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N.B.: When citing this work, cite the original article.

Original Publication:

Micha Milovanovic, Elisabeth Fransson, Claes Hallert and Petter Järemo, Letter: Atrial fibrillation and platelet reactivity, International Journal of Cardiology, 2010.

<http://dx.doi.org/10.1016/j.ijcard.2010.02.030>

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<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-59612>

Atrial fibrillation is associated with increased platelet reactivity

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Running head: Atrial fibrillation and platelet reactivity

Key-words: ADP, atrial fibrillation, flow cytometry, platelets, platelet reactivity, P-selectin, thrombin

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ABSTRACT

Objective: Relationships between platelet reactivity and atrial fibrillation (AF) have not been investigated before. Thus, platelet reactions after stimulation were compared with the presence of sinus rhythm (SR) after a surveillance period exceeding two years after elective electrical cardioversion.

Methods: 33 individuals with non-valvular AF subject to elective electrical cardioversion were consented. Immediately before cardioversion determination of markers (see below) reflecting platelet reactivity was carried out. After an average of 26 ± 8 (SD) months an ECG was analyzed and platelet laboratory analysis were repeated. A flow cytometry technique was used to determine platelet reactivity i.e. surface bound fibrinogen after stimulation. ADP (1.7 and 8.5 $\mu\text{mol/L}$) and a thrombin receptor activating peptide (TRAP-6) (54 and 74 $\mu\text{mol/L}$) were used as platelet agonists.

Results: As the study was brought to an end subjects with AF ($n=18$) had a trend towards lower platelet reactivity. Compared with day 1 it reached statistical significance ($p=0,016$) when using 1.7 $\mu\text{mol/L}$ ADP to activate platelets. Responses towards the other agonists did not change significantly over time. In contrast, after 26 ± 8 (SD) months subjects with SR ($n=15$) had significant lower platelet reactivity when stimulating with both agonists pairs. The p-values for the two ADP dilutions (1.7 and 8.5 $\mu\text{mol/L}$) were $p=0.002$ and $p=0.031$, respectively. The corresponding figures for TRAP-6 (54 and 74 $\mu\text{mol/L}$) proved to be $p=0.042$ and $p=0.006$.

Conclusion: After 26 ± 8 (SD) months patients returning to AF had higher platelet reactivity than those who remained in SR. The finding provides a link between arrhythmia and clot formation.

INTRODUCTION

Atrial fibrillation (AF) is a frequent occurring sustained arrhythmia. It is well established that the disorder is strongly connected with stroke. The mechanism by which the risk for embolic cerebral events is enhanced is not fully established. Sporadic reports indicate that AF is associated with increased levels of circulating platelet-derived substances such as soluble p-selectin [1, 2]. It has been hypothesized that platelet alterations are signs of underlying cardiovascular co morbidity [3]. The current study analyzes platelet reactivity, activity and the inflammatory response in conjunction with elective electrical cardioversion. In order to strengthen the power of the study and to minimize biases for instance from comorbidities and concomitant drug therapies all subjects served as their own controls. Thus, sampling was carried out at the electrical cardioversion and after more than two years. Subsequently, the study relates alterations of laboratory findings to long-term outcome i.e. sinus rhythm (SR) after the observation period.

PATIENTS AND METHODS

Patients

Outpatients (n=35) attending the general cardiology clinic with ECG-defined non-valvular AF were enrolled. They were all judged eligible for elective electrical cardioversion therapy by their responsible cardiologist. All subjects with three exceptions had sinus rhythm after the procedure. Subjects were recruited consecutively and included when laboratory resources were available. Before cardioversion (day 1) markers reflecting platelet reactivity, activity and the inflammatory response (see below) were determined. After 26 ± 8 (SD) months all participants were called back. A new ECG was analyzed and the assays (see below) were repeated. Then 18 and 15 individuals had AF and SR, respectively. The participants were grouped according to heart rhythm at follow up. Table 1 shows patient demography, associated medical conditions and drug prescriptions on day 1. During the study no participant suffered from stroke or a major thrombotic event. Due to non-diagnostic ECG at follow-up two individuals with implanted pacemakers were excluded. We did not apply any other exclusion criteria.

Laboratory

10 mL blood was collected in the morning into tubes containing 3.8% citrate (0.5 mL). Flow cytometry analysis was commenced in duplicate within two hours of blood collection. Details of the assays for determining platelet reactivity have been explained before [4, 5]. In short, platelets were stimulated in whole blood with ADP (1.7 and 8.5

$\mu\text{mol/L}$) and a thrombin receptor activating peptide (TRAP-6) (54 and 74 $\mu\text{mol/L}$). Platelets were identified with a phycoerythrin-conjugated antibody against GPIb (Dako AS, Denmark). A polyclonal fluorescein isothiocyanate-conjugated chicken antihuman fibrinogen antibody (Biopool AB, Sweden) recognized surface bound fibrinogen. The number of platelets (%) with more membrane bound fibrinogen than a predetermined non-stimulated control was used as an investigational parameter. Surface expression of p-selectin was analyzed with a two-color flow cytometry assay. Platelets (%) having more surface bound p-selectin than a preset control were regarded as P-selectin positive. Determination of soluble p-selectin in citrate anticoagulated blood was carried out using a commercial ELISA kit (R&D system, UK). P-selectin samples were stored at -70°C until analysis. Day 1 and recovery samples were analyzed simultaneously. The Regional Ethics Committee approved the study.

Statistics

The data were considered to be normal distributed. Consequently, the paired and unpaired *t*-tests were employed when appropriate. The chi-square test was used to evaluate categorical information. A probability less or equal 0.05 was regarded to denote significance.

RESULTS

Subjects ($n=33$) were grouped according to heart rhythm at follow-up. 18 participants then had AF and the remaining patients ($n=15$) displayed sustained SR. On day 1, the groups did not diverge with respect to age, smoking habits and gender. Furthermore, their heart rates and blood pressures proved to be similar (Table 1). The groups then did not display any diversity in underlying cardiovascular co-morbidities. Subjects having AF after $26\pm 8(\text{SD})$ months had less digitalis ($p=0.016$) at inclusion. Otherwise, the groups did not differ significantly with respect to day 1 drug prescriptions (Table 1).

Experimental platelet data for the two groups having AF and SR after $26\pm 8(\text{SD})$ months are given in Table 2. Generally, platelet responses towards the current agonists (i.e. reactivity) were lower towards the end of the study period. For AF patients with one exception ($1.7 \mu\text{mol/L}$ ADP; $p=0.016$) the differences failed to reach statistical significance. In contrast, compared to study entry, after $26\pm 8(\text{SD})$ months the SR group demonstrated significant lower platelet responses after stimulation with all four antagonists (Table 2). The *p*-values for the two ADP dilutions ($1.7 \mu\text{mol/L}$ and 8.5

$\mu\text{mol/L}$) proved to be $p=0.002$ and $p=0.031$, respectively. Stimulation with TRAP-6 ($54 \mu\text{mol/L}$; $p=0.042$ and $74 \mu\text{mol/L}$; $p=0.006$) yielded comparable results. Platelet counts and platelet activity as estimated from platelet bound and soluble p-selectin were not linked to the arrhythmia (Table 2). However, both groups had lower circulating P-selectin after the surveillance period. For subjects having AF and SR the p-values were $p<0.001$ and $p=0.001$, respectively.

Table 3 compares the two groups having AF and SR after $26\pm 8(\text{SD})$ months after the initial electrical cardioconversion. It demonstrates that the arrhythmia *per se* is associated with increased platelet reactivity. On day 1, no differences were found with respect to platelet reactivity. In contrast, at study end subjects with AF had more reactive platelets. The p-values for the two ADP concentrations ($1.7 \mu\text{mol/L}$ and $8.5 \mu\text{mol/L}$) were $p=0.049$ and $p=0.045$, respectively. Platelet behavior was similar ($p=0.02$) when using TRAP-6 ($74 \mu\text{mol/L}$) as a platelet-activating agent. However, after $26\pm 8(\text{SD})$ months platelet reactions after stimulation with more diluted ($54 \mu\text{mol/L}$) TRAP-6 were higher among patients with AF. The differences, however, failed to reach statistical significance. Platelet activity as estimated from platelet bound and soluble P-selectin was unaffected by the arrhythmia (Table 3).

DISCUSSION

The current work suggests that it is possible that the irregular heart rate of AF affects platelet reactivity. Indeed, subjects who failed to convert to prolonged SR had higher platelet responses after $26\pm 8(\text{SD})$ months when stimulating with various dilutions of ADP and TRAP-6 (Table 3). Thus, it is tenable to hypothesize that platelets provide a link between the arrhythmia and clot formation.

Even if P-selectin has been recognized in endothelial cells it is apparent that the greater quantity if not all soluble P-selectin evolves from platelets [6]. It has been suggested that soluble P-selectin reflects platelet activity over a long period of time. Surface expression of the molecule could be associated with acute activation [7]. In our hands, neither membrane-bound nor circulating p-selectin suggest an excess of platelet activation in AF (Table 3). Thus, platelet activity is unrelated to heart rhythm.

Both groups developed lower platelet reactivity after the surveillance lasting for more than 2 years. AF was associated with less pronounced alterations (Table 2). The current study suggests that for all patients platelet activity as estimated from soluble p-selectin was lower after 26 ± 8 (SD) months (Table 2). It is tenable that platelets with low reactivity circulate less activated. As a consequence, soluble p-selectin decreases. Subjects having chronic AF had warfarin over the entire study time. In contrast, 6 SR rhythm patients did not have anticoagulation (data not shown) at study end. The drug wanes both platelet activity and adhesiveness [8]. It is tenable that long-term warfarin prescriptions affect platelet reactivity as well. Thus, the drug could explain why both groups then demonstrated lower platelet reactivity. However, despite more frequent anticoagulation AF subjects had higher platelet reactivity after 26 ± 8 (SD) months..

The current laboratory study involves 33 individuals each being investigated extensively employing both labor-intensive flow cytometry and ELISA techniques. By necessity, laboratory studies make it difficult to include sample sizes making it possible to adjust for confounders due to co-morbidities and concomitant drug therapies. Thus, in the current study another approach was chosen and each subject served as its own control. This experimental protocol makes confounders less likely because with a high degree of certainty they do not vary substantially over the observation period. Samples for soluble P-selectin i.e. day 1 and 2 years specimen were analyzed simultaneously. Thus, lower values found after more than 2 years are unrelated to assay conditions.

It is evident that subjects returning to AF had higher platelet reactivity than patients remaining in SR. The current work ascertains that the irregular heart rhythm *per se* has an impact upon platelets. In contrast, platelet activity was unrelated to heart rhythm.

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Table 1. Demographic data, heart rate and blood pressure together with associated medical conditions and drug usage on day 1 for the two groups having atrial fibrillation (n=18) and sinus rhythm (n=15), respectively after 26±8(SD) months. The unpaired *t*-test and the chi-square test were employed when appropriate.

	AF after 26±8(SD) months (day 1)	SR after 26±8(SD) months (day 1)	p- value
Age (years)	70±6	66±8	NS
Current smoking	15 %	10 %	NS
Gender (M/F)	14/4	13/2	NS
Heart rate (beats/min)	77±13	82±13	NS
Diastolic blood pressure (mm Hg)	79±13	77±11	NS
Systolic blood pressure (mm Hg)	133±20	133±19	NS
Diabetes	6 %	21 %	NS
Previous hypertension	44 %	36 %	NS
Previous MI	11 %	7 %	NS
Aspirin	6 %	14 %	NS
ACE-inhibitors	17 %	36 %	NS
A₂-blockers	12 %	0 %	NS
β-blockers	78 %	86 %	NS
Digitalis	11 %	57 %	0,016
Diuretics	22 %	43 %	NS
Lipid lowering drugs	33 %	14 %	NS
Warfarin	94 %	86 %	NS

AF = atrial fibrillation

MI = myocardial infarctions

NS = not significant

SR = sinus rhythm

Table 2. Differences with respect to platelet reactivity and activity between subjects having atrial fibrillation (n=18) and sinus rhythm (n=15) 26±8(SD) months after the initial electrical cardioconversion. The paired *t*-test was employed for statistical evaluation.

	AF after 26±8(SD) months (day 1)	AF after 26±8(SD) months (at study end)	p- value		SR after 26±8(SD) months (day 1)	SR after 26±8(SD) months (at study end)	p- value
Platelet counts (x10⁹/L)	236±48	223±52	NS		240±74	252±84	NS
ADP (1.7 µmol/L) (%)*	34±19	19±14	0.016		27±15	10±12	0.002
ADP (8.5 µmol/L) (%)*	63±21	51±26	NS		58±19	31±28	0.031
TRAP-6 (54 µmol/L) (%)*	44±29	29±20	NS		48±33	16±19	0.042
TRAP-6 (74 µmol/L) (%)*	62±26	45±23	NS		61±19	24±23	0.006
Platelet bound p-selectin (%)**	5.5±4.0	5.0±2.6	NS		4.9±3.9	5.2±4.0	NS
Soluble p-selectin (µg/L)	102±41	48±23	>0,001		103±44	58±23	0.001

AF = atrial fibrillation

NS = not significant

SR = sinus rhythm

*percentage fibrinogen positive cells after stimulation

**cells having more surface-bound p-selectin than a predetermined control

Table 3. Differences with respect to platelet reactivity and activity between the two groups having atrial fibrillation (n=18) and sinus rhythm (n=15) 26±8(SD) months after the initial electrical cardioconversion. The comparisons at inclusion (day 1) are shown in the left columns. The unpaired *t*-test was used to evaluate the data statistically.

	AF after 26±8(SD) months (day 1)	SR after 26±8(SD) months (day 1)	p- value		AF after 26±8(SD) months (at study end)	SR after 26±8(SD) months (at study end)	p- value
ADP (1.7 µmol/L) (%)*	34±19	27±15	NS		19±14	10±12	0.049
ADP (8.5 µmol/L) (%)*	63±21	58±19	NS		51±26	31±28	0.045
TRAP-6 (54 µmol/L) (%)*	44±29	48±33	NS		29±20	16±19	NS
TRAP-6 (74 µmol/L) (%)*	62±26	61±19	NS		45±23	24±23	0.020
Platelet bound p-selectin (%)**	5.5±4.0	4.9±3.9	NS		5.0±2.6	5.2±4.0	NS
Soluble p-selectin (µg/L)	102±41	103±44	NS		48±23	58±23	NS

AF = atrial fibrillation

NS = not significant

SR = sinus rhythm

*percentage fibrinogen positive cells after stimulation

**cells having more surface-bound p-selectin than a predetermined control