

Circulating microparticles in patients with chronic hepatitis C and changes during direct-acting antiviral therapy

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Background. Microparticles (MPs) are heterogeneous vesicles derived from membranes of different cells. Between 70 to 90% of MPs detected in blood originate from platelets. The release of MPs is associated with proinflammatory and procoagulant states. Elevated levels of MPs have been found in different diseases. We investigated MPs levels in patients with chronic hepatitis C (CHC) and changes in level during treatment using direct-acting antiviral therapy (DAA).

Patients and Methods. Thirty-six patients with CHC and forty healthy volunteers were included in the study. Concentrations of MPs were determined indirectly by measuring their procoagulant activity in plasma at baseline, end of therapy (EOT), and 12 weeks after EOT when the sustained virological response was assessed (SVR12).

Results. All patients achieved SVR12, which was associated with rapid improvement of markers of liver damage and function as well as liver stiffness ($P=0.002$). MPs levels were significantly higher in CHC patients than in healthy volunteers ($P<0.001$). No statistically significant decrease was found observed between baseline and SVR12 ($P=0.330$). Analysis of subpopulations with minimal fibrosis F0-1 ($P=0.647$), advanced fibrosis F2-4 ($P=0.370$), women ($P=0.847$), men ($P=0.164$) and genotype 1 ($P=0.077$) showed no significant changes during the follow-up period.

Conclusions. MPs levels are higher in CHC patients and remain elevated shortly after achieving SVR. Higher concentrations of MPs in plasma are probably caused by a chronic uncontrolled exaggerated inflammatory response caused by CHC. Longer observation would probably confirm the significance of MPs levels decrease because normalization of liver function, inflammation, and structure after SVR requires more than 12 weeks.

Key words: microparticles, microvesicles, chronic hepatitis C, direct-acting antiviral therapy

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INTRODUCTION

Microparticles

Microparticles (MPs) are heterogeneous vesicles derived from cell membranes by a budding process^{1,2}. They originate from different cell types³ and they carry on the surface antigens of the cells from which they have descended⁴. Between 70 to 90% of MPs detected in the blood are derived from platelets, but other cells such as endothelial, leukocytes, erythrocytes, and even malignant cells could also shed MPs (ref.^{5,6}). They are released during cell activation by both chemical (eg, cytokines, thrombin, cytotoxic chemotherapy, hypercholesterolemia, and tobacco smoke exposure) and physical (eg, shear stress, hypoxia) stimulation⁶. They also shed during apoptosis⁷ and senescence of cells⁸.

The size of MPs ranges from 100 to 1000 nm (ref.⁹). MPs display a broad spectrum of bioactive substances and receptors on their surface and harbor a concentrated set of cytokines, signaling proteins, mRNA, and microRNA (ref.¹⁰). MPs may transfer complete cell surface receptor signaling pathways into the recipient cell that is specific for the MPs releasing cell, exchange genetic information,

or transfer antigen via MHC class II molecules¹¹. They act as a communication tool for extracellular communication and transport between cells⁶. MPs participate in a variety of physiological functions, mainly including the promotion of blood clotting, endothelial repair, angiogenesis, and inflammatory responses, although their role in body physiology is not yet fully understood^{12,13}.

Release of MPs is associated with proinflammatory and procoagulant states¹³. Elevated levels of MPs were founded in many different conditions, for examples: oncological diseases^{4,14,15}, rheumatic disease^{16,17}, cardiovascular disease^{18,19}, sepsis^{20,21}, diabetes²², pulmonary hypertension²³, liver diseases² or chronic infections like HIV (ref.²⁴) or chronic hepatitis C (CHC) (ref.³). Numerous studies have shown that not only the levels but also the cellular origin and composition of circulating MPs are dependent on the type of disease, the disease state, and medical treatment²⁵. For listed reasons MPs are viewed as a potential new biomarker and also as a possible therapeutic target²².

Chronic hepatitis C

According to the World Health Organisation (WHO), an estimated 71 million people have CHC globally and ap-

proximately 399 000 people died from hepatitis C in 2016. Even in the age of direct-acting antivirals (DAA), CHC still presents an enormous worldwide health problem²⁶. Except for liver involvement, CHC is associated with multiple extrahepatic manifestations, most commonly cryoglobulinemia, membranoproliferative glomerulonephritis, diabetes, or lymphoproliferative disease²⁷. Hepatitis C virus (HCV) infection is also associated with increased thrombotic risk even in the absence of cirrhosis^{28,29}. Additionally, studies are linking CHC with a higher risk of atherosclerosis and other cardiovascular diseases^{30,31}. Therapy of chronic hepatitis C was revolutionized with the discovery of DAA, which entered clinical practice in 2011. The successful rate of currently used DAA therapy is 95-100% without significant side effects³².

Only a few studies about MPs in patients with hepatitis C undergoing DAA therapy have been performed^{33,34}. The aims of the following study were: first, compare MPs level between patients with CHC and control group of healthy volunteers; second, evaluate changes of MPs levels during DAA therapy; third, analyzed MPs changes in subpopulations according to gender, genotypes, and fibrosis stage.

PATIENTS AND METHODS

Patient population

Fifty-nine patients were enrolled in the study from 2019 to 2020, but 23 were excluded: 1 HCV RNA negative, 22 lost to follow up (LTFU). The high LTFU rate (37%) was due to the specific nature of the patient population, where 70% were people who inject drugs (PWIDs). After exclusions total of 36 patients from the outpatient clinic of the Department of Infectious Disease of the University Hospital Brno, Czech Republic with CHC, who completed DAA therapy and post-treatment follow-up were included in the prospective study. The study was approved by the local ethical committee and every enrolled patient signed informed consent with participation in the study.

CHC diagnosis was based on the positivity of HCV RNA and determination of HCV genotype. Three following DAA regimes were used: sofosbuvir/velpatasvir, glecaprevir/pibrentasvir, and grazoprevir/elbasvir. The duration of treatment was 8, 12, or 16 weeks. The selection of regime and its length was based on contemporary Czech national guidelines³⁵. Study visits were at baseline, at the end of treatment (EOT), and 12 weeks after the last dose of antivirals to assess achieving sustained virological response (SVR12). The body mass index (BMI) was calculated using the standard formula: the mass in kilograms divided by the square of the height in meters. The control group of 40 healthy volunteers without CHC or other chronic diseases was used for comparison. The age and gender characteristics of the control group were similar to the study population.

Blood collection and laboratory tests

Venous blood was collected on every study visit and analyzed for complete blood count, international normal-

ized ratio (INR), activated partial thromboplastin time (aPTT), fibrinogen, d-dimers, antithrombin, total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), Gamma-glutamyltransferase (GGT), C-reactive protein (CRP), erythrocyte sedimentation rate after 1 h (ESR 1) and 2 h (ESR 2) and PCR HCV RNA. HCV genotype was determined at the baseline. All tests were performed by a local hospital laboratory using commercially available assays.

Microparticle measurement

Venous blood was collected in a tube with 3.2% sodium citrate and within two hours plasma supernatant was centrifuged (15 min 1,500 × g + 2 min 13,000 × g) at room temperature. Obtained plasma samples were rapidly frozen and stored at - 80°C. Before analysis plasma was thawed for 15 min at 37 °C and tested within four h.

A photometric method using ZYMUPHEN™ MP-Activity (Hyphen Biomed, Neuville-sur-Oise, France) was used for measurement of MPs procoagulant activity in plasma using an automated enzyme-linked immunosorbent assay DS2 (DYNEX Technologies Inc., Chantilly, USA). The assay principle is following: The diluted assayed plasma sample, supplemented with calcium, Factor Xa, and thrombin inhibitors, is introduced into one of the microplate wells coated with Streptavidin and biotinylated Annexin V, then incubated. Following a washing step, the Factor Xa-Va mixture containing calcium, then the purified prothrombin, are introduced. When present in the tested sample, microparticles bind to Annexin V and expose their phospholipids surface, thus allowing to FXa-FVa, in presence of calcium, to activate prothrombin into thrombin. The phospholipids concentration is then the limiting factor. There is a direct relationship between the phospholipids concentration and the amount of thrombin generation, which is measured via its specific activity on the thrombin substrate. The reaction is stopped with 2% Citric Acid and Absorbance is measured at 405 nm. MP concentration is calculated using a calibration curve. The reference limit set by the manufacturer was 0-5.0 nM phosphatidylserine equivalent³⁶. The origin of MPs was not examined, only their total concentration in plasma.

Liver stiffness measurement

Transient elastography - FibroScan® (Echosens, Paris, France) device was used for non-invasive measurement of liver stiffness. The stage of liver fibrosis was established based on measured values using the interpretation guide in myFibroscan® mobile application. Transient elastography was performed on every study visit. Not a single liver biopsy was not performed in our patient's group.

As supporting indicators of liver fibrosis were used three scoring indexes: AST to platelet ratio index (APRI), Fibrosis-4 (Fib-4), and the King's score. The formula for the APRI score is [(AST/upper limit of the normal AST range) × 100]/Platelet Count (10⁹/L) (ref.³⁷). The upper limit of the normal (ULN) AST range was set on 36.14 IU/L for women and 51.2 IU/L, which are ULN in our laboratory. The FIB-4 values were calculated using the formula: age (years) × AST [U/L]/(platelets [10⁹/L] ×

(ALT [U/L])^{1/2} (ref.³⁸). The King's score = age(years) × AST (U/L) × INR/platelet count (10⁹/L) (ref.³⁹).

Statistic analysis

For statistical analysis of collected data was used software R developed by R Core team version: 3.5.1 with expansion RStudio® version: 1.2.2019. Correlation between values of MPs and other laboratory values at baseline, EOT and SVR 12 were calculated using Linear Regression Model Using Generalized Least Squares (Table 1 and Fig. 1), Repeated Measures ANOVA (Fig. 2) and Mann-Whitney test for comparison MPs level between control and study group (Fig. 1). The level of statistical significance was set at <0.05. Median, minimum, and maximum measured values are listed in Tables 1 and 2. Most of the values have been rounded to one decimal. INR, aPTT ratio, total bilirubin have been rounded to two decimals and *P*-value to three decimals.

RESULTS

Patient population characteristic

Thirty-six patients completed the whole study. The median age was 39 (16-79) years. Four patients were previously treated with a combination of pegylated interferon and ribavirin without achieving SVR. A genotype composition with other characteristics are listed in Table 1. In one case genotype was not possible to determined due to low viral load (279 IU/mL). The one patient with genotype 4e had African origin, the other 35 were Caucasian.

Risk factors for HCV acquisition were following: intravenous drug abuse 23 (64%), blood transfusion 4 (11%), tattooing 2 (6%), men having sex with men 2 (6%), unknown route of transmission 5 (14%). Most of the patients were treated with glecaprevir/pibrentasvir combination

(78%). This DAA regime is usually only 8 weeks long, which from our experiences ensures better compliance between PWIDs. All patients achieved SVR 12 weeks after EOT.

Liver involvement

A significant decline in liver stiffness was observed in the study population despite a relatively short course

Table 1. Baseline characteristic of patients and DAA treatment

Patient	36
Female	16 (44%)
Male	20 (56%)
Median (Min; Max)	
Age	39 (16; 79)
BMI	25.2 (16.4; 35.4)
HCV viral load (IU/mL)	374,500(279; 10,200,000)
Transient elastography (kPa)	6.3 (2.6; 75)
Liver Fibrosis Stage	
F0-1	22
F2	3
F3	2
F4	9
HCV genotype	
1a	6
1b	16
1a/1b	2
3a	10
4e	1
Undetermined (low viral load)	1
HCV treatment	
Glecaprevir/pibrentasvir	28
Grazoprevir/elbasvir	4
Sofosbuvir/velpatasvir	4

Table 2. Laboratory test and elastography results at baseline, EOT, and SVR12.

	Baseline	EOT	SVR 12	<i>P</i>
Leucocytes (10 ³ /L)	6.6 (3.5-11.9)	7.1 (4-11)	6.9 (2.4-12.3)	0.339
Platelets (10 ³ /L)	241 (30-384)	227.5 (45-443)	227.5 (44-679)	0.226
Hemoglobin (g/L)	147.5 (90.3 -172)	147.5 (101-172)	145 (102-169)	0.991
INR	0.94 (0.79-1.61)	0.92 (0.78-1.55)	0.9 (0.83-1.47)	0.006
aPTT Ratio	1.03 (0.87-1.35)	0.99 (0.85-1.27)	1 (0.85-1.29)	0.016
Antitrombin (%)	102.5 (38-121)	107.5 (45-121)	104.5 (46-121)	0.243
Fibrinogen (g/L)	2.68 (1.54-3.65)	3.19 (1.83-4.67)	3.01 (2.07-4.36)	<0.001
D-dimers (mg/L)	0.24 (0.9-3.05)	0.36 (0.1-5.89)	0.28 (0.1-0.39)	0.563
ESR 1 (mm)	7 (2-60)	6 (2-68)	8 (1-66)	0.574
ESR 2 (mm)	21 (2-86)	26 (2-88)	20 (2-98)	0.192
CRP (mg/L)	1 (1-10.6)	1 (1-12.7)	1.2 (1-6.4)	0.180
Bilirubin (µmol/L)	9.5 (3.8-55.8)	9.75 (2.6-54.5)	8.85 (2.7-30.9)	0.038
ALT (U/L)	95.2 (18.1-332)	20.5 (7.8-68.1)	20.8 (9.6-109.6)	<0.001
AST (U/L)	61.5 (24.1-189.8)	28.3 (14.5-154.2)	28.3 (16.9-104.2)	<0.001
GGT(U/L)	73.5 (9-841.6)	22.8 (7.8-173.4)	21 (9-193.2)	<0.001
APRI	0.5 (0.3-11.5)	0.3 (0.1-6.7)	0.3 (0.1-3.5)	<0.001
FIB-4	80.5 (13.5-2713.9)	22.7 (3.9-929.3)	20.6 (5.9-472.6)	<0.001
King's score	7.9 (2.7-322)	4.4 (1-179)	3.8 (1.5-90.9)	<0.001
Fibroscan (kPa)	6.3 (2.6-75)	5.75 (2.7-75)	5.3 (2.8-75)	0.002

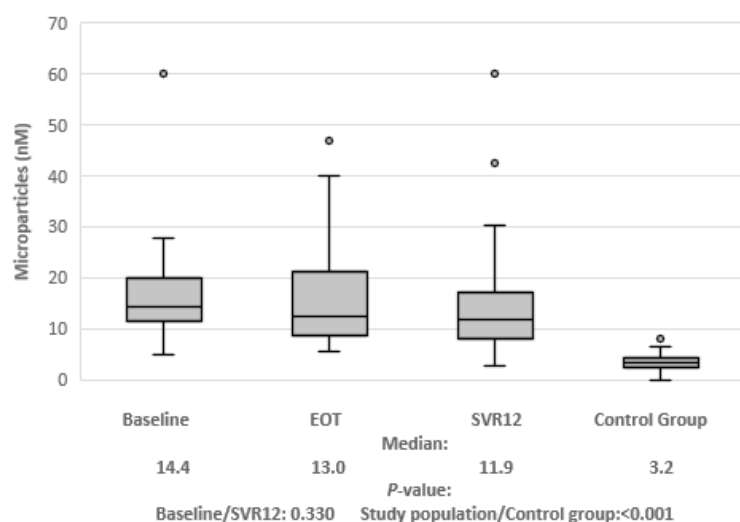


Fig. 1. Microparticles levels in study population and control group.

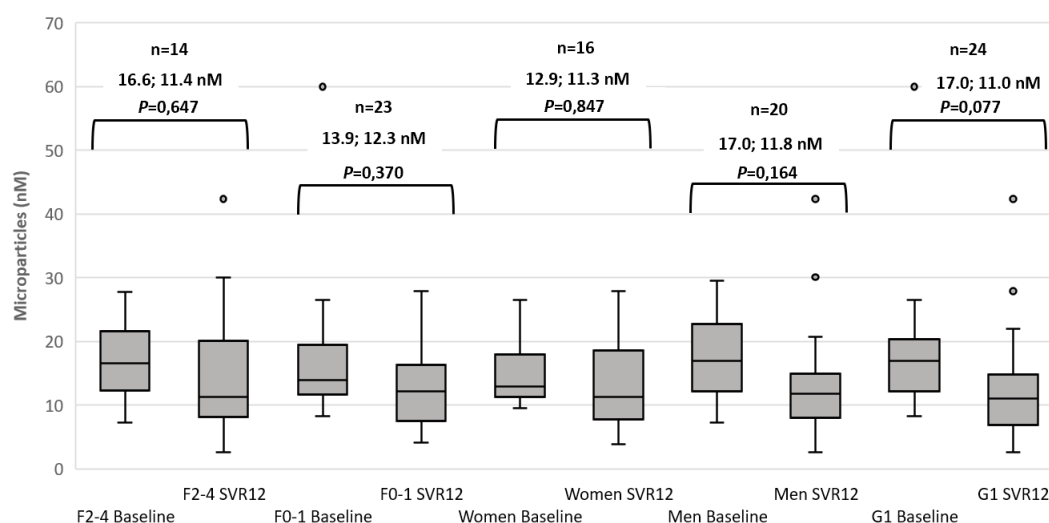


Fig. 2. Microparticles levels at baseline and SVR12 in studied subpopulations.

of post-treatment follow-up. The median value measured by transient elastography dropped from 6.3 kPa to 5.3 kPa during the follow-up period with was usually only 20 (20-28) weeks long. Levels of functional liver test and markers of necroinflammatory damage: total bilirubin, ALT, AST, GGT, INR and aPTT also decreased with statistical significance. The same trend was observed with liver fibrosis staging indexes (APRI, Fib-4, King's score), mostly because they are calculated from identical liver parameters. On the contrary markers of inflammation did not show relevant changes during DAA therapy. Detailed laboratory test results are listed in Table 2.

MPs levels in the study population and control group

The comparison of MPs levels between patients with CHC and healthy volunteers is shown in Figure 1. MPs levels were significantly higher in HCV positive population before treatment (14.4 nM; 3.2 nM; $P<0.001$), at the EOT (13.0 nM; 3.2 nM; $P<0.001$) and at SVR12 visit (11.9; 3.2 nM; $P<0.001$). A decline in MPs levels was observed during the study (14.4 nM; 11.9 nM) but did not reach statistical significance ($P=0.330$).

MPs levels in specific subpopulations

Development of MPs levels between baseline and SVR12 was analyzed in selected subgroups of patients according to gender, presence of significant fibrosis F2-4 (n=14; 16.6 nM; 11.4 nM), or its absence F0-1 (n=23; 13.9 nM; 12.3 nM) and genotype of HCV. In all observed subgroups, MPs levels declined but without a statistically important difference. Most distinct decrease of MPs was found out in genotype 1 patients (n=24; 16.5 nM; 10.2 nM; $P=0.077$) and in men subpopulation (n=20; 16.8 nM; 11.3 nM; $P=0.164$). Results of MPs levels development are summarized in Fig. 2.

DISCUSSION

In our study, it was demonstrated that MPs levels are higher in CHC patients than in HCV-negative volunteers. This finding is in correlation with a similar study done by Kanellopoulou et al.³. Contrary there was not observed a statistically significant decrease in MPs levels during CHC therapy. This could be explained by using DAA

regimens instead of interferon-based regimens, which are usually shorter (8-16 vs. 24-48 weeks) and require only 12 weeks long follow-up to evaluate SVR.

Higher concentrations of MPs in plasma are probably caused by a chronic uncontrolled exaggerated inflammatory response in patients with CHC. This reaction is complex and not fully understood^{40,41}. Commonly used markers like CRP, ESR, or white blood count are not sensitive enough to detect and quantify inflammation during CHC as shown in our study results. Successful DAA therapy with achieving SVR leads to resolution of liver inflammation, which is confirmed by a decrease of liver markers and significant improvement of liver stiffness⁴². After an initial rapid change, improvement of fibrosis, inflammation, and metabolic function of the liver slowing down, but steadily continues for months even years^{42,43}.

Analysis of specific subpopulations showed a decreasing pattern of MPs levels in all groups without reaching statistical significance. The greatest difference was observed in men and genotype 1 subgroup. The concentration of MPs rises with a stage of fibrosis and was higher in men than in women, but again without proving a statistically important difference.

In our previous study, MPs levels were analyzed in HIV-positive patients using the same method of detection⁶. Based on our results, MPs levels of HIV-positive patients both treated (8.9 nM) and untreated (9.0 nM) were higher than in the control group (3.2 nM) but lower than the newly studied HCV positive group. These findings would indicate that inflammatory reaction is generally stronger in HCV positive than in HIV positive.

Our study confirmed once again effectivity of DAA treatment of CHC. All patients achieved SVR. Markers of necroinflammatory damage and liver function decreased significantly. Liver stiffness measured by transient elastography or calculated indirectly using scoring indexes for liver fibrosis showed substantial improvement in a short period.

There are several limitations to our study. First of all, the study population was relatively small (n=36), which is even more prominent in the analysis of selected subpopulations. Secondly, the follow-up period after EOT was short (20-28 weeks). Longer observation would probably statistically confirm the significance of MP levels decrease because normalization of liver function, inflammation, and structure after SVR requires more than 12 weeks⁴⁴. Another limitation arises from the used method of MPs concentration detection by measuring their procoagulant activity. Activation of coagulation cascade during blood collection and sample preparation or the presence of residual platelets can falsely elevate results³⁶. In comparison with flow cytometry it is not possible to identify the origin and size of MPs (ref.⁴⁵).

CONCLUSION

Despite usually non-elevated routine inflammatory markers, CHC causing a significant inflammatory response. Similarly contrary to normal d-dimers levels, plate-

lets are activated during CHC because it is assumed that 70-90% of MPs are originated from platelets. Successful antiviral treatment can improve liver function, liver fibrosis, and as well as the risk of cardiovascular diseases.

The precise role of MPs in the organism and pathophysiology of diseases is still uncertain and further research is needed. Our study of MPs is a part of basic research, which we believe helps establish MPs as a diagnostic marker in the future and may lead to therapeutic application.

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