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Proteomic profiling for biomarker discovery in heparin-induced thrombocytopenia

Tracking no: ADV-2024-012782R1

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Abstract:
New analytical techniques can assess hundreds of proteins simultaneously with high sensitivity, facilitating the observation of their complex interplay and role in disease mechanisms. We hypothesized that proteomic profiling targeting proteins involved in thrombus formation, inflammation, and the immune response would identify potentially new biomarkers for heparin-induced thrombocytopenia (HIT). Four existing panels of the Olink proximity extension assay covering 356 proteins involved in thrombus formation, inflammation, and immune response were applied to randomly selected patients with suspected HIT (confirmed HIT, n=32; HIT ruled-out, n=38; positive heparin/PF4 [H/PF4] antibodies, n=28). The relative difference in protein concentration was analyzed using a linear regression model adjusted for sex and age. To confirm the test results, soluble P-selectin was determined using ELISA in above mentioned patients and an additional second dataset (n=49). HIT was defined as a positive heparin-induced platelet aggregation test (HIPA; washed platelet assay). Among 98 patients of the primary dataset, the median 4Ts score was 5 in patients with HIT, 4 in patients with positive heparin/PF4 antibodies, and 3 in patients without HIT. The median OD of a polyspecific heparin/PF4 ELISA was 3.0, 0.9, and 0.3, respectively. Soluble P-selectin remained statistically significant after multiple test adjustments. The area under the receiver-operating-characteristics-curve was 0.81 for Olink and 0.8 for ELISA. Future studies shall assess the diagnostic and prognostic value of soluble P-selectin in the management of HIT.

Conflict of interest: COI declared - see note

COI notes: The institution of JKH received grant support, consultancy fees, or honoraria from SNSF, Baxter/Takeda, Bayer, CSL-Behring, NovoNordisk, Octapharma, Roche, SOBI, Roche, Sanofi, FOPH, and Swiss Hemophilia Society, outside of the current work. MN received research grants from Bayer Healthcare, Roche diagnostics, Siemens healthineers, Pentapharm, and Bühlmann laboratories, as well as lecture fees from Sysmex, Siemens healthineers, and Euroimmun, outside of the current work. AG reports personal fees from Aspen, grants from Ergomed, grants from Boehringer Ingelheim, personal fees from Bayer Vital, grants from Rovi, grants from Sagent, personal fees from Chromatec, personal fees from Instrumentation Laboratory, grants and personal fees from Macopharma, grants from Portola, grants from Biokit, personal fees from Sanofi-Aventis, grants from Blau Farmaceutics, grants from Prosensa/Biomarin, grants and other from DRK-BSD NSTOB, grants from DRK-BSD Baden-Würtemberg/Hessen, personal fees from Roche, personal fees from GTH e.V., grants from Deutsche Forschungsgemeinschaft, grants from Robert-Koch-Institut, non-financial support from Veralox, personal fees from Dilaflor, non-financial support from Vakzine Projekt Management GmbH, grants from GIZ Else-Körner-Stiftung, non-financial support from AstraZeneca, non-financial support from Janssen Vaccines & Prevention B.V., personal fees from Takeda Pharma, personal fees from Falk Foundation e.V., grants from European Medicines Agency , personal fees from Mylan Germany, outside the submitted work; In addition, Dr. Greinacher has a patent Screening Methods for transfusion related acute lung injury (TRALI) with royalties paid to EP2321644, 18.05.2011 , and a patent Verfahren und Vorrichtung zur Herstellung von Universalplasma. licensed to DE 10 2020 212 609 B3 2022.04.07. TB reports grant support, consultancy fees, honoraria, or support for attending meetings from DFG, Stiftung Transfusionsmedizin und Immunhämatologie e.V, DRK Blutspendedienst, Deutsche Herzstiftung, Ministerium für Wissenschaft, Forschung und Kunst Baden Würtemberg, Gesellschaft für Thrombose- und Hämostaseforschung, Berufsverband Deutscher Internisten, CoaChrom Diagnostica GmbH, Robert Bosch GmbH, Ergomed, Bayer, Bristol-Myers Squibb, Doctrina Med AG, Leo Pharma GmbH, Schöchl medical education GmbH, Mitsubishi Tanabe GmbH, Novo Nordisk GmbH, Swedish Orphan Biovitrium GmbH. All other authors declare that no conflict of interest exists.

Preprint server: No;

Author contributions and disclosures: HN wrote the analysis plan, analyzed, and interpreted the data, and wrote the first manuscript draft. HHC and FC contributed to the design of the study, analyzed and interpreted data, provided infrastructure and reagents, and contributed to the first draft of the manuscript. JH contributed to the analysis plan and interpretation of data. JDS, AG, DAT, AM, WAW, AS, JAKH, BG, PV, TB, and LG collected data. MN designed and implemented the study, collected data, contributed to analysis plan and interpretation of data, and wrote the manuscript. All authors contributed to the interpretation of data, reviewed the manuscript critically, and approved the final version of the manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Detailed data can be obtained upon reasonable request from the corresponding author.

Clinical trial registration information (if any):

Proteomic profiling for biomarker discovery in heparin-induced thrombocytopenia

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Keywords
heparin-induced thrombocytopenia; proteomics; biomarker; SELP protein; P-selectin; inflammation

Data sharing statement
All data is available from the corresponding author upon reasonable request.

Key points

1. This is the first study to apply proteomic profiling to patients with suspected HIT, thus analyzing a large number of potential proteins.
2. Our analysis provided evidence supporting the potential of soluble P-selectin as a promising new biomarker in HIT.

Abstract

New analytical techniques can assess hundreds of proteins simultaneously with high sensitivity, facilitating the observation of their complex interplay and role in disease mechanisms. We hypothesized that proteomic profiling targeting proteins involved in thrombus formation, inflammation, and the immune response would identify potentially new biomarkers for heparin-induced thrombocytopenia (HIT). Four existing panels of the Olink proximity extension assay covering 356 proteins involved in thrombus formation, inflammation, and immune response were applied to randomly selected patients *with suspected HIT* (confirmed HIT, n=32; HIT ruled-out, n=38; positive heparin/PF4 [H/PF4] antibodies, n=28). The relative difference in protein concentration was analyzed using a linear regression model adjusted for sex and age. To confirm the test results, soluble P-selectin was determined using ELISA in above mentioned patients and an additional second dataset (n=49). HIT was defined as a positive heparin-induced platelet aggregation test (HIPA; washed platelet assay). Among 98 patients of the primary dataset, the median 4Ts score was 5 in patients with HIT, 4 in patients with positive heparin/PF4 antibodies, and 3 in patients without HIT. The median OD of a polyspecific heparin/PF4 ELISA was 3.0, 0.9, and 0.3, respectively. Soluble P-selectin remained statistically significant after multiple test adjustments. The area under the receiver-operating-characteristics-curve was 0.81 for Olink and 0.8 for ELISA. Future studies shall assess the diagnostic and prognostic value of soluble P-selectin in the management of HIT.

1 Introduction

Diagnostic workup, assessment of prognosis, and treatment monitoring of heparin-induced thrombocytopenia (HIT) are hampered by a lack of reliable and specific biomarkers. HIT is a severe adverse reaction to heparin, one of the most commonly used anticoagulants [1]. Exposure to heparin can trigger the formation of platelet-activating antibodies against a heparin-platelet factor 4 complex [2–5]. Paradoxically, these antibodies can induce a prothrombotic state, leading to severe thromboembolism, limb loss, and even death [6]. In contrast, patients suspected of having HIT are often treated with dangerous anticoagulants with a high bleeding risk, such as argatroban [7–9]. Thus, misdiagnosis of HIT has severe consequences, including increased morbidity and mortality due to over- or undertreatment [10]. Due to their limited availability and prolonged turnaround times, washed platelet activation assays,

which are regarded as the reference standard, are not suitable for use in the acute phase of HIT [11,12]. The commonly used heparin/PF4 (H/PF4) antibody assays, however, have limited specificity and, therefore, put the patient at risk of overtreatment [13]. Despite recent advancements, including automated H/PF4 antibody assays, prediction models, and machine-learning applications, there is still a diagnostic gap that needs to be addressed [14–17]. Therefore, new biomarkers are a promising tool to develop enhanced diagnostic tests for the diagnosis, prognosis or monitoring of HIT [18].

New analytical techniques enable the simultaneous determination of hundreds of biomarkers with extremely high sensitivity [19]. Proteins are critical mediators in hemostasis mechanisms, contributing to immunological response and inflammation, and venous and arterial thromboembolism [20]. These techniques can help observing the interplay of protein-biomarkers and their role in the mechanism of HIT. Among these techniques, Olink's proximity extension assay (PEA; Uppsala, Sweden) for proteomic profiling stands out for its high sensitivity, low risk of interferences, low specimen volume, and the large number of biomarkers that can be determined simultaneously [21]. This powerful platform has already been used successfully to identify potential biomarkers for a range of diseases, including cardiovascular disease, inflammatory diseases, cancer, and infectious diseases [22–25].

We hypothesize that the application of proteomic profiling using the Olink platform can identify novel biomarkers for the management of HIT, potentially enabling a more accurate diagnosis.

Methods

Study design, setting, and population

The present analysis was conducted in-line with a large prospective cross-sectional study. Three groups of patients were selected out of 120 patients recruited in line with the TORADI-HIT dataset [16,26], or a preceding pilot study [11,27]: (a) confirmed HIT, (b) H/PF4 antibodies present but HIT ruled out, and (c) HIT ruled out, H/PF4 antibodies not present (Figure 1; primary dataset). Patients in each group were randomly selected. An additional, random sample of 50 patients was selected to confirm the findings in a second dataset. Overall inclusion criteria were: (1) suspected HIT: anti-heparin-pf4 (H/PF4) antibody assay ordered OR 4Ts score rated OR hematology consultancy service requested, (2) age ≥ 18 years, and (3) general informed consent. Exclusion criteria were (1) insufficient serum specimen, (2) insufficient clinical data,

113 and (3) did not pass Olink quality control. The TORADI-HIT study recruited patients
114 from 11 study centers in Switzerland, Germany, and the USA [16]. Most patients were
115 included in Inselspital, University Hospital of Bern, Switzerland. Biomarker discovery
116 was done using Olink's proximity extension assay (356 different proteins). The results
117 were verified using ELISA determinations of the proteins (in French Blood
118 Establishment (EFS) Auvergne-Rhone-Alpes, and University Jean Monnet, Mines Saint-
119 Etienne, INSERM, U 1059 SAINBIOSE laboratory). The appropriate ethical committee
120 approved the final protocol. The study was conducted in accordance with the
121 declaration of Helsinki.

122 Definition of patient groups

123 HIT was defined by a positive washed-platelet functional assay, specifically the
124 heparin-induced platelet activation assay (HIPA) [11,16,27]. Multiple studies have
125 demonstrated that washed platelet assays, such as the serotonin release assay and
126 HIPA, exhibit high sensitivity and specificity and strong concordance with clinical HIT.
127 Therefore, the American Society of Hematology (ASH) and the British Committee for
128 Standards in Hematology recommend these assays as reference standards [28,29].
129 Patients with positive heparin/PF4 antibodies were defined by a positive immunoassay
130 (ELISA) but a negative HIPA. HIT-negative patients were defined by a negative ELISA
131 and a negative HIPA.

132 Work-up and laboratory tests

133 Detailed clinical and laboratory data including residual serum samples were collected
134 at diagnosis following a pre-specified protocol. Serum samples were frozen at -80°C.
135 HIPA and H/PF4 immunoassay was conducted within one week after arrival. The
136 laboratory technicians were blinded to the results of the other test and to the clinical
137 information.

138 For the HIPA, serum samples were incubated with 4 different washed platelet
139 donations in the presence of (a) only buffer, (b) 0.2 IU/ml low molecular weight
140 heparin, and (c) 100 IU/ml heparin. All details were published previously [11,16,30]. The
141 test was considered positive if aggregation occurred within 30 min for at least two
142 donors in the presence of 0.2 IU/ml low-molecular-weight heparin, but not in the
143 presence of 100 IU/ml heparin. On each plate, positive and negative controls were
144 also measured.

For the H/PF4 immunoassay, the polyspecific Lifecodes PF4 Enhanced (Immucor, Dreieich, Germany) was performed according to the manufacturer's instructions. Optical density > 0.5 was considered positive. The test was previously validated in our laboratory and external and internal quality controls were performed [11].

Proteomic profiling

To assess the proteomic profile, four existing panels of Olink's (Olink Proteomics Inc., Uppsala, Sweden) proximity extension assay (PEA) were performed by Olink Uppsala: "Cardiovascular II", "Cardiovascular III", "Immune response" and "Inflammation". These panels comprise 356 different proteins involved in thrombus formation and inflammation. A full list of all proteins can be found in supplementary table S3. In short, the PEA recognizes proteins by pairs of oligonucleotide-linked antibodies [21]. If the antibodies bind in proximity to each other the oligonucleotides hybridize, and a new PCR primer sequence is revealed. This DNA barcode is then amplified and detected via quantitative PCR. The cycle threshold value, which is inversely correlated to the protein concentration in the sample, is then normalized and transformed to an arbitrary unit called normalized protein expression (NPX) on a log 2 scale. The quality of the measurements is assured through multiple internal controls (incubation controls, extension controls, and detection controls) as well as sample controls (inter-plate and negative controls), details of which are described elsewhere [31]. This innovative technique has been successfully used to identify various key biomarkers in a broad range of diseases, including venous thromboembolism [22–25,32] . The proteins were then annotated with their corresponding gene using the human protein atlas project [33].

P Selectin ELISA technology assay

The levels of soluble P-selectin (soluble CD62P; corresponding to SELP; minimum detectable concentration: 0.244 ng/mL) were quantified in serum samples using ELISA (IBL International, Hamburg, Germany). Absorbance at 450 nm (for serotonin, 405 nm) was measured using an ELISA plate reader (Magellan Software, Sunrise TM, Tecan Group Ltd, Lyon, France). Results were normalized to 2×10^8 platelets/ml and data were expressed in pg/mL [34].

Statistical analysis

To explore the variability between the different patient groups, a principal component analysis (PCA) using single value decomposition and sparse least square analysis

178 (sPLS) was used. Additionally, to quantify the association between protein levels and
179 the presence of HIT we fitted a linear model to the data using the "stats" package for
180 R. In the model, the NPX value of the different proteins served as the dependent
181 variable while the HIPA status was used as the independent variable. To account for
182 physiological differences among the patients, the model was adjusted for age and sex.
183 The Benjamini-Hochberg method was used to adjust the calculated p-values to
184 account for multiple testing, setting the false-discovery rate at 5%. A heatmap
185 showing the 50 most significantly changed proteins, as well as a volcano plot, were
186 plotted. For the biomarker that showed the highest significance, we created boxplots
187 by thrombosis status and compared the different groups using the Wilcoxon-Rank-
188 Sum test. Finally, to determine the diagnostic usefulness of the biomarker, we
189 performed a receiver-operator characteristics curve (ROC) analysis and calculated the
190 area under the curve (ROC-AUC). Additionally, we performed a multivariable linear
191 regression and ROC-analysis using thrombosis as the dependent variable. All analyses
192 were done in R version 4.1.2.

193 The appropriate ethical committee approved the final protocol (Kantonale
194 Ethikkommission Bern).

195 Results

196 Patient characteristics

197 Out of a random sample of 120 patients, 32 with confirmed HIT were included, 28
198 with H/PF4 detected (without HIT), and 38 without HIT (Figure 1; primary dataset).
199 Overall, 21 were excluded because of insufficient clinical data or leftover sample
200 material; one sample did not pass Olinks quality control. The median 4Ts score was 5
201 in patients with HIT (inter-quartile range [IQR] 4, 6), 4 in patients with positive H/PF4
202 antibodies (IQR 3.75, 4), and 3 in patients without HIT (2, 4). The median H/PF4
203 ELISA was 3.0 (2.4, 3.0) in patients with HIT, 0.9 (0.7, 1.5), and 0.3 (0.2, 0.3) in
204 patients without. Detailed patient characteristics are given in Table 1. From the second
205 dataset comprising 50 patients with suspected HIT, one was excluded because of
206 insufficient data (Figure 1). Among these patients, 12 were HIT-positive, 16 were
207 H/PF4 positive, and 21 were HIT-negative. Detailed data of this second dataset is
208 available in Table S1 of the supplementary material.

Proteomic profile

The primary dataset was used for proteomic profiling. In PCA and sPLS, minor differences between HIPA-positive and HIPA-negative patients were observed. Overlapping clusters were interpreted as a consequence of low patient numbers and similar patient characteristics (patients *with suspected HIT*). Results of the PCA and sPLS are displayed in Figure S1 and S2 of the supplementary material, respectively.

Protein abundance analysis revealed a statistically significant association of 40 proteins with HIT status (8 upregulated, 32 downregulated). Out of these proteins, soluble P-selectin remained statistically significant after multiple test adjustments (false discovery rate 5%; = 1.04, 95% CI 0.63, 1.45). A clustered heatmap is available in Figure 2 and a volcano plot showing adjusted p-values is available in Figure 3. Fold changes with adjusted p-values are available in the supplementary material.

ELISA and additional analyses

An ELISAwas used to determine the serum soluble P-selectin levels both in the primary data set that underwent Olinks PEA and in an additional data set of 49 patients suspected of having HIT. First, we analyzed the first data set and found the following median soluble P-selectin values: 25783 pg/ml (IQR: 21238, 27157) for patients without HIT, 29350 pg/ml (IQR: 22175, 36963) for patients with negative HIPA but positive immunoassay, and 38150 pg/ml (IQR: 33888, 42075) for patients with HIT. There was a statistically significant difference between all groups when compared to the patients without HIT (no HIT vs. Antibody positive: $p = 0.02$; no HIT vs. HIT: $p = <0.01$).

Interestingly, different results are seen when analyzing only patients with thromboembolism: 32423 pg/ml (IQR: 27342, 36437), 33450 pg/ml (IQR: 24900, 34650; p-value = 0.73), and 37750 pg/ml (IQR: 35988, 42925; p-value = 0.13) for HIT negative, antibody positive, and HIT positive patients, respectively.

Similar results were obtained in the second, confirmatory dataset: 24147 pg/ml (IQR: 19627, 24149) in patients without HIT, 31547 (IQR: 24057, 31342; p-value = 0.02) in patients with positive antibodies, and 35048 (IQR: 32038, 38087; p-value = < 0.01) in patients with HIT. In contrast, no significant differences were seen in patients with thromboembolism. Boxplots showing soluble P-selectin levels for both datasets combined are displayed in Figure 4.

ROC-analysis of the soluble P-selectin as measured by Olink in the first cohort showed a ROC-AUC of 0.81 (95% CI: 0.72, 0.90) Similar results were observed with the ELISA (ROC-AUC 0.80; both groups).

ROC-analysis of soluble P-selectin for detecting thrombosis showed a lower ROC-AUC of 0.65 (95% CI: 0.52, 0.77) for the Olink assay and 0.67 (95% CI: 0.55, 0.79) for the ELISA (Figure S3). An additional multivariable linear regression showed a significant association between P-selectin levels and the different patient groups, even when adjusting for the presence of thrombosis, age, and sex (Table S2).

Discussion

We applied the Olink PEA covering 356 proteins involved in thrombus formation, inflammation, and immune response to 98 randomly selected patients with suspected HIT and confirmed the results with an ELISA assay in the patients mentioned above and an additional dataset of 47 patients. Among 40 proteins that were statistically significantly associated with HIT status in protein abundance analysis, soluble P-selectin remained significant after multiple test adjustments. This association was confirmed in a ROC analysis in PEA and ELISA (0.80 and 0.81 respectively). This association was especially apparent in patients *without* thrombosis, suggesting potential usefulness in this group.

To our knowledge, this is the first investigation to apply the PEA technology to patients *with suspected HIT*, thus analyzing a large number of proteins potentially associated with immune-mediated thrombosis. Prior omics-based analyses primarily focused on genetic variants. Four genome-wide association studies (GWAS) investigated the risk factors for HIT and revealed genetic variants associated with various enzymes, the AB0 Complex, and distinct receptor proteins [18,35–37]. However, comprehensive studies including metabolomics, proteomics, and transcriptomics are still missing [18].

Our study suggest that soluble P-selectin holds potential as a diagnostic marker for HIT. P-selectin is a glycoprotein that is expressed in platelets and endothelial cells and is involved in leukocyte adhesion and thrombocyte aggregation [38]. When platelets are activated, P-selectin is mobilized from the α granules to the external membrane [39]. In recent years, this mechanism has been leveraged to develop flow cytometry-based tests for activated platelets in patients suspected of having HIT. However, the diagnostic performance of these tests is limited [27,40]. Besides, soluble P-selectin, which can be released into the bloodstream through proteolytic cleavage or alternative

splicing, has been shown to be elevated in various cardiovascular and thrombotic disorders, including myocardial infarction, venous thrombosis, and COVID-19-related thrombosis [41–45]. Thus, soluble P-selectin appears to be a general marker for platelet activation [46]. Moreover, CD62P-mediated cross-talk between the vessel wall, platelets, monocytes and neutrophils results in the activation of innate immune cells and an increase in the expression of tissue factor. This initial activation of immune cells has the effect of thrombus reinforcement and retardation of subsequent resolution processes [32]. Interestingly, our findings extend decades-old observations on increased values of soluble P-selectin in patients with HIT [47–49]. However, these studies have methodological limitations, and soluble P-selectin was not yet considered a biomarker for HIT.

Our study has several strengths. Most importantly, the patients were randomly selected from a population of *patients with suspected HIT.* This is closely resembling the target population for a potential diagnostic or prognostic test, including not only patients with confirmed HIT but also patients with H/PF4 antibodies, and patients without HIT but with similar presenting diseases. As a consequence, contrasts are less pronounced compared to healthy controls but correspond to realistic clinical settings. In addition, we analyzed a large number of proteins in a relatively large cohort. Besides, the results obtained with the PEA were confirmed with an independent analytical technique (Luminex) and in a second dataset. All these points contribute to the high validity of the study.

However, our study also has some limitations. Firstly, we excluded a certain proportion of patients due to incomplete clinical data or residual sample material. However, we consider these dropouts to be at least "*at random*," *and* thus unlikely to affect the results of the study. Secondly, the population was not consecutive because of the high costs of the PEA tests. We cannot fully exclude that a certain selection bias is present. One might additionally argue that a matching procedure according to age and sex would increase the validity of the results. To account for this, we included age and sex in the regression model. These limitations suggest that our results must be confirmed in an independent, larger cohort of consecutive patients. Such a diagnostic accuracy study would also have to be carried out with test systems that can be used in daily practice (e.g. CLIA). Another limitation is that we have used serum rather than plasma. However, this limitation is minimized due to the differential analysis of the various groups using the same sample preparation process. In addition, a polyspecific rather than a IgG-specific ELISA was employed. However, there are three reasons why

this point did not introduce bias: (1) it is not a diagnostic accuracy study in which the performance of current tests is used as a comparison, (2) several studies have shown that the correlation between polyspecific and IgG-specific immunoassay is very high [13], and (3) this information is not included in the study either as an investigated variable or as an outcome variable.

Our data confirm that soluble P-selectin is a promising new biomarker in patients with HIT. This fits to our current understanding of the mechanism of HIT, which recognizes platelet activation as an important feature. The concept of soluble P-selectin as a general biomarker for platelet activation is supported by comparable observations in many other thromboembolic diseases. Soluble P-selectin might be included in future diagnostic decision support tools, thus adding information about platelet activation. The protein may be particularly useful in the diagnosis of HIT without thrombosis, which is a particularly challenging diagnostic situation. Furthermore, the differential concentration of soluble P-selectin in patients with and without thrombosis suggests its potential use as a prognostic marker. This is consistent with observations suggesting soluble P-selectin as a prognostic marker for thromboembolism in COVID [45]. However, our findings must be confirmed in future studies, prospectively including patients with suspected HIT.

In conclusion, our analysis of 356 proteins associated with thrombus formation, inflammation, and immune response in a representative study of patients with suspected HIT has provided evidence supporting the potential of soluble P-selectin as a promising new biomarker. As this was particularly apparent in patients without thrombosis, a potential application appears not only as a diagnostic but also as a prognostic biomarker. Nevertheless, further validation of our findings in diverse settings and populations is warranted, necessitating prospective studies that include patients with suspected HIT.

Acknowledgments

This study was supported by a research grant from the Swiss National Science Foundation (#179334), the International Society on Thrombosis and Haemostasis (https://www.isth.org/page/toradihit), and the CTU research grants (Clinical Trial Unit, Inselspital, University Hospital). We thank Justine Brodard for implementing the heparin-induced platelet activation at Inselspital, Vincent Benites and Laura Celeste Rotondo for performing all laboratory tests, Anja Stalder and Margret Bachmann-Mac Donald for study management, and residents at all study centers. The authors wish to

acknowledge those individuals who provided technical support throughout our investigations including Charles-Antoine Arthaud, Marie-Ange Eyraud, and Amelie Prier from the 'Etablissement Français du Sang (EFS) Auvergne-Rhone-Alpes', France.

Authorship Contributions

HN wrote the analysis plan, analysed, and interpreted the data, and wrote the first manuscript draft. HHC and FC contributed to the design of the study, analysed and interpreted data, provided infrastructure and reagents, and contributed to the first draft of the manuscript. JH contributed to the analysis plan and interpretation of data. JDS, AG, DAT, AM, WAW, AS, JAKH, BG, PV, TB, and LG collected data. MN designed and implemented the study, collected data, contributed to analysis plan and interpretation of data, and wrote the manuscript. All authors contributed to the interpretation of data, reviewed the manuscript critically, and approved the final version of the manuscript.

Conflict of Interest Disclosures

The institution of JKH received grant support, consultancy fees, or honoraria from SNSF, Baxter/Takeda, Bayer, CSL-Behring, NovoNordisk, Octapharma, Roche, SOBI, Roche, Sanofi, FOPH, and Swiss Hemophilia Society, outside of the current work. MN received research grants from Bayer Healthcare, Roche diagnostics, Siemens healthineers, Pentapharm, and Bühlmann laboratories, as well as lecture fees from Sysmex, Siemens healthineers, and Euroimmun, outside of the current work. AG reports personal fees from Aspen, grants from Ergomed, grants from Boehringer Ingelheim, personal fees from Bayer Vital, grants from Rovi, grants from Sagent, personal fees from Chromatec, personal fees from Instrumentation Laboratory, grants and personal fees from Macopharma, grants from Portola, grants from Biokit, personal fees from Sanofi-Aventis, grants from Blau Farmaceutics, grants from Prosensa/Biomarin, grants and other from DRK-BSD NSTOB, grants from DRK-BSD Baden-Würtemberg/Hessen, personal fees from Roche, personal fees from GTH e.V., grants from Deutsche Forschungsgemeinschaft, grants from Robert-Koch-Institut, non-financial support from Veralox, personal fees from Dilaflor, non-financial support from Vakzine Projekt Management GmbH, grants from GIZ Else-Körner-Stiftung, non-financial support from AstraZeneca, non-financial support from Janssen Vaccines & Prevention B.V., personal fees from Takeda Pharma, personal fees from Falk Foundation e.V., grants from European Medicines Agency , personal fees from Mylan

Germany, outside the submitted work; In addition, Dr. Greinacher has a patent Screening Methods for transfusion related acute lung injury (TRALI) with royalties paid to EP2321644, 18.05.2011 , and a patent Verfahren und Vorrichtung zur Herstellung von Universalplasma. licensed to DE 10 2020 212 609 B3 2022.04.07. TB reports grant support, consultancy fees, honoraria, or support for attending meetings from DFG, Stiftung Transfusionsmedizin und Immunhämatologie e.V, DRK Blutspendedienst, Deutsche Herzstiftung, Ministerium für Wissenschaft, Forschung und Kunst Baden Würtemberg, Gesellschaft für Thrombose- und Hämostaseforschung, Berufsverband Deutscher Internisten, CoaChrom Diagnostica GmbH, Robert Bosch GmbH, Ergomed, Bayer, Bristol-Myers Squibb, Doctrina Med AG, Leo Pharma GmbH, Schöchl medical education GmbH, Mitsubishi Tanabe GmbH, Novo Nordisk GmbH, Swedish Orphan Biovitrium GmbH. All other authors declare that no conflict of interest exists.

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Tables

Table 1: Patient characteristics of the primary dataset

	HIT positive	H/PF4 positive	HIT negative
n	32	28	38
Male sex (%)	22 (68.8)	17 (60.7)	24 (63.2)
Age (median [IQR])	68.5 [64.8, 76.0]	77.0 [55.0, 79.0]	74.0 [54.0, 81.0]
4Ts (median [IQR])	5 [4, 6]	4 [4, 5]	3 [2, 4]
ELISA GTI polyspecific OD (median [IQR])	3.0 [2.4, 3.0]	0.9 [0.7, 1.5]	0.3 [0.2, 0.3]
Setting (%)			
Cardiac surgery	13 (40.6)	3 (10.7)	4 (10.5)
ICU	10 (31.2)	12 (42.9)	14 (36.8)
Others	9 (28.1)	13 (46.4)	20 (52.6)
Thrombocytes G/L (median [IQR])	60 [43, 81]	68 [48, 101]	59 [41, 80]
Thrombosis (%)	15 (57.7)	5 (25.0)	4 (11.8)

Figure 1: Flow of the patients (primary and second dataset)

Figure 2: Clustered heatmap illustrating the z-scores of the 50 most significant proteins, stratified by HIT status (group). The following additional information is shown: sex, setting, and presence of thrombosis.

538 Figure 3: Vulcanoplot showing the differential abundance of proteins between
539 between HIT-patients and non-HIT patients (including Heparin/PF4 antibody
540 positives). The X-axis depicts the fold change (NPX difference) while the Y-axis
541 depicts the $-\log_{10}$(adjusted p-value). Green dots represent a p-value < 0.05,
542 and red dots represents adjusted p-values between 0.05 and 0.3.

543 Figure 4: P-selectin in patients with HIT, positive heparin/PF4 antibodies, and
544 without HIT, depending on the presence of thromboembolism (ELISA, all pa-
545 tients)

546 Figure 5: Receiver-operating characteristic (ROC) curve of P-selectin for the
547 presence of HIT as measured with the Olink assay and ELISA (all patients)

548

Figure 1

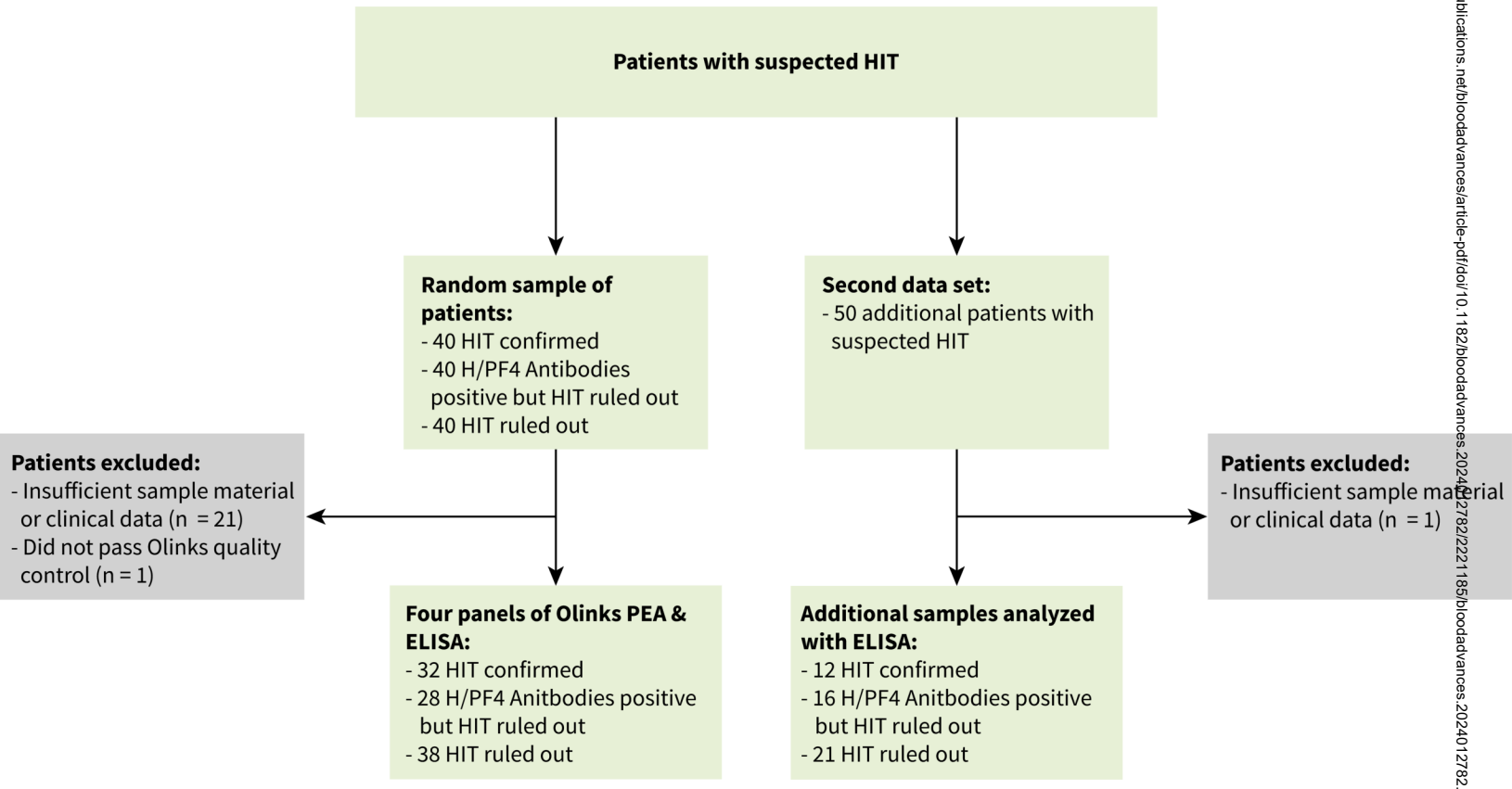

Figure 2

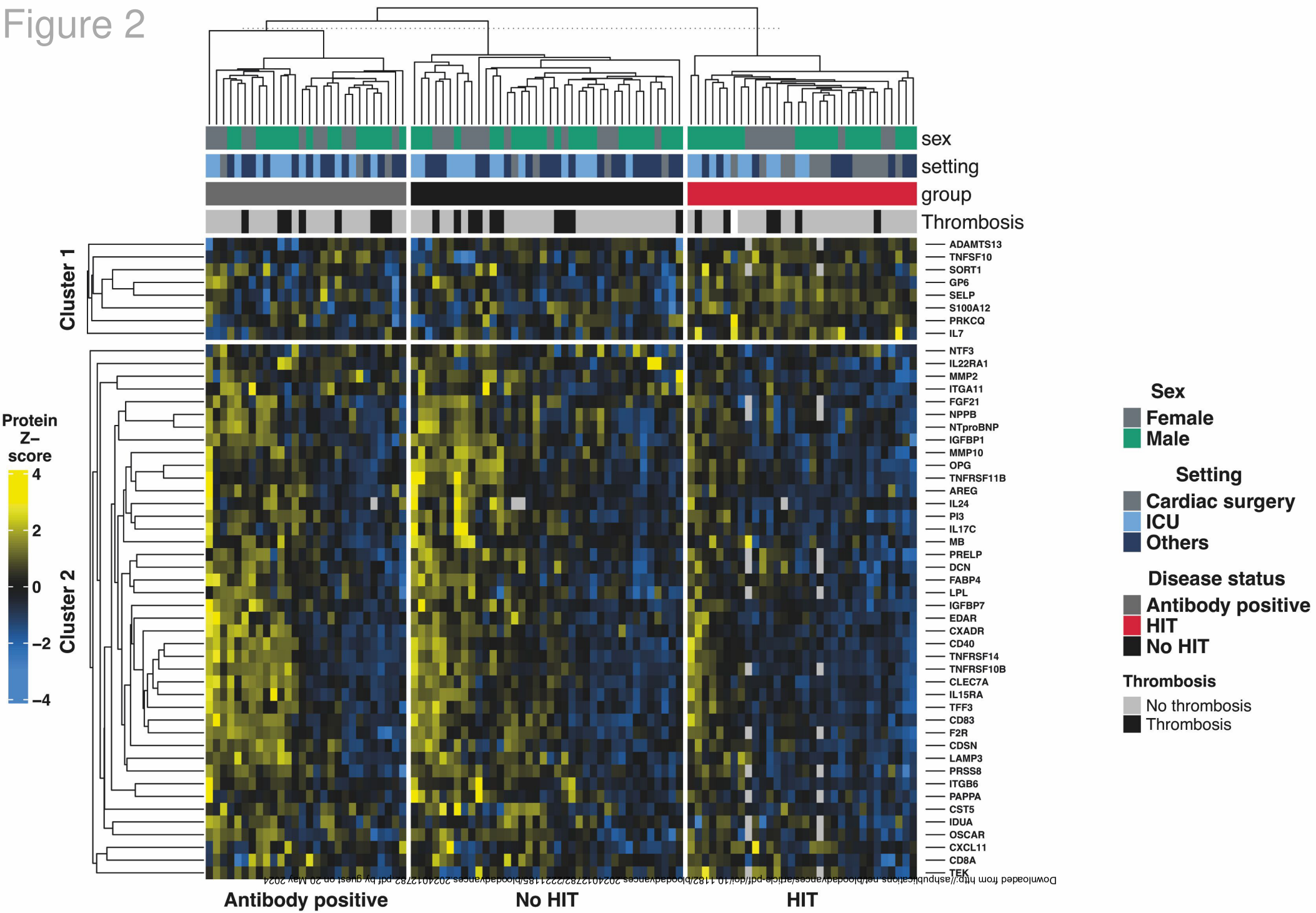

Figure 3

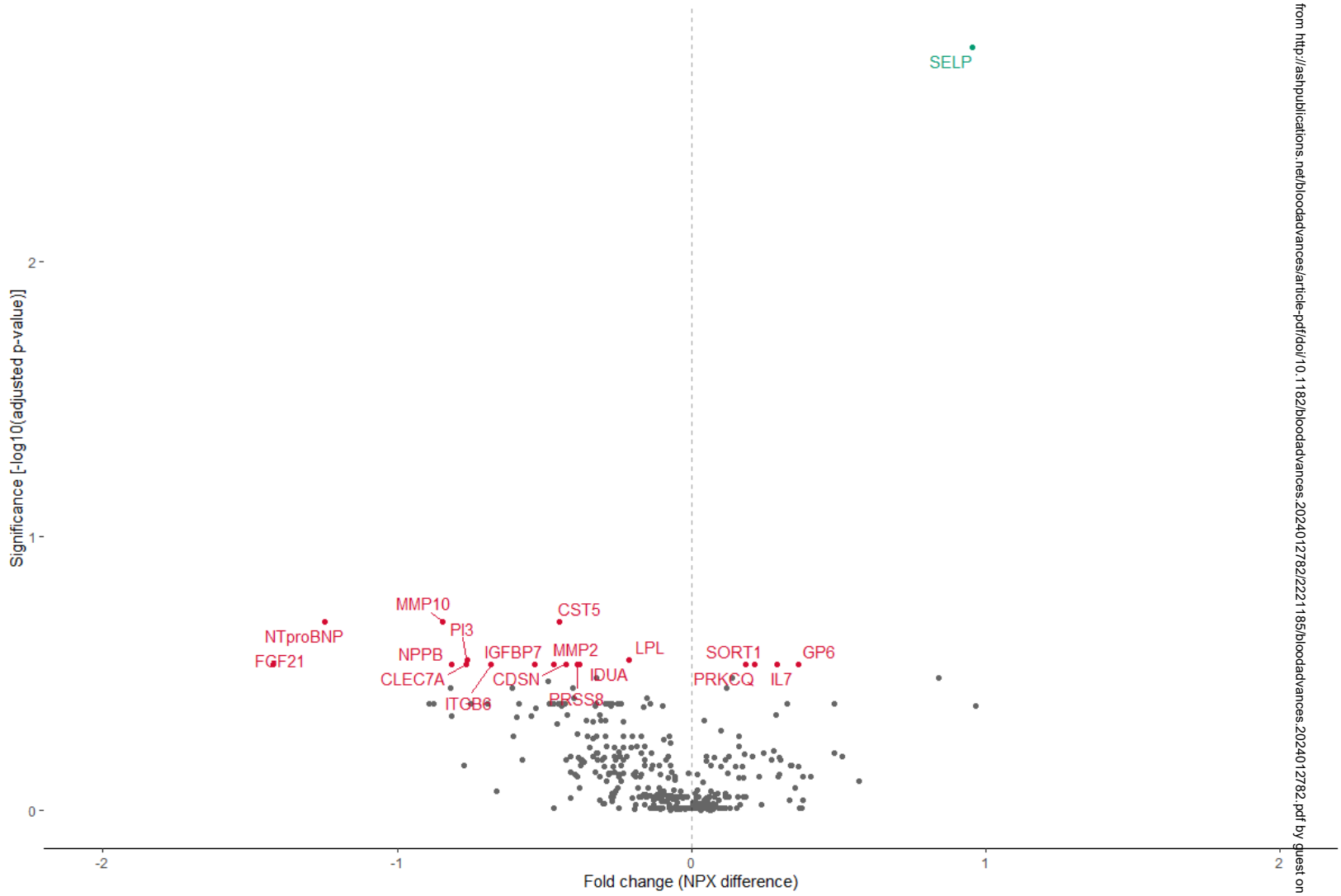

Figure 4

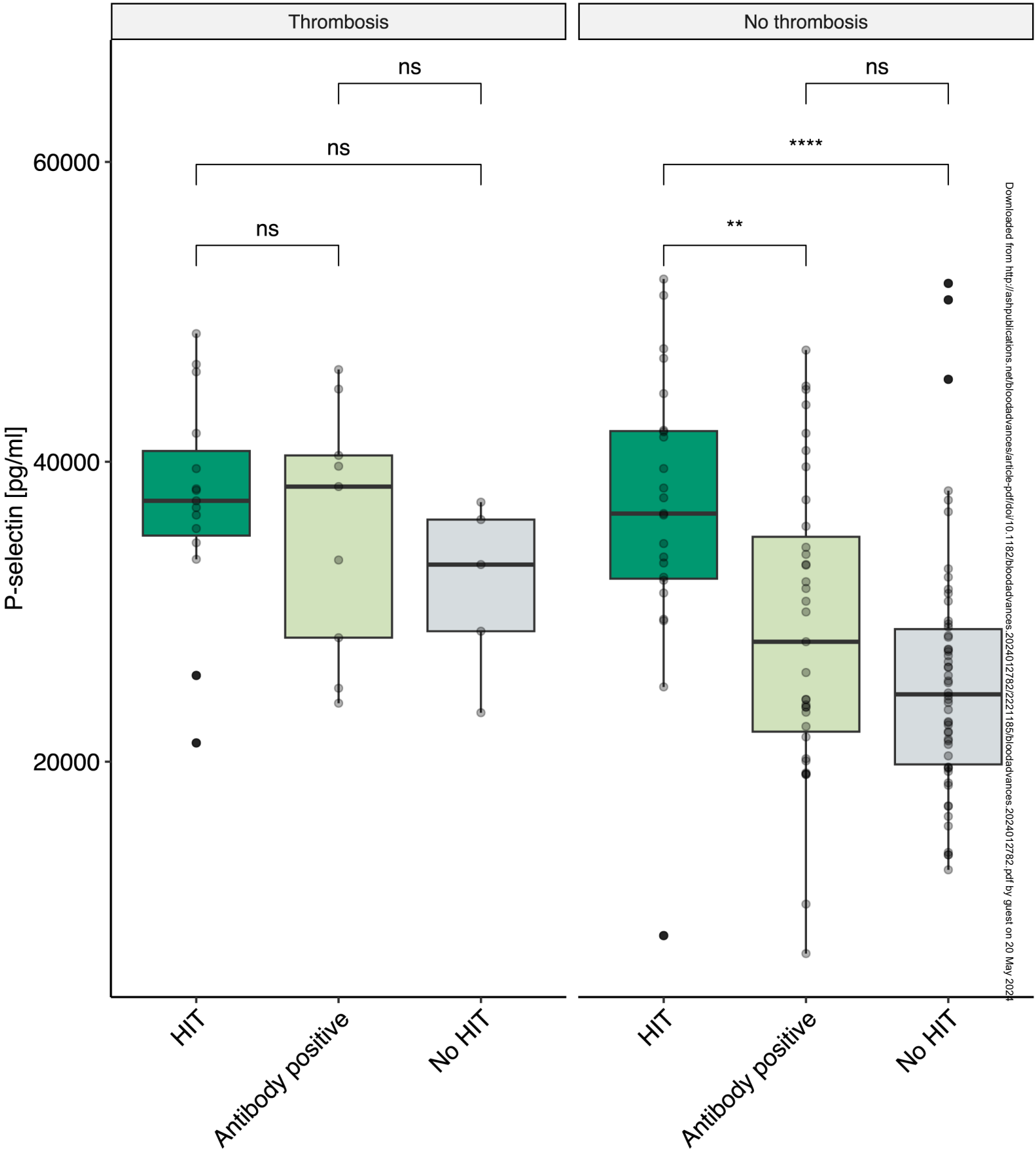

Figure 5