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Full Length Article

Elevated blood plasma levels of tissue factor-bearing extracellular vesicles in patients with atrial fibrillation

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ABSTRACT

Background: The risk of thrombus formation in the left atrial appendage (LAA) in patients with atrial fibrillation (AF) may result from blood stasis, local endocardial changes, and/or changed blood composition. Extracellular vesicles (EVs), especially subtypes exposing tissue factor (TF), have procoagulant capacity. We hypothesized that blood concentrations of TF-bearing EVs and other procoagulant biomarkers are elevated in AF patients, particularly in the LAA lumen.

Methods: From 13 AF patients and 12 controls a venous blood sample was drawn prior to cardiac surgery. Intraoperatively, venous blood and blood directly from the LAA was drawn. Plasma levels of EVs, including TF- and cell type specific antigen-bearing EVs, were measured using a protein microarray platform. Plasma levels of TF, von Willebrand factor (vWF), cell free deoxyribonucleic acid (cf-DNA), procoagulant phospholipids (PPLs), and total submicron particles were also evaluated.

Results: Significantly higher EV levels, including a several-fold higher median level of TF-bearing EVs were measured in AF patients compared with controls. Median concentrations of TF and vWF were approximately 40% and 30% higher, respectively, in the AF group than in the control group, while no significant differences in levels of cf-DNA, PPLs, or total submicron particles were observed. No significant differences in levels of any of the measured analytes were observed between intraoperative venous and LAA samples.

Conclusions: Increased plasma concentrations of TF in AF patients are accompanied and probably at least partly explained by increased levels of TF-bearing EVs, which may be mechanistically involved in increased thrombogenicity in AF patients.

1. Introduction

Atrial fibrillation (AF) is the most common treatment-requiring cardiac arrhythmia [1]. Thromboembolism is considered the most important complication of AF [2], and stroke is five times more frequent in AF patients than in persons with sinus rhythm [3]. In general, thrombus formation results from abnormal changes in blood flow, blood constituents, and/or the blood vessel wall [4], but the relative impacts and potential mutual dynamics of these processes in AF-related thrombosis are not fully understood [5].

It is known that AF induces blood stasis in the left atrium (LA), including the left atrial appendage (LAA), where most intra-atrial

Abbreviations: AF, atrial fibrillation; LA, left atrium; LAA, left atrial appendage; vWF, von Willebrand factor; TF, tissue factor; EV, extracellular vesicle; NET, neutrophil extracellular trap; PPL, procoagulant phospholipid; CABG, coronary artery bypass grafting; PS, phosphatidylserine; V_{pre}, preoperative venous; V_{int}, intraoperative venous; PFP, platelet free plasma; CV, coefficient of variation; cf-DNA, cell free deoxyribonucleic acid; DPBS, Dulbecco's phosphate-buffered saline; PPL-CT, procoagulant phospholipid dependent clotting time; NTA, nanoparticle tracking analysis

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thrombi are formed [5]. While blood stasis is believed to play an important role in the process, it is not accepted as the sole link between AF and risk of thrombosis [5]. Indices of endocardial changes in the LA [6] and the LAA [7] and impaired endothelial function [8] in AF patients have been demonstrated. Associations between AF and increased circulating blood levels of a range of hemostatic markers, including platelet factor 4, β-thromboglobulin, P-selectin, D-dimer, fibrinogen, thrombin-antithrombin complex, and prothrombin fragment 1 + 2 have been identified, indicating platelet and coagulation activation in these patients [9]. However, the underlying mechanisms of these associations are not clear [9]. AF may also induce expression of the procoagulant proteins von Willebrand factor (vWF) and tissue factor (TF) in the LAA endocardium [10].

Thus, local changes in the LAA in AF patients may lead to local endocardial production and release of prothrombotic agents, potentially accumulating in the LAA lumen during blood stasis. Increased venous blood levels of vWF [9] and TF [11] have been identified in AF patients.

Other, more recently introduced potential mediators of thrombogenic processes are extracellular vesicles (EVs) and neutrophil extracellular traps (NETs). EVs are lipid bilayer membrane-bounded particles which can be released from various cell types, including endothelial cells, as a response to physiological or pathological stimuli [12]. EVs contain membranous, cytoplasmic and in some cases nuclear components from their parent cells. Subtypes of EVs exposing TF and procoagulant phospholipids (PPLs), particularly phosphatidylserine (PS), on their surface are established as thrombogenic factors [13]. In blood plasma, EVs coexist with other submicron particles, including lipoproteins and protein aggregates, which complicates EV measurement [14,15]. NETs, which consist of chromatin, histones, proteases, and other intracellular proteins released from neutrophils during vital or suicidal NETosis, form part of the innate defense against infectious pathogens, but have also been linked to sterile inflammation and thrombosis [16]. Inflammation has been linked to initiation and maintenance of AF and, moreover, to AF-related thrombogenicity [17]. Plasma indices of inflammation, C-reactive protein and interleukin-6, have been documented elevated in AF patients [18].

In this study, we aimed to elucidate thrombogenicity of AF by comparing the levels of TF, vWF, PPLs, NETs, total submicron particles, and EVs, including EVs exposing TF and markers of platelet, leukocyte, or endothelial origin, in venous blood and blood drawn directly from the LAA in patients with and without AF. We hypothesized that blood from AF patients contained higher levels of these procoagulant biomarkers than blood from control patients and, furthermore, we hypothesized that in AF patients the blood from the LAA contained higher levels of the procoagulant biomarkers than venous blood.

2. Materials and methods

2.1. Patients

13 patients with known nonvalvular AF (seven with paroxysmal and six with persistent or permanent ongoing AF) and 12 patients without a history of AF referred to the Department of Cardiothoracic Surgery, Aalborg University Hospital, Denmark, for elective aortic valve surgery or coronary artery bypass grafting (CABG) were included in this study which was approved by The North Denmark Region Committee on Health Research Ethics and the Danish Data Protection Agency. Nonvalvular AF was defined as AF without concurrent mitral stenosis or artificial heart valves [19]. With regard to patients receiving antithrombotic treatment, acetylsalicylic acid was continued until surgery, while vitamin K antagonists, direct oral anticoagulants, and clopidogrel were discontinued two days, three days, and five days, respectively, prior to surgery. Routine anaesthetic procedures were employed. No preoperative transfusions including platelets were given to the patients.

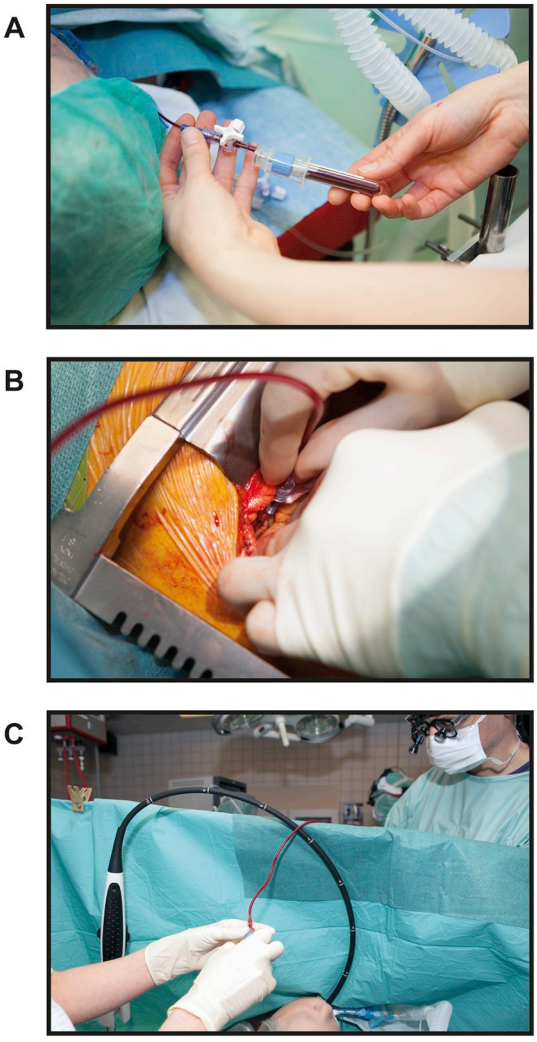

Fig. 1. Blood sampling procedure. (A) Venous blood was sampled through a central venous catheter. (B and C) Blood from the LAA was sampled through a cannula with an extension tube.

2.2. Blood sampling

For sampling of blood for analysis of procoagulant biomarkers 3.5 mL Vacuette 3.2% sodium citrate plastic tubes (Greiner Bio-One, Kremsmünster, Austria) were used. Preoperatively, three tubes of venous blood (V_{pre}) were sampled from a central venous line (Fig. 1A) immediately before commencement of surgery. Three more tubes of central venous blood were sampled intraoperatively (V_{int}) after sternotomy immediately before systemic heparinization. Also, immediately before systemic heparinization three tubes of intracardiac blood were sampled through a cannula inserted in the LAA and a 75 cm Luer Lock Extension tube (Praxisdienst, Longuich, Germany) (Fig. 1B and C). Platelet free plasma (PFP) was prepared for analysis by a double centrifugation at 2500*g* for 15 min at room temperature and stored at −80 °C until analysis, except for the PFP for submicron particle measurements, which were performed on fresh PFP on the day of blood sampling.

2.3. Blood analysis

2.3.1. EV analysis by EV Array

EV Array analysis was performed as described by Bæk and Jørgensen [20]. While no generic label for all types of EVs exists [21], PS and the tetraspanin protein superfamily, including CD9, CD63, and

CD81 [22], are established general markers of main groups of EVs termed microvesicles [23] and exosomes [24], respectively. However, PS also exists on exosomes [12], and tetraspanins have been identified on microvesicles as well [22], hence, in this context, we use the collective term, EVs. Annexin V binds to membranes bearing PS with high affinity [25]. In this study, we used anti-Annexin V and anti-CD9 and anti-CD81 as general markers of EVs. In EV studies, CD146, CD31, and CD62E are well-established markers of endothelial cell origin [26]. CD206 and CD163 are expressed by macrophages and dendritic cells [27], while CD14 is primarily present on monocytes and macrophages [28]. CD41, CD42a, and CD42b are platelet membrane proteins [29]. For capturing the EVs, we used a panel of anti-human antibodies, including anti-CD14, anti-CD16 (BD Biosciences, Mountain View, CA, USA), anti-TF (HTF-1 clone, BD Biosciences, San Jose, CA, USA), anti-TF (TF9-10H10 clone, Millipore, Burlington, MA, USA), anti-CD41 (Biolegend, San Diego, CA, USA), anti-CD42a (LifeSpan BioSciences, Seattle, WA, USA), anti-Annexin V, anti-CD31, anti-CD206, anti-CD42b (R&D Systems Inc., MN, USA), anti-CD62E (Thermo Scientific, Waltham, MA, USA), anti-CD163 (Trillium Diagnostics, Bangor, ME, USA), and a combination of anti-CD63 (Bio-rad, Oxford, UK), anti-CD9, and anti-CD81 antibodies. As detecting antibodies anti-TF (TF9-10H10 clone) and, parallelly, a combination of anti-CD9 and anti-CD81 antibodies were applied. For EV Array with the printing technology and sample volume (10 μL) used in this study, an inter-assay coefficient of variation (CV) of 10.3% and an intra-assay CV of 2.9% has been estimated [24]. Approximately 2.5×10^4 EVs per antibody printing spot are expected to yield a significant signal [30].

2.3.2. Additional analyses

For quantification of TF antigen, we applied the Imubind anti-human Tissue Factor ELISA (Sekisui Diagnostics, Lexington, MA, USA) measuring the absorbance at a wavelength of 450 nm with a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). PFP levels of NETs were estimated using extracellular plasmatic cell free deoxyribonucleic acid (cf-DNA) as a surrogate marker. Plasma was diluted 10-fold and allocated into wells in a 96 well plate (Nunc A/S, Roskilde, Denmark), where either Sytox Green (Thermo Fisher, Carlsbad, CA, USA), a fluorescent DNA dye, or Dulbecco's Phosphate-Buffered Saline (DPBS) was applied to the plasma samples. Samples treated only with DPBS functioned as blanks to eliminate fluorogenic background noise originating from Sytox Green. Calf thymus DNA (Thermo Fisher) was used for a standard curve to determine the concentration of cf-DNA in each sample. After five minutes of incubation at 27 °C, the plates were processed using a FLUOstar Optima microplate reader (BMG Labtech, Ortenberg, Germany). The fluorescence intensity of the DNA binding to the Sytox Green was measured with a 485 nm excitation filter and a 520 nm emission filter.

Plasma content of PPL was evaluated by measuring PPL dependent clotting time (PPL-CT) on a STA Compact Coagulation Analyzer (Diagnostica Stago, Asnières, France), using STA Procoag PPL (Diagnostica Stago).

We recorded intra-assay CVs of TF, cf-DNA, and PPL assays of 6.4, 7.7, and 1.6% respectively, and corresponding inter-assay CVs of 12.6, 15.8, and 5.4%. In all analyses an even inter-assay distribution of AF patient samples and control samples was applied.

vWF antigen levels were measured in one series (same day) using the vWF Ag (Siemens Healthineers, Erlangen, Germany) on a Sysmex CS2100i (Siemens Healthineers) with a within-day CV of 2.9%.

Nanoparticle tracking analysis (NTA) was applied for measurement of submicron particles, using a NanoSight LM10-HS (Malvern Instruments Ltd., Malvern, UK) with a Luca DL-658 M-OEM EMCCD camera (Andor Technology, Belfast, UK) and a 405 nm laser. Settings were chosen as earlier described for PFP samples [15]. In brief, particle tracking duration was set to 60 s, camera shutter to 500, camera gain to 300, and detection threshold to 3. For data processing, we used NanoSight NTA 2.3.

Lipid levels were measured in a Cobas 8000 Modular Analyzer (Roche Applied Science, Penzberg, Germany) with reagents from Roche. Estimated between day CVs on particle concentration and particle size are 6.4 and 9.3%, respectively [15].

2.4. Statistics

For statistical analysis and graph preparation, GraphPad Prism, version 6.01 (GraphPad Software, Inc., La Jolla, CA, USA), was applied.

P-values for pairwise comparison was evaluated using the Wilcoxon matched pairs signed rank test. For comparison of independent groups, we used the Mann-Whitney *U* test. For correlation analysis Spearman's rank correlation was applied.

Two-sided *p*-values were given, and values below 0.05 considered statistically significant. Assuming a marked effect size of 1.2, and choosing 80% as desired level of statistical power, we needed to include 11 patients in both the AF group and the control group, based on power/sample size calculations. Using non-parametric statistics, we considered it suitable to include 12–13 patients in each group.

3. Results

Study population characteristics are given in Table 1. There were no statistically significant differences between the AF patient group and the control group in the continuous data except for a significantly higher LA diameter and INR (international normalized ratio) one day prior to surgery in the AF patient group. While the median INR was only 0.1 higher in the AF group, four AF patients had INR above 1.4, which was the highest INR observed in the control group. Combined results on PFP levels of EVs as measured by EV Array analysis are given in Figs. 2, 3, and 4. PFP levels of TF, vWF, cf-DNA, PPL-CT, and submicron particles are presented in Fig. 5. No significant differences of any of the biomarkers were observed between PFP from the first (A_1) and the third (A_3) tube of blood drawn from the LAA in neither the AF patient group nor the control group. In the following, all given LAA data represent A_1 results. No significant differences between patients with paroxysmal and patients with persistent/permanent AF were observed.

3.1. EVs measured by EV Array

3.1.1. Capture and detection by general EV markers

Levels of EVs captured by anti-Annexin V and detected via binding of CD9/CD81 were significantly higher in the AF patients than in the controls with regard to the V_{pre} and the V_{int} samples with the same tendency observed in the LAA samples ($p = 0.05$) (Fig. 2A). Levels of EVs both captured and detected via binding of tetraspanins were significantly higher in AF patients than in controls with regard to the V_{int} samples with the same trend, although not statistically significant, seen in the V_{pre} ($p = 0.12$) and the LAA ($p = 0.10$) samples (Fig. 2B).

3.1.2. Capture by anti-TF combined with detection by anti-tetraspanin antibodies

Levels of vesicles captured by anti-$TF_{(TF9\text{-}10H10)}$ antibodies and detected via binding of CD9/CD81 were significantly higher in the AF patients than in the controls in all three sample types (Fig. 2C). Levels of vesicles captured by anti-$TF_{(HTF\text{-}1)}$ antibodies and detected via binding of CD9/CD81 were significantly higher in the AF patients than in the controls in the V_{pre} samples with the same trend, although not statistically significant, seen in the LAA ($p = 0.13$) and the V_{int} ($p = 0.09$) samples (Fig. 2D).

3.1.3. Capture by cell type markers combined with detection by anti-tetraspanin antibodies

When EV Array was performed with antibodies against CD42a, CD42b, and CD41 as capturing agents and anti-CD9/CD81 antibodies as detecting agents, no significant differences were observed, although

Table 1
Study population characteristics.

Group	Control (n = 12)	All AF patients (n = 13)	Paroxysmal AF (n = 7)	Persistent/permanent AF (n = 6)
Gender				
Females/males, [No.]	3/9	2/11	2/5	0/6
Age, [years]	67 (48–80)	72 (45–81)	72 (45–81)	72 (67–76)
BMI, [kg/m^2]	27.2 (22.9–36.0)	30.7 (23.6–39.8)	30.7 (24.9–39.3)	27.6 (23.6–39.8)
Echocardiographic data				
left atrial diameter, [mm]*	39 (32–47)	46 (34–58)	42 (34–54)	49 (47–58)
LVEF, [%]	60 (45–71)	60 (35–73)	60 (35–73)	55 (43–60)
LVEDD, [mm]	51 (38–60)	51 (38–57)	49 (38–57)	54 (39–55)
LVESD, [mm]	35 (22–52)	33 (25–44)	30 (25–35)	38 (27–44)
Smoking status				
current/former/never, [No.]	1/8/3	2/6/5	1/3/3	1/3/2
Diabetes mellitus				
insulin-treated/diet or OAD-treated/no diabetes, [No.]	1/5/6	2/1/10	0/1/6	2/0/4
Baseline plasma CRP, [mg/L]	0.8 (< 0.5–48.6)	2,0 (< 0.5–11.0)	1.2 (< 0.5–9.5)	4.5 (< 0.5–11.0)
Baseline hematological data				
Blood leukocytes, [$\times 10^9$/L)	7,7 (4.4–10.8)	7.1 (4.5–10.1)	7.2 (4.5–10.1)	6.9 (4.8–9.4)
Blood platelets, [$\times 10^9$/L)	225 (132–419)	232 (119–363)	179 (126–326)	268 (119–363)
Blood hemoglobin, [mmol/L]	8.6 (7.3–10.6)	8.6 (6.0–9.6)	8.7 (6.0–9.6)	8.6 (6.4–8-8)
Baseline plasma lipids, [mmol/L]				
Total cholesterol	3.1 (1.4–4.9)	3.1 (1.6–4.9)	3,9 (2.9–4.9)	2.7 (1.6–3.2)
Low density lipoprotein cholesterol	1.5 (0.6–3.2)	1.6 (0.6–2.9)	2.0 (1.5–2.9)	1.1 (0.7–1.7)
High density lipoprotein cholesterol	0.9 (0.6–1.5)	1.2 (0.6–1.7)	1.0 (0.7–1.7)	1.2 (0.6–1.7)
Triglyceride	1.3 (0.4–2.1)	0.9 (0.4–2.0)	1.2 (0.5–2.0)	0.8 (0.4–1.0)
Lipid lowering drugs				
Users/non-users, [No.]	8/4	6/7	1/6	5/1
Antihypertensive drugs				
Yes/no, [No.]	7/5	12/1	7/0	5/1
Thromboprophylaxis				
VKA/DOAC/clopidogrel/aspirin, [No.]	0/0/1/8	8/2/1/3	4/1/1/2	4/1/0/1
INR one day prior to surgery*	1.1 (0.9–1.4)	1.2 (1.1–2.5)	1.1 (1.1–2.2)	1.2 (1.1–2.5)
Type of surgery				
Aortic valve surgery/CABG, [No.]	5/7	10/3	7/0	3/3

Continuous data are given as median (range). *Statistically significant difference in continuous data between AF patient group and control group. LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; OAD, oral antidiabetic drug; CRP, C-reactive protein; VKA, vitamin K antagonist; DOAC, direct oral anticoagulant; INR, international normalized ratio; CABG, coronary artery bypass grafting.

there was a modest tendency towards higher levels in AF patient group than in the control group (Fig. 3A, B, and C, $p > 0.17$ in all comparisons). When capture with anti-CD31 and detection via anti-CD9/CD81 antibodies was performed, a significantly higher signal was detected in the AF patients in comparison with the controls in the V_{pre} and the LAA samples, while the corresponding p-value for the V_{int} samples was 0.11 (Fig. 3D). Capture with anti-CD146 antibodies and detection via CD9/CD81-binding resulted in significantly higher signals in the AF patient group than in the control group for all three sample types (Fig. 3E), which was also the case when capture by anti-CD206-antibodies was combined with detection via anti-CD9/CD81 antibodies (Fig. 3G). No differences between the groups were observed, when anti-CD62E antibodies were used as capturing agents and anti-CD9/CD81 antibodies were used as detecting agents (Fig. 3F). When capture was performed using anti-CD163 (Fig. 3H) or anti-CD14 (Fig. 3I) antibodies combined with detection via anti-CD9/CD81 antibodies, there was a trend towards higher levels in the AF patients than in the controls, although p-values were just above the set threshold for statistical significance (0.06 in the V_{pre} samples in both cases).

3.1.4. Capture by cell type markers combined with detection by anti-TF antibodies

When capture by anti-CD42a, anti-CD42b, anti-CD62E, or anti-CD163 antibodies was combined with detection via TF-binding (Fig. 4A, B, F, and H, respectively), no detectable signal was present in the majority of the samples and no significant differences between AF patients and controls were observed. When capture by anti-CD41 antibodies was combined with detection via anti-TF antibodies some signal among the AF patients and the controls, but no convincing tendency of differences between the groups, was observed (Fig. 4C). When capture by anti-CD14 antibodies combined with detection via anti-TF antibodies was performed, significantly higher levels were observed in the AF patients than in the controls in the V_{pre} and the LAA samples. In the V_{int} samples the same trend was observed, but this was not statistically significant ($p = 0.09$) (Fig. 4I). A similar tendency, but less marked, was seen when capture performed by anti-CD31, anti-CD146, or anti-CD206 antibodies, was combined with detection via anti-TF antibodies (Fig. 4D, E, and G, respectively).

3.2. TF

Results on TF antigen levels as measured by ELISA are shown in Fig. 5A. Significantly higher levels of TF were observed in the V_{pre} and the V_{int} samples from the AF patients than in the corresponding samples from the controls. The same tendency was seen in the LAA samples but this was not statistically significant ($p = 0.07$).

In both the control group and the AF patient group there was a tendency towards lower TF antigen levels in the LAA and V_{int} samples than in the V_{pre} samples. When comparing the V_{pre} samples and the V_{int} samples from the AF patient group the difference was statistically significant.

3.3. vWF

Results on the vWF levels are given in Fig. 5B.

Venous vWF antigen concentrations were significantly higher in AF patients than in controls in the V_{pre} as well as in the LAA and the V_{int} samples. No significant differences were observed between the types of blood sample (V_{pre}, LAA, and V_{int}) in neither the AF patient group nor the control group.

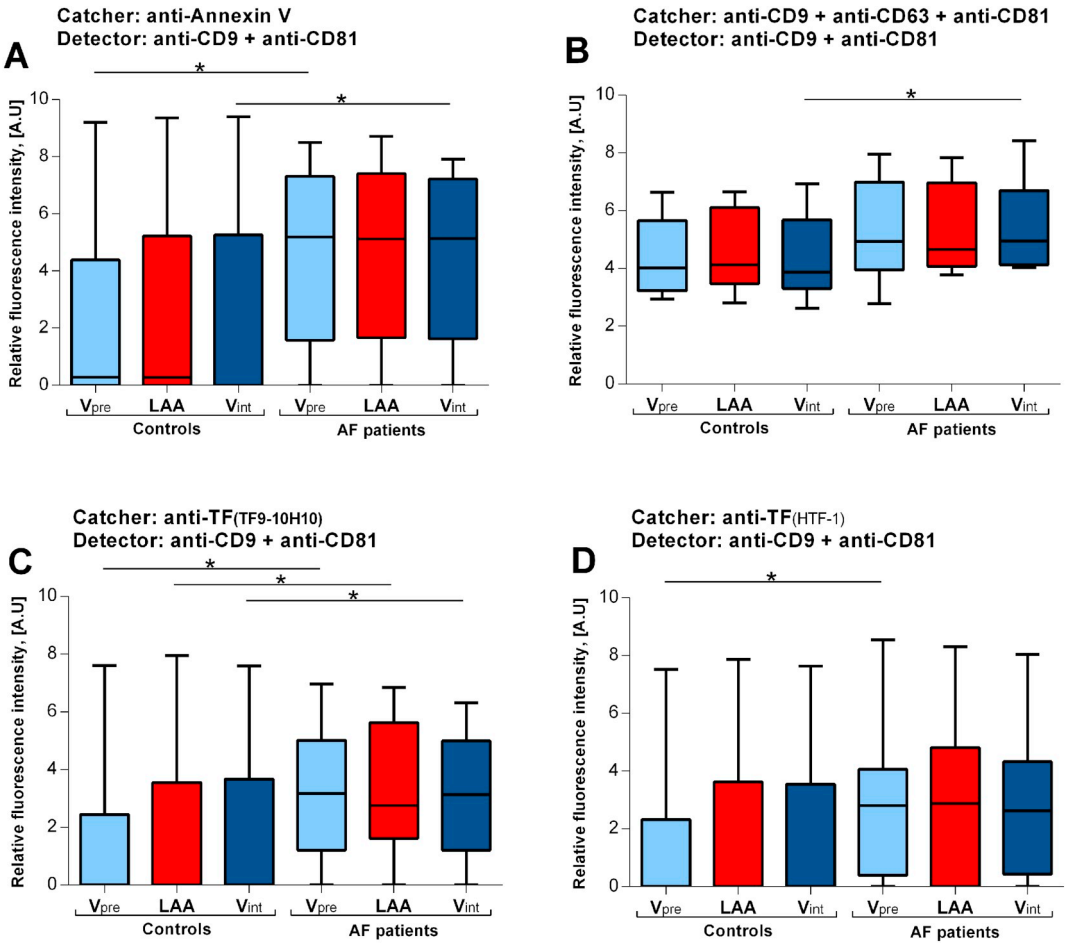

Fig. 2. Extracellular vesicles (EVs) captured by general EV markers or anti-TF-antibodies combined with detection via anti-tetraspanin antibodies. Relative fluorescence intensity in EV Array on platelet free plasma from 13 atrial fibrillation (AF) patients and 12 controls drawn preoperatively from a central vein (V_{pre}), and intraooperatively from the left atrial appendage (LAA), and from a central vein (V_{int}). Catching and detecting agents are indicated above each subfigure (A-D). A.U., arbitrary units; TF, tissue factor. Boxes indicate the first, second (median), and third quartile. Whiskers indicate the range. $^{*}p < 0.05$.

3.4. Cf-DNA

Fig. 5C shows the measured cf-DNA concentrations.

In the control group as well as in the AF patient group, cf-DNA concentrations were significantly higher in the V_{int} and the LAA samples than in the V_{pre} samples. No significant differences were observed between the AF patient group and the control group.

3.5. PPL

PPL-CT results are displayed in Fig. 5D.

No significant differences in PPL-CT were observed between groups or between blood sample types.

3.6. Submicron particles

Results on particle concentrations are given in Fig. 5E and results on mean particle diameters in Fig. 5F. No significant differences were observed between the types of blood sample in neither the AF patient nor the control group, and no significant differences were observed between the controls and the AF patients. Correlation analysis on AF patients and controls together showed that particle concentration significantly correlated with triglyceride concentration (Fig. 6A, $r_s = 0.82$, $p < 0.0001$), and less pronouncedly, yet statistically significantly, with total cholesterol ($r_s = 0.55$, $p < 0.01$, Fig. 6B) and low density lipoprotein (LDL) cholesterol ($r_s = 0.52$, $p < 0.01$, Fig. 6C). For one patient with paroxysmal AF no data on submicron particles were obtained because of technical problems, hence only 12 AF patients are represented in the data on particle size and concentration in Figs. 5 and 6.

4. Discussion

In this study, we measured blood plasma levels of biomarkers related to blood coagulability and endothelial activation in patients undergoing coronary artery bypass or aortic valve surgery. The main novel findings were significantly higher levels of EVs exposing tetraspanins, PS, TF, and markers of endothelial cell and leukocyte origin, including a specific subgroup of TF-bearing EVs derived from cells of the monocyte-macrophage-lineage, in AF patients compared with controls. Thus, increased levels of TF antigen in plasma in AF patients are probably at least partly explained by elevated levels of TF-bearing EVs. No significant differences in levels of any measured procoagulant biomarkers were found between intraoperative blood samples drawn from a central vein and from the LAA.

4.1. EVs and thrombogenicity

EVs have been nominated as plausible contributors to a range of biological processes including coagulation [31]. The presence of PS on EVs facilitates the assembly of coagulation factors and promotes coagulation [23], and subtypes of EVs that in addition to PS expose TF,

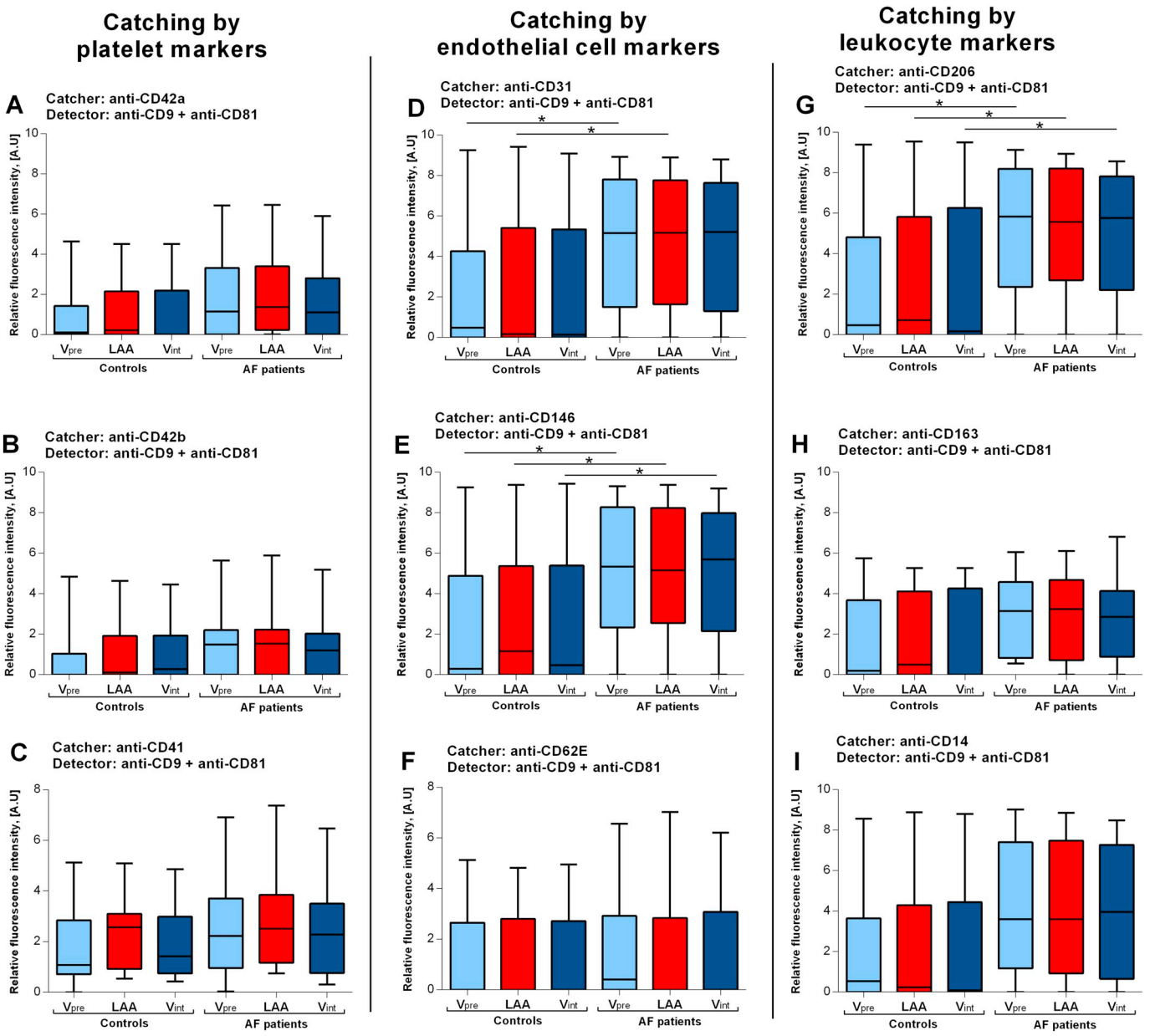

Fig. 3. Extracellular vesicles (EVs) captured by cell type-specific EV markers combined with detection via anti-tetraspanin antibodies. Relative fluorescence intensity in EV Array on platelet free plasma from 13 atrial fibrillation (AF) patients and 12 controls drawn preoperatively from a central vein (V_{pre}), and intraooperatively from the left atrial appendage (LAA), and from a central vein (V_{int}). Catching and detecting agents are indicated above each subfigure (A-I). A.U., arbitrary units. Boxes indicate the first, second (median), and third quartile. Whiskers indicate the range. *$p < 0.05$.

reportedly have the highest level of procoagulant activity [13]. In the circulation, TF exists in its full-length form incorporated in the membranes of EVs and in a soluble alternatively spliced form [32]. It is controversial what range of cell types is capable of synthesizing TF [33], but it has been indicated that monocytes probably account for most TF-exposing EVs in the blood, while endothelial cells, platelets, and neutrophils may contribute their share under some conditions [13]. Basavaraj et al. suggested that conflicting findings on these issues could partly be explained by varying TF-binding capacity of different anti-TF antibody clones [33] and found that the TF9-10H10 clone was suitable for detection of TF on EVs using flow cytometry [33], while the HTF-1 clone detected the lowest proportion of TF-positive EVs [33]. In the present study, using either clone, we observed significant differences in TF-bearing EV levels, comparing AF patients with controls.

4.2. Inter-related inflammatory and procoagulant processes in the LAA

Nakamura et al. demonstrated inflammatory cell infiltration and expression of TF in the endothelium of the LAA in patients with non-valvular AF and a recent thromboembolic event [10]. Furthermore, vWF was overexpressed in the LAA endothelium in AF patients [10]. vWF is a well-established marker of endothelial dysfunction and damage [5], mediating adhesion of platelets to sites of vascular damage [34]. Activated platelets can recruite leukocytes, including monocytes, neutrophils, lymphocytes, and dendritic cells [35]. Cells release EVs upon activation or cell stress [36]. Thus, EVs, including TF-exposing subtypes, released from endothelial cells and/or leukocytes and/or platelets in an inflammatory milieu of the LAA endocardium in AF patients may conceivably contribute to thrombogenicity in these patients. NETosis represents another link between inflammation and coagulation [37]. We hypothesized that blood drawn directly from the

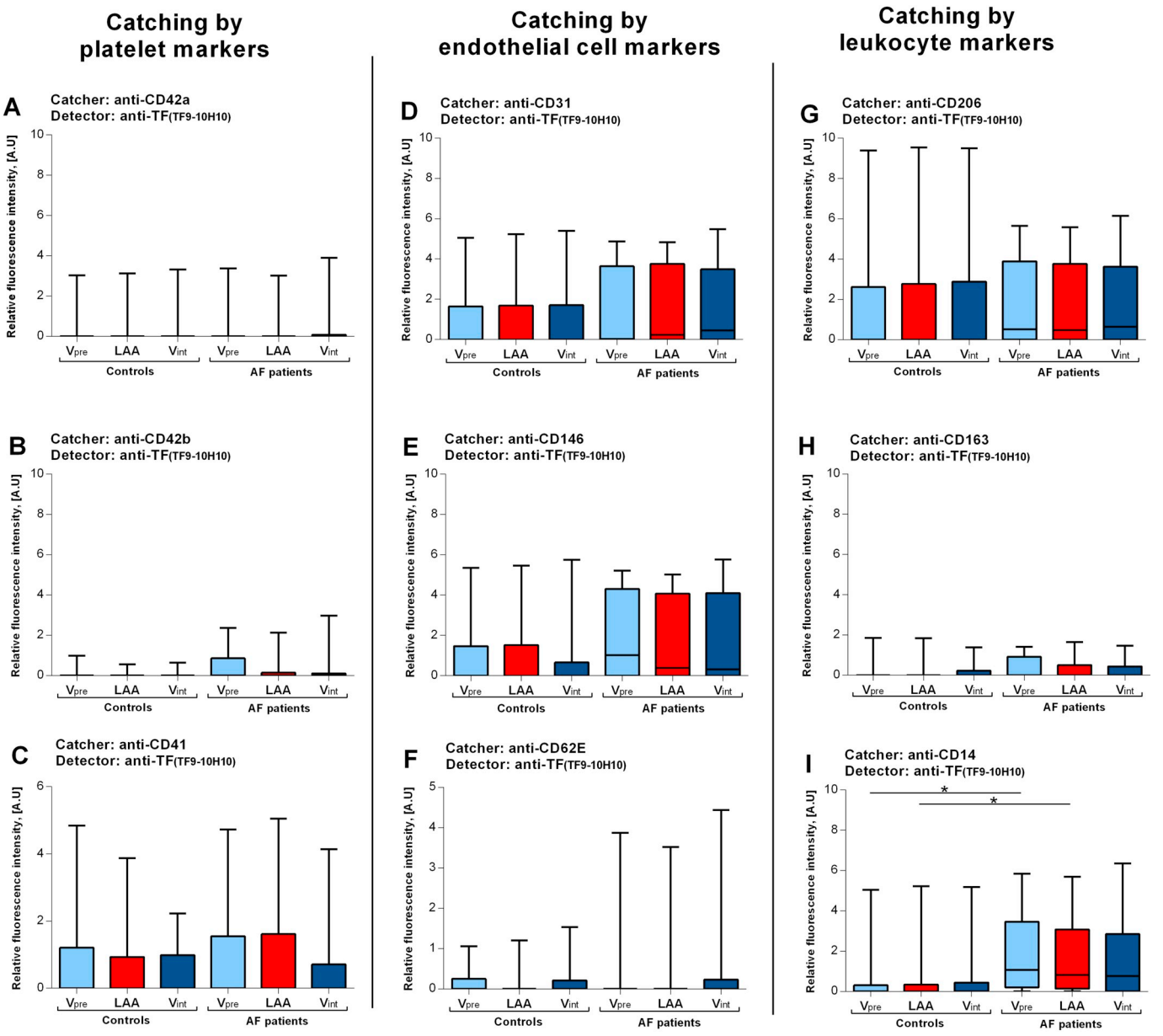

Fig. 4. Extracellular vesicles (EVs) captured by cell type-specific EV markers combined with detection via anti-TF antibodies. Relative fluorescence intensity in EV Array on platelet free plasma from 13 atrial fibrillation (AF) patients and 12 controls drawn preoperatively from a central vein (V_{pre}), and intraooperatively from the left atrial appendage (LAA), and from a central vein (V_{int}). Catching and detecting agents are indicated above each subfigure (A-I). TF, tissue factor; A.U., arbitrary units. Boxes indicate the first, second (median), and third quartile. Whiskers indicate the range. *$p < 0.05$.

LAA would contain elevated levels of EVs, TF, vWF, and NETs (i.e. cf-DNA) in comparison with venous blood. Anatomically, the LAA is a long, tubular, hooked structure with a narrow inlet from the main part of the LA [38], and since AF induces an abnormal flow pattern with reduced flow velocity in the LAA [5], ongoing AF may induce reduced exchange of blood between the LAA and the general circulation, resulting in accumulation of procoagulant agents released from cells in the LAA. Clearly, this hypothesis was not supported by our data since in general the biomarkers in LAA samples and V_{int} samples in AF patients were at the same level (Figs. 2–5). This could indicate that AF is a systemic disease rather than a local, but there will be an unavoidable influx of blood from the main part of LA and thus from the rest of the circulation during blood sampling from the LAA. Shirani et al. found a mean luminal LAA volume of 5.4 mL and a considerable intra-individual variation, the standard deviation being 3.7 mL [7]. Thus, admixture of blood from the rest of the circulation could have camouflaged differences between the compartments in our study.

We did, however, observe higher concentrations of circulating cf-DNA in the V_{int} and the LAA samples than in the V_{pre} samples in AF patients as well as controls. This can probably be ascribed to cell damage and possibly also sterile inflammation induced by sternotomy. The tendency towards lower TF concentration as measured by ELISA in the intraoperative blood samples than in the preoperative samples may possibly reflect an element of consumption or changed configuration of TF in blood due to sternotomy-induced activation of the coagulation system.

4.3. vWF and TF levels in AF patients versus controls

Our data demonstrated significantly higher vWF levels in AF patients than in controls, which is in accordance with previous findings on venous blood by other investigators [9]. Also, we observed significantly

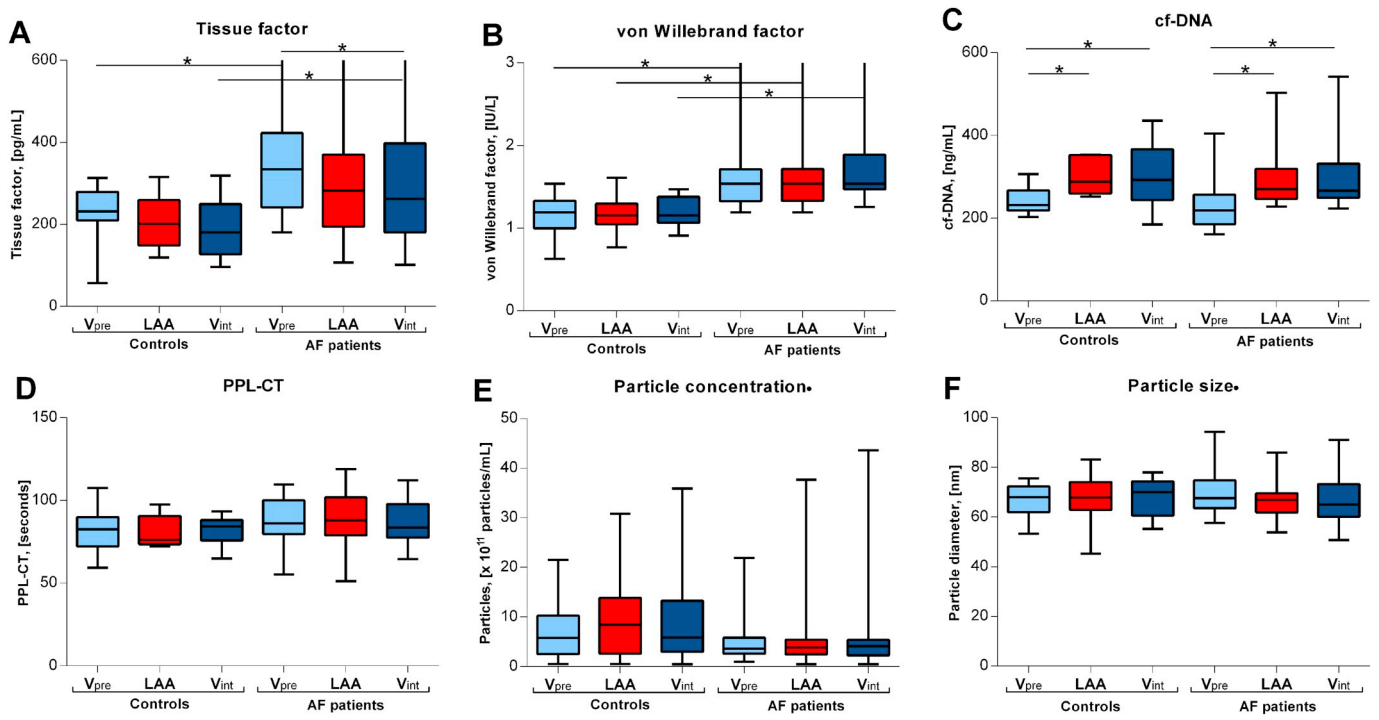

Fig. 5. Tissue factor (TF), von Willebrand factor (vWF), cell free deoxyribonucleic acid (cf-DNA), submicron particle, and procoagulant phospholipid-dependent clotting time (PPL-CT). Analysis was performed on platelet free plasma from 13 atrial fibrillation (AF) patients and 12 controls drawn preoperatively from a central vein (V_{pre}), and intraooperatively from the left atrial appendage (LAA), and from a central vein (V_{int}). The measured parameters are indicated above each subfigure (A–F). •Only 12 AF patients are represented in the data on particle size and concentration. Boxes indicate the first, second (median), and third quartile. Whiskers indicate the range. One patient with paroxysmal AF displayed markedly higher levels of vWF (V_{pre}: 22.12 IU/L; LAA: 21.98 IU/L; V_{int}: 21.84 IU/L) and TF (above the linear range of the ELISA) than the rest of the study participants, hence the omission of upper whiskers in the AF patient group in (A) and (B). *$p < 0.05$.

higher levels of TF in plasma in AF patients than in controls. Previously, Chung et al. observed higher plasma levels of TF in patients with a history of AF than in a healthy control group, while the AF patients did not display higher levels than an additional control group of patients with coronary artery disease [11]. In that study, it was not specified if the AF group included valvular AF patients [11]. In our study, we included nonvalvular AF only, as mitral valve disease per se affects coagulability and causes endocardial damage in the left atrium [39], and furthermore, AF patients and controls had comparable cardiovascular risk factors.

4.4. EV levels in AF patients versus controls

Our EV Array results clearly indicated higher levels of EVs, including TF-bearing EVs, in plasma from AF patients than in plasma from controls. The significantly higher levels of CD206-exposing EVs, CD31-exposing EVs, and CD146-exposing EVs, when anti-tetraspanin antibodies were used as detecting agents suggest that AF is associated with increased levels of EVs derived from cells of the monocyte-macrophage lineage and the endothelial lineage. However, we did not find differences when anti-CD62E antibodies were used as capturing agents. A tendency towards higher concentrations in the AF patient group, although statistically insignificant, was also seen for CD14-exposing EVs and CD163-exposing EVs detected via anti-tetraspanin antibodies, which supports the basis for suggesting that EVs from monocytes and macrophages are more abundant in AF patients than in patients without AF. When anti-TF antibodies were used for detection after capture by cell-type specific antigens, significant differences were seen only for the CD14-positive EVs, consistent with the statement that monocytes are likely to be the main source of TF-bearing EVs in health and disease [13]. A similar trend was observed for CD206-positive EVs which also presumably mostly originate from cells of the monocyte-macrophage

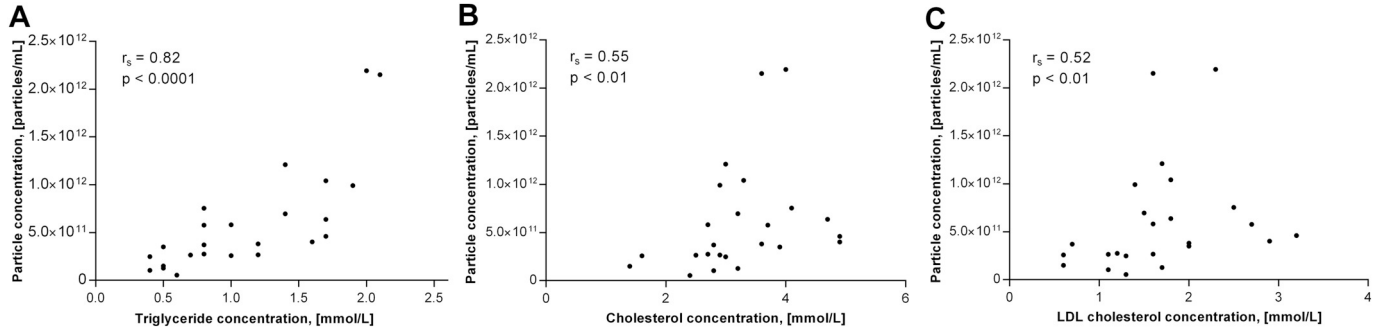

Fig. 6. Correlation between submicron particle concentration and lipid levels in AF patients and controls. (A) Submicron particle concentration plotted against triglyceride concentration. (B) Submicron particle concentration plotted against total cholesterol concentration. (C) Submicron particle concentration plotted against low density lipoprotein (LDL) cholesterol concentration. $n = 24$.

lineage. Also, a modest tendency was observed for some of the endothelial cell marker-bearing EVs, suggesting that the possibility exists that endothelial cells may release TF-bearing EVs as a result of AF.

Scarce literature exists on the potential association between AF and plasma contents of EVs. Azzam et al. found higher concentrations of platelet-derived EVs in valvular AF patients than in healthy control subjects [40]. Choudhury et al. demonstrated higher levels of platelet-derived EVs in nonvalvular AF patients than in healthy control, while no difference was observed between AF patients and a group of control subjects with various cardiovascular diseases, and it was suggested that platelet activation in AF patients results from underlying cardiovascular disease [41]. The modest and statistically insignificant tendency observed for the platelet marker-positive EVs in our study likewise suggests that levels of platelet-derived EVs may to a lesser extent, if at all, be associated with AF. Ederhy et al. [42] demonstrated higher levels of platelet-derived EVs as well as endothelium-derived EVs in nonvalvular AF patients than in a control group *without* cardiovascular risk factors, but similar to a control group *with* cardiovascular risk factors. With regard to PS-positive EVs, Ederhy et al. found that AF patients displayed higher levels than a control group *without* as well as a control group *with* cardiovascular risk factors [42]. This is in line with our data on Annexin V-positive EVs. Interestingly, based on a functional assay, Pourtau et al. [43] described lower EV-associated TF-activity in LA blood from AF patients than in venous blood from healthy subjects. Furthermore, they observed a reduction in EV-associated TF-activity in LA blood 20 min after AF induction in patients that had been in sinus rhythm for ten days. The authors suggested that the decrease could be due to consumption [43].

4.5. Levels of submicron particles and PPLs

NTA is considered a valuable supplement to label-based approaches to EV analysis [21]. However, when applied on PFP, a considerable share of particles detected by NTA is probably accounted for by lipoproteins [44], even in fasting samples [14]. In the present study, submicron particle concentrations correlated with plasma triglyceride concentrations in agreement with recent findings [14,15], and furthermore moderately with LDL as well as total cholesterol concentrations. This indicates that the submicron particle levels were markedly influenced by lipoprotein content, which probably explains why the apparently higher EV concentrations in AF patients than controls are not reflected in the NTA results. Nor did PPL-CT significantly differ between AF patients and controls. Other investigators have also presented data in which higher EV levels were not accompanied by measurable changes in total PS concentrations [45].

4.6. Study limitations

One major limit of the study is the small number of patients but in spite of this we found several significant differences. The study was planned to find larger differences, cf. the power calculation. EV release has been associated with a range of physiological and pathological processes [12], and AF is associated with a range of factors, including hypertension, diabetes mellitus, heart failure, left ventricular hypertrophy, male sex, age, smoking, and obesity [46]. Thus, our results may be influenced by confounders and intermediate variables. The study is too small for stratification but there were no significant differences between the groups (Table 1) except for a higher LA diameter and INR in AF patients. The higher INR levels in the AF patient group were measured the day before the operation, and therefore lower on the day of operation, but some remaining action of the patients' anticoagulant therapy on the results cannot be excluded.

4.7. Clinical perspectives

There is an increasing focus on identification of patients who do and patients who do not require oral anticoagulation, and biochemical biomarkers may potentially be a valuable supplement to clinical risk score systems such as the CHA_2DS_2-VASc score [47]. vWF has been suggested relevant for this purpose and was recently shown to provide some additional prognostic information with regard to cardiovascular events, stroke, and cardiovascular mortality, although the absolute impact on decision-making was found too marginal to institute vWF for risk prediction [48]. Our data substantiated the previous report on elevated levels of TF in plasma in AF patients by Chung et al. [11] and elaborated on that knowledge by demonstrating higher levels of TF-bearing EVs, including CD14-positive TF-bearing EVs. Theoretically, procoagulant capacity of these EVs may be mechanistically involved in thrombogenicity in AF patients. Thus TF-bearing EVs, and subtypes of these, may have a potential as predictors of thromboembolic events in AF patients and in this way they may be relevant candidates for further exploration in prospective studies.

5. Conclusions

Increased plasma concentrations of TF in AF patients are accompanied and probably at least partly explained by increased levels of TF-bearing EVs. TF-bearing EVs may be mechanistically involved in increased thrombogenicity in AF patients and may have a potential as predictive markers of AF-related thromboembolism.

Disclosure statement

The authors report no conflicts of interest, including no funding from any for-profit organizations.

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