Vascular Calcification and not Arrhythmia in Idiopathic Atrial Fibrillation Associates with Sex Differences in Diabetic Microvascular Injury miRNA Profiles

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RESEARCH ARTICLE

Vascular Calcification and not Arrhythmia in Idiopathic Atrial Fibrillation Associates with Sex Differences in Diabetic Microvascular Injury miRNA Profiles

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Abstract: ***Background*:** Atrial Fibrillation (AF) in patients without concomitant cardiovascular pathophysiological disease, is called idiopathic Atrial Fibrillation (iAF). Nonetheless, iAF patients have often times subclinical coronary (micro) vascular dysfunction and, particularly in women, a higher prevalence of subsequent cardiovascular comorbidities. Previously, we identified a plasma miRNA association with diabetes and microvascular injury in Diabetic Nephropathy (DN) patients. Therefore, in this study we assessed whether plasma levels of these diabetic, microvascular injury associated miRNAs reflect microvascular integrity in iAF patients, associated with the presence of paroxysmal arrhythmia or instead are determined by concealed coronary artery disease.

***Methods*:** Circulating levels of a pre-selected set of diabetic, (micro) vascular injury associated miRNAs, were measured in 59 iAF patients compared to 176 Sinus Rhythm (SR) controls. Furthermore, the presence of coronary artery and aortic calcification in each patient was assessed using Cardiac Computed Tomography Angiography (CCTA).

***Results*:** Paroxysmal arrhythmia in iAF patients did not result in significant miRNA expression profile differences in iAF patients compared to SR controls. Nonetheless, coronary artery calcification (CAC) was associated with higher levels of miRNAs-103, -125a-5p, -221 and -223 in men. In women, CAC was associated with higher plasma levels of miRNA-27a and miRNA-126 and correlated with Agatston scores. Within the total population, ascending Aortic Calcification (AsAC) patients displayed increased plasma levels of miRNA-221, while women, in particular, demonstrated a Descending Aorta Calcification (DAC) associated increase in miRNA-212 levels.

***Conclusions*:** Diabetic microvascular injury associated miRNAs in iAF are associated with subclinical coronary artery disease in a sex-specific way and confirm the notion that biological sex identifies iAF subgroups that may require dedicated clinical care.

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1. INTRODUCTION

There is a strong association between Atrial Fibrillation (AF) and vascular disease, as this arrhythmia usually occurs in patients with cardiovascular comorbidities [1, 2] and, particularly in women upon diabetes mellitus [3]. However, in a subset of patients with AF, meticulous phenotyping cannot identify any concomitant cardiovascular disease, a condition called idiopathic AF (iAF). Mechanistic studies in iAF patients have identified several underlying pathophysiological mechanisms that pinpoint myocardial, microvascularperfusion abnormalities in iAF [4], including oxidative stress [5], systemic inflammation [6] and subclinical coronary artery

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disease [7]. Interestingly, in conjunction with female sex, these (micro) vascular comorbidities in iAF have been shown to augment the relative risk and event rates for stroke and transient ischemic attack [8]. Therefore, early monitoring of (micro) vascular integrity in iAF patients could aid to better characterize iAF subgroups that might benefit from dedicated treatment strategies.

MicroRNAs (miRNAs) are short non-coding RNA molecules that fine-tune gene expression and thereby control functionally-related biological pathways [9]. In human plasma, selected miRNAs circulate in levels that are different across (micro) vascular disease phenotypes [10] or biological sex [11]. Following this notion, we recently demonstrated that a subset of circulating miRNAs is associated with diabetic induced microvascular injury in Diabetic Nephropathy (DN) patients and correlated with conventional circulating markers of microvascular injury, such as angiopoietin-2 (Ang-2), soluble thrombomodulin (sTM) and Insulin-like Growth Factor (IGF) [12]. Previous miRNA expression profiling Studies in AF patients demonstrated that miRNA-126 [13] and miRNA-152 [14] levels are associated with prevalent arrhythmia, yet no relationship with vascular integrity or biological sex was established in these studies.

To determine whether circulating miRNAs could also serve as markers of (micro) vascular integrity in iAF, we assessed the association of plasma levels of 48 selected diabetic microvascular disease associated miRNAs which we previously found to be associated with (diabetic) conditions of (micro) vascular dysfunction [12, 15]. Next, because iAF has also been associated with increased subclinical coronary artery disease [7], we determined whether selected circulating miRNA levels were associated with coronary artery disease on Cardiac Computed Tomography Angiography (CCTA). Finally, because diabetes in women is a significant predictor of AF development [3] and women with iAF are at a higher risk of stroke or transient ischemic attack [8], we stratified circulating miRNA levels according to biological sex, to evaluate whether biological sex is a determinant of the association between vascular calcification and circulating miRNAs.

2. METHODS

2.1. Patient Selection

This study was approved by the Institutional Review Board and complied with the ethical principles of the Declaration of Helsinki. All patients gave written informed consent. Of all consecutive patients who underwent CCTA in the Maastricht University Medical Center between January 2008 and March 2011, 115 iAF patients referred for work-up for Pulmonary Vein Isolation (PVI) and 275 age, sex and PROCAM-score-matched [16] SR-controls were selected as previously described [7]. For RNA isolation we selected 235 patients (59 iAF, 176 SR) of whom EDTA-plasma was available.

2.2. Computed Tomography

Non-contrast enhanced coronary calcium scans were performed in all patients as described previously [7]. Scans were performed using a uniform scan protocol (240 to 400 mA (depending on body weight), at 80 to 120 kV) on a 64-slice CT platform (Brilliance, Philips Healthcare, Best, the Netherlands) and second generation dual source CT (Definition Flash, Siemens Healthcare, Forchheim, Germany). Cases and controls were equally distributed among both scanners. Two independent observers determined Calcification of the Coronary Arteries (CAC), ascending (AsAC) and Descending Aorta (DAC) using dedicated vendor specific software. The presence of calcification was defined as Agatston>0; the Agatston score was determined using a 3 mm CT slice thickness and a detection threshold of ≥130 HU involving ≥1mm^2 area/lesion (3 pixels) [17]. In case of ambiguity, final judgment was reached in a consensus conference. Calcium above the origin of the right coronary artery to the end of scan range, or up to the origin of the brachiocephalic artery, was considered to be in the ascending aorta. Calcium present distal from the origin of the left subclavian artery up to the diaphragm was considered to be localized in the descending aorta.

2.3. RNA Isolation and microRNA Profiling

RNA was isolated from 200 µL EDTA-plasma with 800 µL Trizol reagent (Invitrogen, Breda, the Netherlands) using the RNeasy Micro Kit (Qiagen, Venlo, the Netherlands) with an adapted protocol. Briefly, chloroform was added to the plasma/Trizol mixture and centrifuged for 15 min at 15000g. The aqueous phase was combined with 1.5 volumes of 100% ethanol, conveyed to a MinElute Spin column (Qiagen) and centrifuged for 15 s at 18000 g. RNA was washed with 700 µL RWT buffer, twice with 500 µL RPE buffer and centrifuged for 15 s at 18000 g after the first two washing steps and 2 min at 18000 g after the last washing step. RNA was eluted with 15 µL RNase-free water.

MiRNAs were selected based on their previously established association with (micro)vascular dysfunction in profiling studies [12, 15]. (Supplementary Table **1**) For miRNA cDNA synthesis, reverse transcription of total RNA was performed using the miRNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was pre-amplified using Megaplex PreAmp primers pools A V2.1 (Applied Biosystems) according to the manufacturer's protocol. Custom designed Megaplex cards were generated to determine the expression of the selected microRNAs. Megaplex arrays were run and analyzed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The obtained miRNA cycle threshold (Ct)-values were normalized using the median Ct value of the expression of 48 measured miRNAs per patient [18].

2.4. Statistical Analysis

Baseline characteristics are presented as mean ± SD and differences between groups were compared using a student T-test, or number (%) and compared using χ^2-tests. MiRNA-values are presented as mean ± SD and the differences between the groups were assessed using a Student's T-test and general linear modeling to correct age, sex and vitamine K antagonist-use. In addition, miRNA-126 and miRNA-223 levels were corrected for aspirin use since aspirin inhibits platelet activation thereby decreasing the levels of both miRNAs [19].

Table 1. Clinical characteristics of patients with or without idiopathic atrial fibrillation, calcification in the coronary arteries, ascending- or descending aorta.

Patients	SR (n=176)	iAF (n=59)	p-value	CAC- (n=161)	CAC+ (n=71)	p-value	AsAC– (n=184)	AsAC+ (n=51)	p-value	DAC- (n=178)	DAC+ (n=57)	p-value
Age (years)	54.3 ± 9.3	54.4 ± 10.1	0.93	52.6 ± 9.5	57.8 ± 8.1	**<0.001**	52.8 ± 9.8	59.8 ± 5.3	**<0.001**	52.6 ± 9.6	59.9 ± 6.2	**<0.001**
Sex (female)	51 (29.0)	19 (32.2)	0.64	59 (36.6)	10 (14.1)	**0.001**	48 (26.1)	22 (43.1)	**0.02**	49 (27.5)	21 (36.8)	0.18
Atrial fibrillation				37 (23.0)	21 (29.6)	0.29	48 (26.1)	11 (21.6)	0.51	45 (25.3)	14 (24.6)	0.91
AF duration (months)		45.3 ± 49.7		35.5 ± 53.4	58.2 ± 64.6	0.18	42.3 ± 60.3	57.5 ± 58.0	0.45	37.9 ± 54.8	69.3 ± 70.3	0.16
Systolic blood pressure (mm Hg)	127 ± 9	127 ± 13	0.66	126 ± 9	127 ± 11	0.52	127 ± 10	127 ± 10	0.80	127 ± 10	126 ± 9	0.37
Diastolic blood pressure (mm Hg)	80 ± 10	78 ± 12	0.25	79 ± 10	82 ± 11	**0.03**	80 ± 11	79 ± 10	0.67	80 ± 11	79 ± 8	0.93
BMI (kg/m^2)	26.0 ± 3.5	26.1 ± 3.0	0.85	26.0 ± 3.5	26.3 ± 3.1	0.62	26.0 ± 3.4	26.2 ± 3.2	0.79	26.2 ± 3.4	25.8 ± 3.3	0.51
BSA (m^2)	1.9 ± 0.2	2.0 ± 0.2	0.13	2.0 ± 0.3	2.0 ± 0.2	0.53	2.0 ± 0.2	1.9 ± 0.2	0.18	2.0 ± 0.2	1.9 ± 0.2	0.45
Creatinine (umol/L)	84 ± 13	87 ± 13	0.08	83 ± 13	88 ± 12	**0.01**	85 ± 13	82 ± 13	0.14	85 ± 13	83 ± 13	0.23
eGFR ($ml/min/1.73m^2$)	97 ± 24	97 ± 22	0.96	99 ± 25	92 ± 19	0.07	99 ± 26	89 ± 18	**0.02**	99 ± 24	89 ± 21	**0.01**
Total cholesterol (mmol/L)	5.5 ± 1.0	5.5 ± 1.0	0.88	5.5 ± 0.9	5.5 ± 1.0	0.96	5.4 ± 0.9	5.6 ± 1.0	0.42	5.4 ± 1.0	5.7 ± 0.9	0.11
LDL-cholesterol (mmol/L)	3.4 ± 0.9	3.5 ± 0.8	0.19	3.4 ± 0.8	3.5 ± 1.0	0.46	3.4 ± 0.9	3.5 ± 0.8	0.30	3.4 ± 0.9	3.6 ± 0.8	0.06
HDL-cholesterol (mmol/L)	1.3 ± 0.4	1.3 ± 0.4	0.22	1.3 ± 0.4	1.3 ± 0.4	0.45	1.3 ± 0.4	1.4 ± 0.4	0.20	1.3 ± 0.4	1.4 ± 0.5	**0.04**
Triglycerides (mmol/L)	1.7 ± 0.9	1.5 ± 0.9	0.35	1.6 ± 0.9	1.7 ± 0.9	0.27	1.6 ± 0.9	1.7 ± 0.9	0.86	1.7 ± 0.9	1.5 ± 0.7	0.10
Glucose (mmol/L)	5.6 ± 0.7	5.3 ± 0.6	**0.02**	5.5 ± 0.7	5.4 ± 0.8	0.41	5.5 ± 0.8	5.5 ± 0.6	0.91	5.5 ± 0.8	5.6 ± 0.5	0.52
Echocardiography												
Left atrial diameter (mm)	37 ± 5	39 ± 5	**0.003**	37 ± 5	39 ± 5	**0.02**	37 ± 5	39 ± 5	0.11	37 ± 5	38 ± 5	0.47
IVSd (mm)	8.8 ± 1.1	8.5 ± 0.7	0.08	8.6 ± 1.0	8.8 ± 0.9	0.25	8.7 ± 1.0	8.6 ± 1.1	0.73	8.6 ± 1.0	8.8 ± 1.0	0.33
LVPWd (mm)	8.7 ± 1.1	8.4 ± 0.6	**0.02**	8.5 ± 1.1	8.7 ± 0.8	0.21	8.6 ± 1.0	8.5 ± 1.0	0.74	8.5 ± 1.1	8.7 ± 0.8	0.33
EDV (ml)	112 ± 25	118 ± 25	0.23	115 ± 25	114 ± 27	0.96	115 ± 25	110 ± 25	0.29	116 ± 26	108 ± 23	0.27
ESV (ml)	35 ± 12	46 ± 13	**<0.001**	40 ± 13	38 ± 13	0.35	40 ± 14	40 ± 12	0.99	40 ± 14	38 ± 12	0.51
LVEF (%)	60 ± 5	62 ± 5	0.39	61 ± 5	61 ± 5	0.96	60 ± 5	62 ± 5	0.25	61 ± 5	61 ± 4	0.31
CT Agatston Scores												
Coronary artery	27.1 ± 86.7	69.8 ± 154.4	0.04		123.4 ± 168		27.7 ± 89.9	73.3 ± 159.3	0.06	28.8 ± 93.9	65.3 ± 143.4	0.08
Aortic Valve	6.4 ± 25.1	16.8 ± 56.6	0.17	5.6 ± 22.8	17.1 ± 54.9	0.09	3.2 ± 19.9	30.0 ± 63.2	**0.004**	2.6 ± 15.7	29.1 ± 63.7	**0.003**
Ascending aorta	5.4 ± 24.7	7.7 ± 35.9	0.58	2.9 ± 11.0	13.3 ± 47.3	0.07		27.7 ± 54.9		1.8 ± 9.2	19.1 ± 52.4	0.02
Descending aorta	28.2 ± 124.8	44.8 ± 192.2	0.44	18.0 ± 94.3	66.3 ± 218.4	0.08	25.4 ± 133.0	57.4 ± 178.8	0.16		133.4 ± 271.0	

(Table 1) contd….

Patients	SR (n=176)	iAF (n=59)	p-value	CAC- (n=161)	CAC+ (n=71)	p-value	AsAC– (n=184)	AsAC+ (n=51)	p-value	DAC- (n=178)	DAC+ (n=57)	p-value
Medication												
VKA	0 (0.0)	15 (25.4)	**<0.001**	6 (3.7)	9 (12.7)	**0.01**	12 (6.5)	3 (5.9)	0.87	9 (5.1)	6 (10.5)	0.14
Aspirin	21 (12.1)	33 (56.9)	**<0.001**	34 (21.4)	19 (27.5)	0.31	43 (23.8)	11 (22.0)	0.80	42 (24.0)	12 (21.4)	0.69
Beta blocker	28 (16.2)	23 (39.7)	**<0.001**	30 (18.9)	20 (29.0)	0.09	41 (22.7)	10 (20.0)	0.69	39 (22.3)	12 (21.4)	0.89
Digoxin	0 (0.0)	8 (13.8)	**<0.001**	6 (3.8)	2 (2.9)	0.74	5 (2.8)	3 (6.0)	0.27	5 (2.9)	3 (5.4)	0.37
Nondihydropyridine CCB	1 (0.6)	5 (8.6)	**0.001**	1 (0.6)	5 (7.2)	**0.004**	6 (3.3)	0 (0.0)	0.19	3 (1.7)	3 (5.4)	0.14
Statin	19 (10.8)	7 (11.9)	0.82	17 (10.6)	9 (12.7)	0.64	17 (9.2)	9 (17.6)	0.09	20 (11.2)	6 (10.5)	0.88

Data is expressed as mean ± SD or n (%). AF, Atrial Fibrillation; SR, Sinus Rhythm; CAC, Coronary Artery Calcification; AsAC, Ascending Aorta Calcification; DAC, Descending Aorta Calcification; BMI, Body Mass Index; BSA, Body Surface Area; CAD, Coronary Artery Disease; VKA, Vitamin K Antagonist; CCB, Calcium Channel Blocker; ACE, Angiotensin Converting Enzyme; ARB, Angiotensin Receptor Blocker; IVSd, Interventricular Septum diameter; LVPWd, Left Ventricular Posterior Wall diameter; EDV, End Diastolic Volume; ESV, End Systolic Volume; LVEF, Left Ventricular Ejection Fraction.

Table 2. Differential expression of miRNAs in patients with or without calcification in the coronary arteries, ascending- or descending aorta.

miRNA in Patients	Total Population			Female			Male		
	CAC- (n=160)	CAC+ (n=71)	p-value	CAC- (n=58)	CAC+ (n=10)	p-value	CAC- (n=100)	CAC+ (n=61)	p-value
miRNA-27a	1.54± 1.91	1.35 ± 0.75	0.44	1.17 ± 0.66	1.67 ± 0.81	0.013	1.76 ± 2.32	1.31 ± 0.74	0.457
miRNA-103	0.36 ± 0.32	0.50 ± 0.36	0.013	0.30 ± 0.28	0.49 ± 0.51	0.130	0.39 ± 0.33	0.50 ± 0.34	0.150
miRNA-125a-5p	0.41 ± 0.30	0.55 ± 0.52	0.027	0.37 ± 0.27	0.47 ± 0.37	0.371	0.43 ± 0.31	0.56 ± 0.54	0.042
miRNA-126#	26.78 ± 13.76	30.07 ± 13.70	0.05	25.83 ± 12.15	36.47 ± 15.38	0.012	27.33 ± 14.64	29.04 ± 13.14	0.341
miRNA-221	6.66 ± 6.54	9.03 ± 7.56	0.038	6.23 ± 8.68	7.69 ± 4.03	0.782	6.91 ± 4.98	9.25 ± 7.99	0.05
miRNA-223#	397.05 ± 229.29	472.14 ± 215.75	0.035	415.69 ± 227.16	515.57 ± 256.38	0.103	390.54 ± 231.53	465.02 ± 209.96	0.043
	AsAC- (n=184)	**AsAC+ (n=51)**		**AsAC- (n=48)**	**AsAC+ (n=22)**		**AsAC- (n=136)**	**AsAC+ (n=29)**	
miRNA-221	7.76 ± 7.53	5.79 ± 3.48	0.024	6.99 ± 9.61	4.93 ± 1.98	0.124	8.03 ± 6.67	6.42 ± 4.18	0.119
	DAC- (n=178)	**DAC+ (n=57)**		**DAC- (n=49)**	**DAC+ (n=21)**		**DAC-(n=129)**	**DAC+ (n=36)**	
miRNA-212	0.039 ± 0.039	0.034 ± 0.020	0.62	0.028 ± 0.016	0.041 ± 0.027	0.035	0.042 ± 0.045	0.034 ± 0.019	0.346

Comparisons within the total population are adjusted for age, sex, and vitamin-K antagonists, while female and male group comparisons are adjusted for age and the use of vitamin K antagonists. MiRNA expression values marked with# are corrected for aspirin use. MiRNA relative expression data are presented as mean ± SD.

3. RESULTS

3.1. Baseline Characteristics of iAF Patients Compared to SR Controls

Baseline characteristics of the iAF patients and the SR controls are listed in Table **1**. Patients with iAF had a significantly larger left atrial diameter (LAd) and end systolic volume (ESV), while the left ventricular posterior wall diameter (LVPWd) was significantly smaller. Significantly different CAC, AsAC and DAC Agatston scores between groups are listed in Table **1**. Patients with CAC were average older, more often male and had larger LAds while patients with or without aortic calcification were comparable except for their age.

3.2. Coronary Artery Calcification and not Arrhytmia Associates with Sex-Specific Circulating miRNA Profiles in iAF

To investigate whether the presence of paroxysmal arrhythmia in iAF patients associates with circulating levels of the selected panel of 48 miRNAs, we assessed their plasma levels in iAF patients compared to SR controls (Table **2**). After correction for biological sex and age, no plasma miRNAs were found to be associated with a history of AF in the total iAF patient population. Next, to determine the contribution of subclinical-CV comorbidities on circulating miRNA expression, we assessed the association of CAC with plasma miRNAs. In the total population, CAC associated with significantly higher levels of miRNAs-103, 125a-5p, 221 and

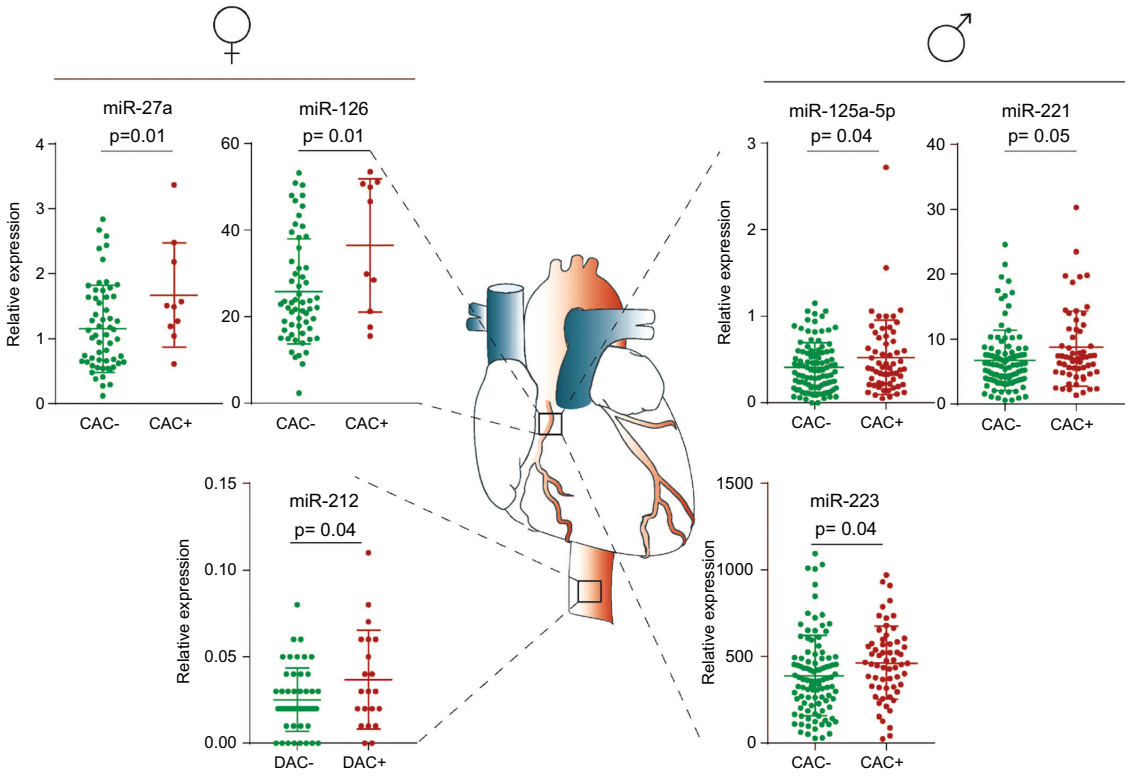

Fig. (1). Differential circulating miRNAs in women and men either with or without Coronary Artery Calcification (CAC), Ascending Aorta Calcification (AsAC) and Descending Aorta Calcification (DAC). Comparisons are adjusted for age, aspirin and Vitamin K antagonist use. Only significantly differently expressed miRNAs are shown.

223, of which the latter three were increased in in men only (Table **2**, Fig. **1**). In contrast, analysis of CAC associated miRNAs in women revealed a significantly increased expression of miRNA-27a and miRNA-126.

3.3. Circulating Levels of miRNA-221and miRNA-212 Levels in Women, Associate with Aortic Calcification

Next, following the identification of coronary calcification associated miRNAs, we examined the relationship between specific miRNAs and aorta calcification. Within the total patient population, the presence of AsAC resulted in lower levels of miRNA-221. Interestingly, upon stratification for biological sex, the AsAC association with miRNA-221 still displayed a trend towards lower levels, although these differences were not significant for both men and women. However, the presence of DAC in women but not in men was associated with higher expression levels of miRNA-212 expression in women.

4. DISCUSSION

This study shows that in iAF, instead of arrhythmia, subclinical CV comorbidities such as CAC, AsAC and DAC, are associated with plasma levels of a selected set of miRNAs. Interestingly, when CAC, AsAC and DAC group comparisons were stratified for biological sex we observed a sex-specific, significant increase in miRNA-27a and miRNA-126 levels in women with a positive CAC score, while the levels of miRNA-125a-5p, miRNA-221 and miRNA-223 were higher in CAC positive men. Furthermore, circulating levels of miRNA-221 were elevated in patients with ascending aorta calcification, while miRNA-212 expression was associated with descending aorta calcification in women only.

Several of the differentially expressed miRNAs in this study have already shown promising potential as biomarker for clinical diagnostics and patients' response to treatment. As such, we previously demonstrated that the plasma levels of miRNA-126 increase, while miRNA-223 levels decrease in DN patients and normalize to healthy control levels, upon a simultaneous pancreas kidney (SPK) transplantation [12]. Furthermore, in type 2 diabetes patients, both miRNA-126 and -223 display a positive response to drug treatment [20] while miRNA-126 was found to predict platelet activation and the response to aspirin therapy [21] Also, miRNA-223 can be used in clinical care because its elevated levels were prognostic for cardiovascular mortality in patients with coronary artery disease (4). Interestingly, plasma levels of miRNA-27a were similarly found to provide additional aid in dedicated patient care because a reduction in its circulating levels was associated with rehospitalization for heart failure patients (5). Therefore, the observed association of these miRNAs with vascular calcification in this study could potentially indicate a pathophysiology of subclinical coronary (micro) vascular dysfunction in iAF patients. Nonetheless, more miRNA expression profiling and functional studies in iAF patients are warranted to validate this relationship.

Studies on the relationship between iAF and circulating miRNAs are limited, although several studies reported changes in circulatory miRNAs in AF. In particular, miRNA-150 has consistently been associated with AF, and was repeatedly found to be decreased in (older) patients with AF

[22-24]. We could not confirm this association in the present study with iAF patients. It could be argued that the lowering of miRNA-150 levels in AF are mostly related to the contribution of CV comorbidities, particularly because miRNA-150 is a marker of advanced atherosclerosis [25]. Therefore, next to its association with arrhythmia in AF, this miRNA-150 change could also be related to the simultaneous prevalence of hypertension [22], heart failure [23] or diabetes [24] in these patient cohorts. Likewise, a similar relationship was identified between AF and plasma levels of miRNA-21 [24], a marker of cardiac fibrosis [26] and miRNA-152 [14] which is decreased in high glucose conditions [27]. Again these miRNAs levels were also not altered in our iAF patients. A possible explanation for this discrepancy could be that our patients were not diabetic and relatively young, whereas previous miRNA-21 expression changes were predominantly observed in older patients with a more severe AF phenotype, and an ensuing state of atrial ischemia and fibrosis [26]. Taken together, it could be argued that the previously identified circulatory miRNA profiles in AF are predominantly determined by an ensuing presence of arrhythmia in conjunction with severe CV comorbidities that are absent or less manifest in our iAF patient cohort.

The observed increase of the identified circulating miRNAs in this study could be explained by looking at their functional role within the vasculature system. For instance, in the setting of atherosclerosis, increased miRNA-103 expression in activated endothelial cells (ECs) represses endothelial KLF4 expression and thereby cellular quiescence [28] whereas an increased endothelial miRNA-212 expression is anti-angiogenic [29]. As such, the higher levels of miRNA-103 and miRNA-212 in this study may suggest a pathophysiology of endothelial dysfunction and inflammation. Furthermore, although miRNA-126 normally favours vascular regeneration [30], the association of increased levels of miRNA-126 with vascular calcification in this study could indicate that, although speculative, more plasma miRNA-126 reflects a vascular regenerative feedback response in these iAF patients. Moreover, miRNAs are distributed among plasma miRNA carriers, such as exosomes [31], circulating high density lipoprotein (HDL) [32] or Argonaute-2 (Ago2) [33]. These plasma miRNA carriers enable the functional delivery of miRNAs at distal sites to pass on a miRNA mediated feedback loop in tissue inflammation, repair and regeneration. The observed increased expression of plasma miRNA-126, -27a, -125a-5p, -212 and -223 could have such carrier dependent impact. For instance, CD34+ peripheral blood mononuclear cells (PBMCs) derived exosomal miRNA-126 was found to be proangiogenic [34] whereas HDL-miRNA-223 transfer repressed endothelial activation and inflammation [35]. Taken together, it could be argued that the observed increase of plasma miRNAs may reflect underlying pathophysiology or a regenerative response.

Diabetes mellitus is a highly significant predictor of AF in women [3]. Furthermore, in iAF patients the relative risk of stroke, Transient Ischemic Attack (TIA) and heart failure are at least doubled in woman compared to man [8] further emphasizing the action of sex-specific mechanisms in cardiovascular pathophysiology. These mechanisms may also involve the action of sex-specific miRNAs. In woman, miRNA expression levels can differ from those in man due to oestrogen-dependent production or as a consequence of a gene-dose effect following incomplete X-chromosome inactivation [36]. This could be possible because the X-chromosome in humans and mice is relatively enriched for miRNAs (118 annotated miRNAs on the human X-chromosome, source: miRBase *vs*. 22) with a two-fold higher miRNA density compared to autosomes. These X-chromosome-located miRNAs (X-linked miRNAs) could potentially display an increased expression in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) from the cardiovascular system due to incomplete X-chromosome inactivation (XCI) [11]. This could not explain that the X-linked miRNA-221 and miRNA-223 in this study were increased in male patients only. Therefore, another explanation for their sex biased miRNA expression could be the simultaneous expression with a sex-biased host gene or the sex specific expression of components of the miRNA processing machinery, such as Dicer or Drosha [11]. Therefore, another interesting mechanism for sexual dimorphism of miRNA expression could be the preferential binding of certain transcription factors (TFs) to the proximal genomic regions of particular sex-biased miRNA genes (sex-biased miRNAs have 15.7 TF binding sites in vicinity compared to 9.1 TF binding sites for non-biased miRNAs) [37]. Since, so far, 33 female specific miRNA-regulating TFs have been identified compared to 56 male specific miRNA regulating TFs [37], it increases the likelihood that this regulatory control will develop differently between men and women.

In this study, women with coronary artery- and aortic calcification displayed higher circulating levels of miRNA-27a, miRNA-212 and miRNA-126 compared to men. Since women display higher levels of miRNA-126 during the ovulatory phase, and oestrogen was demonstrated to increase miRNA-126 expression to control the inflammatory response of ECs, a role for oestrogen in transcription of this vascular-protective miRNA in CAC positive women can be proposed [38]. Furthermore, the observed female-related difference in miRNA-27a expression is consistent with observations that this miRNA is decreased in women with gestational diabetes [39] but increased in women with polycystic ovary syndrome [40], suggesting that miRNA-27a is responsive to a synchronous state of high estrogen and metabolic dysfunction in women. Moreover, in contrast to the studied women in this cohort, CAC positive men displayed significantly higher levels of miRNA-221 and miRNA-223. Interestingly, miRNA-221, is known as a promoter of vascular calcification [41] and regulated by androgen hormone levels [42], while miRNA-223 is a known biomarker of CAC. [43] Therefore, although speculative, the male specific expression of these miRNAs in this cohort could be related to a male-specific cause or outcome of CV comorbidities in iAF patients. Taken together, it could be argued that the association of these sex-specific circulating miRNAs with vascular calcification in IAF patients confirms the notion that biological sex should be taken into account when deciding on vascular prophylactic therapy, including anticoagulation [8, 44].

This miRNA profiling study in iAF patients has several limitations. Measured miRNAs were selected based on their previous association with vascular injury. However, since numerous miRNAs are (mechanistically) involved in CV disease, we cannot exclude more miRNA changes in iAF

than those observed in this study. Furthermore, this study was not followed by miRNA expression validation studies in independent iAF cohorts nor did we conduct intervention studies to evaluate whether the identified miRNAs are the cause or the outcome of (vascular calcification in) iAF. Therefore, future studies to validate these circulating levels of miRNAs in larger iAF patient cohorts are needed. However, since vascular calcification and age in iAF patients both augment coronary calcification [45] and because platelet activation hampers the biomarker potential of miRNA-126 and miRNA-223 [19], a strength of this study is the statistical adjustment of differentially regulated miRNAs for the use of aspirin and vitamin K antagonists.

CONCLUSION

Circulating miRNAs in iAF do not associate with the mere presence of paroxysmal arrhythmia but instead display a sex-specific association with subclinical vascular calcification. This sex specific miRNA profile association with vascular calcification in iAF patients, confirms the existence of sex specific etiologies of vascular disease, and suggests that sex specific strategies for the prevention of vascular complications should be employed.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Institutional Review Board.

HUMAN AND ANIMAL RIGHTS

No animal were used in the study. The reported experiments in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10 ethics/10helsinki/).

CONSENT FOR PUBLICATION

All patients gave written informed consent.

CONFLICT OF INTEREST

One author Joachim E. Wildberger of this manuscript declares institutional relationships with the following companies: Siemens AG, Bayer Healthcare, Philips, GE, all outside submitted work.

All other authors report no relationships that could be construed as a conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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