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Letter to the Editors-in-Chief

First exploratory study on the metabolome from plasma exosomes in patients with paroxysmal nocturnal hemoglobinuria

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ABSTRACT

Introduction: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare disease in which patients are at increased risk of thrombosis. The mechanisms underlying the associated thrombosis risk are still poorly understood, although it is known that Eculizumab, the drug of choice for symptomatic patients, prevents thrombotic events. Exosomes are extracellular vesicles that can carry and disseminate genetic material, tumor biomarkers and inflammatory mediators. To date, the metabolite cargo of plasma exosomes from PNH patients has not yet been explored. In this pilot trial, we compared the metabolome of plasma exosomes from PNH patients with that of healthy subjects in order to provide further insights into this rare disease.

Results: We used a non-targeted metabolomics approach with UPLC-ESI-QTOF-MS/MS and GC–MS platforms. Multivariate analyses revealed the differential occurrence ($p < .001$) of 78 metabolites in plasma exosomes from PNH patients vs healthy control subjects. Remarkably, prostaglandin F2-alpha (6.1-fold), stearoyl arginine (5.3-fold) and 26-hydroxycholesterol-3-sulfate (11.2-fold) were higher in PNH patients vs healthy controls ($p < .001$).

Conclusions: This is the first description on the differential metabolite cargo occurring in plasma exosomes from PNH patients. Our results could contribute to the search for possible prognostic biomarkers of thrombotic risk in patients with PNH. Further research in a larger cohort to validate these results is warranted.

1. Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal hematopoietic stem cell disease with associated pro-inflammatory status and risk of thrombosis [1,2]. This rare disease is caused by a somatic mutation of the phosphatidylinositol glycan anchor biosynthesis class A (PIGA) gene in bone marrow stem cells, resulting in the disruption of the biosynthesis of glycosylphosphatidylinositol (GPI) and thereby a deficiency of all GPI-anchored proteins such as CD55 and CD59 on the cell membrane [3]. This provokes an increased sensitivity of complement in PNH cells, intravascular hemolysis, promotion of inflammatory mediators, and systemic hemoglobin release [2]. The poor survival of PNH patients is associated with the onset of thromboembolic complications, with a relative risk of 10.2 at 8 years [4,5]. Although there are multiple factors contributing to the occurrence of the thrombotic event [2,6,7], platelet activation is likely the main cause of the high incidence of thrombosis associated with PNH [8–10]. Patients with PNH who have suffered a thrombotic event require anticoagulation and are treated with the anti-C5 monoclonal antibody eculizumab, which prevents the spread and recurrence of more thrombotic events in some patients [7].

Exosomes are spherical membrane vesicles with 30–240 nm in diameter that are involved in intercellular communication by modulating the response of recipient cells, which may be located at distance of the secreting cell [11]. Exosomes contain both intracellular and extracellular components, including metabolites resulting from cell uptake, genetic material, proteins and all those interacting cell membrane elements [12]. Increasing evidence supports that the exosome composition might be a reflection of the physiological status, which suggests a potential role of exosomes as surrogate biomarkers for the diagnosis of different diseases [13,14]. In the case of PNH, it has been reported to be associated with an increase in the number of extracellular vesicles released to the blood streaming [15], although others have documented no differences in the number of extracellular vesicles between PNH patients and healthy controls [16] or there is still no evidence of correlation between clone size and the number of extracellular vesicles [17,18]. Moreover, it has been reported that the absence of GPI-anchored membrane proteins affects the composition of red blood cell-derived microvesicles, as well as the composition and concentration of platelet-derived vesicles [19].

The impact of PNH and its treatment (eculizumab) on extracellular vesicles has been studied previously [16,20]. We hypothesized that unraveling the metabolite composition of plasma exosomes from PNH patients may help to better understand the disease. To this purpose, we aimed to explore here for the first time, through a non-targeted metabolomics approach, the metabolome of plasma exosomes derived from PNH patients and compare it with that of healthy individuals.

2. Materials and methods

2.1. Patients and samples

Plasma samples and data from patients with diagnosed PNH included in this study ($n = 9$) were provided by the National DNA Bank Carlos III (www.bancoadn.org, Salamanca, Spain) and processed according to standard operating procedures with the appropriate approval of the Ethical and Scientific Committees (Protocol number 15/0007). Table 1 shows demographic and clinical characteristics of PNH patients.

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Table 1
Demographic and clinical features of selected PNH patients. N.D. Not Determined. LDH is represented as ratio between the actual value and the upper normal reference level.

	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13	Patient 14
Females/males (F/M)	M	F	F	M	M	M	F	M	M
Age (years)	51	13	65	46	58	45	49	63	64
Eculizumab	NO	NO	NO	YES	YES	YES	YES	YES	YES
Treatment other than Eculizumab	Steroids	NO	Anticoagulants	NO	Anticoagulants	NO	NO	NO	NO
Thrombosis									
(YES/NO)	NO	NO	YES	YES	YES	YES	NO	NO	NO
Arterial/venous (AT/VE)	NO	NO	AT	AT	VE	AT	NO	NO	NO
PNH clone in neutrophils (%)	92.2	39.3	97.5	72.7	86.5	99.2	89.3	99.4	97.9
PNH clone in monocytes (%)	96.9	43.4	96.6	81.4	86.7	97.1	94.4	92.8	100
LDH ratio	8.05	2.55	4.48	8.24	0.74	0.82	1.27	1.51	2.62
Direct bilirubin (mg/dL)	0.7	0.0	0.6	0.3	0.6	0.8	0.4	N.D.	0.8
Total bilirubin (mg/dL)	3.0	1.4	1.9	2.3	8.5	2.4	3.3	1.5	1.7
Reticulocytes (%)	< 0.5	4	N.D.	6.9	N.D.	4.7	7.5	9.5	9.2
Hemoglobin (g/dL)	12.3	12.7	7.7	8.9	12.3	11.1	12.1	11.9	10.1

Control blood samples from 5 healthy normoweight subjects (3 males and 2 females, 36 ± 14 years old) were obtained from the Blood Transfusion Centre in Murcia (Centro Regional de Hemodonación, Murcia, Spain) [21].

2.2. Isolation of plasma exosomes and metabolomics analysis

Exosomes were isolated from 500 μL of plasma using the Total Exosome Isolation Kit (ThermoFisher Scientific, Fisher Scientific, Madrid, Spain). The final pellet was resuspended in ultrapure diethyl pyrocarbonate-treated water and analyzed by electron microscopy and western blotting (Supplemental Information and Supplemental Fig. 1).

Untargeted metabolomics was performed at the CEBAS-CSIC Metabolomics Platform (Murcia, Spain) using the combination of gas chromatography (GC) and liquid chromatography (LC), both in tandem with mass spectrometry (MS). The detailed methodology can be found in Supplemental Methods in Supporting Information.

In the case of GC–MS analysis, plasma exosomes (250 μL) were sonicated, extracted either with chloroform/methanol (MeOH) (1:3, v/v) or (2,1, v/v) for polar and non-polar metabolites, respectively, and further centrifuged. The corresponding supernatants were reduced to dryness using a speed vacuum system. Then, samples were derivatized to trimethylsilyl derivatives prior the injection of the sample into a HP 6850 gas chromatograph interfaced with a HP 5977 mass selective detector (MSD) (Agilent Technologies, Waldbrom, Germany) (Supporting Information).

In the case of LC-MS analysis, plasma exosomes (250 μL) were sonicated, extracted with acetonitrile/MeOH (1:1, v:v) and centrifuged. The supernatant was reduced to dryness and further reconstituted in 200 μL MeOH:H_2O (1:1) for polar metabolites and in 200 μL of chloroform:MeOH (2:1, v:v) to dissolve the non-polar metabolites. The analyses of both polar and non-polar samples were carried out using an Agilent 1290 Infinity series LC system coupled to a 6550 I-Funnel Accurate-Mass QTOF (Agilent) with a dual electrospray ionization interface (ESI-Jet Stream Technology) for simultaneous spraying of a mass reference solution that enabled continuous calibration of detected *m/z* ratios (Supplementary Methods).

Feature extraction was carried out on Agilent Profinder B.06.00, which is a stand-alone feature extraction program for LC-MS based profiling analyses (Supplementary Methods). To find compounds by Molecular Features (MFs) was carried out using a pre-filter to take peaks with height greater or equal to 10,000 counts, allowing only −H and +HCOO as negative ions species and +H as positive ions (Supplementary Methods). The definitive files were exported into the Mass Profiler Professional (MPP) 2.0 software package (Agilent, Santa Clara, CA, USA) for statistical analysis. In MPP, the maximum fold change is set to 16 and thus any values over this value will be reported as 16-fold. Unidentified compounds were aligned across the different samples based on their retention times' tolerance and the mass spectral similarity. The MassHunter MSC (Molecular Structure Correlator) program was used to correlate accurate mass MS/MS fragment ions for a compound of interest with one or more proposed molecular structures for that compound (Supplementary Methods).

2.3. Statistical analysis

Multivariate statistical comparisons were performed between healthy controls and PNH patients. As an additional sub-analysis, samples of PNH patients were clustered into three different subgroups, i.e. 'Ecu-no-Thrombosis' ($n = 3$) included patients without thrombosis under treatment with eculizumab, 'Ecu-Thrombosis' ($n = 3$) was formed by patients under treatment with eculizumab who had suffered a thrombotic event, and 'No-Ecu' (n = 3) included patients without eculizumab treatment. Student's *t*-test unpaired analyses were applied with a level of significance of $p < .05$ with Bonferroni Holm Family-wise Error Rate (FWER) multiple testing corrections and a fold-change cut-off of 2.0. Student's *t*-test was mainly focused on the search for the statistical significance of the null hypothesis between control and PNH patients for the three treatments. Tools such as PCA-2D and Hierarchical Heat Map were used to visualize some statistical results, using the final list of statistically significant MFs.

3. Results

Multivariate analyses revealed a differential pattern in the metabolome of plasma exosomes from PNH patients compared to that from healthy controls (Fig. 1). However, a number of these significant metabolites did not yield an accurate mass based putative identification when searched against several databases and on-line libraries. For this reason, despite 78 metabolites were significantly different among groups, only 6 were tentatively identified. Among the identified metabolites differently found in PNH patients and healthy controls, we detected a significant increase (6.1-fold) in the levels of prostaglandin F2-alpha (PGF2α), stearoyl arginine (SA) (5.3-fold) and 26-hydroxycholesterol-3-sulfate (26 HS) (11.2-fold) in PNH patients in comparison with controls ($p < .001$) (Fig. 1). When the analysis was performed among the three sub-groups of patients, we identified differential levels of diacylglycerol (DAG) and cholesterol that were 16.0-fold higher ($p < .001$) (which is the highest score provided by MPP software to highlight significant changes) in those patients under treatment with eculizumab in comparison to those without treatment. In addition, aminoethyl phosphonic acid (AEPA) decreased (16.0-fold) in the group Ecu-Thrombosis in comparison with the group Ecu-no-Thrombosis ($p < .001$).

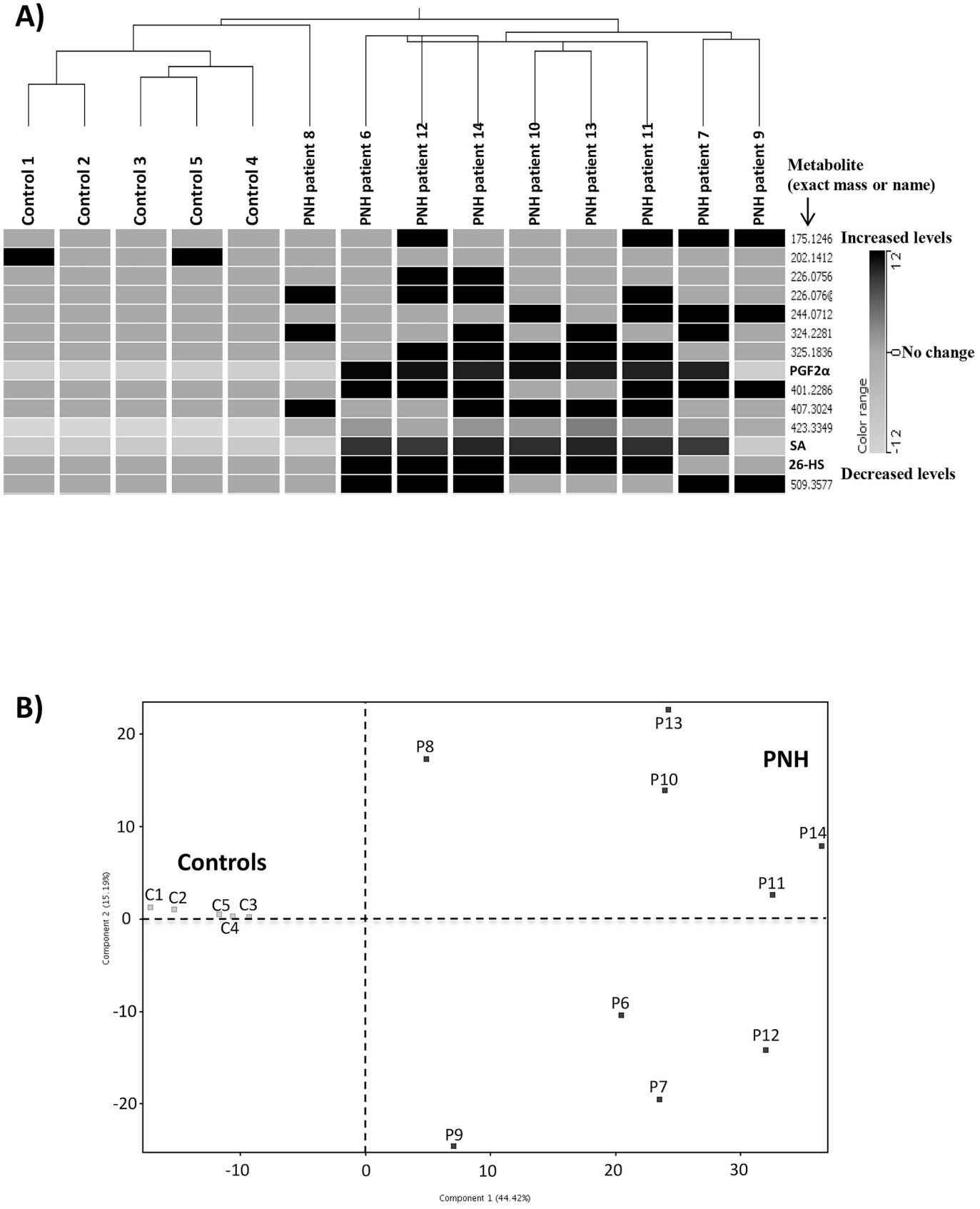

Fig. 1. Analysis of plasma exosome metabolites in patients and controls. (A) Hierarchical heat map visualization of plasma exosome metabolites significantly detected by UPLC–MS in positive ESI. Each row represents a unique feature with a characteristic mass-to-charge ratio and retention time while each column represents one subject. SA, stearoyl arginine; 26-HS, 26-hydroxycholesterol 3-sulfate; PGF2α, prostaglandin F2α. (B) Two-dimensional Principal Component Analysis (PCA scores plot) of plasma exosome metabolites significantly different obtained by UPLC–MS in positive ESI showing the separation between the two study groups. PNH, metabolites in PNH patients; Controls, metabolites in control healthy subjects. The X-axis shows interclass separation while Y-axis illustrates the intra-class variability on Y-axis.

4. Discussion

PNH is a rare disease in which the underlying mechanisms and the associated prothrombotic state of patients are still poorly understood [2]. Overall, the tendency toward thrombosis is not the same in all patients and seems to be multifactorial, i.e. platelet activation, intravascular hemolysis and neutrophil/monocyte activation, where the individual contribution of these factors to the prothrombotic state in PNH remains elusive so far [2,7].

Exosomes are involved in cell communication since their content may influence the function of the recipient cell [22] and also may reflect the status of the patient, especially in diseases with a clear inflammatory component such as PNH [2]. The metabolic profiling of exosomes secreted by different types of cells and also found in a number of physiological fluids has been previously reported [12]. However, this had not been explored in plasma exosomes of patients with PNH so far. Consequently, we hypothesized that the metabolite cargo of exosomes could be related to the onset and/or evolution of this disease. Integrating clinical metabolomics-based biomarker discovery and clinical management of rare diseases is emerging as a valuable translational approach [23]. In the present study, the combination of electrospray chromatography ionization mass spectrometry and multivariate statistical analyses detected differences in the metabolome of plasma exosomes from PNH patients and healthy subjects. Thus, the metabolites differentially present in patients could play a role in PNH.

A number of metabolites have been previously reported to occur in exosomes from different fluids (plasma, milk, urine, etc.) and cell origin (platelets, leukocytes, epithelial, endothelial and tumor cells) such as sphingomyelin, ganglioside GM3, cholesterol, and different aminoacids, among others [12,24]. In our analyses, most of the previously described metabolites were found in both patients and healthy controls (results not shown), but only some of them differentially occurred in PNH patients. However, only a few of these metabolites were identified. The number of metabolites would have dramatically increased with a lower cut-off (i.e. 1.5-fold, or even 1.2-fold as reported in many studies) to establish a significant statistical change. However, in a physiological context, the borderline between 'statistical' and 'clinical' relevance is hard to be established [25]. In addition, the unambiguous identification of many metabolites remains a major challenge in metabolomics, especially when the number of samples is limited [26]. This is the case of the present trial with PNH patients, and we acknowledge that there are many limitations in this type of studies, including the low number of cases for this rare disease and the fact that patients are not homogeneous, i.e. despite we selected our patients considering the neutrophil and monocyte PNH clones (Table 1), for instance, those patients that have not suffered thrombosis at the moment of the blood withdrawal could develop a thrombotic event in the future. In addition, although thrombotic events in patients with PNH usually occur in the venous territory [27], in the patients we have selected there is a higher incidence of arterial thrombosis. Therefore, the influence that this can have on the metabolites detected is unknown. Eventually, both patients and controls were caucasians but they were not age- and sex- matched, as we selected controls available in our laboratory. We want to emphasize that given the rarity of the disease, the number of patients to be divided into age groups was very low.

As the present study is the first report on the metabolome of plasma exosomes from PNH patients, we cannot compare our results with previous studies and consequently this can limit further conclusions. Overall, the finding of a plausible physiological meaning for the different levels of stearoyl arginine in patients vs controls remains elusive. Neither the possible implication for a higher DAG and cholesterol levels in eculizumab-treated patients as well as for lower AEPA levels in the Ecu-Thrombosis vs Ecu-no-Thrombosis groups. In the case of 26-hydroxycholesterol-3-sulfate, higher levels of this steroid in PNH patients could be related to a lower steroid sulfatase activity, whose expression has been associated with the in situ production of cytokines and the degree of atherosclerotic changes in the aorta [28].

Diacylglycerol (DAG) and cholesterol were higher in patients treated with eculizumab. It is known that these patients have significantly greater extravascular haemolysis compared to non-ECU patient. It has been described that during eculizumab treatment, binding of C3 by PNH red blood cells may constitute an additional disease mechanism in PNH, producing a variable degree of extravascular hemolysis [29]. In extravascular hemolysis red blood cells are phagocytized by macrophages in the spleen and liver due to abnormalities in the cell membrane. DAG is a key molecule enhancing phagocytosis [30], and in some cases, macrophages could release cholesterol [31]. Therefore, this increase detected in these molecules could be as a consequence of the macrophage-mediated phagocytosis of the erythrocytes affected by C3 binding.

Inflammation can promote local thrombosis, and thrombosis can amplify inflammation [32], and this interplay may occur in many PNH patients. Remarkably, the increased levels of PGF2α in plasma exosomes from PNH patients vs healthy controls is consistent with a pro-inflammatory state [33]. PGF2α is one of the most abundant prostaglandins formed at sites of inflammation [34] and has been proposed as a chemoattractant for neutrophils recruitment [35]. Since spreading and amplification of thrombosis by neutrophils and extracellular nucleosome networks have been recently suggested [36], we can speculate that plasma exosomes from PNH patients could function as reservoir of circulating PGF2α and thus disseminate from cell to cell major promoters of intravascular blood coagulation and thrombosis. However, it would be necessary to validate PGF2α levels in a larger number of patients with PNH and confirm its association with an increased thrombotic risk, since PGF2α (as well as SA and 26-HS) level was similar to controls in 2 out of 4 patients with thrombosis whereas it was increased in all 5 patients which never experienced a thrombotic event. Moreover, the role of PGF2alpha in platelet aggregation is not well defined, as it might also antagonize this process via different interactions with the TXA2/PGH2 receptor [37]. In addition, PGF2alpha is not only produced at the site of inflammation, but also in other tissues under various physiological and pathological conditions. Therefore, PGF2alpha-carrying exosomes could be actively released by some cell types.

Our study shows a number of limitations, particularly the small sample size. In general, despite exosomes have been suggested to be useful in the diagnosis and prognosis of diseases such as ovarian malignancies and stroke [38,39], however, the analysis of plasma exosomes is the picture of a specific moment, i.e. a precise time-point. In addition, in the present pilot trial, the low number of patients for this rare disease and the lack of identification for a number of other metabolites, a classical limitation in non-targeted metabolomics [40], prevent unequivocal links between plasma exosomes metabolites and patients' status.

5. Conclusions

Our results suggest that plasma exosomes could be a reservoir of (pro-inflammatory) circulating metabolites, such as PG2α, that may influence the pathological status of the PNH patients. Nevertheless, the present study is a first approach on this specific topic and further studies, with larger groups of patients, are needed to validate the metabolites described here as potential surrogate biomarkers of this disease.

Ethics approval and consent to participate

Plasma samples and data from patients with diagnosed PNH included in this study were provided by the National DNA Bank Carlos III (www.bancoadn.org, Salamanca, Spain) with the appropriate approval of the Ethical and Scientific Committees.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Authors' contributions

IMM and JCE were the principal investigators and take primary responsibility for the paper. FV, JEY, RTM, GLG, NB, SE and NGB performed the laboratory work for this study. IMM and JCE coordinated the research and wrote the manuscript, and VV critically reviewed the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.10.001.

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