blood advances

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodadvances@hematology.org

Platelet proteomic profiling in sitosterolemia suggests thrombocytopenia is driven by lipid disorder and not platelet aberrations

Tracking no: ADV-2023-012018R1

Jessica Del Castillo Alferez (Sanquin Research, Netherlands) Anton Tool (Sanquin, Netherlands) Karin van Leeuwen (Sanquin Blood Supply Foundation,) Floris van Alphen (Sanquin Research, Netherlands) Marion Brands (Emma Children's Hospital, Amsterdam University Medical Center (AUMC), University of Amsterdam, Netherlands) Monique Suijker (Emma Children's Hospital, AUMC, University of Amsterdam, Netherlands) Alexander Meijer (Sanquin Research, Netherlands) Arie Hoogendijk (Sanquin Research, Netherlands) Taco Kuijpers (Department of Blood Cell Research, Sanquin Research, Amsterdam, Netherlands)

Abstract:
Sitosterolemia is a rare autosomal-recessive genetic disorder in which patients develop hypercholesterolemia, and may exhibit abnormal hematologic and/or liver test results. In this disease, dysfunction of either ABCG5 or ABCG8 results in intestinal hyperabsorption of all sterols, including cholesterol and more specifically plant sterols or xenosterols, as well as in the impaired ability to excrete xenosterols into the bile. It remains unknown how and why some patients develop hematologic abnormalities. Only a few unrelated patients with hematologic abnormalities at the time of diagnosis have been reported. Here, we report on two unrelated pedigrees who were believed to have chronic immune thrombocytopenia as most prominent feature. Both consanguineous families showed recessive gene variants in ABCG5, that were associated with disease by in-silico protein structure analysis as well as clinical segregation. Hepatosplenomegaly was absent. Thrombopoietin levels and megakaryocyte numbers in bone marrow were normal. Metabolic analysis confirmed the presence of strongly elevated plasma levels of xenosterols. Potential platelet proteomic aberrations were longitudinally assessed following dietary restrictions combined with the administration of the sterol absorption inhibitor ezetimibe. No significant effects on platelet protein content before and after onset of treatment were demonstrated. Although we cannot exclude that lipotoxicity has a direct and platelet-specific impact in patients with sitosterolemia, our data suggest that the thrombocytopenia is neither caused by a lack of megakaryocytes nor driven by proteomic aberrations of the platelets themselves.

Conflict of interest: No COI declared

COI notes:

Preprint server: No;

Author contributions and disclosures: Contribution: J.D.C.A., A.J.H. and T.W.K. wrote the manuscript; A.T.J.T. and M.B. performed cell isolation and clinical laboratory analysis; K.v.L. performed DNA analysis J.D.C.A. and F.P.J.v.A. performed mass spectrometry analysis; J.D.C.A, A.J.H., A.B.M. and T.W.K. analyzed the data; T.W.K., M.H.S, and M.M.B. diagnosed and treated the patients, and analyzed clinical data; all authors read and approved the manuscript.

Non-author contributions and disclosures: Yes; The authors thank Masja de Haas and Leendert Porcelijn for the Flow Cytometry analysis. The authors are grateful to the patients and their family members for participation in this study. J.D.C.A is supported by the SYMPHONY consortium, which received funding from the Netherlands Organization for Scientific Research (NWO) in the framework of the NWA-ORC Call grant agreement NWA.1160.18.038. funded.

Agreement to Share Publication-Related Data and Data Sharing Statement: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD041248

Clinical trial registration information (if any):

Platelet proteomic profiling in sitosterolemia suggests thrombocytopenia is driven by lipid disorder and not platelet aberrations

Jessica Del Castillo Alferez [1], Anton T.J. Tool [2], Karin van Leeuwen [3], Floris P.J. van Alphen [3], Marion M. Brands [4], Monique H. Suijker [5], Alexander B. Meijer [1], Arie J. Hoogendijk [1], Taco W. Kuijpers [2,6] *

[1] Department of Molecular Hematology, Sanquin Research, Amsterdam, the Netherlands;

[2] Department of Blood Cell Research, Sanquin Research, Amsterdam, The Netherlands;

[3] Department of Research facilities, Sanquin Research, Amsterdam, The Netherlands;

[4] Department of Pediatric Metabolic Diseases, Emma Children's Hospital, Amsterdam University Medical Center (AUMC), University of Amsterdam, Amsterdam, The Netherlands;

[5] Department of Pediatric Hematology, Emma Children's Hospital, AUMC, University of Amsterdam, Amsterdam, The Netherlands.

[6] Department of Pediatric Immunology, Rheumatology and Infectious Diseases, Emma Children's Hospital, AUMC, University of Amsterdam, Amsterdam, The Netherlands.

Corresponding author:

Arie J. Hoogendijk, Department of Molecular Hematology (Y328), Sanquin Research, Plesmanlaan 125, 1066 CX Amsterdam, the Netherlands;

Tel: +31-20-512 7186 (department)

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD041248

Taco W. Kuijpers, Department of Blood Cell Research, Sanquin Research, Amsterdam, The Netherlands, 1066 CX Amsterdam, the Netherlands; Department of Pediatric Immunology, Rheumatology and Infectious Diseases, Emma Children's Hospital, AUMC, University of Amsterdam, Amsterdam, The Netherlands

Abstract

Sitosterolemia is a rare autosomal-recessive genetic disorder in which patients develop hypercholesterolemia, and may exhibit abnormal hematologic and/or liver test results. In this disease, dysfunction of either ABCG5 or ABCG8 results in intestinal hyperabsorption of all sterols, including cholesterol and more specifically plant sterols or xenosterols, as well as in the impaired ability to excrete xenosterols into the bile. It remains unknown how and why some patients develop hematologic abnormalities. Only a few unrelated patients with hematologic abnormalities at the time of diagnosis have been reported. Here, we report on two unrelated pedigrees who were believed to have chronic immune thrombocytopenia as most prominent feature. Both consanguineous families showed recessive gene variants in ABCG5, that were associated with disease by in-silico protein structure analysis as well as clinical segregation. Hepatosplenomegaly was absent. Thrombopoietin levels and megakaryocyte numbers in bone marrow were normal. Metabolic analysis confirmed the presence of strongly elevated plasma levels of xenosterols. Potential platelet proteomic aberrations were longitudinally assessed following dietary restrictions combined with the administration of the sterol absorption inhibitor ezetimibe. No significant effects on platelet protein content before and after onset of treatment were demonstrated. Although we cannot exclude that lipotoxicity has a direct and platelet-specific impact in patients with sitosterolemia, our data suggest that the thrombocytopenia is neither caused by a lack of megakaryocytes nor driven by proteomic aberrations of the platelets themselves.

Key Points

- Two pedigrees with suspected chronic immune thrombocytopenia had ABCG5 variants causing sitosterolemia- a metabolic lipid disorder.
- The platelet proteomic landscape remains longitudinally stable in mutant ABCG5 protein before and after medical intervention.

Introduction

Hematological disease affecting the number of platelets is a common manifestation of numerous underlying conditions. Thrombocytopenia can be caused by underdevelopment, immune-mediated processes, chemotherapy, infection-related and genetic defects, amongst others. An accurate identification of the causes of thrombocytopenia is necessary for its adequate management yet this can be challenging given the high degree of heterogeneity of severity and clinical manifestations[1].

Inherited Thrombocytopenia consists of a group of disorders with defects in genes regulating megakaryocyte differentiation and platelet production [2,3]. While previously considered to be a rare disorder, it is now thought that the frequency of inherited thrombocytopenia may be underestimated[4]. The variable clinical expression of inherited thrombocytopenia contributes to its underdiagnosis[5]. Since some patients are asymptomatic and the relatively mild bleeding symptoms in others can frequently be overlooked until a low platelet count is detected often as part of a routine blood test.

Mediterranean stomatocytosis can exhibit macrothrombocytopenia and mild hemolysis with red cell stomatocytosis[6,7]. This condition is the hematological manifestation of sitosterolemia, a rare autosomal recessive metabolic disorder that is characterized by the accumulation of dietary sterols[8,9]. Sitosterolemia is caused by mutations in *ABCG5* or *ABCG8*, two genes encoding members of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter family[10], which form a dimeric complex transporting plant xenosterols out of the cell[4]. ABCG5 and ABCG8 are expressed in hepatocytes and enterocytes where they play a fundamental role in lipid metabolism by mediating sitosterol efflux and preventing sterol accumulation[11]. Loss of function of this complex leads to clinical variable phenotypes, such as premature atherosclerosis, atherosclerosis, splenomegaly, cardiovascular disease and xanthoma formation in most cases, and rarely in purely hematological defects marked by hemolytic anemia or platelet dysfunction and thrombocytopenia[12]. Hematological

abnormalities (hemolytic anemia and macrothrombocytopenia) may be present in 25-35% of patients, in whom it is usually associated with the main clinical features, as occurs in the 70% of the cases.[13]

In healthy individuals, cholesterol levels are rather stable and hardly influenced by daily intake. In contrast, individuals affected by sitosterolemia show increased intestinal absorption and decreased biliary excretion of dietary sterols, hypercholesterolemia, and premature coronary atherosclerosis[14]. Sitosterolemia is the only form of hypercholesterolemia that will respond to dietary restriction of foods rich in plant sterols such as vegetable oils, margarine, wheat germs, nuts, seeds, avocado and chocolate[15,16]. Hypercholesterolemia in patients with sitosterolemia is also responsive to bile acid sequestrants but not to statins[17]. Hence, medical intervention consists of a sterol-free diet and the sterol absorption inhibitor, ezetimibe[18,19].

Hematological abnormalities (hemolytic anemia and/or macrothrombocytopenia) are present in a minority of patients with sitosterolemia[13,20]. It has been suggested that the hematologic abnormalities in sitosterolemia are caused by the accumulation of circulating sterols in blood cell membranes[21]. How xenosterol accumulation affects platelet numbers in sitosterolemia and why in only some individuals is not understood. Bleeding abnormalities and macrothrombocytopenia in the mouse model for sitosterolemia are thought to be due to direct plant sterol incorporation into the platelet membrane and premature clearance[21]. Still, it has also been suggested that direct lipotoxicity exerted to circulating cells and blood components could account for the low platelet numbers[22,23]. Nevertheless, the impact of sitosterolemia in the platelet content remains unknown. Recent advancements in mass spectrometry based platelet proteomics have provided novel insights that have improved our understanding of biological processes regulating platelets in health and disease[24]. In this study, we aimed to characterize the effect of *ABCG5* mutations for their impact on the platelet proteome and assess the effect of the aforementioned medical intervention. Therefore, we

characterized the platelet proteome in the hematological presentation of sitosterolemia in four individuals from two unrelated pedigrees presenting ABCG5 mutations. Patients were followed for a year after receiving ezetimibe treatment and a dietary restriction of sterols, after which time sitosterol levels decreased and platelet counts increased. We assessed the effect of medical intervention in the platelet content by comparing before and after treatment time points using mass spectrometry-based proteomics to better understand the hematological presentation of sitosterolemia.

Methods

Blood Collection and platelet preparation

Heparinized venous blood was collected from four healthy donors and four homozygous sitosterolemia patients before and after treatment with informed consent , following Dutch regulations and the declaration of Helsinki. Platelets were prepared as previously described[25]. In short, platelet-rich plasma (PRP) was obtained by centrifuging whole blood for 20 minutes at 120g. To isolate platelets, PRP was centrifuged for 10 minutes at 2000*g*. The pellet was resuspended in a buffer comprising 36 mmol/L citric acid, 103 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L EDTA, 5.6 mmol/L D-glucose, and 10% (vol/vol) ACD-A (BD, Plymouth, UK) at pH 6.5. Around 100×10^{10} cells were lysed in 8M Urea, 100mM Tris and sonicated for 10 minutes. 5-20 ug of proteins were reduced and alkylated with DTT and IAM followed by Trypsin digestion. Peptides were desalted with Empore-C18 StageTips and eluted with 0.5% (vol/vol) acetic acid, 80% (vol/vol).

Mass spectrometry and data analysis

LC-MS/MS was performed as described previously[26]. Platelets samples were analyzed in triplicate. All data were acquired using Xcalibur software. Raw files were processed using MaxQuant (2.0.1.0) and the uniprot database containing reviewed proteins only (2040 entries, downloaded on April 8th,

2021). Output files were further analyzed in R. (v.2022.02.3). Contaminants and reverse values were filtered out. Proteins were included in analysis if they were present in at least 60% of control samples or patients in this study. Missing values were replaced with random values from a downshifted normal distribution (width=0.3 standard deviations and down shift =1.8 standard deviations of the non-missing data). Statistical analysis was performed using the limma package; Proteins were considered significant when presenting an absolute log2fold change of > 1 and a P-value < 0.05. Results were censored for comparisons in which all conditions contained imputed values to limit potential false positives due to the imputation strategy. Gene ontology enrichment and

Heparinized venous blood was collected from healthy donors (Sanquin Blood Supply) and patients (AMC) with informed consent, in accordance with Dutch regulations.

Results

Patient mutation and hematological parameters

We identified two families with sitosterolemia, initially diagnosed with chronic immune thrombocytopenia (cITP). Targeted Next Generation Sequencing panel testing showed homozygous gene variants in *ABCG5* **(Figures 1A and 1B)** . These mutations are predicted to cause a splice defect (c.775-3C>G) and a premature stop codon (c.1336C>T) before the transmembrane coils in pedigrees A and B, respectively (**Figure 1C**). Pedigree A shows the first family with three affected homozygous siblings: A-II-1 (16 years old), A-II-2 (14 years old), A-II-3 (9 years old) and one unaffected (**Figure 1A)**. Pedigree B, shows the second family with 1 affected homozygous sibling: B-II-1 (6 years old) and one unaffected **(Figure 1B)**. In both pedigrees, parents were consanguineous and heterozygous for the variants. Only individuals homozygous for the *ABCG5* gene variant presented sitosterol levels higher than 500 μmol/L (**Figure 1D**), with similar abnormalities in campesterol and cholestanol levels (**Figures 1E and 1F**).

Any bleeding diathesis in our patients was absent, but patients presented a platelet count below $100x10^9$/L (**Figure 1G**) and a mean platelet volume larger than 12 fL (**Figure 1H**). We did not observe

altered thrombopoietin (TPO) levels in any of the patients (**Figure 1I**). Moreover, bone marrow samples of propositus B-II-1 showed normal numbers of megakaryocytes (**Supplementary Table1**). Patients did not present hemolytic anemia nor other sitosterolemia-related symptoms such as cardiovascular complications. Hepatosplenomegaly was also absent.

Effect of Ezetimibe and dietary sterol restriction on sitosterol levels and thrombocytopenia

Treatment with the sterol absorption inhibitor ezetimibe combined with a sterol-poor diet was prescribed. We performed longitudinal monitoring after starting ezetimibe treatment combined with dietary restrictions for ~1 year. This treatment regimen significantly reduced sitosterol blood levels (**Figure 2A**, *P*-value=1.81×10^{-7}) and simultaneously increased platelet counts significantly (**Figure 2B**, *P*-value=0.017). As expected, sitosterol levels and platelet count were inversely correlated with a Pearson coefficient of -0.63 (**Supplementary Figure 1A**). After a year of follow-up, all affected individuals are without complaints to date. Notably, the index case in pedigree B (B-II-1) exhibited a more robust response to treatment and was the only patient that reached a normal mean platelet volume (**Figure 2C**). On the other hand, index cases in pedigree A exhibited more modest changes and only A-II-3 reached a normal platelet count. Treatment in our patients reduced the lipid levels to subnormal concentrations while normalizing hematological parameters at the same time.

Depth and stability of platelet proteomic profiling

The protein products of ABCG5 and ABCG8 genes form a dimeric ATP-binding cassette protein ('sterolin') [11]. ABCG5 and ABCG8 serve specifically to exclude the non-cholesterol sterol entry at the intestinal level and are involved in sterol excretion at the hepatobiliary level[11]. To investigate the effect of elevated plasma sterols on the platelet protein content we opted for a proteomics approach to analyze the platelets before and ~1 year after treatment of all patients (homozygous subjects) and to compare them with healthy controls. Firstly, we inspected the depth of the measured platelet proteome. Plotting the Label-Free Quantification (LFQ) intensities against the ranked abundance

illustrated the dynamic range of the platelet proteome spanned more than 10 orders of magnitude (**Figure 3A**). Main platelet proteins such as integrin alpha 2b (ITGA2B), as well as staple alpha granule components thrombospondin-1 (THBS1) and platelet factor 4 (PF4) were abundantly present. Low-abundant proteins included hornerin (HRNR) and (PTDSS1). Notably, no sterolins were detected in control or patient platelets, corroborating that its function is mostly relevant at the level of the digestive tract. Gene ontology term enrichment on all quantified proteins confirmed the over-representation of platelet-associated terms such as platelet aggregation and platelet alpha granules (**Figure 3B**). Next, the sample variance and reproducibility were examined. The standard deviation was calculated and compared to the relative median LFQ intensities. (**Figure 3C**). The majority of proteins (99%) presented a standard deviation smaller than 2, including potential co-purified plasma proteins carbonic anhydrase 1 (CA1) and hemoglobulins (HBA1,HBB). Proteins with the highest observed standard deviations included low abundant proteins and potential blood contaminants such as red cell-derived spectrin alpha erythrocytic 1 (SPTA1). Profilin-1 (PFN1) and Actin cytoplasmic 1 (ACTG1) presented the lowest abundance. Further examination of typical platelet proteins across the dynamic range of measured proteins confirmed stability (**Figure 3D**). Together, these findings show the robustness of the proteomics method and highlight the comparability of the platelet specimens from (pediatric) sitosterolemia patients with healthy (adult) controls.

Assessment of the effect of ezetimibe and dietary sterol restriction on platelet proteomes

Bleeding abnormalities and macrothrombocytopenia in sitosterolemia are thought to be predominantly due to direct plant sterol incorporation into the platelet membrane, resulting in platelet hyperactivation and premature clearance [21]. To investigate the underlying mechanism responsible for the platelet aberrations in sitosterolemia, in particular its impact on the proteome, we first compared the platelet proteome in our patients before treatment with platelets from healthy controls, using label-free proteomics. The patients' platelet proteome exhibited a differential abundance of 30 proteins, including CD151, EFEMP1, and von Willebrand factor (VWF) which were

lower in patients (**Figure 4A**; Supplementary Table2). CD151 is a membrane known to form a complex with integrins and to regulate cell adhesion and migration[27]. Notably no other membrane proteins detected were differentially expressed (Supplementary Figure 2). Conversely, several proteins in the platelet proteome were significantly upregulated at the time of diagnosis, including alpha-2-macroglobulin (A2M) and apolipoproteins APOA1, APOA2, APOE. Hence, some proteins that appear to be more abundant in patient platelets were potential plasma contaminants, or derived from other contaminating blood components (*e.g.* the hemoglobin alpha and beta chains [HBA1, HBB] and erythrocyte-derived carbonic anhydrase 1 [CA1]). Gene ontology enrichment analysis revealed that cholesterol/sterol transfer activity, lipoprotein particle receptor and protein lipid complex binding molecular functions were increased in patients regardless of treatment (Supplemental Figures 3A-2C). These data suggest a cellular response to the high plasma lipid levels which, despite significantly dropping after treatment, remained higher than normal levels

Next, we compared the platelet proteomes before and ~1 year after treatment (**Figure 4B**). Only 19 proteins showed significant changes in their abundance, representing only 1.01% of the total proteins reliably quantified. Notably, HSP90AB2P was significantly downregulated. Gene ontology enrichment analysis for biological process revealed that response to toxic substance and lipoprotein remodeling were increased (Supplementary Figures 3D-2F). Lastly, we compared the proteome of the patients ~1 year after treatment against controls. Highlighted by the largely unaffected granule content (**Figure 4A-C**), the patient platelet proteome remained stable longitudinally while sitosterol levels decreased and platelet counts increased, even to normal ranges in patients A-II-3 and B-II-1.

Prompted by the limited impact on the proteome, we explored if there were networks of co-varying protein trends in play. To this end, we used weighted gene co-expression network analysis (WGCNA) to define and analyze co-regulated and interconnected protein profiles[28]. WGCNA resulted in eight modules containing 61-614 proteins. We combined pairwise Pearson coefficients with module

allocations to visualize a correlation map of proteins in this study and annotated the median expression of each module per grouped condition (**Figure 4D**). Modules 1 and 2 were the largest ones, while module 8 was the smallest. Module 8 was most different between controls and patient treatment conditions. These proteins were annotated for their biological processes as proteins within the same modules likely share biological functions (**Figure 4E**). This revealed modules 1, 2 and 8 enriched for mitochondria, cytoskeleton and blood particles, respectively. Given that module 1 enriched for mitochondria-related terms and the fact that lipid catabolism happens in the mitochondria, we examined the intensity profiles of proteins involved in fatty acid beta-oxidation as annotated in the Reactome[29] database (**Figure 4F**). Several members of this pathway presented lower levels than controls. Some notable examples are medium-chain specific acyl-CoA dehydrogenase (ACADM), very long-chain specific acyl-CoA dehydrogenase (ACADV), Acyl-coenzyme A thioesterase 9 (ACOT9) and propionyl-CoA carboxylase beta chain (PCCB). These data suggest a potential impact of the high sterol levels on the mitochondrial level, particularly affecting fatty acid beta-oxidation.

Based on the observation that the median intensities of module 8 were higher both pre- and post-treatment in patients, than in healthy controls (Figure 4D), we inspected this module more closely. This module was largely composed of proteins enriching for acute inflammatory response, blood microparticles, extracellular matrix and other potential copurified proteins. Comparing the protein levels of platelets after treatment with controls showed that apolipoproteins APOA1, APOA2, APOA4 and APOE remained at an increased expression level, suggesting prevalent microparticles such as lipoproteins and chylomicrons (**Figure 4G**). Plasma proteins such as S100A9 and complement-related proteins C3, C4A:C4B, C4BPA , known to be involved in inflammation, were also in module 8 and showed higher levels in patients than controls irrespective of the treatment, which makes inflammation very unlikely as supported by low CRP levels (data not shown).

Individual proteomic trends

Principal component analysis showed patients did not group based on pedigree nor treatment, suggesting individual trends could provide further insight (Supplementary Figure 4A). To identify inter-individual differences on the platelet proteome as a response to Ezetimibe, the pre- and post-treatment proteomes were compared per individual (**Figures 5A-5D).** Curiously, index cases A-II-3 and B-II-1 who presented the best response to treatment and reached a normal platelet count, showed the least amount of proteins changing over time with only three to six proteins changing. This suggested that thrombocytopenia in our patients with sitosterolemia is not associated with proteomic aberrations. In total, 129 different proteins were significantly regulated with most proteins changing in index cases A-II-1 and A-II-2. Nonetheless, to gain insight into the biological processes that might be affected by the lipid content, we performed enrichment analysis on the proteins that characterized individual trends (**Figure 5E**), as exemplified by VDAC2 and VDAC3, in individuals A-II-1 and B-II-1. (**Figure 5A and 5D**). Interestingly, GO terms associated mostly with the mitochondria, as well as endoplasmic reticulum, transporter activity and inflammatory response. As expected, there was a noticeable overlap with the enrichment analysis of the WGCNA modules. Based on these observations, we plotted some of the mitochondrial proteins showing individual trends (**Figure 5F**). Several of these proteins showed distinct expression profiles in patients before and after treatment. Remarkably, this included Acyl-coenzyme A thiosterase 9 mitochondrial (ACOT9), which is involved in the catabolism of fatty acids, as well as proteins that are involved in mitochondrial respiration and ATP synthesis: ATP synthase subunit gamma (ATP5F1C) and cytochrome b-c1 complex subunits 1 and 2 (UQCRC1 , UQCRC2). Similar trends could be appreciated in these proteins, marked by an increase in A-II-2 and B-II-1 after treatment; a decrease in A-II-1, and no changes in A-II-3. Together these observations point at individual heterogeneity.

Discussion

Sitosterolemia is an autosomal disorder caused by mutations in *ABCG5* or *ABCG8* [14]. Dysfunction of either ABCG5 or ABCG8 results in intestinal hyperabsorption of all sterols, including cholesterol and plant sterols, and impaired ability to excrete sterols into bile[6]. Here, we characterized the platelet proteome of four individuals from two unrelated pedigrees, who presented the hematological presentation of sitosterolemia using label-free quantitative MS. The effect of treatment on platelets was assessed by comparing the platelets isolated from patients with sitosterolemia before and after treatment. Limited proteomic changes across patients were found, highlighting that thrombocytopenia is not associated with proteomic aberrations in the platelets. As reviewed previously[12,13,30,31], more than 100 sitosterolemia cases have been reported since the first description in 1974[8]. A handful of patients presenting with hematologic abnormalities only have been reported thus far, some of whom had been misdiagnosed as ITP or hemolytic anemia, for which splenectomy was performed[13,32,33]. To our knowledge, this is the first study to report the proteomic characterization of platelets in the context of sitosterolemia and treatment effects by diet restrictions combined with ezetimibe.

Here, patients were prescribed ezetimibe combined with a low sterol diet and followed for ~1 year. Sitosterol levels dropped and platelet numbers increased. Our findings in two unrelated pedigrees are in line with studies performed in experimental models where dietary and genetic components of macrothrombocytopenia have been assessed. Both Abcg5-/- and Abcg8-/- mice develop macrothrombocytopenia when fed a high sterol diet[34–36]. However, when fed a sterol-poor diet, these hematological parameters normalized comparable with those of wild-type mice[36]. Hematological parameters also normalize in Abcg5-/- mice when treated with the sterol absorption inhibitor ezetimibe[36–38]. Changes in MPV in our patients were not as clear upon treatment as in the mice model.

The platelet protein content was neither considerably different from the normal platelet proteome nor did we observe major proteomic alterations upon treatment, even though platelet count increased. These observations were supported by the fact that the patients presented no bleeding diathesis even when their platelet count was lower than normal levels. Moreover, no significant differences were observed when comparing the content of platelets alpha nor dense granules highlighting the lack of platelet dysfunction.

As expected from the documented tissue expression pattern, both ABCG5 and ABCG8 were not detected in platelets. Available tissue expression data provide evidence that ABCG5 and ABCG8 are exclusively expressed in the liver, as well as small and large intestine.[39] This corroborates their main function in the digestive tract and the liver, resulting in a change in the microenvironment in sitosterolemia[11]. Our data and several other studies point to the causative role of the microenvironmental abnormality instead of intrinsic platelet defects as the cause of the hematological presentation of sitosterolemia.

We observed interesting trends at the level of mitochondrial proteins. Firstly, mitochondrial proteins made up the largest module of covarying proteins which showed overall lower trends in our patients. It has been previously proposed that phytosterols can target the mitochondria[40]. Sitosterol in the mitochondria have been reported to alter cholesterol transport and metabolism, for example by competing with cholesterol and affecting the maintenance of mitochondrial membrane stability[41,42]. Secondly, we observed lower levels of proteins involved in fatty acid beta-oxidation such as ACADM, ACADVL and PCCB in our patients. In accordance, in vitro experiments performed on cardiomyocytes supplemented with phytosterol induced a reduction in metabolic activity and cell growth[43]. In addition, lipotoxicity has been previously suggested to promote mitochondrial dysfunction and lead to impaired TCA cycle related enzymatic processing[44]. Curiously, PCCB deficiency can give rise to propionic acidemia, in which patients may also develop thrombocytopenia, anemia and

neutropenia[45]. Lastly, we observed individual trends in proteins involved in mitochondrial metabolism and respiration such as acyl-CoA thioestherase ACOT2 , ATP synthase and cytochrome subunits (UQCRC1, UQCRC2). In some in vitro experiments, beta sitosterol has shown to induce mitochondrial modifications such as cytosolic release of cytochrome C and to associate with an apopototic mode of death[46]. Interestingly, differential expression of mitochondrial proteins such as thioesterase ACOT2 have been proposed to play compensatory roles contributing to heterogeneity in clinical severity in subjects with metabolic diseases such as ACADM[47]. Together, these data could indicate potential lipotoxicity at play.

Whether hematological effects are caused from sitosterol accumulation (the most abundant accumulating xenosterol) or more bioactive, less abundant xenosterols, is as yet unknown. Xenosterol accumulation has been reported to affect the plasma membrane[13,35,36]. We do not have data on the lipid content on the platelets . Future studies could make use of lipidomic strategies to evaluate lipid content in blood cells. The mechanism of how xenosterolemia could affect platelet metabolism, particularly in the mitochondria as well as platelet clearance also remains to be investigated. Taken together, with the normal megakaryocyte number and morphology, as well as the normal TPO levels, we propose that the platelet development itself is not abnormal. Instead proplatelet release, platelet clearance or lipotoxicity are likely to be the driving factor of thrombocytopenia in sitosterolemia.

Acknowledgments:

The authors thank Masja de Haas and Leendert Porcelijn for the Flow Cytometry analysis. The authors are grateful to the patients and their family members for participation in this study. J.D.C.A is supported by the SYMPHONY consortium, which received funding from the Netherlands Organization for Scientific Research (NWO) in the framework of the NWA-ORC Call grant agreement NWA.1160.18.038. funded.

Authorship:

Contribution: J.D.C.A., A.J.H. and T.W.K. wrote the manuscript; A.T.J.T. and M.B. performed cell isolation and clinical laboratory analysis; K.v.L. performed DNA analysis J.D.C.A. and F.P.J.v.A. performed mass spectrometry analysis; J.D.C.A, A.J.H., A.B.M. and T.W.K. analyzed the data; T.W.K., M.H.S, and M.M.B. diagnosed and treated the patients, and analyzed clinical data; all authors read and approved the manuscript.

Conflicts-of-interest disclosure:

The authors have no conflicts of interest to declare.

ORCID profiles:

J.D.C.A., 0000-0002-2305-0743. T.W.K., 0000-0002-7421-3370.

A.J.H. 0000-0002-3082-2289

References

1. de Vos TW, Winkelhorst D, de Haas M, Lopriore E, Oepkes D. Epidemiology and management of fetal and neonatal alloimmune thrombocytopenia. *Transfus Apher Sci*. 2020;59(1).

doi:10.1016/J.TRANSCI.2019.102704

2. Rocca B, Ranelletti FO, Maggiano N, Ciabattoni G, De Cristofaro R, Landolfi R. Inherited macrothrombocytopenia with distinctive platelet ultrastructural and functional features. *Thromb Haemost*. 2000;83(1):35-41. doi:10.1055/S-0037-1613753/ID/JR1613753-11

3. Nurden P, Stritt S, Favier R, Nurden AT. Inherited platelet diseases with normal platelet count: phenotypes, genotypes and diagnostic strategy. *Haematologica*. 2021;106(2). doi:10.3324/HAEMATOL.2020.248153

4. Bastida JM, Benito R, Janusz K, et al. Two novel variants of the ABCG5 gene cause xanthelasmas and macrothrombocytopenia: a brief review of hematologic abnormalities of sitosterolemia. *J Thromb Haemost*. 2017;15(9):1859-1866. doi:10.1111/JTH.13777

5. Huang CC, Niu DM, Charng MJ. Genetic Analysis in a Taiwanese Cohort of 750 Index Patients with Clinically Diagnosed Familial Hypercholesterolemia. *J Atheroscler Thromb*. 2022;29(5):639. doi:10.5551/JAT.62773

6. Rees DC, Iolascon A, Carella M, et al. Stomatocytic haemolysis and macrothrombocytopenia (Mediterranean stomatocytosis/macrothrombocytopenia) is the haematological presentation of phytosterolaemia. *Br J Haematol*. 2005;130(2):297-309. doi:10.1111/J.1365-2141.2005.05599.X

7. von Behrens WE. Splenomegaly, macrothombocytopenia and stomatocytosis in healthy Mediterranean subjects (splenomegaly in Mediterranean macrothrombocytopenia). *Scand J Haematol*. 1975;14(4):258-267. doi:10.1111/J.1600-0609.1975.TB02425.X

8. Bhattacharyya AK, Connor WE. Beta-sitosterolemia and xanthomatosis. A newly described lipid storage disease in two sisters. *J Clin Invest*. 1974;53(4):1033-1043. doi:10.1172/JCI107640

9. Wang Z, Cao L, Su Y, et al. Specific macrothrombocytopenia/hemolytic anemia associated with sitosterolemia. *Am J Hematol*. 2014;89(3):320-324. doi:10.1002/AJH.23619

10. Sun Y, Wang J, Long T, et al. Molecular basis of cholesterol efflux via ABCG subfamily

transporters. *Proc Natl Acad Sci U S A*. 2021;118(34). doi:10.1073/PNAS.2110483118/VIDEO-1

11. Klett EL, Lee MH, Adams DB, Chavin KD, Patel SB. Localization of ABCG5 and ABCG8 proteins in human liver, gall bladder and intestine. *BMC Gastroenterol*. 2004;4. doi:10.1186/1471-230X-4-21

12. Yoo E-G. Sitosterolemia: a review and update of pathophysiology, clinical spectrum, diagnosis, and management. *Ann Pediatr Endocrinol Metab*. 2016;21(1):7. doi:10.6065/APEM.2016.21.1.7

13. Bastida JM, Girós ML, Benito R, Janusz K, Hernández-Rivas JM, González-Porras JR. Sitosterolemia: Diagnosis, Metabolic and Hematological Abnormalities, Cardiovascular Disease and Management. *Curr Med Chem*. 2019;26(37):6766-6775. doi:10.2174/0929867325666180705145900

14. Berge KE, Tian H, Graf GA, et al. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science*. 2000;290(5497):1771-1775. doi:10.1126/SCIENCE.290.5497.1771

15. Bazerbachi F, Conboy EE, Mounajjed T, et al. Cryptogenic Cirrhosis and Sitosterolemia: A Treatable Disease If Identified but Fatal If Missed. *Ann Hepatol*. 2017;16(6):970-978. doi:10.5604/01.3001.0010.5290

16. Niu DM, Chong KW, Hsu JH, et al. Clinical observations, molecular genetic analysis, and treatment of sitosterolemia in infants and children. *J Inherit Metab Dis*. 2010;33(4):437-443. doi:10.1007/S10545-010-9126-2

17. Ajagbe BO, Othman RA, Myrie SB. Plant Sterols, Stanols, and Sitosterolemia. *J AOAC Int*. 2015;98(3):716. doi:10.5740/JAOACINT.SGEAJAGBE

18. Buonuomo PS, Iughetti L, Pisciotta L, et al. Timely diagnosis of sitosterolemia by next generation sequencing in two children with severe hypercholesterolemia. *Atherosclerosis*. 2017;262:71-77. doi:10.1016/J.ATHEROSCLEROSIS.2017.05.002

19. Deng L, Xu J, Chen W, et al. Remediation of ABCG5-Linked Macrothrombocytopenia With

436 Ezetimibe Therapy. *Front Genet*. 2021;12. doi:10.3389/FGENE.2021.769699/FULL
437 20. Zhou Z, Su X, Cai Y, et al. Features of chinese patients with sitosterolemia. *Lipids Health Dis*.
438 2022;21(1):1-10. doi:10.1186/S12944-021-01619-1/FIGURES/2
439 21. Kanaji T, Kanaji S, Montgomery RR, Patel SB, Newman PJ. Platelet hyperreactivity explains the
440 bleeding abnormality and macrothrombocytopenia in a murine model of sitosterolemia.
441 *Blood*. 2013;122(15):2732-2742. doi:10.1182/BLOOD-2013-06-510461
442 22. Bao L, Li Y, Deng SX, Landry D, Tabas I. Sitosterol-containing Lipoproteins Trigger Free Sterol-
443 induced Caspase-independent Death in ACAT-competent Macrophages *. *J Biol Chem*.
444 2006;281(44):33635-33649. doi:10.1074/JBC.M606339200
445 23. Slatter DA, Aldrovandi M, O'Connor A, et al. Mapping the Human Platelet Lipidome Reveals
446 Cytosolic Phospholipase A 2 as a Regulator of Mitochondrial Bioenergetics during Activation.
447 *Cell Metab*. 2016;23(5):930-944. doi:10.1016/j.cmet.2016.04.001
448 24. Burkhart JM, Gambaryan S, Watson SP, et al. What can proteomics tell us about platelets? *Circ*
449 *Res*. 2014;114(7):1204-1219. doi:10.1161/CIRCRESAHA.114.301598
450 25. Kuijpers TW, Tool ATJ, van der Bijl I, et al. Combined immunodeficiency with severe
451 inflammation and allergy caused by ARPC1B deficiency. *J Allergy Clin Immunol*.
452 2017;140(1):273-277.e10. doi:10.1016/j.jaci.2016.09.061
453 26. Sprenkeler EGG, Henriet SSV, Tool ATJ, et al. MKL1 deficiency results in a severe neutrophil
454 motility defect due to impaired actin polymerization. *Blood*. 2020;135(24):2171-2181.
455 doi:10.1182/blood.2019002633
456 27. Lau LM, Wee JL, Wright MD, et al. The tetraspanin superfamily member CD151 regulates
457 outside-in integrin αIIbβ3 signaling and platelet function. *Blood*. 2004;104(8):2368-2375.
458 doi:10.1182/BLOOD-2003-12-4430
459 28. Langfelder P, Horvath S. WGCNA: An R package for weighted correlation network analysis.
460 *BMC Bioinformatics*. 2008;9(1):1-13. doi:10.1186/1471-2105-9-559/FIGURES/4
461 29. Fabregat A, Jupe S, Matthews L, et al. The Reactome Pathway Knowledgebase. *Nucleic Acids*

Res. 2017;46:649-655. doi:10.1093/nar/gkx1132

30. Pek SLT, Dissanayake S, Fong JCW, et al. Spectrum of mutations in index patients with familial hypercholesterolemia in Singapore: Single center study. *Atherosclerosis*. 2018;269:106-116. doi:10.1016/J.ATHEROSCLEROSIS.2017.12.028

31. Xia Y, Duan Y, Zheng W, et al. Clinical, genetic profile and therapy evaluation of 55 children and 5 adults with sitosterolemia. *J Clin Lipidol*. 2022;16(1):40-51. doi:10.1016/j.jacl.2021.11.015

32. Qin M, Luo P, Wen X, Li J. Misdiagnosis of sitosterolemia in a patient as Evans syndrome and familial hypercholesterolemia. *J Clin Lipidol*. 2022;16(1):33-39. doi:10.1016/J.JACL.2021.11.004

33. Frederiksen TC, Mortensen MB, Kanstrup HL. Seventeen years of misdiagnosis in rare dyslipidaemia: a case report ofsitosterolaemia in a young female. *Eur Hear J Case Reports*. 2021;5(5). doi:10.1093/EHJCR/YTAB188

34. Wilson DW, Oslund KL, Lyons B, et al. Inflammatory dilated cardiomyopathy in Abcg5-deficient mice. *Toxicol Pathol*. 2013;41(6):880-892. doi:10.1177/0192623312466191

35. Chase TH, Lyons BL, Bronson RT, et al. The mouse mutation “thrombocytopenia and cardiomyopathy” (trac) disrupts Abcg5: a spontaneous single gene model for human hereditary phytosterolemia/sitosterolemia. *Blood*. 2010;115(6):1267-1276. doi:10.1182/BLOOD-2009-05-219808

36. Kruit JK, Drayer AL, Bloks VW, et al. Plant sterols cause macrothrombocytopenia in a mouse model of sitosterolemia. *J Biol Chem*. 2008;283(10):6281-6287. doi:10.1074/JBC.M706689200

37. Cornier M-A, Dabelea D, Hernandez TL, et al. The Metabolic Syndrome. *Endocr Rev*. 2008;29(7):777-822. doi:10.1210/er.2008-0024

38. Rees DC, Iolascon A, Carella M, et al. Stomatocytic haemolysis and macrothrombocytopenia (Mediterranean stomatocytosis/macrothrombocytopenia) is the haematological presentation of phytosterolaemia. *Br J Haematol*. 2005;130(2):297-309. doi:10.1111/J.1365-

2141.2005.05599.X

39. Uhlen M, Zhang C, Lee S, et al. A pathology atlas of the human cancer transcriptome. *Science (80-)*. 2017;357(6352):eaan2507. doi:10.1126/SCIENCE.AAN2507

40. Panov A, Kubalik N, Brooks BR, Shaw CA. In vitro effects of cholesterol β-D-glucoside, cholesterol and cycad phytosterol glucosides on respiration and reactive oxygen species generation in brain mitochondria. *J Membr Biol*. 2010;237(2-3):71-77. doi:10.1007/S00232-010-9307-9

41. Mlayeh L, Chatkaew S, Léonetti M, Homblé F. Modulation of Plant Mitochondrial VDAC by Phytosterols. *Biophys J*. 2010;99(7):2097. doi:10.1016/J.BPJ.2010.07.067

42. Chun RL, Zhou Z, Ru XL, et al. beta-sitosterol decreases irradiation-induced thymocyte early damage by regulation of the intracellular redox balance and maintenance of mitochondrial membrane stability. *J Cell Biochem*. 2007;102(3):748-758. doi:10.1002/JCB.21326

43. Danesi F, Ferioli F, Fiorenza Caboni M, et al. Phytosterol supplementation reduces metabolic activity and slows cell growth in cultured rat cardiomyocytes. *Br J Nutr*. 2011;106(4):540-548. doi:10.1017/S0007114511000626

44. Piccolis M, Bond LM, Kampmann M, et al. Probing the Global Cellular Responses to Lipotoxicity Caused by Saturated Fatty Acids. *Mol Cell*. 2019;74(1):32-44.e8. doi:10.1016/j.molcel.2019.01.036

45. Pena L, Franks J, Chapman KA, et al. Natural history of propionic acidemia. *Mol Genet Metab*. 2012;105(1):5-9. doi:10.1016/J.YMGME.2011.09.022

46. Choi YH, Kong KR, Kim YA, et al. Induction of Bax and activation of caspases during beta-sitosterol-mediated apoptosis in human colon cancer cells. *Int J Oncol*. 2003;23(6):1657-1662. doi:10.3892/IJO.23.6.1657/HTML

47. Martines ACMF, Gerding A, Stolle S, et al. Transcriptome analysis suggests a compensatory role of the cofactors coenzyme A and NAD+ in medium-chain acyl-CoA dehydrogenase knockout mice. 2019;9(1):14539. doi:10.1038/s41598-019-50758-0

514

515

516

Figure Legends

Figure 1. Patients' genetics and hematological characteristics before treatment. A-B) Family Pedigrees: double line shows a consanguineous union; filled symbol, affected individuals; half-filled symbols, heterozygous unaffected carrier; open symbols, not affected. A) Pedigree A with propositus A-II-1,A-II-2,A-II-3. B) Pedigree B with propositus B-II-1 C) Molecular annotation of *ABCG5* variants reported here. D-F)Sterol levels in plasma. D) Sitosterol. E) Campesterol. F) Cholestanol. G) Platelet count. H) Mean platelet volume. I) Thrombopoietin (TPO). Dotted lines show the normal plasma parameters.

Figure 2 Longitudinal Hematological parameters before and after treatment. Patients were followed for ~1 year with samples collected throughout this time. A) Sitosterol plasma levels. B) Platelet count C) Mean platelet volume

Figure 3 Proteomic depth and stability . A) Dynamic range of quantified proteins (LFQ intensities are shown). Gene ontology term enrichment network of all quantified proteins. Node size is proportional to the number of proteins associated with the GO annotation. Node color intensity denotes p-values while edge width denotes term similarities based on Jaccard's similarity. C) Scatterplot of standard deviation and mean proteins abundances. D) LFQ intensities of proteins covering the range of measurement. Lines denote mean intensities

Figure 4. Proteomic landscape of platelets from controls, patients before and after treatment. A) Volcano plot comparing platelet proteomes of patients with sitosterolemia before treatment and healthy controls. Upregulated proteins in patients are shown in orange, while downregulated proteins are shown in blue. B) Volcano plot comparing proteomic profiles of patients before and after treatment. Upregulated proteins after treatment are shown in red. C) Volcano plot comparing

platelet proteomes of patients with sitosterolemia after treatment and healthy controls. Upregulated proteins in patients are shown in red, while downregulated proteins are shown in dark blue A-C) Circles are drawn around granule derived proteins: Purple= Alpha granules; Green= Dense granules. Difference in expression is shown on the x-axis and the logarithmic p-value (-log(p-value)) is shown on the y-axis. Dotted lines indicate the threshold of significance with the horizontal line marking a -log10 (adj.p-value) of 1, and the vertical dotted lines marking a log2(fold change) of 1. For complete list of significantly regulated proteins, please refer to the Supplementary Tables. D). Heatmap of Pearson correlation coefficient of the pairwise comparison of all quantified proteins in this study. Row and column splits are based on WGCNA defined functional modules, which are numbered. Color gradients denote coefficients (Purple: -1, White: 0, Orange:1). Heatmap of median module intensity per condition is annotated below. Colors reflect LFQ intensity. E) Gene ontology (GO) term enrichment on functional modules. Color indicates module, while node size indicates amount of proteins with a GO term annotation and edge width denote GO term similarities based on Jaccard's similarity. F-G) Box plots showing protein intensities (LFQ) of a selection of proteins in module 8, associated with an inflammatory response (F) or blood microparticles (G). Black dots represent individual measurements

Figure 5. Proteomic landscape of platelets from controls, patients before and after treatment

Volcano plot comparing proteomic profiles before and after treatment of individual patients A)Index case A-II-1. B) Index case A-II-2. C) Index case A-II-3. D) Index case B-II-1. Upregulated proteins in patients are shown in red, while downregulated proteins are shown in dark blue. Difference in expression is shown on the x-axis and the logarithmic p-value (-log(p-value)) is shown on the y-axis. Dotted lines indicate the threshold of significance with the horizontal line marking a -log10 (adj.p-value) of 1, and the vertical dotted lines marking a log2(fold change) of 1. E)Gene ontology (GO) term enrichment on functional modules. Color indicates module, while node size indicates amount of proteins with a GO term annotation and edge width denote GO term similarities based on Jaccard's

similarity. F) Box plots showing protein intensities (LFQ) of a selection of mitochondrial proteins in Black dots represent individual measurements

Figure 1

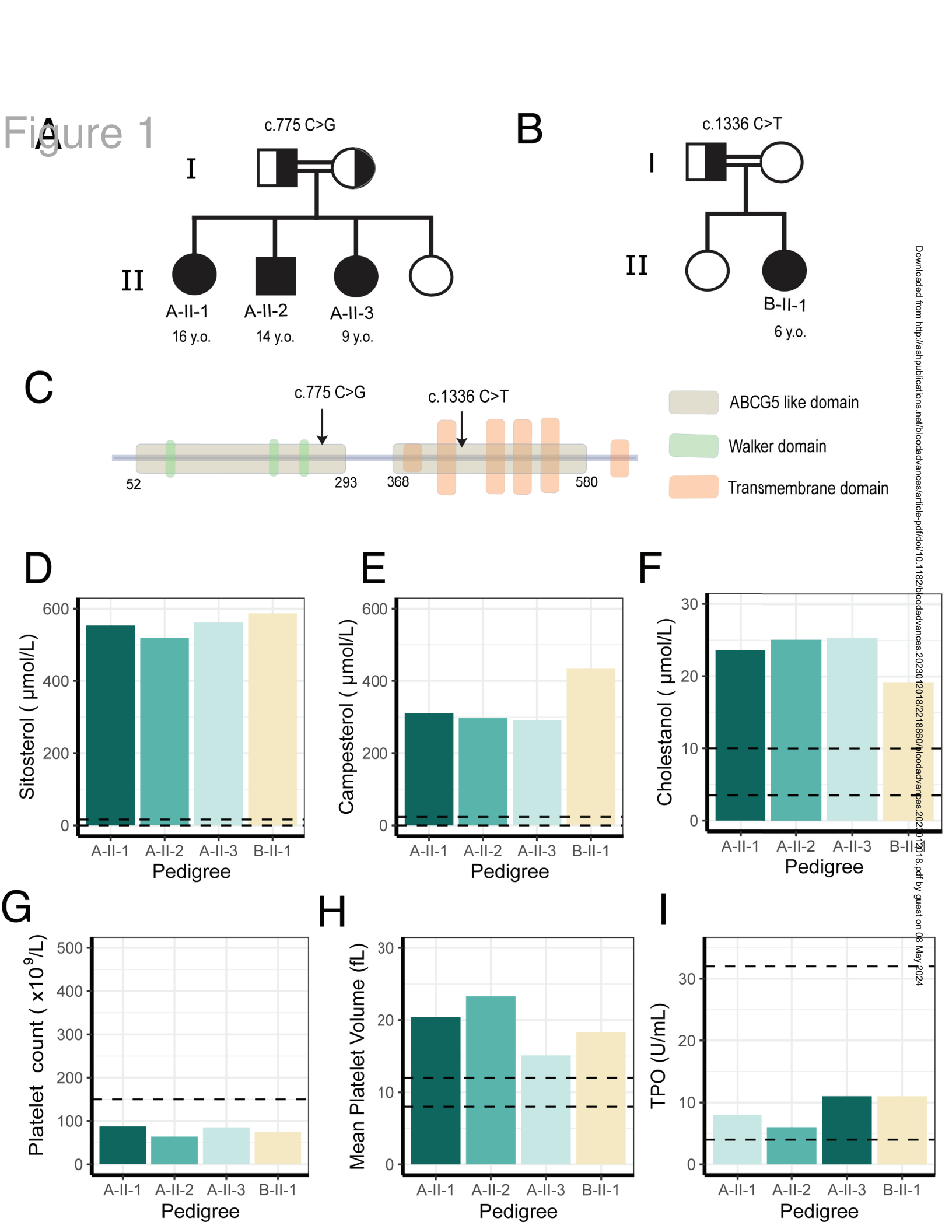

Figure 2

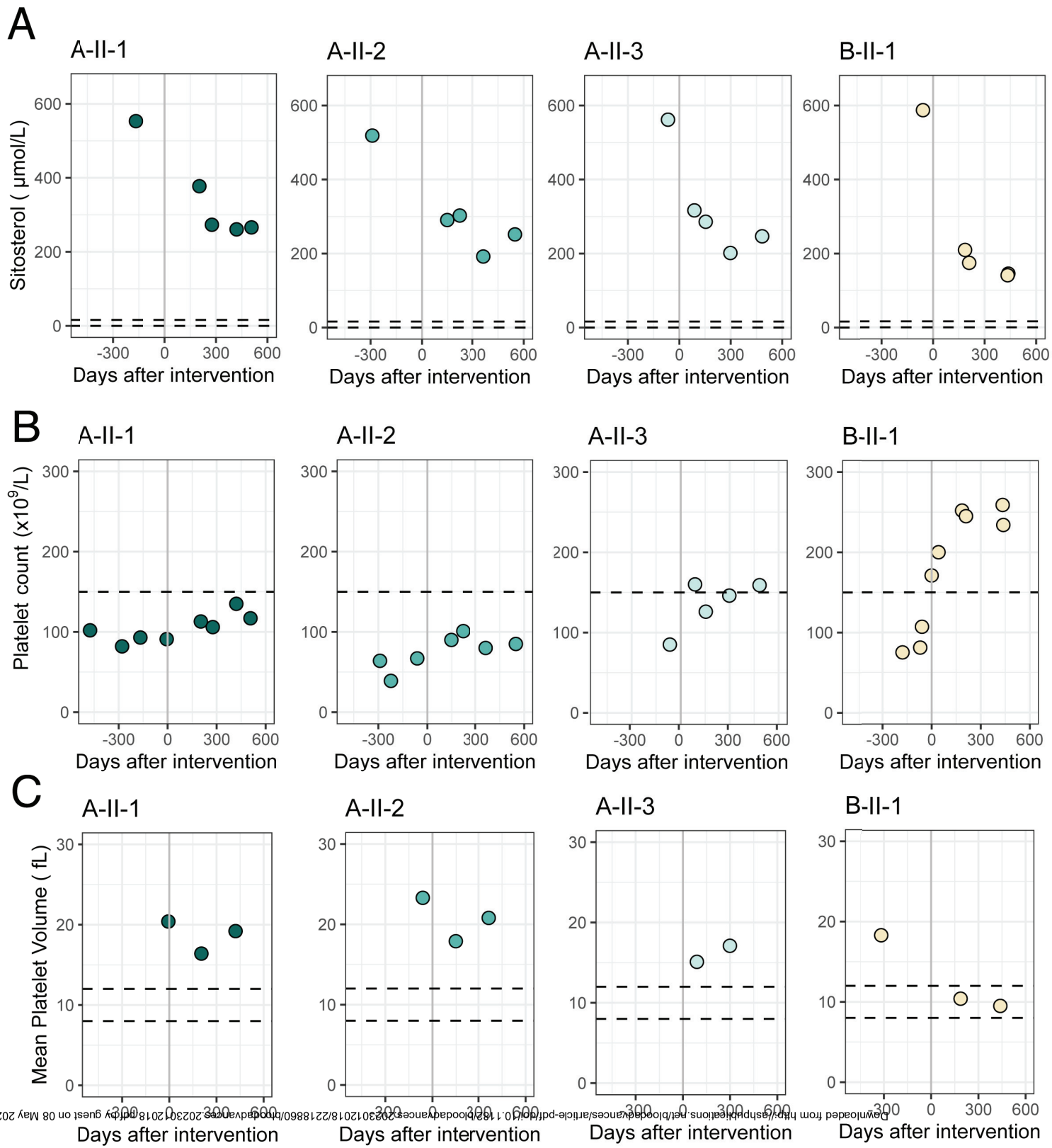

Figure 3

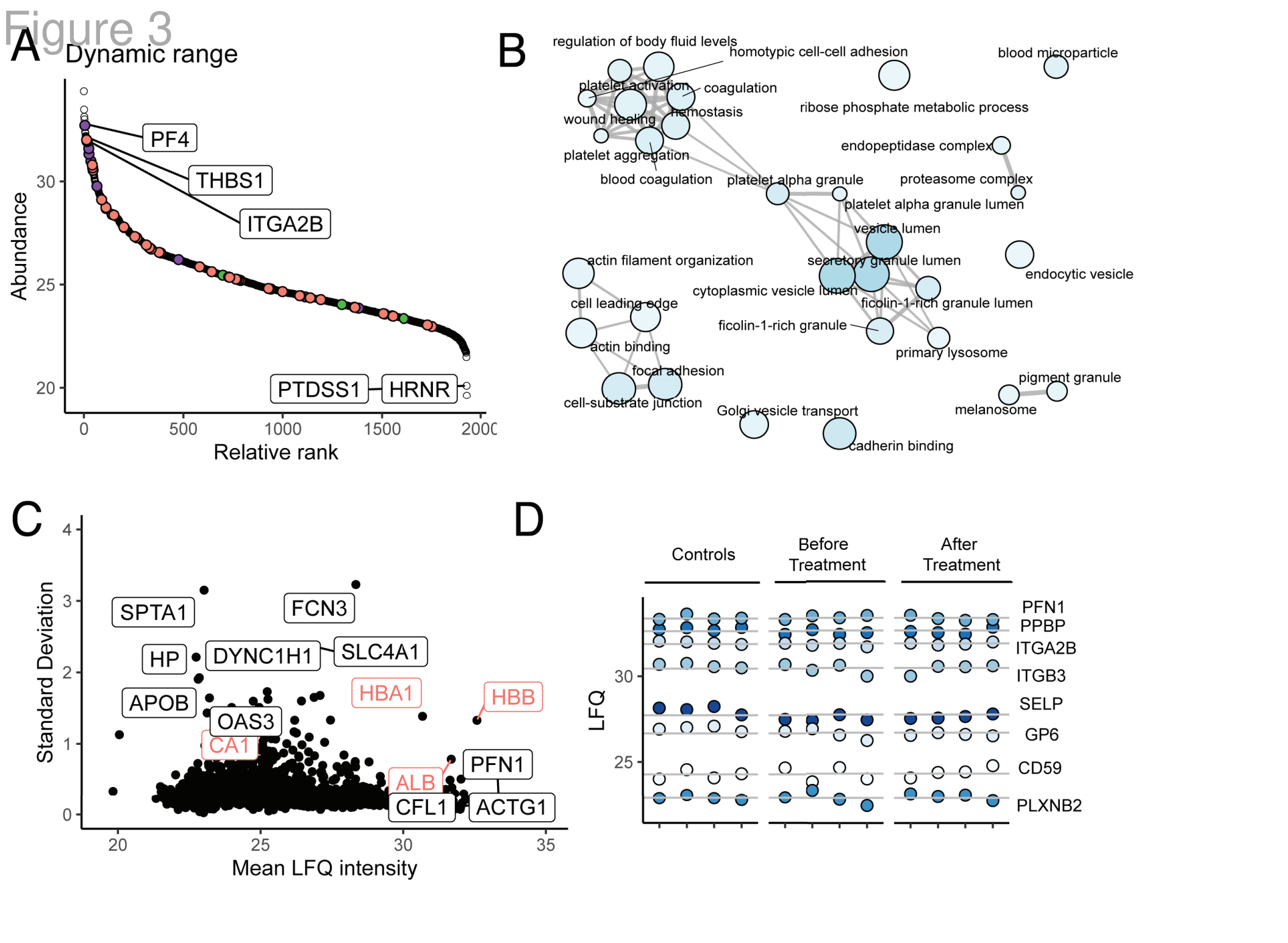

Figure 4

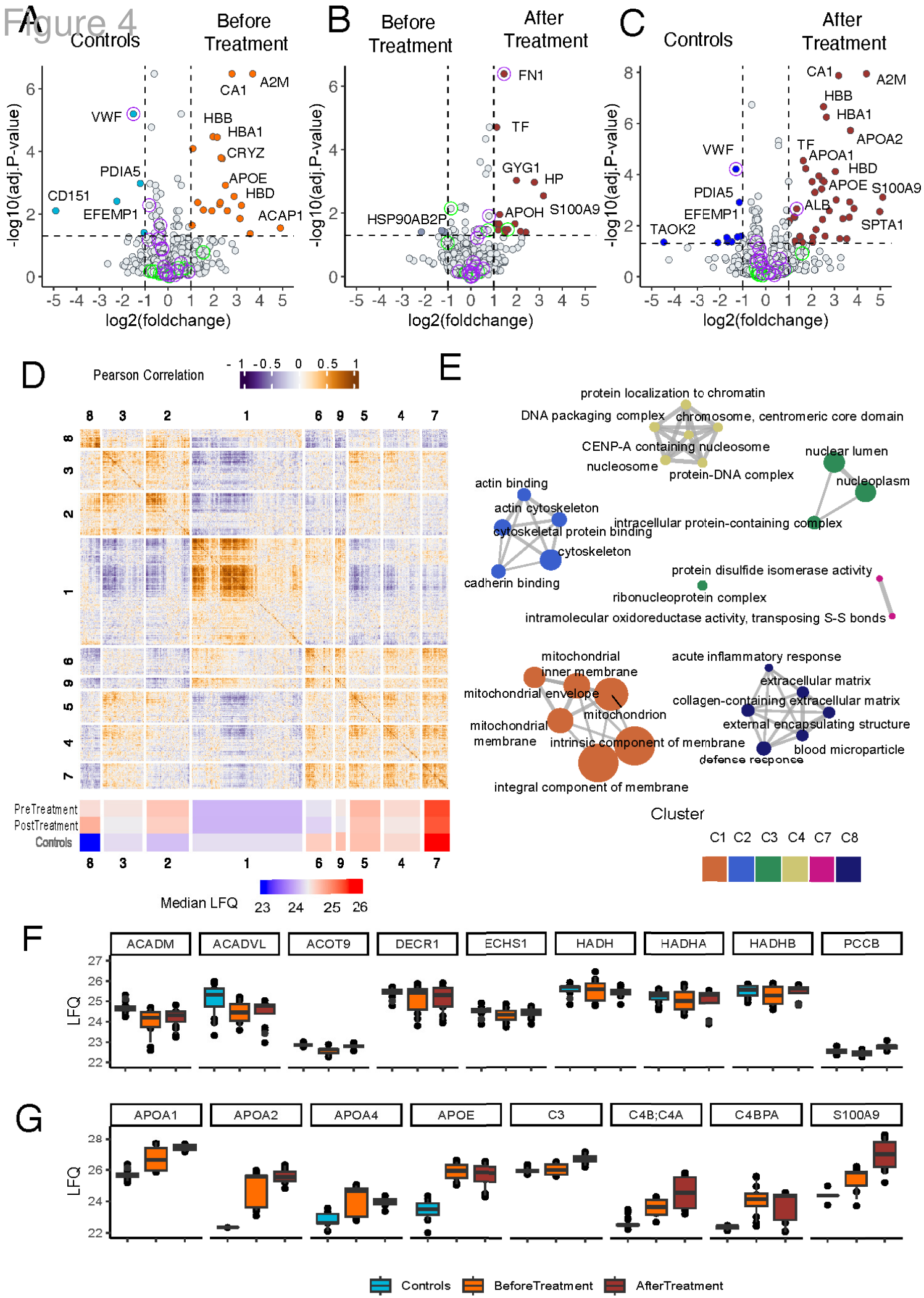

Figure 5