Gender and tachycardia: independent modulation of platelet reactivity in patients with atrial fibrillation

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Abstract

Background Female patients with atrial fibrillation (AF) experience increased risk of thromboembolism compared to males, an observation that is reflected by its inclusion in the CHA₂DS₂-VASc score. New onset AF (often associated with tachycardia) also confers upon patients increased thromboembolic risk. The mechanisms underlying this risk are uncertain, but new onset AF is associated with profound impairment of platelet nitric oxide (NO) signalling. Given that cardiovascular responses to catecholamines are gender-dependent, and that the presence of tachycardia in new onset AF may represent a response to catecholaminergic stimulation, we explored the potential impact of gender and tachycardia on platelet aggregation and NO signalling.

Methods Interactions were sought in 87 AF patients between the extent of adenosine diphosphate (ADP)-induced platelet aggregation, the anti-aggregatory effects of the NO donor, sodium nitroprusside, gender, and admission heart rate. The potential impact of platelet expression of thioredoxin-interacting protein (Txnip) was also evaluated.

Results Analysis of covariance confirmed the presence of physiological antagonism between platelet ADP and NO responses \( F(1, 74) = 12.212, P < 0.01 \), while female sex correlated with impaired NO responses independent of platelet aggregability \( F(2, 74) = 8.313, P < 0.01 \). Admission heart rate correlated directly with platelet aggregation \( r = 0.235, P < 0.05 \), and inversely with NO response \( r = -0.331, P < 0.01 \). Txnip expression varied neither with gender nor with heart rate. Conclusions These results indicate that gender and heart rate are independent determinants of platelet function. Prospective studies of the putative benefit of reversal of tachycardia on restoration of normal platelet function are therefore a priority.

Keywords: Atrial fibrillation; Gender; Heart rate; Nitric oxide; Platelet aggregation

1 Introduction

Clinical factors associated with increased thromboembolic risk in atrial fibrillation (AF) have been identified epidemiologically and formalised into stroke risk algorithm.¹² However, the physiological bases underlying these categories of risk are as yet incompletely understood. In particular, while platelet hyperaggregability has been observed in the context of AF,³⁴ its potential role in determining thromboembolic risk remains unclear, studies have so far failed to establish a correlation between platelet reactivity and clinical scores of thromboembolic risk.

Among factors which have been shown to modulate platelet aggregability are the interactions between ambient pro-aggregatory stimuli (such as plasma catecholamine concentrations),⁵ and anti-aggregatory autacoids such as nitric oxide (NO). We have previously shown that the inhibitory response of platelets to NO varies widely in patients with AF,⁶ and is particularly impaired in ‘new onset’ AF. Mechanisms to account for this were not fully explored, although theoretically, sources of impaired response to NO might include increased reactive oxygen species (ROS) generation,⁷ and/or oxidation/depletion of the haeme component of soluble guanylate cyclase (sGC) (see Chirkov and Horowitz⁸ for review).

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Recent onset AF is often associated with relatively rapid ventricular response,\(^\text{[9]}\) symptomatic of acute increased sympathetic stimulation. In this context, elevated plasma catecholamine concentrations could contribute to platelet hyper-reactivity through direct pro-aggregatory effects,\(^\text{[5]}\) as well as through increased ROS generation,\(^\text{[10]-[12]}\) and resultant scavenging of NO. Pro-inflammatory effects of catecholamine release measured in myocardium occur predominantly in ageing females,\(^\text{[13]}\) hence there may in theory be gender-specific aspects to this phenomenon.

Another possible mechanism accounting for the interaction between heart rate and impaired NO response in new onset AF is the stimulation of inflammation. This could, for example, occur via oxidant-stimulated increased expression of the pro-inflammatory protein, thioredoxin-interacting protein (Txnip, see Chong, \textit{et al.}\(^\text{[14]}\) for review), which appears to suppress NO responses.\(^\text{[15]}\)

We have therefore performed further evaluations of the physiological data from Standard vs. Atrial Fibrillation spESific managemeNT studY (SAFETY) in order to delineate potential mechanisms underlying the interactions of tachycardia with impaired platelet NO signalling.

2 Methods

2.1 Patient selection

The investigation was conducted as a single centre mechanistic sub-study of the SAFETY, an investigation of non-pharmacological management strategies in patients hospitalized with AF.\(^\text{[16]-[17]}\) Patients were considered for inclusion if they were admitted to hospital due to AF. Exclusion criteria for SAFETY were age < 45 years, primary diagnosis of valvular heart disease, scheduled catheter ablation of AF, pre-existing NYHA class III–IV heart failure with a documented left ventricular ejection fraction (LVEF) < 45%, alcohol-induced AF and terminal illness requiring palliative care. Patients receiving P\(_2\)Y\(_{12}\) receptor antagonists were also excluded from the current sub-study because of potential impact of such agents on capacity to measure platelet response to NO. The study was approved by the institutional Ethics of Human Research Committee. Written informed consent was obtained in all cases.

2.2 Clinical data

All patients underwent standardized clinical assessment and routine biochemical investigation. Additional cardiac investigations were resting ECG (which was used for measures of admission heart rate) and transthoracic echocardiography: LVEF was calculated from biplane images using Simpson’s method.\(^\text{[18]}\)

2.3 Platelet aggregometry

Platelet aggregometry was performed using whole blood impedance aggregometry as previously described.\(^\text{[19]}\) Briefly, venous blood was collected under vascular stasis from an antecubital vein using a 21G butterfly with a 20 mL syringe into 10 ML tubes containing 1: 10 volume of acid citrate anticoagulant (2 parts 0.1 mol/L citric acid to 3 parts of 0.1 mol/L trisodium citrate). Aggregation was induced with ADP (2.5 µmol/L), and responses were recorded for electrical impedance (Ω) via a computer interface system (Aggrolink, Chrono-log, Havertown, Pennsylvania, USA). The NO donor sodium nitroprusside (SNP, 10 µmol/L) was used to measure platelet response to NO. Inhibition of aggregation by SNP was evaluated as percentage of maximal aggregation in the absence of sodium nitroprusside.

2.4 Platelet thioredoxin-interacting protein determination

Platelet Txnip content was determined as previously reported.\(^\text{[15]}\) Briefly, EDTA-anticoagulated blood was centrifuged to obtain platelet rich plasma, which was smeared onto untreated slides and fixed using 4% (w/v) paraformaldehyde in PBS, then stored at −70°C until assayed. Slides were blocked using 20% (v/v) goat serum in PBS, followed by Txnip detection using rabbit polyclonal anti-human vitamin D3 upregulated protein 1 (VDUP-1) (Invitrogen, USA), 1% (w/v) BSA in PBS and incubating overnight at 2–4°C. Secondary detection was performed using FITC-conjugated swine anti-rabbit polyclonal IgG (Dako, Denmark), as well as primary detection of platelet CD41 using RPE-conjugated mouse monoclonal anti-human CD41 (Dako, Denmark) in PBS. Fluorescence was developed using ‘fluorescent mounting medium’ (Dako, Denmark) and images acquired at 400× using an Axio Scope.A1 microscope with apotome and AxioVision 4.8 software (Carl Zeiss, Germany). Images were analysed for densitometric fluorescence using AxioVision LE software. The intra-assay coefficient of variation (CV) was 8.5% and the inter-assay CV was 18.6%.

2.5 Statistical methods

Data were analysed by evaluating potential univariate followed by multivariate correlates of (a) platelet response to NO and (b) platelet response to ADP. Clinical factors evaluated were age, sex, LVEF, duration of AF (with “new onset” AF defined on the basis of de novo detection), heart rate on admission ECG and CHA2DS2-VASc score. Analysis of covariance was utilized to evaluate the ADP: NO response relationship in different patient cohorts. Patient characteristics were compared by non-paired t-test, Mann-
Whitney U test or $\chi^2$ test as appropriate. All data for normally distributed parameters are expressed as mean ± SE unless otherwise stated. Skewed data are expressed as median and interquartile range (IQR). Data were analysed using the IBM SPSS Statistics 20 and GraphPad Prism 6 software packages.

3 Results

The clinical and pharmacological characteristics of the study cohort have been reported previously.\(^6\) Of the total cohort, 85.1% were in AF at the time of initial ECG recording. A breakdown of clinical parameters assessed across gender can be observed in Table 1.

Females displayed lower incidence of known coronary artery disease (CAD), higher LVEF, relatively preserved renal function, and higher CHA$_2$DS$_2$VASc scores when compared with males.\(^2\) Pharmacotherapy did not differ significantly across gender, though a trend towards increased statin use in females was noted (Table 2). In particular, there was no significant difference between gender as regards anti-platelet treatment (e.g., aspirin)\(^{20}\) or with agents that increase platelet NO response, as previously documented with statins and angiotensin-converting enzyme (ACE) inhibitors.\(^{19,21}\)

We previously reported that platelet aggregation is increased and NO response decreased in female, compared with male AF patients.\(^6\) Currently, we have evaluated the relationship between these parameters in a gender-specific manner analysis of covariance (ANCOVA, Figure 1), in order to determine whether the differing NO response can be explained by physiological antagonism.\(^{22}\) While physiological antagonism was present, female sex represented an aggregation-independent basis for impaired platelet NO response ($P < 0.01$).

We have also previously reported an inverse correlation between admission heart rate and platelet response to NO ($r = -0.331, P < 0.01$),\(^6\) while extent of ADP-induced platelet aggregation was positively correlated ($r = 0.235, P < 0.05$). Evaluation by ANCOVA (Figure 2) indicated that the heart

Table 1. Clinical characteristics of the study population, by gender.

<table>
<thead>
<tr>
<th></th>
<th>Male ($n = 45$)</th>
<th>Female ($n = 42$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs</td>
<td>72 (63, 81)</td>
<td>73 (65, 82)</td>
<td>NS</td>
</tr>
<tr>
<td>Age ≥ 75 yrs</td>
<td>20 (44.4%)</td>
<td>19 (45.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Comorbidities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>3 (6.7%)</td>
<td>4 (9.5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension</td>
<td>27 (60.0%)</td>
<td>32 (76.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>13 (28.9%)</td>
<td>8 (19.0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Prior stroke/TIA</td>
<td>5 (11.1%)</td>
<td>4 (9.5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Known coronary artery disease</td>
<td>20 (44.4%)</td>
<td>9 (21.4%)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Clinical presentation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Admission heart rate, beats/min</td>
<td>85 (72, 134)</td>
<td>96 (70, 136)</td>
<td>NS</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>58 (49, 60)</td>
<td>62 (58, 68)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma creatinine, $\mu$mol/L</td>
<td>90 (76, 109)</td>
<td>75 (67, 91)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma CRP, mg/L</td>
<td>27.0 (11.0, 46.0)</td>
<td>14.0 (5.4, 45.0)</td>
<td>NS</td>
</tr>
<tr>
<td>CHA$_2$DS$_2$VASc Score</td>
<td>3 (1, 4)</td>
<td>4 (2, 5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>New onset AF</td>
<td>11 (24.4%)</td>
<td>11 (26.2%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as $n$ (%) or median (IQR). AF: atrial fibrillation; CRP: C-reactive protein; IQR: inter quartile range; LVEF: left ventricular ejection fraction; NO: not significant; TIA: transient ischaemic attack.

Figure 1. Evaluation of the impact of gender upon the physiological antagonism between pro-aggregatory ADP and anti-aggregatory NO responses in AF patients. ANCOVA demonstrated physiological antagonism: NO response varied inversely with extent of aggregation [F(1, 74) = 12.212, $P < 0.01$]. (1) Independent impact of female sex: diminished NO response per unit ADP-induced platelet aggregation [F(2, 74) = 8.313, $P < 0.01$]; (2) A non-significant trend [F(1, 74) = 2.244, $P = 0.138$] towards diminution of gender differences (as regards platelet NO response) at greater ADP responses. ADP: adenosine diphosphate; AF: atrial fibrillation; ANCOVA: analysis of covariance; NO: nitric oxide.
rate/aggregation relationships did not vary according to duration of AF (similar analysis of the heart rate/NO relationships can be found here).\(^6\)

The potential gender-specificity of the interaction between heart rate and aggregability parameters was also evaluated by ANCOVA (Figure 3). These analyses indicated that for all levels of heart rate, females were hyperaggregable compared to males, and that for all levels of heart rate, females displayed diminished platelet NO response compared to males.

In our previous report,\(^6\) we speculated that plasma ‘surges’ in catecholamine levels could foster the development of oxidative stress, thus contributing to impaired platelet NO response. Tn Xinp is a proinflammatory protein that interacts reciprocally with NO signalling (see Chong et al.\(^{14}\) for review); we sought to explore whether platelet Tn Xinp expression interacted with new onset AF and/or gender in the context of potential catecholamine-mediated (i.e., tachycardia) oxidative stress (Figure 4). Neither duration of AF nor gender significantly correlated with platelet Tn Xinp expression.

**Figure 2.** Evaluation of the impact of duration of AF on the interaction between tachycardia and extent of platelet aggregation.

ANCOVA confirms that aggregation tends to increase \((P = 0.071)\) with tachycardia. However, the relationship does not vary significantly according to duration of AF \([F(1, 83) = 0.192, P = 0.662]\). As previously reported,\(^6\) there was a significant \((r = 0.235, P < 0.05)\) relationship overall between heart rate and aggregability. AF: atrial fibrillation; ANCOVA: analysis of covariance.

**Figure 3.** ANCOVA evaluation of the impact of gender on the interaction between tachycardia and extent of platelet aggregation (A) and platelet response to NO (B). While increasing heart rate was associated with both incremental aggregation \((P < 0.05)\) and diminished NO response \((P < 0.05)\), these relationships did not vary significantly according to gender \([F(1, 83) = 0.702, P = 0.405; F(1, 73) = 0.049, P = 0.825, \text{respectively}]\). ANCOVA: analysis of covariance; NO: nitric oxide.

**4 Discussion**

We have recently demonstrated an association between new onset AF and impaired platelet NO response.\(^6\) The current data demonstrate that the relationship between heart rate and NO response does not vary significantly according to whether AF is of recent onset or chronic. Therefore, the data are strongly consistent with the concept that the tachycardia commonly associated with new onset AF is closely linked with the suppression of platelet NO signaling. One potential mechanism to account for the current observations would be the precipitation of AF in the presence of increased sympathetic discharge,\(^{23}\) with associated redox stress and NO scavenging.

We evaluated the relationship between heart rate and platelet Tn Xinp content because non-laminar flow may promote expression of this pro-inflammatory protein.\(^{24,25}\) However, no relationship was found. These data imply that impairment of platelet NO response in the presence of tachycardia is not mediated via increased Tn Xinp expression.

AF in females engenders increased stroke risk when compared to males,\(^{26,27}\) although the precise mechanisms for this incremental risk remain uncertain. Furthermore, differential stroke incidence in females (compared to males) is independent of anticoagulant therapy.\(^{28,29}\) The specific thromboembolic risk represented by gender has even been
incorporated into the CHA2DS2-VASc stroke risk score, and endorsed by current AHA and ESC guidelines.[2,30,31]

In the current analysis, we postulated that tachycardia might contribute to the impaired NO response, and that differential biochemical response to tachycardia in females might underlie attenuation of NO signaling. Evaluation of heart rate was limited to that on admission ECG: this may not have been completely representative of heart rate around the time of admission, especially in the presence of AF. However, ECG data provided an unbiased if limited sample of patients’ heart rates. In any event, the tachycardia: NO response relationship was gender-independent (Figure 3), nor did heart rate vary according to gender (Table 1). The potential explanations for impaired NO responses in females therefore include both physiological antagonism (that is, a consequence of platelet hyperaggregability to ADP),[22] and/or greater susceptibility of ageing females[32–39] to redox stress in response to catecholamine release.[10–12] As demonstrated by the analysis shown in Figure 1, impaired NO responses in females is actually independent of ADP response. In fact, female patients in the current study had a lower prevalence of prior coronary artery disease than males, and would otherwise be expected to exhibit better-preserved NO signaling.[40,41]

There are some limitations to the present study. Of necessity, the sample size of the cohort and the short follow-up period preclude any evaluation and/or conclusions being drawn as regards clinical outcomes. Additionally, determination of heart rate and blood sampling was not always concurrent: the substantial impact of heart rate upon platelet reactivity remained significant despite this. Lastly, ADP was the only agonist used to induce platelet aggregation (as opposed to collagen, for example): this was decided upon on the basis that ADP-induced platelet aggregation is reversible, thus facilitating the evaluation of inhibitory signalling pathways (such as those activated by NO).

In conclusion, while both tachycardia and female gender are associated with impaired NO response in AF patients, they appear to be entirely independent of one another. The potential clinical advantages of reversal of tachycardia in terms of restoration of normal platelet homeostasis have not been adequately explored to date, but should now be evaluated.

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