were treatment-experienced but achieved SVR. Six (86%) patients with genotype 1, 14 (93%) with genotype 3, and four (67%) with genotype 4 achieved SVR. None of these patients developed any serious adverse event and no patient discontinued treatment due to adverse events. No patient developed any thromboembolic event during treatment or follow-up period.

PMP counts at baseline

The median PMPs at baseline were 510 counts/ μL in CHC patients, who were then classified into cases with high (≥ 500 counts/ μL) and low (< 500 counts/ μL) PMPs. Univariate analysis revealed that CHC patients with high compared to those with low PMPs were more frequently smokers (93% vs. 36%, $P=0.001$). ALT levels showed a trend to be higher in cases with high than low PMPs levels ($P=0.085$). No other statistically significant difference in demographic or laboratory

Table 1 Demographic, clinical and laboratory characteristics in chronic hepatitis C patients with platelet microparticle (PMP) levels ≥ 500 and < 500 counts/ μL

| Patient characteristics | Total (N=28) | PMPs<500 (N=14) | PMPs ≥ 500 (N=14) | P |
|--------------------------------|------------------|------------------|------------------------|-------|
| Age (years) | 28 (22-54) | 35 (28-53) | 34 (22-54) | 0.946 |
| Males, n (%) | 18 (64) | 7 (50) | 11 (79) | 0.123 |
| BMI (kg/m ²) | 24 (19-34) | 22 (19-33) | 25 (19-34) | 0.227 |
| Smokers, n (%) | 18 (64) | 5 (36) | 13 (93) | 0.001 |
| IVDU, n (%) | 13 (46) | 5(36) | 8 (57) | 0.272 |
| Genotype, n (%) | | | | 0.725 |
| 1 or 4 | 13 (46) | 7 (50) | 6 (42) | |
| 3 | 15 (54) | 7 (50) | 8 (57) | |
| HCV RNA ($\times 10^3$ IU/mL) | 450 (7.5-8000) | 435 (7.5-4500) | 521 (8.5-8000) | 0.667 |
| AST (IU/L) | 38 (19-549) | 32 (19-299) | 47 (23-549) | 0.265 |
| ALT (IU/L) | 54 (28-532) | 40 (28-435) | 75 (31-532) | 0.085 |
| ALP (U/L) | 71 (39-163) | 61 (39-163) | 79 (43-105) | 0.401 |
| GGT (U/L) | 35 (9-205) | 31(9-100) | 43 (15-205) | 0.427 |
| Bilirubin (mg/dL) | 0.4 (0.2-1.2) | 0.5 (0.2-1.2) | 0.4 (0.2-1.2) | 0.649 |
| WBC ($\times 10^3$ /L) | 7.1 (2.5-12.4) | 7.1 (3.4-10.3) | 7.1 (2.5-12.4) | 1.000 |
| Hemoglobin (g/dL) | 14.1 (11.3-17.1) | 13.4 (11.9-16.9) | 14.5 (11.3-17.1) | 0.306 |
| Platelets ($\times 10^3$ /L) | 260 (80-420) | 253 (80-420) | 280 (200-420) | 0.285 |
| PT (sec) | 11.9 (10.3-16.9) | 11.8 (10.3-16.9) | 12.0 (10.9-13.2) | 0.458 |
| APTT (sec) | 33 (28-55) | 32 (27-55) | 33 (28-42) | 0.899 |
| Fibrinogen (mg/dL) | 288 (133-424) | 265 (133-345) | 299 (176-424) | 0.285 |
| D-Dimers | 384 (170-3500) | 397(170-863) | 336(190-3500) | 0.734 |
| Liver stiffness (kPa) | 6.1 (2.9-20.9) | 5.6 (2.9-12.2) | 6.7 (4.8-20.9) | 0.227 |
| SVR, n (%) | 24 (86) | 12 (86) | 12 (86) | 1.000 |

Quantitative values are expressed as median (range)

BMI, body mass index; IVDU, intravenous drug users; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, γ -glutamyl-transpeptidase; WBC, white blood cells; PT, prothrombin time; APTT, activated partial thromboplastin time; SVR, sustained virological response

characteristics was observed between CHC patients with low and high PMPs (Table 1).

Median PMPs levels at baseline were higher in CHC patients (510 counts/ μ L) compared with NAFLD patients (166 counts/ μ L, $P < 0.001$) and HV (316 counts/ μ L, $P = 0.007$) (Fig. 1A). In contrast, median PMP levels did not differ significantly between NAFLD patients and HV ($P = 0.242$). The median baseline PMP levels were higher in smokers ($n = 18$) compared to non-smokers ($n = 10$) with CHC (794 vs 260 counts/ μ L, $P = 0.006$). Higher PMPs were also found in smokers compared to non-smokers in patients with NAFLD (254 vs. 108 counts/ μ L, $P = 0.05$) and HV (436 vs. 161 counts/ μ L, $P = 0.003$). Among smokers from all groups, CHC patients had higher PMPs compared with NAFLD patients ($P = 0.001$) and HV ($P = 0.024$) (Fig. 1B). Likewise, among non-smokers from

all groups, CHC patients had higher PMP levels compared with NAFLD patients ($P = 0.014$) and HV ($P = 0.075$) (Fig. 1B).

PMP levels during antiviral treatment

During antiviral treatment, PMP levels declined significantly from baseline to both EOT (median decline: 372 counts/ μ L, $P = 0.035$) and 24 weeks after EOT (SVR24) (median decline: 106 counts/ μ L, $P = 0.006$) (Table 2, Fig. 1A). PMP levels showed a numerical reduction from EOT to SVR24, but this change did not reach statistical significance in all CHC patients ($P = 0.163$).

None of the CHC patients changed smoking habits during antiviral treatment or post-treatment follow up. The decline of PMP levels during treatment was mainly observed in

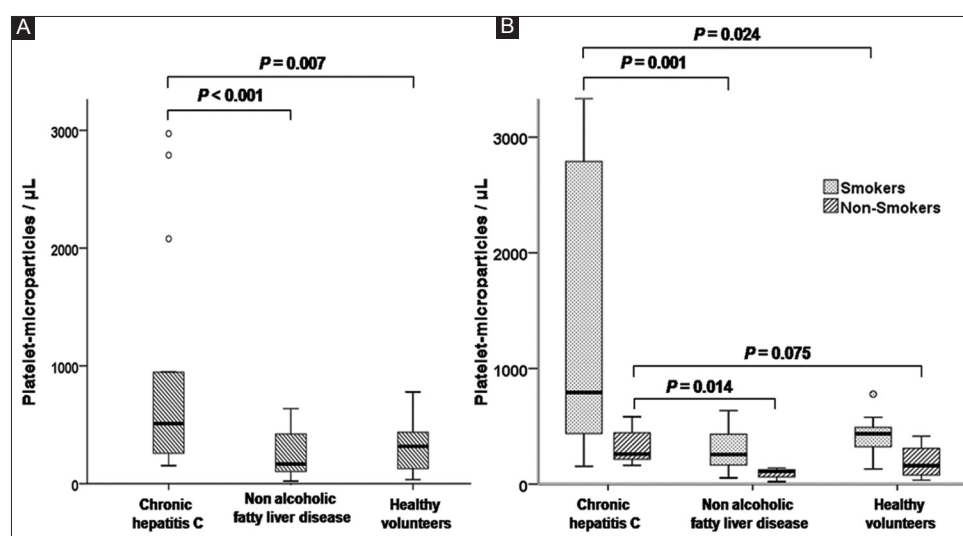


Figure 1 Results of platelet microparticle levels in 28 patients with chronic hepatitis C, 20 patients with non-alcoholic fatty liver disease, and 20 healthy volunteers. Boxes and whisker plots express medians, interquartile range and overall ranges. (A) All patients, (B) smokers vs. non-smokers

Table 2 Platelet microparticle (PMP) levels at different time points during antiviral treatment in chronic hepatitis C patients

| | PMPs (counts/ μ L) | | | P-values for PMP changes | | |
|--------------|------------------------|---------------|---------------|--------------------------|--------------|-------------------|
| | Baseline | EOT | SVR24 | Baseline to EOT | EOT to SVR24 | Baseline to SVR24 |
| All patients | 510 (153-5184) | 265 (57-1583) | 186 (20-886) | 0.035 | 0.163 | 0.006 |
| Smokers | 794 (153-5184) | 298 (75-1408) | 186 (20-886) | 0.004 | 0.382 | 0.009 |
| Non-smokers | 260 (161-582) | 186 (57-1583) | 165 (54-772) | 0.449 | 0.225 | 0.600 |
| P^* | 0.006 | 0.837 | 0.910 | | | |
| SVR | 510 (153-5184) | 265 (75-1583) | 186 (20-886) | 0.062 | 0.179 | 0.018 |
| No SVR | 509 (215-4369) | 252 (57-914) | 182 (165-198) | 0.465 | 0.655 | 0.180 |
| P^{**} | 0.874 | 0.538 | 1.000 | | | |
| Genotype 1/4 | 493 (161-5184) | 195 (57-1338) | 178 (20-772) | 0.398 | 0.686 | 0.025 |
| Genotype 3 | 551 (153-4831) | 298 (75-1583) | 186 (42-886) | 0.048 | 0.221 | 0.087 |
| P^{***} | 0.928 | 0.731 | 1.000 | | | |

Quantitative values are expressed as median (range). * P -values for comparisons between smokers and non-smokers; ** P -values for comparisons between patients with and without SVR; *** P -values for comparison between patients with genotype 1/4 and patients with genotype 3

EOT, end-of-treatment; SVR24, 24 weeks post-treatment; SVR, sustained virological response

smokers, in whom PMPs declined significantly from baseline to EOT ($P=0.004$) and SVR24 ($P=0.009$) (Table 2, Fig. 2B), while no significant decline was again observed from EOT to SVR24 ($P=0.382$). In contrast, in non-smokers, there was no significant change of PMPs even between baseline and EOT ($P=0.449$) or SVR24 ($P=0.600$) (Table 2, Fig. 2B).

In relation to response to treatment, only patients who achieved SVR had a significant decline in PMPs from baseline to SVR24 ($P=0.018$). In this subgroup, PMPs declined from baseline to EOT with the change being close to statistical significance ($P=0.062$), which was reached in the change of PMPs from baseline to SVR24 ($P=0.018$). In contrast, in the patients without SVR, PMP levels did not show any statistical difference from baseline to EOT ($P=0.465$) or SVR24 ($P=0.180$) (Table 2).

At SVR24, PMP levels became similar between smokers and non-smokers CHC patients (Table 2, Fig. 2B). In addition, no significant difference was found in PMP levels at EOT or SVR24 between CHC and NAFLD patients ($P=0.102$ and $P=0.667$, respectively) or HV ($P=0.614$ and $P=0.426$, respectively).

Discussion

PMPs may be used as prognostic markers of cardiovascular disease. It has been demonstrated that PMP levels follow the pattern of platelet activation during and after an acute coronary syndrome, as they decrease after initiation of antithrombotic treatment [16]. Local increase of PMP values has been measured in coronary arteries in patients with myocardial infarction and reduction was detected after successful revascularization [8]. PMP levels have been correlated with thromboxane B₂, platelet activated factor, endothelin-1 and neutrophil-to-lymphocyte ratio in patients with coronary intermediate lesions suggesting platelet activation and endothelial dysfunction [2].

Investigators suggested therefore that PMP levels reflect platelet activation [17] and their presence in circulation represents serious procoagulant risk [6,18].

Previous studies have shown that CHC may be associated with increased cardiovascular mortality [13,19], a high risk of coronary artery disease [20,21], ischemic stroke and atherosclerosis [22,23]. Furthermore, a positive association between CHC and carotid atherosclerotic plaques was observed [24]. However, the results are still conflicting and some investigators recommend long-term large-scale prospective studies to further confirm the association between HCV status and atherosclerosis [24].

In the current study, we demonstrated that PMP concentrations at baseline were higher in CHC patients compared with two control groups, patients with NAFLD and HV. Smoking was found to be a risk factor for high PMP levels in all groups examined but the highest levels were detected in CHC patients who smoked. The above findings suggest that both CHC and smoking can be procoagulant factors perhaps having a synergistic effect when both are present. Patients with hypertension, diabetes and cardiovascular diseases were excluded from our cohorts by the design of the study. Only smoking has remained to be an atherogenic factor along with CHC and emerged to be related with high PMP values. No other parameter correlated to high levels of PMPs at baseline analysis.

A significant decline in PMP levels was observed at EOT mainly in smokers with CHC. In fact, smokers with CHC benefited most from antiviral treatment as the reduction in PMPs was more obvious in this subgroup. Even though the most elevated PMP levels were observed in smokers at initiation of treatment, PMP values became similar to controls and to non-smokers both at EOT and at the end of follow up.

PMPs continued to decrease and the lowest values were recorded at 24 weeks following EOT in patients with SVR. In fact, at the end of the 24-week post-treatment period, PMPs

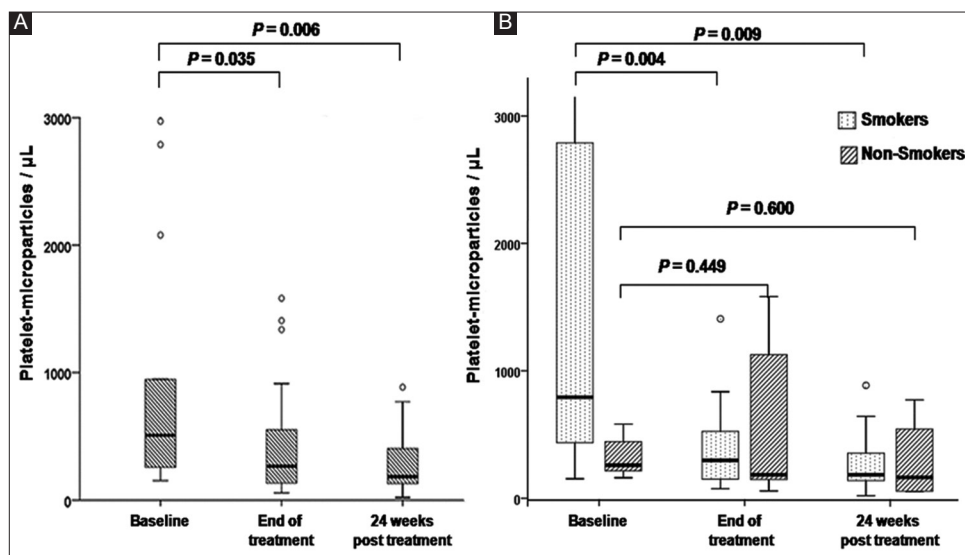


Figure 2 Results of platelet microparticle levels in 28 patients with chronic hepatitis C at different time points during antiviral treatment. Boxes and whisker plots express medians, interquartile range and overall ranges. (A) All patients, (B) smokers vs. non-smokers

Summary Box

What is already known:

- Microparticles are heterogeneous submicron vesicles released from different cell types
- Platelet microparticles are involved in the process of atherosclerosis by activating the endothelial cells and aggregating leukocytes in the vascular intima
- Chronic hepatitis C is associated with insulin resistance and many studies have suggested an association with cardiovascular events and/or high risk for atherosclerosis

What the new findings are:

- Patients with chronic hepatitis C have higher levels of platelet microparticles than patients with non-alcoholic fatty liver disease or healthy controls
- Antiviral treatment in chronic hepatitis C patients can decrease platelet microparticle levels implying a potential beneficial effect in the atheromatic process
- Smokers with chronic hepatitis C seem to benefit most from antiviral treatment

of CHC patients became similar to those of control groups suggesting a deactivation of platelets leading to clearance of PMPs from the blood circulation. It is of note that the decline in PMPs was not maintained in patients without SVR suggesting a withdrawal of the beneficial effect of antiviral treatment in this subgroup. No differences of PMP reduction were observed between different HCV genotypes. Both HCV genotype subgroups 1 or 4 and 3 followed similar patterns of PMP decline after antiviral treatment, but patients with HCV genotypes 1 and 4 experienced a greater decline in PMPs compared to genotype 3 patients.

The pathogenesis of the potential atherogenic role of HCV has not as yet been fully understood. Systemic chronic inflammation [25], increased oxidative stress [26], hepatic steatosis leading to reduced insulin sensitivity [23] and endothelial dysfunction [27] may be involved. In addition, a direct effect of the virus may play a role as HCV RNA was detected in both carotid plaques and brain tissue [28,29]. Recently, some biomarkers involved in atherogenic process such as C-reactive protein, soluble vascular cell adhesion molecule-1 and soluble E-selectin were reported to be higher in CHC patients compared with controls [30]. However, no study so far has evaluated PMPs as markers of platelet activation at CHC patients before and after treatment.

Our study has some limitations. First, the relatively small number of patients may have resulted in some type II errors thus not allowing a proper evaluation of the role of several factors, like HCV genotype and severity of liver fibrosis, on PMP decline. Moreover, the exclusion of specific subgroups of

NAFLD patients (e.g. cases with diabetes and cardiovascular diseases) may have resulted in selection of NAFLD patients with milder liver disease severity than in other NAFLD cohorts, which might have affected the PMP levels in this setting.

In conclusion, our study evaluated a marker of platelet and endothelial cell activation, and showed that platelets are activated in CHC patients, particularly in smokers, but they can be deactivated by successful antiviral treatment. Since the inhibition of platelets persisted after EOT only in patients with SVR, it is reasonable to assume that such a potential beneficial effect on the atherosclerotic process is not related to the type of treatment itself but to the HCV clearance, and, thus, it will also be provided by the new direct antivirals which offer a chance for cure in almost all HCV patients.

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