JIM Original Article

doi: 10.1111/joim.13502

Plasma lipopolysaccharide-binding protein is a biomarker for future venous thromboembolism: Results from discovery and validation studies

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Abstract. Jensen SB, Latysheva N, Hindberg K, Ueland T. Plasma lipopolysaccharide-binding protein is a biomarker for future venous thromboembolism: Results from discovery and validation studies. *J Intern Med.* 2022;**292:**523–535.

Background. Effect-size underestimation impedes biomarker identification. Long follow-up time in prospective studies attenuates effect-size estimates for transient biomarkers, while disease category–specific biomarkers are affected by merging of categories. Venous thromboembolism (VTE) encompasses deep vein thrombosis (DVT) and pulmonary embolism (PE).

Objectives. (i) To re-analyze untargeted proteomic data to identify biomarker candidates for future VTE that differ between DVT and PE and are attenuated by extended time between sampling and VTE. (ii) To perform targeted candidate validation.

Patients/Methods. A VTE case-control discovery study and a nested case-control validation study were derived from the general population surveyed in 1994–95. Plasma was obtained at study enrollment, and VTE events were registered until 2007. Untargeted proteomic data were re-analyzed for candidate discovery. Lipopolysaccharide-binding protein (LBP) was validated by enzyme-linked immunosorbent assay.

Results. Elevated LBP was discovered as a candidate DVT biomarker in women with less than 3 years between blood sampling and DVT. In the validation study, the odds ratio (OR) for DVT was 2.03 (95% confidence intervals [CI]: 1.53–2.74) per standard deviation (SD) increase in LBP for women with less than 3 years between blood sampling and DVT. Adjustment for age, body mass index, and C-reactive protein attenuated the OR to 1.79 (95% CI: 1.25–2.62) per SD. In the validation study, we observed an OR for VTE of 0.47 (95% CI: 0.28–0.77) for men in the 25th to 50th percentiles when compared to the lowest quartile.

Conclusions. We discovered and validated increased LBP as a predictive biomarker for DVT in women. We found an increased VTE risk for men in the lowest quartile of LBP.

Keywords: biomarkers, proteomics, regression dilution bias, sex differences, thromboembolism, venous, venous thrombosis

Introduction

Venous thromboembolism (VTE) is a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE is a complex disease that affects 1–2 per 1000 individuals each year with serious short- and long-term complications [1–3]. The past decades have revealed numerous risk factors for VTE, and because most PEs are suspected to be a result of the embolization of a DVT, it has been expected that risk factors for VTE would contribute equally to the risk of DVT and PE [4, 5]. However, several risk factors bear unequal risk of DVT and PE. This was first described for carriers of the factor V Leiden (FVL) gene variant, where carriers of the variant had substantially higher risk of DVT than PE, a phenomenon known as the FVL paradox [4–6]. Among others, elevated body mass index (BMI) and estrogen use entail a higher risk of DVT than of PE [5, 7–9]. The cumulative lifetime risk of incident VTE is similar for men and women [10–12]. The main sex differences in VTE

risk are reproductive risk factors (e.g., oral contraceptives and pregnancy) for women, and male sex is a strong risk factor for VTE recurrence after unprovoked VTE [10–13].

Baseline measurements are commonly used to measure the strength of associations between risk factors and disease incidence. In prospective studies, single measures of variables are subject to random measurement error from short- and long-term biologic variations, which increase as the time between sampling and the outcome extends. This information bias leads to underestimation of the risk association due to regression dilution bias [14].

We previously published an untargeted mass spectrometry (MS)–proteomic profiling in VTE to identify predictive biomarkers [15]. In the previous study, we did not include the analysis of DVT and PE as separate categories nor did we systematically consider the effect of extended time between blood sampling and the occurrence of VTE. In this study, we re-analyzed the MS-based proteomic dataset to discover predictive biomarkers for VTE with different effect size between DVT and PE and affected by regression bias. Because female reproductive factors are a major contributor to sex difference in VTE risk [10, 11] and estrogen signaling augments inflammatory gene expression [16–18], we performed sex-stratified analysis and validated our discovery of plasma lipopolysaccharide-binding protein (LBP) as a predictive biomarker of DVT in women in a larger nested case-control study.

Materials and methods

The source population

The Tromsø Study is a single-center prospective cohort study with repeated health surveys of the inhabitants of the municipality of Tromsø, Norway [19]. The fourth survey was conducted from 1994 to 1995, where all inhabitants aged 25 years or older were invited to participate. At a 77% participation rate, a total of 27,158 individuals were included in the study and followed from the date of inclusion until an incident VTE, migration, death, or end of follow-up (1 September 2007). All first lifetime VTE events were identified using the hospital discharge diagnosis registry, the autopsy registry, and the radiology procedure registry from the University Hospital of North Norway, which is the only hospital that provided VTE treatment in the Tromsø region. Trained personnel confirmed and recorded each VTE by extensive medical record review, as previously described [20]. A VTE was confirmed if the presence of signs and symptoms of PE or DVT was combined with objective confirmation by a diagnostic procedure (i.e., compression ultrasonography, venography, spiral computed tomography, perfusion ventilation, scan, pulmonary angiography, or autopsy), resulting in treatment initiation (unless contraindications were specified). The VTEs were classified as PE with or without concurrent DVT or as DVT only. During the follow-up period (1994–2007), 462 individuals experienced a VTE event. The study outline is presented in Fig. 1.

Discovery study cohort

From the source population, we established a nested case-control study of 100 VTE cases and 100 controls. Cases were prioritized according to the shortest time from blood sampling to VTE, and a control matched on age and sex was randomly sampled from the source cohort. Case-control sample pairs—where both plasma samples passed quality control for MS proteomics—were included in our discovery study [15].

Validation study cohort

For each of the 462 VTE cases, two age-, sex-, and index date–matched controls were randomly sampled from the source population (n = 924). The index date was defined as the date of the VTE event, meaning that the controls had to be living in Tromsø and be without a VTE diagnosis at the time of the VTE event in the corresponding case. From this population, 52 cases and 90 controls did not have plasma samples of adequate quality available for the analyses, thus leaving 410 VTE cases and 834 controls for the final analyses in our nested case-control study.

Ethics approval

The regional committee of medical and health research ethics approved the study, and all participants provided written consent

Plasma samples

Nonfasting blood samples were drawn from an antecubital vein into 5 ml vacutainer tubes containing EDTA as an anticoagulant. Blood samples were processed within 1 h by centrifugation at

Fig. 1 *The study outline: Plasma samples from a venous thromboembolism (VTE) biomarker discovery cohort were subjected to untargeted MS3-based plasma proteomic profiling (a). We aimed to identify biomarker candidates that were different between deep vein thrombosis (DVT) and pulmonary embolism (PE), which were subjected to time-dependent regression attenuation, and sex difference was assessed (a). A larger nested case-control study that partly overlaps the discovery study was established for validation. The absolute lipopolysaccharide-binding protein (LBP) levels in plasma samples from the validation study were determined by dual-binder enzyme-linked immunosorbent assay (ELISA), and LBP was evaluated as a biomarker for VTE (b).*

3000 g for 10 min at 22°C, and plasma samples were collected and frozen at −70°C in 1 ml aliquots. All plasma samples were generated at the same unit and stored by the Tromsø Study. For samples included in the discovery study, samples were shipped on dry ice to Proteomic Sciences (Cobham, UK) and processed as previously described [15].

Measurements

Physical measurements. Baseline characteristics including age, sex, and anthropometrics were collected by physical examination at study enrollment. Height and weight were measured with subjects wearing light clothing and no shoes. BMI was calculated as the weight in kilograms divided by the square of height in meters (kg/m^2).

MS-based proteomic profiling. Sample quality control, processing, data acquisition, and analysis for the MS-based discovery study has been comprehensively described previously [15]. Briefly, plasma samples were depleted for albumin and IgG and digested with trypsin. Samples were multiplexed using TMT10 reagents and fractioned before analysis in duplicate by liquid chromatography-MS3 using an EASY-nLC 1000 system coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Peptide-level quantification was processed and normalized by a previously described strategy [21] to allow relative protein-level quantification across all multiplexed samples. Details about MS data analysis were provided previously [15]. Peptides with measurements in more than a third of the samples were used in pairwise correlation analysis.

Plasma measurement of LBP by an enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of LBP and C-reactive protein (CRP) (cat# DY870 and DY1707, RnD systems, Minneapolis, MN) in a set-up that combined a CiBi SELMA (CiBio, Jena, Germany), an EL406 washer/dispenser (Biotek, Winooski, VT), and a Synergy H2 microplate reader (Biotek). Optical density (OD) at 450 nm was used for quantification by comparison to a dilution series of a standard provided by the manufacturer. OD at 540 nm was used for wavelength correction. The intra- and interassay coefficients of variation were less than 10% for both assays.

Statistical analysis

Multivariate linear regression was performed to identify DVT- and PE-biomarker candidates with significantly different protein levels between cases and controls in the discovery study. Results are expressed as point estimates with 95% confidence intervals (CIs) for beta coefficients standardized according to the standard deviation (SD) in the control group. The model included age and BMI as covariates. For the validation study, logistic regression models were used to calculate odds ratios (ORs) with 95% CI for VTE, DVT, and PE by LBP modeled as a continuous and discretized categorical variable based on the distribution of LBP in the control population. In models with LBP as a continuous variable, the risk estimates are expressed as ORs per SD increase in LBP according to the SD in the control group. In categorical analysis, the results are with respect to the indicated reference group. The multivariable models included age, BMI, CRP, and sampling date as covariates. A minimum of five cases were required for regression analysis.

Regression dilution bias introduced by extended time between blood sampling and occurrence of the outcome was evaluated by restricting the cases included in the analyses. Cases were included if they were diagnosed before a given maximum time allowed between blood sampling and VTE. We assessed 0.1-year increments in the maximum time allowed until the full follow-up time was reached. For DVT in women, a maximum time of 3 years was used based on observation in the discovery study. Statistical analysis was performed in R (version 4.1.0) using standard packages. Pearson's correlation coefficient was used for correlation analyses.

Results

The baseline characteristics of the study participants and the characteristics of the VTE events in the discovery and validation studies are summarized in Table 1. Participants who developed VTE had slightly higher CRP levels and a higher BMI, while LBP levels were similar between the groups (Table 1). The median age at the VTE event was 69.2 years, and the proportion of women was 52%. DVTs made up 62.7% of all VTE events where 58.2% were classified as provoked.

We re-analyzed data from our untargeted VTE biomarker discovery study [15] to identify

Table 1. *Baseline characteristics of the discovery and validation study participants*

	DVT	PE	Controls	DVT[a]
Discovery study				
Participants	55	25	88	11
Median age (range)	68 (28–83)	66 (33–80)	65 (28–83)	63 (28–81)
Sex (male, *n* [%])	21 (38)	11 (44)	39 (44)	0
BMI (mean ± SD)	27.0 ± 4.2	27.3 ± 3.8	24.7 ± 3.5	28.4 ± 5.0
Years to event (mean [range])	3.77 (0.10–6.80)	3.94 (0.70–6.80)		1.78 (0.93–2.95)
Unprovoked	25 (45%)	9 (36%)		4 (36%)
LBP (AU)	194 ± 70	144 ± 31	176 ± 55	246 ± 98
CRP[b] (mg/L)	2.29 ± 1.72	1.96 ± 1.45	2.11 ± 1.79	3.53 ± 2.55
Validation study				
Participants	253	157	834	29
Median age (range)	62 (25–89)	62 (26–90)	62 (25–90)	67 (28–89)
Sex (male, *n* [%])	118 (47)	79 (50)	389 (47)	0
BMI (kg/m^2) (mean ± SD)	26.9 ± 4.36	27.5 ± 4.73	26.1 ± 4.1	26.6 ± 4.53
CRP[b] (mg/L) (mean ± SD)	2.20 ± 1.64	1.89 ± 1.55	1.82 ± 1.54	3.61 ± 2.07
LBP (μg/mL) (mean ± SD)	6.13 ± 2.61	6.15 ± 2.49	6.09 ± 2.25	8.66 ± 3.54
Years to event (mean [range])	6.60 (0.09–12.80)	8.10 (0.65–12.60)		1.60 (0.16–2.97)
Unprovoked (*n* [%])	101 (40)	72 (46)		8 (28)

Abbreviations: AU, arbitrary units; BMI, body mass index; CRP, C-reactive protein; DVT, deep vein thrombosis; LBP, lipopolysaccharide-binding protein; PE, pulmonary embolism; SD, standard deviation.
[a]Female participants diagnosed with a DVT within 3 years after blood sampling.
[b]Summary of enzyme-linked immunosorbent assay measurements.

biomarker candidates that were not identified as VTE biomarker candidates due to opposing effect sizes for DVT and PE, and regression attenuation caused by extended time between blood sampling and VTE (Fig. 1a). The discovery study—which included 55 DVT and 25 PE cases—identified LBP as a candidate biomarker with opposing effect sizes between DVT and PE (Fig. 2a,b). LBP levels were elevated in women who suffered from a DVT within 3 years of blood sampling (Fig. 2c). Contrarily, women who later suffered from a PE had lower levels of LBP than the control population (Fig. 2d). We found no association between plasma LBP levels and DVT or PE risk in men (Fig. 2e,f). In analysis restricted to 3 years of follow-up, the OR was 2.5-fold higher (OR 2.54, 95% CI 1.10, 3.38) for DVT in women per 1 SD increase in plasma LBP levels. High Pearson's correlation coefficients between eight peptides assigned to LBP attested acceptable data quality (Fig. S1).

Plasma LBP levels were measured by ELISA in the validation study (Fig. 1b). The Pearson's correlation coefficient for plasma LBP measurements by MS-based proteomics and ELISA was 0.72. The distribution of baseline characteristics across quartiles of LBP levels in the validation study is shown in Table 2. The median age, BMI, and mean CRP levels increased across quartiles of LBP. LBP plasma levels correlated with BMI (Pearson's r = 0.13) and CRP (Pearson's r = 0.50).

The ORs for VTE and subgroups of VTE across quartiles of plasma LBP levels are shown in Table 3. We found no association between LBP and VTE in women when the full follow-up time of 12.8 years was used (Table 3). The OR for VTE was 0.47 (95% CI 0.28, 0.77) for men in the 25th to 50th percentiles (Q2) when compared to the lowest quartile (Q1) (Table 3) with comparable ORs for DVT (OR 0.49, 95% CI 0.27,0.87), PE (OR 0.44, 95% CI 0.20, 0.92), unprovoked VTE (OR 0.36, 95% CI 0.17,0.73), and provoked VTE (OR 0.49, 95% CI 0.30, 1.05), also after adjustment for age, BMI, and CRP (Tables S1–S3). Adjustment for sampling date did not affect the results.

To investigate the effect of regression dilution bias from extended time between blood sampling and occurrence of VTE, we plotted the ORs for VTE among subjects with high (highest quartile)

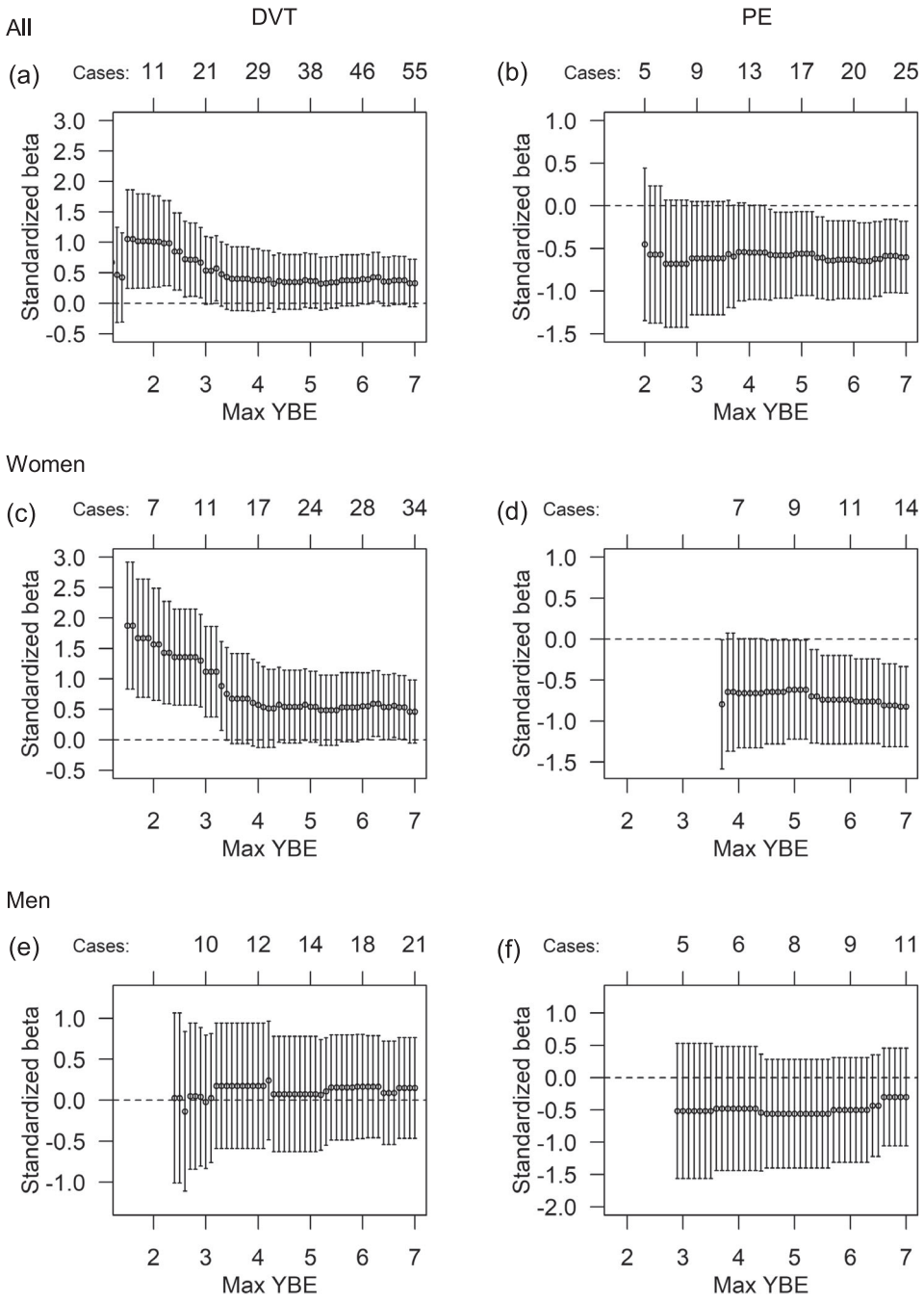

Fig. 2 *Mass spectrometry–based measurement of the relative plasma levels of lipopolysaccharide-binding protein was used to calculate the follow-up-time-dependent standardized beta coefficients for deep vein thrombosis (DVT) (a) and pulmonary embolism (PE) (b) using linear regression with adjustment for age and body mass index. Sex-stratified analysis is shown for women (c and d) and men (e and f). The maximum allowed time in years from blood sampling to venous thromboembolism event (YBE) is indicated on the x-axis. The number of cases included in the analysis is indicated above the plot. Point estimates with 95% confidence intervals are shown.*

Table 2. *Distribution of baseline characteristics in the validation study according to quartiles of plasma levels of LBP*

Percentile	<25%	25%–50%	50%–75%	>75%
Range (μg/mL)	0.84–4.68	4.68–5.75	5.75–7.14	7.14–21.58
Participants	334	281	310	319
Median age (range)	59 (25–85)	60 (25–90)	62 (26–84)	66 (25–89)
Sex (male)	166	131	144	145
BMI (kg/m^2) (mean ± SD)	25.8 ± 3.8	26.2 ± 3.9	26.3 ± 4.3	27.3 ± 4.8
CRP (mg/L) (mean ± SD)	1.2 ± 1.1	1.4 ± 1.2	1.8 ± 1.3	3.0 ± 1.8

Abbreviations: BMI, body mass index; CRP, C-reactive protein; SD, standard deviation.

versus low (lowest quartile) plasma levels of LBP as a function of time between blood sampling and the VTE events (Fig. 3). We also plotted the OR for VTE among male subjects with low LBP levels (lowest quartile) versus subjects in Q2 (Fig. S2). As shown in Fig. 3b, the increased OR for VTE for women with high plasma levels of LBP was substantially attenuated by extended time between blood sampling and VTE events. In contrast, the increased risk for VTE for men with low LBP levels (Q1) compared to men in the Q2 did not change substantially as a function of the time between blood sampling and occurrence of VTE (Fig. S2).

Finally, we limited the cases included in analysis to women diagnosed with DVT within 3 years after blood sampling. Baseline characteristics for this group are presented in the rightmost column of Table 1. We found a strong linear association between LBP and DVT in women (OR 2.03 per 1 SD, 95% CI 1.53, 2.74). This OR was attenuated to 1.79 (95% CI 1.25, 2.62) after adjustment for age, BMI, and CRP. Age-stratified analysis indicated a stronger association in older (>60 years) individuals (Table S4). Due to the overlap between the discovery and validation cohorts, the confirmatory finding in the validation study could be driven by the individuals already included in the discovery study. Therefore, we performed sensitivity analysis, where participants in the hypothesis-generating discovery study were excluded from analysis. The baseline characteristics are presented in Table S5. Based on the 18 women who experienced a DVT within 3 years after blood sampling, the OR for DVT was 1.84 (95% CI 1.22, 2.73) per 1 SD increase in LBP. The OR was unchanged (1.82 95% CI 1.20, 2.73) after adjustment for age and BMI but was attenuated to 1.48 (95% CI 0.88, 2.46) after further adjustment for CRP. Adjustment for sampling date did not affect the results.

Discussion

We re-analyzed data from our untargeted VTE biomarker discovery study to identify LBP as a plasma biomarker candidate for future DVT in women. We validated our finding in a larger nested case-control study. Women had a twofold higher OR for DVT for each SD increase in LBP plasma level when follow-up time was limited to 3 years. The association between LBP and DVT in women was attenuated, but remained significant, after adjustment for age, BMI, and CRP. In contrast, we observed increased risk of VTE for men in the lowest quartile of LBP compared to men in the second quartile.

The association between DVT and inflammation has been intensely debated over the past decade, to outline the direction of the association and the causal pathway [22]. Clearly, the classical hallmarks of inflammation are often present because of acute DVT, and case-control studies have shown increased plasma levels of inflammatory markers after the incidence of DVT [23, 24]. The causative role of inflammation in VTE has been addressed in several prospective studies, most commonly through the inflammatory marker CRP, with conflicting results [25–34]. In general, prospective studies with long follow-up time find no association between CRP levels at baseline and the risk of VTE [26–29, 32]. However, studies with short follow-up and studies that consider the time-dependent risk attenuation by regression dilution bias tend to find an association between CRP and the risk of VTE [29, 31, 33, 34]. It was previously demonstrated that elevated CRP in women, but not men, may contribute to obesity-mediated VTE [33]. Furthermore, using a case-crossover study design, Grimnes et al. have shown that acute inflammation—as reflected by elevated CRP—may be a trigger for VTE [34]. Similarly, a

Table 3. *OR with 95% CI for overall VTE and subgroups according to quartiles and 1 standard deviation increase in LBP plasma levels*

Variable	#Cases	#Controls	Model 1	Model 2	Model 3
VTE women					
Per SD	213	445	1.08 (0.92,1.26)	1.04 (0.89,1.22)	0.99 (0.82,1.19)
Q1	57	113	Ref.	Ref.	Ref.
Q2	42	110	0.76 (0.47,1.22)	0.73 (0.45,1.17)	0.73 (0.45,1.17)
Q3	53	111	0.95 (0.60,1.49)	0.91 (0.57,1.44)	0.87 (0.55,1.40)
Q4	61	111	1.09 (0.70,1.70)	0.99 (0.63,1.57)	0.90 (0.55,1.48)
DVT women					
Per SD	135	445	1.12 (0.94,1.33)	1.10 (0.92,1.32)	1.02 (0.83,1.26)
Q1	35	113	Ref.	Ref.	Ref.
Q2	29	110	0.85 (0.48,1.49)	0.82 (0.47,1.44)	0.83 (0.47,1.45)
Q3	34	111	0.99 (0.58,1.70)	0.95 (0.55,1.63)	0.88 (0.51,1.54)
Q4	37	111	1.08 (0.63,1.83)	1.01 (0.59,1.74)	0.84 (0.46,1.52)
PE women					
Per SD	78	445	1.00 (0.78,1.27)	0.94 (0.72,1.20)	0.92 (0.68,1.23)
Q1	22	113	Ref.	Ref.	Ref.
Q2	13	110	0.61 (0.28,1.25)	0.56 (0.26,1.16)	0.56 (0.26,1.16)
Q3	19	111	0.88 (0.45,1.71)	0.84 (0.43,1.66)	0.86 (0.43,1.71)
Q4	24	111	1.11 (0.59,2.11)	0.94 (0.49,1.83)	0.99 (0.48,2.03)
VTE men					
Per SD	197	389	0.96 (0.81,1.13)	0.94 (0.79,1.11)	0.89 (0.74,1.07)
Q1	68	98	Ref.	Ref.	Ref.
Q2	32	98	0.47 (0.28,0.77)	0.47 (0.28,0.78)	0.46 (0.27,0.76)
Q3	47	96	0.71 (0.44,1.12)	0.69 (0.43,1.11)	0.66 (0.41,1.06)
Q4	50	97	0.74 (0.47,1.18)	0.68 (0.42,1.09)	0.60 (0.36,0.99)
DVT men					
Per SD	118	389	0.90 (0.72,1.10)	0.88 (0.71,1.09)	0.83 (0.66,1.05)
Q1	43	98	Ref.	Ref.	Ref.
Q2	21	98	0.49 (0.27,0.87)	0.49 (0.27,0.89)	0.48 (0.26,0.86)
Q3	26	96	0.62 (0.35,1.08)	0.62 (0.35,1.08)	0.59 (0.33,1.03)
Q4	28	97	0.66 (0.38,1.14)	0.60 (0.34,1.06)	0.53 (0.29,0.97)
PE men					
Per SD	79	389	1.05 (0.83,1.31)	1.01 (0.79,1.27)	0.96 (0.74,1.24)
Q1	25	98	Ref.	Ref.	Ref.
Q2	11	98	0.44 (0.20,0.92)	0.44 (0.20,0.92)	0.43 (0.19,0.90)
Q3	21	96	0.86 (0.45,1.63)	0.82 (0.42,1.57)	0.78 (0.40,1.51)
Q4	22	97	0.89 (0.47,1.68)	0.78 (0.40,1.50)	0.69 (0.34,1.38)

Note: Model 1: no adjustment. Model 2: adjusted for age and BMI. Model 3: adjusted for age, BMI, and CRP.
Abbreviations: BMI, body mass index; CRP, C-reactive protein; DVT, deep vein thrombosis; PE, pulmonary embolism; LBP, lipopolysaccharide-binding protein; SD, standard deviation.

high neutrophil-to-leucocyte ratio is an alternative measure of inflammation and is associated with increased risk of VTE within a 3-year follow-up with similar risk estimates for DVT and PE [35]. The large prospective HUNT2 study found an association between CRP and VTE within 1 year of blood sampling, but no association between a panel of inflammatory cytokines and VTE in the same population [29, 36]. Altogether, these findings suggest that shorter periods with an augmented inflammatory state and long-term low-grade inflammation increase VTE risk. In support of this notion, the

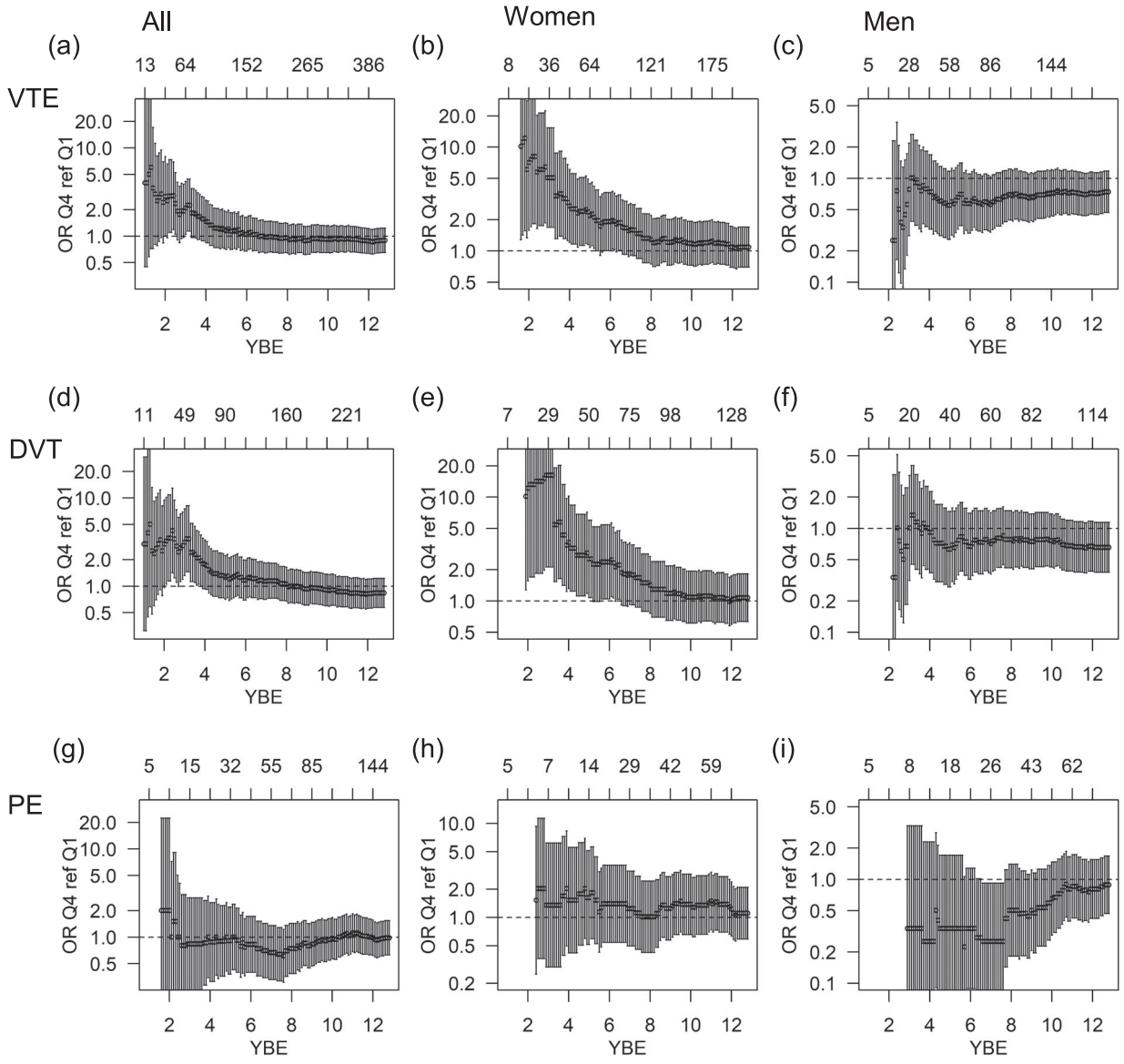

Fig. 3 *Plots of estimated odds ratios with 95% confidence intervals shown as whiskers for overall venous thromboembolism (VTE), deep vein thrombosis (DVT), or pulmonary embolism (PE) as a function of the maximum time from blood sampling to VTE events. Subjects with plasma lipopolysaccharide-binding protein (LBP) levels in the highest quartile (Q4) were compared to those with LBP levels in the lowest quartile (Q1). The number of VTE events included in the analysis are depicted above the plots.*

risk of VTE in association with autoimmune diseases is more pronounced during flare-ups, and the strong provoking factors for VTE—infection, cancers, and surgery—all share the feature of inflammation [37–39].

Through an untargeted approach, we identified LBP as a biomarker for future DVT in women. To the best of our knowledge, this is a novel finding, and the association between LBP and VTE has not been studied previously. LBP is correlated with

age, BMI, and CRP, which is consistent with other studies and suggests confounding [40–44]. While age and BMI did not affect the risk estimates, the attenuation of risk by CRP may be expected since both CRP and LBP are synthesized in the liver in response to inflammatory cytokines with a prominent role for IL6 [45, 46]. Importantly, the risk estimate remained significant after CRP adjustment. We observed that men with low LBP levels were at an increased risk of VTE, while we observed no effect of regression dilution bias from extended time between blood sampling and VTE event. These findings merit validation in future studies and are coherent with the notion of a lower basal VTE risk in women than in men—which is temporarily elevated by exposure to reproductive factors—and with male sex as a risk factor for VTE reoccurrence [10–13].

LBP is essential for TLR4-dependent immune activation and synergizes with estrogen receptor signaling to enhance IFN-I production in plasmacytoid dendritic cells [47, 48]. Estradiol enhances TLR-mediated inflammatory responses and has been suggested to mediate immunological differences between men and women [16, 17]. DVT is characterized by sterile inflammation, which may be enforced by synergy between LBP and estrogen to explain the observed sex difference.

LBP has been suggested as a biomarker for estrogen receptor α activation [49], and elevated LBP levels have been associated with several inflammation-driven VTE-related diseases, such as cardiovascular diseases [40, 42–44]. Consistent with our study, many studies have shown correlations between plasma LBP levels and VTE-related risk factors such as CRP, BMI, and age [40–44, 50]. We suggest including LBP in a recently published biomarker panel for VTE risk assessment for menopausal hormone therapy [51].

LBP is commonly used as a biomarker for the lipopolysaccharide (LPS) load in blood where the main sources of LPS are gut leakage and infection. Infection is a well-established trigger for VTE, while recent proteomic and metabolomic studies have indicated a role for gut microbiota in hypercoagulability and VTE development [52, 53]. Experimental studies have shown that LPS promotes fibrin amyloidosis, which may be reversed by LBP, and alters clot structure and density [54]. Alteration in fibrin structure may offer a plausible mechanistic link between LBP plasma levels and VTE risk where embolization potential could be affected, helping to explain the unequal risk between DVT and PE.

A strength of this study lies in the study design where the temporal sequence of blood sampling and outcome eliminated the risk of reverse causation. Furthermore, the source cohort was recruited from a single-center survey of the general population with a 77% participation rate, which limits selection bias. Additionally, our results were unaffected by adjustment for the time since blood sampling in the regression models. This indicates minimal bias from the time of blood sampling, which occurred over a 10-month period.

The hypothesis-generating untargeted discovery preceding targeted validation is a strength of this study. This design generated the hypothesis of LBP as a DVT biomarker in women. This hypothesis was confirmed both in the validation study all together and in the discovery study-independent subcohort that provided validation in an independent sample set.

The high correlations between two complementary techniques used to measure LBP in plasma limited information bias from measurement error and is a strength of the study. Additionally, the two techniques were orthogonal, and the discovery study took advantage of the improved quantitative capacity of MS3 [55, 56].

The limited number of women from which plasma samples were obtained less than 3 years before the occurrence of DVT may limit the confidence in this finding. We addressed this limitation by sensitivity analysis that successfully validated the association between women's DVT and LBP in the discovery study-independent subcohort, but we cannot eliminate the risk of residual confounding. It is a limitation of the study that the suggested association between PE and low LBP levels in women from the discovery study was not validated, possibly because the limited number of women who later suffered from PE in the discovery study may have caused a false positive identification, which could in part be augmented by random measurement error in the MS study. In addition, we did not have information on oral contraceptive use in women, although we expect this would not affect our results based on the age of the women with DVT in our study. Lastly, our observation of

increased VTE risk for men in the lowest quartile of LBP was not found in the discovery study. The limited power of the discovery study, particularly after sex stratification, combined with the use of a statistical approach suited for linear associations may explain this. Our findings should be validated in an independent source population.

Conclusion

In conclusion, we used a prospective study design combined with a two-step biomarker identification pipeline to reveal LBP as a biomarker for future VTE. Untargeted proteomics identified LBP as a candidate biomarker for women's DVT. The finding was validated in a larger nested case-control study that permitted validation in a sample set that was independent of the discovery cohort. In the validation study, we found that men in the lowest quartile of LBP were at an increased VTE risk.

Acknowledgments

The authors thank Professor Sigrid K. Brækkan and Professor John-Bjarne Hansen for their invaluable contributions to this manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

Author Contributions

Thor Ueland proofread the manuscript.

Data sharing and data accessibility

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

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: Eight peptides identified by MS2 and quantified by MS3 were used to determine the plasma levels of LBP in the discovery cohort. The lower panels show scatterplots of each peptide pair, and the corresponding Pearson correlation coefficients are shown in the upper panels. The panels in the diagonal show the histogram of the peptide distribution and the aminoacid sequence determined by MS2.
Figure S2: Plots of estimated odds ratios with 95% confidence intervals shown as whiskers for VTE, DVT, or PE as a function of the maximum time from blood sampling VTE events. Male subjects with plasma LBP levels in the lowest quartile (Q1) were compared to those with LBP levels in the second quartile (Q2, reference category). The number of VTE events are depicted above the plots.
Table S1: OR with 95% CI for VTE according to quartiles of plasma LBP levels.
Table S2: OR with 95% CI for DVT according to quartiles of plasma LBP levels.
Table S3: OR with 95% CI for PE according to quartiles of plasma LBP levels.
Table S4: OR with 95% CI for DVT according to one standard deviation increase in LBP plasma levels. Women with less than three years between blood sampling and DVT were stratified by age.
Table S5: Baseline characteristics of the women in the discovery study independent validation study. Cases were restricted to women diagnosed with a DVT within three years after blood sampling. ■

