Multiplex protein screening of biomarkers associated with major bleeding in patients with atrial fibrillation treated with oral anticoagulation

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Background: Oral anticoagulants (OAC) in patients with atrial fibrillation (AF) prevent thromboembolic events, but are associated with significant risk of bleeding.

Objectives: To explore associations between a wide range of biomarkers and bleeding risk in patients with AF on OAC.

Method: Biomarkers were analyzed in a random sample of 4200 patients, 204 cases with major bleedings, from ARISTOTLE. The replication cohort included 344 cases with major bleeding and 1024 random controls from RE-LY. Plasma samples obtained at randomization were analyzed by the Olink Proximity Extension Assay cardiovascular and inflammation panels and conventional immunoassays. The associations between biomarker levels and major bleeding over 1 to 3 years of follow-up were evaluated by random survival forest/Boruta analyses and Cox regression analyses to assess linear associations and hazard ratios for identified biomarkers.

Results: Out of 268 proteins, nine biomarkers were independently associated with bleeding in both cohorts. In the replication cohort the linear hazard ratios (95% confidence intervals) per interquartile range were for these biomarkers: TNF-R1 1.748 (1.456, 2.098), GDF-15 1.653 (1.377, 1.985), EphB4 1.575 (1.320, 1.880), suPAR 1.548 (1.294, 1.851), OPN 1.476 (1.240, 1.757), OPG 1.397 (1.156, 1.688), TNF-R2 1.360 (1.144, 1.616), cTnT-hs 1.232 (1.067, 1.423), and TRAIL-R2 1.202 (1.069, 1.351).

Conclusions: In patients with AF on OAC, GDF-15, cTnT-hs, and seven novel biomarkers were independently associated with major bleedings and reflect pathophysiological processes of inflammation, apoptosis, oxidative stress, vascular calcification, coagulation, and fibrinolysis. Investigations of the utility of these markers to refine
INTRODUCTION

Atrial fibrillation (AF) is associated with a five-fold increased risk of thromboembolisms, mainly stroke, independently of other risk factors. Oral anticoagulants (OAC) significantly reduce the risk of thromboembolic events but, unfortunately, are also associated with a significant risk of major bleeding complications. Accurate assessment to balance the risk of stroke and systemic embolic events (S/SEE), against the risk of major bleeding is therefore an important therapeutic goal in the clinical management of these patients.5

Age, prior hemorrhage, severe renal disease, and anemia (hemoglobin) have been independently associated with an increased risk of major bleeding in patients with AF.6 Using different combinations of these mainly clinical variables has resulted in at least five validated risk scores for better prediction of bleeding events in patients with AF. However, in recent years circulating protein biomarkers have been shown to add substantial incremental information about bleeding risk in patients with AF on OAC treatment. These biomarkers encompass markers of oxidative stress, myocardial injury, renal impairment, anemia, inflammation, and coagulation and fibrinolytic activity.8 To improve the prognostication of major bleeding in patients with AF we recently identified the strongest clinical variables and biomarkers associated with the outcome—that is, growth differentiation factor 15 (GDF-15), cardiac Troponin T (cTnT-hs), and hemoglobin analyzed by conventional methods. These biomarkers together with age and history of prior bleeding were included in the novel ABC bleeding score.9 This bleeding score improved the discrimination for prediction of major bleeding compared to risk scores based on clinical variables and performed correct reclassification of bleeding risk.9,10

Proximity Extension Assay (PEA; Target 96, Olink Proteomics) is an example of a new analytical high-throughput technology that allows simultaneous measurements of hundreds of biomarkers in 1 µl plasma. This technology is useful for screening multiple protein biomarkers for associations with cardiovascular disease (CVD) and responses to treatment.11 In the present study, we used three Target 96 multiplex immunoassays—CVD II, CVD III, and Inflammation—including 276 proteins, all with proposed involvement in cardiovascular disease, metabolism, inflammation, and immunity. Our main purpose was to identify novel prognostic biomarkers and potential pathophysiological processes associated with major bleeding events in patients with AF treated with OACs. Identification of biomarkers associated with bleeding can help with risk prediction and the development of novel therapies that might mitigate the risk of bleeding in patients with AF taking OACs.

ESSENTIALS

- Biomarkers associated with bleeding can help with risk prediction in patients with atrial fibrillation (AF) on oral anticoagulants (OACs).
- New analytical technologies allow measurements of hundreds of biomarkers in small plasma volumes.
- In a targeted proteomic approach we screened plasma from 5568 patients with AF on OAC.
- Nine out of 268 investigated biomarkers were significantly and independently associated with the risk of bleeding.

METHODS

2.1 Patient population

2.1.1 Identification cohort

The Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation (ARISTOTLE) was a double-blind, double dummy, randomized clinical trial that enrolled 18,201 patients with AF and at least one CHADS2 risk factor for stroke or systemic embolism between December 2006 and April 2010. Exclusion criteria included conditions other than AF that required anticoagulation (e.g., prosthetic heart valve) and severe renal insufficiency (serum creatinine >2.5 mg/dl [221 µmol/L] or calculated creatinine clearance <25 ml/min). Patients were randomized to warfarin (n = 9081) or apixaban (n = 9120). The patients were followed for 1 to 3 years (median 1.74). Details of the ARISTOTLE trial have previously been published.4,12

2.1.2 Replication cohort

The Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) trial was a prospective, multicenter, randomized trial comparing two blinded doses of dabigatran with open label warfarin that enrolled 18,113 patients with AF between December 2005 and March 2009. Exclusion criteria included severe heart valve disorder, recent stroke, creatinine clearance less than 30 ml/min, or active liver disease.3,13 Patients were followed for 1 to 3 years (median 1.96).
In both trials, patients at certain centers participated in the biomarker substudies and provided, at randomization, venous blood samples. EDTA plasma was immediately centrifuged and frozen in aliquots and stored at –70°C until analyzed centrally at the Uppsala Clinical Research Center (UCR) Laboratory, Uppsala University, Uppsala, Sweden. In both trials ethics committee approval was obtained for all investigational sites, and all patients provided written informed consent.

2.1.3 | Outcome assessment

The primary safety endpoint in both the ARISTOTLE and RE-LY trials was major bleeding, adapted from the International Society on Thrombosis and Haemostasis (ISTH) definition.12-14 A blinded Clinical Events Committee reviewed and centrally adjudicated all suspected bleeding events in both studies. Major bleeding was defined as acute or subacute clinically overt bleeding accompanied by at least one of the following: (1) decrease in hemoglobin level of ≥2 g/L; (2) a transfusion of at least two units of packed red blood cells; and/or (3) fatality or occurrence in a critical area or organ: intracranial, intraspinal, intraocular, pericardial, intraarticular, intramuscular with compartment syndrome, or retroperitoneal.

2.1.4 | Multimarker screening study design

The identification of biomarkers associated with major bleedings was based on a random sample of 4200 patients from the ARISTOTLE biomarker substudy (N = 14,780 patients with available measurements on cTnT-hs, NT-proBNP [N-terminal pro-B-type natriuretic peptide], GDF-15, and cystatin C), the identification cohort, including 204 patients with and 3996 patients without major bleedings during follow-up. Replication was performed by a case-cohort design and included all 344 cases with major bleedings and a random sample of 1024 patients without major bleeding in the RE-LY biomarker substudy (N = 8549 patients with available measurements on cTnT-hs, NT-proBNP, and GDF-15).

2.2 | Biochemical analyses

The plasma concentrations of high-sensitivity cTnT-hs, NT-proBNP, and GDF-15 were determined by Roche immunoassays using a Cobas Analytics e601 (Roche Diagnostics). Interleukin 6 (IL-6) was analyzed using the high-sensitivity sandwich ELISA immunoassay (R&D Systems Inc.) and Cystatin C with the ARCHITECT system ci8200 (Abbott Laboratories) using the particle-enhanced turbidimetric immunoassay (PETIA) from Gentian. Estimated glomerular filtration rate (eGFR) was calculated based on centrally determined creatinine levels using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation.15

2.2.1 | Proteomic profiling

The proteomic analyses were performed at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala University, without information on any other data. The determinations were performed with the high-throughput PEA technique using the Target 96 Multiplex CVD II96x96, CVD III96x96, and Inflammation96x96 panels (Olink Proteomics, www.olink.com/products), which together simultaneously measured 276 selected proteins in plasma potentially related to CVD and inflammation. The PEA technology uses pairs of antibodies equipped with DNA reporter molecules.11 When binding to their correct targets, antibody pairs give rise to new DNA amplicons each ID-barcoding their respective antigens. The amplicons are subsequently quantified using the Fluidigm BioMark™ HD real-time PCR platform. Inter-plate variability was adjusted by intensity normalization with the plate median as the normalization factor. For data analysis Olink NPX Manager software was used. The results provide relative values, normalized protein expression (NPX) data, which are log2 transformed and one-unit-higher NPX represents a doubling of the measured protein concentration. The PEA assays have high reproducibility and repeatability with mean intra-assay and inter-assay coefficients of variation around 8% and 12%, respectively, and average inter-site variation at 15%.11 A good correlation between biomarkers analyzed with the PEA technique and analyzed by conventional immunoassays has previously been shown.16 The protein markers in the identification cohort included three panels, CVD II, CVD III, and Inflammation, and are detailed in Tables S1 and S2. Of the 276 PEA proteins, 10 were available on more than one panel, resulting in 266 unique markers. As initial results in the identification cohort identified biomarkers from the CVD II and CVD III panels as more strongly associated with the outcome major bleeding, the inflammation panel was omitted in the replication subset.

2.3 | Statistical analyses

A random survival forest algorithm17 was used to evaluate the simultaneous association between variables and major bleeding. The evaluation included levels of 263 PEA markers, four conventional markers (NT-proBNP, cTnT-hs, GDF-15, and IL-6), renal function (cystatin C in the ARISTOTLE and CKD-EPI in the RE-LY studies, respectively), and 13 clinical characteristics (randomized treatment, age, gender, body mass index [BMI], smoking, hypertension, diabetes, hemoglobin, previous myocardial infarction, stroke/transient ischemic attack [TIA], peripheral artery disease, heart failure, and previous bleeding). The total number of biomarkers analyzed with the random survival forest algorithm was therefore 268 (5 analyzed by conventional analyses + 266 biomarkers analyzed with PEA excluding 3 PEA duplicate biomarkers that were analyzed by conventional analyses). The number of trees was 5000, splits were done according to a maximally selected statistic criterion, and the
variables were ranked according to their scaled permutation variable importance, measured as the change in Harrell’s C-index before and after permuting the variable of interest. The scaling is done over the trees and not over the variables and therefore does not yield values between 0 and 1. Subjects with all PEA markers missing were excluded, in ARISTOTLE 316 patients. In RE-LY there was none. There were only a few partially missing values and these were singly imputed using multivariate imputations by chained equations. An identical approach was used in the RE-LY evaluation, with a total of 184 PEA markers. The largest proportion of missing values for clinical variables was 0.5% in ARISTOTLE and 2% in RE-LY; for the PEA biomarkers 6% in ARISTOTLE and 0.1% in RE-LY. A Boruta analysis was used to confirm which of the variables in the random survival forest analysis had a larger than random association with outcomes. In short, the Boruta analysis performs multiple runs of random survival forest comparing all variables to random variables, which are shuffled copies of the original variables. Variables performing better than the maximum random variable importance are classified as confirmed, variables performing worse are rejected, and variables that cannot be confirmed or rejected are classified as tentative. In the Boruta analysis the number of trees was lowered to 2000 due to performance issues and a maximum of 100 random survival forests were run. Biomarkers with a top ranking in the random survival forest analysis and confirmed in the Boruta analysis in both cohorts, were considered to have confirmed association with the risk of major bleeding.

The pairwise correlation between PEA biomarkers and established conventional biomarkers was assessed by the Spearman correlation.

Cox regression analyses were performed including each of the established standard immunoassays (naturally log-transformed) and the PEA biomarkers, one at a time, assuming a linear association with the log hazard rate. In the validation cohort, weighted Cox regression analyses were done, in which each subject was given a weight corresponding to the reciprocal of the sampling probability. Thus, the cases were given a weight of 1.0 and the randomly sampled controls were given a weight of 1/0.1716528.

On the inflammation panel, 16 of the proteins had more than 80% of the measurements below the limit of detection and these were not candidates for inclusion in the Cox regression models. The Cox regression analyses were performed in an unadjusted model and a model adjusted for baseline clinical characteristics (age, gender, BMI, smoking, hypertension, diabetes, prior myocardial infarction, prior stroke/TIA, peripheral artery disease, heart failure, prior bleeding, and randomized treatment) and adjusting also for renal function (cystatin-C) and the established markers of bleeding risk (GDF-15 and cTnT-hs). Results were presented as the relative hazard for an interquartile difference of each marker with corresponding 95% confidence intervals and P-values. Thus, the hazard ratio can be interpreted as the relative hazard comparing the two biomarker values defining the inner 50% of the distribution, that is, the third versus the first quartile. The incremental discriminative value for each biomarker was illustrated by the C-index. All analyses were done using the R environment for statistical computing, version 3.3.1 using the ranger package.

3 | RESULTS

3.1 | Baseline characteristics

Baseline characteristics of the identification and validation cohorts are shown in Tables 1 and 2, respectively. In both studies, patients who experienced major bleeding events during follow-up were at baseline older and had more previous cardiovascular events including prior bleedings. At baseline, these patients also had higher concentrations of the cardiovascular biomarkers, NT-proBNP, cTnT-hs, and GDF-15. The inflammation marker IL-6 was also higher in this group of patients with AF. Slightly lower baseline levels of hemoglobin were found in patients with bleeding events during follow-up.

Complete lists of all 266 biomarkers and their relative concentrations (NPX values) in patients with or without bleeding events and limit of detection (LOD) are presented in Tables S1 and S2 for the identification and replication cohorts, respectively.

3.2 | Identification substudy

In the ARISTOTLE cohort, 13 clinical variables and 268 biomarkers were analyzed in the random survival forest analysis and the 50 variables with highest variable importance are presented in Figure S1A in supporting information. In this cohort osteopontin (OPN), age, and GDF-15 were identified as having the strongest association with major bleeding. According to the corresponding Boruta analysis, age and the two top biomarkers were followed by 14 biomarkers which had confirmed or tentatively confirmed importance for predicting bleeding events: cTnT-hs, interleukin-17 receptor A (IL-17RA), tumor necrosis factor receptor 1 (TNF-R1), ephrin type-B receptor 4 (EphB4), cystatin C, trefoil factor 3 (TFF3), TNF-R2, TNF-related apoptosis inducing ligand receptor 2 (TRAIL-R2), CD 40, soluble urokinase plasminogen activator receptor (suPAR), spondin-2 (SPON2), C-C motif chemokine 17 (CCL17), lymphotoxin-beta receptor (LTLBR), and osteoprotegerin (OPG) (Figure 1). The linear association with bleeding events for each of these individual biomarkers were investigated by unadjusted (Figure S2A) and adjusted for clinical characteristics (Table S3) Cox regression analyses.

3.3 | Replication substudy

The variable importance for the 50 highest ranked variables in the random survival forest including 12 clinical variables and 186 biomarkers in the RE-LY cohort are shown in Figure S1B. The random survival forest and Boruta analyses identified age and 28 biomarkers with confirmed importance for bleeding, of which 9 biomarkers were also identified in the ARISTOTLE cohort (Figure 2 and Table 3).
Variables with strongest association to major bleeding events were age, TNF-R1, TNF-R2, GDF-15, hemoglobin, interleukin 18 (IL-18) binding protein, fibroblast growth factor 23 (FGF-23), TRAIL-R2, and troponin (cTnT-hs).

The linear associations between these biomarkers and major bleeding by unadjusted (Figure S2B) and adjusted for clinical variables Cox regression analyses are shown in Table S4.

### 3.4 Biomarkers associated with bleeding in both cohorts

According to the random survival forest and Boruta analyses the following nine biomarkers were confirmed in both cohorts: GDF-15, cTnT-hs, OPN, EphB4, TNF-R1, TNF-R2, suPAR, TRAIL-R2, and OPG (Table 3). The linear associations between the identified nine biomarkers and major bleeding by unadjusted and adjusted Cox regression analyses in both cohorts are shown in Table 3. The C-index varied in the ARISTOTLE cohort between 0.685 and 0.715 and in the RE-LY cohort between 0.668 and 0.694 by adding one of the nine identified biomarkers to the baseline model including the clinical characteristics—age, gender, BMI, smoking, hypertension, diabetes, prior myocardial infarction, prior stroke/TIA, peripheral artery disease, heart failure, prior bleeding, and randomized treatment. The associations of the nine prognostic biomarkers with bleeding by splines are shown in Figure S3A,B.

The correlation of the nine identified biomarkers and established cardiovascular biomarkers, NT-proBNP, cTnT-hs, GDF-15, and renal function (cystatin C) are shown in Table 4. TNF-R1, TNF-R2 and TRAIL-R2, GDF-15, EphB4, and suPAR were moderately correlated with renal function (cystatin C) in both studies, rho ≥0.5. TNF-R1, TRAIL-R2, and suPAR also correlated with GDF-15 in both studies.

### 4 DISCUSSION

In this study we used a targeted proteomic approach to identify and confirm new biomarkers indicating risk of bleeding based on one random sample and one case-cohort in patients with AF treated with oral anticoagulation. Within each cohort, the strength of the biomarkers’ association with the risk of bleeding were ranked according to two different principles, each capturing different aspects...
of the association. The random survival forest and Boruta algorithms evaluated the biomarkers simultaneously while the Cox regression analyses evaluated the biomarkers individually. We confirmed that the biomarkers GDF-15 and cTnT are strongly and independently related to the risk of major bleeding in these patients. In addition, we were also able to identify seven novel biomarkers—OPN, OPG, TNF-R1, TNF-R2, TRAIL-R2, EphB4, and suPAR—out of 268 investigated proteins to be significantly and independently associated with the risk of bleeding. These nine novel biomarkers represent different pathophysiological processes and warrant further investigations concerning their utility for assessment of bleeding risk and identification of new therapeutic targets that might prevent bleeding in patients with AF treated with OAC.

4.1 Identified biomarkers and pathophysiological implications

The identified biomarkers represent a broad array of pathophysiological processes. We attempt, therefore, to put our findings into perspective of these pathways.

The cytokine GDF-15 is secreted by a broad range of cells upon hypoxia and oxidative stress and is strongly associated with cardiovascular disease. It is a marker of cellular aging and inflammatory activity, and a major risk indicator of hemorrhages in patients with AF treated with OAC and in patients with acute coronary syndrome (ACS). The underlying mechanism of the association of GDF-15 and risk of major bleeding is not fully understood and has yet to be revealed. Beyond its inflammatory activity, one possible process leading to enhanced bleeding risk might be that upon cellular stress and tissue damage, such as vascular vulnerability, GDF-15 is secreted.

In the present study OPN and TNF-R1 were strong predictors of major bleeding in both cohorts. OPN is a secreted multifunctional glucophosphoprotein that plays major roles in physiological as well as in pathophysiological processes. Several vascular cell types, such as monocytes/macrophages, fibroblasts, endothelial cells, vascular smooth muscle cells, and myocytes, upregulate and secrete OPN. OPN has also been suggested to be involved in and serve as a biomarker for vascular calcification. Similar to OPN, OPG, another member of the TNF receptor superfamily, is a modulator of vascular calcification and correlated with coronary calcium scores in patients with acute coronary disease, which is a risk factor for non–coronary artery bypass grafting–related major bleeding in ACS. In patients with ACS treated with dual antiplatelet therapy, OPG was recently identified as an independent biomarker of bleeding events.

| TABLE 2 Baseline characteristics and concentrations of established biomarkers in the validation cohort, the RE-LY trial |
| Baseline characteristics | Randomly selected patients N = 1024 | Cases with major bleeding N = 344 |
| Age | 72.0 (66.0–77.0) | 76.0 (71.0–80.0) |
| Gender/female | 383 (37.4%) | 115 (33.4%) |
| Body mass index (BMI) | 27.9 (25.0–31.2) | 27.3 (24.3–30.9) |
| Current smoker | 80 (7.8%) | 22 (6.4%) |
| Hypertension | 814 (79.5%) | 279 (81.1%) |
| Diabetes | 212 (20.7%) | 100 (29.1%) |
| Prior myocardial infarction | 172 (16.8%) | 74 (21.5%) |
| Prior stroke | 204 (19.9%) | 71 (20.6%) |
| Peripheral arterial disease | 36 (3.5%) | 20 (5.8%) |
| Heart failure | 298 (29.1%) | 99 (28.8%) |
| Warfarin | 343 (33.5%) | 117 (34.0%) |
| NT-proBNP (ng/L) | 816.0 (382.2, 1452.5) | 935.0 (468.8, 1705.8) |
| cTnT-hs (ng/L) | 11.9 (7.6, 19.0) | 16.9 (10.7, 28.3) |
| GDF-15 (ng/L) | 1455.0 (1083, 2122.0) | 2056.5 (1434.2, 3055.5) |
| Cystatin C (mg/L) | 1.0 (0.8, 1.2) | 1.1 (0.9, 1.4) |
| eGFR (ml/min) | 65.2 (54.3, 74.2) | 59.7 (49.0, 70.2) |
| IL-6-hs (ng/L) | 2.3 (1.4, 3.8) | 2.8 (1.8, 5.1) |
| CRP (mg/L) | 2.5 (1.2, 5.4) | 3.2 (1.4, 7.5) |
| Hemoglobin (g/dl) | 14.3 (13.2, 15.3) | 13.4 (12.4, 14.7) |

Note: Continuous variables presented as median (IQ1–IQ3). Categorial variables are presented as numbers (percentage).

Abbreviations: CABG, coronary artery bypass grafting; CRP, C-reactive protein; cTnT-hs, cardiac troponin T measured with a high-sensitivity assay; eGDFR, estimated glomerular filtration rate; GDF-15, growth differentiation factor-15; IL-6, interleukin 6; NT-proBNP, N-terminal pro-B-type natriuretic peptide; PCI, percutaneous coronary intervention.
Not much attention has been focused on the role of OPN in AF. However, very recently, OPN was found to induce atrial fibrosis and to be significantly associated with incident AF. It is also strongly associated with future ischemic stroke in patients with AF during anticoagulant treatment. Recently, it was shown that humans express multiple OPN isoforms that have different functional effects. However, the importance of these isoforms in vascular pathophysiology, CVD, and AF is still elusive.

The strong association of TNF-R1 and bleeding risk assessment in patients with AF treated with OAC identified in the present study has not been previously reported. TNF-alpha binds mainly to TNF-R1, which belongs to the TNF receptor superfamily and is expressed on all tissues in the body including cardiomyocytes. Upon activation of TNF-R1 the induced signaling pathways mainly result in apoptosis and necrosis. Protease cleavage by ADAM17 of the receptor releases the soluble form (sTNF-R1) and increased sTNF-R1 plasma levels are independently associated with higher risk of renal disease progression, cardiovascular events, and mortality in patients with diabetes. In a large population-based cohort, individuals with high levels of sTNF-R1 had increased risk of myocardial infarction, cardiovascular death, and stroke, and the plasma levels were suggested as surrogate markers of arterial injury. The TNF alpha/TNF-R1 system also induces release of endothelial cell-derived extracellular vesicles, which participate in vascular damage by contributing to apoptosis and promoting inflammation of the endothelial cells. Another so-called death receptor, TRAIL-R2 has been attributed to be the main apoptosis-inducing receptor. It induces apoptosis in macrophages, vascular smooth muscle cells, and endothelial cells and inflammatory activity of atherosclerotic plaques, and thereby contributes to the plaque vulnerability phenotype.

The association between OPN and bleeding risk found in our two large cohorts of patients with AF may reflect effects of calcified
arteries on bleeding rather than the arrhythmia. Infiltration of calcium into the vessel wall may lead to weakening of the wall by reducing compensatory compliance and the biological and mechanical hemostatic capacity of the vessel. Thus, this may limit vascular ability to appropriately respond to trauma and thereby predispose the vessel to bleeding.

Activation of the TNF alpha/TNF-R1 system and TRAIL-R2 can induce endothelial cell damage and apoptosis of the vessel wall, which provides a possible mechanism of bleeding propensity.

EphB4 is a member of the Eph tyrosine kinase receptor family, which constitutes the largest receptor tyrosine kinase (RTK) family in the genome. The Eph–ephrin signaling acts as a global cell positioning system and the EphB4/EphrinB2 system plays an important role in vascular development and angiogenesis. The Eph receptor–ephrin signaling is also involved in inflammatory processes such as monocyte adhesion, transmigration through vascular endothelium, and atherosclerotic plaque development.

Matrix metalloproteinases (MMPs) and members of the disintegrin and metalloproteinase (ADAM) family, predictors of incident AF, participate in the regulation of Eph functions and signaling upon Eph–ephrin interaction often results in cell detachment and repulsion. We have previously described that members of the Eph RTK family, including EphB4, can be proteolytically cleaved in their ectodomain by tissue factor/factor VIIa, the main initiator of blood coagulation, leading to cell repulsion. Thus, the Eph family of RTKs are novel co-receptors and proteolytical substrates of the coagulation system with consequences on cellular functions beyond blood coagulation. The activation of blood coagulation in AF might lead to proteolytic cleavage of EphB4 and a soluble form measured in plasma. To our knowledge, the present study is the first to show an association between EphB4 and AF including bleeding events in AF patients. Whether EphB4 is merely a specific biomarker of bleeding propensity reflecting increased expression on vascular cells in AF...
whether it, considering its complex biology, plays a causative role in AF remains to be elucidated.

The inflammatory biomarker suPAR is involved in the development of atherosclerosis and is associated with the presence and severity of coronary artery disease, cardiovascular death, and myocardial infarction.\(^{50,51}\) uPAR is expressed on a variety of cells, including leukocytes and endothelial cells, and localizes active uPA to the cell surface, which forms an extracellular proteolytic enzyme system.\(^{52}\) The uPA system cleaves plasminogen into plasmin and thereby plays a mandatory role in fibrinolysis by dissolution of fibrin clots and degradation of blood clotting factors and extracellular matrix.\(^{53,54}\) Inflammatory cytokines cleave uPAR from the cell surface into a soluble form that is engaged in innate immune responses through regulation of cell adhesion and migration.\(^{54}\) An elevated plasma level of suPAR has also been suggested as a biomarker of heart failure and of incident AF.\(^{55,56}\) In the present study suPAR was found to have a strong prognostic value in prediction of future bleeding events in patients with AF on OAC, confirming the modulatory role of fibrinolysis on the hemostatic balance. These results are in accordance with previous studies, which clearly demonstrated

| Biomarker | ARISTOTLE | RE-LY |
|-----------|-----------|-------|
|           | Hazard ratio (95% CI) | Hazard ratio (95% CI) |
| OPN       | 1.988 (1.667, 2.372) | 1.777 (1.525, 2.071) |
| GDF-15    | 1.731 (1.496, 2.001) | 2.056 (1.772, 2.385) |
| EphB4     | 1.378 (1.220, 1.557) | 1.968 (1.683, 2.302) |
| TNF-R1    | 1.894 (1.605, 2.236) | 1.752 (1.383, 2.521) |
| cTnT-hs   | 1.596 (1.404, 1.814) | 1.457 (1.284, 1.654) |
| TNF-R2    | 1.765 (1.490, 2.090) | 1.494 (1.255, 1.778) |
| suPAR     | 1.777 (1.493, 2.116) | 1.830 (1.566, 2.137) |
| TRAIL-R2  | 1.307 (1.191, 1.436) | 1.290 (1.163, 1.432) |
| OPG       | 1.650 (1.388, 1.963) | 1.793 (1.535, 2.093) |

Notes: The hazard ratios correspond to a comparison of the third and the first sample quartiles or, since the association is assumed linear, an interquartile difference.

The Cox regression model is adjusted for clinical characteristics—age, gender, BMI, smoking, hypertension, diabetes, prior MI, prior stroke/TIA, prior PAD, prior HF, prior bleeding and randomized treatment, renal function, cystatin C, and the biomarkers GDF-15 and cTnT-hs.

Abbreviations: BMI, body mass index; CI, confidence interval; cTnT-hs, cardiac troponin T measured with a high-sensitivity assay; EphB4, ephrin type-B receptor 4; GDF-15, growth differentiation factor-15; HF, heart failure; MI, myocardial infarction; OPG, osteoprotegerin; OPN, osteopontin; PAD, peripheral artery disease; RF, random survival forest; suPAR, soluble urokinase plasminogen activator receptor; TIA, transient ischemic attack; TNF-R, tumor necrosis factor receptor; TRAIL-R2, TNF-related apoptosis inducing ligand receptor 2.

| Biomarker | GDF-15 | NT-proBNP | cTnT-hs | Cystatin C |
|-----------|--------|-----------|---------|-----------|
|           | ARI RE-LY | ARI RE-LY | ARI RE-LY | ARI RE-LY |
| GDF-15    | 1 1 | 0.35 0.37 | 0.49 0.44 | 0.52 0.49 |
| cTnT-hs   | 0.49 0.44 | 0.38 0.40 | 0.49 0.44 | 0.52 0.49 |
| TNF-R1    | 0.55 0.55 | 0.29 0.28 | 0.45 0.40 | 0.64 0.61 |
| OPN       | 0.40 0.35 | 0.28 0.26 | 0.34 0.32 | 0.37 0.33 |
| suPAR     | 0.53 0.50 | 0.33 0.32 | 0.40 0.33 | 0.55 0.48 |
| EphB4     | 0.42 0.41 | 0.22 0.19 | 0.38 0.29 | 0.51 0.49 |
| TNF-R2    | 0.49 0.48 | 0.22 0.23 | 0.39 0.31 | 0.59 0.56 |
| TRAIL-R2  | 0.63 0.60 | 0.39 0.41 | 0.49 0.42 | 0.64 0.60 |
| OPG       | 0.44 0.41 | 0.25 0.28 | 0.33 0.34 | 0.29 0.16 |

Abbreviations: cTnT-hs, cardiac troponin T measured with a high-sensitivity assay; EphB4, ephrin type-B receptor 4; GDF-15, growth differentiation factor-15; NT-proBNP, N-terminal pro-B-type natriuretic peptide; PEA, Proximity Extension Assay; OPG, osteoprotegerin; OPN, osteopontin; suPAR, soluble urokinase plasminogen activator receptor; TNF-R, tumor necrosis factor receptor; TRAIL-R2, TNF-related apoptosis inducing ligand receptor 2.
that elevated D-dimer levels, reflected as a marker of fibrinolysis, are associated with increased bleeding events in AF patients.\textsuperscript{57} GDF-15, suPAR, EphB4, and the death receptors were moderately correlated with cystatin C. Renal impairment is a well-known risk factor for CVD and thromboembolism but also bleeding propensity, which has been attributed to an imbalance in the coagulation system.\textsuperscript{58,59} suPAR, TNF-R1, and TRAIL-R2 also correlated moderately with GDF-15, thus further indicating that inflammatory as well as coagulation processes and endothelial dysfunction are involved.

This is the first study to report a protein multimarker approach in screening of new biomarkers of importance for bleeding events in patients with AF and OAC. The identification of nine biomarkers independently associated with bleeding confirms the hypothesis that molecular biomarkers can be potentially useful to indicate the underlying biology and pathways of importance for adverse events in cardiovascular disease, including AF. A multimarker screening may also identify future targets for therapies to reduce bleeding or prevent thromboembolism without increasing bleeding. Important molecular mechanisms by which these biomarkers may confer bleeding risk are cellular aging, inflammation and innate immune responses, vascular remodeling and calcification, endothelial cell damage, apoptosis, fibrosis, coagulation activity, and fibrinolysis. The exact mechanisms remain, however, elusive. At least OPN and suPAR are not only markers of bleeding, but also markers of specific pathological processes, such as fibrosis in the heart, underlying incident AF. Impaired vessel walls in combination with decreased coagulation propensity, due to OAC, and activation of fibrinolysis in patients with AF at older age might be one plausible explanation for these biomarkers as indicators of future bleeding. In the clinical setting GDF-15 has already clearly improved the risk prediction beyond clinical risk factors. Whether the newly identified biomarkers may add clinical value to the assessment of major bleeding risks in patients with AF needs to be validated.

4.1.1 Strengths and limitations

The strength of this study is that it is based on cross-validation of proteomic findings in two large materials from two clinical cohorts with complete follow-up and independent ascertainment of all outcome events. Considering the problem of multiplicity and mass-significance when evaluating the 268 biomarkers, it was alleviated by using a random survival forest and Boruta algorithm, as well as comparing the results in two independent cohorts. We based our selection of important biomarkers on a non-linear machine learning approach using the same criteria as we have previously published.\textsuperscript{60} The random survival forest and Boruta algorithms allow for non-linear associations and complex interactions among the variables. In the Cox regression analyses, on the other hand, we assumed a linear association between the log relative hazard of major bleedings and each marker one at a time, making it possible to estimate average adjusted hazard ratios in a more conventional way.

There were differences between the two cohorts in regard to study design, random sample versus case cohort, and to the number of bleeding events, with 204 in the identification cohort and 344 in the replication cohort. The PEA inflammation panel was not applied in the validation cohort. These differences may have influenced the results to some degree.

The results in this study were obtained in individuals with AF enrolled in clinical trials evaluating antithrombotic therapy, which may limit external generalizability.

The biomarker assays used in the PEA technique result in values expressed in relative units, NPX values. The PEA technique is excellent for screening purposes but for clinical applications absolute concentrations will be required. Prior validation studies have, however, clearly demonstrated that biomarkers analyzed with the PEA technique have good further investigation concordance with established immunoassays.\textsuperscript{16}

5 CONCLUSIONS

In two well-characterized clinical trial cohorts of patients with AF on OAC we confirmed, out of 268 screened biomarkers, the importance of GDF-15 and cTnT-hs and identified seven novel biomarkers that contribute to prognostication of the risk of major bleedings. These biomarkers showed association with inflammatory processes (GDF-15, OPN, OPG, TNF-R1, TNF-R2, TRAIL-R2, EphB4, and suPAR), vascular remodeling and calcification (GDF-15, OPG, OPN), endothelial cell damage and cell survival (TNF-R1, TNF-R2, TRAIL-R2), coagulation (EphB4), and fibrinolysis (suPAR). Further in-depth studies are required to elucidate the underlying biological processes for the risk of major bleeding in AF. Whether any individual or a combination of these novel biomarkers might improve prognostication of bleeding in the clinical setting and guide future therapies that may reduce bleeding warrant further investigations using quantitative assays in these and other cohorts.

CONFLICTS OF INTEREST

Dr. Siegbahn: institutional research grants from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb/Pfizer, GlaxoSmithKline, Roche Diagnostics and consultancy fees from Olink Proteomics. Mr. Lindbäck: institutional research grants from Boehringer Ingelheim, Bristol-Myers Squibb and Pfizer. Dr. Hijazi: lecture fees from Boehringer Ingelheim, Roche, Bristol-Myers Squibb, and Pfizer; consulting fees from Merck Sharp & Dohme, Roche, Bristol-Myers Squibb, and Pfizer. Dr. Åberg: none. Dr. Alexander: institutional research grants from Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Cryolife, CSL Behring, Ferring, Glaxosmithkline, and XaTek and consulting fees/honoraria from AbbVie, Bristol-Myers Squibb, Cryolife, Glaxosmithkline, Pfizer, and Portola. Dr. Elkelboom: honoraria and institutional research grants from AstraZeneca, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb/Pfizer, Daiichi Sankyo, GlaxoSmithKline, Janssen, Sanofi Aventis, and Eli Lilly, as well as a personnel award from the Heart and Stroke Foundation. Dr. Lopes: institutional research grant and consulting fees from Bristol-Myers Squibb; institutional research grant from GlaxoSmithKline; consulting
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**AUTHOR CONTRIBUTIONS**

AS and LW conceived the research and planned the design of the study. AS, JL, ZH, TP, JO, and LW performed formal analysis of the study results. JL performed the statistical analyses. AS wrote the manuscript. All coauthors critically revised the manuscript. All coauthors have approved the final manuscript for submission.

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