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Cholesterol and Phospholipid Distribution Pattern in the Erythrocyte Membrane of Patients with Hepatitis C and Severe Fibrosis, before and after Treatment with Direct Antiviral Agents: A pilot Study

Charalampos PAPADOPOULOS[a], Maria PANOPOULOU[b], Theodora MYLOPOULOU[b], Konstantinos MIMIDIS[b], Ioannis TENTES[a], Konstantinos ANAGNOSTOPOULOS[a]

[a]Laboratory of Biochemistry, School of Medicine, Democritus University of Thrace, Alexandroupolis, Greece

[b]Laboratory of Microbiology, School of Medicine, Democritus University of Thrace, Alexandroupolis, Greece

[c]Department of Internal Medicine, School of Medicine, Democritus University of Thrace, Alexandroupolis, Greece

ABSTRACT

Background: *Objectives: Hepatitis C virus requires and induces changes in liver lipidome for its life cycle. In addition, alterations in plasma and erythrocyte lipidome are observed during a range of chronic liver diseases.*

Methods: *A total of six subjects (three males and three females) were included in our study. All subjects were HCV positive according to virus RNA detection. Erythrocyte ghosts were prepared from blood and collected upon diagnosis and also at the end of the treatment with Direct Antiviral Agents (DAA). Lipids were extracted from the erythrocyte ghosts, and cholesterol and phospholipids were analyzed by thin layer chromatography. A semi-quantitative estimation of cholesterol (CHOL), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and sphingomyelin (SM) was performed by densitometric analysis of the chromatographs.*

Address for correspondence:
Konstantinos Anagnostopoulos, PhD
Laboratory of Biochemistry, School of Medicine, Democritus Universisty of Thrace, 68100 Alexandroupolis, Greece
Tel./Fax: +30 25510 30502, +30 6908463899
Email: kanagnos@med.duth.gr, kanagno@gmail.com

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Results: *After the antiviral treatment, PE percentage decreased, whereas the PC/PE and CHOL/PE ratio increased significantly. There were also other weaker differences for CHOL, PI, PS, PC and SM. Before DAA there was a very weak correlation between ALT and PC/PE ratio. In contrast, there was a steep negative correlation between these two parameters after DAA.*

Conclusions: *Red blood cell lipid composition and especially the PC/PE ratio could be a candidate real time biological marker for inflammation resolution during hepatitis C treatment.*

Keywords: erythrocyte, membrane, lipids, inflammation, hepatitis C.

INTRODUCTION

Hepatitis C is an infection of the liver caused by the hepatitis C virus. According to the World Health Organization (WHO), 3% of the world population has been infected by HCV. Hepatitis C can be either acute or chronic. Chronic hepatitis C may progress to cirrhosis and subsequently to hepatocellular carcinoma (1).

In chronic liver diseases there are many metabolic alterations affecting patients' nutritional status (2), including changes in liver lipid metabolism and lipidome. In hepatitis C, these changes accommodate the viral life cycle (3, 4). Alterations in hepatic lipid metabolism trigger changes in plasma lipidome and the latter may relate to liver histopathology (5, 6).

Red blood cell lipid pattern is also affected in chronic liver diseases (7–9). It has been observed to correlate with liver steatosis and steatohepatitis (10). In addition, Tanaka *et al* found that ribavirin and interferon treatment increased PE, SM and CHOL/PE ratios and decreased PC and PC/SM ratios in the erythrocyte membrane of patients with hepatitis C (11). However, these alterations might be attributed to the entrapment of ribavirin in erythrocytes (12, 13).

Both inflammation and oxidative stress modify the lipid composition of erythrocyte membrane (14–16). In patients with hepatitis C, viral clearance seems to attenuate fibrosis (17), inflammation (18) and oxidative stress (19). Whether this effect reflects upon erythrocyte membrane lipid composition is an issue that certainly merits investigation.

We speculate that if hepatitis C infection and subsequent fibrosis modify the pattern of the erythrocyte membrane cholesterol and phospholipids, this event may indicate liver tissue damage and resolution before and after treatment of the disease, respectively.

The aim of this study has been to investigate the erythrocyte membrane cholesterol and phospholipid pattern in patients with hepatitis C virus and severe fibrosis, before and after treatment. A potential application as a novel reliable biomarker shall also be considered.

MATERIALS AND METHODS

Patients

Three men and three women aged 61.5±8.64 years were included in our study. All subjects were HCV positive according to virus RNA detection. Blood was collected at the beginning and end of treatment. Every patient had fibrosis ≥F3, as defined through FIBROSCAN, and was treated with DAAs (Table 1). HCV infection was defined through polymerase chain reaction (PCR) of viral serum RNA detection. The protocol was approved by the appropriate Scientific Committee.

TABLE 1. Basic characteristics of patients participated in this study

Age (years)	61.5±8.64
Gender (males/females)	3/3
HCV genotype (1/2/3/4)	4/1/1/0
Fibrosis stage (F3-F4)	6

Isolation of erythrocyte membrane "ghosts"

Three milliliters of blood containing EDTA was centrifuged at 200 g for 10 minutes at 4oC. Plasma and buffy coat were removed. Then, the erythrocyte pellet was washed with cold saline solution and centrifuged at 200 g for 10 minutes at 4°C.

Erythrocytes were diluted 1:10 (v/v) with cold hemolysis solution (Tris 1 mM–NaCl 10 mM – EDTA 1 mM pH 7.2) and, after incubation for 30 minutes at 4°C with continuous shaking, they were centrifuged at 15000 rpm in HERMLE 323 K centrifuge, 220.80 VO2 rotor, at 4°C for 16 minutes.

The pellet was collected, and the last step was repeated for as many times as needed, until the final pellet was milky-white, signifying hemoglobin removal. Samples were kept at -80°C until analysis. Total erythrocyte membrane protein was estimated with the Bradford assay using bovine serum albumin as standard (20).

Lipid isolation

An aliquot of the sample (300-500 μL) was mixed with an equal volume of ice-cold trichloromethane/methanol (2/1 v/v). The two phases were separated by centrifugation (2000 rpm for 10 minutes at 4°C, in Sorvall® Dupont MC12V centrifuge, in rotor F-12/M.18) and the organic phase was collected (20) and kept at 4°C. The procedure was repeated once more, and the resulting two organic phases were pooled and washed with an equal volume of ice-cold deionized water. The final organic phase was collected, and the solvent was evaporated at 37°C in an Eppendorf® thermomixer.

The remaining lipid was suspended in 100 μL of solvent (trichlomethane/methanol 2/1 v/v) at 4°C and analyzed by thin layer chromatography (TLC).

Thin layer chromatography

Thin layer chromatographic analysis of erythrocyte membrane lipids was done on a 10X10- cm chromatographic plate (TLC Silica gel 60 F254 (Merck KGaA 64071 Darmstadt, Germany) using a mixture of chloroform/methanol/acetic acid/water (60/50/1/4) (v/v/v/v). Before loading the samples, the plate was desiccated at 150° C for 10 minutes and pre-run with the developing solvent mixture as above.

After sample separation, the plate was dried with hot air and then placed in a closed container of vaporized iodine. Lipids appeared as dark yellow bands against a lighter background.

Lipids were identified by comparison with lipid standards, namely cholesterol, phosphatidyl-ethanolamine, phosphatidyl-inositol, phosphatidyl-serine, phosphatidyl-choline and sphingomyelin all purchased from Sigma-Aldrich.

Digital pictures of the chromatographs were taken, the areas of individual spots were estimated using Gel Analyser® software and expressed as "percentage of total lipid quantified" in the sample. Ratios of lipids were also calculated. Every sample was analyzed in triplicate.

Statistical analysis

Statistical analysis was done with the R programming language v. 3.6 (21). Bayesian analysis was performed using JAGS 4.3.0 (22).

Testing for difference between means was performed by the Bayes Factor package, using a Jeffreys prior on the variance and a Cauchy prior with scale=1 on the effect size. Bayesian linear regression was done using JAGS assuming a wide normal prior for the slope and intercept and a lognormal distribution for the error.

RESULTS

Since the sample size was small, we used a Bayesian approach for statistical analysis, which is much more suited to provide meaningful results for small datasets. Bayesian analysis does not assume large sample sizes and smaller datasets can be analyzed while retaining statistical power and precision (23, 24).

In Table 2, the mean and median before and after DAA are shown. Also, the lower and upper 95% posterior intervals for the effect size (difference between means) is given, along with the probability that the means difference is not zero.

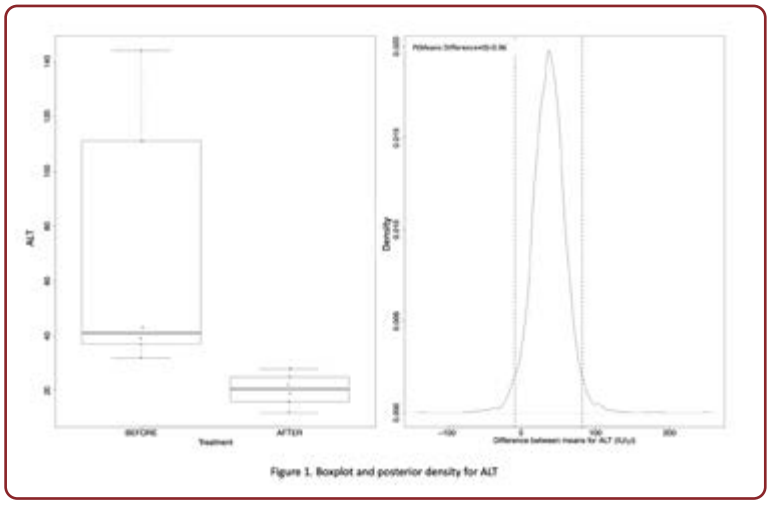

FIGURE 1. Boxplot and posterior density for ALT

TABLE 2. Mean and median values before and after direct antiviral treatment, lower and upper edges of the 95% posterior density and probability that the difference between the means before and after treatment differs from zero

	Before DAA		After DAA		Lower 95% posterior density	Upper 95% posterior density	P (means difference≠0)
Parameter	Mean	Median	Mean	Median			
ALT (U/μL)	67.67	41.00	20.33	20.50	-6.29	82.41	0.96
AST (U/μL)	65.50	49.00	27.83	29.00	-7.12	66.85	0.95
RBC/mL	4.53	4.49	4.33	4.36	-0.13	0.43	0.87
Hb (g/dL)	14.02	13.95	13.23	13.15	-0.02	1.27	0.97
MCH (pg)	31.15	31.10	30.30	30.45	-0.08	1.4	0.96
MCHC (g/dL)	34.30	34.35	33.45	33.50	0.14	1.29	0.99
MCV (fl)	90.37	91.15	90.27	92.55	-2.03	2.19	0.53
CHOL	31.25	32.00	36.38	36.50	-14.82	7.22	0.78
PE	18.17	17.60	14.03	15.05	-1.09	7.69	0.94
PI	4.57	4.45	3.48	3.60	-2.56	4.35	0.70
PS	6.03	5.15	2.33	0.00	-5.08	10.7	0.78
PC	17.45	16.35	19.35	19.85	-6.45	3.45	0.74
SM	22.33	23.05	23.90	23.55	-8.54	6.19	0.64
PC/PE	0.97	0.94	1.38	1.38	-0.61	-0.10	0.99
PC/SM	0.86	0.68	0.85	0.76	-0.41	0.44	0.49
CHOL/PE	1.77	1.70	2.96	2.31	-2.66	0.81	0.88
CHOL/SM	1.54	1.40	1.60	1.44	-0.96	0.87	0.54
PC/(PE+SM)	0.44	0.40	0.51	0.48	-0.21	0.10	0.77
(PI+PS)/SM	0.55	0.40	0.24	0.14	-0.38	0.87	0.79

RBC=red blood count, Hb=hemoglobin, MCH=mean corpuscular hemoglobin, MCHC=mean corpuscular hemoglobin concentration, MCV=mean corpuscular volume, CHOL=cholesterol, PE=phosphatidyl-ethanolamine, PI=phosphatidyl-inositole, PS=phosphatidyl-serine, PC=phosphatidyl-choline, SM=sphingomyelin

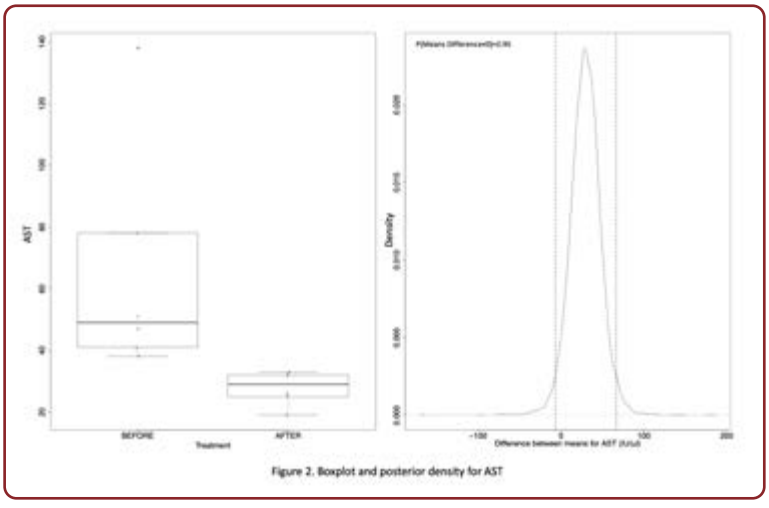

Figure 2. Boxplot and posterior density for AST

FIGURE 2. Boxplot and posterior density for AST

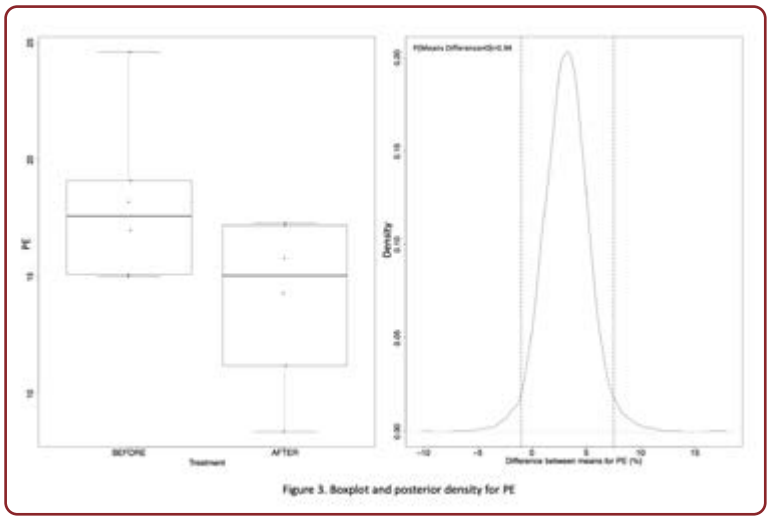

FIGURE 3. Boxplot and posterior density for PE

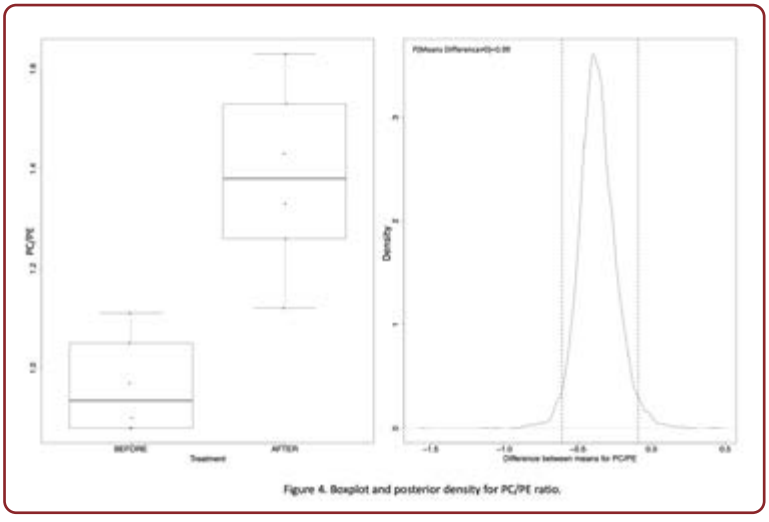

FIGURE 4. Boxplot and posterior density for PC/PE ratio

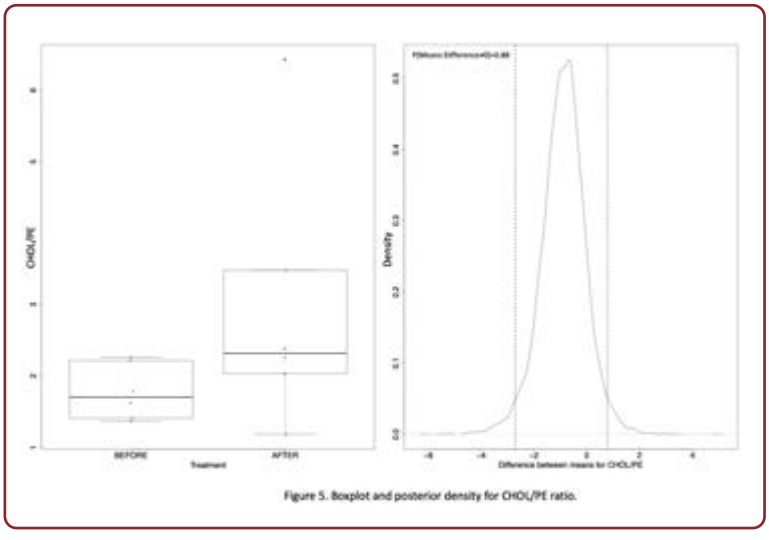

FIGURE 5. Boxplot and posterior density for CHOL/PE ratio

This probability can be considered significant if it is far from 0.50. The most significant differences between means before and after treatment, are for the liver function markers ALT ($P_{mean\ diff\neq0}=0.96$) and AST (Pmean diff≠0 =0.95), shown in Figures 1 and 2, respectively. For hematological markers, very significant mean differences appear for hemoglobin ($P_{mean\ diff\neq0}=0.97$), MCH ($P_{mean\ diff\neq0}=0.96$) and MCHC ($P_{mean\ diff\neq0}=0.99$). The highest probability of mean difference for the lipids is for PE ($P_{mean\ diff\neq0}$ =0.94) shown in Figure 3. For lipid ratios, the highest difference is for the PC/PE ratio ($P_{mean\ diff\neq0}=0.99$) and also for the CHOL/PE ratio ($P_{mean\ diff\neq0}=0.88$), as depicted in Figures 4 and 5, respectively. In the aforementioned Figures 1-5, the boxplot and posterior distributions of the difference between means are shown for AST, ALT, PE, PC/PE and CHOL/PE ratio. The boxplots and posterior densities for all remaining measured parameters are also shown in the supplementary material.

When Bayesian linear regression of the PC/PE ratio *versus* ALT was performed for all patients before and after DAA, there was a significant shift in the pattern of regression (Figure 6). Before treatment, there is very little correlation between these two parameters, and change in ALT is correlated with very little change in the PC/PE ratio. Things are radically different after treatment, where an increase in ALT causes a steep decrease in this ratio.

CONCLUSION

Hepatitis C virus infection dramatically affects the kinetics of lipid trafficking and distribution *via* blood circulation among tissues within the human organism. The liver is the major organ displaying lipid infiltration and subsequent fibrosis as part of the progress of the disease. On the other hand, the erythrocyte is the most abundant cell in the blood, ubiquitous and available to interact with all organs. Under this perspective, the lipidome of the erythrocyte membrane may also be affected by the irregular systemic lipid profile that is evident in the course of the disease. In the present study, we sought to determine the phospholipid and cholesterol patterns in a group of patients with hepatitis C in terms with other clinical parameters at two endpoints of the disease (upon diagnosis and after treatment with DAAs).

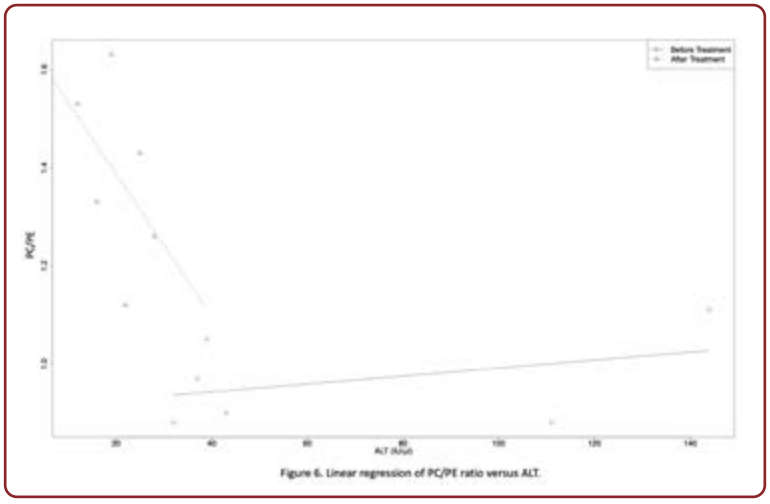

FIGURE 6. Linear regression of PC/PE ratio *versus* ALT

After antiviral treatment there was a decrease in AST and ALT levels (Figures 1 and 2, respectively), as expected from the resulting improved liver function. The PE levels decreased (Figure 3), while PC/PE and CHOL/PE ratios increased (Figures 4 and 5, respectively). We speculate that the observed increase of PC/PE in our patients may correlate with an improvement in liver histopathology, since the increase in this ratio was accompanied by a decrease in AST and ALT levels. The PC/PE ratio was strongly negatively correlated with ALT levels after treatment. Before treatment, this correlation was very weak, as shown in Figure 6. Arendt *et al* found that the PC/PE ratio in erythrocyte membrane was lower in NAFLD and NASH patients than healthy subjects, and this decrease followed a similar decrease in the PC/PE ratio of the liver (9). Furthermore, mice lacking phosphatidylethanolamine N-methylotransferase (PEMT) and fed a low-PC diet, display a low PC/PE ratio in the liver subsequently developing steatohepatitis (25). In other animal models of steatohepatitis, low PC/PE ratio was observed in liver endoplasmic reticulum (26) as well as in mitochondria (27). Finally, Dinkla *et al* reported that systemic inflammation can reduce PC, but not PE, content in the erythrocyte membrane, through secretory phospholipase IIA – independent hydrolysis to lysophosphatidylcholine (LPC) (16). This finding may indicate that the increase of PC/PE ratio in erythrocyte observed after antiviral treatment may possibly correlate to a remission of liver inflammation. Another explanation may be a PC exchange between plasma lipoproteins and the erythrocyte (28). However, molecular mechanisms explaining the absence of correlation of the PC/PE ratio to ALT levels before treatment, require further examination. It is possible that liver damage repair depends on PC/PE ratio to a higher extent than liver damage formation does. Alternatively, liver PC/PE ratio is not reflected upon erythrocyte to the same extent during the different stages of liver damage.

Regarding the increase of CHOL/PE ratio in patients with hepatitis C after successful treatment, we hypothesize that it is an adaptation of erythrocytes to the new metabolic environment, formed either by liver damage regression or eradication of hepatitis C virus. Dawaliby *et al* showed that eukaryotic cells preserve a mechanism for maintaining a dynamic balance between cholesterol and phosphatidylethanolamine content (29). However, since human mature erythrocytes do not contain organelles for de novo synthesis of lipids, we speculate that the alteration of CHOL/PE ratio possibly indicates changes in plasma lipidome and/or changes in intercellular lipid transport.

Other lipid ratios were examined as potential markers of phospholipid peroxidation (13), as shown in Table 2. However, no significant differences in these ratios before and after antiviral treatment were found.

No other quantitative differences in the levels of cholesterol and phospholipids were observed in the present study. Although hematological parameters changed after treatment, no significant correlation with lipid composition was observed.

In conclusion, antiviral treatment increased the PC/PE ratio in patients with hepatitis C and severe fibrosis. Therefore, red blood cell lipid composition could be a candidate real time biological marker for hepatitis C treatment. Although the sample size is small, Bayesian analysis is well suited to the analysis of such samples. This study was undertaken as an initial proof-of concept. This study will be expanded to a greater number of hepatitis C patients and other paradigms of liver disease, both *in vivo* and *in vitro*. ❑

Conflicts of interests: none declared.
Financial support: none declared.

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