Received: 18 July 2025 | Accepted: 24 November 2025

DOI: 10.1111/bjh.70278

BJHaem
BRITISH JOURNAL OF HAEMATOLOGY

RESEARCH LETTER

Plasma proteomic profiles of patients during oral anti-coagulant treatment with vitamin K antagonists

To the Editor,

Although effective, oral anti-coagulants (OACs) are associated with severe adverse events in patients with atrial fibrillation (AF) and with venous thromboembolism (VTE), including major bleeding and breakthrough strokes or VTE.[1,2] Direct oral anti-coagulants (DOACs) present an improved safety profile, but vitamin K antagonists (VKAs) remain the only treatment option for certain populations (e.g. with mechanical heart valves, anti-phospholipid syndrome, DOAC contraindication or failures). The frequency of these adverse events calls for research towards individualization of anti-coagulant therapy.

Targeted proteomics with stable isotope-labelled internal standards enables multiplexed absolute quantification of hundreds of proteins in plasma. It allows identification of biomarkers to improve risk prediction, tailor treatment or provide novel mechanistic insight.[3,4] It is used for these scopes in various cardiovascular diseases, for example, myocardial infarction, which can ultimately lead to individualized treatment.[5]

In this proof-of-concept study, we investigated using targeted proteomics to quantify over 100 plasma proteins in a large cohort of patients who initiated VKA treatment. We aimed to (1) characterize protein measurements in the entire cohort and (2) assess the assay's ability to detect differences across known clinically relevant subgroups, to lay the groundwork for further analysis. We used data from the Biomarkers in the Leiden Etiology and Epidemiology of bleeding in vitamin K antagonists Drug users Study (BLEEDS), a population-based cohort including 16 570 patients starting VKA at three anti-coagulation clinics in the Netherlands between 2012 and 2014.[6] Baseline characteristics and follow-up information on occurrence of adverse events were collected from the electronic patient records of the anti-coagulation clinic. Plasma was collected from the blood sample taken for international normalized ratio (INR) analyses in 83% (see details of sample and data collection in the 'Supplementary Methods' in Supporting Information).

To characterize protein measurements of the cohort, a case-cohort study design was established for efficiency reasons, including a random sample of the entire cohort at baseline (371 patients, i.e. the subcohort) and all the cases who developed an adverse event during follow-up with plasma available (i.e. 250 cases of major bleeding, 58 stroke patients and 10 recurrent VTE patients). Quality controls included two sample replicates from three BLEEDS patients and three sample replicates from two healthy controls. To assess the assay's ability to detect differences in known clinically relevant subgroups (age, sex, indication for VKA treatment, INR of the sample, use of anti-platelet therapy, cholesterol-lowering therapy, diabetes and hypertension), we evaluated protein profiles in the subcohort only to avoid influence of the adverse events. Fold changes between subgroups were calculated and p-values of the Wilcoxon rank-sum test were corrected for multiple testing (Benjamini–Hochberg adjusted p-value ≤0.05). Proteins with an absolute fold change ≥15% and p-adjusted ≤0.05 were considered significantly different (see 'Supplementary Methods' in Supporting Information; Figure S1 for details on study design and statistical analysis). Results on the association of protein levels with adverse events during treatment were beyond the scope of this initial assessment analysis and will be reported separately.

A panel of 269 proteins was used for quantification (Table S1). This panel was previously developed and applied.[7,8] It covers various pathways including the coagulation and complement system, apolipoproteins and proteins related to inflammation. Sample preparation, measurements and data inspection were performed as previously described[7,8] and summarized in the 'Supplementary Methods' in Supporting Information.

We quantified proteins in 683 samples from the BLEEDS case-cohort, including replicates from three patients (taken 7, 8 and 66 days apart). One sample was omitted due to a technical error. Of the 269 proteins, 139 were above the lower limit of quantitation in ≥10% of the samples and were considered quantifiable, while 130 did not (Table S1). Thirty-three (97% of included) coagulation and fibrinolytic proteins were successfully detected and 23 (68%) quantified. All 20 complement factors and 14 apolipoproteins were quantified. Mean protein concentration ranged from 1.4 fmol/μL (adhesion G protein-coupled receptor F5) to 453735.5 fmol/μL (albumin). There were no differences in the number of quantified proteins by case status (Figure S2). Protein abundances were normalized to eliminate the variability in sample protein content. Using

unsupervised clustering, there was no grouping according to patients' characteristics or available sample information, indicating no batch effect (Figures S3 and S4). The healthy control and BLEEDS replicates showed very good correlation (Pearson's coefficient ranged 0.997–0.999 and 0.999–0.938, respectively, Figures S5 and S6).

The subcohort was composed of 371 patients randomly sampled at baseline from the BLEEDS (Tables S2 and S3). The mean age was 70 years (standard deviation 12), and 54% (199) were men. The main treatment indication was AF (69%, 257) followed by VTE (22%, 80). Patients with VTE as an indication presented distinct proteomic profiles compared

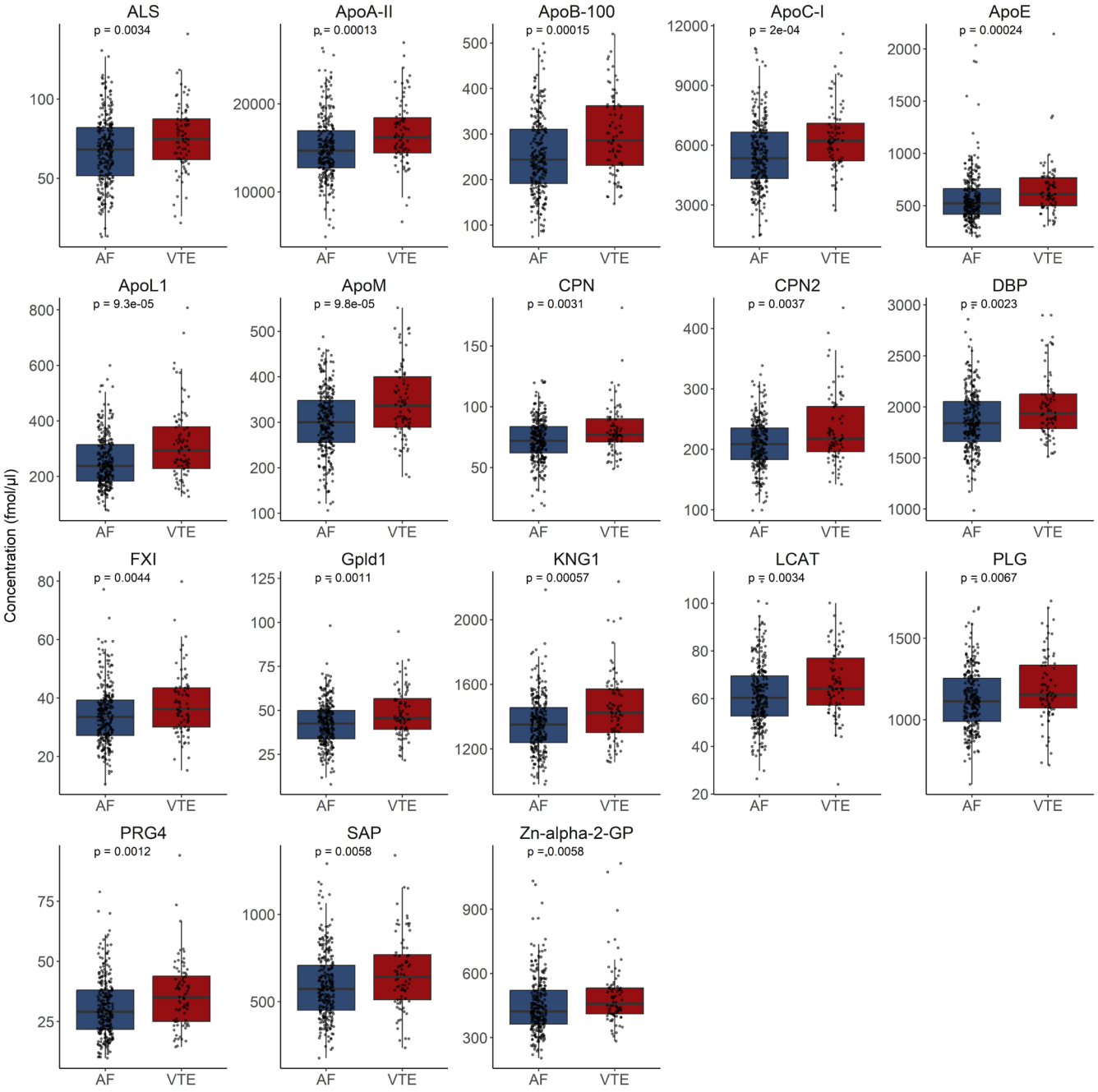

FIGURE 1 Distinct protein profiles in patients with VTE as VKA indication compared to AF patients. Protein levels of 18 significantly different proteins comparing patients treated with VKA for VTE (red) with AF patients (blue). Boxplots represent the median and interquartile range, and the dots are the individual levels. *p*-Values of comparison using Wilcoxon rank-sum test are reported. AF, atrial fibrillation; ALS, insulin like growth factor binding protein complex acid labile subunit; ApoA-II, apolipoprotein A II; ApoB-100, apolipoprotein B-100; ApoC-I, apolipoprotein C I; ApoE, apolipoprotein E; ApoL1, apolipoprotein L1; ApoM, apolipoprotein M; CPN, carboxypeptidase N catalytic chain; CPN2, carboxypeptidase N subunit 2; DBP, vitamin D-binding protein; FXI, coagulation factor XI; Gpld1, phosphatidylinositol glycan-specific phospholipase D; KNG1, kininogen 1; LCAT, phosphatidylcholine sterol acyltransferase; PLG, plasminogen; PRG4, proteoglycan 4; SAP, serum amyloid P component; VKA, vitamin k antagonists; VTE, venous thromboembolism; Zn-alpha-2-GP, zinc alpha 2 glycoprotein.

with AF patients. Eighteen proteins differed between the two groups (Figure 1; Figures S7A and 8). Levels of apolipoprotein (Apo) A-II, B-100, C-I, E, L1 and M were higher in VTE than in AF patients. Levels of proteoglycan-4 (PRG-4), factor (F)XI, plasminogen and kininogen-1 were also higher in patients receiving VKA for VTE. No differences in protein profiles were observed between pulmonary embolism (PE) and deep vein thrombosis (DVT) patients (Figure S7B).

Ageing was associated with 55 different proteins, of which 30 had fold change ≥15% (Figure 2A). Protein levels were mostly decreased in males compared with females (39 decreased proteins vs. 4 increased). Levels of pregnancy zone protein, sex hormone-binding globulin and adiponectin were ≥15% lower in males than in females (Figure 2B). The differences in protein profiles due to ageing also varied by sex (Figure S9).

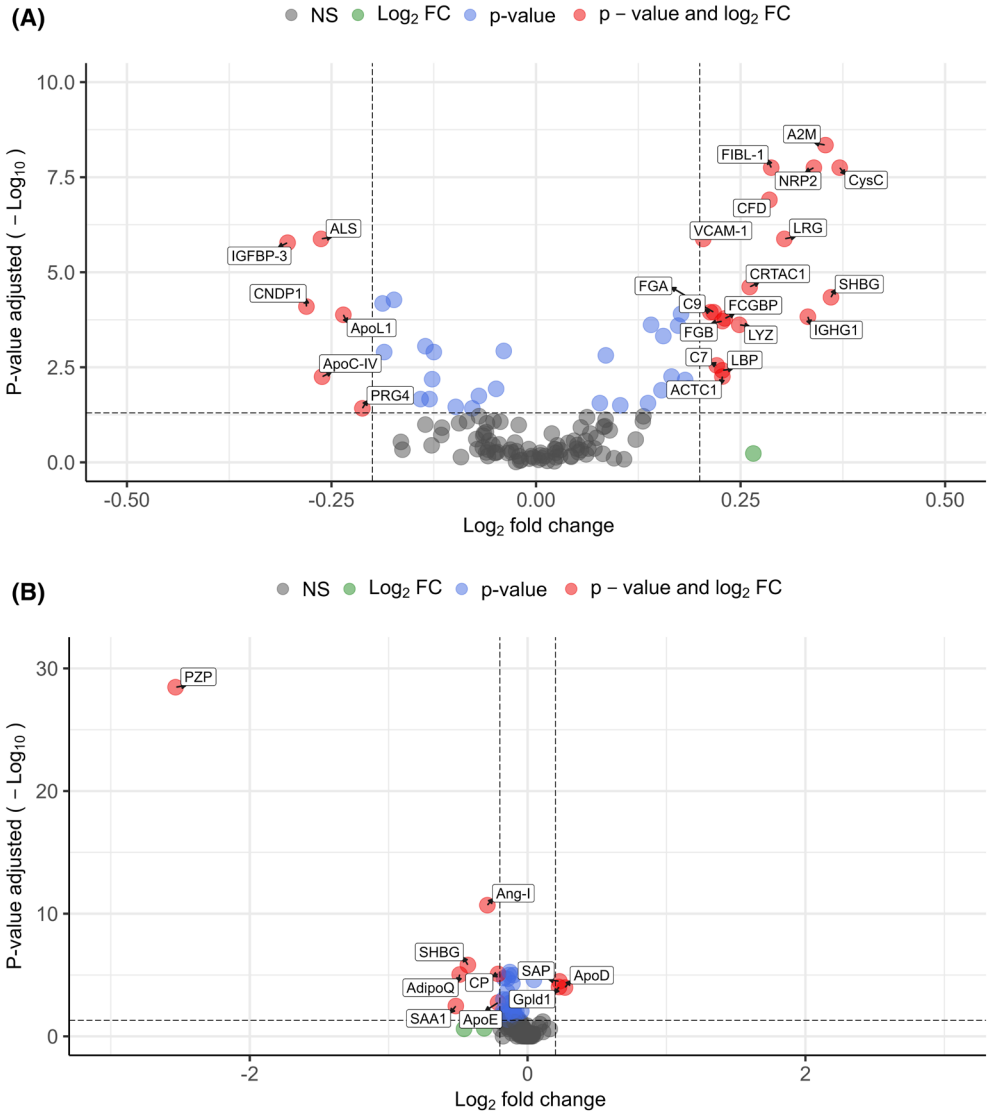

FIGURE 2 Protein profiles by age (A) and sex (B). In this plot, each dot represents a protein. The fold change on the *x*-axis (ratio of the mean protein level in one group to that in the other group) is plotted against the significance of this difference on the *y*-axis (expressed as $-\log_{10}$ of the *p*-value from the Wilcoxon rank-sum test, adjusted for false discovery rate). The most significant proteins appear at the top of the plots. (A) Older patients (≥70 years old) are compared with younger patients (<70 years old); protein levels are increased (left side) or decreased (right side) in older patients compared with younger patients. (B) Male patients are compared with female patients; protein levels are increased (right side) or decreased (left side) in male patients compared with female patients. A2M, alpha 2 macroglobulin; ACT, alpha 1 anti-chymotrypsin; ACTC1, actin alpha cardiac muscle 1; AdipoQ, adiponectin; ALS, insulin-like growth factor-binding protein complex acid labile subunit; ANG, angiotensinogen; ApoC-IV, apolipoprotein C IV; ApoD, apolipoprotein D; ApoE, apolipoprotein E; ApoL1, apolipoprotein L1; ApoM, apolipoprotein M; B2M, beta 2 microglobulin; C7, complement component C7; C9, complement component C9; CFD, complement factor D; CNDP1, beta Ala His dipeptidase; CP, ceruloplasmin; CRTAC1, cartilage acidic protein 1; CysC, cystatin C; FC, fold change; FCGBP, IgGFc-binding protein; FGA, fibrinogen alpha chain; FGB, fibrinogen beta chain; FIBL-1, fibulin 1; Gpld1, phosphatidylinositol glycan specific phospholipase D; IGFBP-3, insulin-like growth factor-binding protein 3; IGHG1, Ig gamma 1 chain C region; LBP, lipopolysaccharide-binding protein; LRG, leucine-rich alpha 2 glycoprotein; LUM, lumican; LYZ, lysozyme C; NRP2, neuropilin 2; NS, not significant; PRG4, proteoglycan 4; PZP, pregnancy zone protein; SAA1, serum amyloid A 1 protein; SAP, serum amyloid P component; SHBG, sex hormone-binding globulin; TIMP-2, metalloproteinase inhibitor 2; VCAM-1, vascular cell adhesion protein 1.

Other comorbidities and comedications showed a minor impact on the plasma proteome of VKA patients. In patients with diabetes, levels of four proteins were lower than in patients without (Figure S10). Anti-platelet use, hypertension and type of VKA were associated minor differences in protein profiles (Figure S11A–C). As expected, users of cholesterol-lowering medications had lower levels of ApoB-100 and ApoE compared with non-users (Figure S11D). Patients with elevated INR had lower levels of FII (Figure S11E) and the INR correlated with vitamin K-dependent coagulation proteins levels (Figure S12).

We found that the VKA indication (VTE or AF) was associated with 18 different protein levels, in particular apolipoproteins and coagulation-related proteins. Increased levels of ApoE and C-I were previously associated with an increased VTE risk.[9] Elevated apolipoproteins levels may reflect lower prevalence of cholesterol-lowering medications (16% in VTE vs. 46% in AF patients). Moreover, we observed increased levels of PRG-4 in VTE patients, a glycoprotein which binds to neutrophils in plasma.[10] We found no difference in protein profiles between DVT and PE patients. A previous study identified five proteins as signature of PE,[11] but these were not included in our assay and could not be validated. Differences in protein profiles between VTE and AF patients might be confounded by age and sex, which had a major impact on the plasma proteome. For example, ageing was associated with lower levels of apoC-I and PGR-4. In our results, comorbidities and comedication had a minor impact on protein profiles of VKA patients. This underlines the importance of considering OAC indication, age and sex in studies focusing on associations (causal or predictive) of these biomarkers with clinical events.

Our results confirm the robustness of targeted quantitative proteomics with stable-isotope labelled internal standards, as a method for biomarker discovery. We quantified ≥100 proteins in 683 samples. Nonetheless, 130 proteins were not quantifiable. Several proteins in the panel do not normally circulate in plasma which were still included as disease markers. Moreover, the assay was developed and validated under optimal pre-analytical conditions, not always reproducible in clinical settings.[7] Consequently, more proteins are included than are usually quantifiable to avoid missing potential biomarkers. Using multiple proteotypic peptides per protein could improve the number of quantified proteins; however, this would increase analytical time and costs.

A strength of this study is the large and unselected population of VKA users, with sufficient statistical power to evaluate protein profiles in subgroups. We were able to quantify 139 proteins in a small volume of plasma. As limitations, 131 proteins could not be quantified and protein content varied between samples, requiring normalization. Finally, validation in a DOAC cohort is warranted as DOACs are the most common OACs.

In conclusion, quantitative targeted proteomics is a reliable method for highly multiplexed protein quantification. While immunoassays measurements correlate well with targeted proteomics, they are less sensitive and specific.[12] Other technologies, specifically aptamer-based SOMAScan[13] and antibody-based Olink[14] platforms, report measuring larger numbers of proteins; however, studies have shown discrepancies using these technologies, also when compared with immunoassays.[15] Mass spectrometry can measure more proteins, and the panel used in this study has been thoroughly validated[7] showing good correlations with immunoassays and activity assays.[12] We established that proteomic profiles of VKA patients were mainly determined by age, sex and VKA indication. Elevated levels of apolipoproteins and PRG-4 warrant further exploration as potential VTE signature. These findings lay the groundwork for future studies assessing the potential of this technology to predict treatment-related adverse events and elucidate their mechanisms.

KEYWORDS

anti-coagulants, coumarins, proteomics, sex differences

AUTHOR CONTRIBUTIONS

NvR, SCC, BJMvV and YM designed the research and obtained funding. NvR collected the samples and the clinical data. YM, CHB and DRG supervised the proteomics measurements. EC and YM analysed the data and wrote the manuscript. EC, BJMvV, MHAB, MW, DRG, CHB, SCC, NvR and YM revised the paper for important intellectual content.

ACKNOWLEDGEMENTS

The authors would like to thank all patients who participated in the BLEEDS. C.H.B. is grateful for support from the Segal McGill Chair in Molecular Oncology at McGill University and for support from the Warren Y. Soper Charitable Trust for the Warren Y. Soper Clinical Proteomics Centre at the Jewish General Hospital (Montréal, Quebec, Canada). C.H.B. is also grateful for support from the Alvin Segal Family Foundation for the Segal Cancer Proteomics Centre at the Jewish General Hospital (Montréal, Quebec, Canada). This work was done under the auspices of a Memorandum of Understanding between McGill and the U.S. National Cancer Institute's International Cancer Proteogenome Consortium (ICPC). ICPC encourages international cooperation among institutions and nations in proteogenomic cancer research in which proteogenomic datasets are made available to the public. This work was also done in collaboration with the U.S. National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC). DRG and the work performed at the University of Victoria-Genome BC Proteomics Centre was supported by funding to the Pan-Canadian Proteomics Centre (PCPC) from Genome Canada and Genome British Columbia, through the Genomics Technology Platform (GTP) program for operations and technology development (264PRO) and operations support from Genome BC for the BC Proteomics Centre (374PRO).

FUNDING INFORMATION

The BLEED study was funded by the Dutch Heart Foundation (2011 T12) Center for Translational Molecular Medicine (01 C-201) and the Dutch Heart Foundation (99.165). This work and EC are supported by the Netherlands Thrombosis Foundation (2019-01).

CONFLICT OF INTEREST STATEMENT

M.H.A.B. has received consultancy fees from VarmX B.V (all fees to the institution). C.H.B. is the CSO of MRM Proteomics, Inc., and the V.P. of Proteomics at Molecular You. Y.M. is the Chief Bioinformatics Officer of MRM Proteomics, Inc. The remaining authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon reasonable request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Eleonora Camilleri[1]
Bart J. M. van Vlijmen[2,3]
Mettine H. A. Bos[2,3]
Manfred Wuhrer[4]
David R. Goodlett[5]
Christoph H. Borchers[6,7,8,9]
Suzanne C. Cannegieter[1,2,3]
Nienke van Rein[1,2,10]
Yassene Mohammed[4,5,7]

[1]*Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands*
[2]*Division of Thrombosis and Hemostasis, Department of Internal Medicine, Leiden University Medical Center, Leiden, The Netherlands*
[3]*Einthoven Laboratory for Vascular and Regenerative Medicine, Leiden University Medical Center, Leiden, The Netherlands*
[4]*Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands*
[5]*UVic-Genome British Columbia Proteomics Centre, University of Victoria, Victoria, British Columbia, Canada*
[6]*Segal Cancer Proteomics, Centre, Lady Davis Institute for Medical Research, McGill University, Montreal, Canada*
[7]*Gerald Bronfman Department of Oncology, Jewish General Hospital, Montreal, Canada*
[8]*Division of Experimental Medicine, McGill University, Montreal, Canada*
[9]*Department of Pathology, McGill University, Montreal, Canada*
[10]*Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, The Netherlands*

Correspondence
Yassene Mohammed, Center for Proteomics and Metabolomics, Leiden University Medical Center, C7-Q, Albinusdreef 2, 2333ZA, P.O. Box 9600, Leiden 2300 RC, The Netherlands.
Email: y.mohammed@lumc.nl

ORCID

Eleonora Camilleri https://orcid.org/0000-0002-9502-2434

REFERENCES

1. Ruff CT, Giugliano RP, Braunwald E, Hoffman EB, Deenadayalu N, Ezekowitz MD, et al. Comparison of the efficacy and safety of new oral anticoagulants with warfarin in patients with atrial fibrillation: a meta-analysis of randomised trials. Lancet. 2014;383(9921):955–62.
2. Nv R, Heide-Jørgensen U, Lijfering WM, Dekkers OM, Sørensen HT, Cannegieter SC. Major bleeding rates in atrial fibrillation patients on single, dual, or triple antithrombotic therapy. Circulation. 2019;139(6):775–86.
3. Doerr A. Mass spectrometry-based targeted proteomics. Nat Methods. 2013;10(1):23.
4. Gillette MA, Carr SA. Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. Nat Methods. 2013;10(1):28–34.
5. Motiwala SR. From proteomics to therapeutics. J Am Coll Cardiol. 2019;74(12):1554–6.
6. van Rein N, Lijfering WM, Bos MH, Herruer MH, Vermaas HW, van der Meer FJ, et al. Objectives and design of BLEEDS: a cohort study to identify new risk factors and predictors for major bleeding during treatment with vitamin K antagonists. PLoS One. 2016;11(12):e0164485.
7. Gaither C, Popp R, Mohammed Y, Borchers CH. Determination of the concentration range for 267 proteins from 21 lots of commercial human plasma using highly multiplexed multiple reaction monitoring mass spectrometry. Analyst. 2020;145(10):3634–44.
8. Mohammed Y, Kootte RS, Kopatz WF, Borchers CH, Büller HR, Versteeg HH, et al. The intestinal microbiome potentially affects thrombin generation in human subjects. J Thromb Haemost. 2020;18(3):642–50.
9. Orsi FA, Lijfering WM, Van der Laarse A, Ruhaak LR, Rosendaal FR, Cannegieter SC, et al. Association of apolipoproteins C-I, C-II, C-III and E with coagulation markers and venous thromboembolism risk. Clin Epidemiol. 2019;11:625–33.
10. Das N, Schmidt TA, Krawetz RJ, Dufour A. Proteoglycan 4: from mere lubricant to regulator of tissue homeostasis and inflammation: does proteoglycan 4 have the ability to buffer the inflammatory response? Bioessays. 2019;41(1):e1800166.
11. Ten Cate V, Prochaska JH, Schulz A, Koeck T, Pallares Robles A, Lenz M, et al. Protein expression profiling suggests relevance of noncanonical pathways in isolated pulmonary embolism. Blood. 2021;137(19):2681–93.
12. Mohammed Y, van Vlijmen BJ, Yang J, Percy AJ, Palmblad M, Borchers CH, et al. Multiplexed targeted proteomic assay to assess coagulation factor concentrations and thrombosis-associated cancer. Blood Adv. 2017;1(15):1080–7.
13. Ngo D, Sinha S, Shen D, Kuhn EW, Keyes MJ, Shi X, et al. Aptamer-based proteomic profiling reveals novel candidate biomarkers and pathways in cardiovascular disease. Circulation. 2016;134(4):270–85.
14. Assarsson E, Lundberg M, Holmquist G, Bjorkesten J, Thorsen SB, Ekman D, et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. PLoS One. 2014;9(4):e95192.

15. Eldjarn GH, Ferkingstad E, Lund SH, Helgason H, Magnusson OT, Gunnarsdottir K, et al. Large-scale plasma proteomics comparisons through genetics and disease associations. Nature. 2023;622(7982):348–58.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.