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Full Length Article

A prospective cohort study to identify and evaluate endotypes of venous thromboembolism: Rationale and design of the Genotyping and Molecular Phenotyping in Venous ThromboEmbolism project (GMP-VTE)

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ABSTRACT

Several clinical, genetic and acquired risk factors for venous thromboembolism (VTE) have been identified. However, the molecular pathophysiology and mechanisms of disease progression remain poorly understood. This is reflected by uncertainties regarding the primary and secondary prevention of VTE and the optimal duration of antithrombotic therapy. A growing body of literature points to clinically relevant differences between VTE phenotypes (e.g. deep vein thrombosis (DVT) versus pulmonary embolism (PE), unprovoked versus provoked VTE). Extensive links to cardiovascular, inflammatory and immune-related morbidities are testament to the complexity of the disease. The GMP-VTE project is a prospective, multi-center cohort study on individuals with objectively confirmed VTE. Sequential data sampling was performed at the time of the acute event and during serial follow-up investigations. Various data levels (e.g. clinical, genetic, proteomic and platelet data) are available for multi-dimensional data analyses by means of advanced statistical, bioinformatic and machine learning methods. The GMP-VTE project comprises $n = 663$ individuals with acute VTE (mean age: 60.3 ± 15.9 years; female sex: 42.8%). In detail, 28.4% individuals ($n = 188$) had acute isolated DVT, whereas 71.6% subjects ($n = 475$) had PE with or without concomitant DVT. In the study sample, 28.9% ($n = 129$) of individuals with PE and 30.1% ($n = 55$) of individuals with isolated DVT had a recurrent VTE event at the time of study enrolment. The systems-oriented approach for the comprehensive dataset of the GMP-VTE project may generate new biological insights into the pathophysiology of VTE and refine our current understanding and management of VTE.

1. Background

Venous thromboembolism (VTE), a condition which encompasses deep vein thrombosis (DVT) and pulmonary embolism (PE), is responsible for up to 300,000 deaths annually in the United States, and over half a million deaths per year in Europe [1]. The first symptom of PE is sudden death in one quarter of cases and of the patients diagnosed with acute VTE, 10–30% die within the first month after diagnosis [2]. VTE is associated with a high risk of recurrence (≈33% within 10 years of the initial event [2]), and can also entail quality of life-reducing or lethal long-term sequelae, such as the post-thrombotic syndrome (PTS) or chronic thromboembolic pulmonary hypertension (CTEPH) [3]. Moreover, VTE is also prone to underdiagnosis since it frequently remains asymptomatic [3].

VTE is widely considered to be the result of the excessive expression of coagulation factors, causing fibrin-rich thrombi to develop that can subsequently propagate and embolize downstream. Different factors influence this hypercoagulability, as was first recognized by Virchow [4]. Predisposing factors include genetic risk variants such as the autosomal dominant factor V Leiden, factor II (prothrombin) G20210A or various type I and II mutations of antithrombin, protein C and protein S [5]. In non-O blood type individuals, von Willebrand factor (VWF) and factor VIII levels are approximately 25% higher [6], elevating the risks of both arterial and venous thrombosis. A variety of conditions,

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including cirrhotic liver disease and nephrotic syndromes, are known to either promote the expression of coagulation factors or to inhibit endogenous anticoagulants [7]. Links between VTE and comorbid conditions such as cancer [8], inflammatory bowel disease (IBD) [9], cardiovascular diseases such as atrial fibrillation [10] and congestive heart failure [11], and autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis [12] have also been reported, suggesting that a diverse range of physiological systems interact with the coagulation cascade. Lastly, a host of so-called 'provoking' risk factors have also been established to contribute to the risk of VTE: examples of these are surgery, trauma, pregnancy, prolonged immobilization, and oral contraceptive use [13].

While it is evident that the pathogenesis of VTE is multifactorial, the knowledge of the biological processes underlying different manifestations of VTE is still limited. We know that VTE tends to originate in the calf veins, but it is not clear why in some patients calf thrombi spontaneously resolve while in others they propagate [14]. Further, it is not clear whether PE is always the result of a precipitating DVT, or whether it can develop in situ, aggravated by local processes such as ischemia and inflammation [15,16]. Despite recent advances, the role of platelets in venous thrombosis and their interactions with neutrophil extracellular traps (NETs), leukocytes and other cell types, still remains obscure. Too little is known about which molecularly distinct phenotypes of VTE are circumscribed by the term 'unprovoked'. Finally, it is of great interest whether differences are to be discovered in the proteomes of patients who develop recurrent VTE, versus those who do not. Gaining a deeper understanding of such differences is essential to provide more specific and individualized care to patients.

The Genotyping and Molecular Phenotyping in Venous ThromboEmbolism (GMP-VTE) project aims to fill these lacunae in information by analyzing genetic and extensive targeted proteomic expression data on VTE patients, in the acute phase and during sequential follow-up investigations. With this data, clinical phenotypes of VTE can be contrasted to elucidate the genetic components and molecular mechanisms that are involved in their development and the sequelae associated with them. In addition, supervised and unsupervised clustering methods may be useful to identify novel endophenotypes and molecular risk profiles of individuals with VTE.

2. Methods

2.1. Study aims

The goal statement of the GMP-VTE project is fourfold: by analyzing the genomic and proteomic expression data of VTE patients, we intend (1) to identify proteins that could potentially influence disease development and prognosis of VTE, (2) to improve the biological understanding of VTE, (3) to potentially define additional subtypes of VTE and (4) to evaluate new potential therapeutic targets. The ultimate aim underlying these goals is to improve treatment and secondary prevention of VTE and its sequelae.

2.2. Study design

The GMP-VTE project consists of four key components: (1) clinical data, including comprehensive information on the clinical profile, the VTE phenotype and routine laboratory data, (2) proteomic data, (3) genomic data and (4) a functional characterization of human platelets. Analytic methods and quality control strategies are clarified in detail in subsequent sections.

The GMP-VTE project has a prospective study design and comprises a baseline visit, containing information and biomaterial collected at time of enrollment, i.e. during the diagnostic workup of the acute VTE event, and follow-up visits, with information and/or biomaterial collected at 3, 6, 12 and 24 months post-index event (see Fig. 1). Proteomic and genomic profiling was performed in all individuals who had given explicit consent for both, while platelet function testing was performed in a subset of individuals (see section: Platelet function analysis).

2.3. Study participants

2.3.1. Patient recruitment and enrolment

Confirmed VTE patients were recruited from the VTEval (NCT02156401) [17] and FOCUS BioSeq [18] projects, two prospective, observational cohort studies with comprehensive biobanking, which enrolled individuals with DVT and PE, respectively. The VTEval project is a single-center study conducted at the Johannes Gutenberg University Medical Center Mainz in Mainz, Germany, while FOCUS BioSeq is a multi-center study conducted nationwide in Germany. In brief, both studies collected comprehensive clinical information and venous blood samples from acute VTE patients (and individuals with clinical suspicion of VTE in VTEval), and aimed to analyze and discover clinical risk factors and molecular markers for acute and recurrent VTE, including subclinical and clinical sequelae. Both studies employed all-comer designs, subject to inclusion and exclusion criteria. To be enrolled, patients had to be at least 18 years of age, had to have received an objective diagnosis via an imaging modality, and had to have provided written informed consent (see: Data protection and ethical procedures). Exclusion criteria specific to the FOCUS BioSeq study were a history of CTEPH and an incidental diagnosis of VTE. The individual study designs for both studies are described in detail elsewhere [17,18].

2.4. Data protection and ethical procedures

The GMP-VTE's parent studies (i.e. VTEval and FOCUS Bioseq) were approved by the independent ethics committee of each participating study site. The studies were designed and executed in accordance with all local legal and regulatory requirements, especially the General Data Protection Regulation (EU 2016/679) and the Declaration of Helsinki (2013, 7th revision). All GMP-VTE subjects provided written informed consent to participate in the parent studies. Written informed consent was obtained for biomaterial and blood sampling, genetic analysis, and sharing of data with research partners. Access to data entry systems and study database was regulated by password protection and enlisted user assignment. All data were pseudonymized prior to analysis or data sharing. The steering committees of both progenitor studies gave consent for the establishment of the joint GMP-VTE project, which adheres to all legal requirements of both studies.

2.5. Clinical information

2.5.1. Clinical definitions

In the GMP-VTE project, a provoked VTE was defined as VTE in the presence of at least one of the following risk factors: cancer (i.e., active cancer and/or therapy in the last 6 months), immobilization in the last 30 days, invasive surgery in the last 30 days, trauma in the last 30 days, long-distance travel (> 4 h) in the last 30 days, current pregnancy or puerperium, and current oral contraceptive use. Further sub-categorizations, i.e. VTE provoked by a transient risk factor or by a persistent risk factor, were made pursuant to the most recent recommendations issued by the Scientific Standardization Committee (SSC) of the International Society of Thrombosis and Hemostasis (ISTH) [13].

2.5.2. Comprehensive phenotyping of VTE

Detailed diagnostic reports allowed for the specific categorization of VTE by anatomical location, enabling investigations into phenotypic subtypes based on, inter alia, thrombus propagation (i.e., number of contiguous thrombosed venous segments), sidedness (e.g., unilateral or bilateral PE) or cardiac involvement (e.g., signs of right ventricular dysfunction in radiological report). In addition, a large set of relevant clinical variables, including detailed history of risk factors and comorbidities, were determined by conducting an extensive standardized

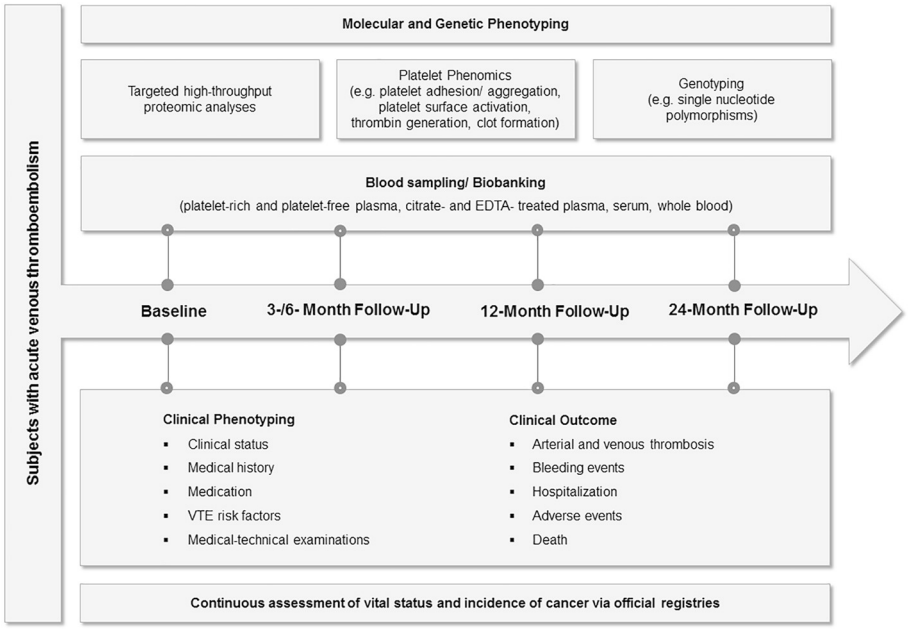

Fig. 1. GMP-VTE project cohorts.

investigation upon enrolment of each patient, including a detailed anamnesis based on an electronic case report form, and various anthropometric and hematological measurements.

2.5.3. Diagnostic modalities

All DVT patients received whole-leg compression ultrasonography with color Doppler, while imaging in PE patients was conducted using computed tomographic pulmonary angiography or ventilation/perfusion (V/Q) scintigraphy. Patients in whom PE was confirmed were additionally examined with ultrasonography. All diagnoses were independently confirmed by board-certified angiologists or radiologists connected to the relevant study sites.

2.5.4. Medication use

All medication use, irrespective of indication, was charted during baseline and follow-up investigations based on medical records and anamnestic information. Medication use was entered into the database using the Anatomical Therapeutic Chemical (ATC) classification system [19].

2.5.5. Clinical outcome

The clinical outcome of study participants is monitored in multiple ways (Fig. 1). Study participants receive standardized computer-assisted telephone interviews at regular intervals (3, 12 and 24 months after the index event), in which they are asked about the incidence of any adverse events since their last visit or call, and are asked questions relating to their therapeutic regimen and overall well-being. All answers are recorded in standardized forms. To minimize the potential for recall bias, incident adverse events are checked against medical records, insofar as these are available. In addition, study participants receive invitations for in-hospital or outpatient follow-up investigations, which are planned to occur at 3/6, 12 and 24 months post-index event. During this follow-up investigation, participants receive a full physiological examination, and blood samples are obtained for analysis. Lastly, mortality and incident cancer information is retrieved from local registry offices and official cancer registries. The specific clinical endpoints that are recorded for the GMP-VTE project are recurrent VTE, major and clinically relevant non-major bleeding, and arterial thrombotic events. This latter endpoint encompasses myocardial infarction, stroke and transient ischemic attack. Detailed definitions of all clinical endpoints are included in the Supplemental Appendix.

2.6. Data management

2.6.1. Harmonization of VTEval and FOCUS Bioseq projects

While study design and data acquisition are widely comparable between VTEval and FOCUS Bioseq, there are certain differences in study design-related aspects: most notably, FOCUS Bioseq focuses on individuals with acute PE (with or without concomitant DVT), whereas VTEval enrolled individuals with clinical suspicion of DVT and/or PE. Individuals enrolled in VTEval in whom acute VTE was objectively ruled out or who had an incidental VTE finding were excluded from the GMP-VTE project. During a detailed harmonization process, a shared set of variables was defined for use in the GMP-VTE project.

2.6.2. Quality control

A centralized data management team coordinated data harmonization and quality control. During data entry, integrity checks were in place to minimize entry failures; any missing data or inconsistencies were reported back to the respective site and clarified by the responsible investigator. The quality control included plausibility checks according to predefined procedures. The clinical dataset was recoded before scientific database storage. Biomaterials and data are only available for research purposes in this form (i.e., double pseudonymized).

2.7. Biobanking

Biomaterial (e.g. venous blood) obtained from all patients at the study centers was preprocessed for biobanking in specially trained laboratories according to uniform standard operating procedures (SOPs) [17,18]. Subsequently, all specimens were centrally stored in temperature-monitored freezers at −80 °C in the Biomaterial Bank Mainz (BMBM), University Medical Center Mainz, Germany.

2.8. Proteomic analyses

To comprehensively evaluate phenotype-associated differences in blood plasma proteome profiles of individuals with VTE, proximity extension assay (PEA)-based 96-plex immunoassay panels were utilized

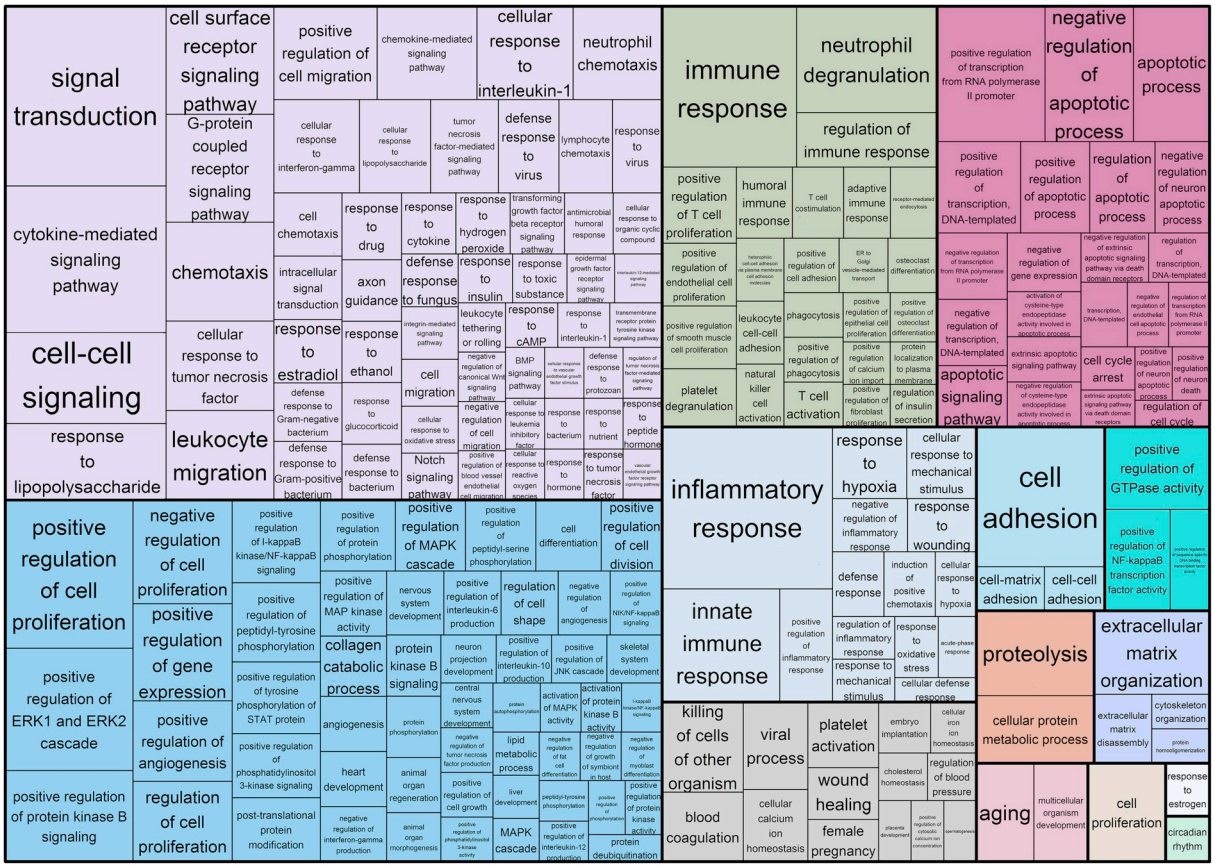

Fig. 2. Biological processes covered by the proteins analyzed in the GMP-VTE project.
Figure generated using REVIGO (http://revigo.irb.hr/). Taking an automatically generated list of gene ontology (GO) terms from the Uniprot database associated with each of the 444 profiled proteins as input, this "TreeMap" represents a clustered overview of biological processes covered by the GMP-VTE project. Each rectangle represents a biological process; the size of each rectangle is proportional to the number of proteins (i.e. frequency of a GO term in the input list) implicated in this process, and the larger squares (each with a distinct color) signify thematic 'superclusters'. Clustering was done based on Resnik similarity. Only processes with at least 6 associated proteins are shown in this figure.

(Proseek Multiplex 96 × 96; Olink Bioscience, Uppsala, Sweden). The initial characterization comprised five commercially available protein panels, each providing the relative quantification of 92 proteins (i.e. cardiovascular II, cardiovascular III, cardiometabolic, inflammation, and immune response panels) [20–22]. Limited overlaps in protein assays between panels were used for interplate comparisons and resulted in the quantification of 444 unique proteins per individual. The proteins cover diverse biological processes including inflammation, adaptive immune response, cytokine-mediated signaling, lymphocyte and mast cell activation, chemotaxis, coagulation, platelet activation, cell adhesion, proteolysis, extracellular matrix organization, blood vessel morphogenesis, angiogenesis, cellular metabolism, and response to hypoxia, in various areas implicated in disease (see also Fig. 2).

2.8.1. Proximity extension assay technology

Multiplex immunodetection of proteins by the PEA technique provides high specificity, while high sensitivity is achieved by real-time quantitative PCR (qPCR)-based signal amplification during detection [20–22]. To eliminate potential false-positive signals by antibody cross-reactivity, the technique uses pairs of antibodies linked to single-stranded DNA oligonucleotides (PEA probes) that bind to the same target antigen molecule. Upon hybridization after simultaneous binding in proximity on the same antigen molecule, one strand is extended by a DNA polymerase, creating a unique amplifiable DNA template sequence (i.e. an amplicon) acting as surrogate marker for a specific antigen. After unspecific pre-amplification, these amplicons are quantified by qPCR using protein-specific primer pairs, with the final number of PCR amplicons being proportional to the initial concentration of antigen in the sample. The resulting threshold cycle value (Fluidigm Real-Time PCR Analysis Software, Version 4.3.1, San Francisco, USA) for each protein is transformed to normalized protein expression (NPX) units using software (Olink® NPX Manager, Version 1.1.4.0, Uppsala, Sweden) supplied by the manufacturer. NPX units represent relative quantifications of protein concentrations on a log2-scale (i.e. an increase by 1 NPX represents a duplication of the protein concentration).

2.8.2. Relative protein quantification

EDTA-treated plasma samples of 663 individuals were thawed, aliquoted for each measurement into 96-well PCR plates, refrozen and stored at −80 °C until actually being measured. PEAs were then performed according to the user manual provided by the manufacturer. In brief, 1 μL of an EDTA-treated plasma sample is mixed with 3 μL incubation mix, containing the 92 probe pairs, in a 96-well PCR plate and subsequently incubated overnight at 4 °C. For DNA elongation and pre-amplification, 96 μL of extension mix are added to each sample, containing PEA enzyme and PCR reagents. These mixtures are incubated for 5 min at room temperature and then processed in an Applied Biosystems 2720 thermal cycler (Applied Biosystems Inc., Foster City, CA, USA; 20 min at 50 °C followed by 17 cycles). For quantification, 2.8 μL of each PCR product are mixed with 7.2 μL detection mix in a new 96-well PCR plate, from which 5 μL are transferred onto a primed 96.96 Dynamic Array™ IFC (Fluidigm, South San Francisco, CA, USA). After transferring 5 μL of the unique primer solutions for each protein onto the array chip, chip loading is finalized in the IFC controller HXTM

(Fluidigm, South San Francisco, CA, USA). Quantification itself is performed on a BioMark™ HD real-time PCR platform (Fluidigm, South San Francisco, CA, USA), running software supplied by the manufacturer (Olink Wizard for GenEx, MultiD Analyses AB, Göteborg, Sweden). Detailed information on internal quality controls, control samples and inter-plate normalization is presented in the Supplemental Appendix.

2.8.3. Duplicate measurements

Each of the five protein panels generates the relative expression levels of 91 (Cardiovascular III and Inflammation) or 92 (Cardiovascular II, Immune Response, and Cardiometabolic) proteins, respectively. Twelve proteins (i.e. CCL3, CXCL1, CCL11, FGF21, FGF23, IL-5, IL-10, IL-18, MCP-1, OPG, SCF, and uPA) were measured in duplicate and one protein (IL-6) in triplicate due to overlap between panels. For each protein existing in duplicate or triplicate, the measurement with the largest number of NPX units above the limit of detection (LOD) and minimum coefficient of variation in the pooled control samples was chosen for further analysis.

2.8.4. Processing of protein data

Preprocessing of proteomic data included transformation of proteins with skewed distribution, outlier detection, and missing value imputation. For each protein, a lower limit of detection was calculated based on the negative controls and subtracted from the measured NPX values. To ensure approximate normality, the NPX units of all proteins were subjected to different transformations (i.e., none, log, or square root), and kept in the form associated with the smallest absolute skewness.

All variables were checked for outliers after variable transformation. Univariate outlier detection flagged a sample if its distance to the median of all samples was greater than five times the (constant-adjusted) median absolute deviation, with the constant set to 1.4826 [23]. For multivariate outlier detection, the first two components of a principal components analysis (applied separately to each of the five PEA panels) were used and all samples with a distance greater than five times the median absolute deviation from the centroid (median of the first two principal components) were flagged as outliers. Outliers according to this definition were set to missing and subsequently imputed. Missing values were imputed using a Gibbs sampler based imputation method (GSimp [24], https://github.com/WandeRum/GSimp).

2.9. Platelet function analysis

2.9.1. Sample selection

Platelet function characteristics were analyzed in a subgroup of 180 randomly selected study participants recruited at the University Medical Center Mainz. Fresh citrated whole blood was analyzed within 2 h after blood withdrawal from baseline and follow-up visits of study participants. Detailed information on the platelet analysis procedures, including the timing of their initiation and duration of each procedure, are provided in Supplemental Table 1.

2.9.2. Analysis of biomaterial

Depending on the type of test, whole blood, platelet-rich plasma (PRP), platelet poor plasma (PPP) or platelet-free plasma (PFP) were used. PRP was isolated by centrifugation of whole blood at 200 $\times g$ for 10 min; PPP was obtained by 15 min of centrifugation at 2,000 $\times g$; PFP was obtained by centrifugation of whole blood for 5 min at 2,000 $\times g$. The collected PPP was further centrifuged for 10 min at 11,000 $\times g$. Five different assays of platelet function were carried out, enabling investigations into platelet adhesion, aggregation, activation and clot formation: A platelet function analyzer (PFA)-200 system (Siemens Healthcare, Marburg, Germany) was used to measure platelet adhesion and aggregation after collagen/adenosine diphosphate (ADP) and collagen/epinephrine (EPI) stimulation in whole blood at high shear rates. Platelet aggregation after adding different platelet agonists (i.e. ADP, EPI, collagen, arachidonic acid and thrombin receptor activating peptide) was measured in PRP using light transmission aggregometry (LABiTec, Ahrensburg, Germany). Calibrated automated thrombogram (CAT) assay (Thrombinoscope BV, Maastricht, the Netherlands) was used to measure thrombin generation in PRP (with adjusted platelet concentration to 150,000 platelets/μL using autologous PPP) after exposure to a trigger (e.g., tissue factor). To understand the contribution of platelets, thrombin generation in PFP was also investigated. Activation-dependent platelet surface antigens (i.e. P-selectin, CD63, fibrinogen, tissue factor and Annexin V) at resting conditions and PAC-1 after activation of whole blood with collagen/ADP trigger, were analyzed by flow cytometry using a BD Accuri C6 (BD Biosciences, San Jose, CA). Finally, rotational thromboelastometry (ROTEM; Werfen, Barcelona, Spain) was used to dynamically assay several parameters of clot formation in whole blood. The full set of tests produced a total of 26 variables of platelet characteristics in the context of VTE.

2.10. Genomic analysis

2.10.1. Genotyping

Within 2 h after blood draw, EDTA plasma was prepared and the blood clot stored at −20 °C until DNA isolation was carried out. For genotyping, high quality DNA was salted out [25] for 610 individuals, solved in Tris-EDTA (TE) buffer and stored at −80 °C. Genotyping was performed using the Illumina Infinium® Omni2.5Excome-8 v1.3 BeadChip according to the manufacturers' protocol using an automated workflow (Life & Brain GmbH, Bonn). Genotypes were called using GenomeStudio 2.0 (Illumina, Inc., San Diego) and the supported Illumina cluster file. Quality control and filtering was done using GenomeStudio 2.0 (Illumina, Inc., San Diego) and PLINK1.9 [26,27] according to basic quality parameters [28].

2.10.2. SNP selection

Selected SNP sets were prepared for genetic investigations of VTE-related mechanisms. Variants with known associations to VTE were extracted from databases using the GWAS Catalog [29] (www.ebi.ac.uk/gwas/) and PheGenI [30] (www.ncbi.nlm.nih.gov/gap/phegeni), filtering by disease-related search terms. For a better understanding of interactions between genetic variations and proteomic expression, SNPs were selected in accordance with possible or proven relationships. The SNP panel data set included variations with an established modulating effect on the target proteins, matched to the GTEx eQTL database [31] (gtexportal.org). Additional SNPs were selected based on their genomic location as follows: SNPs at the genomic locus of the target proteins itself, as well those within the range of 2000 base pairs (bp) upstream and 500 bp downstream from the locus, were included (dbSNP [32], build 150, hg 19). Furthermore, genes whose gene products are or could be involved in disease-relevant mechanisms were identified by combining database searches in OMIM [33,34], GO annotations of EBI-QuickGO [35,36] and Reactome [37] to form a comprehensive gene dataset. When SNPs were missing on the Omni 2.5 chip, these were replaced by proxies (with $R^2 > 0.98$), reducing redundant variants in linkage disequilibrium ($R^2 > 0.9$) to one using SNIPA, LDlink and PLINK1.9 [38–40].

2.11. Concept for statistical analysis

The overarching objective of the analyses planned for the GMP-VTE project is to explore the molecular mechanisms underlying specific VTE phenotypes with an innovative, multidimensional perspective on the pathophysiology of VTE. As described above, this is achieved by high-throughput multiplex technology that covers broad spectra of mechanisms within specific biological systems. These mechanisms are hypothesized to be involved in venous thrombosis, and proteins will be explored for significant under- or overexpression in a given phenotype. Alternatively, proteomic expression profiles for specific VTE

phenotypes will be investigated in isolation, e.g. by studying agnostically derived clustering structures in the data.

To fully leverage the data encapsulated by the project, advanced statistical and machine learning methods will be applied: The GMP-VTE project, by design, has a number of properties that will define the statistical techniques used for analyses. Firstly, due to the observational nature of the biodatabase, methods will be applied that can account for the presence of potential confounding variables, such as multivariable regression analysis. Secondly, the data contained in the project is high-dimensional, and in many analyses the number of variables (e.g. specific proteins) will be greater than the number of individuals included ($p > n$). The fact that many markers will have some degree of intercorrelation induces a multicollinearity problem that needs to be addressed by the methods used. Finally, appropriate methods should be selected to prevent overfitting in this setting. This setting suggests a combination of regularized regression techniques (e.g. least absolute shrinkage and selection operator regularization, LASSO method) and supervised (e.g. random forest, boosting) or unsupervised machine learning methods (e.g. hierarchical clustering, k-means clustering) for the analysis. In the interest of time and reproducibility, methods that necessitate little hyperparameter optimization will be preferred.

2.12. Implementation

The GMP-VTE project was initiated by the departments of Clinical Epidemiology and Clinical Studies of the Center for Thrombosis and Hemostasis (CTH) of University Medical Center, Mainz, Germany, in concert with Disease Genomics, Research & Development, Pharmaceuticals of Bayer AG. A steering committee, comprising senior researchers from both groups, was ultimately responsible for the design and execution of the project. Both partners have access to pseudonymized raw data, and actively collaborated on the development of interesting research directions and projects. Complete academic freedom, including decisions pertaining to the design, execution and publication of research projects, was guaranteed via contractual agreement.

The parent studies of the GMP-VTE project were registered online, at clinicaltrials.gov (VTEval: NCT02156401) and germanctr.de (FOCUS: DRKS00005939), respectively.

3. Results

3.1. Participants with proteomic, genomic, and platelet phenotype information

Protein profiling was conducted at baseline in the total sample of $N = 663$ individuals with a VTE event, and follow-up measurements were made at 3, 12 and 24 months post-index event (see also: Fig. 1). Genotyping was performed in 610 out of 663 individuals. Platelet phenotyping was performed in 180 individuals at baseline, and in 35 individuals in follow-up. Sample sizes may be further expanded in future.

3.2. Baseline characteristics of participants of the GMP-VTE project

A sample of N = 663 individuals with an acute VTE event at the time of study enrolment was included in the GMP-VTE project. A phenotype-stratified overview of baseline characteristics for all individuals is shown in Table 1. Of the subjects with PE, 71.6% ($n = 475$) were diagnosed with an acute PE with ($n = 315$, 72.9%) or without ($n = 117$, 27.1%) concomitant DVT. The remaining 188 subjects (28.4%) were diagnosed with acute isolated DVT. Individuals with PE were older than those with DVT and had a slightly lower proportion of women. As shown in Table 1, individuals with PE had a higher body mass index (BMI) and were less often current smokers. DVT individuals more often had an active cancer at the time of study inclusion. Among PE patients, 12.5% ($n = 56$) had a history of PE, and 24.2% ($n = 107$) a history of DVT. Among DVT patients with a recurrent VTE episode, history of DVT was far more frequently observed than history of PE.

Table 1
Baseline characteristics of the patients included in the GMP-VTE project, stratified by phenotype.

Characteristic	Pulmonary embolism[a] ($n = 475$)	Deep vein thrombosis ($n = 188$)
Age in years, mean (SD)	61.8 (15.8)	56.7 (15.6)
Body mass index (kg/m^2), median (IQR)	28.4 (24.9–32.4)	27.1 (23.9–30.9)
Female sex, n (%)	200 (42.1%)	84 (44.7%)
Smoking (current), n (%)	59 (13.8%)	43 (23.5%)
VTE risk factors		
Active cancer, n (%)	54 (11.8%)	27 (14.8%)
History of VTE	129 (28.9%)	55 (30.1%)
Deep vein thrombosis, n (%)	107 (24.2%)	54 (29.5%)
Pulmonary embolism, n (%)	56 (12.5%)	10 (5.5%)
Pregnancy, n (%)	2 (0.4%)	3 (1.6%)
Recent immobilization, n (%)	76 (16.7%)	26 (14.2%)
Recent surgery, n (%)	35 (7.7%)	8 (4.4%)
Recent trauma, n (%)	18 (4.0%)	10 (5.5%)
Recent travel, n (%)	49 (10.9%)	28 (15.4%)
Thrombophilia, n (%)	13 (3.7%)	10 (5.6%)
Comorbidities		
Arterial hypertension, n (%)	252 (56.2%)	65 (36.1%)
Atrial fibrillation, n (%)	24 (5.4%)	4 (2.2%)
Congestive heart failure, n (%)	27 (6.2%)	4 (2.2%)
Coronary artery disease, n (%)	34 (7.8%)	10 (5.5%)
Diabetes mellitus, n (%)	60 (13.5%)	20 (10.9%)
History of stroke, n (%)	30 (6.7%)	4 (2.2%)
Pulmonary arterial hypertension, n (%)	6 (1.4%)	1 (0.6%)
Laboratory parameters		
D-dimer (mg/L), median (IQR)	4.3 (2.03–10.59)	1.6 (0.83–4.38)
C-reactive protein (mg/L), median (IQR)	21.85 (7.53–58.5)	7.1 (2.9–16.88)
Leukocytes (per nL), median (IQR)	9.07 (7.01–11.6)	7.32 (5.84–9.51)

Abbreviations: IQR: interquartile range; VTE: venous thromboembolism; SD: standard deviation.
[a] ± deep vein thrombosis.

3.3. Platelet function analysis subsample

Of the 180 samples (female sex: 40.0%) designated for platelet function testing, $n = 38$ (21.1%) were patients with isolated DVT and $n = 142$ (78.8%) were derived from patients with acute PE with ($n = 100$, 73%) or without ($n = 37$, 27%) concomitant DVT. The mean age of study participants was 60.9 (± 16) years, and mean BMI was 29.1 (± 6.1) kg/m^2. Active cancer was present in $n = 20$ participants (11.5%). The clinical profile of this subsample did not differ relevantly from the overall sample as illustrated in Supplemental Table 2.

4. Discussion

By combining detailed phenotypic and genotypic information with extensive targeted proteomic expression data, collected both in the acute phase of disease and during follow-up, the GMP-VTE project enables granular investigations into the molecular pathophysiology and disease progression of the various clinical manifestations of VTE. This project, the first of its kind with this size and scope in the field of VTE, has the potential to contribute to an increased biological understanding of what constitutes particular manifestations of VTE (e.g., unprovoked VTE), identify new potential therapeutic or diagnostic targets, and to determine whether new subtypes of VTE could be identified to better guide therapy (e.g., the duration of anticoagulation treatment). Current clinical practice is guided by clinical risk scores and diagnostic tests with limited diagnostic accuracy [41–44], and therapeutic

recommendations are still routinely revised since pharmacologic treatment and prophylaxis are far from optimized. In certain phenotypes, such as isolated distal DVT and subsegmental PE, guideline recommendations are to monitor but not anticoagulate [45,46], even though the clinical consequences of such a strategy are not well-investigated. Finally, the underlying mechanisms that predispose cancer patients [8] and those with arterial thromboembolic [10,11], inflammatory or autoimmune disease to VTE should be elucidated, so that better preventive strategies for such patients may be devised.

While genomic studies have become common in VTE [47], proteomic analysis, certainly at the scale envisaged in this project, is still relatively uncharted in the field of VTE. The proximity extension assay technology, in particular, has not been applied to this pathology before. Previous proteomics-based studies in VTE, which focused on identifying diagnostic biomarkers by comparing VTE patients to control individuals from population cohorts [48,49] or on pathophysiological mechanisms [50], have used various forms of mass spectrometry for protein identification. Mass spectrometrical analysis identifies peptides or peptide sequences, which can be used to identify and differentiate between known proteins. In these untargeted approaches, any number of proteins can theoretically be detected; the primary limiting factors in this regard are knowledge, technical knowhow (e.g. of data mining, for the identification step) and the low sensitivity of mass spectrometry to low abundance proteins, which frequently turn out to be significant hurdles in practice [51]. Other technical difficulties further impede the widespread use of mass spectrometry [51]. By contrast, the PEA technology constitutes a targeted, specific antibody-based approach. The fact that one knows which proteins are detectable ahead of time benefit the certainty with which one can describe the output and, more importantly, the use of techniques to specifically amplify protein expression signals improves the sensitivity of the assay [20]. This also enables the accurate detection of low abundance proteins.

Genotypic information incorporated in the GMP-VTE project was not necessarily collected for the purpose of identifying new SNPs or genetic loci predisposing individuals to the development of specific VTE phenotypes. Instead, established genetic risk factors, e.g. Factor V Leiden or the prothrombin mutation G2021A, will be combined in genetic risk scores, which can be incorporated in analyses of protein expression data. In this manner, the variation in protein expression due to the phenotype of interest can be distinguished from that arising from heritability.

The contribution of platelets to venous thrombosis is increasingly recognized and supported by evidence from laboratory studies as well as randomized clinical trials [52–55]. Platelet-leukocyte interactions, in particular, have recently become recognized as key contributors to venous thrombosis. In mice, it was discovered that recruited neutrophils scan for activated platelets. In mice deficient in P-selectin glycoprotein ligand-1 (PSGL-1) neutrophil adhesion, rolling, extravasation and NET formation, and thus thromboinflammation, a mechanism which may be necessary for thrombus propagation [56], were significantly reduced [52]. Conversely, NETs, meshes of neutrophil granule proteins and chromatin, entrap platelets and activate them, further exacerbating thrombosis [53]. P-selectin and PSGL-1 antagonist therapy were demonstrated to have comparable efficacy to enoxaparin in reducing both venous thrombus burden and inflammation in several non-human primate models [54]. In addition, antiplatelet therapy reduced the risk of recurrent VTE by 42% in a placebo-controlled trial in humans [55]. This evidence supports the notion that platelets are relevantly implicated in human VTE, and with the GMP-VTE project we hope to contribute to the understanding of their involvement by extensively assessing platelet characteristics and platelet function in different human VTE phenotypes.

In summary, the GMP-VTE project allows investigations into VTE phenotypes from various perspectives, both in acute disease and follow-up settings. Since no biological system exists in a vacuum, applying a holistic, systems-oriented approach to research might help to advance our understanding of VTE. In future, the GMP-VTE project may yet be extended to discover further relevant mechanisms, e.g. by incorporating measurements of microRNA, dsDNA or DNA-histone complexes, or by the exploration of epigenetics.

Declaration of Competing Interest

Vincent ten Cate, Thomas Koeck, Marina Panova-Noeva, Steffen Rapp, Jürgen H. Prochaska, Michael Lenz, Andreas Schulz, Lisa Eggebrecht, M. Iris Hermanns and Thomas Münzel declare no conflicts of interest. Stefan Heitmeier, Thomas Krahn, Volker Laux and Kirsten Leineweber are employees of Bayer AG. Stavros Konstantinides reports grants and personal fees from Bayer AG, during the conduct of the study; grants from Boehringer Ingelheim, grants and personal fees from Actelion, grants and personal fees from Daiichi-Sankyo, grants and personal fees from BTG, personal fees from MSD and personal fees from Servier, outside the submitted work. Philipp S. Wild reports grants from Bayer AG and from the German Federal Ministry of Education and Research, during the conduct of the study; grants and personal fees from Boehringer Ingelheim, grants from Philips Medical Systems, grants and personal fees from Sanofi-Aventis, grants and personal fees from Bayer Vital, grants from Daiichi Sankyo Europe, personal fees from Bayer Health Care, personal fees from Astra Zeneca, personal fees and non-financial support from Diasorin and non-financial support from I.E.M., outside the submitted work.

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Author contributions

PSW and SVK are the principal investigators of the VTEval and FOCUS Bioseq studies, which formed the basis of the GMP-VTE project.

PSW conceptualized the GMP-VTE project.

MIH and TK were responsible for the targeted proteomics (proximity extension assay) measurements.

TK and ML performed the quality control and preprocessing of the proteomic data.

SR designed and supervised the genomic analysis.

MPN designed and supervised the platelet function testing component.

PSW, SVK and JHP supervised and directed the planning, collection and processing of clinical information for all study participants.

AS designed the concept for the statistical analyses, and is the lead statistician for the GMP-VTE project.

ML is responsible for the bioinformatic analyses of the collected data.

VTC, ML, TK, SR, MPN, JHP and PSW drafted the manuscript.

All authors contributed intellectual content, critically reviewed and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.07.019.

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