Original Article

Subtle renal dysfunction and bleeding risk in atrial fibrillation: symmetric dimethylarginine predicts HAS-BLED score

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Received March 19, 2015; Accepted July 1, 2015; Epub August 1, 2015; Published August 15, 2015

Abstract: Background: Risk of substantial haemorrhage represents a critically important limitation to effective antithrombotic treatment in patients with atrial fibrillation (AF). While it is known that this risk is increased in anticoagulated patients either in the presence of anti-aggregatory drugs or concomitant renal insufficiency, there are currently few data on the potential interactions between endogenous platelet aggregability and bleeding risk. Objective: We therefore evaluated in a cohort of AF patients: (1), the putative relationship between platelet aggregability and HAS-BLED score; (2), the potential biochemical bases for such a relationship. Methods: Patients were included as part of SAFETY, a randomised controlled trial evaluating outpatient management of AF patients. Platelet response to ADP was evaluated via whole blood impedance aggregometry; clinical and biochemical correlates of platelet aggregation were sought via univariate and multivariate analysis. Results: Platelet aggregation correlated inversely (r=-0.220, p<0.05) with HAS-BLED score. Univariate biochemical correlates of decreased platelet aggregation were plasma concentrations of symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA). On multivariate analyses, plasma SDMA concentration (β=-0.318, p<0.01), platelet content of thioredoxin-interacting protein (Txnip, β=0.261, p<0.05) and plasma thrombospondin-1 (TSP-1, β=0.249, p<0.05) concentration were predictive of platelet ADP response. Consistent with previous reports, plasma SDMA concentrations were strongly and inversely correlated with estimated glomerular filtration rate (eGFR, r=-0.780, p<0.001). Conclusions: These data therefore suggest that (1), physiologically impaired, like pharmacologically impaired, platelet aggregability may increase bleeding risk in anticoagulated AF patients; (2), the biochemical basis for this may include impaired effects of nitric oxide (via Txnip, TSP-1) but also concomitant renal dysfunction.

Keywords: Atrial fibrillation, platelet aggregation, thrombospondin-1, thioredoxin-interacting protein, symmetric dimethylarginine

Introduction

Almost all patients with atrial fibrillation (AF) are at increased risk for thromboembolic events, which can be reduced by the use of oral anticoagulants: NOACs, engenders some increase in the risk of major bleeding. The clinical factors predictive of bleeding risk on (warfarin) anticoagulation have been delineated [2-5], but little is known of the physiological and biochemical bases for such observations.

One potential basis for bleeding risk is excessive anticoagulation: this is generally avoided
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via effect monitoring for warfarin and by dosage adjustment with the various NOACs. However, many bleeds occur despite apparently optimal anticoagulant dosage [5-8]. In these circumstances, individual predisposition to bleeding must be considered. Of the various components of the HAS-BLED score (hypertension, abnormal renal/liver function, stroke, bleeding history, labile INR, elderly, drugs/alcohol), only renal and hepatic dysfunction constitute potential bases for anticoagulant effect to be increased. Indeed, renal insufficiency was identified as a significant determinant of major and minor bleeding events in sub-analyses of the ROCKET-AF [9], ARISTOTLE [10], and RE-LY [11] trials for patients receiving either warfarin or NOACs. Severe hepatic impairment is a contraindication for the use of NOACs [12] in patients with AF or for prevention of venous thromboembolism.

Platelet aggregability also may modulate bleeding risk in such patients. For example, pharmacoologically impaired platelet aggregability represents a well-defined basis for incremental bleeding risk in anticoagulated patients: in trials involving treatment of AF, concomitant use of aspirin/clopidogrel with warfarin or NOACs increases bleeding risk [13, 14]. Furthermore, attempts to utilise NOACs in combination with aspirin and other anti-aggregatory agents in treatment of acute coronary syndromes have been limited by bleeding complications [15-17]. On the other hand, few data are available regarding the potential impact of physiological variability in platelet aggregability regarding bleeding risk in anticoagulated patients: this area represents the objective of the current study. It was observed that extent of platelet aggregation correlated inversely with bleeding risk. Univariate followed by multivariate analyses were subsequently performed to identify clinical and biochemical correlates of diminished platelet aggregability.

Materials and methods

Patient selection

The investigation was conducted as a prospective single center mechanistic sub-study of the recently reported Standard vs. Atrial Fibrillation spEcific managemenT studY (SAFETY) [18, 19], an investigation of non-pharmacological management strategies in patients hospitalized with AF. Patients were considered for inclusion if they were admitted to hospital due to chronic 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 P2Y$_{12}$ receptor antagonists were also excluded from the current sub-study because of potential impact of such agents on capacity to measure platelet response to ADP. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease formula [20]. The study complied with the Declaration of Helsinki and was approved by the Ethics of Human Research Committee of The Queen Elizabeth Hospital. Written informed consent was obtained in all cases.

Clinical data

All patients (n=83) underwent standardized clinical assessment and routine biochemical investigation. Additional cardiac investigations were resting electrocardiogram (ECG, which was used for measures of admission heart rate) and transthoracic echocardiography: LVEF was calculated from biplane images using Simpson’s method [21]. Thromboembolic risk was assessed using the CHA$_2$DS$_2$VASC (congestive heart failure, hypertension, aged ≥75 years, diabetes mellitus, prior stroke/transient ischemic attack, vascular disease, aged 65-74 years, sex category: female) score [22] and bleeding risk was assessed using the HAS-BLED score [23]. During 12 months follow up from study enrolment, no patients experienced ischemic strokes, while nine patients experienced ischemic strokes, while nine patients experienced clinically relevant (TIMI major [24]) bleeding events.

Physiological and biochemical investigations

Blood samples were obtained following admission for biochemical/physiological investigations as follows:

Platelet aggregometry: Platelet aggregometry was performed using whole blood impedance aggregometry as previously described [25]. Briefly, venous blood was collected from an antecubital vein into 10 ml tubes containing 1:10 volume of acid citrate anticoagulant (2 parts 0.1 M citric acid to 3 parts of 0.1 M trisodium citrate). Aggregation was induced with ADP
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Clinical correlates of platelet aggregability

Clinical and biochemical factors were evaluated for their potential influence on platelet aggregability. Patient characteristics were compared by non-paired t-test, Mann-Whitney U test or chi-square ($\chi^2$) test as appropriate. Correlates between clinical parameters and aggregation were evaluated by ANOVA. All data for normally distributed parameters are expressed as mean ± standard error of the mean unless otherwise stated. Skewed data are expressed as median and interquartile range, and linearization of such data was performed by logarithmic transformation or square root conversion. Data were analyzed using the IBM SPSS Statistics 20 and GraphPad Prism 6 software packages.

**Results**

**Clinical correlates of platelet aggregability**

Clinical characteristics (Table 1) and pharmacotherapy (Table 2) were typical for an elderly chronic AF cohort. As previously documented [28-30], platelet response to ADP was more
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| Table 2. Pharmacotherapy of chronic atrial fibrillation patients admitted to hospital |
| Pharmacological profile (n=83) |
| **Anti-thrombotic therapy** | |
| Aspirin, n (%) | 27 (32.5) |
| Warfarin, n (%) | 49 (59.0) |
| **Rate and/or rhythm control therapy** | |
| Anti-arrhythmics, n (%) | 22 (26.5) |
| β-receptor antagonists, n (%) | 48 (57.8) |
| Digoxin, n (%) | 29 (34.9) |
| Calcium channel blockers, n (%) | 24 (28.9) |
| **RAAS inhibitors** | |
| ACE inhibitors, n (%) | 26 (31.3) |
| Angiotensin receptor antagonists, n (%) | 23 (27.7) |
| **Other medications** | |
| Statins, n (%) | 44 (53.0) |
| Metformin, n (%) | 13 (15.7) |
| Nitrates, n (%) | 11 (13.3) |

Note: ACE = angiotensin-converting enzyme.

pronounced in female compared to male AF patients (9.9 [6.8, 11.6] Ω vs. 7.2 [5.6, 9.4] Ω, p<0.05). Although there is a theoretical basis for impairment of platelet aggregability by aspirin therapy [31], no significant interaction was observed within this cohort (p=ns, ANOVA). Extent of platelet aggregation correlated inversely with HAS-BLED scores (Figure 1), though no significant correlation was observed with CHA$_2$DS$_2$VASc scores (data not shown).

Biochemical correlates of platelet aggregability

Biochemical correlates of ADP-induced platelet aggregation are depicted in Figure 2. Plasma ADMA and SDMA both correlate with diminished platelet aggregability, whereas eGFR, plasma TSP-1 and platelet Txnip content correlated with increased platelet aggregability. Additionally, plasma ADMA (r=-0.428, p<0.001) and plasma SDMA (r=-0.780, p<0.001) concentrations were both strongly and inversely correlated with eGFR.

**Multivariate determinants of platelet aggregability in atrial fibrillation**

Clinical and biochemical univariate correlates of ADP-induced platelet aggregation were subjected to backward stepwise multiple logistic regression (Table 3). Despite the hyperaggregable response to ADP in females, gender did not represent a multivariate determinant of response. Plasma SDMA concentration, rather than eGFR per se, constituted a strong independent predictor of poor platelet aggregation in response to ADP. Conversely, both plasma TSP-1 concentrations and platelet Txnip content were independently associated with increased platelet response to ADP.

**Relationship between determinants of aggregation and HAS-BLED scores**

Given that SDMA clearance is largely renal [32], we evaluated the possibility that SDMA predominantly reflected renal function. Indeed, there was a strong direct correlation between plasma SDMA and eGFR (r=-0.780, p<0.001), while the correlation between plasma ADMA and eGFR was somewhat weaker (r=-0.428, p<0.001). Plasma SDMA concentrations, and eGFR, were also significant correlates of HAS-BLED scores (Figure 3).

**In vitro effects of symmetric dimethylarginine**

Additional studies were performed in order to delineate whether the observed relationship between plasma SDMA concentrations and diminished platelet aggregation (Figure 2B) might reflect a previously undetected anti-aggregatory effect of SDMA. In vitro studies revealed that SDMA in supra-physiological concentrations (≥30 µM) potentiated ADP-induced platelet aggregation in whole blood (Figure 4). No evidence of an anti-aggregatory effect was observed.

**Discussion**

Given that pharmacological inhibition of platelet aggregation engenders increased bleeding
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risk among anticoagulated AF patients [13, 14], we have currently tested the hypothesis that physiological variability in platelet aggregability also might exert a similar influence. To this end, we tested the hypothesis that decreased platelet aggregability corresponded to increased HAS-BLED score. In a cohort of AF patients from SAFETY [18, 19], a significant relationship was indeed found, although the size of the study precluded the comparison of actual bleeding rates.

We sought to identify biochemical bases for impaired platelet aggregability in this cohort. It emerged that elevated SDMA levels, acting presumably as a surrogate for (mildly) impaired renal function rather than on the basis of intrinsic interaction with platelet function, strongly and independently predicted impaired platelet aggregability. Furthermore, both plasma TSP-1 concentration and platelet Txnip levels corresponded with increased platelet aggregability. Therefore the current findings shed new light on the potential bases for bleeding risk in AF patients.

It is well-known that among patients anticoagulated with warfarin, bleeding risk increases sharply as renal function decreases, and that there is a less marked increase with Apixaban [10]. The majority of the relevant events occurred in patients with moderate renal insufficiency, whereas renal function was generally well-preserved in the current cohort. Nevertheless, SDMA levels correlated closely and inversely with eGFR, and SDMA appeared devoid of intrinsic anti-aggregatory effect. Consistent with the current findings, it was recently shown that SDMA levels represented a strong independent predictor of bleeding events in warfarin or Apixaban-treated patients [33]. However, it must be noted that SDMA exerts some pro-oxidant effects [34, 35], and it therefore is possible that its direct actions on vasculature, rather than platelets, may engender bleeding risk.

Table 3. Multivariate correlates of platelet aggregability in chronic atrial fibrillation patients

<table>
<thead>
<tr>
<th>Factor</th>
<th>β</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma SDMA concentrations</td>
<td>-0.318</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Platelet Txnip content</td>
<td>0.261</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Plasma TSP-1 concentrations</td>
<td>0.249</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Note: SDMA = symmetric dimethylarginine; Txnip = thioredoxin-interacting protein; TSP-1 = thrombospondin-1.

Figure 2. Biochemical correlates of platelet aggregability in chronic atrial fibrillation. (A) Plasma asymmetric dimethylarginine (ADMA, r=-0.226, p<0.05); (B) Plasma symmetric dimethylarginine (SDMA, r=-0.308, p<0.01) concentrations correlated inversely with ADP-induced aggregation; (C) Estimated glomerular filtration rate (eGFR, r=0.250, p<0.05); (D) Plasma thrombospondin-1 (TSP-1) concentrations (r=0.262, p<0.01), and (E) platelet thioredoxin-interacting protein (Txnip) content (r=0.298, p<0.01) were all direct correlates of ADP-induced aggregation.
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TSP-1 has not previously been implicated as a modulator of bleeding risk. However, TSP-1 release from platelet α-granules occurs during platelet activation [36] and TSP-1 suppresses NO signalling by inhibiting its activation of soluble guanylate cyclase: this would be expected to impact on platelet aggregability [37, 38]. Thus, the direct relationship is not surprising.

Similarly, Txnip is a pro-inflammatory molecule [39], the expression of which is suppressed by NO [27, 40]; hence increased Txnip should theoretically lead to hyperaggregability. However, this relationship has not previously been documented.

It must of course be acknowledged that in a study of this size there is no likelihood of being able to evaluate actual bleeding rates. However, the current results are consistent with the (admittedly limited) available clinical data.

The main potential clinical implications of our findings are that, (1) even mild renal dysfunction is likely to constitute a basis for increased bleeding risk in AF patients; (2) pharmacological suppression of Txnip expression [39], for example with calcium channel antagonists [41], or angiotensin-converting enzyme (ACE) inhibitors [42], in AF patients may reduce thromboembolic risk, but increase that of bleeding during anticoagulation.

Acknowledgements

This investigation was supported by NHMRC Program Grant 519823 and the NHMRC Centre of Research Excellence to Reduce Inequality in Heart Disease. Simon Stewart, Jocasta Ball and Cher-Rin Chong are supported by the NHMRC. Jeffrey Isenberg is supported by the NIH grants P01 HL103455-01, 1R01HL108954-01, 1R01HL112914-01A1 and 2R01HL089658, and also by the Vascular Medicine Institute, the Institute for Transfusion Medicine and the Hemophilia Center of Western Pennsylvania.

Disclosure of conflict of interest

None.

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