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Targeted proteomics for evaluating risk of venous thrombosis following traumatic lower-leg injury or knee arthroscopy

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Abstract

Introduction: Patients with lower-leg cast immobilization and patients undergoing knee arthroscopy have an increased risk of venous thrombosis (VT). Guidelines are ambiguous about thromboprophylaxis use, and individual risk factors for developing VT are often ignored. To assist in VT risk stratification and guide thromboprophylaxis use, various prediction models have been developed. These models depend largely on clinical factors and provide reasonably good C-statistics of around 70%. We explored using protein levels in blood plasma measured by multiplexed quantitative targeted proteomics to predict VT. Our aim was to assess whether a VT risk prediction model based on absolute plasma protein quantification is possible.

Methods: We used internal standards to quantify proteins in less than 10 µl plasma. We measured 270 proteins in samples from patients scheduled for knee arthroscopy or with lower-leg cast immobilization. The two prospective POT-(K)CAST trails allow complementary views of VT signature in blood, namely pre and post trauma, respectively. From approximately 3000 patients, 31 patients developed VT who were included and matched with double the number of controls.

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Results: Top discriminating proteins between cases and controls included APOC3, APOC4, APOC2, ATRN, F13B, and F2 in knee arthroscopy patients and APOE, SERPINF2, B2M, F13B, AFM, and C1QC in patients with lower-leg cast. A logistic regression model with cross-validation resulted in C-statistics of 88.1% (95% CI: 85.7–90.6%) and 79.6% (95% CI: 77.2–82.0%) for knee arthroscopy and cast immobilization groups respectively.

Conclusions: Promising C-statistics merit further exploration of the value of proteomic tests for predicting VT risk upon additional validation.

KEYWORDS

blood proteins, prediction, proteomics, quantitative evaluation, reference standards, venous thrombosis

1 | INTRODUCTION

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are two manifestations of venous thrombosis (VT), which is associated with considerable mortality and morbidity.^{1–3} Several genetic and environmental factors play a role in the development of VT.^{4,5} VT is in general characterized with an incidence rate of 0.1–0.2% per year in the general population.¹ Patients with lower-leg cast immobilization and patients undergoing knee arthroscopy have a 56-fold and 16-fold increased risk of developing VT respectively, in the first three months compared with the general population.^{6,7}

Recent studies have shown that thromboprophylactic treatment with low-molecular-weight heparin (LMWH) does not prevent VT following lower-leg casting or during the first 8 days after knee arthroscopy.⁸ Despite LMWH treatment, 1.4% and 0.7% still develops VT, respectively, which was not lower than the incidence in the control groups.⁸ These patients must have a strong propensity to develop VT to which thromboprophylactic treatment may need to be adjusted to either longer duration or higher dosage. For this, estimating an individual's VT risk is required, for which several prediction models have proven valuable (Table S1).^{9–14} These models mostly depend on clinical information and patient history.^{15–17} We assessed whether the prediction can be improved using protein absolute concentrations measured in blood.

We used targeted mass spectrometry-based proteomics to quantify blood plasma proteins and studied the association with subsequent VT in lower-leg casting and knee arthroscopy patients.^{18–21} Our approach is multiplexed and requires relatively low amount/volume of sample, typically less than 10 μ L plasma and over 100 target proteins.^{22,23} The purpose of this study was to assess the possibility of using precise plasma protein abundances in predicting venous thrombosis after knee arthroscopy or after lower leg injury.

ESSENTIALS

- Venous thrombosis (VT) risk prediction depends largely on clinical factors and allows reasonably good C-statistics of around 70%.
- Quantitative mass spectrometry-based proteomics with internal standard allows measuring absolute protein concentration in multiplex using low sample volume.
- 167 proteins were quantified in 10 μ L plasma from two prospective cohorts with complementary views of VT signature in blood, namely pre and post trauma.
- Using cross validation and bootstrapping, discriminating proteins predicted VT risk with C-statistics >78% demonstrating a potential for proteomics VT prediction test.

2 | METHODS

2.1 | Patients and sample collection

We used samples acquired from two large multicenter randomized clinical trials, designed to study the effectiveness of thromboprophylaxis for prevention of symptomatic VT, i.e., the Prevention of Thrombosis after Lower Leg Plaster Cast (POT-CAST) trial and the Prevention of Thrombosis after Knee Arthroscopy (POT-KAST) trial. Study details have been described elsewhere⁸ and the general characteristics of participants are summarized in Table 1. In short, between March 2012 and January 2016, patients aged 18 years or older, admitted to the emergency department who were treated with lower-leg cast immobilization (POT-CAST trial), and patients aged 18 years or older who had an indication for knee arthroscopy – such as meniscectomy, removal of loose bodies and diagnostic arthroscopy – were included (POT-KAST trial).

TABLE 1 General characteristics of patients included in the current study to evaluate the protein signature of VTE

	Knee arthroscopy		Lower-leg injury	
	Cases (N = 8)	Controls (N = 16)	Cases (N = 23)	Controls (N = 46)
Sex				
Male, n (%)	4 (50.0)	8 (50.0)	13 (56.5)	26 (56.5)
Female, n (%)	4 (50.0)	8 (50.0)	10 (43.5)	20 (43.5)
Age				
Median in years (25th–75th percentile)	50.5 (50.0–58.8)	51.0 (49.0–60.3)	55.1 (46.3–59.1)	53.4 (45.1–60.7)
BMI				
Median in years (25th–75th percentile)	28.7 (25.7–31.2)	27.0 (24.6–30.2)	27.9 (24.5–30.3)	26.4 (24.9–30.1)
Comorbidity				
Yes, n (%)	1 (12.5)	2 (12.5)	6 (72.7)	5 (11.4)
No, n (%)	7 (87.5)	14 (87.5)	16 (27.3)	39 (88.6)
Smoking				
Current smoker, n (%)	0 (0.0)	3 (18.8)	9 (42.9)	12 (27.3)
Non-smoker, n (%)	2 (25.0)	11 (68.8)	5 (23.8)	12 (27.3)
Former smoker, n (%)	6 (75.0)	2 (12.5)	7 (33.3)	20 (45.5)
Current use of oral contraceptives				
Yes, n (%)	1 (12.5)	1 (6.3)	2 (9.1)	1 (2.3)
No, n (%)	7 (87.5)	15 (93.8)	20 (90.9)	43 (97.7)
Malignancy in last 5 years				
Yes, n (%)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)
No, n (%)	7 (87.5)	16 (100.0)	22 (100.0)	44 (100.0)
Surgery in past 2 months				
Yes, n (%)	0 (0.0)	1 (6.3)	6 (28.6)	4 (9.1)
No, n (%)	8 (100.0)	15 (93.8)	15 (71.4)	40 (90.9)
Immobility in last 2 months (for at least 4 days)				
Yes, n (%)	1 (12.5)	0 (0.0)	3 (14.3)	1 (2.3)
No, n (%)	7 (87.5)	16 (100.0)	18 (85.7)	43 (97.7)
VT in first degree relatives				
Yes, n (%)	2 (25.0)	0 (0.0)	4 (21.1)	5 (12.5)
No, n (%)	6 (75.0)	16 (100.0)	15 (78.9)	35 (87.5)
Varicose veins				
Yes, n (%)	2 (25.0)	5 (31.3)	4 (19.0)	4 (9.1)
No, n (%)	6 (75.0)	11 (68.8)	17 (81.0)	40 (90.9)
ABO-blood type^a				
Homozygote O, n (%)	3 (37.5)	8 (50.0)	7 (30.4)	19 (45.2)
Heterozygote O, n (%)	4 (50.0)	7 (43.8)	13 (56.5)	21 (50.0)
Homozygote non-O, n (%)	1 (12.5)	1 (6.3)	3 (13.0)	2 (4.8)
FII20210 prothrombin mutation				
No polymorphism, n (%)	8 (100.0)	16 (100.0)	22 (95.7)	42 (100.0)
Heterozygote, n (%)	0 (0.0)	0 (0.0)	1 (4.3)	0 (0.0)
Factor V Leiden				
No polymorphism, n (%)	7 (87.5)	16 (100.0)	19 (82.6)	41 (97.6)
Heterozygote, n (%)	1 (12.5)	0 (0.0)	4 (17.4)	1 (2.4)

(Continues)

TABLE 1 (Continued)

	Knee arthroscopy		Lower-leg injury	
	Cases (N = 8)	Controls (N = 16)	Cases (N = 23)	Controls (N = 46)
rs2066865				
No polymorphism, n (%)	4 (50.0)	7 (43.8)	11 (47.8)	22 (52.4)
Heterozygote, n (%)	3 (37.5)	6 (37.5)	10 (43.5)	18 (42.9)
Homozygote, n (%)	1 (12.5)	3 (18.8)	2 (8.7)	2 (4.8)
rs2289252				
No polymorphism, n (%)	3 (37.5)	3 (18.8)	5 (21.7)	20 (46.5)
Heterozygote, n (%)	4 (50.0)	10 (62.5)	14 (60.9)	17 (39.5)
Homozygote, n (%)	1 (12.5)	3 (18.8)	4 (17.4)	6 (14.0)
LMWH assignment				
Yes, n (%)	5 (62.5)	7 (43.8)	9 (42.9)	20 (45.5)
No, n (%)	3 (37.5)	9 (56.3)	12 (57.1)	24 (54.5)

^aABO-blood type is stratified based on number of alleles (genes): i.e. in case of 'homozygote O' there are two alleles of blood type O, in case of 'heterozygote O' there is one allele of blood type O and one allele of a blood type other than O (A or B), in case of 'homozygote non-O' there are two alleles of blood type other than O.

Each trial included approximately 1500 patients, randomized to either low molecular weight heparin or no treatment (ratio 1:1). All patients completed a questionnaire on risk factors for VT at time of inclusion. In patients with lower leg injury, blood was drawn upon presentation at the emergency department, and in knee arthroscopy patients, blood was drawn within 4 hours before they underwent surgery. Citrate plasma and EDTA plasma was collected, centrifuged at 2500 g for 10 min, and then transferred to micro centrifuge tubes. All samples were stored at -80°C within 4 hours after blood collection. The medical ethics committee at Leiden University Medical Center approved both trials (Table 2).

2.2 | Study design

In a case-control design, nested in these trials, cases were patients who developed VT within 3-months following inclusion (31 patients in total, i.e., 23 from the POT-CAST and 8 from the POT-KAST trial). Controls (ratio 1:2), were patients who did not develop VT, i.e., 46 from the POT-CAST and 16 patients from the POT-KAST trial (Figure 1). Controls were matched on age and sex, and to account for sampling storage variation, on the hospital of inclusion and the time and date of sample storage (in this order). For all analyses, EDTA samples were used. For a sensitivity analysis, for validation and comparison with EDTA samples, we also included 10 citrate plasma samples (from POT-CAST and POT-KAST patients, with and without venous thrombosis).

2.3 | Sample preparation

Targeted mass spectrometry-based proteomics assays were developed previously for 270 proteins covered by 274 surrogate peptides

(Table S2). The assays development is part of our efforts to build a documented library of Multiple Reaction Monitoring (MRM) assays for blood plasma proteins.^{22,24,25} The peptides for the MRM assays were selected by PeptidePicker²⁵ and synthesized and validated as previously described.^{26,27} The sample preparation was performed in an automated manner using a Tecan Freedom Evo 150 robot. The Urea-based preparation protocol used was developed and applied previously.²⁸ For the current study, the digests were prepared through simultaneous denaturation and reduction of the homogenate with 9 M urea/20 mM dithiothreitol for 30 min at 37°C . Denatured proteins were alkylated with iodoacetamide (40 mM final concentration) for 30 min at room temperature, and then samples were diluted to reach a final urea concentration of 0.55 mM prior to tryptic digestion. Digestion was carried out at a 10:1 substrate:enzyme ratio using Tosyl phenylalanyl chloromethyl ketone -TPCK-treated trypsin (Worthington) for 18 h at 37°C . After digestion, samples were acidified with aqueous 1% formic acid (FA), and a chilled heavy labeled synthesized internal standard (SIS) peptide mixture was added. Samples were concentrated *via* solid phase extraction (10 mg Oasis HLB cartridges; Waters), using the manufacturer's recommended protocol. Finally, the samples were eluted with 55% acetonitrile (ACN)/0.1% FA (300 μl) and lyophilized to dryness. The dried samples were rehydrated in 0.1% formic acid to a final concentration of 1 $\mu\text{g}/\mu\text{l}$ for LC/MRM-MS analysis.

2.4 | Targeted proteomics

The samples were separated on-line with a RP-UHPLC column (EclipsePlusC18 RRHD 150 x 2.1 mm i.d., 1.8 μm particle diameter; Agilent) maintained at 50°C . Peptide separations were performed at 0.4 ml/min over a 56 min run, *via* a multi-step LC gradient (1.5–81% 2–80% mobile phase B; mobile phase B: 0.1% FA in ACN). The

TABLE 2 Top discriminating proteins between VTE cases and controls following traumatic lower-leg injury or knee arthroscopy

Protein names	UniprotKB accession	Gene names	Increase or decrease in cases relatively to controls
Knee arthroscopy			
Apolipoprotein C-III (Apo-CIII) (ApoC-III) (Apolipoprotein C3)	P02656	APOC3	Decrease
Attractin (DPPT-L) (Mahogany homolog)	O75882	ATRN	Decrease
Apolipoprotein C-IV (Apo-CIV) (ApoC-IV) (Apolipoprotein C4)	P55056	APOC4	Decrease
Apolipoprotein C-II (Apo-CII) (ApoC-II) (Apolipoprotein C2) [Cleaved into: Proapolipoprotein C-II (ProapoC-II)]	P02655	APOC2	Decrease
Coagulation factor XIII B chain (Fibrin-stabilizing factor B subunit) (Protein-glutamine gamma-glutamyltransferase B chain) (Transglutaminase B chain)	P05160	F13B	Decrease
Prothrombin (EC 3.4.21.5) (Coagulation factor II) [Cleaved into: Activation peptide fragment 1; Activation peptide fragment 2; Thrombin light chain; Thrombin heavy chain]	P00734	F2	Decrease
Plaster cast			
Apolipoprotein B-100	P04114	APOB	Increase
Apolipoprotein E (Apo-E)	P02649	APOE	Increase
Alpha-2-antiplasmin (Alpha-2-AP) (Alpha-2-plasmin inhibitor) (Alpha-2-PI) (Serp F2)	P08697	SERPINF2	Increase
Beta-2-microglobulin [Cleaved into: Beta-2-microglobulin form pl 5.3]	P61769	B2 M	Increase in female, steady in male
Afamin (Alpha-albumin) (Alpha-Alb)	P43652	AFM	Increase
Coagulation factor XIII B chain (Fibrin-stabilizing factor B subunit) (Protein-glutamine gamma-glutamyltransferase B chain) (Transglutaminase B chain)	P05160	F13B	Increase
Complement C1q subcomponent subunit C	P02747	C1QC	Decrease in male, steady in female

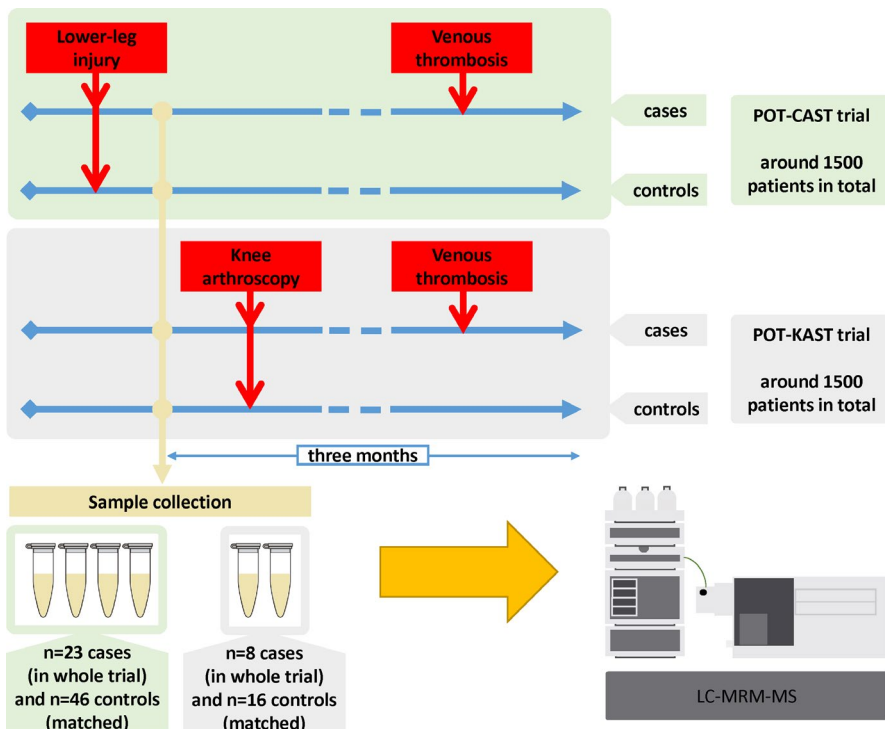


FIGURE 1 Overview of sample collection and measurement (the number of persons in the figure does not reflect the real number of patients in the trials)

exact gradient was as follows (time point in min, solution B %): 0 min, 2%; 2 min, 7%; 50 min 30%; 53min, 45%, 53.5 min, 80%; 55.5 min, 80%; 56 min, 2%. A post-column equilibration of 4 min was used after each sample analysis. The LC system was interfaced to a triple-quadrupole mass spectrometer (Agilent 6495/Agilent 6490) via a standard-flow ESI source, operated in the positive ion mode. The MRM acquisition parameters used for the quantitation were as follows: 3500 V capillary voltage, 300 V nozzle voltage, 11 L/min sheath gas flow at a temperature of 250°C, 15 L/min drying gas flow at a temperature of 150°C, 30 psi nebulizer gas pressure, 380 V fragmentor voltage, 5 V cell accelerator potential, and unit mass resolution in the first and third quadrupoles. For optimal peptide collision induced dissociation, peptide-specific collision energy values had previously been determined experimentally.

2.5 | Determination of protein concentration

The data was inspected using MassHunter Quantitative Analysis (version B.07.00; Agilent) and Skyline Version 3.1.²⁶ This involved peak inspection to ensure accurate selection, integration, and uniformity (of peak shape and retention time) of the measured peptides. For each peptide, the relative peak area ratios of the endogenous to the heavy labeled peptide were calculated. This ratio and the known concentration of internal standard were used to calculate the concentration of the endogenous peptide in the sample by comparison to a standard curve.²⁶ The criteria used for the standard curve regression analysis were $1/x^2$ regression weighting, <20% deviation in a given level's precision and accuracy for each concentration level.²⁶

2.6 | Data and statistical analysis

We used Ward agglomerative method to perform the hierarchical clustering on the scaled and centered protein concentration values. Various methods for feature selection were used to determine top predictors including iterative random forest classification²⁹ for weighted selection, least absolute shrinkage and selection operator - LASSO,³⁰ grouped-lasso penalties - GAMSEL,³¹ multiple logistic regression, as well as non-parametric Wilcoxon rank-sum test. We compared the performance of the different methods to conclude that non-parametric Wilcoxon rank-sum test outperformed the others. Comparison was based on assessing the strength of discrimination between cases and controls in a simple logistic regression discriminator. Logistic regression modeling was also used to calculate the area under the curve or C-statistics with the top predictors using cross validation with 80% and 20% of the data entries for training and testing, respectively. While we primarily used cross validation, parallel to that we also applied validation by bootstrapping for comparison. Here, instead of dividing the data entries into 80% and 20% training and testing sets, the training set is generated using sampling with replacement with the same number of

entries as the whole data set, while the testing set is generated from the remaining entries that were not selected for training. For cross validation and bootstrapping, we used 100 repeats which were enough for the results to converge. When compared to 200 and 500 and 1000 repeats there were no significant changes in the reported AUC. We ensured that the training set included always at least one female and one male patient. We handled the two clinical situations separately in our analysis. Because sex-specific profiles based on plasma protein levels were previously reported in human and mouse,³²⁻³⁶ we investigated whether stratification according to sex was necessary. For that, we identified top discriminating proteins between female and male subjects, including patients and controls, and we assessed the strength of the discrimination by C-statistics based on cross validation as described before. All data analysis steps and visualization were performed in R statistical software tool version 3.6.2. This study is exploratory, and the purpose of all feature selection methods was to reach an ordered list of variables from which the top discriminators are used in the final logistic regression model. These top discriminators are not affected by a false discovery rate correction. Rather than evaluating each protein individually, as one would do in a mechanistic study, we have evaluated the prediction models themselves using cross validation and bootstrapping and by providing the corresponding confidence intervals.

3 | RESULTS

We used a quantitative targeted proteomics panel that covers 270 proteins, which included the proteins that we were able to quantify in plasma in previous experiments.^{22,23,27} We were able to detect 197 proteins and quantify 167 proteins in our samples (Table S2). For the quantification we have considered all protein concentrations within 50% and above of their lower limit of quantification. The measured proteins included many coagulation and complement factors, covering 64 proteins in the complement and coagulation cascade, of which 45 were successfully quantified. Figure 2 summarizes the determined concentrations from all 93 EDTA and 10 citrate plasma samples. In Supplementary Protein Concentration Report we included box and whisker plots for each protein individually along with the Wilcoxon rank-sum test results.

3.1 | Hierarchical clustering

In the hierarchical clustering in Figure 2, citrate plasma samples were grouped together. The clustering shows that almost all determined protein concentrations using citrate plasma are of lower levels compared to EDTA plasma samples. Median protein concentration ranged between 72% and 94% in citrate plasma of that of EDTA plasma. In addition, various proteins could only be measured in EDTA samples. This is mainly attributed to these samples containing a stronger anticoagulant that allows for less intrinsic protease and

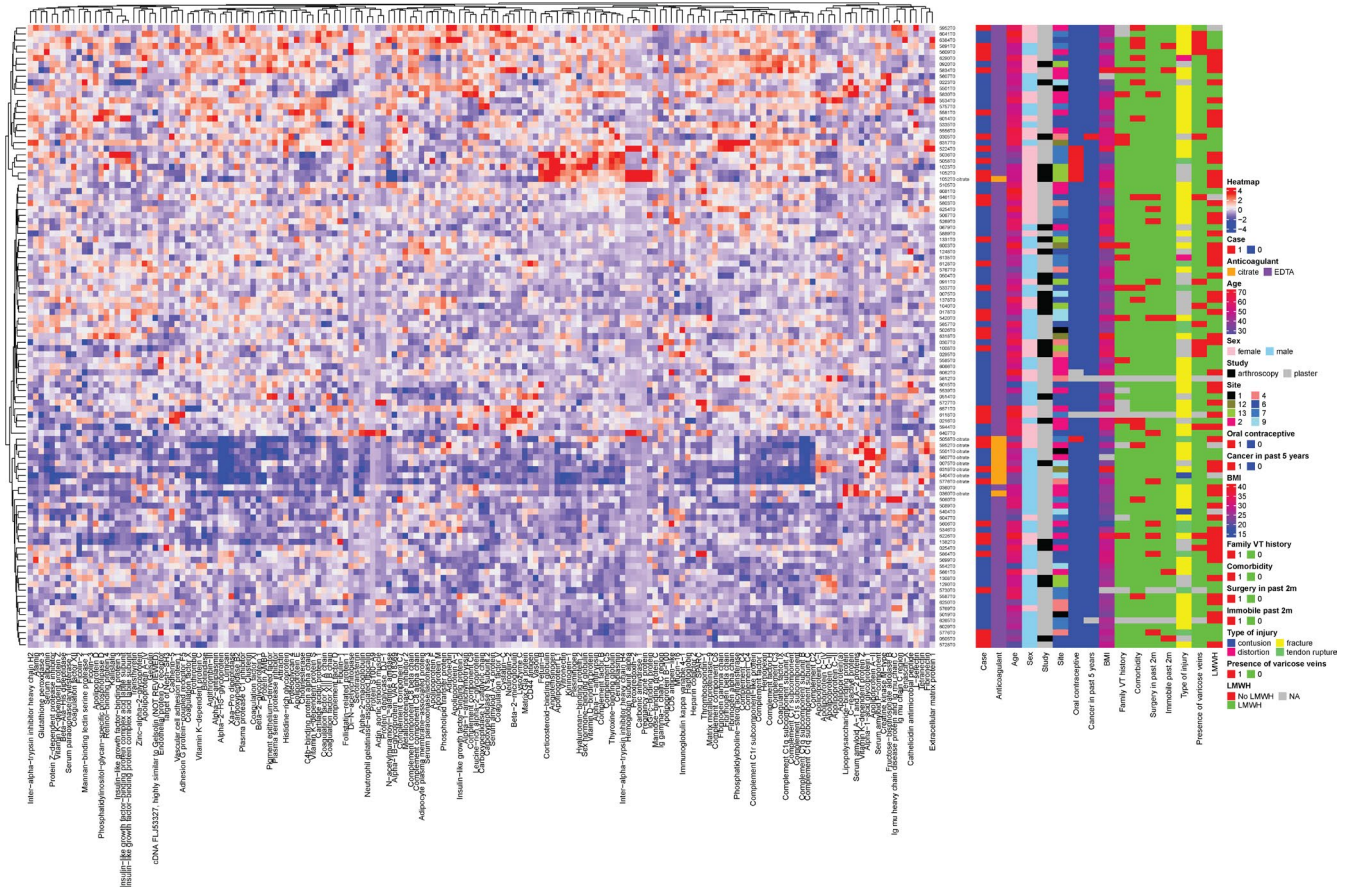


FIGURE 2 Heatmap and hierarchical clustering of quantified proteins in all samples. The hierarchal clustering of patients is shown in the left side and of proteins on the top. The heatmap in the middle shows scaled protein concentrations (relative to mean of measured samples of each protein). Sample annotations are included on the right side along with the associated color legend. Annotations include case: with red for patients with VTE and blue for no VTE patients; anticoagulant used: citrate indicated in orange and EDTA in purple; patient age: on a color scale from 30 to 70 (blue to red); patient sex with pink refers to female and sky-blue to male; study: either knee arthroscopy in black or lower-leg injury with plaster cast in grey; site of sample collection (numbered); use of oral contraceptive with red indicating recorded use and blue for no use; cancer/malignancy in last 5 years with 1 indicating yes in red and 0 indicating no in blue; BMI: on a color scale from 15 to 40 (blue to red); family VT history indicating whether there were VT incidents in first degree relatives with red indicating yes, green no, and grey unknown; comorbidity: with red indicating yes, green no, and grey unknown; surgery as well as immobility in the past 2 months: both with red for yes, green for no, and grey for unknown; type of injury of lower-leg injury patients: with blue for contusion, yellow for fracture, dark pink for distortion, and green for tendon rupture; presence of varicose veins: with red indicating yes, green no, and grey unknown; and finally LMWH indicating wither LMWH was used in green, not used in red, while grey indicating missing values. The clustering shows that citrated plasma samples were grouped together. No grouping is associated with the sample collection site. A strong cluster constitutes female individuals who used oral contraceptives. Furthermore, samples were partially grouped according to sex

peptidase activities during the initial sample handling. Nonetheless, excellent correlations between protein levels in citrate and EDTA plasma samples were determined (Figure S1). Looking closely at Figure 2, we did not recognize any cluster associated with the sample collection site, which indicates that there was no, or negligible batch effect related to collection procedure.

A second strong cluster constituted female individuals who used oral contraceptives, which left a strong signature on plasma protein abundances leading to clear grouping of these subjects. Interestingly, this cluster showed a stronger grouping effect than treatment group (i.e., knee arthroscopy or cast immobilization). Furthermore, samples were partially grouped according to sex. Hence, sexual dimorphism

protein profiles can be caught to some degree by the measured protein abundancies.

3.2 | Feature selection for best discrimination

After evaluating various strategies as mentioned in the Methods section, we concluded that the simplest strategy performed best. While random forest feature selection resulted in a protein set more suited for (unsupervised) hierarchical clustering, the feature selection based on Wilcoxon rank-sum test resulted in best discrimination using a regression model. In a simple yet robust approach, top

discriminating proteins were used in the predictors for each of the two clinical groups. As the number of discriminating proteins can vary; we studied the top 15 proteins and built our final regression models using a subset. Figure S2 shows the change in the C-statistics of the predictors for POT-CAST as well as POT-KAST based on consecutive addition of one discriminator at a time. Best discrimination was achieved using the selected discriminators as in Figure S2 and detailed below.

We investigated sexual dimorphism in our results, and a relatively good discrimination between male and female subjects, independently whether these were case or control subjects, was possible. Figure S3 shows a heatmap along with unsupervised hierarchical clustering based on the top 10 discriminators, which allowed a C-statistic of 94.3% (95% CI: 93.4–95.3%). Therefore, further analyses were stratified for sex.

3.3 | Venous thrombosis after knee arthroscopy and discrimination based on plasma protein profiles

Eight cases and 16 controls were included from the POT-KAST trial. This accounted for all cases present in the trial along with two matched controls per case. Figure S2-A represents the top 15 discriminating proteins as obtained by Wilcoxon rank-sum test along with the change in C-statistics as we include additional proteins. Figure 3 represents the six most discriminating proteins between cases and controls, stratified by sex, showing that cases had reduced levels of three apolipoproteins (C-II, C-III, C-IV), attractin, coagulation factor XIII, and prothrombin, i.e. APOC2, APOC3, APOC4, ATRN, F13B, and F2. Including these six proteins in a logistic regression model resulted in C-statistics of 88.1% (95%CI: 85.7–90.6%), calculated with repeated cross validation and similar values were reported with bootstrapping. Table 3 lists the regression coefficients and Figures S4 and S5 represent the hierarchal clustering obtained using these top discriminating proteins.

3.4 | Predicting venous thrombosis after lower-leg cast immobilization using plasma protein profiles

From the POT-CAST trial, 23 cases and 46 controls were included. Similar to POT-KAST, this accounted for all cases present in the trial ($n = 1519$) along with two matched controls per case. Figure S2-B represents the top 15 discriminating proteins as obtained by Wilcoxon rank-sum test and illustrates how C-statistics change with the inclusion of top discriminating proteins. A logistic regression using the seven top discriminating proteins resulted in C-statistics of 79.6% (95% CI: 77.2–82.0%) estimated using cross validation, similar values were reported with bootstrapping. Figure 4 represents these seven proteins, showing different trends in cases versus controls in the abundance of apolipoprotein B-100, apolipoprotein E, alpha-2-antiplasmin, beta-2-microglobulin, afamin, coagulation factor XIII B

chain, and complement C1q subcomponent subunit C, i.e., APOB, APOE, SERPINF2, B2 M, AFM, F13B, and C1QC. Table 3 lists the coefficients of the logistic regression model using these top discriminators. In comparison, using clinical predictors to discriminate between cases and controls such as age, sex, first-degree family history of VT event, comorbidity, presence of varicose veins, BMI, and previous surgery within 2 months before blood collection, all of which were part of various VT risk models such as L-TRIP,¹⁶ the AUC was 63.2% (95% CI: 59.8–66.5%). We have also assessed the performance of discrimination when combining these clinical predictors with proteomics. Except for sex, the various possible combinations of the clinical predictors with the proteomic top discriminating proteins did not improve the classification performance over using protein abundances alone. When using a binary sex predictor alongside the abundances of the top seven discriminating proteins a slight improvement in C-statistic to from 79.6 to 81.0% (95% CI: 78.7–83.3%) was observed (Figure 4). Figures S6 and S7 represent the hierarchal clustering obtained using top discriminating proteins in lower-leg cast immobilization patients.

4 | DISCUSSION

Using quantitative targeted proteomics, we profiled 93 samples from the POT-(K)CAST trials. From approximately 3000 patients, all 31 patients who developed VT were included and matched with controls. Logistic regression models resulted in C-statistics of 88.1% and 79.6% for knee arthroscopy and lower leg cast immobilization groups, respectively.

This work relayed on two prospective studies, in which blood was sampled in patients before the thrombotic event (Figure 1). To that end, large cohorts are necessary to find sufficient number of cases, and this explains the low number of cases despite collecting more than 3000 samples. Ultimately, external validation is crucial to validate our results, nonetheless it is important to bear in mind that the current samples were collected in a multicentric effort at different sites, and that we used cross-validation as well as bootstrapping for our models. To reach stronger statistics than what we have in our work, for example 70 cases, a cohort of 10,000 patients would be necessary, which would possibly require multinational efforts making it quite challenging.

4.1 | Quantitative targeted mass spectrometry to measure protein abundances

Targeted protein quantification with internal standards is suited for longitudinal and multicentric studies because it references comparability to spiked-in internal standards. This was essential for the translational aspect of our initial question; namely can precise blood plasma protein abundances be used to assess VT risk? We used targeted proteomics despite it not being widely available as a routine analysis technique in hospitals, which can be seen as a limiting factor

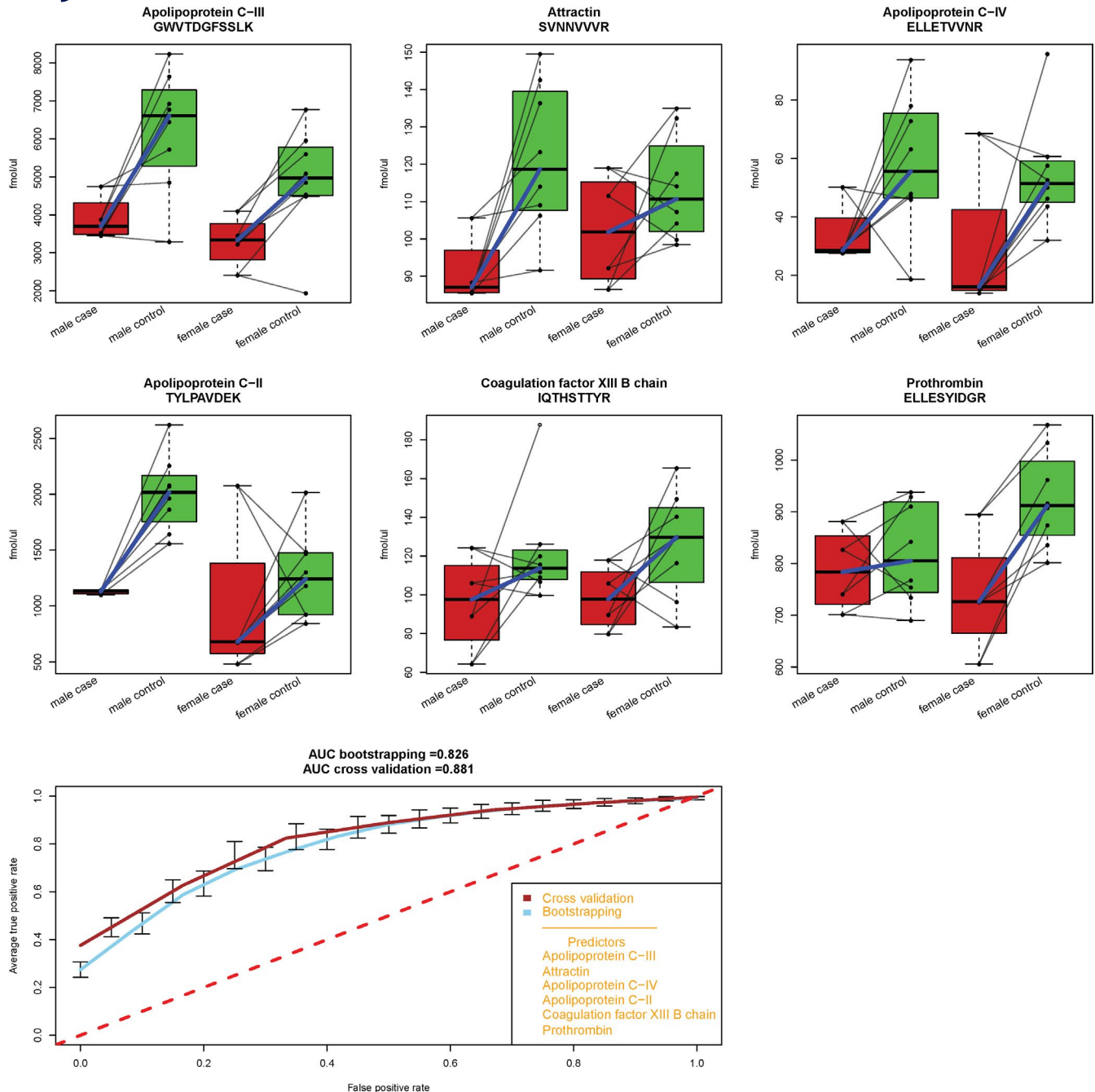


FIGURE 3 Top discriminating proteins in POT-KAST and associated prediction model. Six discriminating proteins between cases and controls showed cases had reduced levels of APOC2, APOC3, APOC4, ATRN, F13B, and F2. A logistic regression model average AUC of 88.1% (95% CI: 85.7–90.6%, calculated with repeated cross validation with 100 repeats, and 80/20% training/testing sets)

for the generalizability of our work. Nonetheless, by identifying the few best proteins that are sufficient to predict risk, we envision that these can be used in a panel that does not necessary depend on mass spectrometry. We showed previously how targeted mass spectrometry-based proteomics results correlates well with antibody as well as activity assays.³⁷

The proteomics community has demonstrated the ability to detect (not quantify) up to 912 proteins in plasma.³⁸ Here, we used a panel for 270 plasma proteins which were quantifiable in plasma

in previous experiments.^{22,23,27} Many assay development efforts, including ours, are performed under optimal conditions for sample collection and processing and therefore we do not expect to be able to quantify all proteins in real application. The panel used covered 64 proteins in the complement and coagulation cascade, of which 45 were successfully quantified (Figure 2). We were able to quantify many procoagulant and anticoagulant factors, as well as fibrinolysis proteins. This included coagulation factors II, V, IX, X, XI, XII, and XIII, as well as fibrinogen, prothrombin, thrombin-antithrombin complex,

TABLE 3 Coefficients of the logistic regression for POT-KAST and POT-CAST

	Cross validation				Bootstrapping			
	Median	Mean	95% CI of mean		Median	Mean	95% CI of mean	
			Lower limit	Upper limit			Lower limit	Upper limit
Top discriminating proteins in POT-KAST and associated prediction model (Figure 3)								
(Intercept)	-25.828	-27.895	-29.491	-26.299	-18.462	-19.753	-21.483	-18.023
Apolipoprotein C III	-40.113	-35.235	-39.004	-31.466	-23.934	-23.837	-28.05	-19.624
Attractin	-41.372	-43.984	-46.625	-41.342	-30.096	-30.274	-32.902	-27.647
Apolipoprotein C IV	6.278	0.967	-2.451	4.386	1.308	-2.024	-5.617	1.57
Apolipoprotein C II	-17.837	-15.555	-17.94	-13.169	-15.452	-13.505	-16.112	-10.898
Coagulation factor XIII B chain	-9.605	-9.309	-11.249	-7.368	-7.752	-8.693	-11.439	-5.946
Prothrombin	-22.532	-20.426	-22.767	-18.085	-18.984	-15.224	-18.436	-12.012
Top discriminating proteins in POT-CAST and associated prediction models (Figure 4)								
(Intercept)	-1.279	-1.332	-1.367	-1.298	-1.316	-1.461	-1.533	-1.389
Apolipoprotein B 100	1.088	1.116	1.073	1.159	1.138	1.245	1.132	1.359
Apolipoprotein E	-0.002	-0.012	-0.044	0.021	-0.012	0.052	-0.034	0.138
Alpha 2 antiplasmin	0.585	0.63	0.584	0.675	0.602	0.717	0.605	0.829
Beta 2 microglobulin	1.353	1.41	1.362	1.458	1.515	1.671	1.561	1.782
Afamin	0.276	0.277	0.237	0.317	0.35	0.349	0.257	0.442
Coagulation factor XIII B chain	-0.381	-0.282	-0.341	-0.222	-0.391	-0.305	-0.458	-0.152
Complement C1q subcomponent subunit C	-1.549	-1.605	-1.65	-1.56	-1.698	-1.812	-1.907	-1.718

and von Willebrand factor.³⁹⁻⁴⁵ Antithrombin, protein C, and protein S were also quantified.⁴⁶⁻⁴⁸ Quantified fibrinolysis related proteins associated with VT risk included alpha2-antiplasmin, fibrinopeptide A, plasmin-alpha2-antiplasmin, plasminogen, tissue plasminogen activator, and Carboxypeptidase B2 (thrombin-activatable fibrinolysis inhibitor - TAFI).⁴⁹⁻⁵² However, three proteins associated with VT risk were detectable but not quantifiable, coagulation factor VII, thrombomodulin and p-selectin. Three additional proteins of interest, factor VIII, tissue factor pathway inhibitor, and plasminogen activator inhibitor-1 were not detected. Upon examination, we concluded that alternative surrogate peptides could improve quantifiability. Previously, we quantified coagulation factor VII using a different, longer proxy peptide; i.e. VAQVIIPSTYVPGTTNHDIALLR³⁷ versus VSQYIEWLQK in the current work. Longer sequences are harder to synthesize, however in this case the longer peptide is better. Another example is Factor VIII, which is a protein with high number of natural sequence variants - around 490 known and predicted. The large number of variants makes it hard to find suitable proteotypic peptides. The peptide used LHPHYSIR contains few sites with various possible sequence variants; P2172L/Q/R, T2173A/I, H2174D, and R2178C/H/L. Although we always attempt to use peptides with no or insignificant (known) modifications and variances, our view on existing PTMs and variances is continuously updated with new published studies. Therefore, using multiple proteotypic peptides per protein is advantageous,³⁷ yet with a large number

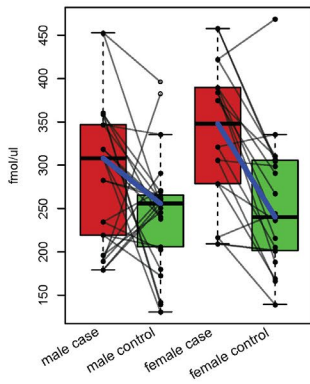
of targets, instrumentation speed is a limiting factor. Our panel required one-hour cycle per sample. Using longer gradient and adding additional fractionation steps allows measuring more targets, but increases cost.⁵³ Tissue factor pathway inhibitor can be quantified in a two-dimensional liquid chromatography method but not in a one-dimensional method.⁵³ In summary, balancing study objectives and number of multiplexed peptide assays is essential. For our objective, i.e. investigating predictability of VT risk using precise plasma protein concentrations, the panel used demonstrated the possibilities and future works may improve on our results.

4.2 | Cross validation and bootstrapping

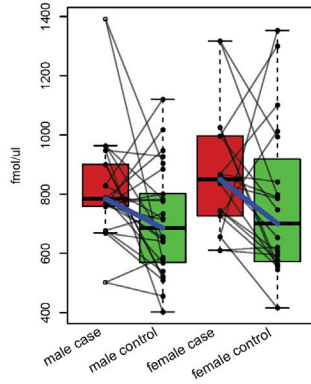
The POT-(K)CAST trials form a suitable testbed for developing a prediction model allowing two essential views of VT signature in blood, pre and post trauma. Cases from each cohort have their own control group and applying the same analytical and data analysis approaches allowed good comparability.

Validation in an independent cohort is important for testing prediction models. However, the low incidence rate in VT cases in general, makes it infeasible to replicate prospective trials like POT-(K)CAST. We opted therefore to perform computational approaches using cross validation and bootstrapping to assess predictability. Both approaches reported similar results as can be seen in Figures 3

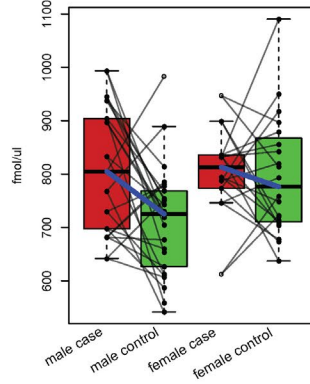
Apolipoprotein B-100
FPEVDVLTK



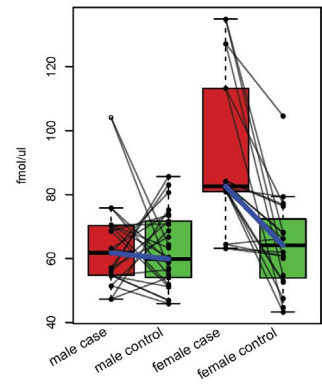
Apolipoprotein E
LGPLVEQGR



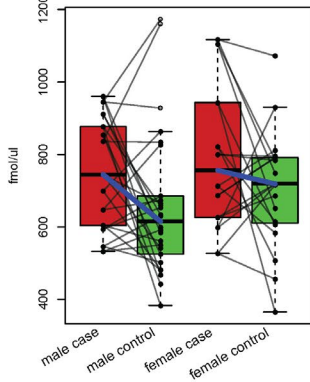
Alpha-2-antiplasmin
LGNQEPGGQTALK



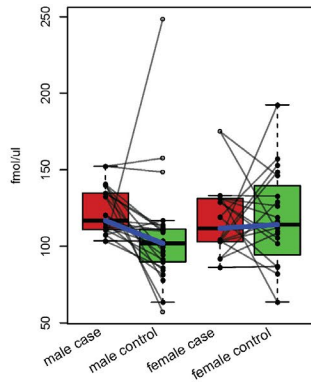
Beta-2-microglobulin
VNHVTLSPK



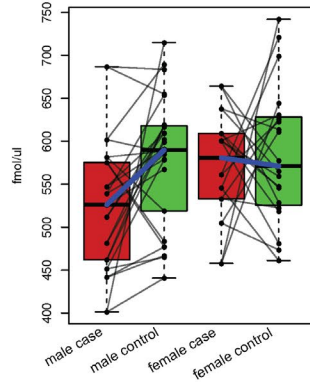
Afamin
DADPDTFFAK



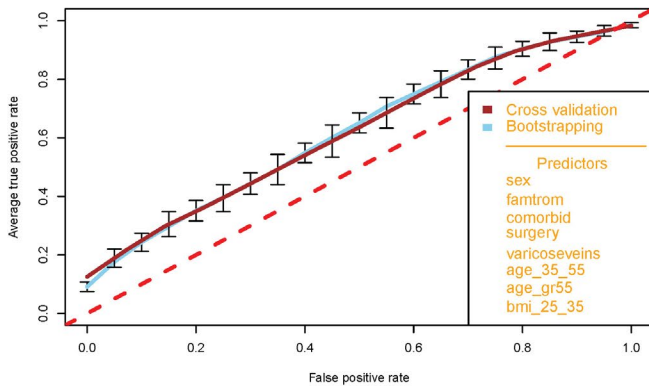
Coagulation factor XIII B chain
IQTHSTTYR



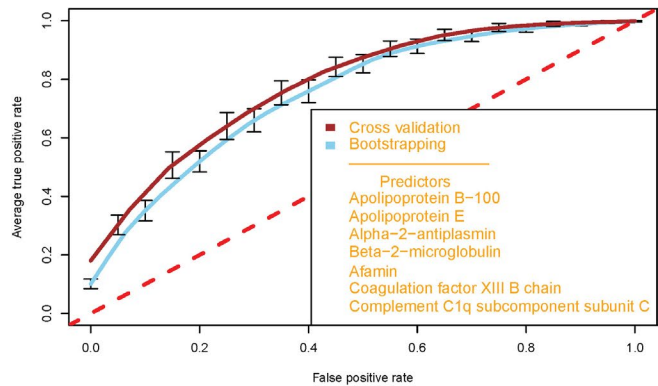
Complement C1q subcomponent subunit C
FQSVFTVTR



AUC bootstrapping =0.627
AUC cross validation =0.632



AUC bootstrapping =0.757
AUC cross validation =0.796



AUC bootstrapping =0.781
AUC cross validation =0.81

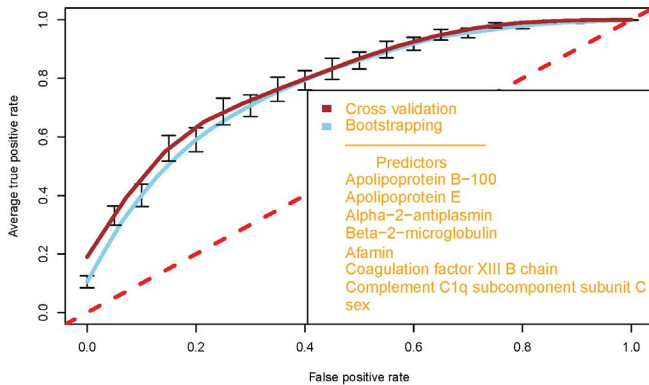


FIGURE 4 Top discriminating proteins in POT-CAST and associated prediction models. Seven proteins showed different trends in cases versus controls included APOB, APOE, SERPINF2, B2 M, AFM, F13B, and C1QC. A logistic regression these proteins resulted in an average AUC of 79.6% (95% CI: 77.2–82.0%, calculated with repeated cross validation with 100 repeats, and 80/20% training/testing sets). A logistic regression using clinical predictors (L-TRiP¹⁶) resulted in AUC of 63.2% (95% CI: 59.8–66.5%). Including sex with the top seven discriminating proteins resulted in a C-statistic of 81.0% (95% CI: 78.7–83.3%). In the three prediction models, the used predictors are mentioned in the legend. For the first prediction model we used clinical data alone, for the second protein abundances alone were used, and for the third we used protein abundance in addition of whether there was a surgery. famtrom: refer to history of VT in first degree relatives (Table 1)

and 4. Nonetheless, these computational approaches do not usually account for all variations in sample collection, storage, or other similar factors that are considered in an external validation using a separate cohort. However, because sample collection was multicentric, some of the aforementioned variabilities are inherited. The main goal of our work is to investigate whether, in principle, precise plasma protein profiling can be used to predict VT. To that end, cross validation and bootstrapping provided a good initial internal validation and further external validation as well as testing of each individual predictor should follow.

4.3 | Discriminating proteins in relation to VT after knee arthroscopy

Despite the low number of cases and controls in the POT-KAST we were able to pinpoint a discriminating profile based on the determined protein concentration (Figure 3). Having three apolipoproteins in the top discriminators indicates a relation between the levels of these proteins in blood plasma and the risk of VT. Apolipoproteins C-II, C-III, and C-IV are well characterized and are all constituent of circulating (triglyceride-rich) lipoproteins, are all relatively abundant in plasma, and are produced mainly in the liver, with C-II and C-IV being both part of the so-called APOE/C1/C4/C2 gene cluster⁵⁴ and CIII element of the APOA1/C3/A4/A5 gene cluster.⁵⁵ In both clusters, genes are coordinately expressed (i.e., share liver regulatory elements), and as such it is of interest that APOA4 (Figure 3) and APOE are part of the protein signature specific for patients that develop VT after arthroscopy or plaster cast, respectively (compare Figure 3 and Figure 4). As these C apolipoproteins are constituent of lipoproteins, their plasma levels are known to be associated with the risk of developing cardiovascular disease, in particular arterial disease.⁵⁶ While associations of C apolipoproteins with VT risk have not been reported in literature before, we recently found in case-control study that plasma C apolipoproteins (and also APOE) levels associate with the plasma levels of a number of pro-coagulant factors, suggesting a link between C apolipoproteins and VT.^{57,58}

Prothrombin and factor XIIIb are both well-described coagulation factors, are also abundant in plasma, and are mainly produced in the liver. Prothrombin and Factor XIIIb are involved in fibrin network formation upon (vascular) injury, with factor XIIIb, in complex with Factor XIIIa, being activated by thrombin (i.e. the cleaved and activated form of prothrombin). Plasma prothrombin level is known

to be associated with increased risk of VT.⁵⁹ Although less characterized, elevated Factor XIII (XIIIa and b in complex) levels are also associated with the risk of VT.^{60,61} Recently, a study found a relation between factor XIIIb and sex, i.e. factor XIIIb levels were lower in male VT patients than in females.⁶² As already mentioned above, we found previously that plasma C apolipoproteins associate with a panel of pro-coagulation factors and among these is prothrombin.⁵⁸ Of the six proteins that contributed to discrimination between cases and controls in knee arthroscopy patients, attractin is the most poorly characterized. Attractin has been reported to be involved in initial immune cell clustering during inflammatory response and to have a critical role in normal myelination in the central nervous system.⁶³ No link with vascular disease, including VT, has been described before, and therefore we can only speculate whether this protein relates to VT in a way beyond being solely a discriminating protein, i.e., a marker of something else.

4.4 | Discriminating proteins in relation to VT in patients with lower-leg cast immobilization

We measured an increase of APOB in cases compared to controls in the POT-CAST subjects. APOB has multiple functions in maintaining the homeostasis of liver-derived lipoproteins, especially of low-density lipoprotein (LDL). APOB is a component of LDL and ensures the binding of LDL to its receptor. In this way, it is able to regulate plasma levels of LDL and it has been identified to play a role in the pathophysiology of arterial thrombosis.^{64–66} A direct association between APOB and VT is not known, but one previous study showed that APOB/LDL is able to bind von Willebrand Factor (vWF) and regulate the proteolytic cleavage of vWF by ADAMTS13. When vWF is cleaved, it becomes less procoagulant.⁶⁷ For beta-2-microglobulin, afamin, and complement C1q-subcomponent subunit C, that are all relatively abundant in plasma, literature does not provide clues on how these proteins may relate to VT. In contrast, alpha-2-antiplasmin is a well-documented inhibitor of plasmin, i.e. the enzyme that degrades fibrin networks as present in thrombosis. Of interest, alpha-2-antiplasmin may be cross-linked to fibrin by factor XIII.⁶⁸ Alpha-2-antiplasmin levels have been investigated for association with the risk of VT, though associations were modest (OR 1.2) in particular as compared to other proteins involved in regulation of fibrin degradation (PAI-1, tissue-type plasminogen activator – tPA, thrombin-activatable fibrinolysis inhibitor – TAFI⁶⁹) and that are also part of the

MRM-assay used in the present study. As mentioned above, apolipoprotein E like the C apolipoproteins associates with VT in the MEGA case-control study, supporting some role for triglyceride-rich lipoprotein associated proteins in VT. Apolipoprotein E plays an important role in the distribution of vitamin K, which is necessary for the synthesis of coagulation factor II, VII, IX, X and protein C, S and Z.⁷⁰⁻⁷² Other studies state that certain genotypes of apoE are associated with higher risk of VT.⁷³

4.5 | Prediction of VT using absolute quantitation of blood plasma proteins

Mass spectrometry-based proteomics is viewed largely as a high throughput method to elucidate and study disease and biological processes. We opted to answer the question whether we can use proteomics to predict VT, without in-depth exploration of mechanistic insights. Our results should not be interpreted in a mechanistic way, which requires a different type of modeling and analysis. With the exception of coagulation factor XIIIb, the protein discriminators in the knee arthroscopy cohort are different from patients with cast immobilization after a traumatic lower-leg injury. While this may warrant a future emphasis on studying factor XIIIb in the context of VT in orthopedic interventions, it is important to point out that blood collection in both groups took place at different time points in respect to trauma (Figure 1). Factor XIIIb showed increase in abundances when blood was drawn after trauma, i.e. POT-CAST, and its abundance was decreased when blood was drawn before trauma, i.e. POT-KAST, both compared to own matched controls. It is probable that in POT-CAST patients trauma-induced coagulopathy had started leading to increase of various procoagulants in the systemic circulation.⁷⁴ This is not the case in POT-KAST patients as those are at rest situation awaiting intervention, knee arthroscopy, which occurred post blood sampling. Previously, we studied the procoagulant state and found that patients with lower-leg injury had higher levels of procoagulant factors, while in knee arthroscopy patients these levels remained steady.⁷⁵ This suggests that quantitative proteomics has predictive value both in plasma reflecting a person's steady state, as in plasma in a person in whom a severe event has just taken place. Mechanistic associations between Factor XIIIb and the other discriminating proteins and the differences in plasma profile before and after trauma for VT cases is a matter of a future additional experiment.

We also examined all possible combinations of top discriminating proteins as predictors and the relationship to the prediction strength evaluated by the C-statistics. We evaluated a minimum C-statistics of 59.9% for POT-CAST and 75.6% for POT-KAST patients. A maximum C-statistics for POT-CAST patient prediction of 82.5% was obtained when APOB, SERPINF2, B2 M, and C1QC were considered. For prediction in the POT-KAST cohort, a maximum of 95.6% was achieved with APOC3, ATRN, and F2. When all proteins are included as predictors, the confidence intervals of the beta value of APOC4

for POT-KAST and of APOE for POT-CAST patients overlapped zero (Table 3). While these proteins are discriminating cases from control subjects in the corresponding cohort, and therefore are important to consider in our discussion, when combined with other top discriminating proteins their contribution to the regression model is reduced or eliminated. In summary, while optimizing the number of predictors is possible, the fact that the worst models still have predictive values supports our conclusion about the possibility to use precise protein concentration to predict VT.

5 | CONCLUSIONS

We presented initial results on the possibilities of predicting VT using accurate measurement of plasma protein concentrations by targeted quantitative proteomics. The results were demonstrated in samples from the POT-(K)CAST trials for assessing the risk of VT following traumatic lower-leg injury or knee arthroscopy. While these initial results, assessed by internal cross validation as well as bootstrapping, demonstrated the principle of prediction of VT using protein abundances determined by targeted MRM proteomics, further external validation, especially due to the low sample size, is necessary. We showed how to measure 100+ proteins in low volumes of EDTA (<10 µl) and found a possible protein signature for VT. Protein concentration values from EDTA and citrate plasma showed very good correlation suggesting that our results in EDTA plasma is transferable to citrate plasma, both being the most common anticoagulant used in collecting plasma samples. Improving and extending the panel we used to quantify even more proteins is possible, yet focusing on the translational aspect is key. Ultimately, one does not want to measure hundreds of proteins to predict risk, rather quantifying only a few best predicting proteins should be sufficient. We followed this logic and showed that it is possible to perform prediction using up to seven proteins. Our results suggest quantitative targeted proteomics as a rapid promising low cost extension to current methods of blood testing.

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CONFLICT OF INTEREST

C.H.B. is the Chief Scientific Officer of MRM Proteomics, Inc., the co-founder and Chief Technology Officer of Creative Molecules, Inc. and Chief Technology Officer of Molecular You. The other authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

S.C.C. designed the current study and set up the original clinical trials. C.E.T., B.N. and R.A.A. collected the samples. Y.M. and C.H.B. performed the proteomics analysis and collected the data. Y.M. performed the data analysis, generated the figures, and wrote the initial draft of the manuscript. Y.M., C.E.T., B.N., R.A.v.A, CHB, F.R.R., B.v.V. and S.C.C. interpreted the results and wrote the manuscript. All authors read and contributed to the final version of the text.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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