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Subtype-specific plasma signatures of platelet-related protein releasate in acute pulmonary embolism

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ABSTRACT

Introduction: There is evidence that plasma protein profiles differ in the two subtypes of pulmonary embolism (PE), isolated PE (iPE) and deep vein thrombosis (DVT)-associated PE (DVT-PE), in the acute phase. The aim of this study was to determine specific plasma signatures for proteins related to platelets in acute iPE and DVT-PE compared to isolated DVT (iDVT).

Methods: Within the Genotyping and Molecular Phenotyping of Venous ThromboEmbolism (GMP-VTE) Project, a multicenter prospective cohort study of 693 confirmed VTE cases, a highly sensitive targeted proteomics approach based on dual-antibody proximity extension assay was applied. LASSO-regularized logistic regression analysis selected 33 and 30 of 135 platelet-related candidate proteins in iPE and DVT-PE vs. iDVT, respectively.

Results: All regulated proteins were well associated with six prominently released platelet proteins and the majority showed specificity for iPE and DVT-PE compared to iDVT. While iPE-specific proteins were assigned to be predominantly released via shedding mechanisms and extracellular vesicles, granule secretion was identified as a major release mechanism assigned to DVT-associated PE-specific proteins. Network analysis demonstrated three interconnected clusters of specifically regulated proteins in iPE linked to immunoreceptor signaling, pathogen clearance and chemotaxis, whereas for DVT-associated PE one cluster linked to tissue remodeling and leukocyte trafficking. Machine learning-based analysis reveals specific plasma signatures and differential release mechanisms of proteins related to platelets in acute iPE and DVT-associated PE.

Conclusion: These data suggest that the platelet protein releasate contributes to the differential regulation of plasma proteins in acute PE compared to iDVT, which may be associated with different platelet activation patterns.

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1. Introduction

Platelets crucially mediate the recruitment of leukocytes to the inflamed vessel wall in the pathogenesis of atherothrombosis [1,2]. Here, they act as cellular linkers to enable the inflammatory crosstalk between immune and endothelial cells at high arterial shear stress. Immune and endothelial cell activation is modulated by stimulated platelets via direct receptor-mediated interaction and/or release of immunomodulatory and pro-inflammatory molecules and extracellular vesicles (EV). However, the contribution of platelets in the development of venous thromboembolism (VTE) and its subtypes pulmonary embolism (PE) and deep vein thrombosis (DVT), initiated by local inflammatory responses at the activated endothelium at low shear stress, is less elucidated.

Experimental VTE in mice demonstrated that platelets interact with von Willebrand factor (VWF)-exposing endothelial cells and form conjugates with leukocytes via glycoprotein (GP) Ibα, as well as trigger endothelial recruitment and leukocyte-dependent coagulation [3]. In a similar venous thrombosis model induced by flow restriction, deficiency of complement factor 3 but not 5 reduced tissue factor (TF)-triggered thrombus and fibrin formation accompanied by diminished platelet recruitment and reactivity *in vitro* [4–6]. Another murine model of venous thrombosis induced by silencing of the natural anticoagulants antithrombin and protein C preferentially affected large veins of the head and was dependent on tissue factor and platelets too [7]. These experimental data suggest that platelets contribute to thromboinflammation in the venous system by triggering inflammatory and coagulation processes.

In humans, the ASPIRE and WARFASA trials demonstrated that blocking of platelet thromboxane A_2 synthesis by low-dose aspirin monotherapy substantially reduces recurrent thrombotic events of both PE and DVT phenotypes in patients with incident unprovoked VTE [4–6]. Recently, we demonstrated that platelets from patients with acute VTE exhibit more exocytosis of dense granules and lysosomes accompanied by higher plasma levels of thromboxane B_2, but less platelet-dependent thrombin generation than patients with excluded VTE, independent of aspirin therapy [8]. Non-aspirin users with acute VTE presented with shorter collagen/epinephrine-mediated closure time in the platelet function analyzer compared to individuals with excluded VTE. These results revealed differential platelet activation and reactivity properties in acute VTE, which are associated with a distinct proportion of low-responders to aspirin. Epidemiological studies provide evidence for a role of increased expression of the megakaryocyte and platelet-selective immunoglobulin receptor glycoprotein GPVI, caused by the common single nucleotide polymorphism rs1613662, on the increased risk of VTE [9].

Activated platelets can release more than 300 proteins, which are involved in the regulation of hemostatic, inflammatory and angiogenic responses of platelets, leukocytes and vascular cells [10]. Major sources of the platelet protein releasate are secreted granule cargos, proteolytically cleaved or shed surface receptors and membrane-bound proteins as well as platelet-derived EV. The platelet α-granule secretome covers the majority of released platelet proteins, which are synthesized in megakaryocytes or endocytosed from plasma.

Recent advances in mass spectrometry techniques allow a comprehensive quantification of between 2000 and 6000 proteins in resting, inhibited and activated highly purified human platelets [11] with estimated copy numbers from 3700 proteins [12]. Advanced ELISA-based assays and mass spectrometry approaches enable the qualitative and quantitative assessment of platelet-released proteins in plasma and isolated platelets, respectively [13]. Studies with ELISA-based quantitation of prominent soluble platelet activation markers demonstrated increased levels of soluble P-selectin and platelet factor 4 in plasma from patients in acute and chronic VTE [14,15].

However, a comprehensive analysis of platelet-associated plasma proteins in large and well-studied cohorts of VTE is still missing. A recent plasma protein profiling study of patients with acute VTE from our group identified overlapping plasma proteomes in PE subtypes within the acute phase, but also a specific protein signature for isolated PE (iPE) associated with non-canonical pathways preferentially involved in atherosclerosis and arterial thrombosis [16].

Using machine learning-based analysis of targeted plasma protein profiling data from 541 patients with VTE [17], we aimed to identify plasma protein signatures related to the putative platelet releasate that are specific for the PE subtypes iPE and DVT-associated PE when compared to isolated DVT (iDVT).

2. Materials and methods

2.1. Study participants

Data were collected as part of the Genotyping and Molecular Phenotyping of Venous Thromboembolism (GMP-VTE) project, a multicenter prospective cohort study initiated by the University Medical Center of Johannes Gutenberg University Mainz, Germany [17]. The GMP-VTE study encompasses patients from two previously registered prospective cohort studies: *VTEval* (ClinicalTrials.gov identifier: NCT02156401) and *FOCUS Bioseq* (German clinical trials registry identifier: DRKS00005939). The studies comply with the Declaration of Helsinki (2013, 7th revision), Good Epidemiological Practices, the locally appointed legal and regulatory requirements, including General Data Protection Regulation (EU 2016/679). All patients signed the written informed consent for biomaterial and blood sampling, genetic analysis and sharing of data with research partners.

The GMP-VTE study comprises 693 patients with acute VTE enrolled at the time or shortly after the imaging-validated diagnosis. Because cancer represents a distinct entity of VTE, patients with active cancer ($n = 84$) were excluded. In addition, participants without proteomic measurements ($n = 27$) and those who did not undergo imaging for DVT ($n = 41$) were excluded from this investigation. The PE study groups iPE (99 patients) and DVT-associated PE (282 patients) were compared with 160 patients suffering from iDVT (Supplementary Fig. 1). Suspected DVT and PE cases were checked by whole-leg color Doppler ultrasonography and computer tomographic pulmonary angiography or ventilation/perfusion scintigraphy, respectively. Concomitant DVT in patients with PE was diagnosed by additional whole-leg imaging. All diagnoses were adjudicated and validated by board-certified medical specialists (e.g. angiologists, radiologists).

2.2. Protein profiling

Venous blood was collected during the acute event of VTE, anticoagulated with ethylenediaminetetraacetic acid (EDTA) and centrifuged at 200 ×*g* for 10 min followed by centrifugation at 2000 ×*g* for 15 min at room temperature within 60 min after blood collection to minimize artificial protein degradation. The platelet-poor plasma was immediately stored at −80 °C in the Biomaterial Bank of the University Medical Center Mainz. Profiling of high and low abundant plasma proteins was conducted using proximity extension assay (PEA) technology (Olink Biosciences, Uppsala, Sweden), a targeted proteomics technology that integrates oligonucleotide-labeled antibodies and quantitative real-time PCR amplification, providing normalized expression (NPX) values [18]. 444 proteins were measured utilizing five Olink panels (trade names: Cardiovascular II (CVDII), Cardiovascular III (CVDIII), Cardiometabolic, Inflammation, Immune Response). Plasma samples were thawed at 30 °C for 10 min and immediately processed for PEA analysis. The details of the protein profiling methodology and quality control procedures are described elsewhere [17].

2.3. Identification of platelet-related plasma proteins included in targeted proteome panels

444 proteins from the five targeted proteomics assay panels were compared with platelet proteins identified based on advanced mass spectrometry studies [11,12,19–24], gene databases (Kyoto Encyclopedia of Genes and Genomes (KEGG) [http://www.kegg.jp/], Reactome [https://reactome.org/], Gene Set Enrichment Analysis [GSEA] [https://www.gsea-msigdb.org/g], BioCarta [https://maayanlab.cloud/]) and a systematic literature search in the PubMed database (https://pubmed.ncbi.nlm.nih.gov; Fig. 1), resulting in the identification of total 135 platelet-related proteins included in the PEA panels for further analysis.

These 135 platelet-related proteins were selected and classified into validated (based on literature) and putative groups of platelet release mechanisms. Validated platelet release mechanisms comprise granule secretion and membrane shedding and/or extracellular vesicles (EV), whereas putative EV release was arbitrarily defined for platelet proteins for which the release mechanism was not specifically described in the literature.

2.4. Association analysis of platelet-related plasma proteins with platelet-prominent proteins included in targeted proteome panels

To support the specificity of the identified plasma proteins as platelet-derived, all selected proteins were additionally related to six predefined prominent platelet proteins, i.e. glycoprotein Ibα (GPIbα, *GP1BA*), P-selectin *(*CD62P*, SELP)*, CD40 ligand (CD40L, *CD40LG*), platelet-derived growth factor subunit β (PDGF-β, *PDGFB*), stromal cell-derived factor 1 (SDF-1α, C-X-C Motif Chemokine Ligand 12 [*CXCL12*]) and lysosomal associated membrane protein 3 (LAMP-3/*LAMP3*, CD63/*CD63*), by penalized linear regression. These proteins are abundantly expressed by human platelets and known to be released upon platelet activation (supplemental Methods) [25].

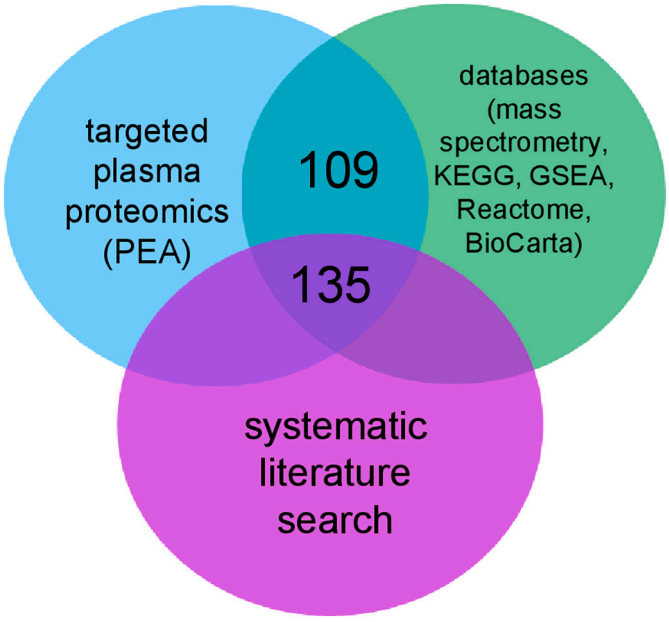

Fig. 1. Study design and workflow. 109 proteins associated with platelets were selected from 444 proteins of five PEA panels based on state-of-the-art mass-spectrometry studies [11,12,19–24], KEGG, Reactome, GSEA and BioCarta databases. Additional 26 platelet-related proteins were identified based on a systematic literature search using PubMed database, resulting in total 135 platelet-related proteins identified in the five PEA panels. Abbreviations: GSEA – gene set enrichment analysis, KEGG – Kyoto Encyclopedia of Genes and Genomes, PEA – proximity extension assay.

GPIbα (*GP1BA*), part of the GPIb-V-IX receptor complex, is crucially involved in VWF-mediated platelet recruitment to the injured endothelium and platelet-leukocyte conjugation in murine VTE models [26]. A disintegrin and metalloproteinase (ADAM) 17 has been demonstrated to cleave GPIbα into the soluble extracellular domain glycocalicin in response to stress or strong stimuli [27]. P-selectin (*SELP*), CD40L (*CD40LG*) and LAMP-3 (*LAMP3, CD63*) are located in the membrane of α-granules and dense granules, lysosomes, respectively. They are translocated via exocytosis to the surface of activated platelets, therefore attributed to the function class “membrane receptors/secretory proteins” and assigned to the release mechanism “shedding and EV”. CD40L (*CD40LG*) is enzymatically cleaved by MMP-2 and MMP-9 to soluble CD40L upon strong platelet activation by collagen and/or thrombin [28]. However, the shedding mechanisms for P-selectin (*SELP*) and LAMP-3 (*LAMP3*, *CD63*) are not known yet. Nevertheless, these granule-membrane proteins can be released via platelet-derived EV, which are still present in platelet-poor plasma [29], but their characterization in acute VTE remains elusive [30]. The growth factor PDGF-β (*PDGFB*) and the chemokine SDF-1α (*CXCL12*) are abundantly stored in α-granules and released via secretion [31,32]. The initial phenotype-specific selections of proteins were ranked by number of ‘hits’ in relation to these prominent platelet proteins and visualized as bar charts.

2.5. Statistical analysis

Highly skewed protein expression levels were transformed to approximate normality by square root or natural logarithmic transformations. Missing values resulting from chip failures were imputed by random forest using only proteomic data, using the ‘missForest’ R package. Technical outliers in the proteomic data, defined as being four median absolute deviations away from the median, were winsorized. Normalized protein expression levels were computed for each VTE phenotype, quantifying phenotype-specific expression as percent standard deviation change relative to the global expression average (i.e., the mean expression level per protein across the full study sample), and visualized as a hierarchically clustered heatmap (Fig. 2). For the identification of particularly phenotype-specific proteins, least absolute shrinkage and selection operator (LASSO)-penalized logistic regression models were used. LASSO regularization is a form of penalized maximum likelihood estimation that results in shrinkage of estimated coefficients (in this case, log odds ratios) towards zero. In this manner, it is a selection algorithm, as variables with coefficients of exactly zero are omitted from the model. It is particularly well-suited to high-dimensional settings, i.e., settings in which the number of predictors is large, and may even exceed the number of observations, in which case traditional maximum likelihood estimation fails. The amount of shrinkage of the log odds ratios is controlled by the parameter λ, the value of which was determined by leave-one-out cross-validation (LOOCV). LOOCV is a procedure in which the model is created on the basis of all of the data except one observation (n-1), and the prediction error is determined on the left-out single observation. This is repeated as many times as there are observations, and the prediction error is then averaged across folds. This procedure is repeated for a sequence of λ values, and the λ value at which the average prediction error is minimized, is considered optimal. LOOCV was used to select the penalty parameter λ associated with the minimum average prediction error on the holdout folds, indicating generalizability to external samples. Non-linear relationships were captured by incorporating fractional polynomial transformations for all included predictors. As adjustment covariates, age, sex, body mass index (BMI), obesity, the modification of diet in *renal* disease-based estimated glomerular filtration rate [33] (MDRD-eGFR), white blood cell (WBC) count, antiplatelet agent intake, provoked vs. unprovoked etiology of the acute VTE event, chronic heart failure and the cardiovascular risk factors (CVRF), smoking and diabetes mellitus were included. To denote the results of the penalized regression models, the λ Ratio (LR), a measure of predictive robustness of each

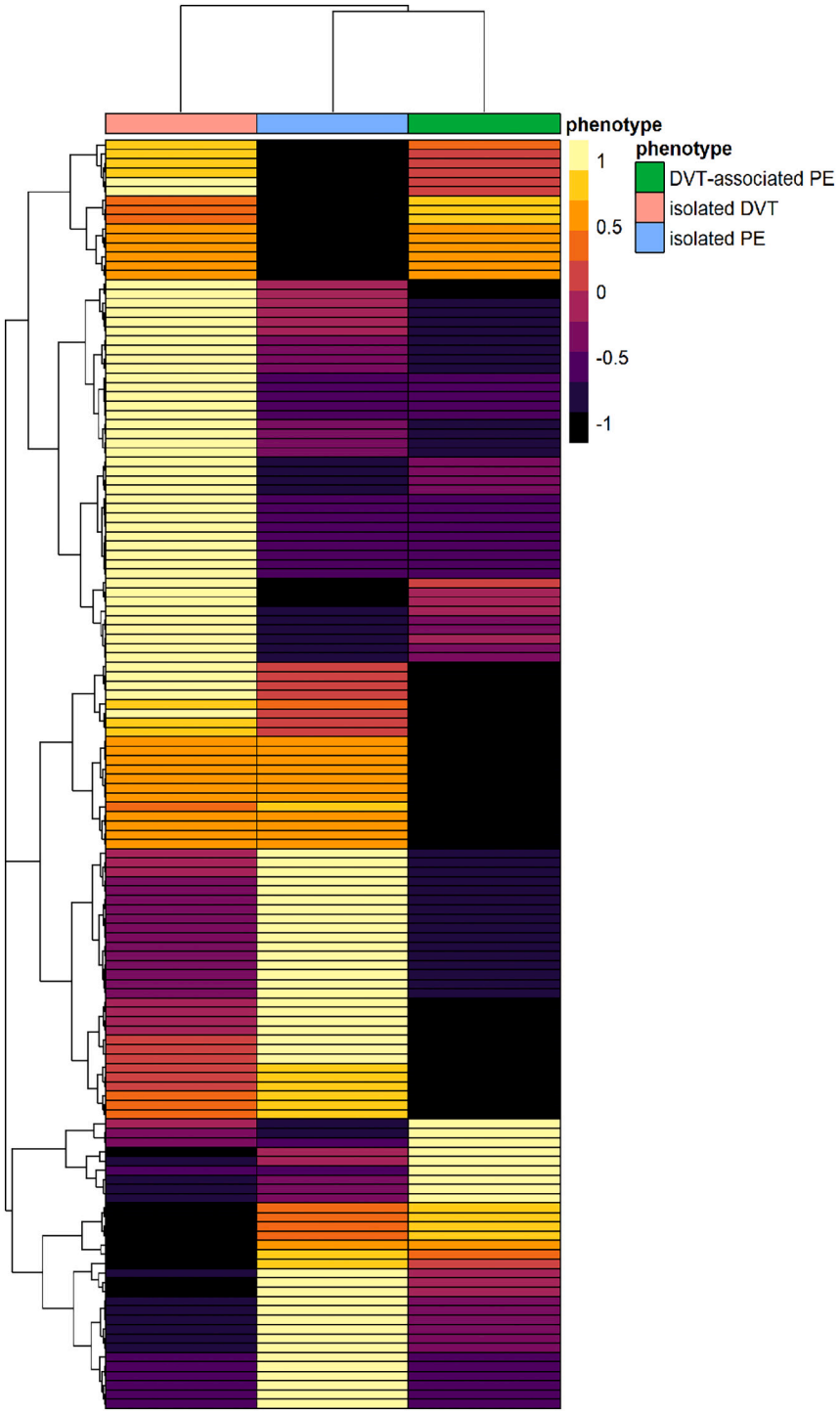

Fig. 2. Hierarchically clustered heatmap of mean protein expression within VTE phenotypes. Columns represent protein expression in different VTE-subtypes, each row represents the measurement of a single protein. Abbreviations: DVT – deep vein thrombosis, i – isolated, PE – pulmonary embolism.

protein, was calculated as the ratio of the λ at which the variable's coefficient was shrunk to zero vs. the LOOCV-selected λ. All data preprocessing and analysis were conducted using the statistical software package R, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Clinical characteristics

The clinical characteristics of patients with acute VTE at baseline are presented in Table 1. In contrast to iPE (51.5 % females), the iDVT (41.2 % females) and DVT-associated PE group (42.2 % females) had a higher prevalence of males. Groups were similar in age and BMI. The history of VTE was less prevalent in the iPE group. Active smoking was more prevalent in iDVT compared to patients with PE, whereas arterial

Table 1
Patient characteristics at baseline stratified by VTE-phenotype.

Variable	iDVT (n = 160)	iPE (n = 99)	DVT-PE (n = 282)
Female sex	41.2 % (66/160)	51.5 % (51/99)	42.2 % (119/282)
Age (years)	61.2 (17.4)	61.2 (17.4)	61.8 (15.5)
BMI [kg/m^2]	26.62 (22.81/30.77)	26.57 (23.39/29.39)	29.35 (25.15/33.18)
Risk factors of VTE			
VTE history	33.3 % (53/159)	16.3 % (15/92)	34.8 % (93/267)
DVT history	32.7 % (52/159)	13.0 % (12/92)	30.1 % (80/266)
PE history	5.7 % (9/158)	5.3 % (5/95)	14.7 % (39/266)
Recent immobilization	14.4 % (23/160)	11.5 % (11/96)	14.8 % (40/271)
Recent travel	16.9 % (27/160)	10.5 % (10/95)	12.0 % (32/267)
Pregnancy, current	1.9 % (3/160)	0 % (0/98)	0.7 % (2/282)
Recent surgery	4.4 % (7/160)	8.3 % (8/96)	4.0 % (11/272)
Diagnosed thrombophilia	5.8 % (9/155)	1.3 % (1/75)	3.3 % (7/213)
Recent trauma	6.9 % (11/159)	3.1 % (3/96)	4.8 % (13/269)
Symptomatology			
Wells score PE	0.75 (1.06)	3.97 (2.48)	5.24 (2.12)
Wells score DVT	1.45 (1.19)	0.21 (1.05)	1.08 (1.24)
Cardiovascular risk factors			
Arterial hypertension	35.7 % (56/157)	51.0 % (49/96)	60.5 % (159/263)
Diabetes	9.4 % (15/160)	15.6 % (15/96)	14.6 % (38/261)
Smoking	25.0 % (40/160)	12.0 % (11/92)	14.4 % (37/257)
Obesity	31.8 % (47/148)	20.2 % (20/99)	43.3 % (122/282)
Comorbidities			
Atrial fibrillation	2.5 % (4/158)	5.2 % (5/96)	5.0 % (13/261)
Congestive heart failure	1.9 % (3/158)	9.5 % (9/95)	5.9 % (15/256)
Chronic kidney disease	3.8 % (6/157)	7.4 % (7/95)	5.7 % (15/261)
Chronic liver disease	3.8 % (6/156)	2.1 % (2/94)	7.3 % (19/259)
Chronic lung disease	11.2 % (18/160)	16.7 % (16/96)	10.2 % (28/274)
Coronary artery disease	5.7 % (9/159)	7.4 % (7/94)	7.8 % (20/257)
History of stroke	0.6 % (1/159)	4.2 % (4/95)	8.0 % (21/262)
Peripheral artery disease	3.2 % (5/156)	11.4 % (5/44)	3.0 % (5/166)
Antithrombotic medication			
Clopidogrel (B01AC04*)	1.9 % (3/160)	5.1 % (5/99)	2.8 % (8/281)
ASA (B01AC06*)	12.5 % (20/160)	29.3 % (29/99)	34.5 % (97/281)
Clopidogrel plus ASA	12.5 % (20/160)	32.3 % (32/99)	35.9 % (101/281)
Heparins (B01AB*)	58.8 % (94/160)	50.5 % (50/99)	60.5 % (170/281)
FXa inhibitors (B01AF*)	10.0 % (16/160)	28.3 % (28/99)	32.0 % (90/281)
Anticoagulants (B01AB*, B01AF*, VKA/B01AA*)	69.4 % (111/160)	74.7 % (74/99)	81.9 % (230/281)
Laboratory analysis			
Troponin I [pg/ml]	2.20 (1.50/4.30)	10.00 (2.37/38.90)	16.70 (4.40/60.10)
NT-proBNP [pg/ml]	102.3 (56.0/207.1)	190.5 (101.3/688.9)	369.6 (116.8/1337.2)
D-dimer [mg/l]	1.44 (0.76/4.01)	2.25 (1.15/7.32)	4.85 (2.68/11.98)
CRP [mg/l]	6.20 (2.70/14.67)	14.56 (3.90/63.23)	24.00 (9.03/53.33)
Platelet count [/nl]	237.0 (188.4/289.6)	239.0 (203.5/284.2)	235.0 (186.0/287.7)
WBC count [/nl]	7.41 (6.04/9.48)	8.59 (6.81/11.41)	9.36 (7.10/11.61)
RBC count [/pl]	4.52 (0.56)	4.39 (0.53)	4.45 (0.61)

hypertension and diabetes were more frequent in patients with PE. The proportion of VTE patients suffering from atrial fibrillation, congestive heart failure, chronic kidney disease, coronary artery disease and history of stroke were higher in PE groups than for iDVT. Peripheral artery disease was most frequent in iPE (rather low numbers) compared to the other VTE phenotypes. Patients with acute PE at the time of blood drawing received about 3-fold more antiplatelet drugs, i.e. acetylsalicylic acid (ASA), clopidogrel (discontinued after VTE diagnosis [34]), as well as factor (F) Xa inhibitors than patients with acute iDVT. Troponin I, N-terminal pro b-type natriuretic peptide (NT-proBNP) and C-reactive protein (CRP) indices were elevated in the PE groups. Blood cell counts were similar among the groups.

Categorical variables are presented as absolute and relative frequencies; continuous variables are presented as mean ± SD when normally distributed or as median interquartile ranges when non-normally distributed. "Recent" means within last 30 days before enrolment. *ATC classification system codes. Abbreviations: ASA - acetylsalicylic acid, BMI – body mass index, CRP – C-reactive protein, CVRF – cardiovascular risk factor, F – factor, i – isolated, DVT – deep vein thrombosis, DVT-PE – deep vein thrombosis associated pulmonary embolism, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, MCV – mean corpuscular volume, NT-proBNP - N-terminal pro b-type natriuretic peptide, PE – pulmonary embolism, RBC – red blood cells, VKA – vitamin K antagonists, VTE – venous thromboembolism, WBC – white blood cells.

3.2. Identification of platelet-related plasma proteins in targeted proteomics assay panels

135 proteins were selected from 444 plasma proteins of five targeted proteomics assay panels as platelet-associated based on state-of-the-art mass spectrometry databases of human platelets, KEGG, Reactome,

GSEA, BioCarta databases and systematic literature research (Fig. 1). Supplemental Table 1 summarizes the extracted platelet-related proteins with estimated copy numbers [12], functional classes (UniProtKB) [11], assumed release mechanisms (i.e. granule secretion, shedding, [putatively] EV), granule compartment of secretory proteins and corresponding references.

3.3. Machine learning-based selection of platelet-related plasma proteins in PE subtypes compared to iDVT

LASSO-regularized logistic regression models with fractional polynomial transformations accounting for linear and non-linear relations were used to identify differentially expressed plasma proteins related to platelets in the two subtypes of PE, iPE and DVT-associated PE, when compared to iDVT (Fig. 3). 33 proteins were selected for iPE with an area under the receiver operating characteristic curve (AUC) of 0.94 (LOOCV AUC: 0.75) and 30 proteins were selected for DVT-associated PE with an AUC of 0.89 (LOOCV AUC: 0.77), indicating good discrimination between phenotypes based on the expression levels for the selected proteins. The majority of the 20 selected proteins more highly expressed in iPE vs iDVT (Fig. 3A, B), whereas 17 of 30 selected proteins were less highly expressed in DVT-associated PE (Fig. 3C, D) compared to iDVT. Supplemental Tables 2 and 3 present all model specifications and estimates in detail.

All regression models were adjusted for age, sex, arterial hypertension, diabetes, smoking, obesity, atrial fibrillation (AF), congestive heart failure (CHF), chronic inflammatory disease (CID), chronic kidney disease (CKD), chronic liver disease (CLD), coronary artery disease (CAD),

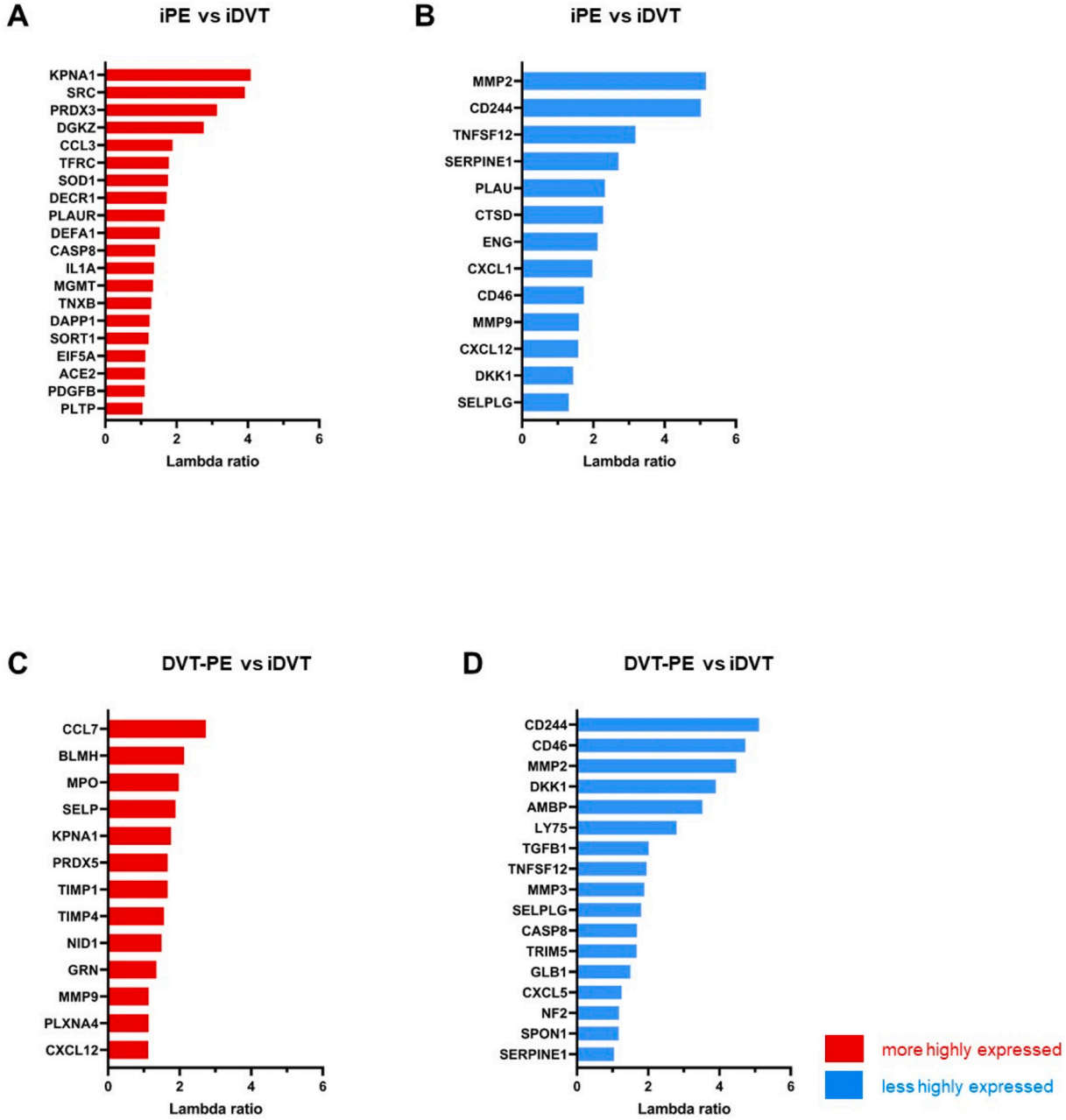

Fig. 3. Machine learning-based analysis of regulated plasma proteins related to platelets for PE subtypes compared to iDVT. LASSO-regularized logistic regression with fractional polynomial transformation selected more or less highly expressed plasma proteins associated with platelets for iPE (A, B) and DVT-associated PE (C, D) vs iDVT. 33 proteins were identified for iPE (Fig. 3A, B) with an area under the receiver operating characteristic curve (AUC) of 0.94 (LOOCV AUC: 0.75) and 30 proteins were identified for DVT-associated PE (Fig. 3C, D) with an AUC of 0.89 (LOOCV AUC: 0.77).

history of stroke, pulmonary arterial hypertension (PAH), baseline antiplatelet agent use, baseline anticoagulant use, platelet count, red blood cell count (RBC), leukocyte count (WBC). Abbreviations: AUC – area under the receiver operating characteristic curve, DVT-PE – deep vein thrombosis-associated pulmonary embolism, eGFR (MDRD) - Estimated glomerular filtration rate (modification of diet in renal disease), EV – extracellular vesicles, iDVT – isolated deep vein thrombosis, iPE – isolated pulmonary embolism, LASSO – least absolute shrinkage and selection operator.

3.4. Association between regulated platelet-related proteins and prominent platelet activation markers in PE subtypes

The targeted proteomics panels include a subset of well-known platelet activation markers, i.e. GPIbα (*GP1BA*), P-selectin (*SELP*), CD40L (*CD40LG*), PDGF-β (*PDGFB*), SDF-1α (*CXCL12*) and LAMP-3 (*LAMP3*, *CD63*), which are abundantly expressed in and released by human platelets upon activation [12,35]. LASSO-regularized regression analysis demonstrated that all selected platelet-related proteins are associated with at least one of the prominent platelet-activation markers (Fig. 4). 88 % of regulated proteins in iPE (vs iDVT) and 93 % of regulated proteins in DVT-associated PE (vs iDVT) were associated with at least 3 prominent platelet activation markers, supporting their likely platelet origin. Full details of the analysis are provided in Supplemental Tables 4 and 5.

3.5. Specifically regulated plasma proteins linked to platelets in iPE and DVT-associated PE

In both PE subtypes, 11 shared proteins were selected (Fig. 5), suggesting similar involvement of these platelet-related proteins in both PE phenotypes. Notably, caspase-8 (*CASP8*), SDF-1α (*CXCL12*) and matrix metalloproteinase (MMP)-9 (*MMP9*) demonstrated an inverse expression direction in the two PE subtypes. The majority of the selected platelet-related proteins was specifically regulated in iPE (22 of total 33 regulated proteins) and DVT-associated PE (19 of total 30 regulated proteins) compared to iDVT (Fig. 5), indicating that platelet activation patterns may differ between the PE subtypes in the acute phase. Full details of the analysis can be checked in Supplemental Tables 6–9.

3.6. Release mechanisms of platelet-related proteins specifically selected in PE subtypes

Release mechanisms of selected proteins related to platelets were defined based on literature and databases reporting on protein release mechanisms of activated platelets and functional classes/protein localization (Supplemental Table 1). Release via secretion of platelet α-granules and lysosomes was identified for 36 % (8 of 22 proteins) of specifically regulated proteins in iPE, whereas in DVT-associated PE platelet degranulation was found for the majority (63 %, 12 of 19 proteins) of specifically selected proteins (Fig. 6A). All these proteins were assigned to the functional class “secretory proteins” (Supplemental Table 1).

Plasma proteins selected by multiple LASSO-regularized regression models are presented as specifically regulated (more and less highly expressed) in iPE (B) and DVT-associated PE (C) and shared regulated (more and less highly expressed) in iPE (D) and DVT-associated PE (E), including the attribution to the release mechanisms: granule secretion in purple (functional class: secretory proteins), shedding/EV in blue (functional classes: membrane receptor and channels) and putatively EV in cyan (functional classes: protein kinases and phosphatases, mitochondrial proteins, signaling and adapter proteins, proteasomal proteins, transcription and translation, other nuclear proteins, other metabolism). All regression models were adjusted for age, sex, arterial hypertension, diabetes, smoking, obesity, atrial fibrillation (AF), congestive heart failure (CHF), chronic inflammatory disease (CID), chronic kidney disease (CKD), chronic liver disease (CLD), coronary artery disease (CAD), history of stroke, pulmonary arterial hypertension (PAH), baseline antiplatelet agent use, baseline anticoagulant use, platelet count, red blood cell count (RBC), leukocyte count (WBC). Abbreviations and symbols: ↓ - less highly expressed in PE vs iDVT. DVT-PE – deep vein thrombosis-associated pulmonary embolism, EV – extracellular vesicles, iDVT – isolated deep vein thrombosis, iPE – isolated pulmonary embolism, LASSO – least absolute shrinkage and selection operator.

Isolated PE-specific secretory proteins included urokinase-type plasminogen activator (*PLAU*), cathepsin D (*CTSD*), C-X-C motif chemokine 1 (*CXCL1*), C—C motif chemokine 3 (*CCL3*), the antimicrobial protein neutrophil defensin 1 (*DEFA1*), interleukin1α (*IL1A*), the extracellular matrix glycoprotein tenascin-X (*TNXB*) and the platelet-derived growth factor subunit β (*PDGFB*) (Fig. 6B). Secretory proteins specifically regulated in DVT-associated PE included the α-1-microglobulin/bikunin precursor (*AMBP*), the monocyte chemotactic protein 3 (MCP-3, *CCL7*), transforming growth factor β-1 (*TGFB1),* myeloperoxidase (*MPO*), MMP-3 (*MMP3)*, the metalloproteinase inhibitors (*TIMP*) 1 and 4, β-galactosidase (*GLB1*), platelet activation stimulator nidogen-1 (*NID1*), a regulator of cell growth granulin (*GRN*), chemokine CXCL5 (*CXCL5*) and spondin-1 *(SPON1)* (Fig. 6C).

Among proteins assigned to the functional class “membrane receptors” or granule membrane proteins assigned to the functional class “membrane receptors/secretory proteins”, i.e. P-selectin (*SELP*), CD40 ligand (CD40L, *CD40LG*), lysosome-associated membrane glycoprotein

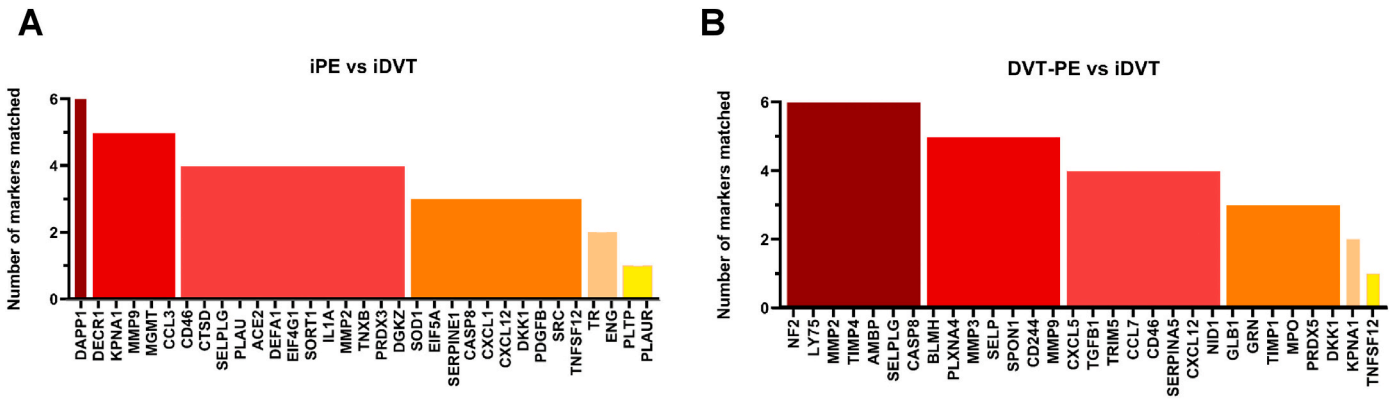

Fig. 4. Associations between regulated platelet-related proteins and six prominent platelet activation markers of the PEA panels. LASSO-regularized logistic regression analysis was applied to determine associations. The number of platelet prominent proteins, which associated with each regulated platelet-related are presented for iPE (A) and DVT-associated PE (B) vs. iDVT as “number of markers matched”. All LASSO-regularized selected proteins associated with at least one prominent platelet activation marker.

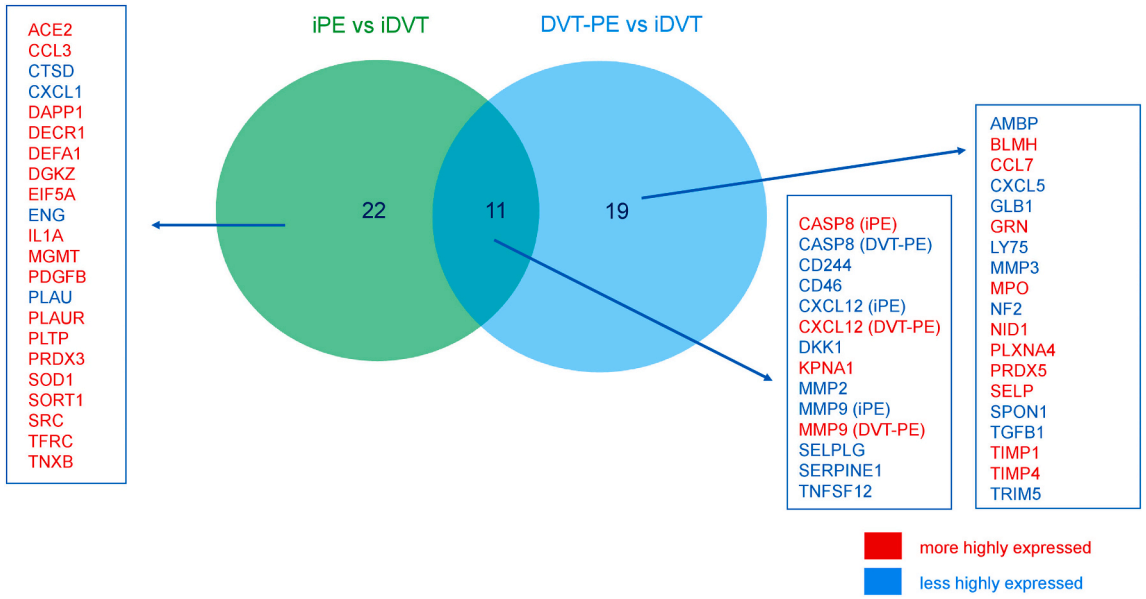

Fig. 5. Specific and shared regulation of platelet-related proteins in PE subtypes compared to iDVT. The Venn diagram includes the number of more and less highly expressed plasma proteins related to platelets, which shows specific and overlapping regulation in iPE and DVT-associated PE vs. iDVT.

3 (LAMP-3, *CD63*), lectin-like oxidized LDL receptor 1 (LOX-1, *OLR1*), tumor necrosis factor ligand superfamily, member 12 (*TNFSF12*) and 14 (*TNFSF14*), we assumed release via shedding and/or EV (Supplemental Table 1). For all other function classes putative release via EV was assumed (Supplemental Table 1).

For iPE, 23 % (5 of 22 proteins) of specifically expressed proteins were likely to be released via shedding and EV (Fig. 6). These comprise the adhesion promoter under static conditions endoglin (*ENG*), the transferrin receptor protein 1 (*TFRC*), urokinase plasminogen activator surface receptor (*PLAUR*), sortilin (*SORT1)* and angiotensin-converting enzyme 2 (*ACE2)* (Fig. 6B).

41 % (9 of 22) represented specific proteins putatively released via EV in iPE compared to iDVT, which belong to the functional classes "protein kinases and phosphatases", i.e. proto-oncogene tyrosine-protein kinase (*SRC*), "mitochondrial proteins", i.e. thioredoxin-dependent peroxide reductase (*PRDX3*), 2,4-dienoyl-CoA reductase, mitochondrial (*DECR1*), "signaling and adapter proteins", i.e. diacylglycerol kinase zeta (*DGKZ*), methylated-DNA-protein-cysteine methyltransferase (*MGMT*), dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide (*DAPP1*), "other metabolism", i.e. superoxide dismutase [Cu—Zn] (*SOD1*), phospholipid transfer protein (*PLTP*), and "transcription and translation", i.e. eukaryotic translation initiation factor 5A-1 (*EIF5A*) (Fig. 6B).

In contrast, only 16 % (3 out 19) and 21 % (4 out 19) of specifically expressed proteins in DVT-associated PE were assigned to platelet release via shedding/EV and putatively EV, respectively (Supplemental Fig. 2B). Lymphocyte antigen 75 (*LY75*), *SELP* and Plexin-A4 (*PLXNA4*) belong to the functional class "membrane receptors and channels" associated with a release via shedding. Proteins potentially released by EV belong to the classes "other metabolism", i.e. bleomycin hydrolase (*BLMH*), "proteasomal proteins", i.e. tripartite motif-containing protein 5 (*TRIM5),* "mitochondrial proteins", i.e. peroxiredoxin-5 (*PRDX5*) and "signaling and adapter proteins", i.e. merlin (*NF2*). The release mechanisms of shared between PE subtypes proteins are provided in Fig. 6D, E.

Overall, these data suggest differences in the release profile of platelet-related proteins between the PE subtypes in comparison to iDVT. In iPE, platelet-related proteins may be predominantly released via shedding from the platelet surface and via EV, whereas in DVT-associated PE platelet degranulation seems to be the more prominent platelet release mechanism of regulated proteins.

3.7. Network analysis of specific platelet-related protein signatures in iPE and DVT-associated PE

Protein-protein interaction (PPI) network analysis demonstrated differential interconnected clusters of specifically regulated proteins related to platelets in plasma from patients with acute iPE and DVT-associated PE compared to iDVT (Fig. 7). 73 % (16 of 22 proteins) of specifically selected proteins in iPE were suggested in four clusters, of which three were interconnected. All proteins in these clusters were more highly expressed with the exception of *ENG*, *CTSD*, *PLAU* and *CXCL1* (Fig. 7A, Supplemental Fig. 2A). For DVT-associated PE one cluster was created with 47 % (9/19) of specifically selected proteins, representing all granule cargo proteins (Fig. 7B). Here, the majority of secretory proteins related to platelets was more highly expressed (67 %, 6 of 9 proteins) compared to iDVT (Supplemental Fig. 2B).

4. Discussion

Recent studies provide evidence that clinical characteristics and outcomes differ between PE phenotypes and iDVT [36–38]. Our cohort of 541 patients with acute VTE after exclusion of active cancer demonstrated a higher prevalence of arterial hypertension, diabetes and chronic inflammatory, atherosclerotic and cardiovascular diseases in iPE and DVT-associated PE compared to iDVT. The greater inflammatory expansion and cardiovascular burden in PE subtypes were also reflected by higher levels of CRP, troponin I and NT-proBNP, respectively.

Medication prescription patterns differed accordingly, with antiplatelet therapy, i.e. ASA and clopidogrel overrepresented among patients with PE. On the one hand, this could be explained with prophylactic antiplatelet medication due to suspicion of myocardial infarction for patients with acute PE when initially presented to the hospital. On the other hand, the higher preponderance of cardiovascular disease could be associated with the higher proportion of antiplatelet agents in the PE groups, which raised the hypothesis that differences in platelet activity exist between PE and iDVT.

Therefore, the plasma proteome was analyzed by a targeted antibody-based PEA technology, covering 444 proteins, to compare the profile of regulated proteins, which are associated with platelet releasate of the PE subtypes iPE and DVT-associated PE with iDVT. Of 135 extracted platelet proteins, machine learning analysis by LASSO-

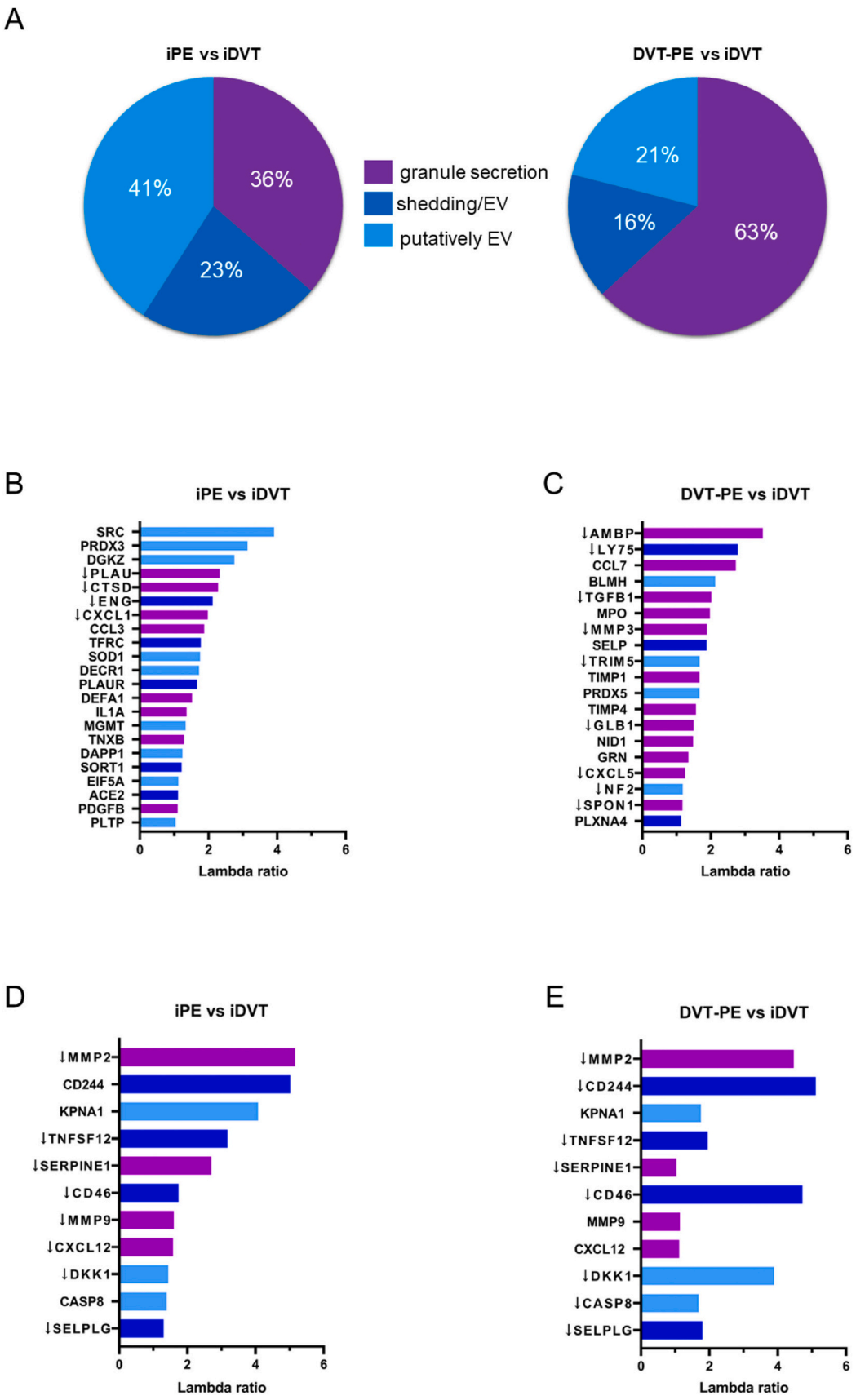

Fig. 6. Distribution, regulation and assigned release mechanisms of platelet-related plasma proteins in PE subtypes compared to iDVT. Distribution of release mechanisms assigned for specifically expressed platelet-related plasma proteins (A).

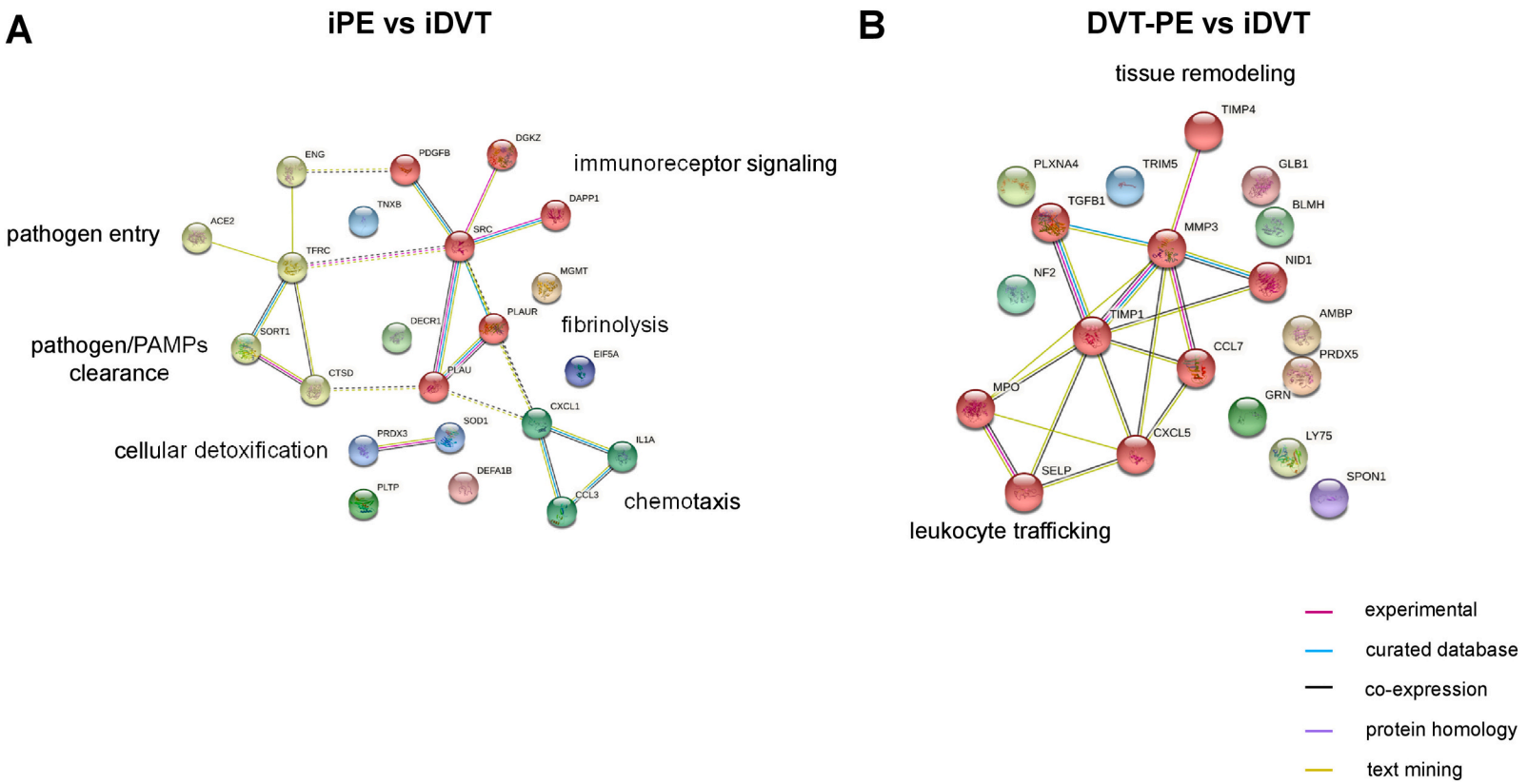

Fig. 7. STRING network analysis of specifically regulated platelet-related proteins associated with iPE and DVT-associated PE compared to iDVT. Colored solid lines represent connections between proteins based on experiments, databases, co-expression information, protein homology and text mining. Abbreviations: PAMP – pathogen-associated molecular pattern molecule.

regularized logistic regression models selected 24 % for iPE and 22 % for DVT-associated PE, which reflected differential protein profiles compared to iDVT. To validate the selected proteins as likely platelet-derived, the selections were related to six prominent platelet activation markers. Notably, all regulated proteins demonstrated a good association with these six prominent platelet activation markers, supporting their likely platelet origin in the plasma of patients with acute PE compared to iDVT. The platelet prominent chemokine and pro-angiogenic factor SDF-1α (*CXCL12*) with autocrine and paracrine functions involved in vascular remodeling was selected by LASSO-regularized regression analysis to be regulated in both PE subtypes. In contrast to iPE, SDF-1α demonstrated a more highly expression in DVT-associated PE than in iDVT, suggesting a potentially distinct role in vascular inflammation and atherogenesis [32].

This investigation revealed that both PE subtypes present with specific plasma protein profiles, which are associated with platelets. In iPE, LASSO-regularized regression and PPI network analysis resulted in 4 clusters of up to 6 functionally interacting proteins based on 22 specifically expressed platelet-related proteins compared to iDVT. The main cluster is linked to adhesive, pattern recognition and immune receptor signaling, including the prototype of Src family kinases (SFK) c-Src, which crucially transfer ligand signaling via the immunoreceptor tyrosine-based activation motif (ITAM)-associated platelet receptors Fcγ receptor IIA, GPVI, CLEC-2 (hemi-ITAM) and GPIbα [39,40].

In addition, the urokinase-type plasminogen activator (uPA, *PLAU*) receptor (uPAR, *PLAUR*) is also linked to Src signaling [41] and involved in plasmin generation, highlighting its role in immunity and fibrinolysis [42]. Leukocyte-ligated uPA has been shown to support lysis of venous thrombi [43]. Whether the uPAR-uPA axis on platelets also contributed to venous fibrinolysis remains to be investigated. The phosphatidylinositol 3,4,5-trisphosphate-binding protein dual adaptor for phosphotyrosine and 3-phosphoinositides (*DAPP1*), as well as the diacylglycerol kinase isoform ζ (*DGKZ*), are regulated by Src and implicated in the negative regulation of GPVI-mediated platelet activation [44,45].

Recently, angiotensin-converting enzyme 2 (ACE-2, *ACE2*) and the transferrin receptor (*TFRC*) have been identified as entry receptors for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [46]. However, there are still controversial reports, whether SARS-CoV-2 interacts with platelets via ACE-2 [47]. Pathogen entry is closely linked to processing and clearance of pathogens/pathogen-associated molecular patterns with a potential contribution of sortilin-1 (*SORT1*) [48], which also mediates targeting of the aspartic protease cathepsin-D (*CTSD*) to lysosomes [49]. In lysosomes, cathepsin-D participates in autolysosomal degradation [50]. Cathepsin-D is also stored in platelet lysosomes [22], secreted upon platelet activation and involved in local degradation of extracellular matrix components [51].

The neutrophilic antimicrobial protein α-defensin (*DEFA1B*), located in platelet α-granules and released by activated platelets, induces the formation of misfolded fibrinogen and thrombospondin-1 like structures, which activates human platelets via the integrin $\alpha_{IIb}\beta_3$ [52]. The chemokines Gro-1 (*CXCL1*), MIP-α (*CCL3*) and the cytokine IL-1α (*IL1A*) are abundant secretion products of platelet α-granules, driving the activation and pro-inflammatory response of innate immune cells [53]. IL-1α has been demonstrated to be activated by thrombin, which is highly generated on procoagulant platelets [54]. The interconnected thiol-specific peroxidase peroxiredoxin 3 (*PRDX3*) and superoxide dismutase-1 (*SOD1*) protect against cellular oxidative stress induced by peroxides, which have been shown to mediate venous thrombosis and enhanced platelet activity in aging mice [55].

In contrast to iPE, DVT-associated PE presented with one cluster of 9 directly interacting plasma proteins related to platelets involved in tissue remodeling and leukocyte trafficking. The tissue inhibitors of matrix metalloproteinases TIMP1 and TIMP4, which are prominent effectors of tissue remodeling, were more highly expressed in DVT-associated PE than in iDVT and are secreted from platelet α-granules. Higher plasma levels of both TIMP1 and TIMP4 have been reported to be associated with type 2 diabetes, arterial hypertension and myocardial infarction [56,57], which is in line with the higher prevalence of these CVRF and major cardiovascular events in DVT-associated PE than in iDVT in our study.

P-selectin predominantly shed from the platelet surface after α-granule exocytosis as soluble P-selectin, is a reliable marker of *in vivo* platelet activation [58,59]. Soluble P-selectin, which plays an important role in platelet and leukocyte adhesion to the activated vessel wall [60], has been reported to be elevated in the circulation from patients with DVT [14,61] and to be a risk factor of VTE recurrence [15]. In our study, machine learning-based approaches differentiated a higher expression of P-selectin in the plasma from patients with DVT-associated PE compared to iDVT, suggesting a relation with DVT disease severity. In line with the importance of leukocyte recruitment via adhesive sP-selectin interactions, the chemokines MCP-3 (*CCL7*) and ENA-78 (*CXCL5*), preferentially activating and attracting leukocytes [62–64], presented with more highly expression in the plasma of DVT-associated PE compared to iDVT, too.

Accordingly, myeloperoxidase (*MPO*), mediating the production of hypochlorous acid, was also upregulated in this PE subtype. Hypochlorous acid has been shown to trigger the microbicidal activity of neutrophils [65] and to increase platelet P-selectin surface expression and ROS formation [66]. Furthermore, hypochlorus acid-modified proteins are potent platelet agonists [67].

Recently, we published that VTE phenotypes studied within the GMP-VTE cohort also differ in platelet function [34]. LASSO-regression analysis adjusted for age, sex, cardiovascular and VTE risk factors, comorbidities and antithrombotics, demonstrated that platelets from patients with iPE and DVT-associated PE show lower capacity of aggregation and tissue factor-triggered thrombin generation compared to iDVT *in vitro*. Interestingly, surface tissue factor was positively, thrombin peak height and spontaneous platelet aggregation in platelet-rich plasma were negatively linked in iPE compared to the iDVT phenotype, while thrombin velocity in platelet-rich plasma and platelet aggregation in response to ADP and epinephrine were negatively linked in DVT-associated PE in relation to iDVT. These results suggest, that 1) lower platelet reactivity *in vitro* may be associated with higher platelet activation *in vivo* during the acute phase of PE compared to iDVT and 2) PE-subtypes share common but also exhibit different platelet activation/reactivity patterns, supporting our data of platelet-related plasma protein profiles.

Although we attributed distinct platelet-related proteins to the release via EV, i.e. microparticles, exosomes and apoptotic bodies [68], this study did not address the quantification and characterization of EV in the plasma of VTE phenotypes. There is accumulating evidence that cancer-associated VTE is linked to increased plasma levels of microparticles, including platelet-derived microparticles [69,70]. In non-cancer patients, a significant increase in platelet-derived microparticles was only observed with recurrent but not with initial or unprovoked VTE compared to healthy blood donors [71,72]. The distribution of EV in the different VTE phenotypes remains to be elucidated in future studies.

Using LASSO-regression analysis we identified a good relation between regulated proteins in PE subtypes and at least 3 prominently released platelet proteins, i.e. GPIbα, P-selectin, CD40L, PDGF-β, SDF-1α and LAMP-3, analyzed in the PEA panels. The role of these prominent platelet proteins as soluble biomarkers of platelet activation in acute VTE has been only limited addressed. An increase in soluble P-selectin plasma levels with predictive potential has been established in patients with VTE and its phenotypes, while soluble CD40L was not significantly altered [15,61,73,74]. Because these prominent platelet proteins are also known to be released by other cell types, especially activated endothelial cells, and the analyzed PEA panels did not include the platelet selective protein GPVI, we cannot conclude that the regulated proteins are predominantly released by platelets. Additional studies are needed to specify the impact of different cell types on the release of these

platelet-likely proteins in PE. Furthermore, while optimized and standardized methods of blood and plasma sample processing were used for biobanking and PEA analysis, we cannot completely exclude artificial protein degradation processes *ex vivo*, which affect the composition of the plasma proteome.

In summary, our data indicate that iPE and DVT-associated PE exhibit specific and different plasma signatures that are involved in platelet-related immunothrombosis and thromboinflammatory processes when compared to iDVT. Moreover, platelet activation protein profiles appear to differ between PE subtypes, with a preponderance of secreted proteins in DVT-associated PE as opposed to proteins more likely to be shed or released into plasma by EV in iPE. Thus, platelets may contribute to the regulation of distinct plasma protein levels in the acute phase of PE, which differs between PE subtypes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2022.10.005.

Declaration of competing interest

S.H., C.G., S.S., and K.L. are employees of Bayer AG. The study was sponsored inter alia by Bayer AG. The sponsors had no role in the design or conduct of the research. H.T.C. received research funding outside the present study from Bayer and received outside the present study honoraria for consultation and/or advisory board participation, from Bayer, Alveron, Galapagos, Portola and Alexion. All reimbursements were transferred to the CARIM institute. H.T.C. and H.M.H.S. are shareholders with Coagulation Profile, a university spinoff small diagnostic company not involved in the present study. P.S.W. has received research funding outside the present study from Boehringer Ingelheim, Sanofi-Aventis, Bayer Healthcare, Daiichi Sankyo Europe and Novartis and received outside the present study honoraria for lectures or consulting from Boehringer Ingelheim, Bayer HealthCare, Evonik, AstraZeneca and Sanofi-Aventis. P.S.W. is principal investigator of the DIASyM research core (BMBF 161L0217A).

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