

Digoxin and Platelet Activation in Patients With Atrial Fibrillation: In Vivo and In Vitro Study

Daniele Pastori, MD, PhD;* Roberto Carnevale, PhD;* Cristina Nocella, MSC; Simona Bartimoccia, MSC; Marta Novo, MSC; Vittoria Cammisotto, MSC; Silvia Piconese, PhD; Maria Santulli, MD; Fortunata Vasaturo, MD; Francesco Violi, MD;† Pasquale Pignatelli, MD, PhD;‡ Atherosclerosis in Atrial Fibrillation (ATHERO-F) Study Group‡

Background—Digoxin use was shown to be associated with an increased risk of cardiovascular events in atrial fibrillation (AF). We hypothesized that digoxin may affect cardiovascular risk by increasing platelet activation.

Methods and Results—Post hoc analysis of a prospective study of anticoagulated patients with AF. Patients were divided into 2 groups balanced for age, sex, and cardiovascular risk factors: digoxin users (n=132) and nonusers (n=388). Urinary excretion of 11-dehydro-thromboxane B₂ (TxB₂), a marker of platelet activation, and serum digoxin concentration (SDC) were measured. In vitro experiments were performed on platelets from healthy subjects and AF patients, which were incubated with scalar doses of digoxin (0.6–2.4 ng/mL) with or without prestimulation with a sub-threshold of collagen. Median 11-dehydro-TxB₂ was 105.0 (interquartile range, 60.0–190.0) ng/mg creatinine, and median SDC was 0.65 (interquartile range, 0.40–1.00) ng/mL. Urinary 11-dehydro-TxB₂ and SDC were correlated ($r_s=0.350$, $P<0.001$). Patients in the upper tertile of SDC showed higher 11-dehydro-TxB₂ compared with non-digoxin users ($P=0.019$). In vitro study showed an increased basal platelet activation in patients with AF compared with healthy subjects. Digoxin (2.4 ng/mL) induced calcium mobilization, PAC-1 (procaspase-activating compound 1) and platelet aggregation in AF patients but not in healthy subjects. After pretreatment with a sub-threshold of collagen, digoxin dose-dependent induced calcium mobilization, arachidonic acid release, TxB₂ biosynthesis, PAC-1 and soluble platelet selectin expression, and platelet aggregation, which were inhibited by antibody against digoxin.

Conclusions—We found a significant in vivo correlation between SDC and platelet activation. Supratherapeutic SDC increased in vitro platelet aggregation via calcium-related phospholipase A₂ phosphorylation. Our findings may have clinical implications for AF patients treated with digoxin. (*J Am Heart Assoc.* 2018;7:e009509. DOI: 10.1161/JAHA.118.009509.)

Key Words: atrial fibrillation • digoxin • platelet aggregation • thromboxane

Digoxin is still widely used for acute and chronic heart rate control in patients with atrial fibrillation (AF), particularly in those patients not achieving a good heart rate

or who are unable to tolerate therapy with β -blockers, or when heart failure coexists.¹

Recent studies raised concerns on the use of digoxin in AF because of a putative increased risk of cardiovascular and all-cause mortality in patients given digoxin.^{2,3} However, current evidence on this issue is still controversial,⁴ given the lack of a randomized trial specifically aimed at testing digoxin safety in AF. Furthermore, the biologic mechanism underlying such association is still elusive.

Two previous studies performed in patients with heart failure investigated the relationship between serum digoxin concentration (SDC) and cardiac adverse events, showing that SDC above the therapeutic range (ie, >1.2 ng/mL) was associated with an increased risk of ventricular arrhythmias and mortality.^{5,6} It is also noteworthy that in the DIG (Digitalis Investigation Group) trial, SDC in the range of 0.5 to 0.7 ng/mL was associated with the lowest risk of all-cause mortality, while higher SDC (1.6–2.0 ng/mL) was associated with increased mortality.⁷

From the I Clinica Medica, Department of Internal Medicine and Medical Specialties (D.P., C.N., S.B., M.N., V.C., S.P., F.V., P.P.), Department of Medical-Surgical Sciences and Biotechnologies (R.C.), and Department of Experimental Medicine (M.S.), Sapienza University of Rome, Italy; IRCCS Neuromed, Pozzilli (IS), Italy (C.N.).

*Dr Pastori and Dr Carnevale contributed equally to this work.

†Dr Violi and Dr Pignatelli are co-senior authors.

‡A complete list of the ATHERO-AF (Atherosclerosis in Atrial Fibrillation) Study Group can be found in the appendix at the end of the article.

Correspondence to: Pasquale Pignatelli, MD, PhD, I Clinica Medica, Viale del Policlinico 155, Roma, 00161, Italy. E-mail: pasquale.pignatelli@uniroma1.it
Received April 19, 2018; accepted October 1, 2018.

© 2018 The Authors. Published on behalf of the American Heart Association, Inc., by Wiley. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

Clinical Perspective

What Is New?

- Serum digoxin is associated with increased urinary excretion of thromboxane B₂ in patients with atrial fibrillation, and in vitro experiments showed that digoxin increased platelet function in prestimulated platelets.

What Are the Clinical Implications?

- Patients should be kept in the therapeutic range of digoxin to avoid not only proarrhythmic side effects related to digoxin but also an increase in platelet activation that may lead to thrombotic complications.

Digoxin acts by inhibiting the sodium-potassium ATPase pump on the membrane of cardiac myocytes, causing an increase in sodium and intracellular calcium, resulting in increased myocardial contractility. Similarly, digoxin binds a specific sodium-potassium ouabain-like receptor present on the platelet surface.⁸ In vitro studies demonstrated that ouabain, a glycoside with analogous characteristics as digoxin, increases platelet reactivity to other agonists such as thrombin by increasing calcium mobilization.^{9,10} Also, digoxin was demonstrated to increase platelet calcium mobilization⁸ and expression of platelet-binding receptors such as P-selectin,¹¹ but direct evidence of the effect of digoxin on platelet activation is still lacking.

These effects may be particularly relevant in patients with AF in whom digoxin use could exacerbate platelet activation, which is associated with an increased cardiovascular disease incidence.¹²

Table 1. Baseline Characteristics of Patients With AF Taking or Not Taking Digoxin Balanced for Risk Factors

	Digoxin Users (n=132)	Non-Digoxin Users (n=388)	P Value
Age, y	75.2±7.2	74.6±7.8	0.480
Women, %	47.7	46.9	0.920
Hypertension, %	92.4	87.4	0.152
Diabetes mellitus, %	25.0	19.8	0.219
Heart failure, %	29.5	22.4	0.101
History of stroke/TIA, %	18.2	14.7	0.334
History of MI, %	22.7	19.3	0.451
11-dehydro-TxB ₂ (ng/mg creatinine)	101.0 [60.0–174.5]	110.0 [60.0–183.75]	0.602*

AF indicates atrial fibrillation; MI, myocardial infarction; TIA, transient ischemic attack; TxB₂, Thromboxane B₂.

*Mann-Whitney test.

We hypothesized that the association between digoxin use and cardiovascular disease may be dependent on digoxin's ability to activate platelets. To test this hypothesis (1) we investigated in vivo the relationship between platelet activation and SDC in a population of patients with nonvalvular AF treated or not with digoxin; and (2) we performed an in vitro study incubating platelets with scalar doses of digoxin to explore the possible interplay between digoxin and platelet aggregation and the underlying mechanism.

Materials and Methods

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

In Vivo Study

This is a post hoc analysis from an observational prospective cohort study including 815 patients with AF, 171 of whom were treated with digoxin.¹³ Of these, 14 patients were excluded, as they were taking aspirin in addition to vitamin K antagonists, and 25 patients did not have a urine sample (not mandatory for the study). Thus, the study group included 132 patients with AF taking digoxin. To compare urinary 11-dehydro-thromboxane B₂ (TxB₂) excretion, we included a group of 388 patients with AF not treated with digoxin and balanced for cardiovascular risk factors, as shown in Table 1.

Inclusion and exclusion criteria have been previously described.¹³ For all patients, baseline medications were collected, including type and dose of each drug taken by patients.

The study protocol was approved by the Sapienza University Institutional Review Board and was conducted in accordance to the declaration of Helsinki. Patients gave informed consent to participate in the study. The study was registered at ClinicalTrials.gov (NCT01882114).

Laboratory Analysis

After overnight fasting, blood samples obtained from patients with AF and healthy subjects (HS) after supine rest for at least 10 minutes, were taken into tubes with 3.8% sodium citrate and centrifuged at 300g for 10 minutes to obtain supernatant and immediately stored at –80°C until use. All products, when not differently specified, are from Sigma Aldrich.

Serum Digoxin Concentration

SDC was measured on the AxSYM analyzer (Abbott Laboratories, Abbott Park, IL) using the Digoxin III Reagent pack (Ref.

6L07, rev. September 2010, Abbott, Wiesbaden, Germany). The sensitivity of the AxSYM Digoxin II assay was calculated to be 0.3 ng/mL.

In Vitro Study

To evaluate platelet activation we analyzed $[Ca^{2+}]_i$ mobilization, which is the “primum movens” of platelet activation, and expression of PAC-1 (procaspase-activating compound 1), which is an antibody that recognizes an epitope of the glycoprotein IIb/IIIa of activated platelets, at/or near the platelet fibrinogen receptor. Finally, we measured platelet aggregation, which is part of the sequence of events leading to thrombus formation. The above reported markers of platelet activation were studied in platelets isolated from patients with AF (n=3, 2 men and 1 woman) and HS (n=5, 3 men and 2 women). Then, to study the pathway involved in digoxin-mediated platelet activation, we studied platelets from HS to avoid confounding factors from concomitant drugs potentially influencing platelet function such as those taken by patients with AF. Hence, to mimic the platelet preactivation found in patients with AF, we treated platelets from HS with subthreshold concentrations of collagen, as previously reported by Nocella et al.¹⁴

Platelet Aggregation

Venous blood was drawn in trisodium citrate (3.8%, 1/10 [v:v]) from healthy subjects (n=5) and patients with AF not treated with digoxin (n=3) who had fasted for at least 12 hours. To obtain platelet-rich plasma (PRP), blood was centrifuged for 15 minutes at 180g at room temperature, and the supernatant PRP was separated.¹⁵ To avoid leukocyte contamination, only the top 75% of the PRP was collected. PRP samples were treated with digoxin (0.6–2.4 ng/mL), ouabain (4 μ mol/L) and/or pretreated with a subthreshold concentration (STC) of collagen (Mascia Brunelli, 0.25 μ g/mL) as a primer. STC concentration of agonists was defined as the highest concentration that elicited <20% platelet aggregation. Based on a previous work showing an increased cardiovascular risk at 1.2 ng/mL,⁶ we used 3 SDCs, 1 within the therapeutic range (0.6 ng/mL), 1 threshold value (1.2 ng/mL), and 1 above the therapeutic range (2.4 ng/mL).

To evaluate the specific pathways of digoxin-mediated platelet activation, samples were treated with the antibody antidigoxin (4 μ mol/L), or the phospholipase A₂ (PLA₂) inhibitor arachidonyl trifluoromethyl ketone (AACOCF3) (14 μ mol/L) or the calcium chelator ethylenediaminetetraacetic acid (EDTA) (2 mmol/L) (20 minute at 37°C before activation).

Platelet aggregation was performed on a Bio/Data 8-channel platelet aggregometer (PAP-8E BioData) using

siliconized glass cuvettes under continuous stirring at 180 g, using techniques based on the method of Born.¹⁶ After stimulation with agonists, samples were centrifuged for 3 minutes at 300 g. Supernatants were stored at –80°C for analysis of 11-dehydro-TxB₂ and pellets were stored at –80°C for analysis of cytosolic PLA₂ (cPLA₂) phosphorylation and arachidonic acid production.

Western Blot Analysis of cPLA₂ Protein Phosphorylation

Platelet pellets were suspended in a 2X Lysis buffer (5 mmol/L EDTA, 0.15 mol NaCl, 0.1 mol Tris pH 8.0, 1% triton, and 10 μ g/mL of protease and phosphatase inhibitors cocktail). The protein concentration of each lysate was determined by Bradford assay. Equal amounts of protein (30 μ g/lane) were solubilized in a 2X Laemmli sample buffer containing 20% of 2-mercaptoethanol and were electrophoretically separated on a 10% SDS-polyacrylamide gel and then electro-transferred to nitrocellulose membranes. After blocking with bovine serum albumin 5% (Sigma Aldrich, Saint Louis, MO), the membranes were incubated overnight at 4°C with polyclonal anti-p-cPLA₂ antibody, raised against an amino acid sequence recognizing the phosphorylation in the Ser505 site of cPLA₂, or polyclonal anti-cPLA₂ antibody antibodies, and as loading control mouse monoclonal anti- β -actin antibody (Santa Cruz Biotechnology, Dallas, TX). Subsequently, the membranes were incubated with secondary antibody (Santa Cruz Biotechnology; 1:5000), and then the immune complexes were detected by enhanced chemiluminescence substrate. Densitometric analysis of the bands was performed using Image J software, and signal density was normalized to β -actin density.

Platelet and Urinary Thromboxane

Platelet TxA₂ generation was analyzed as previously described¹⁴ by evaluating its stable metabolite TxB₂ by ELISA commercial kit (Cusabio Technology, Houston, TX) and expressed as pg/mL $\times 10^8$ cells.

The excretion of the stable urinary metabolite 11-dehydro-TxB₂ was measured by an enzyme-linked immunosorbent assay commercial kit (Cusabio Technology). Data are expressed as pg/mg creatinine. Intra- and interassay coefficients of variation were 4.0% and 3.6%, respectively.

Measurement of $[Ca^{2+}]_i$ Mobilization

Platelet $[Ca^{2+}]_i$ mobilization was measured in PRP incubated for 20 minutes at 37°C with 8 μ mol/L FLUO 4-acetoxymethyl ester (FLUO 4-AM; Invitrogen, Waltham, MA) in dimethyl sulfoxide. One milliliter of Tyrode buffer was added, and the mixture was analyzed by the Epics XL-MCL Cytometer

(Coulter Electronics, Hiialeah, FL) equipped with an argon laser at 488 nm. The basal fluorescence intensity corresponding to FLUO 4-AM was measured for 1 minute in the platelet population identified in the gate as CD61-positive events. Digoxin (0.6–2.4 ng/mL), ouabain (4 μ mol/L) or an STC of agonists alone or digoxin 2.4 ng/mL in presence of an STC of agonists with antidigoxin, EDTA, or AACOCF3 were added and data acquired for 1 minute.

Platelet Arachidonic Acid Assay

Arachidonic acid (AA) was analyzed in platelets by gas chromatography as previously described.^{17,18} Briefly, AA was processed for direct transesterification with acetyl chloride, which allowed for the derivatization of both free and esterified fatty acid as methyl ester. These samples were added with methyl heptadecanoate as internal standard and identification, precision, and accuracy were evaluated using mixtures of authentic methylated fatty acid standards and a control plasma pool as previously described.¹⁹ Analyses were performed on an Agilent 7820A Plus Gas Chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a G4513A automatic liquid sampler and a flame-ionization detector (GC-FID). Separation was carried out on a 100-m capillary column (Supelco, SP-2560 100 m 3 0.25 mm inner diameter, 0.20 mm thickness; Sigma Aldrich, Milan, Italy).

Flow Cytometry Analysis of PAC-1

Platelet suspension (200 μ L, 2×10^8 /mL) was incubated for 30 minutes at 37°C with or without digoxin (0.6–2.4 ng/mL), ouabain (4 μ mol/L) or STC of agonists alone or digoxin 2.4 ng/mL in the presence of an STC of agonists with antidigoxin, EDTA, or AACOCF3. Platelets were then fixed with (2%) paraformaldehyde (0.1% bovine serum albumin) for 60 minutes at room temperature. The suspension was treated with monoclonal antibody (20 μ L) for 60 minutes at 4°C. The unbound monoclonal antibody was removed by centrifugation at 300g for 3 minutes (twice) after the addition of phosphate-buffered saline (0.1% bovine serum albumin). Fluorescence intensity was analyzed on BD FACSCanto Flow Cytometer (Coulter Electronics) equipped with an argon laser at 857 nm. For every histogram, 50 000 platelets were counted to evaluate the percentage of positive platelets. Antibody reactivity was reported as mean fluorescence intensity.

sP-Selectin Assay

In the supernatant of above reported experiments we evaluated sP-selectin levels by a commercial immunoassay (DRG International, Springfield, NJ) and values were expressed as ng/mL. Intra- and interassay coefficients of variation were <10%.

Statistical Analysis

Categorical variables were reported as counts and percentages. Continuous variables were expressed as mean \pm SD or as median and interquartile range or median and minimum-maximum values. We tested the independence of categorical variables by χ^2 test. Student *t* test was used to compare means and Mann-Whitney *U* test to compare medians.

The Spearman rank-order correlation test (r_s) was used to perform correlation analysis.

Groups comparison was performed by the Kruskal-Wallis test.

Only *P* values lower than 0.05 were considered statistically significant. All tests were 2-tailed, and analyses were performed using computer software packages (SPSS-22.0, IBM, Armonk, NY).

Results

In Vivo Study

Clinical characteristics of patients with AF according to digoxin treatment are reported in Table 1. Mean age of patients on digoxin was 75.2 ± 7.2 years, and 47.7% were women. The 2 groups were balanced for the main cardiovascular risk factors (Table 1).

Median SDC was 0.65 ng/mL (interquartile range, 0.40–1.00, min-max 0.30–2.45 ng/mL), whereas median urinary 11-dehydro-TxB₂ was 105.0 (interquartile range, 60.0–190.0) ng/mg creatinine. A significant correlation was found between SDC and urinary 11-dehydro-TxB₂ ($r_s=0.350$, $P<0.001$). In particular, when we divided SDC into tertiles, we found that patients in the highest tertile of SDC, corresponding to a median value of 1.17 ng/mL, had significantly higher 11-dehydro-TxB₂ levels compared with those in the first tertile (Table 2; $P<0.001$).

Of note, patients in the first 2 tertiles of SDC showed similar 11-dehydro-TxB₂ levels compared with patients not treated with digoxin, while patients in the third tertile had significantly higher 11-dehydro-TxB₂ levels compared with digoxin nonusers ($P=0.019$).

Vivo Study

Digoxin and platelet activation in HS and AF patients

Basal levels of [Ca²⁺]_i mobilization, PAC-1 expression, and platelet aggregation were higher in AF patients compared with HS (Figure 1A through 1C). After incubation of platelets with digoxin (2.4 ng/mL), we found a significant increase in [Ca²⁺]_i mobilization, PAC-1 expression, and platelet aggregation compared with unstimulated platelets only in platelets from patients with AF, whereas digoxin alone did not affect platelet activation in samples from HS (Figure 1A through 1C). No

Table 2. Levels of Urinary 11-Dehydro-TxB₂ According to Tertiles of SDC

Digoxin Tertiles	SDC (ng/mL) (Median, IQR)	11-Dehydro-TxB ₂ (ng/mg Creatinine) (Median, IQR)	P Value for 11-Dehydro-TxB ₂
First tertile	0.29 [0.20–0.40]	76.0 [50.0–130.0]	Ref.
Second tertile	0.60 [0.58–0.74]	100.0 [61.25–150.0]	0.213
Third tertile	1.17 [1.00–1.50]	148.5 [87.5–200.0]	<0.001

IQR indicates interquartile range; SDC, serum digoxin concentration; TxB₂, thromboxane B₂.
Kruskal–Wallis Test for 11-dehydro-TxB₂: $P < 0.001$.

effect was seen with 0.6 to 1.2 ng/mL of digoxin (data not shown).

In Vitro Study on Healthy Subjects

Patients with AF usually have cardiovascular comorbidities and take several different drugs that can potentially affect platelet function, and present with a basal increased platelet activation compared with HS, we performed in vitro experiments in platelets from HS (n=5, males 3, females 2, age 33.8 ± 4.1 years) pretreated with an STC of collagen (0.25 μ g/mL) in order to mimic the preactivation found in platelets from AF.

As no effect was seen with 0.6 to 1.2 ng/mL of digoxin (data not shown), in each in vitro experiment we reported only the effect of 2.4 ng/mL (first column).

[Ca²⁺]_i Mobilization

[Ca²⁺]_i mobilization is a fundamental step for platelet activation.²⁰ Compared with STC alone, digoxin up to 2.4 ng/mL or ouabain significantly increased [Ca²⁺]_i mobilization (Figure 2A). Treatment with antidigoxin and with EDTA significantly inhibited [Ca²⁺]_i mobilization (Figure 2A). Digoxin alone did not induce any change in [Ca²⁺]_i mobilization (Figure 2A).

PAC-1 Expression

PAC-1 is an antibody that recognizes an epitope of the glycoprotein IIb/IIIa of activated platelets at/or near the platelet fibrinogen receptor.

While digoxin alone had no effects on IIb/IIIa expression, incubation of platelets stimulated with an STC of collagen (0.25 μ g/mL) with scalar concentrations of digoxin (0.6–2.4 ng/mL) induced a dose-dependent increase of sP-selectin compared with STC alone (Figure 2B); similar findings were observed with ouabain as positive control.

Conversely, pretreatment of platelets with the antibody against digoxin or AACOCFF3, a specific PLA₂ inhibitor and/

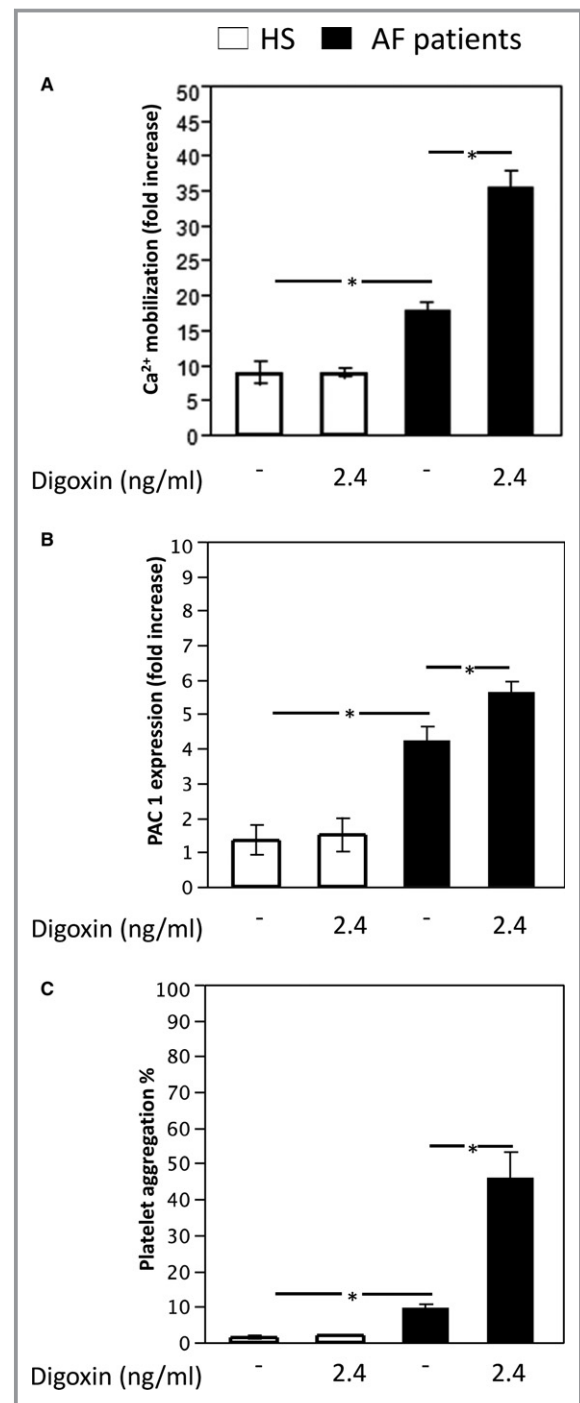


Figure 1. Comparison of in vivo digoxin-induced platelet activation in HS and patients with AF. **A**, [Ca²⁺]_i mobilization in platelets incubated with or without digoxin alone (2.4 ng/mL) in patients with AF (n=3) or HS (n=5) ($*P < 0.001$). **B**, glycoprotein IIb/IIIa expression (PAC-1) in platelets incubated with or without digoxin (2.4 ng/mL) in patients with AF (n=3) or HS (n=5) ($*P < 0.001$). **C**, Platelet aggregation in platelet-rich plasma incubated with or without digoxin (2.4 ng/mL) in patients with AF (n=3) or HS (n=5) ($*P < 0.001$). AF indicates atrial fibrillation; HS, healthy subjects; PAC-1, procaspase-activating compound 1.

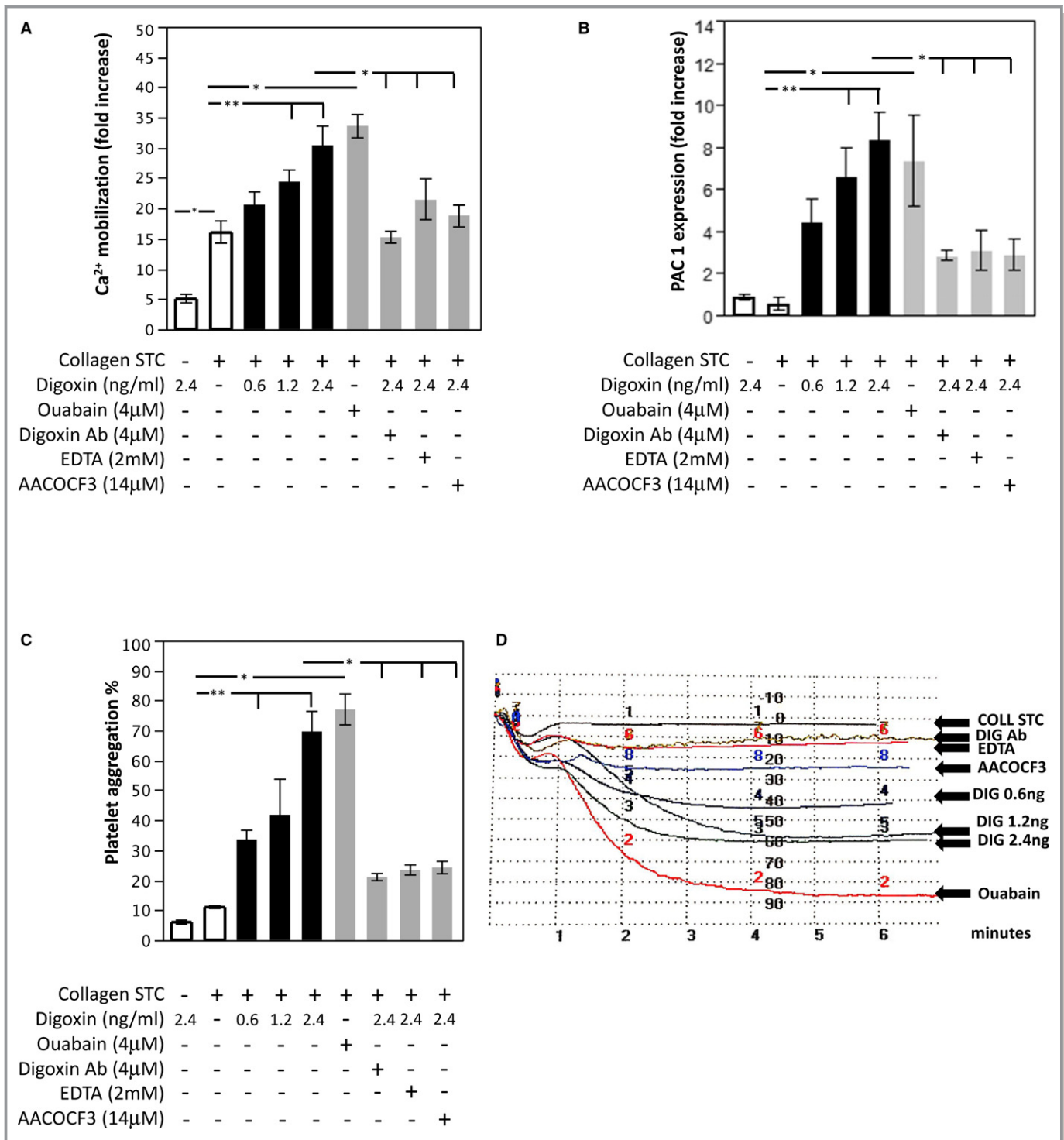


Figure 2. In vitro digoxin-induced platelet activation in HS. **A**, $[Ca^{2+}]_i$ mobilization in platelets incubated with digoxin alone (2.4 ng/mL) or prestimulated with an STC of collagen (0.25 μg/mL) and activated with scalar concentrations of digoxin (0.6–2.4 ng/mL) or ouabain in the presence or not of antibody against digoxin or EDTA (* P <0.05, ** P <0.001, n =3 experiments). **B**, IIb/IIIb expression (PAC-1) in platelets incubated with scalar concentrations of digoxin (0.6–2.4 ng/mL) or ouabain and prestimulated with an STC of collagen (0.25 μg/mL) in the presence or not of antibody against digoxin, EDTA, or AACOCF3 (n =3 experiments) (* P <0.05, ** P <0.001). **C**, Platelet aggregation in platelet-rich plasma incubated with digoxin alone (2.4 ng/mL) or prestimulated with an STC of collagen (0.25 μg/mL) and activated with scalar concentrations of digoxin (0.6–2.4 ng/mL) or ouabain and in the presence or not of antibody against digoxin, EDTA, or AACOCF3 (n =5 experiments) (* P <0.05, ** P <0.001). **D**, Representative tracing of platelet aggregation. AACOCF3 indicates arachidonyl trifluoromethyl ketone; EDTA, ethylenediaminetetraacetic acid; HS, healthy subjects; PAC-1, procaspase-activating compound 1; STC, subthreshold concentration.

or EDTA, a calcium chelator, significantly inhibited digoxin-induced IIb/IIIa expression (Figure 2B).

Platelet Aggregation

While digoxin alone had no effect on platelet aggregation, incubation of platelets with an STC stimulated with scalar concentrations of digoxin (0.6–2.4 ng/mL) induced a dose-dependent increase of platelet aggregation compared with STC alone (Figure 2C and 2D); similar findings were observed with ouabain as positive control. Increase of platelet aggregation was already observed with 0.6 ng/mL digoxin but reached significance with 1.2 ng/mL (Figure 2C and 2D). Conversely, pretreatment of platelets with antidigoxin or AACOCF3, a specific PLA₂ inhibitor, and/or EDTA, a calcium chelator, significantly inhibited digoxin-induced platelet aggregation (Figure 2C and 2D).

TxB₂ biosynthesis, granule release (sP-selectin), cPLA₂ phosphorylation, and AA

To further study the potential mechanism of platelet activation, we evaluated TxB₂; granule release, as assessed by sP-selectin; PLA₂ phosphorylation, a key enzyme for the release of eicosanoids from the platelet membrane²¹; and AA production.

Digoxin alone had no effect on TxB₂ biosynthesis, P-selectin release, PLA₂ phosphorylation, and AA increase, while incubation of platelets stimulated with an STC of collagen (0.25 μg/mL) and scalar concentrations of digoxin (0.6–2.4 ng/mL) showed a dose-dependent increase of TxB₂ production, P-selectin release, PLA₂ phosphorylation, and AA release compared with STC alone (Figure 3A through 3E). Similar results were observed by treating platelets with ouabain (Figure 3A through 3E). Pretreatment of platelets with antidigoxin or AACOCF3 or EDTA, significantly inhibited digoxin-induced platelet activation (Figure 3A through 3E).

Discussion

In this study, we showed a direct correlation between serum digoxin and urinary excretion of 11-dehydro-TxB₂, a marker reflecting platelet activation and cyclooxygenase activity in patients with AF. We also demonstrated in vitro that digoxin induces platelet aggregation via calcium-related PLA₂ phosphorylation and TxB₂ biosynthesis.

The data presented here are in keeping with an earlier study comparing 16 patients with AF taking digoxin (mean digoxin level=0.93 ng/dL) with 14 patients not taking digoxin.¹¹ The first group had significantly increased levels of a marker of platelet activation such as P-selectin. However, it was unclear if platelet changes were related to digoxin

concentration and the mechanism potentially accounting for such association was not investigated.

In our study, we found a significant correlation between SDC and urinary excretion of 11-dehydro-TxB₂, suggesting an interplay between digoxin levels and platelet activation. Of note, in this regard, patients in the first 2 tertiles of SDC (ie, <1 ng/mL), showed a similar degree of platelet activation compared with patients not receiving digoxin. Conversely, those in the upper tertile of SDC disclosed a significantly higher excretion of 11-dehydro-TxB₂ compared with non-digoxin users.

Based on this, we performed an in vitro study experiment using the circulating concentration of digoxin detected in digoxin-treated patients experiencing cardiovascular disease.⁷

We found that digoxin significantly increased platelet aggregation and TxB₂ biosynthesis in platelets from patients with AF. Hence, incubation of digoxin alone (without pretreatment of platelet with a subthreshold of agonist) activated platelets from patients with AF but not from HS, suggesting that digoxin may act as “second hit” in enhancing platelet function in conditions where a basal increased platelet activation is already present. Thus, platelets from patients with AF present a certain degree of basal activation attributable to the concomitant presence of multiple cardiovascular comorbidities, such as hypertension and diabetes mellitus, which are known to be associated with increased platelet activation.^{22,23} This result is in keeping with a previous work showing no difference in ex vivo and in vitro platelet activation in 20 HS randomized to receive digoxin or placebo for 10 days.²⁴

To explore a potential underlying mechanism, we focused on calcium mobilization, which is crucial for AA metabolism and TxB₂ biosynthesis.²⁰ We found that digoxin induced platelet calcium mobilization in a dose-dependent fashion, which was inhibited by treatment with a specific antibody against digoxin.

Then, we investigated the calcium-mediated PLA₂ phosphorylation, which represents the step forward leading to TxB₂ production, and glycoprotein IIb/IIIa activation via AA release.²¹ We found that digoxin specifically induced platelet PLA₂ phosphorylation, resulting in AA mobilization and TxB₂ biosynthesis. This effect was digoxin-related as confirmed by the similar results obtained with the digoxin-like compound ouabain. Furthermore, the pretreatment of samples with a specific antibody against digoxin completely suppressed platelet activation. Moreover, the specific inhibitor of PLA₂, namely, AACOCF3, almost completely inhibited digoxin-induced platelet activation and TxB₂ biosynthesis, reinforcing the hypothesis that digoxin-induced platelet activation was dependent on calcium mobilization and eventually PLA₂ activation.

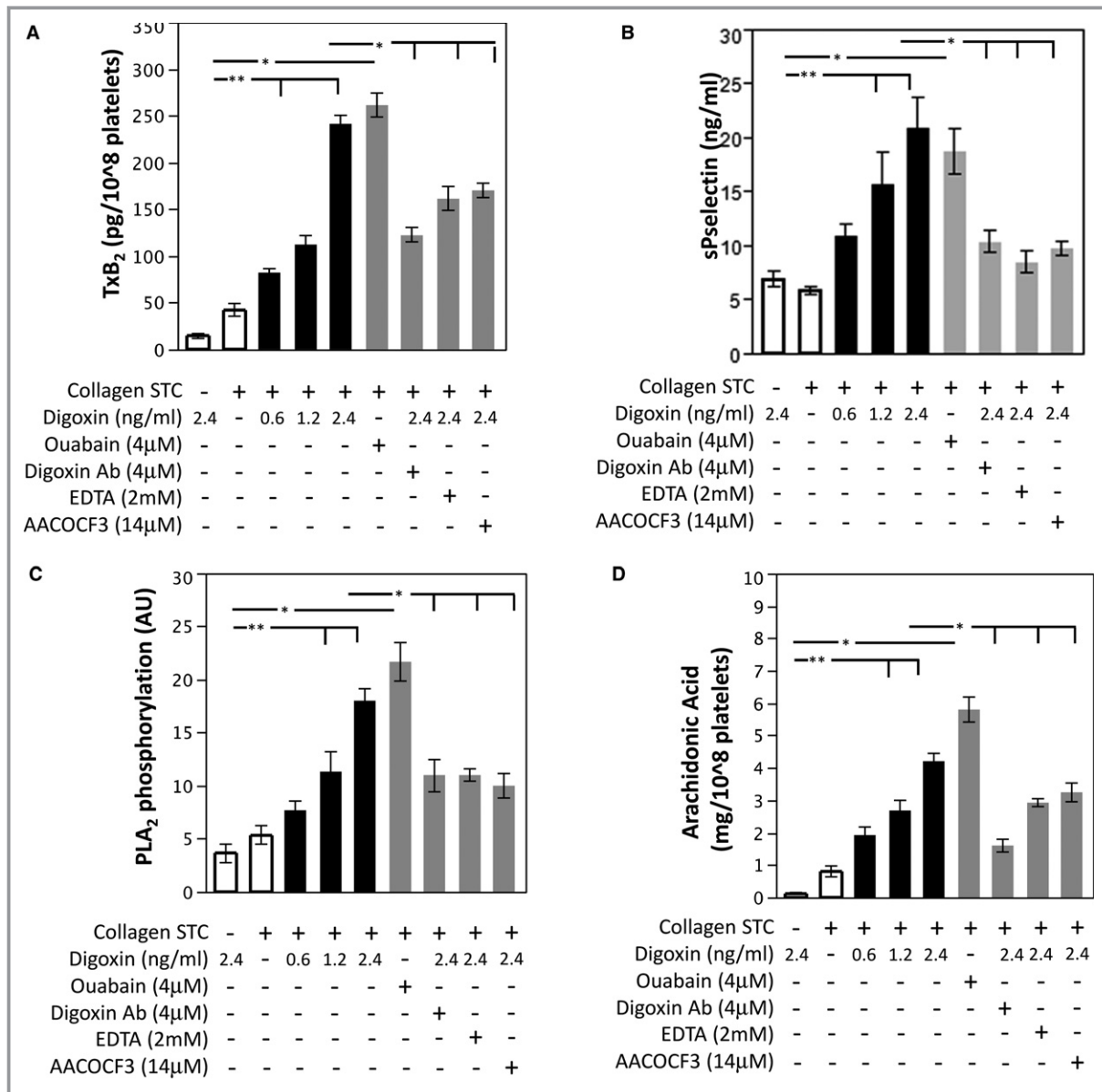


Figure 3. Mechanisms of digoxin-induced platelet activation. **A**, 11-dehydro-TxB₂ production from platelets incubated with scalar concentrations of digoxin (0.6–2.4 ng/mL) or ouabain and prestimulated with an STC of collagen (0.25 μg/mL) in the presence or not of antibody against digoxin, EDTA, or AACOCF3 (n=5 experiments in healthy subjects) (*P<0.05, **P<0.001). **B**, sP-selectin release in platelets incubated with scalar concentrations of digoxin (0.6–2.4 ng/mL) or ouabain and prestimulated with an STC of collagen (0.25 μg/mL) in the presence or not of antibody against digoxin, EDTA, or AACOCF3 (n=3 experiments) (*P<0.05, **P<0.001). **C**, cPLA₂ phosphorylation was analyzed in platelets incubated with digoxin alone (2.4 ng/mL) or prestimulated with an STC of collagen (0.25 μg/mL) and activated with scalar concentrations of digoxin (0.6–2.4 ng/mL) or ouabain in the presence or not of antibody against digoxin, EDTA, or AACOCF3 (*P<0.05, **P<0.001; n=3 experiments in healthy subjects). **D**, Arachidonic acid production in platelets incubated with digoxin alone (2.4 ng/mL) or prestimulated with an STC of collagen (0.25 μg/mL) and activated with scalar concentrations of digoxin (0.6–2.4 ng/mL) or ouabain in the presence or not of antibody against digoxin EDTA or AACOCF3 (*P<0.05, **P<0.001; n=3 experiments in healthy subjects). **E**, Representative western blot of cPLA₂ phosphorylation. AACOCF3 indicates arachidonyl trifluoromethyl ketone; Ab, antibody; cPLA₂, cytosolic phospholipase A₂; EDTA, ethylenediaminetetraacetic acid; STC, subthreshold concentration; TxB₂, thromboxane B₂.

Our study has clinical implications. We found that SDCs exceeding the therapeutic range (ie, >1.2 ng/mL), favor in vitro activation only in primed platelets, indicating the need of

substimulated platelets to obtain extensive aggregation. This finding reinforces the importance of keeping patients in the therapeutic range of SDCs to avoid not only proarrhythmic side

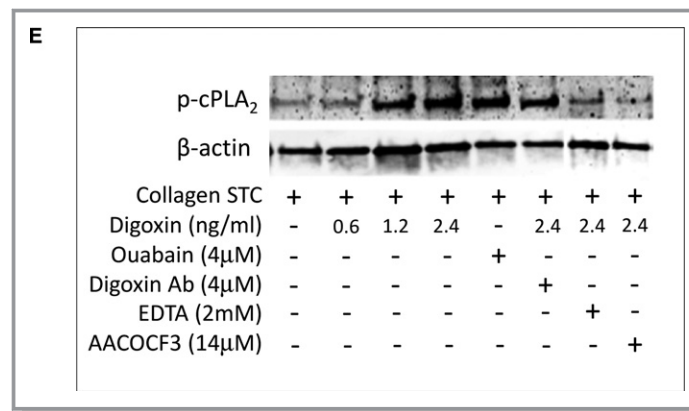


Figure 3. Continued

effects related to digoxin but also an increase in platelet activation that may lead to thrombotic complications. Indeed, our findings provide novel insight into the increased risk of cardiovascular disease observed in AF with an SDC >1.2 ng/mL.

This study presents limitations that need to be mentioned. Despite the fact that we found a significant correlation between SDC and urinary TxB₂, we cannot exclude that other pathways may be involved in the digoxin-related platelet activation. Moreover, we included only Caucasian patients with AF, and, in addition, the relationship between digoxin and platelet function may be different in other cardiovascular settings, such as in patients with heart failure in sinus rhythm.

In conclusion, we found a significant correlation between digoxin and platelet activation in vivo in patients with AF treated with vitamin K antagonists. Digoxin induced in vitro platelet TxB₂ formation via calcium mobilization, PLA₂ phosphorylation, and eventually AA release. Monitoring digoxin concentration and platelet activation may be relevant to reduce the cardiovascular risk in patients with AF.

Appendix

Members of the ATHERO-AF study group: Mirella Saliola, Marco Antonio Casciaro, Tommaso Bucci, Alessandra D'Amico, Tiziana Di Stefano, Patrizia Iannucci, and Elio Sabbatini.

Disclosures

None.

References

- Eichhorn EJ, Gheorghiadu M. Digoxin. *Prog Cardiovasc Dis*. 2002;44:251–266.
- Ouyang AJ, Lv YN, Zhong HL, Wen JH, Wei XH, Peng HW, Zhou J, Liu LL. Meta-analysis of digoxin use and risk of mortality in patients with atrial fibrillation. *Am J Cardiol*. 2015;115:901–906.
- Qureshi W, O'Neal WT, Soliman EZ, Al-Mallah MH. Systematic review and meta-analysis of mortality and digoxin use in atrial fibrillation. *Cardiol J*. 2016;23:333–343.
- Ziff OJ, Lane DA, Samra M, Griffith M, Kirchhof P, Lip GY, Steeds RP, Townend J, Kotecha D. Safety and efficacy of digoxin: systematic review and meta-analysis of observational and controlled trial data. *BMJ*. 2015;351:h4451.
- Rathore SS, Curtis JP, Wang Y, Bristow MR, Krumholz HM. Association of serum digoxin concentration and outcomes in patients with heart failure. *JAMA*. 2003;289:871–878.
- Adams KF Jr, Patterson JH, Gattis WA, O'Connor CM, Lee CR, Schwartz TA, Gheorghiadu M. Relationship of serum digoxin concentration to mortality and morbidity in women in the digitalis investigation group trial: a retrospective analysis. *J Am Coll Cardiol*. 2005;46:497–504.
- Adams KF Jr, Butler J, Patterson JH, Gattis Stough W, Bauman JL, van Veldhuisen DJ, Schwartz TA, Sabbah H, Mackowiak JI, Ventura HO, Ghali JK. Dose response characterization of the association of serum digoxin concentration with mortality outcomes in the digitalis investigation group trial. *Eur J Heart Fail*. 2016;18:1072–1081.
- Le Quan-Sang KH, David-Dufilho M, Kerth P, Pernollet MG, Frisk-Holmberg M, Meyer P, Devynck MA. Changes in platelet free Ca²⁺ concentration after chronic digoxin treatment. *Fundam Clin Pharmacol*. 1987;1:125–134.
- Oshima T, Ishida T, Matsuura H, Ishida M, Ishibashi K, Ozono R, Watanabe M, Kajiyama G, Kanbe M. Lack of effect of ouabain on calcium homeostasis in rat platelets: comparative study with human platelets. *Am J Physiol*. 1994;266:R651–R657.
- Roevens P, de Chaffoy de Courcelles D. Ouabain increases the calcium concentration in intracellular stores involved in stimulus-response coupling in human platelets. *Circ Res*. 1990;67:1494–1502.
- Chirinos JA, Castellon A, Zambrano JP, Jimenez JJ, Jy W, Horstman LL, Willens HJ, Castellanos A, Myerburg RJ, Ahn YS. Digoxin use is associated with increased platelet and endothelial cell activation in patients with nonvalvular atrial fibrillation. *Heart Rhythm*. 2005;2:525–529.
- Pastori D, Pignatelli P, Farcomeni A, Cangemi R, Hiatt WR, Bartimoccia S, Nocella C, Vicario T, Bucci T, Carnevale R, Lip GY, Violi F. Urinary 11-dehydrothromboxane b2 is associated with cardiovascular events and mortality in patients with atrial fibrillation. *Am Heart J*. 2015;170:490–497.e491.
- Pastori D, Farcomeni A, Bucci T, Cangemi R, Ciacci P, Vicario T, Violi F, Pignatelli P. Digoxin treatment is associated with increased total and cardiovascular mortality in anticoagulated patients with atrial fibrillation. *Int J Cardiol*. 2015;180:1–5.
- Nocella C, Carnevale R, Bartimoccia S, Novo M, Cangemi R, Pastori D, Calvieri C, Pignatelli P, Violi F. Lipopolysaccharide as trigger of platelet aggregation via eicosanoid over-production. *Thromb Haemost*. 2017;117:1558–1570.
- Ferroni P, Speziale G, Ruvolo G, Giovannelli A, Pulcinelli FM, Lenti L, Pignatelli P, Criniti A, Tonelli E, Marino B, Gazzaniga PP. Platelet activation and cytokine production during hypothermic cardiopulmonary bypass—a possible correlation? *Thromb Haemost*. 1998;80:58–64.
- Saito T, Welker RD, Fukui H, Herberman RB, Chirigos MA. Development of hyporesponsiveness to augmentation of natural killer cell activity after multiple doses of maleic anhydride divinyl ether: association with decreased numbers of large granular lymphocytes. *Cell Immunol*. 1985;90:577–589.

17. Iuliano L, Monticolo R, Straface G, Zullo S, Galli F, Boaz M, Quattrucci S. Association of cholesterol oxidation and abnormalities in fatty acid metabolism in cystic fibrosis. *Am J Clin Nutr*. 2009;90:477–484.
18. Ginanni Corradini S, Zerbinati C, Maldarelli F, Palmaccio G, Parlati L, Bottaccioli AG, Molinaro A, Poli E, Boaz M, Serviddio G, Mennini G, Corsi A, Bianco P, Rossi M, Iuliano L. Plasma fatty acid lipidome is associated with cirrhosis prognosis and graft damage in liver transplantation. *Am J Clin Nutr*. 2014;100:600–608.
19. Iuliano L, Pacelli A, Ciacciarelli M, Zerbinati C, Fagioli S, Piras F, Orfei MD, Bossu P, Pazzelli F, Serviddio G, Caltagirone C, Spalletta G. Plasma fatty acid lipidomics in amnesic mild cognitive impairment and Alzheimer's disease. *J Alzheimers Dis*. 2013;36:545–553.
20. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol*. 2010;30:2341–2349.
21. Harizi H, Corcuff JB, Gualde N. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends Mol Med*. 2008;14:461–469.
22. Minuz P, Patrignani P, Gaino S, Degan M, Menapace L, Tommasoli R, Seta F, Capone ML, Tacconelli S, Palatresi S, Bencini C, Del Vecchio C, Mansueto G, Arosio E, Santonastaso CL, Lechi A, Morganti A, Patrono C. Increased oxidative stress and platelet activation in patients with hypertension and renovascular disease. *Circulation*. 2002;106:2800–2805.
23. Santilli F, Davi G, Consoli A, Cipollone F, Mezzetti A, Falco A, Taraborelli T, Devangelio E, Ciabattoni G, Basili S, Patrono C. Thromboxane-dependent CD40 ligand release in type 2 diabetes mellitus. *J Am Coll Cardiol*. 2006;47:391–397.
24. Pettersen E, Hagberg IA, Lyberg T, Gjesdal K. Do cardiac glycosides affect platelet function? A flow cytometric study in healthy volunteers. *Eur J Clin Pharmacol*. 2002;58:181–186.