Different circulating biomarkers in women and men with paroxysmal atrial fibrillation: results from the AF-RISK and RACE V studies

Ruben R. De With1†, Vicente Artola Arita 1†, Bao-Oanh Nguyen1, Dominik Linz2,3,4,5,6, Hugo Ten Cate17,8,9, Henri Spronk7,8,9, Ulrich Schotten3,4, Anton Jan van Zonneveld10, Ömer Erkünner2,3, M. Agustina Bayón1, Anders S. Schmidt11,12, Justin G.L.M. Luermans2,3, Harry J.G.M. Crijns12, Isabelle C. Van Gelder1, and Michiel Rienstra1

1Department of Cardiology, University of Groningen, University Medical Centre Groningen, Groningen, Hanzeplein 1, 9713 GZ, The Netherlands; 2Department of Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands; 3Department of Cardiology, Radboud University Medical Centre, Nijmegen, The Netherlands; 4Centre for Heart Rhythm Disorders, University of Adelaide and Royal Adelaide Hospital, Adelaide, Australia; 5Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; 6Departments of Biochemistry, Thrombosis Expertise Center (TEC) Maastricht, Maastricht, The Netherlands; 7Internal Medicine, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands; 8Maastricht University Medical Center+1, Maastricht, The Netherlands; 9Department of Internal Medicine (Nephrology), Eindhoven Laboratory for Vascular and Regenerative Medicine, Leiden University Medical Center, Leiden, The Netherlands; 10Department of Internal Medicine, Randers Regional Hospital, Randers, Denmark; 11Centre for Emergency Medicine, Institute for Clinical Medicine, Aarhus University, Aarhus, Denmark; 12Centre for Emergency Medicine, Institute for Clinical Medicine, Aarhus University, Aarhus, Denmark

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Aims
The clinical risk profile of atrial fibrillation (AF) patients is different in men and women. Our aim was to identify sex differences in blood biomarkers in patients with paroxysmal AF.

Methods and results
Sex differences in 92 blood biomarkers were measured in 364 patients included in our discovery cohort, the identification of a risk profile to guide atrial fibrillation therapy (AF-RISK) study, assessed by multivariable logistic regression and enrichment pathway analysis. Findings were subsequently confirmed in 213 patients included in our validation cohort, the Reappraisal of Atrial Fibrillation: Interaction between HyperCoagulability, Electrical remodelling, and Vascular Destabilisation in the Progression of AF (RACE V) study. In the discovery cohort, mean age was 59 ± 12 years, 41% were women. CHA2DS2-VASc-score was 1.6 ± 1.4. A total of 46% had hypertension, 10% diabetes, and 50% had heart failure, predominantly with preserved ejection fraction (47%). In women, activated leukocyte cell adhesion molecule (ALCAM) and fatty acid binding protein-4 (FABP-4) were higher. In men, matrix metalloproteinase-3 (MMP-3), C-C motif chemokine-16 (CCL-16), and myoglobin were higher. In the validation cohort, four out of five biomarkers could be confirmed: levels of ALCAM (P = 1.73 × 10−4) and FABP-4 (P = 2.46 × 10−4) and adhesion biological pathways [false discovery rate (FDR) = 1.23 × 10−4] were higher in women. In men, levels of MMP-3 (P = 4.31 × 10−8) and myoglobin (P = 2.10 × 10−4) and markers for extracellular matrix degradation biological pathways (FDR = 3.59 × 10−4) were higher.

Conclusion
In women with paroxysmal AF, inflammatory biomarkers were more often higher, while in men with paroxysmal AF, biomarkers for vascular remodelling were higher. Our data support the clinical notion that pathophysiological mechanisms in women and men with AF may differ.

Trial registration
Clinicaltrials.gov identifier NCT01510210 for AF-RISK; Clinicaltrials.gov NCT02726698 for RACE V.

† The first two authors contributed equally to the study.

\* Corresponding author. Tel: +31 50 3611327; fax: +31 50 3614391. E-mail address: m.rienstra@umcg.nl

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Keywords

Atrial fibrillation • Blood biomarkers • Sex differences • Inflammation • Vascular disease

**Graphical Abstract**

*What's new?*
- In patients with paroxysmal atrial fibrillation (AF), inflammation biomarkers are more expressed in women in comparison to men, and vascular remodelling biomarkers are more expressed in men in comparison to women.
- In patients with paroxysmal AF, enrichment of cell adhesion pathways in women compared to men, and enrichment of extracellular matrix organization pathways in men compared to women were observed.
- Blood biomarker analysis may contribute to a personalized medicine approach in patients with paroxysmal AF.

**Introduction**

Atrial fibrillation (AF) is the most common sustained arrhythmia around the world and the number will increase due to ageing populations and active search for early diagnosing. The development of AF is driven by risk factors including, but not limited to, ageing, obesity, and underlying cardiovascular risk factors and diseases. The cumulative prevalence of AF over the years is high and comparable in both sexes. However, women and men with AF differ regarding age and presence of comorbidities; women with AF are older, have more hypertension, valvular heart disease, and heart failure with preserved ejection fraction, and men with AF present more often coronary artery disease. Whether the age difference or clustering of comorbidities are causative of the difference in clinical risk profile of AF...
between women and men is yet to be determined. Other factors such as sex hormones or differentially expressed blood biomarkers representing distinct biological pathways may also play a role.1,2

Blood biomarkers can be seen as representation of distinct biological pathways and may differ between men and women with AF. C-reactive protein (CRP), an inflammatory biomarker, and NT-pro-B-type natriuretic peptide (NT-proBNP), a biomarker indicating cardiac stretch, have been shown to differ in women and men. CRP has been associated with AF incidence in men, whereas NT-proBNP has been associated with incident AF in women.3 NT-proBNP and fibroblast growth factor 23, a hormone regulating biomarker associated with AF, have been suggested to help to identify those at risk for AF;4 NT-proBNP and Cancer Antigen 125 (CA-125) have been associated with AF in patients without any concomitant disease.5 Therefore, biomarkers may help to find guidance for a personalized approach to patients with AF.2–4

Our aim was to identify sex differences in blood biomarkers in patients with paroxysmal AF, to provide an insight into potential sex-specific pathophysiological mechanisms in a well-phenotyped AF population.

Methods

Study population

Patients included in the identification of a risk profile to guide atrial fibrillation therapy (AF-RISK) study were used as discovery cohort. The methods of the AF-RISK study have previously been described.6 In short, AF-RISK was a prospective, multicentre, observational study including patients with history of AF, performed in The Netherlands between May 2011 and March 2016. Inclusion criteria were patients aged ≥18 years who presented at either the inpatient or outpatient cardiology clinic with paroxysmal AF (total AF history <2 years, or total AF history <3 years in case of ≤2 AF episodes of ≤48 h per month terminating spontaneously) or persistent AF (total AF history <2 years, and total persistent AF duration >7 days and <1 year) in whom a rhythm control strategy was preferred. Exclusion criteria were patients with history of heart failure >3 years, severe valvular disease, contra-indication for oral anticoagulation, acute coronary syndrome <1 month, or post-operative AF. In total, 386 patients had paroxysmal AF and were in sinus rhythm at the moment of blood sampling: from this amount, 366 (94%) had blood biomarker results available and were included for the current analysis.

Patients included in the Reappraisal of Atrial Fibrillation: Interaction between HyperCoagulability, Electrical remodelling, and Vascular Destabilisation in the Progression of AF (RACE V) study were used as validation cohort.4 In short, RACE V is an ongoing investigator-initiated, prospective, multicentre registry aiming to include 750 patients in multiple centres in The Netherlands. Inclusion criteria were patients aged ≥18 years with paroxysmal AF, a maximum AF history of 10 years since diagnosis at the moment of inclusion, a maximum CHA2DS2-VASC score of 5, and no other indication for anticoagulation drugs (e.g. mechanical valve prosthesis). Patients had to have at least two documented episodes of paroxysmal AF in the past year or one documented episode with at least two symptomatic episodes in the past year suspected to be AF without documentation. In patients with a Medtronic pacemaker, atrial high rate episodes (AHREs) >190 beats per minute lasting >6 min were qualified as AF episodes. Patients with other types of pacemakers, defibrillators or cardiac resynchronization therapy could not participate due to differences in AHRE algorithm and/or incompatibility with the type of home-monitoring. Further exclusion criteria were patients with a history of persistent AF, currently on amiodarone, current pregnancy or a life expectancy <2.5 years, patients with AF caused exclusively due to transient triggers (e.g. postoperative, due to infection), patients with a previous pulmonary vein isolation (PVI), or intention to undergo PVI, or diagnosed congenital heart disease. In total, 247 patients had available blood samples; from this amount, a total of 34 (14%) were excluded because of AF at the moment of sampling. Samples from the remaining 213 patients were used for the current analyses.

Both AF-RISK and RACE V were performed in concordance with the Declaration of Helsinki. The Institutional Review Board approved both protocols. AF-RISK was registered at Clinicaltrials.gov (Clinicaltrials.gov identifier NCT01510210), as well as RACE V (Clinicaltrials.gov identifier NCT02726698) and all patients gave written informed consent.

Blood biomarkers

An electrocardiogram was performed to assess the heart rhythm prior to blood sampling. Blood sampling was performed in a similar fashion at baseline in both cohorts. EDTA anticoagulated plasma was obtained from ethylenediaminetetraacetic acid tubes and was stored at −80 ºC. Multiplex immunoassay by proximity extension assay (PEA) technology (Olink Bioscience, Uppsala, Sweden) was used to measure 92 biomarkers from the Olink cardiovascular panel III (full list shown in Supplementary material online, Table S1). The PEA technology uses a homogeneous assay that uses pairs of antibodies equipped with DNA reporter molecules. In the kits, 92 oligonucleotide-labelled antibody probe pairs are allowed to bind to their respective target if present in the sample. A PCR reporter sequence is then amplified, and subsequently detected and quantified using real-time PCR. The assay was performed in a homogeneous 96-well format without any need for washing steps. Internal controls were added to each sample and include two immunoassay controls, one extension control and one detection control. Samples for which one or more of the internal control values deviate from a pre-determined range were flagged and removed before statistical analysis. PEA results do not provide absolute concentration of the proteins; instead, proteins are expressed as normalized protein expression on a log2-transformed concentrations where a larger number represents a higher protein level in the sample, typically with the background level at around zero.

Limit of detection (LOD) was defined as 3 standard deviations above background and reported in pg/mL for all assays for which recombinant protein antigen was available. Four biomarkers (bleomycin hydrolase, spondin-1, elafin, and cathepsin D) had >10% of the values below LOD and were therefore excluded. The remaining 88 biomarkers were used for the analyses.

Comorbidities

Heart failure was defined as one of the following: (i) history of heart failure admission, regardless of the left ventricular ejection fraction (LVEF); (ii) LVEF <45%; (iii) LVEF >45%, an elevated NT-proBNP (>400 ng/L) with either structural heart disease (history of left ventricular hypertrophy or wall diameter ≥11 mm or septum diameter ≥11 mm) or diastolic dysfunction (average annular e’ < 8 cm/s, and deceleration time > 220 ms, and average E/e’ > 8) on echocardiography.7 Hypertension was defined by a systolic blood pressure >140 mmHg or diastolic blood pressure ≥90 mmHg, or use of antihypertensive medication. Diabetes mellitus was defined by use of antidiabetic drugs. Coronary artery disease was defined as history of myocardial infarction, percutaneous coronary intervention or coronary artery bypass grafting.
Sex differences in blood biomarkers were tested by univariable and multivariable logistic regression. In the multivariable logistic regression model additional adjustment for obesity, age, heart failure, and coronary artery disease was performed based on differences found between women and men at baseline and knowledge from previous literature. The final model was tested for significant interactions. Odds ratios (ORs) per standard deviation with 95% confidence intervals (CIs) were given. Biomarkers with higher values in men were expressed as OR vs. women, biomarkers higher in women were presented as OR vs. men. Biomarkers found in the discovery cohort were subsequently tested by univariable and multivariable logistic regression in the validation cohort.

Enrichment pathway analyses were performed for blood biomarkers with higher values in women in comparison to men. The median value of each biomarker in women was divided by the median value of the same biomarker in men to produce a sex difference ratio per biomarker. This ratio was then transformed into percentage (Supplementary material online, Figure S1). Biomarkers found in the discovery cohort were subsequently tested in the validation cohort.

Confirmed biomarkers in the validation cohort were additionally enriched in a network analysis using STRING to identify relevant biological pathways in which the biomarkers are involved. STRING is a database that provides assessment of physical and functional protein interactions which contribute to common biological processes. This knowledge derives from databases and text-mining highly calibrated, such as Gene Ontology (GO) Resource using high level groupings established by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps. Biomarkers that entered the pathway analysis were two-layer enriched.

In both multivariate logistic regression and pathway analyses, a multiple testing correction was performed using a Bonferroni correction for the 88 biomarkers that were tested. A P-value < 5.68 × 10^-4 (0.05/88) was considered statistically significant. Additionally, pathway enrichment underwent false discovery rate (FDR) correction for multiple testing. Pathways with higher FDR were selected as main representative processes.

### Table 1 Baseline characteristics AF-RISK discovery cohort

| Characteristic                  | Total population (N = 364) | Women (N = 150) | Men (N = 214) | P-value |
|--------------------------------|-----------------------------|------------------|---------------|---------|
| Age (years)                    | 59 ± 12                     | 60 ± 12          | 58 ± 12       | 0.030   |
| History of AF (months)         | 6 (2–18)                    | 5 (2–17)         | 6 (2–20)      | 0.329   |
| Heart failure                  | 182 (50%)                   | 66 (44%)         | 116 (54%)     | 0.070   |
| HFpEF                          | 171 (47%)                   | 65 (43%)         | 106 (50%)     | 0.289   |
| HFrEF                          | 11 (3%)                     | 1 (1%)           | 10 (5%)       | 0.059   |
| Hypertension                   | 167 (46%)                   | 76 (51%)         | 91 (43%)      | 0.164   |
| Diabetes mellitus              | 35 (10%)                    | 12 (8%)          | 23 (11%)      | 0.471   |
| Coronary artery disease        | 21 (6%)                     | 6 (4%)           | 15 (7%)       | 0.260   |
| Peripheral artery disease      | 9 (3%)                      | 3 (2%)           | 6 (3%)        | 0.741   |
| Stroke or TIA                  | 23 (6%)                     | 10 (7%)          | 13 (6%)       | 0.830   |
| COPD                           | 23 (6%)                     | 7 (5%)           | 16 (8%)       | 0.382   |
| CHA2DS2-VASc score<sup>a</sup> | 1.6 ± 1.4                   | 2.3 ± 1.3        | 1.1 ± 1.2     | <0.001  |
| EHRA class<sup>b</sup>         |                             |                  |               | 0.296   |
| I                              | 110 (30%)                   | 34 (23%)         | 76 (36%)      |         |
| II                             | 204 (56%)                   | 94 (63%)         | 110 (51%)     |         |
| III                            | 49 (14%)                    | 22 (15%)         | 27 (13%)      |         |
| Height (cm)                    | 178 ± 10                    | 170 ± 7          | 184 ± 7       | <0.001  |
| Weight (kg)                    | 88 ± 18                     | 81 ± 17          | 92 ± 17       | <0.001  |
| BMI (kg/m<sup>2</sup>)         | 28 ± 5                      | 28 ± 6           | 27 ± 5        | 0.129   |
| Obesity (BMI > 30)             | 99 (27%)                    | 43 (29%)         | 56 (26%)      | 0.633   |
| Blood pressure (mmHg)          |                             |                  |               |         |
| Systolic                       | 131 ± 18                    | 134 ± 20         | 128 ± 15      | 0.004   |
| Diastolic                      | 78 ± 9                      | 78 ± 11          | 78 ± 8        | 0.693   |
| PQ time (ms)                   | 165 ± 25                    | 161 ± 24         | 168 ± 25      | 0.007   |
| Left atrial volume (mL)        | 67 ± 21                     | 62 ± 19          | 69 ± 21       | 0.002   |
| Left atrial volume index (mL/m<sup>2</sup>) | 33 ± 10          | 33 ± 10          | 32 ± 10       | 0.696   |
| LV ejection fraction (%)       | 57 ± 4                      | 58 ± 3           | 57 ± 5        | 0.016   |

Data are mean (standard deviation), number of patients (%), or median (interquartile range).

AF, atrial fibrillation; BMI, body mass index; COPD, chronic obstructive pulmonary disease; EHRA, European Heart Rhythm Association class for symptoms; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; LV, left ventricular; TIA, transient ischaemic attack.

<sup>a</sup>The CHA2DS2-VASc score assesses thromboembolic risk. C, congestive heart failure/LV dysfunction; H, hypertension; A2, age ≥75 years; D, diabetes mellitus; S2, stroke/transient ischaemic attack/systemic embolism; V, vascular disease; A, age 65–74 years; Sc, sex category (female sex).

<sup>b</sup>In 363 patients, EHRA class data was available.
Results

Patient characteristics

Clinical characteristics of patients from the discovery cohort are shown in Table 1. Patient characteristics were comparable to the entire AF-RISK cohort (data not shown). In short, mean age was 59 ± 12 years, 150 (41%) were women. CHA2DS2-VASc-score was 1.6 ± 1.4. A total of 182 (50%) had heart failure [171 (47%) with preserved and 11 (3%) with reduced ejection fraction] and 167 (46%) hypertension. Women compared to men were slightly older (60 ± 12 vs. 58 ± 12 years, P = 0.03) and had slightly higher LVEF (58 ± 3 vs. 57 ± 5, P = 0.01).

Baseline characteristics from the validation cohort are shown in Table 2. Compared to the discovery cohort, the proportion of women was comparable in the validation cohort (41%). CHA2DS2-VASc-score was 1.9 ± 1.3. A total of 62 (29%) had heart failure [60 (28%) with preserved and 2 (1%) with reduced ejection fraction] and 101 (47%) hypertension. Within the validation cohort, women compared to men were older (66 ± 9 vs. 63 ± 10 years, P = 0.01), had less often heart failure (22 vs. 38%, P = 0.01) and had more often obesity (39 vs. 21%, P = 0.008). Patients in the validation were older in comparison to the discovery cohort (64 ± 9 vs. 59 ± 12 years, P < 0.01) and had longer history of AF (29 vs. 6 months, P < 0.01).

Biomarker analysis

The multivariable logistic regression in the discovery cohort showed that levels of activated leucocyte cell adhesion molecule (ALCAM, P = 4.03 × 10^-4) and fatty acid binding protein-4 (FABP-4, P = 4.48 × 10^-4) were higher in women. While levels of matrix metalloproteinase-3 (MMP-3, P = 6.46 × 10^-3), C-C motif chemokine-16 (CCL-16, P = 4.17 × 10^-4), and myoglobin (P = 2.34 × 10^-8) were higher in men (Figure 1).
The five biomarkers found in the discovery cohort were univariably tested in the validation cohort and all but CCL-16 (univariably OR 1.090, 95% CI 0.829–1.432, \( P = 0.537 \)) were confirmed to be differently expressed between sexes (Table 3). Based on differences on baseline characteristics and knowledge from previous literature, it was adjusted for obesity, age, and heart failure; after this adjustment, only FABP-4 remained higher in women (OR 7.442, 95% CI 3.680–15.051, \( P = 2.32 \times 10^{-8} \)). MMP-3 (OR 8.403, 95% CI 4.329–16.393, \( P = 3.12 \times 10^{-10} \)) remained higher in men.

In the pathway analysis, six biomarkers in women and eight in men were statistically significant (Supplementary material online, Figure S2), which included the biomarkers from the multivariate logistic regression analysis in the discovery cohort. These biomarkers were subsequently tested in the validation cohort; six remained statistically significant in women, FABP-4, ALCAM, NT-proBNP, contactin-1 (CNTN1), metalloproteinase inhibitor 4 (TIMP4), and integrin beta-2 (ITGB2); three remained statistically significant in men matrix, extracellular phosphoglycoprotein (MEPE), myoglobin, and MMP3 (Supplementary material online, Table S2 and Figure S3).

After a two-layer protein enrichment, in women compared to men, pathways with higher FDR under GO analysis showed cell-cell adhesion (FDR = 1.23 \times 10^{-8} ), integrin-mediated signalling pathway (FDR = 3.83 \times 10^{-8} ), and cell adhesion (6.13 \times 10^{-8} ); moreover, cell adhesion molecules (FDR = 5.19 \times 10^{-12} ) pathways resulted under KEGG analysis. In men, extracellular matrix organization (FDR 3.59 \times 10^{-9} ) pathway resulted from GO analysis without any results under KEGG analysis (Figure 2).

**Table 3**  
Multivariate logistic regression results of biomarkers in validation cohort

| Biomarker   | OR     | 95% CI       | P-value     |
|-------------|--------|--------------|-------------|
| MMP-3       | 6.289a | 3.257–12.195 | 4.31 \times 10^{-8} |
| CCL-16      | NS     |              |             |
| Myoglobin   | 3.135a | 1.712–5.747  | 2.10 \times 10^{-4} |
| ALCAM       | 3.165  | 1.735–5.774  | 1.73 \times 10^{-4} |
| FABP-4      | 5.975  | 3.030–11.78  | 2.46 \times 10^{-7} |

aOdds ratios are expressed vs. women.

ALCAM, activated leucocyte cell adhesion molecule; CCL-16, C-C motif chemokine-16; CI, confidence interval; FABP-4, fatty acid binding protein-4; MMP-3, matrix metalloproteinase-3; NS, not statistically significant; OR, odds ratio; SD, standard deviation.

**Discussion**

The aim of this study was to identify sex differences in blood biomarkers in patients with AF. We identified five biomarkers that were differently expressed between sexes with paroxysmal AF. In a validation cohort, four out of five markers were confirmed to be differently expressed between sexes.

**Blood biomarkers in women**

In women, ALCAM and FABP-4 were higher. Cell adhesion molecules, like ALCAM, are involved in leucocyte recruitment in case of tissue damage. In patients with stroke, ALCAM has been associated with long-term mortality.\(^9\) Also, Lim et al.\(^10\) previously showed that higher levels of ALCAM were associated with early recurrence after AF ablation. Moreover, cell adhesion mechanisms increase the adhesiveness of platelets and leucocytes increasing the risk of thrombogenesis even when in sinus rhythm.\(^11\) FABP-4 is mainly expressed in adipose tissue and represents around 6% of the total protein adipocytes. It has been associated with a systemic pro-inflammatory state, development of atherosclerosis and metabolic syndrome. In the presence of coronary artery

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**Figure 1** Blood biomarker sex differences in discovery cohort. ALCAM, activated leucocyte cell adhesion molecule; CCL-16, C-C motif chemokine-16; CI, confidence interval; FABP-4, fatty acid binding protein-4; MMP-3, matrix metalloproteinase-3; OR, odds ratio; SD, standard deviation.
In men, levels of MMP-3 and myoglobin were higher. MMP-3 is part of the family of matrix metalloproteinases that are involved in extracellular matrix degradation and deposition. MMP-3 has been associated with vascular remodelling, including atrial stiffening and coronary artery disease and has also been suggested as potential therapeutic target in atherosclerosis. Moreover, Yue et al. concluded that the excess of proteins involved in extracellular matrix biological pathways may lead to tissue fibrosis, contributing to vascular remodelling: this affects mechanical and electrical function, and therefore can promote AF. Different studies have reported contrasting results between the association of MMP-3 and LAVI (left atrial volume indexed). LAVI is comparable between men and women in the current analysis; we could speculate that the association between MMP-3 and LAVI is not present in this population. However, a conclusive statement of this association cannot be drawn since levels of biomarkers are relative measures from the population, making them not comparable to absolute measures. Myoglobin can be detected in case of muscle degradation. Recurrent episodes of silent ischaemia, also in patients with subclinical coronary artery disease may be the underlying substrate for myocardial myoglobin release. In addition, higher muscle mass in men could contribute to the observed outcome. The combination of MMP-3 and myoglobin may indicate that in men, vascular remodelling plays an important role in AF substrate formation. Prevalence of clinical coronary artery disease was, however, not different between sexes in our discovery nor validation research.
cohorts. When corrected for differences in underlying disease, MMP-3 remained associated with higher values in men. This could indicate that subclinical vascular disease is more prominent in men (Graphical abstract). This in accordance with findings from the Rotterdam study which described subclinical atherosclerosis as an independent risk factor for new-onset AF but not only in men. Subclinical atherosclerosis, which may be present in many patients with AF, was, however, not routinely assessed in our discovery cohort. Since the biomarker panel used in this analysis did not include CA-125, our results cannot be compared to previous findings of this biomarker.

**Strengths and limitations**

Limitations of the current analysis include the use of a biomarker panel with relative values, which impairs comparison with absolute values of other cohorts. Also, this was a cross-sectional study which precludes definite conclusions regarding cause–effect relations. In addition, the AF duration of the validation cohort is longer than in the discovery cohort, implying greater atrial remodelling substrate and differentiated expression of blood biomarkers. Furthermore, information regarding frequency of menstrual cycles in women was not collected, which could have provided an insight on the association of hormones and biomarker expression in women in pre- and post-menopausal periods. Lastly, residual confounding may have affected results, despite adjustment-analysis. Strengths of the current analysis are the well-phenotyped cohorts and the availability of a large number of biomarkers representative of multiple biological pathways. Furthermore, the use of two analytical approaches and two independent cohorts yielded synergic results.

**Conclusion**

In conclusion, in this exploratory analysis, we identified biomarkers differentially expressed in women and men with paroxysmal AF. In a validation cohort, four out of five biomarkers were confirmed. In women with paroxysmal AF, inflammatory biomarkers were higher, while in men with paroxysmal AF biomarkers for vascular remodeling were higher. Our data suggest that pathophysiological mechanisms in women and men with AF may differ. This advocates more research on sex differences in AF and endorses a personalized medicine approach, taking sex differences into account.

**Supplementary material**

Supplementary material is available at Europace online.

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**Conflict of interest**: H.T.C. has received research support from Bayer and Pfizer and is consultant for Alveron and shareholder with Coagulation profile. J.G.L.M.L. has a consultancy agreement with Medtronic. All remaining authors have declared no conflicts of interest.

**Data availability**

The data underlying this article cannot be shared publicly due to the privacy of individuals that participated in the study.

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